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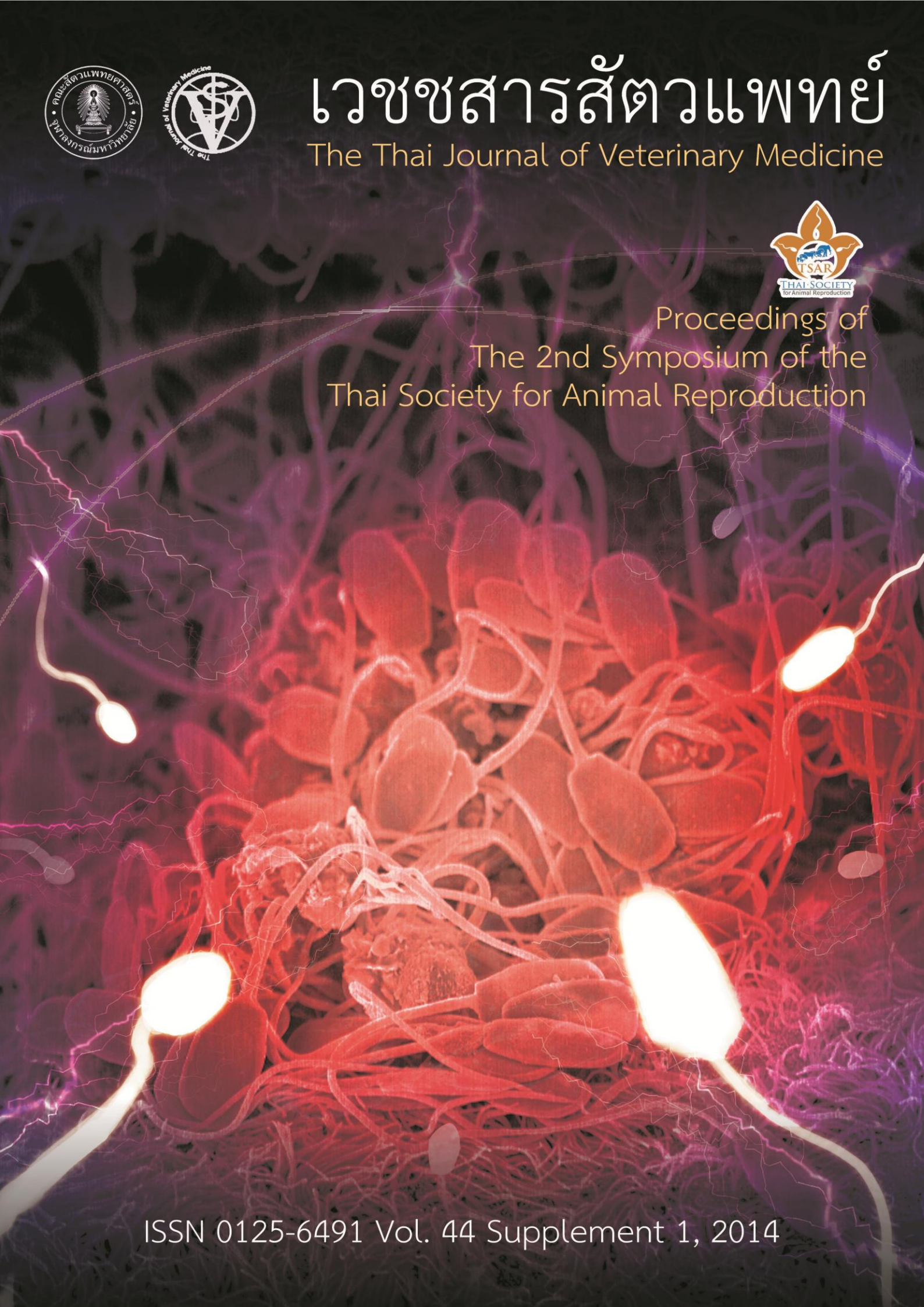
The Thai Journal of Veterinary Medicine

Proceedings of
The 2nd Symposium of the
Thai Society for Animal Reproduction



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INSTRUCTIONS TO AUTHORS

The Thai Journal of Veterinary Medicine publishes articles reporting interdisciplinary investigations concerning veterinary and animal sciences, at all levels of resolution, from basic to clinical, molecular to behavioral, and opinions that are of general interest to the broad community of veterinarians and biological scientists. Clinical or pathological investigations, protocols and reviews will also be considered for publication if they provide significant insight into the structure or function, the pathophysiology of a disease, or its treatment.

In the Journal's Table of Contents, published articles will be shown under one of the appropriate Section titles listed below

SECTIONS

Editorials A limited amount of space will be available for comments about important scientific points or subjects of topical interest, and will be by invitation only.

Reviews and Minireviews will be either by invitation, or submission. The latter will be reviewed by experts in the same manner as other submitted manuscripts.

Original Articles should be novel research findings and provide strong evidence for the conclusions. The manuscripts suitable for publication in TJVM should be of extreme importance to scientists in the field as well as interesting to researchers in other disciplines.

Short Communications These are short communications that describe outstanding new discoveries. This decision will be based on whether the paper reports particularly important findings that are likely to have a high impact in the field of work.

Clinical or Pathological Reports These are short reports of original clinical or pathological findings whose importance mean that they will be of interest to veterinarians.

Diagnostic Forum is a regular feature of TJVM. This includes Ultrasound Diagnosis, ECG Quiz, Ophthalmology Snapshot and What Is Your Diagnosis, all of which will be by invitation only.

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Each manuscript should be accompanied by a signed cover letter in which the corresponding Author states: "The work described has not been submitted elsewhere for publication, in whole or in part". All submissions to *TJVM* must contain experiments that conform to the ethical standards issued by the National Research Council. If the studies deal with animal experiments, the authors certify in the *Materials and Methods* section, that the procedures have been approved by the Authors' Institution's Ethic Committee, and care was taken to minimize the number of animals used. If the ethical standard governing the reported research is different from those guidelines indicated above, the authors must provide information in the submission cover letter about which guidelines and oversight procedures were followed.

Authors should only use abbreviations sparingly and should always define the abbreviation when first used in the text by placing it in parentheses after the full term. The abbreviations should then be used consistently thereafter and appear at least twice in the text. Drug names should be the official or approved names; trade names or common names may be given where the drug is first mentioned. Trade names should be capitalised and the manufacturer's name and country given in parenthesis thereafter. The doses of the drugs should be given as unit weight/unit body weight, e.g. mmol/kg or mg/kg. Symbols for physical units should be restricted to the Systems Internationale (S.I.) Units.

Where possible, Authors should also include a list of three or more potential reviewers for their manuscript, with contact information.

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The manuscript should have a uniform style and be submitted exactly as the author wishes it to appear in print. It should consist of subdivisions in the following sequence.

1) Title page
2) Abstract
3) Text

4) Acknowledgments
5) References
6) Tables
7) Figures legends
8) Figures

Start each subdivision on a new page.

Title Page. The first page of the manuscript should include:

- Title of paper
- Full name of author(s)
- Institutional affiliations and complete mailing address
- The exact number of pages, figures and tables in the article
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Abstract. Submit an abstract of around *250 words* that will serve in lieu of a concluding summary. The abstract must be written in complete sentences and succinctly state the objectives, experimental design of the paper, principal observations, and conclusions; it should be intelligible without reference to the rest of the paper.

Keywords. Four to six key words should be included (alphabet ordering).

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- Original articles: The text should be presented in the following order:

(i) *Introduction.* This should provide the scientific rationale for the research that is reported. No results should be presented.

(ii) *Materials and Methods.* Procedures used in the research should be described in sufficient detail to permit the replication of the work by others. Previously published procedures should be referenced and briefly summarised. The source of all materials, including animals and human tissue, must be provided.

(iii) *Results.* This section presents findings without discussion of their significance. Subsections should be used in order to present results in an organized fashion. The findings may be assisted by high quality illustrations, as necessary, to adequately document the work. Figures should be referred to in the text as Fig.1, Figs. 1, 3-4, etc., and tables as Table 1, Table 1, 3-4, etc.

(iv) *Discussion.* This section presents the Authors' interpretations of their findings and an assessment of their significance in relation to previous work. Repetition of material presented in the Results section should be avoided.

- Short Communication and Clinical or Pathological Reports: These should not exceed 4 pages (approximately 2,000 words in total, including spaces) Follow the instructions for Original Articles with the exception that results and discussion are combined.

- Reviews: Reviews should have an introductory section, followed by several information presentation sections and end with a conclusion section. Section headings should be used to organize the presentation of information.

Acknowledgements. It is the corresponding Author's responsibility to ensure that individuals who are acknowledged for assistance or for providing comments on the manuscript are agreeable to being acknowledged in this way.

References

(a) In the text, references should be quoted as the name of the first author and year in chronological order. Multiple authors are indicated by "et al.", except when there are only two authors, in which case both names are written.

Examples:

..... (Garthwaite and Garthwaite, 1995; Morris, 2000).

..... by Nagy et al. (1999[a,b]).

Clarkson et al. (2004) stated.....

(b) The reference list should be on a separate page at the end of the manuscript, in alphabetical order and arranged as follows: authors' names and initials, year, title of the article, abbreviated title of the journal, volume, first and last page numbers.

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Article in a periodical:

Nagy ZA, Esiri MM and Cato AM 1999[a]. Cell cycle markers in the hippocampus in Alzheimer's disease. Acta Neuropath. 94(2): 6-15.

Chapter in a book:

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An entire book:

Sodikoff CH 1995. Laboratory Profiles of Small Animal Diseases. A Guide to Laboratory Diagnosis. 2nd ed. St. Louis: Mosby 178 pp.

Electronic information:

Dumble LJ 1996. "The third world and infertile women." [Online]. Available: http://www.maff.gov.uk. Accessed July 12, 1997.

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(d) Unpublished or submitted experiments by one of the authors may be mentioned only in the text, not in the References. Initials, as well as surnames, must be given for authors whose unpublished experiments are quoted: (M.L. King, unpublished observations)

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Figure 1 Typical lesions of...

Table 1 Statistical analysis showing....

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Proceedings of
The 2nd Symposium of the Thai Society for Animal Reproduction

(Joint Symposium of the Thai Society for Animal Reproduction and Society for Reproduction and Development)

March 20-21, 2014

Organized by

The Thai Society for Animal Reproduction

Department of Obstetrics, Gynaecology and Reproduction,
Faculty of Veterinary Science, Chulalongkorn University

Society for Reproduction and Development

Proceedings of the 2nd Symposium of the Thai Society for Animal Reproduction

March 20-21, 2014

Opening Ceremony

Venue: Demonstration Room, 60th year Building,
Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

Opening Address

By *Professor Dr. Kei-ichiro Maeda*
President of Society for Reproduction and Development

I am pleased to send my congratulations to the Thai Society for Animal Reproduction (TSAR) for your second meeting. I was honored to attend the first TSAR meeting in October 29, 2011. In the SRD-TSAR joint session, you had 3 SRD members to give talks on their expertise. I witnessed that the meeting had a great success with a lot of stimulating presentations and active discussions. I hope the 3 SRD members giving talks in the TSAR 2014 will stimulate the discussion again.

Thailand and Japan have been keeping a good relationship in every aspect of activities, economically, socially and culturally. We have started a new relationship in reproductive science. I am sure that the present meeting will foster and strengthen the relationship.

Kei-ichiro Maeda, DVM, PhD
The President
Society for Reproduction and Development

Welcome Message

By *Associate Professor Dr. Kaitkanoke Sirinarumitr*
President of the Thai Society for Animal Reproduction

It is my great pleasure to you all a very warm welcome on behalf of the Thai Society for Animal Reproduction (TSAR) and the organizing committee of the joint Symposium of Thai and Japanese for Animal Reproduction and Development (SRD). I am pleased to welcome all of you who participating in the meeting of the Joint Symposium of TSAR and SRD during 20th-21st March 2014, at the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

The TSAR was established in 2010. The first meeting was arranged during 29th-30th September 2011, and it was very successful with 19 speakers, 8 oral presentations and more than 20 poster presentations with over 150 participants. As we knew, Thailand had a big flooding during October to December 2011. TSAR conferences and other activities were stopped and not continued for 2 years.

In this year 2014, it is a very great opportunity to continue our second meeting, and it is very glad to have the collaboration with the SRD again. The joint symposium will dedicate to provide scientific forum for scientists, veterinarians, graduate students and veterinary students who are interested in animal reproduction to attain, present, meet each other, and exchange knowledge. We hope that the joint Symposium of TSAR and SRD will be the start point that TSAR can collaborate to other international Animal Reproduction Societies in the near future.

In conclusion, I wish you all will enjoy the meeting and best wish for the productive and successful conference. I wish you every success and have a very pleasant stay with us in Thailand. I hope that our joint symposium will be strengthened and held again next year.

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The 2nd Symposium of the Thai Society for Animal Reproduction

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Program of the 2nd Symposium of the Thai Society for Animal Reproduction

Venue: Demonstration Room, 60th year Building,
Faculty of Veterinary Science, Chulalongkorn University,
Bangkok, Thailand

Date: Thursday March 20, 2014 Language: English

08.00-08.30	Registration
08.30-09.00	Opening Ceremony
09.00-09.30	The Roles of Estrogen Receptors on Estrus Behavior, Follicle Development and Artificial Insemination *Sayamon Srisuwatanasagul*
09.30-10.00	Infectious Causes of Infertility in Swine in Thailand: Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Aujeszky's Disease Virus (ADV), Porcine Parvovirus (PPV) and Porcine Circovirus Type 2 (PCV2) *Padet Tummaruk*
10.00-10.30	Coffee Break
10.30-11.00	Cryopreservations of Buffalo Oocytes *Rangsun Parnpai*
11.00-11.30	Current Concept in Open- and Closed-cervix Pyometra in Dog *Kaywalee Chatdarong*
11.30-12.00	Estrus Synchronization in Gilts by Altrenogest and hCG *Kampon Kaeoket*
12.00-13.00	Lunch
13.00-13.30	Cutting Edge of Bovine Reproductive Technology: Production of Prion Gene KO Cow to Prevent Spontaneous BSE *Noboru Manabe*
13.30-14.00	Recent Progress on Pig Genetic Resources: Cryopreservation of Gametes and Gonadal Tissues *Kazuhiro Kikuchi*

14.00-14.30	Efficient Production of Sexed Embryos by *In Vitro* Fertilization with X-sorted Sperm and *In Vivo* Matured Oocytes in Dairy Cows *Satoko Matoba*
14.30-15.00	Coffee Break
15.00-15.30	Targets and Roles of Progesterone during the Periovulatory Period in the Bitch *Muhammad Zahid Tahir*
15.00-16.30	Oral Presentation
	Optimization of Pre-processing Procedures for Canine Mammary Tumor Oligonucleotide Microarray Meta-analysis *Kaj Chokeshai-u-saha*, Thanida Sananmuang*
	Cholesterol Loaded Cyclodextrin Improves Sperm Survival in Tris-Based Extender with Less Egg Yolk used in Buffalo Bulls Semen *Mehboob Ahmed, Abdul Sattar, Sajid Iqbal, Qaisar Shahzad,* *Muhammad Zahid Tahi[1], Muhammad Hammad Fayyaz, Mushtaq Ahmad**
	PCR Detection of Porcine Circovirus Type 2 DNA in the Ovarian and Uterine Tissues of Gilts with Reproductive Disturbances *Pachara Pearodwong*, Rachod Tantilertcharoen, Komkrich Teankum,* *Padet Tummaruk*
	Dynamic Changes of GFRα-1 Expressing Testicular cells in Pre- and Post- Pubertal Cats *Narong Tiptanavattana, Araya Radtanakatikanon, Supranee Buranapraditkun, Mongkol Techakumphu, Theerawat Tharasanit**
	Effect of Immunization Against Gonadotropin Releasing Hormone (Improvac®) on Reproductive Functions in Rusa Deer Stags (*Cervus timorensis*) *Orasa Phraluk*, Nikorn Thongtip, Worawidh Wajjwalku*
	Canine Prostatic Specific Esterase (CPSE) in Association with Age and Prostatic Volume *Kanchanarut Mankong, Junpen Suwimonteerabutr, Kaywalee Chatdarong, Suppawiwat Ponglowhapan**
18.00-20.30	**Welcome Reception at Novotel, Siam square, Bangkok**

Date: Friday March 21, 2014 Language: Thai

08.30-09.00	Toll-like Receptor 2 and 4 in Healthy and Infected Canine Endometrium: A Clinical Approach *Sroisuda Chotimanukul*
09.00-09.30	Defence Mechanism in Bovine Endometrium: Current Concepts *Theerawat Swangchan-Uthai*
09.30-10.00	Expression of Cytokines in Porcine Endometrium after Artificial Insemination: A Clinical Implication *Jatesada Jiwakanon*
10.00-10.30	Coffee Break
10.30-11.00	Artificial Insemination in Horse *Theerawat Tharasanit*
11.00-11.30	Artificial Insemination in Asian Elephant (*Elephas maximus*) *Nikorn Thongtip*
11.30-12.00	Artificial Insemination in the Giant Panda *Boripat Siriaroonrat*
12.00-13.00	Lunch
13.00-13.30	Luteinizing Hormone and Follicle Stimulating Hormone Receptors: It's Function and Clinical Implication *Suppawiwat Ponglowhapan*
13.30-14.00	Artificial Insemination in Goats: An Update *Sukanya Leethongdee*
14.00-14.30	*In Vitro* Culture of Feline Embryos: A Review *Thanida Sananmuang*
14.30-15.00	Coffee Break
15.00-15.30	Reproduction in Sea Turtle *Kaitkanoke Sirinarumitr*
15.30-16.00	Reproduction in Whipray *Nantarika Chansue*
16.00-16.30	Artificial Fertilization in Nile Tilapia *Oreochromis niloticus* *Janenuj Wongtavatchai*
16.30-17.00	Snake Reproductive System *Taksa Vasaruchapong*

Review Article

Inducing Infectious Causes of Infertility in Swine in Thailand: Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Aujeszky's Disease Virus (ADV), Porcine Parvovirus (PPV) and Porcine Circovirus Type 2 (PCV2)

Padet Tummaruk[1]* Pachara Pearodwong Em-on Olanratmanee

Abstract

The present study aims to review the prevalence of important reproductive diseases in swine commercial herds in Thailand with special emphasize on porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease virus (ADV), porcine parvovirus (PPV) and porcine circovirus type 2 (PCV2). Retrospective data in swine commercial herds in Thailand found that PRRSV and ADV were detected in approximately 80% and 5% of the pigs, respectively. Furthermore, in the replacement gilts, PRRSV, ADV and PPV were detected in 88%, 4% and 99%, respectively. Interestingly, in the gilts culled due to reproductive failure, the prevalence of PRRSV, ADV and PPV was 74%, 28%, and 86%, respectively. Additionally, recent studies found that artificial insemination (AI) with semen spiked with PCV2 in naïve sows causes reproductive failures, i.e., mummified fetuses and stillborn piglets. The reproductive failure associated with PCV2 can also be manifested as irregular return to estrus, abortion, and low litter size at birth. The review addressed that PPV is an enzootic disease in all of the herds and the replacement gilts are commonly exposed to PPV rather early in their lives. Replacement gilts were an important source of introducing PRRSV into the breeding herds. The prevalence of ADV was higher in gilts culled due to reproductive disturbance than in healthy gilts. Immunization of replacement gilts against PRRSV, PPV and maybe also PCV2, along with the elimination of ADV is the important issue in the swine breeding herds in Thailand.

Keywords: reproduction, health, disease, acclimatization, pig

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Introduction

In general, the viral pathogens causing a large impact to the swine industry in Thailand during the last decade include classical swine fever virus (CSFV), foot and mouth disease virus (FMDV), porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease virus (ADV), porcine parvovirus (PPV) and porcine circovirus type 2 (PCV2). The last four pathogens also contribute to reproductive disorders in gilts and sows (Maldonado et al. 2005). Nowadays, co-infection of these pathogens is commonly observed in the modern swine industry (López-Soria et al. 2010; Tummaruk and Tantilertcharoen, 2012). The co-infection in pigs may cause complicated clinical signs, e.g., porcine respiratory disease complex (PRDC) and post-weaning multisystemic wasting syndrome (PMWS) (Opriessnig et al. 2007). Although the influence of these complex diseases is well established in nursery and fattening pigs, information related to their influences on reproductive problems in gilts and sows are limited. Studies on the association among PPV, PRRSV, ADV and PCV2 may be important for investigation to understand the causes of reproductive failure in gilts raised in Thai swine herds (Tummaruk et al. 2009a). Therefore, this review aims to investigate the prevalence of viruses causing reproductive disorders from different groups of pigs in commercial swine herds in Thailand with special emphasis on PRRSV, ADV, PPV and PCV2.

Porcine reproductive and respiratory syndrome virus (PRRSV)

Porcine reproductive and respiratory syndrome (PRRS), caused by the PRRSV, is one of the most important diseases in the pig production industry throughout the world (Zimmerman et al., 2006; Tummaruk et al., 2013). PRRS virus is an enveloped RNA virus that is divided into two genotypes, i.e., European (EU or type 1) and North American (NA or type 2) (Meng, 2000). The virus can infect many organs including the lung, liver, spleen, tonsil, lymph node, and uterus of pigs and remain in the tissue for many days (Benfield et al., 2000; Laohasittikul et al., 2004; Olanratmanee et al., 2011). PRRSV infection also causes reproductive failures in gilts and sows, e.g., increased return rate and abortion rate, decreased farrowing rate, increased percentage of mummified foetuses per litter, and increased percentage of stillborn piglets per litter (Chung et al., 1997; Olanratmanee et al., 2013). In addition, the number of weak-born piglets and sow mortality rate are also increased (Zimmerman et al., 2006). The major economic losses caused by the PRRSV in herds is mainly due to a decrease in the number of piglets weaned per sow per year, long farrowing intervals, and an increase in the replacement rate (Brouwer et al., 1994). Many management strategies including biosecurity system, acclimatisation of replacement gilts, serological profiling, and vaccination with inactivated and/or modified live virus (MLV) vaccines have been used to minimise the economic loss due to PRRS virus infection (Thanawongnuwech and Suradhat, 2010; Olanratmanee et al., 2013). However, in practice, a high variation in gilt and sow reproductive performance in the herds where PRRS-MLV vaccines have been implemented is still commonly found (Alexopoulos et al., 2005; Martelli et al., 2007; Olanratmanee et al., 2013). In Thailand, the PRRSV has been detected since 1989 (Damrongwatanapokin et al., 1996). Nowadays, the PRRSV of both genotypes 1 and 2 have been identified (Amonsin et al., 2009).

Our recent study on the herds monitoring data revealed that the percentage of PRRSV-positive pigs in swine commercial herds in Thailand during 2004-2007 was 79% (4,492/5,664 pigs) (Tummaruk and Tantilertcharoen, 2012). Nevertheless, the proportion of PRRSV-positive pigs differed among years and herds. The prevalence of PRRSV was significantly increased year after year from 2004 to 2007 (from 65% to 88%). The prevalence of PRRSV was higher in fatteners (84%), gilts (83%), sows (82%) and boars (79%) than in nursery pigs (48%) (Tummaruk and Tantilertcharoen, 2012).

A serological survey in replacement gilts indicated that 88% of the gilts had antibody titer against PRRSV (Tummaruk and Tantilertcharoen, 2012). The S/P ratio of the PRRSV-seropositive gilts varied from 0.428 to 3.673. The percentages of PRRSV-seropositive gilts before and after acclimatization were 84.0% and 92.0%, respectively. However, the percentage of PRRSV-seropositive gilts varied among herds from 55% to 100%. More specifically, in the pregnant gilts, the S/P ratio of PRRSV can vary from 0.03 to 3.7; 87% of them were PRRSV-seropositive (Tummaruk and Tantilertcharoen, 2012).

Our recent study based on PRRSV PCR detection in Thailand found that the strain of PRRSV isolated during 2005 to 2010 was genotype 2 (54.5%), genotype 1 (31.0%) and mixed genotypes (14.5%) (Tummaruk et al., 2013). It was found that PRRSV was detected by PCR in the tissue samples more frequently than the semen and serum samples. The prevalence of PRRSV was high in the nursery pigs. A high prevalence of PRRSV was found in the hot season, indicating that climatic factors may also contribute to the prevalence of PRRSV in Thailand (Tummaruk et al., 2013).

Tummaruk and Tantilertcharoen (2012) found that the number of PRRSV-seropositive gilts was lower in those culled due to abnormal vaginal discharge than those culled from anestrus. The replacement gilts are an important source of introducing PRRSV into the breeding herds. However, only antibody titer (S/P ratio) might not be a good indicator for the existence of PRRSV in tissues or blood circulation of the pigs (Thanawongnuwech and Suradhat 2010; Olanratmanee et al. 2011). Olanratmanee et al. (2011) demonstrated that the virus could be found in the uterine tissue of the gilts with either high or low antibody titer. PRRSV antibody titer differed considerably among the herds.

The reason might be due to genetic variation of PRRSV among the herds. Since the antibody formation of PRRSV was greatly affected by genetic variation and amino acid sequence of PRRSV (Kim et al., 2009), therefore, only antibody titers may not be enough to examine the PRRSV circulation within the herds. Nevertheless, the antibody titer of PRRSV, in many PRRSV non-vaccinating herds in Thailand, is intensively examined in replacement gilts for several times prior to being introduced to the breeding houses. In some breeding herds, PRRS modified live-virus vaccine is used in the replacement gilts to control PRRSV (Cho and Dee 2006). However, the use of PRRS modified live-virus vaccine should be carefully considered due to cross-protection among different strains of PRRSV still is controversial; the shedding of virus from vaccinated pigs was commonly observed during the first few weeks after vaccination (Alexopoulos et al. 2005; Scortti et al. 2006; Kim et al. 2009; Thanawongnuwech and Suradhat 2010). Furthermore, in some cases, co-infection of PRRSV and PPV and/or ADV might possibly occur in the replacement gilts. This may cause a more complicated situation and lead to inferior subsequent reproductive performance in the gilts, because PRRSV has been regarded as an immune-suppressive pathogen (Thanawongnuwech and Suradhat 2010). Recently, Olanratmanee et al. (2011) demonstrated that the PRRSV antigen could remain in the female reproductive tract of the replacement gilts for several months (up to 11 months of age). In this case, the postponement of first mating in gilts should be considered. These findings indicated that the health status of the replacement gilts was an important issue which should be considered before first mating decision.

Aujeszky's disease virus (ADV)

In 1995, a serological survey on glycoprotein I (gI) of ADV from 15 swine herds in Thailand indicated that 98% (597/608 samples) of the pig samples are positive (Wongwatcharadumrong and Platt 1995). It is known that the gI of ADV indicates natural infection (Mengeling et al. 1997). Therefore, monitoring of ADV gI-positive pigs in the herd is an important key for ADV elimination program. At the present time, the prevalence of ADV in Thailand has been declined because ADV vaccine is extensively used, together with the surveillance of ADV-gI is routinely performed. However, the prevalence of ADV causing different types of reproductive failure in gilts has never been investigated in Thailand. Recently, Tummaruk and Tantilertcharoen (2012) demonstrated that the percentage of ADV seropositive pigs from selected commercial herds was 5.3% (70/1332 pigs). However, the percentage of ADV-seropositive pigs varied among herds from 0 to 18%. Furthermore, it was found that the prevalence of ADV was higher in sows (11.9%), boars (4.6%) and nursery pigs (3.2%) than in gilts (0.0%) and fatteners (0.9%) (Tummaruk and Tantilertcharoen, 2012). Across the herds, the prevalence of ADV also varied among years. The prevalence of ADV was 3.8%, 3.4%, 8.5% and 2.3% from 2004 to 2007, respectively (Tummaruk and Tantilertcharoen, 2012). In replacement gilts, the antibody titer against ADV was found in 4.0% from a randomly selected gilts (Tummaruk and Tantilertcharoen, 2012). Of the ADV-seropositive gilts, 2 out of 8 were observed before acclimatization, while the rest was observed after acclimatization (during pregnancy).

Porcine parvovirus (PPV)

In general, PPV antigen can be detected in the sow's serum after infection up to 10 days (Miao et al. 2009). PPV antibody titer varies between 1:32 and 1:512 after vaccination. However, it may reach 1:40960 within 19 days after challenging with field-strained PPV (Józvik et al. 2009). A high level of PPV antibody titer is commonly observed in both gilts and sows under field conditions. This is unlikely to be the result of PPV vaccination. Instead, it is associated with herd size, parity number and reused of storage open vials vaccine (Oravainen et al. 2005). In the Thai swine herds, the replacement and pregnant gilts were PPV-seropositive with a titer of ≥1:128 (Tummaruk and Tantilertcharoen, 2012). Of these gilts, 99.0% had high PPV antibody titer (>1:512) and 97.0% had very high PPV titer (≥1:4096). High PPV titer was found in the gilts culled due to abnormal vaginal discharge more than the others (Tummaruk and Tantilertcharoen, 2012). Under field conditions, the PPV antibody titer ranged from 1:32 to 1:32768 (Tummaruk and Tantilertcharoen, 2012). It was found that 86.0% of the gilts had PPV antibody titer of >1:512 (Tummaruk and Tantilertcharoen, 2012). Besides, 72% of them had PPV antibody titer of ≥1:4096. Interestingly, under field conditions, 75.5% of the culled gilts were exposed to at least two viruses, 18.9% of them were exposed to all the three viruses and 45.9% of them were exposed to both PRRSV and PPV.

Porcine circovirus type 2 (PCV2)

Porcine circovirus type 2 (PCV2) is a non-enveloped single stranded DNA virus containing 3 open reading frames(ORF), i.e., ORF1, ORF2, and ORF3 (Olvera et al., 2007). Up to date, the virus has been divided into at least five subtypes, i.e., PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e (Jantafong et al., 2011; Trible et al., 2012; Buapaichit et al., 2013). In general, a single infection of PCV2 rarely induces clinical symptoms in pigs, however the virus is usually act as a primary causative agent of porcine circovirus-associated disease (PCVAD) (Opriessnig and Hulbur, 2012; Meng, 2013). PCVAD is referred as clinical symptoms involving PCV2 infection in pigs including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), enteric diseases, respiratory diseases, and reproductive disorders (Segalés, 2012). The clinical symptoms of PCVAD are varied depending on many factors, e.g., age, breed, immune systems, and concurrent infections

(Opriessnig et al., 2006; Opriessnig and Hulbur et al., 2012; Shen et al., 2012). Madson et al. (2009) found that artificial insemination (AI) with semen spiked with PCV2 in naïve sows causes reproductive failures, i.e., mummified fetuses and stillborn piglets. Interestingly, the virus can also be detected in up to 88% of the live-born piglets in sows experimentally challenged with PCV2 during AI (Madson et al., 2009). In addition, reproductive failure associated with PCV2 can also be manifested as irregular return to estrus, abortion, and low litter size at birth (Madson and Opriessnig, 2011). The PCV2 antigen is commonly detected in the myocardium of the dead fetuses by immunohistochemistry (IHC) (West et al., 1999). In Thailand, a study on PCV2 detection in PWMS pigs indicate that PCV2 DNA could be detected from formalin-fixed paraffin-embedded (FFPE) tissues of the pigs (Kiatipattanasukul-Bunlunara et al., 2002). Tummaruk et al. (2009) found that PCV2 antibody was detected in 10% of the PCV2 non-vaccinated gilts culled due to reproductive disturbance (e.g., anestrus and abnormal vaginal discharge). Furthermore, a co-infection between PCV2 and other viruses (e.g., porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), and Aujezsky's disease virus (ADV)) has also been reported (Kim et al., 2004).

General discussion

The present review provided information concerning with antibody titers against the selected reproductive diseases in the pigs raised in Thailand. It was found that most of the replacement gilts were exposed to PRRSV (84%), PPV (97%) and ADV (4%) before entering the breeding houses. Furthermore, up to 75.5% of the culled gilts were exposed to at least two viruses, and almost 20% of them were exposed to all the three selected viruses. The data in the culled gilts indicated that they had a relatively delayed age at first mating (265.5 days) and low ADG (461.3 gram/day) (Tummaruk and Tantilertcharoen, 2012). The gilts with a poor growth performance, as well as those with a delayed age at first mating might have health problems and/or had exposed to extremely hot and humid climates during their growing periods. Tummaruk et al. (2009b) demonstrated that the replacement gilts reared under tropical climate attained puberty at approximately 200 days of age, which is about 2 weeks later than those in Europe and North America (Karlbom 1982; Patterson et al. 2010). Furthermore, it has been demonstrated that the gilts with a superior ADG attained puberty earlier than those with inferior ADG (Tummaruk et al. 2009b). These data indicated that the health status of the gilts might, partially, influence their reproductive functions and subsequent reproductive performance.

Conclusions

Most of the replacement gilts were exposed to PRRSV (84%), PPV (97%) and ADV (4%) before entering the breeding house. PPV was an enzootic disease in all of the selected herds; the replacement gilts were commonly exposed to PPV rather early in their lives. Replacement gilts were an important source of introducing PRRSV into the breeding herds. The prevalence of ADV was higher in gilts culled due to reproductive disturbance than in healthy gilts. Recent data on PCV2 indicated that the PCV2 potentially caused reproductive failure in swine. Thus additional studies on PCV2 should be addressed. Practically, immunization of replacement gilts against PRRSV, PPV, and maybe also PCV2 along with the elimination of ADV was the important issue which should be addressed in the swine breeding herds in Thailand.

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Review Article

Current Concept in Open- and Closed-cervix Pyometra in Dogs

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Abstract

Pyometra is the most common uterine disease in intact bitches. Types of pyometra are categorized according to patency of the cervix at clinical presentation; open- and closed-cervix pyometra. Bitches affected with open-cervix pyometra usually develop less severe illness than those with open-cervix pyometra, thus, have more opportunity to be recovered and successfully bred after uterine contents are released. Understanding of the mechanism to control cervical patency would be beneficial to develop a medical treatment aiming to release uterine content. Recent findings in the bitches with pyometra demonstrated that leukocyte invasion and prostaglandin E2 play roles to modify extracellular matrix, i.e. collagen and glycoaminoglycans (GAGs) in the cervical tissue, resulting in the relaxation of the cervix. Bitches with open-cervix pyometra had higher number of neutrophil infiltration which led to collagen degradation and cervical relaxation as a consequence. Hyaluronan was found to be the major GAGs in the cervical tissue but differences were not observed between the bitches with open- and closed-cervix pyometra. In contrast, keratan and heparin sulfate, the two types of sulfated GAGs were different between the two types of pyometra. Prostaglandin E2 subtype 4 receptor expressed higher in the cervical tissue of the bitches with open-cervix pyometra than those with closed-cervix pyometra. Although regulation of cervical relaxation has been studied widely in other species, reports in dogs are rare. This review gathers recent findings of cervical function studies in the bitches with pyometra.

Keywords: cervix, collagen, glycosaminoglycans, prostaglandin, pyometra

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Introduction

Cervical function is dynamic in intact bitches both in physiological and pathological conditions. Cervical dynamics during estrous cycle have been observed in young bitches (Silva et al., 1995) and queens (Chatdarong et al., 2002). In normal bitches during the estrous cycle, it is associated with hormonal profile: the opening was observed radiographically in concurrent with the maximal estradiol:progesterone ratio, while the closure commonly occurred prior to the end of estrus with blood progesterone concentration approximately 60 nmol/L (Silva et al., 1995). The time when the cervix is open and closed varies individually. In some cases, cervical closure takes place early, resulting in unsuccessful sperm entry via vaginal insemination (Verstegen et al., 2001).

Pyometra is the most common uterine pathology in bitches in which dynamics of cervical patency are observed but the control mechanism is not clearly understood. Open-cervix pyometra is characterized by the presence of vaginal discharge whereas it is none in closed-cervix pyometra. Pyometra generally affects bitches at old age with a range between 4 months to 16 years (Johnston et al., 2001). Although ovariohysterectomy is the definite treatment, keeping intact uteri for future fertility is the utmost prospect in some valued bitches with young age. Cervical patency is a key factor to select method of treatment. The low dosage of prostaglandin F2 alpha given repeatedly alone or in combination with dopamine agonists, i.e. anti-progestin (aglepristone) has been reported to induce cervical relaxation and allow drainage of uterine content (Verstegen et al., 2008). Within 90 days of treatment with a combination of prostaglandin and aglepistone (anti-progestin effects), 87% successful rate was reported in bitches with open-cervix pyometra but the mechanism was not clearly known (Fieni et al., 2006). In closed-cervix pyometra, the use of prostaglandin F2 alpha is risky as it may induce uterine rupture (Pretzer, 2008). Understanding of the regulation of cervical patency is, therefore, beneficial to facilitate success of medical treatment for canine pyometra.

Relationship of cervical patency and leukocyte infiltration

Changes of cervical structure during pregnancy and parturition have been described as four phases (Word et al., 2007). The initial phase is so-called the softening phase: the cervix begins to be soft in its texture. The second phase is the cervical ripening characterized by a decrease in tissue collagen concentration and an increase in hydrophilic glycoaminoglycans (Leppert, 1995). The third phase is the cervical dilation phase characterized a massive invasion of leukocytes, occurring at laboring. The fourth phase occurs after parturition revealing rapid recovery of cervical structure involving resolution of inflammation, loss of tissue hydration and re-formation of the dense connective tissue and structural integrity of the cervix (Word et al., 2007). A Significant increase of leukocytes in cervical tissues during parturition has been reported in several species (Owiny et al., 1995; Winkler et al., 1999). The previous study in women demonstrated that the degree of cervical dilation appeared to be correlated with the extent of neutrophil infiltration, which produced cllagenolytic enzymes such as metalloproteinases (collagenases). The accumulation of collagenases led to increases of collagen turnover and degradation of newly synthesized collagen, finally, resulted in decrease collagen content in the cervical tissue (Cawston, 1996; Winkler et al., 1999). Increased leukocyte infiltration associated with connective tissue remodeling has also been reported in the cows during estrus (Breeveld-Dwarkasing et al., 2003). In the bitches, no significant differences of leukocytes invasion in the cervical tissues among stages of the estrous cycle (Kunkitti et al., 2011) were found whereas neutrophil infiltration appeared to be involved in cervical dilation in bitches with open-cervix pyometra (Kunkitti et al., 2011; Tamada et al., 2012). Moreover, positive correlation between the number of neutrophils in the cervical stroma and degraded collagenous fiber in the cervix with increase of the cervical patency has been reported (Tamada et al., 2012).

Relationship of cervical patency and sex steroid hormone receptors

Sex steroid hormones have been involved in cervical structure remodeling. In the rats, reduction of estrogen levels led to increases of cervical cell apoptosis (Ramos et al., 2002), and induced eosinophil invasion (Luque et al. 1998). In the bitches, the radiographic study demonstrated that open of the cervix has been observed on 2.6 ± 2.8 days before LH peak, which is concurrent with the day of the maximal value of estrogen: progesterone ratio. Closure of the cervix occurred at 6.7 ± 1.4 days after LH peak (Silva et al., 1995). These data implied that there was a relationship between hormonal profiles and cervical patency. Study of estrogen receptor alpha and progesterone receptor in the bitch cervical tissue was then performed (Kunkitti et al., 2011). During proestrus and estrus, the increase of progesterone receptor immuno- histochemical expression was coincided with cervical dilation, whereas estrogen receptor alpha expression did not seem to relate to cervical opening in the normal bitches (Kunkitti et al., 2011). In bitches with pyometra, estrogen receptor alpha and progesterone receptor was lower than that in normal bitches (Kunkitti et al., 2011). The estrogen receptor alpha in the bitches with open-cervix pyometra was higher during diestrus than that in anestrus, whereas in the bitches with closed-cervix pyometra, it was lower during diestrus compared to anestrus (Kunkitti et al., 2011). Moreover, no correlation between cervical patency and mRNA expression of estrogen receptor alpha and progesterone receptor was found in the bitches with

pyometra (Tamada et al., 2012). Cervical dilation in normal bitches and bitches with pyometra was likely controlled by different mechanisms.

Relationship of cervical patency and extracellular matrix

Cervical tissue is composed of connective tissue and smooth muscle, supported by extracellular matrix (ECM). The ECM is a network composed of collagen bundles glycoproteins, proteo- glycans, glycosaminoglycans (GAGs) and water (Fosang et al., 1990). Among these, collagen is the most abundant protein in the ECM which provides structural support (Kjaer, 2004). In the ewe, the proportion of collagen to smooth muscle is an important determinant of the structural integrity of the cervix that relates closely to cervical function (Kershaw et al., 2007). The polymeric GAGs are made up of repeating disaccharides to form a family of related molecular species. They are classified into two groups: sulfated and non-sulfated. The sulfated GAGs (S-GAGs) are chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and heparin sulfate (HS), and the non-sulfated GAG is hyaluronan (HA) and hyaluronic acid (Hileman et al., 1998). The major GAGs present in the human cervix are CS, DS, HA, although KS and HS are also found to a lesser extent (Osmers et al., 1993). The major component of GAGs in the ovine cervix is HA (Kershaw et al., 2005). It was postulated that cervical dilation was initiated by the hydration of cervical tissues stimulated by the increase of HA (Cabrol et al., 1987). Moreover, cervical relaxation is mediated by the reorganization of collagen bundles and decrease in the concentration of collagen (Feltovich et al., 2005). During estrus, the bovine cervix relaxes, accompanied by the increase of its water content (Tsiligianni et al., 2001). In this respect, the content of HA, a hydrophilic GAG is thought to influence cervical hydration (Takemura et al., 2005). Another important role of HA is inducing of interleukin-1, a strong inducer of polymorphonuclear leukocytes migration (Ito et al., 1987). Leukocyte infiltration I thought to play a fundamental role in cervical relaxation by increasing collagenase enzyme and collagen degradation (Ito et al., 1987).

In addition to HA, sulfate GAGs (CS, DS, HS and KS) also influence the cervical relaxation in many species. For example, a decrease in DS in human (Osmers et al., 1993) implicated in the reorganization of collagen (Uldbjerg et al., 1983) as DS has the high affinity to the collagen (Engvall and Ruoslahti, 1977). Types of GAGs in the cervical tissue vary among species. In the sheep cervix, only DS was found (Kershaw-Yong et al., 2009) whereas in the rat cervix, DS, HS and CS were presented (Cubas et al., 2010; Akgul et al., 2012). The proportion of collagen to smooth muscle was higher in the bitches with open- compared to those with closed-cervix pyometra. Hyaluronan was also the predominant GAG in the canine cervix (Linharattanaruksa et al., 2013c). In the bitches with pyometra, KS and HS were lower in open- compared to closed-cervix pyometra (Linharattanaruksa et al., 2013c). The lower combined amount of KS and HS (Linharattanaruksa et al., 2013c) and increased neutrophil density (Kunkitti et al., 2011) observed in the bitches with open-cervix pyometra may act to collagen rearrangement causing collagen degradation (Linharattanaruksa et al., 2013c) and cervical relaxation as the final outcome.

Relationship of cervical patency and prostaglandin E2

Prostaglandin E2 (PGE2) has been postulated as the central mediator between cervical relaxation and GAGs (Ji et al., 2008). Prostaglandin E2 exerts its role via G-protein-coupled receptors in the cell membrane, so-called prostaglandin E receptor (EP). There are four subtypes of EP which are EP1, EP2, EP3 and EP4, depending on gene encoding (Narumiya et al., 1999). EP1 and EP3 involve contractile activity of muscle whereas EP2 and EP4 are relaxatory (Coleman et al., 1994). Therefore, PGE2 induce contractility or relaxation of the muscle according to which receptor subtypes are expressed. The expression of EP2 and EP4 mRNA in the sheep cervix throughout the estrous cycle suggested that PGE2 bound to these receptors on smooth muscle and fibroblast cells in the cervical tissue in order to stimulate the relaxation of smooth muscle and GAG synthesis, respectively (Kershaw-Yong et al., 2009). In pregnant rats, EP4 mRNA was peak on the day of parturition, suggesting that PGE2 induced cervical relaxation through EP4 receptor (Chien and Macgregor, 2003). In the bitches with pyometra, mRNA expressions of EP2 and EP4 were not different between those with open- and closed-cervix pyometra (Linharattanaruksa et al., 2013b) whereas immunohistochemical expression of EP4 protein was higher in the bitches with open-cervix pyometra than those with closed-cervix pyometra (Linharattanaruksa et al., 2013a).

Conclusions

Regulation of cervical patency of bitches with pyometra is likely different from the normal cyclic bitches. EP4 is suggested to initiate GAG synthesis, particularly KS and HS, accompanied with neutrophil invasion and collagen degradation in the cervical tissue, resulting in the relaxation of the cervix of bitches and clinically observed vaginal discharge, respectively.

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Review Article

Toll-like Receptor 2 and 4 in Healthy and Infected Canine Endometrium: A Clinical Approach

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Abstract

In female reproductive tract, the uterus should be sterile or at least clear of pathogenic bacteria, but it may be contaminated with bacteria during sexual intercourse and around the time of parturition. Moreover, the bacteria in the vagina may be able to spread to the uterus in some period of the estrous cycle, resulting in the development of uterine infection. Pyometra (chronic uterine inflammation with accumulation of pus in the uterus) is the most important pathological condition of the uterus due to bacterial infection in bitches. Uterine pathology due to pyometra can be also responsible for pregnancy loss that has been related to infertility in bitches. To protect against invading pathogens, the canine endometrium has evolved innate immune mechanisms. Toll-like receptors (TLRs) are the cellular components of the afferent arm of the innate immune system. TLR2 and TLR4 are the best understood in terms of responses to Gram-positive bacteria and Gram-negative bacteria respectively. The study of TLR2 and TLR4 in canine uterus is the beginning in the understanding of canine uterine infection. Furthermore, the understanding of the mechanisms and factors that control TLR2 and TLR4 expression in canine uterus may lead to the development of new therapeutic drugs for the clinical application.

Keywords: Toll-like receptor, endometrium, pyometra, bitch

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Introduction

The canine female reproductive tract is open to the outside environment thus it must consist of effective defense mechanisms to prevent the infection. Many cases of inflammatory disease in the reproductive tract of dogs and cats are considered to be caused by infectious agents (Schultheiss et al., 1999). In the dog, pyometra is the most important pathological condition of the uterus as a result of uterine infection (Ishiguro et al., 2007). Furthermore, there are several important functions of female reproductive tract including the receptacle of sperm, the site of embryo implantation and fetal development. To accommodate these functions the female reproductive tract must have both innate and adaptive immune systems. However, the innate immune system is the most universal, the most rapidly acting and important type of immunity (Beutler, 2004). Interestingly, the female reproductive tract is also influenced by the sex hormones. The hormonal levels fluctuate during the estrous cycle which may influence uterine immunity and the susceptibility to the uterine infection. Thus, sex hormones play a very important role in controlling host immune system in the reproductive tract, discriminating this from other mucosal organs (Entrican and Wheelhouse, 2006).

Toll-like receptors in female reproductive tract

In the endometrium, mucosal epithelial cells are the first line of defense against potentially pathogenic microorganisms by the development of the innate immune mechanisms. The innate immune system can immediately inhibit microorganism growth (Fahey et al., 2005). Rapid innate immune defense mechanisms against infection involved the recognition of invading pathogens by pattern recognition receptors (PRRs) attributed to the Toll-like receptors (TLRs). The member of the TLR family recognize distinct pathogen-associated molecular patterns (PAMPs) produced by various bacterial, viral and fungal pathogens. TLRs then play the important role in activating intracellular signaling pathways after PAMP engagement (Werling and Coffey, 2007). TLRs signals initiate acute inflammatory responses. Following results, such as influx of neutrophils and activation of macrophages, lead to the destroying of pathogens directly (Takeda et al., 2003; Pasare and Medzhitiv, 2004). Recognition of pathogens by TLRs also triggers activation of adaptive immune system, making this receptor family an important link between innate and adaptive immune system (Werling and Jungi, 2003; Linde et al., 2007). The signals for stimulation of adaptive immune system are widely supplied by dendritic cells. In the periphery, immature dendritic cells have a high capacity for endocytosis that allows antigen uptake. These cells are stimulated by different pathogen components to undergo maturation and express many of TLRs. Furthermore to regulating the evolvement of adaptive immune system, TLRs activation may be directly related to induction of antimicrobial function. TLRs are likely to activate the secretion of antimicrobial peptides, consequently controlling the direct killing of pathogens at the surface epithelium. Many pathogen components such as lipopolysaccharide or lipoprotein trigger apoptosis of macrophages and endothelial cells. The induction of apoptosis may restrict the expansion of pathogens by localizing cell death at the area of infection (Takeda et al., 2003). In addition, TLRs are complicit in not only immune responses but also more general cellular homeostasis (Takeda and Akira, 2004; Hopkins and Sriskandan, 2005; Linde et al., 2007). Of the 13 TLRs identified, TLR4 and TLR2 are the best understood in terms of responses to Gram-negative bacteria and Gram-positive bacteria respectively (Darville et al. 2003). TLR4 is the first mammalian TLR identified and it is, consequently, the best described of the family (Linde et al., 2007). TLR4 is the signaling receptor for lipopolysaccharide (LPS), the endotoxic component of Gram-negative bacteria. Meanwhile, TLR2 is reported to detect lipoteichoic acid from Gram-positive bacteria (Savva and Roger, 2013). In female reproductive tract, receptors that recognize conserved PAMPs present on microorganisms are expressed in epithelial cells (Lea and Sandra, 2007). The immunohistochemical study of female reproductive tract in human indicated that TLR2 and TLR4 are differentially presented in the epithelium of various parts of the female reproductive tract. For example, TLR2 is expressed dominantly in cervical tissues and fallopian tubes, followed by ectocervix and endometrium. Conversely, TLR4 is expressed dominantly in the upper parts of the female reproductive tract (uterine tube, uterus) and the lack of this receptor is found in ectocervix and vagina. It is probably that the distribution of TLR in the female reproductive tract indicates the immunotolerance to the commensal microorganisms in lower parts of the female reproductive tract and the intolerance to commensal microorganisms in the upper part of the female reproductive tract (Fazeli et al., 2005). Nevertheless, both mRNA and protein for TLR2 and TLR4 have been detected in the epithelium of vagina in another study (Pivarcsi et al., 2005). The differences of the results may be due to the difference of specimen, techniques and physiological status (Yu et al., 2009).

Toll-like receptors and sex hormones regulation

In human, reproductive cycle changes under the control of sex hormones. These changes influence a wide range of genes in the uterus and ovary, that act to prevent these tissues against pathogens invasion whereas concurrently preparing them for ovulation, menstruation and implantation by regulate the anatomical and histological characteristics of the uterus. Sex hormones are also related to the influx and localization of leukocytes in the uterus (Spornitz, 1992; Yeaman et al., 1997; Aflatoonian and Fazeli,

2008). Estradiol has proinflammatory functions in the uterus and has been related to an influx of leukocytes in the mouse at estrus (De and Wood, 1990; Lea and Sandra, 2007). In rodents, estradiol may play a role in the recruitment of leukocytes as more macrophage infiltrates the endometrium when estradiol concentration is higher (Herath et al., 2006b; Khan et al., 2009). In rat uterus, estradiol stimulates epithelial cells to produce factors with broad-spectrum anti-bacterial function (Fahey et al., 2005). Conversely, progesterone has been related to anti-inflammatory function by inhibits the immune response to make the uterus more susceptible to spontaneous infection (Lewis, 2003; Herath et al., 2006a). Elevated blood progesterone concentrations inhibit production of chemokines in uterus for influx of monocytes and neutrophils (Critchley et al., 2001), and inhibit both peripheral blood and uterine neutrophil phagocytic functions (Dhaliwal et al., 2001). Moreover, in human, cattle and rodents, progesterone inhibits uterine immune response by decreasing the proliferative function of lymphocytes. Consequently, the antigen-specific immunity changed by sex hormones appears to play an important role in protecting the uterus from infectious (Sugiura et al., 2004). While, in the period of increased uterine receptivity, epithelial cells exhibit increased expression of TLR and changed production of specific antimicrobial peptides, therefore improving the capability to both recognize and respond to PAMPs on microorganisms. Ovulation, implantation and menstruation are widely characterized by alters in the type and distribution of both immune and non-immune cells in the uterus (Lea and Sandra, 2007). Aflatoonian et al. (2007) found that the lowest amount of TLR2-6, 9 and 10 genes in the endometrial tissue is expressed at proliferative and menstrual phase of the reproductive cycle. Estrogen levels are higher at proliferative phase compared to secretory phase. Meanwhile, the level progesterone is relatively higher at secretory phase compared to proliferative phase. This may indicate the suppressing effect of estrogen and a stimulating effect of progesterone on TLR expression in the endometrium.

In addition, Hirata et al. (2007) found that TLR2-4, 9 in the human endometrium being high in the perimenstrual phase and low in the periovulatory phase. It has been observed that the proliferative phase of the reproductive cycle, particularly day 1 to 7, is the major risk factor for ascending infection by pathogens. In human, menstrual blood and shed endometrium could be a favorable environment for bacterial growth. Hence, increased TLRs expression in menstrual phase might be a possible immune defense mechanism of the uterus. The menstrual phase is a stage when numerous leukocytes are recruited in the endometrium. Leukocytes are the major source of interferon gamma in the endometrium, and interferon gamma up-regulates TLR4 expression in the stromal cells of endometrium. Accordingly, TLRs and leukocytes may coordinately prevent the uterus from pathogen invasion. In contrast, decreased expression of TLRs in periovulatory phase might protect inappropriate inflammatory response of the uterus evoked by pathogens contaminants with upcoming sperm. From the previous studies indicate that sex hormones may control the expression and function of TLRs in the female reproductive tract, and consequently regulate or influence innate and adaptive immune system to prevent against pathogens whereas supplying a suitable environment which supports the fetus (Aflatoonian and Fazeli, 2008).

Toll-like receptor 2 and 4 in canine endometrium

In the last few years, studies on TLRs has been made in identifying TLRs in different domestic animal species which compose of cattle, buffalo, sheep, goat, horse, pig, chicken, cat and also dog (Kannaki et al., 2011). Many cases of inflammatory disease in the reproductive tract of dogs are considered to be caused by infectious agents. In the bitch, the most important pathological condition of the uterus due to uterine infection is pyometra (Schultheiss et al., 1999; Ishiguro et al., 2007). Gene transcriptions of TLR2 and TLR4 by real-time PCR have been studied in endometrium of diestrous dogs and dogs with pyometra. These genes were up-regulated by the action of LPS from *Escherichia coli* in pyometra cases (Silva et al., 2010). Furthermore, the protein expression of TLR4 and TLR2 have been reported in healthy and infected canine endometrium (Chotimanukul and Sirivaidyapong 2011, 2012; Silva et al., 2012). Silva et al. (2012) described the transcription of TLR genes in endometrium of bitches throughout the estrous cycle. This finding indicated that the TLR-mediated immune surveillance is the key component of defense mechanism in canine endometrium. Interestingly, the expression of TLR4 in endometrial surface epithelium was absent in estrus. Low expression of TLR4 might prevent an unfavorable inflammatory response of the endometrium evoked by microbial contaminants with upcoming sperms after mating (Friberg et al., 1987; Svenstrup et al., 2003; Hirata et al., 2007; Chotimanukul and Sirivaidyapong, 2011). Similarly, the decrease transcription and expression of TLR2 and TLR4 were found during early diestrus to promote implantation. On the other hand, the high concentration of progesterone in this period may weaken the defense mechanisms and could be involved with the increased susceptibility to bacterial infection (Tsumagari et al., 2005, Silva et al., 2012). These findings might be related to the occurrence of pyomertra in this stage. The extremely high levels of TLR4 expression were found in pyometra dogs. This indicated the effect of the innate immune defense mechanism against bacterial infection in pyometra dogs (Chotimanukul and Sirivaidyapong, 2011). And, these findings also reflected the up-regulation of TLR2 and TLR4 genes in endometrium, In addition, TLR2 and TLR4 activation also triggers prostaglandin synthesis which further regulates the local

inflammatory response (Silva et al., 2010, 2012). Nevertheless, TLR2 was not highly expressed in the endometrial surface epithelium as was found with TLR4 expression in pyometra dogs (Chotimanukul and Sirivaidyapong 2011, 2012). This result may indicate a protective role for TLR2 in canine uterine infection which can decrease the uterine pathology due to unfavorable inflammation and improved balance between protective and pathological inflammatory responses to maintain the uterine homeostasis (Si-Tahar et al. 2009, Chotimanukul and Sirivaidyapong, 2012).

Clinical application

Antibiotic have been proven to be powerful tool in the control of infectious disease. However, the use of even very powerful antibiotic has been accompanied by the emergence of pathogens with multidrug resistance. Accordingly, the development of non-antibiotic agents may contribute to combat against invading pathogens (Kaisho and Akira, 2002). Knowledge about functions of TLRs and their ligands may lead to the development of new therapeutic approaches to treat a wide spectrum of diseases including infectious diseases (Akira, 2009) by interfering in the TLR activation cascade (Werling and Coffey, 2007). In addition, the TLRs may be involved in the pathogenesis of inflammation via the recognition of host products. So, reagents that inhibit the action of TLRs may work as anti-inflammatory drugs (Kaisho and Akira, 2002). Inhibitors of TLR responses or TLR antagonists may be effective for treatment of endotoxin shock as well as inflammatory diseases. Sepsis is a systemic response to infection caused by LPS from Gram-negative bacteria. More recently, TLR4 antagonists have been developed. These compounds may be promising for the treatment of sepsis (Akira, 2009; Savva and Roger, 2013). These new therapeutic drugs may be adjusted to treat the canine uterine infection in the future.

Conclusions

TLRs are key mediators and the cellular components of the innate immune system. TLR2 and TLR4 are the best characterized of Gram-positive and Gram-negative bacteria respectively. The expression of TLR2 and TLR4 are found in a different endometrial cell types. The different levels of TLR4 expression seems to be related to physiological changes in the distinct microenvironment of endometrium, leukocytes populations and sex hormones. TLRs expression in canine endometrium reflects the effect of defense mechanism against bacterial infection. On the other hand, it is may indicate a protective role in order to maintain the endometrial homeostasis. For the clinical application, the understanding of mechanisms and factors that control TLR2 and TLR4 expression in canine endometrium will aid in the development of the new adjuvants in order to treat and prevent the bacterial infection effectively.

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Review Article

Expression of Cytokines in Porcine Endometrium after Artificial Insemination: A Clinical Implication

Jatesada Jiwakanon

Abstract

In pig, deposition of extender diluted semen into the uterine body during artificial insemination result in immunoactivation characterized by leukocyte infiltration as well as up- and down-regulation of immunoregulatory cytokines in the endometrium. Balance of endometrial cytokine expression, mediated immune reaction against pathogens and meanwhile immune tolerance to the allogenic spermatozoa, was assumed to be the key mechanism of pregnant success after insemination. In this article, uterine reaction to intrauterine stimulation, role of seminal plasma on uterine cytokine expression and seminal plasma used in artificial insemination will be reviewed and discussed.

Keywords: estrogen receptor, progesterone receptor, endometrium, gilt

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Introduction

In pigs, as in other species, deposition of semen into the female reproductive tract induces a series of immunological reactions resemblingly inflammatory reaction with a cascade of cellular and molecular changes. It has been postulated that the semen induced immunological reactions contribute fertilization, embryo development and successful pregnancy by mean of; microorganism elimination, sperm selection, and activation of immune tolerance towards paternal antigen.

Uterine reaction to intrauterine stimulation

At natural mating or insemination, high volume of semen is deposited into the cervix/uterine body. Deposition of semen into the reproductive tract leads to an inflammatory response characterized by influx of polymorphonuclear leucocytes (PMNs), macrophages, dendritic cells and T cells as well as alter local cytokine expression such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), IL-10, transforming growth factor (TGF)-β1 (O'Leary et al., 2004; Rodriguez-Martinez et al., 2010; Jiwakanon et al., 2011; Jalali et al., 2013). It is likely that the inflammatory response to semen contributes to the removal of sperm by the innate immune system and meanwhile prevents acquired immune responses against sperm.

Matthijs et al., (2003) showed that amount of inseminated volume appeared stimulated neutrophil phagocytic activity in the uterine lumen, e.g. volume of 20 ml instead of 80 ml, more non-phagocytosed spermatozoa were found in the uterine backflow. However, for successful of conception, volume of 80-100 ml with at least 0.5 billion spermatozoa are needed for insemination in pig (Mezalira et al., 2005). Therefore, induction of uterine immunological reactions by inseminated volume probably cannot be avoided in the conventional artificial insemination.

Semen is composed of spermatozoa and seminal plasma (SP). Spermatozoa are antigenic in females (Vaiman et al., 1978; Lander et al., 1990; Tripathi et al., 1999). Phagocytosis of boar spermatozoa by neutrophils collected from peripheral blood of sow was demonstrated in vitro (Matthijs et al., 2000). The elimination of spermatozoa started immediately with semen transport. It has been shown that only a small fraction of the inseminated spermatozoa reach the utero-tubal junction and lower part of the oviduct where they remain viable (Mburu et al., 1997) and reach final maturation before fertilization. Approximately 25 % of the inseminated spermatozoa, and up to 70 % of the volume, are expelled retrogradely within a couple of hours (Steverink et al., 1998). Large numbers of spermatozoa are lost by intra-uterine neutrophil phagocytosis (Lovell and Getty, 1968; Matthijs et al., 2003).

Boar SP contains various substances, e.g. oestrogen that affect uterine contraction (Claus, 1990), and proteins that interact with the spermatozoa and the uterine environment (Topfer-Petersen et al., 2008; Rodriguez-Martinez et al., 2010). Data from humans and rodents show that SP can modulate a variety of immunological functions (see review by Thaler, 1989; Kelly and Critchley, 1997; Robertson and Sharkey, 2001). According to in vitro studies, boar SP appears to contain fractions that influence the immune cell response in both suppressive (Stanek et al., 1985; Veselsky et al., 1991; Dostal et al., 1997) and stimulatory (Leshin et al., 1998; Yang et al., 1998) ways. SP proteins have been shown to both stimulate (Rodriguez-Martinez et al., 2005) and suppress (Rozeboom et al., 1999) migration of PMNs into the uterine lumen of sows. Many studies later, therefore, try to recover its role on immunoregulatory process including delicate cytokine response within the pig endometrium, as will be discuss later on in this review.

Porcine uterine cytokines

It is obvious that dual role for immunomodulation, i.e. acceptance of allogeneic tissue and immune protection against pathogens, must be controlled. A major variable influencing the ensuring immune response in a tissue is the expression of cytokines (Constant and Bottomly, 1997; Santana and Rosenstein, 2003; Kaiko et al., 2008). It was showed that various cytokines secrete by cells of the porcine endometrium and changing of its expression can be detected in different stages of oestrous cycle and pregnancy (Chabot et al., 2004). Most important of immune tolerance seems to be a switch from a pro-inflammatory type of reaction within uterine tissues toward and anti-inflammatory state. Cytokines of interest in studies on the consequence of insemination will be summarized.

IL-1, IL-6 and GM-CSF are major pro-inflammatory cytokines which promote inflammation and stimulate an acute phase response. At the cellular level, IL-1 is critical for neutrophil recruitment by eliciting expression of chemokines and adhesion molecules (Brandolini et al., 1996; Fukumura et al., 1996; Jobin and Gauthier, 1997; Parsey et al., 1998). IL-6 contributes to the early inflammatory response but also affects the T-cell response towards a TH type 2, and is, in combination with TGF-β, involved in the development of TH17 cells, as recently reviewed (Dienz and Rincon, 2009). GM-CSF is implicated as local mediator activating and maintaining of macrophages, granulocytes and dendritic cells in the endometrium (Robertson et al., 1996)

IL-10 is considered to be a potent suppressor of the effector functions of macrophages, T cells and NK cells (Moore et al., 2001). It acts to terminate the inflammatory response and limits inflammation-induced tissue changes by de-activating macrophages and inhibiting their synthesis of pro-inflammatory cytokines and chemokines (de Waal Malefyt et al., 1991; Fiorentino et al., 1991). A role of IL-10 during pregnancy was observed, in mice, by Robertson et al. (2006), who found that IL-10 modulates resistance to inflammatory stimuli by down-regulating pro-inflammatory cytokines in the uterus and placenta.

The transforming growth factor β (TGF-β) is a multifunctional cytokine that influences numerous cellular processes. TGF-β is traditionally regarded as an anti-inflammatory agent (Shull et al., 1992; Kulkarni et al., 1993; Gorelik and Flavell, 2000) and was recently also found to be an important regulator of T-cell differentiation (Bommireddy and Doetschman, 2007; Rubtsov and Rudensky, 2007; Sundrud and Rao, 2007). At the site of implantation, TGF-β is believed to play a significant role in establishing a maternal tolerance to the semi-allogenic conceptus (see review Godkin and Dore, 1998).

Role of seminal plasma on the uterine cytokine

It has been shown in gilts that boar SP induced pro-inflammatory cytokines IL-6 and GM-CSF up-regulation in the endometrial tissue at 34 hours after intrauterine infusion accompanied by macrophage and leukocyte activation (O'Leary et al., 2004), which generally regarded as inflammatory responses after insemination. The stimulatory effect of seminal plasma on IL-6 released from the porcine endometrial stromal cells as early as 3 hours after incubation was also showed, in vitro study (Madej et al., 2012). Long-term modulatory effect of SP on uterine cytokine expression was recently studied, Jalali et al. (2013) found that pro-inflammatory cytokine GM-CSF level in the uterine epithelial cells was significantly down-regulated at 6 days after infusion with SP compared with the cycling gilts. Significantly high level of IL-10 mRNA was observed in stromal cells. At 35-40 h after insemination, Jiwakanon et al. (2011) found that insemination with seminal plasma maintained immunoregulatory cytokine TGF-β1 level in the porcine endometrium whereas BTS lower those levels indicated immune stimulation. Taken together, the results indicated that contents in SP are probably key factor modulated appropriated expression of specific cytokines, i.e. immune activated cytokines, i.e. IL-6 and GM-CSF, for dangerous material clearing and at the same time activate immune tolerated cytokines, i.e. IL-10 and TGF-β1, for allogeneic spermatozoa protection and semi-allogeneic conceptus implantation later on. However, controversial result was also found. Taylor et al (2009) found that SP and semen extender (AndrohepTM) both increased mRNA level of cytokine IL-8, TNF-α, IL-10, and TGF-β at 3 hours after intrauterine infusion, whereas spermatozoa added into those fluid depressed those activation effect. This because of windows of time or animal factor different has to be reminded. Boar seminal plasma contains several immunoregulatory cytokines, i.e. IL-6, IL-10, and TGF-β1 (O'Leary et al., 2011; Jiwakanon and Dalin, 2012). In pig, different immunoactivated and immune-suppressive fractions found in the SP, as mentioned above, probably represent immunoregulatory cytokine functions. It has been demonstrated, in mice, that seminal protein consist of TGF-β1 which can stimulated uterine epithelial cell production of GM-CSF in vitro (Tremellen et al., 1998). In pig, no clear evidence showed whether the seminal cytokines mediated uterine cytokine response. However, the appropriate leukocyte activation in the porcine endometrium for success implantation/pregnancy seemed to be mediated by SP cytokine.

Benefic of SP for AI

Artificial insemination (AI) with fresh semen is generally used in the pig industry. Although, farrowing rates and litter size of AI comparable to natural mating have been observed for pigs (Flowers and Alhusen 1992). Increases in farrowing rate and litter size were reported when seminal plasma was administered before AI plus natural mating (Flowers and Esbenshade, 1993). A beneficial effect on conception rate was also observed when mating with an intact boar was supplemented by seminal plasma provided by a vasectomized boar (Mah et al., 1985). Intrauterine infusion with heat-killed semen during the previous oestrus can also increase litter size (Murray and Grifo, 1986). In addition, Rozeboom et al. (2000) showed that fertility of subsequent AI can be impaired when semen is deposited into an inflamed environment created by an earlier AI, and this impairment was offset by inclusion of seminal plasma in the subsequent insemination. Moreover, using 4-6 pre-pubertal gilts, O'Leary et al. (2004) found that intrauterine infusion of seminal plasma 1-2 h before AI increased the number of viable embryos at days 5 and 9. These positive effects could be explained either by exert physiological effect of spermatozoa as seen for frozen-thawed semen (Kaeoket et al., 2011; Gomez-Fernandez et al., 2012) or deviated/prevent immune reaction on spermatozoa directly (Alghamdi et al., 2004; Harris et al., 2006) or in the female environment as already mentioned above. Commercially used of synthetic seminal plasma to improve reproductive efficiency in sows and gilts have been documented (Garcia Ruvalcaba et al., 2009).

However, the results of SP supplement on reproductive performance still inconsistency. Lessard et al. (2003) could not detected differences on number of live embryos when whole dead semen was infused after the previous conventional insemination compared with by following BTS alone. Farrowing rate was not improved when frozen-thawed semen doses were supplemented with 10% seminal plasma. Comparatively, there was no significant effect of addition of TGF-β, the major cytokine found in SP, to semen extender on total or live foetuses per litter, implantation rate or foetal survival in gilts (Rhodes et al., 2006). Therefore, immunomodulatory effects of seminal content probably improve reproductive performance when inflammation makes fertilization, implantation or even pregnancy establishment problematic.

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Review Article

Defence Mechanism in Bovine Endometrium: Current Concepts

Theerawat Swangchan-Uthai

Abstract

After parturition, the endometrium undergoes extensive changes as a part of involution in order to prepare the uterus for the next pregnancy. Bovine postpartum uteri are susceptible to bacterial contamination that leads to endometritis and poor fertility. Significant bacteria responsible for endometritis are *Escherichia coli* and their endotoxin lipopolysaccharide (LPS). Understanding the molecular mechanisms of detecting and responding to the microbes which influence local uterine function is needed to improve the strategies of reproductive management and develop new diagnostic tools and treatments in bovine herd health. Recently, the development of techniques such as Microarrays and Next generation sequencing for exploring global genes associated with the endometrium response to infection under the developed *in vivo, in vitro* and *ex vivo* systems enable us to analysis the whole transcripts within cells or tissue simultaneously. Followed by carefully validation of the selected genes using real-time quantitative PCR and protein analysis, this approach provided us useful information to understand the complex interactions that underlie both healthy and disease conditions of the postpartum bovine endometrium.

Keywords: endometrium, endometritis, defence mechanism, postpartum, cow

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Introduction

In recent years, a decline in bovine fertility has created enormous problems especially in the dairy industry worldwide. Many groups of researchers have attempted to understand the underlining causes of this phenomenon. To date, the exact answer remains unclear but it must have a multifactorial basis including both genetic and environmental components. One of the main factors for the decreased fertility during the past few decades is thought to be the extreme genetic selection for milk production in dairy cattle, resulting in the era of high yielding dairy cows which also experience reproductive problems. A strong relationship between milk yield and fertility of high genetic merit multiparous Holstein/Friesian cows has been observed. As yield increased, the interval to first service, the proportion of cows with abnormal P_4 profiles and the interval to conception all increased while first-service and second-service conception rates, and overall conception rates decreased significantly (Nebel and McGilliard, 1993; Roche et al., 2000; Lucy and Crooker, 2001; Snijders et al., 2001; Wathes et al., 2001).

A growing body of evidence has indicated that the postpartum period in dairy cattle, especially the period of uterine involution, is a critical time in which the cow's reproductive tract can easily be affected by local and metabolic factors, resulting in apparent delay in the uterine repair process and subsequently affecting fertility (Swangchan-Uthai et al., 2013). This is believed to occur as the consequence of unresolved inflammation during involution (Chapwanya et al., 2009). Both clinical and sub-clinical endometritis are associated with subfertility characterised by poor herd reproductive performance. This can be reflected in a prolonged interval from calving to conception, an increased number of services per conception, an increased proportion of animals which fail to conceive at all and an increased culling rate unless these conditions are effectively managed (Fourichon et al., 2000; Gilbert et al., 2005). Chronic endometrial inflammation has not only an effect on ovarian function (Sheldon, 2004a), but also produces an endometrial environment which is not suitable for embryo survival (Hill and Gilbert, 2008). However, the precise effects of these defence mechanisms toward the multifunctional properties of the endometrial cells remain unclear. Therefore, a greater understanding is needed of the mechanisms used by the postpartum endometrium in order to detect and respond to the microorganisms or their toxins.

Various genomic approaches have been introducing to study biology of cell and tissue in several systems including endometrial biology (Sherwin et al., 2006). Considering with the reproducibility, cost and ease of use, the most currently used of genomic techniques to identify differential genes and their expression levels between RNA samples in cows are Microarrays and Next generation sequencing. Recently, the large scale gene expression studies of bovine uterus (Wathes et al., 2009; Foley et al., 2012; Valour et al., 2013) and validated studies of the candidate genes using real-time quantitative PCR and protein analysis enable us understand the molecular defence mechanism associated with bacterial clearance, endometrial repair and resolution of inflammation in the postpartum cows.

Culture of endometrial cells has been previously reported in several species, for example, mice (Lavranos and Seamark, 1989), pigs (Allen and Wright, 1984), humans (Ishihara et al., 1988) and cows (Fortier et al., 1988). This approach is complementary to the use of *in vivo* studies which have drawbacks, mainly complexity of the systems, poor reproducibility, difficulty of intervention, and ethical issues. The endometrial cells grown in these culture systems generally exhibit a monolayer layer appearance and lack of cell polarity and the ability to stabilise their *in vivo* morphology (Byers et al., 1986; Findlay et al., 1990). In recent years, increasing attention has been directed to the development of endometrial culture systems using advanced technologies, for example, a three-dimensional culture system using extracellular matrix gel (Matrigel) (Cherny and Findlay, 1990; Classen-Linke et al., 1998) or fibrin-agarose gel (Wang et al., 2011), co-culture systems containing endometrial cells with leukocytes (Ho et al., 2006) and *Ex vivo* culture of intact endometrium (Borges et al., 2012). Thus, this article aims to review the defence mechanism of bovine endometrium in the recent *In vivo*, *Ex viv*o and *In vitro* studies and its impact to fertility in postpartum cows. In addition, clinical aspects of postpartum uterine disease and future considerations were also discussed.

Economic impact of uterine infection in the dairy industry

The financial impact of uterine disease on dairy farms is a major issue. The economic losses from uterine infection are derived from subfertility and infertility, prolonged days open and Calving Interval, increased culling for failure to conceive, milk withdrawal and the cost of treatment (Bartlett et al., 1986; Dobson and Smith, 1998; LeBlanc et al., 2002b). Sheldon et al. (2009a) estimated the annual cost of uterine disease in the European Union at €1.4 billion and in the United States at $650 million. The cost of a single case of metritis is approximately €292 in Germany (Drillich et al., 2001). Moreover, Whitaker *et al* (2000) reported the average incidence of treatments relating to fertility in one year at 24% of cows in southern England. In Greece, extended days open can cost on average €5 per day (Valergakis et al., 2007), while previous studies in Canada and the United States estimated the cost at $4.7 per day (Plaizier et al., 1997) and $4.9 per day (Gonzalez-Recio et al., 2004), respectively. Thus, it is useful to know the levels of reproductive disease on each farm. Prompt diagnosis of uterine disease is essential to identify cows at risk

for metritis and endometritis to provide sufficient time for treatment and improve the strategies of reproductive management.

Postpartum uterine conditions

During the calving process, the surface epithelium of both the caruncular and intercaruncular regions undergoes extensive sloughing in association with infiltration of leukocytes, necrosis of the caruncular stalk and placental separation (Gier and Marion, 1968; Leslie, 1983). Endometrial repair must therefore follow parturition to allow the endometrium to return to normal and prepare to conceive again. The process of endometrial regeneration as investigated by histological changes is superficially complete by 25 days postpartum, but the deeper endometrial layers are not fully restored until 6 to 8 weeks (Marion and Gier, 1959).

After calving, four concomitant events are needed to be completed: uterine involution, regeneration of the endometrium, resumption of ovarian function, and elimination of bacterial contamination for preparing the uterus to support a new conceptus (Sheldon, 2004b). Uterine involution is also associated with a drastic reduction in uterine size and reorganization of the endometrium with a concomitant lochial discharge over the first 14 days postpartum (Dobson and Kamonpatana, 1986). Uterine bacterial contamination is a normal sequel to calving and is almost universal in dairy cows at the rate of 80-90% (Foldi et al., 2006). Healthy cows are normally able to clear microbes from the uterus within the first 2 to 3 weeks postpartum, starting at 48 h when leukocytes accumulate in the uterine lumen (Bondurant, 1999). However in 10-20% of contaminated cows will fail to eliminate the microbes so will suffer from infection and chronic uterine inflammation (Borsberry and Dobson, 1989; LeBlanc et al., 2002b). Therefore, histological findings of the bovine postpartum endometrium included the erosion of the epithelial lining, haemorrhage and inflammation in the stromal area with increased immune cells, i.e. polymorphonuclear neutrophils (PMNs), lymphocytes and plasma cells or the quiescent uterus with no inflammation (Chapwanya et al., 2009; Sheldon, 2011).

Postpartum uterine disease in dairy cows can comprise retained fetal membranes, puerperal metritis, pyometra, clinical endometritis and subclinical endometritis. The definitions were reviewed by Sheldon et al. (2006). **"Puerperal metritis"** is defined as an animal with an abnormally enlarged uterus and a foetid watery red-brown uterine discharge associated with fever and other signs of illness within 21 day after calving. The cows that do not show systemic illness but have uterine enlargement and purulent uterine discharge within 21 days after calving are classified as having **"clinical metritis"**. The term **"clinical endo- metritis"** is stated as having purulent uterine discharge (>50% pus) in the vagina at 21 day or more after parturition or having mucopurulent vaginal discharge after 26 days postpartum. The presence of more than 18% PMNs in uterine cytology at day 21 to 33 or more than 10% PMNs at day 34 to 47 after parturition is classified as **"subclinical endometritis"**. Lastly, **"pyometra"** is defined as the accumulation of pus within the uterine lumen together with a persistent CL and a closed cervix. The incidence of uterine infection in postpartum cows has been reviewed by Sheldon et al. (2009a). Approximately 40% of postpartum cows develop puerperal metritis, 20% show clinical metritis signs at 3-week after calving, and ~30% experience chronic inflammation of the uterus (subclinical endometritis). In field practice, the precise diagnosis of these uterine diseases must be performed by a combination of an observation of uterine discharge, vaginoscopy, rectal palpation and endometrial cytology should be considered (LeBlanc et al., 2002a; Gilbert et al., 2005).

Uterine defence mechanisms

The endometrium needs a special mucosal immunity which is continuously dealing with several antigens, i.e. sperm, conceptus and microorganisms following both insemination and parturition. It must selectively provide a differentially appropriate response of either acceptance or defence. Post-breeding inflammation is not common in cows. Previous studies demonstrated that mating either fresh semen or frozen semen in cattle had little effect of inflammatory stimulation within endometrium as bull semen contain high concentration of spermatozoa with less seminal plasma or diluent compared to horses and pigs. In the cow pregnant uterus, a fetus grows in a sterile environment with non-invasive epitheliochorial placenta in both caruncular and intercaruncular area of the endometrium (Wathes and Wooding, 1980). The regulation of embryo-maternal immune response was described elsewhere (Mitko et al., 2008; Bauersachs and Wolf, 2013; Spencer et al., 2013; Valour et al., 2014). As soon as parturition taking place, microbes enter the uterus. The endometrium is defended against the microorganisms and their toxins not only by physical barriers but also by production of several effectors such as inflammatory mediators, acute phase proteins (APPs), antimicrobial peptides (AMPs) and complement components (see Fig. 1).

Female reproductive tract epithelium normally provides physical and chemical barrier including the release of antimicrobial substances such as beta-defensins, Mucin-1 (MUC1) and S100A8/ S100A9 that prevent adhesion and colonization of bacteria (Kunimi et al., 2006; Zasloff, 2007). Apart of those secretions and other elements, endometrium provides professional immune cells (e.g. neutrophils, natural killer (NK) cells, macrophages and lymphocytes) which contribute to protection against infectious agents (Quinn et al., 2011).

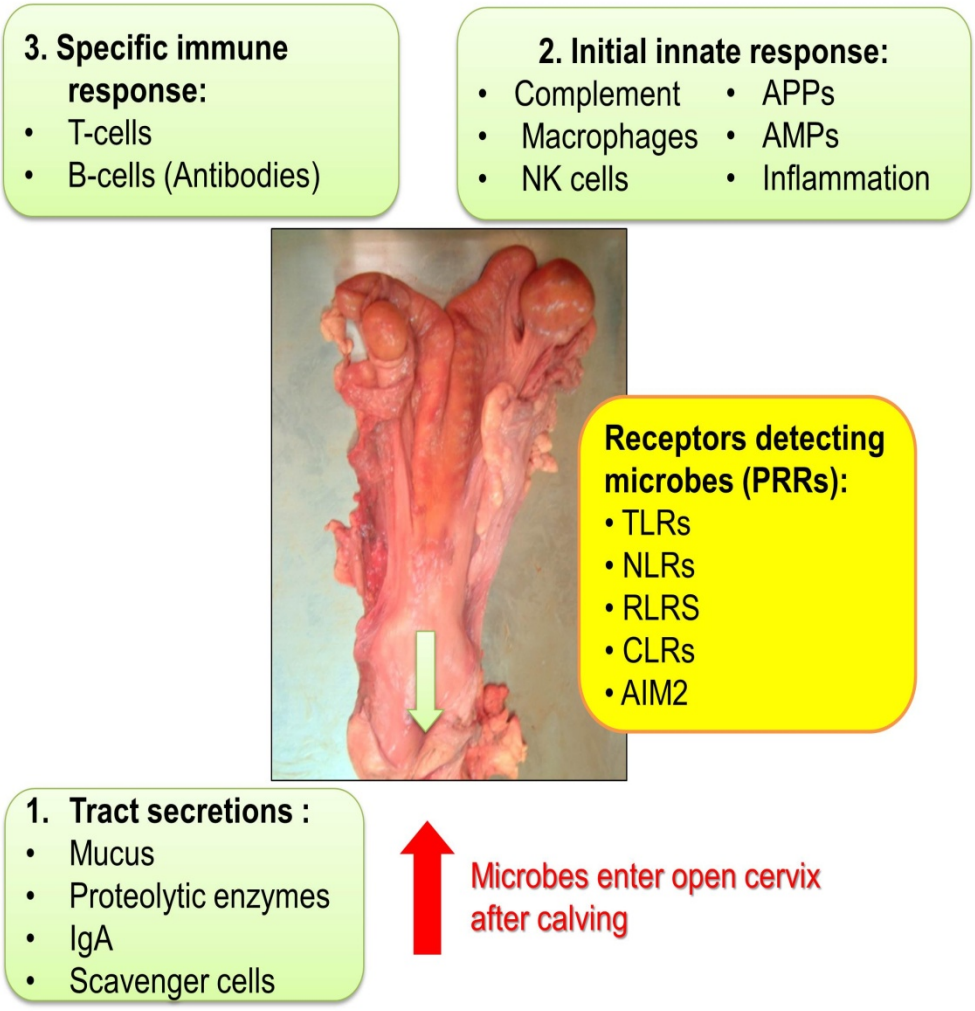

Figure 1 Summary of immune responses generated following contamination after calving. Adapted from Wathes (2010). PRRs: pattern recognition receptors, TLRs: Toll-like Receptors, NLRs: Nucleotide oligomerisation domain-like receptors, RLRs: Retinoic acid-inducible gene-1-like receptors, CLRs: C-type lectin receptors, AIM2: Absent in melanoma 2, APPs: Acute Phase Proteins, AMPs: Antimicrobial peptides, NK; Natural Killer cells.

One important difference between innate and adaptive immunity is the time factor: innate immunity acts rapidly (within minutes for the activation of complement and macrophage phagocytosis), while adaptive immunity requires more time (a week or more for activation of T- and B-lymphocytes and cell proliferation) (Playfair and Bancroft, 2004). Therefore, within the uterine endometrium, the innate immunity and mucosal defence systems are more desirable than acquired immunity (King et al., 2003; Gilbert et al., 2005).

Economically important uterine disease in cattle is commonly associated with *Escherichia coli (E. coli), Trueperella (Arcanobacterium) pyogenes (T. pyogenes), Fusobacterium necrophorum* and *Prevotella* species (Olson et al., 1984; Cullor, 1992). Previous studies showed that *E. coli* and *T. pyogenes* were predominant pathogens previously isolated from clinical cases of uterine disease (Dohmen et al., 2000; Williams et al., 2005a), and are thought to be the primary causes of infertility (Foldi et al., 2006). *E. coli* was identified as the dominant isolated bacteria from 2 weeks postpartum cows with confirmed uterine pathology. This suggested that cows with ongoing *E. coli* infection 10-21 d postpartum are at risk of developing uterine disease (Williams et al., 2005b). In the lochia of dystocia and retained placenta cows, there are high numbers of *E. coli* and high concentrations of their pathogenic ligand, lipopoly-saccharide (LPS) (Dohmen et al., 2000). Recently, a specific strain of *E. coli*, termed as endometrial pathogenic *E. coli* (EnPEC) has been discovered. They were more adherent and invasive for endometrium. The released LPS can initiate more Prostaglandin PGE_2 and Interleukin-8 (IL8) in endometrial treated cells compared to LPS from non-pathogenic *E. coli* (Sheldon *et al.* 2010). Moreover, *E. coli* appears to favour the development of uterine infections by *T. Pyogenes* and virus such as Bovine herpesvirus 4 (Dohmen et al., 2000; Williams et al., 2007; Donofrio et al., 2008).

Previous studies indicated the negative effects of both intrauterine and circulating LPS on bovine reproduction. Local LPS in the uterine lumen stimulated inflammation and caused cell damage, leading to infertility (Sheldon et al., 2009b) . Intrauterine LPS can enter the blood circulation during calving or severe endometritis. This can result in fever, suppression of GnRH and LH secretion from the pituitary gland, and interference with the oestrous cycle (Williams et al., 2008a). Moreover, the LPS also has direct impact on the ovarian health by reducing granulosa cell oestradiol secretion, resulting in non-ovulation of the dominant follicle in some cows treated with LPS (Williams et al., 2008b).

An influx of PMNs from peripheral blood into the endometrium and uterine lumen is an immediate response to the presence of microorganisms and their toxins as the result of chemokines produced by endometrial cells (Zerbe et al., 2003; Silva et al., 2008; Sheldon et al., 2009a). PMNs and macrophages were found in the endometrium of animals which had been infused with LPS or live *E. coli* (Sheldon et al., 2010). Santos *et al.* (2009) and Swangchan-Uthai et al. (2013) also showed a high correlation between PMN counts and inflammatory scores in uterine lavage and endometrial histology. However, the uterine immune response is generated not only by immune cells but also by the endometrial epithelial and stromal cells. Indeed, the epithelial cells are the first line of defence against microbes in the uterine lumen as they provide key receptors to detect several antigens. Thus, primary cell cultures of bovine endometrium and endometrial explant cultures have been extensively developed to study the roles of these cells in defence mechanisms (Leung and Wathes, 1999; Herath et al., 2006; Davies et al., 2008; Donofrio et al., 2008).

Recognition of microorganisms

Mammalian innate immunity especially in the uterus is highly dependent on the expression of pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) of microorganisms including Toll-like Receptors (TLRs, transmembrane receptor) and NOD (nucleotide oligomerisation domain)-like receptors, Retinoic acid-inducible gene-1-like receptors, the C-type lectins and absent in melanoma 2 (AIM2). Initial recognition is mainly achieved by the family of TLRs (Werling and Jungi, 2003) which function as detectors of fungi, viruses and bacteria (Akira and Takeda, 2004; Beutler, 2004). TLR expression in mammals has been reported in whole endometrium, endometrial epithelial cells and other epithelial cells (Strober, 2004; Young et al., 2004). The bovine endometrium expressed TLR 1 to 10 with differential expression in either stromal (TLRs 1 to 7 and 9) or epithelial cells (TLRs 1 to 4, 6, 7, 9, and 10) (Davies et al., 2008). Various studies (Herath et al., 2006; Davies et al., 2008; Herath et al., 2009b; Swangchan-Uthai et al., 2012; Chapwanya et al., 2013) indicated that bovine endometrial cells expressed the TLR4/CD14/MD2 receptor complex which is necessary to detect *E.coli* and their LPS in the uterine lumen of postpartum cows with bacterial infection, while a recent study showed an activation of both TLR4 and TLR2 by *E.coli* or LPS in endometrial epithelial cells (Fu et al., 2013).

Activated TLRs subsequently stimulate the production of proinflam- matory cytokines, chemokines, ROS, NO and PGs via signal transduction pathways or inflammatory activations involving Myeloid differentiation primary response gene (MyD88), CD14, Interleukin-1 receptor-associated kinases IRAK-1, IRAK-4, IRAK-M and TNF receptor associated factor 6 (TRAF-6), activating mitogen-activated protein kinase (MAPK) and the transcription Nuclear factors NFκB (Ghosh et al., 1998; Li and Verma, 2002; Takeda and Akira, 2005; Froy et al., 2007; Monie et al., 2009). However, the pathways activated after TLR stimulation can be organ and species specific.

Table 1 Summary of elements related to the defence of the endometrium against pathogenic bacteria or their endotoxin LPS.

Functions	Genes#	Protein	References
Sensors for LPS	TLR4	TLR4	(Herath et al. 2006, Davies et al. 2008, Herath et al. 2009b, Swangchan-Uthai et al. 2012, Chapwanya et al. 2013, Fu et al. 2013)
	CD14		(Herath et al. 2006, Swangchan-Uthai et al. 2012)
	MD2		(Herath et al. 2006)
	TLR2		(Fu et al. 2013)
Transcription factor	MyD88		(Fu et al. 2013)
	TICAM2		(Fu et al. 2013)
	IRF3		(Fu et al. 2013)
	NFKB1		(Chapwanya et al. 2009, Fu et al. 2013)
Mediators (Cytokines and Chemokines)	IL1A		(Chapwanya et al. 2009, Gabler et al. 2009, Herath et al. 2009b)
	IL1B	IL1B	2012, Swangchan-Uthai et al. 2012, Chapwanya et al. 2013, Fu et al. 2013)
	IL6	IL6	al. 2011, Borges et al. 2012, Fu et al. 2013, Turner et al. 2014)
	IL12A		(Chapwanya et al. 2009)
	IL1R2		(Herath et al. 2009b)
	IL1RN		(Gabler et al. 2009)
	TNF		Chapwanya et al. 2013, Fu et al. 2013)
	IFNB		(Fu et al. 2013)
	IFNG		(Chapwanya et al. 2009)
	IL8	IL8	Uthai et al. 2012, Chapwanya et al. 2013, Fu et al. 2013, Turner et al. 2014)
	CXCL5		(Fischer et al. 2010, Swangchan-Uthai et al. 2012)
	NOS2		(Herath et al. 2009b)
AMPs and APPs	LAP		(Davies et al. 2008, Swangchan-Uthai et al. 2012, Chapwanya et al. 2013)
	TAP		(Davies et al. 2008, Chapwanya et al. 2013)
	BNDB4		(Davies et al. 2008)
	S100A8	S100A8	(Swangchan-Uthai et al. 2012)
	S100A9	S100A9	(Swangchan-Uthai et al. 2012)
	S100A12		(Swangchan-Uthai et al. 2012)
	DEFB5		(Davies et al. 2008, Chapwanya et al. 2009)
	HP		(Chapwanya et al. 2009)
	SAA3		(Chapwanya et al. 2009, Chapwanya et al. 2013)
	MUC-1		(Davies et al. 2008)
Endocrine		PGE_2	(Herath et al. 2006, Herath et al. 2009a, Swangchan-Uthai et al. 2012)
		PGF_{2a}	(Herath et al. 2006, Herath et al. 2009a, Swangchan-Uthai et al. 2012)
	PLA_2G6		(Herath et al. 2009a)
	PTGS2		(Herath et al. 2006)
	PTGES1		(Swangchan-Uthai et al. 2012)
	PTGES2		(Swangchan-Uthai et al. 2012)
	PTGES3		(Gabler et al. 2009)
	L-PGDS		(Gabler et al. 2009)
	AKR1B5		(Swangchan-Uthai et al. 2012)
Tissue reconstruction, inflammatory resolution and restoration of uterine homeostasis	MMP1		(Swangchan-Uthai et al. 2012)
	MMP13		(Swangchan-Uthai et al. 2012)
	SAA1/2		(Foley et al. 2012)
	GATA2		(Foley et al. 2012)
	IGF1		(Foley et al. 2012)
	SHC2		(Foley et al. 2012)
	SERPINA14		(Foley et al. 2012)
	TOLLIP		(Fu et al. 2013)

#Genes in boldface decreased in infected animal or LPS challenged-endometrium; remainder increased.

Following activation with LPS, the bovine endometrial epithelial cells expressed downstream TLR4 signalling mRNAs via MyD88 dependent and independent pathways with signal transduction intermediator TICAM1, subsequently leads to the activation of NF-κB as well as an activation of IRF3 and induces the expression of Interferon IFN-β (Fu et al., 2013). While TLR4 recognises LPS from Gram-negative bacteria, TLR2/TLR1 and TLR2/TLR6 were shown to detect lipopeptides from Gram-positive bacteria in bovine endometrial cells, resulted in an activation of MAPK (ERK1/2 or p38) and NF-κB followed by the accumulation of pro-inflammatory cytokine IL6 and the chemokine IL8 (Turner et al., 2014).

The bovine endometrium contribute to the defence mechanisms of the uterus

The previous studies used global gene analysis and real-time qPCR techniques to show a major induction of mRNA expression of inflammatory mediators and pro-resolution components in postpartum bovine endometrium. Together with previous evidence, the key elements that regulate the defence mechanisms of endometrium against pathogenic bacteria or their endotoxin LPS in postpartum dairy cows are summarised in Table 1.

The endometrial cells respond to LPS by initiating a consequent expression of specific target genes (Swangchan-Uthai et al., 2012; Chapwanya et al., 2013; Fu et al., 2013) leading to subsequent stimulation of the production of immune mediators for clearing the infection. To date, several mediators or mRNA expression of bovine endometrial cells in the presence of bacterial LPS have been discovered, including Tumour necrotic factor α (*TNF)*, Interleukins IL-*1A, -1B,-6,-8, -12A, -1R2, -1RN* and nitric oxide synthase *(NOS2)*, *IFNG, CXCL5* and several AMPs such as *DEFB5* (Defensin, beta 5), *BNBD4 (*Defensin, beta 4A), *TAP* and *LAP* (Tracheal and Lingual Antimicrobial Peptides), S100 family (*S100A8, -A9, -A12*), *HP* (Haptoglobin), *SAA3*(Serum Amyloid A-3) and *Mucin-1* (Beutler et al., 2003; Takeda and Akira, 2004; Herath et al., 2006; Davies et al., 2008; Chapwanya et al., 2009; Herath et al., 2009a; Bryant et al., 2010; Fischer et al., 2010; Galvao et al., 2011; Chapwanya et al., 2012; Swangchan-Uthai et al., 2012; Chapwanya et al., 2013; Fu et al., 2013).

The function of both TNFα and IL1B is to mediate inflammatory responses, induction of other pro-inflammatory cytokines and chemokines such as IL1 and CXCL5, respectively as well as stimulate the production of AMPs, APPs and recruit neutrophils, macrophages and T-lymphocytes into an inflammatory site (Harada et al., 1994; Baggiolini, 2001; Hehlgans and Pfeffer, 2005). IL1B was identified in both glandular and luminal endometrial epithelium, endometrial stroma and endothelial cells in the bovine reproductive tract (Paula-Lopes et al., 1999). It stimulates the action of vascular endothelial cells and promotes influx of inflammatory cells into the extravascular space (Dinarello, 2002). In addition, Herath *et al.* (2009b) and Gabler *et al.* (2009) showed that infertile cows with subclinical or clinical endometritis had higher transcripts of *IL1A*, *IL1B*, *IL1RN* and their receptor *IL1R2*, compared with fertile animals during the early postpartum period. Recently, a study using the *Ex vivo* model of explants of bovine endometrium showed an accumulation of IL1B, IL6 and chemokine IL8 in response to *E.coli* or LPS which was dependent on the stage of oestrus, i.e. the highest release of those chemicals was found in the endometrial explants collected from pre-ovulation stage cows with high oestradiol and low progesterone concentrations (Borges et al., 2012).

Bovine APPs are divided into two classes: type 1 APPs (e.g. SAA) are stimulated by IL6, while type 2 APPs (e.g. HP) are stimulated by IL1 (Baumann and Gauldie, 1994; Streetz et al., 2001). Recently, studies provided evidence of *HP* and *SAA* mRNA expression also in bovine endometrium (Chapwanya et al., 2009; Wathes et al., 2009; Fischer et al., 2010). Bovine endometrial epithelial cells expressed both SAA and HP in response to *E. coli (Chapwanya et al., 2013)*. Chapwanya *et al.* 2012 also measured the levels of HP and SAA in serum and found that circulating levels of SAA increased over the three postpartum time points (15, 30, 60 d).

Regarding the production of AMPs of endometrium, the expression of LAP, TAP, DEFB5 and other bovine β-defensins as well as S100 family (such as S100A8 and S100A9) were indicated in bovine endometrium as an important part of mucosal immunity (Cormican et al., 2008; Swangchan-Uthai et al., 2013). In agreement with previous studies in other types of tissue, the production of these proteins can be stimulated by bacterial LPS and cause prominent immune responses (Yang et al., 2001; Vogl et al., 2007; Davies et al., 2008; Leclerc et al., 2008; Swangchan-Uthai et al., 2012; Chapwanya et al., 2013). Many lines of evidence showed that the S100 proteins take part in various processes of the innate immune response by protecting cells against invasive microorganisms, regulating adhesion of leukocytes to the endothelium and extracellular matrix during the inflammation, and deactivating microorganisms with a zinc-chelating mechanism (Striz and Trebichavsky, 2004). These proteins are released by immune and epithelial cells immediately after host pathogen interaction (Striz and Trebichavsky, 2004), anti-inflammation (Ikemoto et al., 2007; Lim et al., 2008), wound healing (Thorey et al., 2001) and involving in the activation of cytokines, chemokines and inflammatory mediators through LPS-binding TLR4/MD2 complexes. Chapwanya et al. (2012) suggested that the uncontrollable inflammation of the reproductive tract after calving is one of the main issues in initiating puerperal metritis and endometritis. Recent studies that investigated the mechanisms which are capable of controlling these potent inflammatory mediators were performed. Fu et al. (2013) showed an increased expression of *TOLLIP*, the molecule that blocks MyD88 dependent signalling (Zhang and

Ghosh, 2002) in *E.coli* or LPS treated bovine endometrial epithelial cells. While the study in human endometrium (Macdonald et al., 2011) showed that lipoxin A4, a mediator that can elicit anti-inflammatory effects and pro-resolution by induction of the clearance of neutrophils and monocytes from inflamed tissue, was up-regulated after treatment with the inflammatory stimulus phorbol myristate acetate. Lastly, the results from analysis of the entire transcriptome of bovine endometrium collected from day-15 and day-30 postpartum identified five candidate genes as follows: Serum Amyloid A-2 (*SAA1/2)*, Insulin-like growth factor 1 (*IGF1)*, GATA binding protein 2 (*GATA2)*, Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin) member 14 (*SERPINA14*) and Src homology 2 domain containing transforming protein C2 (*SHC2*). These genes functionally associated with endometrium inflammatory resolution, tissue repair and restoration of uterine homeostasis. In addition, our recent findings indicate that LPS may influence uterine tissue repair mechanisms directly through up-regulation of matrix metalloproteinases *MMP1* and *MMP13* genes (Swangchan-Uthai et al., 2012). These proteolytic enzymes encode fibrillar collagenases, enzymes that play the main role in the separation of bovine placenta (Fecteau et al., 1998). The MMPs are essential participants in the extracellular matrix disarrangement which is a normal event that is important tissue remodelling (Hirata et al., 2003). Recently, the study of bovine gene expression profiles during gestation demonstrated that *MMP1* and *MMP13* gene expression was likely to be down-regulated in postpartum endometrium which has returned to normal and is then able to support another pregnancy (Kizaki et al., 2008). The expression of *MMP1, 3*, *9*, and *13* using microarray in the endometrium also showed up-regulation in SNEB (Severe negative energy balance) cows relative to control cows (Wathes et al., 2009). Moreover, SNEB cows produce low levels of plasma IGF-I as a result of liver damage (Wathes et al., 2011). These may suggest that uterine infection in concert with NEB status may delay the mechanism of uterine repair by alteration of IGF bioavailability and MMP levels in the endometrial environment which influenced tissue remodelling by causing cells to exit the cell cycle, inducing apoptosis, and glandular involution (Alexander et al., 1996; Boudreau et al., 1996; Wathes et al., 2011). Postpartum uterine infections including subclinical or clinical endometritis increased prostaglandin (PG) secretion, delayed resumption of oestrous cyclicity and reduced postpartum fertility (Del Vecchio et al., 1992; Bekana et al., 1996; Grigsby et al., 2003; Mateus et al., 2003). From previous findings, LPS affected arachidonic acid metabolism and biosynthesis of PGs by increasing the activity of *PLA2G6*, *PTGS2*, *PTGES* but reducing the activity of *AKR1B1*, a potent PGF synthases (Herath et al., 2006; Herath et al., 2009a; Swangchan-Uthai et al., 2012). In the normal luteolytic mechanism, a pulsatile mode of ovarian oxytocin release is essential to drive the pulsatile secretion of $PGF_{2\alpha}$ via OTRs in the late luteal phase (reviews by Wathes and Lamming (1995)). Several pulses of $PGF_{2\alpha}$ on about day 16-17 of the oestrous cycle are needed for luteolysis, resulting in the resumption of oestrous cycle (Kindahl et al., 1976). In contrast, the uterine infection raised the baseline of $PGF_{2\alpha}$ metabolite but with no pulsatile pattern (Bekana et al., 1996; Mateus et al., 2003). Swangchan-Uthai et al. (2012) showed that LPS up-regulated the secretion of $PGF_{2\alpha}$ in mixed endometrial cell culture. It also stimulated $PGF_{2\alpha}$ in bovine endometrial explant-culture without altering OTR mRNA expression (Leung et al., 2001). These different phenomena between cyclical regression of the CL and the infection may explain why the increase in $PGF_{2\alpha}$ in the absence of OTR would not initiate luteolysis, since $PGF_{2\alpha}$ pulses are required (McCracken et al., 1999).

Clinical management of postpartum uterine disease

Originally faecal contamination was thought to be the primary cause of endometritis. However, studies now indicate that clinical endometritis is associated with several risk factors, including trauma of the female genital tract (by assisted calving, primiparity, male offspring, twins and caesarean section operation) and disruption of the physical/functional barriers to infection (retained placenta, stillbirth, delayed uterine involution, endometrial dysfunction and perturbation of ovarian cycles) (Potter et al.; Bonnett et al., 1993; Opsomer et al., 2000; Sheldon et al., 2002; Kim and Kang, 2003; Han and Kim, 2005; Herath et al., 2009a).

During reproductive examinations before the breeding period, the challenge is to identify cows with postpartum uterine disease that are truly at risk of fertility problems and to make a proper treatment in a suitable time. Thus, a practical case definition based on diagnostic criteria that identify those cows at risk of uterine disease associating with impaired fertility is needed (LeBlanc, 2003). Metritis is a severe inflammation involving all layer of the uterus, whereas endometritis is inflammation limited to the endometrium. Therefore, diagnosis of metritis is relatively easy by clinical examination and rectal palpation. It is characterised by an atonic enlarged uterus with a foetid watery uterine discharge together along a systemic illness and fever (Sheldon et al., 2006). Clinical and subclinical endometritis may be more difficult to recognize as they do not involve systemic signs and rectal palpation alone could not identify cows with subfertility related to endometritis (LeBlanc, 2003). Some cows showed the presence of a purulent vaginal discharge, which is able to predict the severity of inflammation using an endometritis clinical score (Sheldon and Dobson, 2004). Cows with subclinical endometritis may show a significant reduction of reproductive performance in the absence of overt clinical signs but can be identified by assessment of the % of neutrophils in uterine cytology

or the presence of uterine fluid using ultrasonography (Kasimanickam et al., 2004).

Dubuc et al. (2010) proposed that endometritis diagnostic criteria between the use of purulent vaginal discharge and cytology may reflect different manifestations of uterine disease. The risk factors for purulent vaginal discharge are twinning, dystocia, metritis and elevated serum HP, whereas the risk factors for cytological endometritis included low body condition score, hyperketonemia and increased HP. However using either cytology or vaginal exudates, cows diagnosed with endometritis showed subsequent fertility problems including subfertility, extended calving to conception intervals and an increased number of services per pregnancy. During reproductive examination, the precise diagnosis of these uterine diseases must be performed by a combination of an observation of uterine discharge, vaginoscopy, rectal palpation and endometrial cytology (LeBlanc et al., 2002a; Gilbert et al., 2005). The development of early diagnostic and prognostic information for monitoring the health status of a dairy herd, blood proteins such as APPs (SAA, HP and α1-acid glycoprotein) have been shown to reflect the inflammatory responses of several diseases including uterine infection (Murata et al., 2004). However, these markers of bovine endometritis such as all have their own limitations for use in practice (Fischer et al., 2010). For instance, the levels of serum SAA and HP reflected neither their gene expression levels nor the degrees of inflammation in endometrium of postpartum cows (Chapwanya et al., 2012). It is suggested that the expression of APPs were increased not only by bacterial contamination but also in addition to physiological changes associated with normal parturition and uterine involution (Uchida et al., 1993; Sheldon et al., 2001; Humblet et al., 2006). Therefore, the use of newly developed techniques such as ultrasonography, cytobrush and Metricheck in combination with a development of novel potentially biological markers such as IL8 and S100 protein family should be considered (Kasimanickam et al., 2004; McDougall et al., 2007; Barlund et al., 2008; Chan et al., 2010; Ghasemi et al., 2012; Swangchan-Uthai et al., 2013).

Generally, the principle of therapy to treat metritis and endometritis is to resolve inflammatory changes that disturb fertility by reducing the load of pathogenic bacteria and promoting uterine defence and repair. Treatment of mild or subclinical cases should be done between 26 days postpartum and the end of the voluntary waiting period before first service, whereas treatment of more severe cases must be immediate and aggressive (LeBlanc, 2003). Drugs of choice for the therapy of uterine inflammation and infection are divided into two categories: (i) antibiotics, e.g. parenteral or intrauterine routes of tetracycline and cephalosporin and (ii) hormones, e.g. $PGF_{2\alpha}$, oestradiol benzoate, oxytocin and GnRH. The effectiveness of treatment depended on the postpartum interval, the presence of a CL, the type of pathogenic bacteria and the uterine environment at treatment (Janowski et al., 2001; LeBlanc et al., 2002b; Sheldon et al., 2004; Azawi, 2008; Galvao et al., 2009a; Galvao et al., 2009b).

Future considerations

The dairy industry is tending to shift towards larger farms, and must remain profitable. Many groups have shown evidence of the association between a decline of cows' fertility in recent decades with increased genetic selection for milk yield and it is unlikely that breeders will stop using milk production traits as dominant items in genetic selection for the next decade. However, there should be some consideration given for other traits to be available for vets and farmers, in particular in fertility and immune health. We suggest that parameters representing an optimal uterine environment should be included, for example, selection of daughters with less susceptibility to infection or indeed ones that can control the extreme inflammation induced by LPS from pathogenic *E.coli* bacterium.

Postpartum uterine pathology comprises inflammation and infection, where unresolved inflammation can lead to delayed recovery of the healthy reproductive tract before next breeding. This is one of the main issues in initiating puerperal metritis and endometritis. The challenge for veterinarians and researchers is to improve the strategy of clinical decisions and treatment of these cows with poor uterine conditions. The standard focus on the pathology of the "unresolved uterine inflammation" towards a clinical examination and molecular approach should be carried out. In addition, the chief inflammatory mediators that were revealed in this article are TNFα, IL1B and antimicrobial proteins of the S100 family. A further investigation of the mechanisms which are capable of controlling these potent inflammatory mediators should be carried out. A new line of prophylactic and therapeutic tools for inflammation of endometritis such as inhibitors of TLR4, TNFα or S100A8 should be tested.

Conclusions

Postpartum endometritis are responsible for economically important uterine disease in the dairy industry. The endometrium is a main site not only for carrying the conceptus throughout pregnancy but also supporting immune responses against both pathogenic microorganisms and their toxins. This article demonstrated that the bovine endometrium utilises several mechanisms including physical barriers and activation of inflammatory mediators, APPs, AMPs and complement for defence against potential pathogens through receptors TLR4-CD14-MD2 complex (with possible TLR2). The mechanisms of pro-resolution of inflammation were also described. However, the scope of this context is focused on the response of endometrial cells against LPS which is a component of gram-negative bacteria, despite the fact that other pathogenic microorganisms

including gram-positive bacteria, anaerobic bacteria and viruses can also cause inflammation of the bovine endometrium (Sheldon et al., 2009b). However, recently a growing body of evidence has indicated that a range of microbes and their products also have the capacity to stimulate immune responses in either similar pathways as used by LPS or others (Jacca et al., 2013; Turner et al., 2014). The current findings are thus possibly also relevant to the mechanism of action of non-LPS molecules.

For veterinarians in the field, it is of the highest interest to detect at-risk cows with puerperal metritis after calving and subclinical endometritis before breeding. Further studies are required to develop prompt diagnostic tools using newly discovered proteins as biological markers for susceptible cows. Moreover, an indicator for unresolved endometrial inflammation that affects fertility is still required. Underlying factors such as the development of NEB can also compromise these mechanisms. This information is needed to improve the strategies of reproductive management in the modern dairy industry.

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Review Article

Targets and Roles of Progesterone during the Periovulatory Period in the Bitch

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Abstract

In the bitch, the periovulatory period is characterized by the luteinization of preovulatory follicles, causing a significant increase in progesterone (P4) concentrations in the blood, the follicular fluid and probably the oviductal fluid. Moreover, the key events such as oocyte maturation, fertilization and early embryonic development take place within the oviducts while the plasma P4 levels are significantly higher. This paper is aimed to review the existing knowledge on patterns of P4 secretion, mechanisms of P4 action with special emphasis on spatio-temporal expression of various P4 receptors and regulation of key ovarian and oviductal functions by P4.

Keywords: progesterone, receptor, ovary, oviduct, bitch

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Peculiarities of progesterone secretion

Preovulatory luteinization

The terminal follicular growth is accompanied by acquisition of LH receptors leading to metaplasia of granulosa into luteal cells. In the bitch, an early acquisition of LH receptors, also termed as preovulatory luteinisation, has been reported. Compared with bovine, where LH receptors appear in 1cm follicles (50% of the ovulatory diameter: Monniaux et al., 2009), the LH binding sites become detectable in canine follicles of 1mm (20% of the ovulatory diameter: Saint-Dizier et al., 2008). Consequently, plasma P4 concentrations rise just before, concomitantly or just after the start of the LH surge (Concannon, 2011). Indeed, P4 measures around 2 $ng.ml^{-1}$ at LH peak, 6-10 $ng.ml^{-1}$ around ovulation, achieves a plateau around 40-60 $ng.ml^{-1}$ during diestrus and finally fall below 2 $ng.ml^{-1}$ in the 24 h preceding parturition (Concannon et al., 1989; De Gier et al., 2006; Chastant-Maillard et al., 2011).

Follicular microenvironment

Follicular fluid originates from the secretions of somatic cells, exudation of extra-vascular plasma and accumulation of various steroids (E2, P4). In contrast to other mammals in which E2 dominates the periovulatory follicular environment, the preovulatory luteinisation in the bitch leads to significantly high P4 in the follicular fluid exposing the oocytes and embryos to a steroid-rich microenvironment. It has been demonstrated that mean P4 concentrations in canine follicular fluid increase from 3000 $ng.ml^{-1}$ (before the LH peak) to 11000 $ng.ml^{-1}$ (after the LH peak). Compared with plasma P4 levels, the intrafollicular P4 levels were about 100-1500 and 850-2500 times higher, before and after the LH peak, respectively (Fahiminiya et al., 2010).

Oviductal microenvironment

The oviductal fluid originates, in part, by the release of follicular contents in the oviduct, the oviductal secretions and by the excretory factors from serum. In the bovine oviduct, P4 concentrations were found to be higher than in the peripheral blood, particularly on the ipsilateral side of ovulating ovary. While the plasma P4 concentrations were 4-6 $ng.ml^{-1}$, their levels reached around 500 $ng.ml^{-1}$ in the oviductal fluid during the luteal phase (Wijayagunawardane et al., 1998). In the bitch, owing to the high P4 in plasma and follicular fluid, a P4 rich oviductal microenvironment might be expected. Nevertheless, no such data is available in literature.

Mechanisms of action of progesterone

Most of the known biological effects of P4 are mediated through classic (genomic) signalling pathways, through the binding of P4 to its nuclear receptor (PR). This process is relatively slow and involves the synthesis of new mRNA and proteins over a time scale of hours to days. In addition to the genomic effects, steroids may also exert rapid (non-genomic) actions that are insensitive to inhibitors of transcription and demonstrable in cells devoid of nuclear receptors. This involves the activation of intracellular signalling pathways within seconds (alterations in ion fluxes and intracellular free calcium concentrations), minutes (interaction with other second messengers) or over a prolonged period of several hours in the case of oocyte maturation (Thomas and Das, 1997).

Nuclear P4 receptors (PR)

PR is a member of the ligand-inducible DNA-binding super-family of nuclear transcription factors. Nuclear receptors consist of a modular domain structure containing an amino- (N) and a carboxyl- (C) terminal. The N-terminal includes AF-1 and AF-3 while the C-terminal includes the AF-2 transcriptional activation domains. In between the N- and C-terminal, there is a DNA-binding domain (DBD) and a ligand-binding domain (LBD) (Conneely et al., 2003).

In most mammalian species, the PR gene consists of two alternative promoters and translation start sites, resulting in two PR isoforms: the full-length (120 kDa) PRB and the N-terminally truncated (94 kDa) PRA. More precisely, both isoforms are encoded by the same gene but PRB contains an additional 164 amino acids at the N terminal. Indeed, it is due to the AF-3 domain located in this extended N-terminal region that PRB can recruit a number of co-activators which PRA cannot (Li and O'Malley, 2003). In addition, a less common isoform (PRC) has been reported which is the N-terminally truncated (60kDa) form of both PRB and PRA (see Gellersen et al., 2009 for modular structures of these receptors).

P4 being liposoluble, enters in the cells by simple diffusion through the plasma membrane. Once inside the target cells, it binds to PR and induces a conformational change in the latter thus transforming it from an inactive (non-DNA-binding) to an active (DNA-binding) form. Consequently, the heat shock proteins (HSPs) that were bound to the receptor in the absence of hormone are lost, leading to dimerization of PR. Subsequently, the activated PR dimer binds to specific DNA sequences within that promoter region, referred to as hormone response elements (HRE). The bound PR alters the transcription by direct action on transcriptional machinery or by interactions with co-activators or co-repressors. Thus, variable hormone effects are produced at cellular and tissue levels (Conneely et al., 2003; Chabbert-Buffet et al., 2005).
Selective ablation of PRA and PRB in knockout (KO) mice has given insight into the distinct physiological roles contributed by each isoform. PRAKO mice display severe uterine hyperplasia and ovarian abnormalities with no alterations in mammary and thymus development. On the opposite, PRBKO mice demonstrate reduced mammary and thymus development but normal uterine or ovarian functions

(Mulac-Jericevic and Conneely, 2004). From a general point of view, PR is expressed in the hypothalamus, ovary, corpus luteum, oviduct, uterus and mammary glands. In the canine uterus, PR was detected in all cell types including myometrial, stromal, glandular and epithelial cells (Vermeirsch et al., 2000). Moreover, biochemical and immunohistochemical studies demonstrated a variation in PR expression throughout the estrous cycle. In the canine oviduct, PR was detected in various tissue layers of ampulla and fimbriae (Vermeirsch et al., 2002). In a recent study, conducted in our laboratory, PR was localized in the nuclei of epithelial, stromal and muscular cells in the ampulla, isthmus and UTJ of canine oviducts at precise times around ovulation (Tahir et al., 2012). Likewise, the relative expression of PR showed a stage-dependent variation in the ampulla and isthmus (Tahir et al., 2013).

Membrane P4 receptors

P4 binding moieties with characteristics of membrane receptors have been classified in 2 families i.e. membrane associated P4 receptors (MAPR) and progestin and adipoQ receptors (PAQR). The MAPR family consists of P4 receptor membrane component 1 (PGRMC1) and very much related PGRMC2; both containing a single membrane-spanning domain. On the other hand, PAQR family consists of membrane progestin receptors (mPR's) containing three isoforms (mPRα, mPRβ and mPRγ), all containing seven trans-membrane domains.

P4 receptor membrane components (PGRMC1 and 2)

PGRMC1 and 2 are cytochrome-related, highly homologous proteins and believed to have diverged from a common ancestral gene. They have been recognized as extracellular/surface proteins which localise to the plasma membrane. PGRMC1 has been published under several synonyms (25-Dx, Hpr6, Ratp28, mPR, VemaA and IZA) in different biological contexts (Cahill, 2007). Both PGRMC1 and 2, like other MAPR family members, contain an N-terminal transmembrane (TM) domain and a putative cytoplasmic cytochrome b5 (Cyt b5) domain. Between the TM and Cyt b5 domains, is located the putative SH3 target sequence. The Cyt b5 domain is highly conserved, the SH2 target sequence is present at C-terminal while the putative SH3 target sequence is totally absent from PGRMC2 (see Gellersen et al., 2009 for details).

PGRMC1 is predominantly located in intracellular membranes however perinuclear or extracellular localisation has also been documented. Immunolocalization studies have demonstrated an exclusively cytoplasmic (bovine cumulus cells: Aparicio et al., 2011) or cytoplasmic and nuclear (bovine oviduct: Luciano et al., 2011) expression. PGRMC1 has been localized to the tissues of the male and female reproductive tracts including granulosa and luteal cells (human: Engmann et al., 2006; rat: Peluso et al., 2006), uterus and placenta (mouse and human: Zhang et al., 2008), sperms (fish: Thomas et al., 2005; pig: Losel et al., 2004) and cumulus oocyte complexes (cattle: Luciano et al., 2010; Aparicio et al., 2011). Contrary to PGRMC1, PGRMC2 is preferentially expressed in placenta (Gerdes et al., 1998). Moreover, its sub-cellular localization may vary from cytoplasmic (bovine cumulus cells: Aparicio et al., 2011) to cytoplasmic and nuclear (macaque endometrium: Keator et al., 2012; bovine oviduct: Saint-Dizier et al., 2012). PGRMC1 expression was down-regulated in the uterus after P4 treatment (mouse: Zhang et al., 2008) or in the endometrium during the mid-secretory phase (woman: Kao et al., 2002; macaque: Ace and Okulicz, 2004). Unlike PGRMC1, virtually nothing is known about the regulation of PGRMC2. In the very first report of PGRMC1 and 2 expression in the bitch, Tahir et al., 2013 demonstrated a stage-specific variation in the relative and protein expression of both PGRMCs in the canine oviduct.

Membrane progestin receptors (mPRα, β and γ)

The novel mPR's family, discovered in 2003, functions as G-protein coupled receptors (GPCR's). These closely related cell surface receptors mediate their actions on various cytoplasmic signalling cascades by binding to P4 (Fernandes et al., 2008). The first family member (mPRα), originally cloned from spotted seatrout ovaries, is believed to initiate oocyte maturation (Zhu et al., 2003a). Subsequently, two other isoforms (mPRβ, mPRγ) were identified in human and a variety of other species (Zhu et al., 2003b). mPR's, like other members of PAQR family, consist of a core region containing seven trans-membrane domains. The PAQR family includes 11 mammalian members out of which mPRα, β and γ are named as PAQR7, PAQR8 and PAQR5, respectively (see Gellersen et al., 2009 for proposed structures of these receptors).

The mPR's are highly but divergently regulated in different tissues with α isoform being predominantly expressed in reproductive tissues, the β isoform in neural tissues and mPRγ in kidney and gastrointestinal tract in human (Zhu et al., 2003b) and in the corpus luteum in the rat (Cai et al., 2005). In the bitch, mPR's were first characterized in the oviduct and showed an increase in gene expression at Day7 post-ovulation, the time of early embryonic development in the bitch (Tahir et al. 2013). Although the exact roles of mPR's remain to be established in bitches, they have been attributed major roles as intermediaries in progestin induction of oocyte maturation in fish (Thomas, 2008) and porcine (Qiu et al., 2008). Data on the regulation of the expression of mPR's is scarce and somewhat contradictory. In human endometrium, the mRNA expression of mPRα increased while that of mPRγ decreased along with the post-ovulatory rise in P4 (Fernandes et al., 2005). Similarly, *in vitro* studies in primary culture of human myometrial cells also demonstrated that P4 up-regulates the expression of mPRα and not mPRβ

(Karteris et al., 2006). In mice oviduct, the expression of mPRβ mRNAs was down-regulated by P4 whereas it did not vary for mPRγ. Conversely, in human oviduct, mPRγ mRNA expression was down-regulated around ovulation whereas mPRβ mRNAs did not vary across the menstrual cycle (Nutu et al., 2009).

Regulation of ovarian functions

The ovary is the principal site of synthesis and secretion of P4 which in turn also regulates its function. The presence of PR in most follicular cell types and in the corpus luteum and development of PRKO mice have revealed the involvement of P4 in various ovarian functions.

Steroidogenesis

Steroidogenesis is a multi-step process by which cholesterol is converted to biologically active steroid hormones. The pattern of steroidogenesis varies during the cycle and is regulated by several factors. The positive regulators of steroidogenesis include P4, E2, growth hormone, activin A, amphiregulin and insulin. P4 auto-regulates its synthesis in rat (Ruiz et al., 1985) and cow (Kotwica et al., 2004). It also stimulates the gene expression for StAR protein, P450scc and 3β-HSD enzymes thus increasing its own synthesis (Rekawiecki et al., 2005). In the bitch, the main source of P4 is the ovary (Concannon et al., 1989) while small amounts of P4 can also be secreted by the adrenal cortex (Frank et al., 2004). Moreover, during the first half of gestation, ovarian P4 secretion is independent of luteotropic support from pituitary. Meanwhile, the second half is dependent on prolactin (PRL) and possibly gonadotropic hormones (Kowalewski, 2012).

Folliculogenesis

Folliculogenesis involves the transformation of primordial germ cells into oocytes and the growth and differentiation of follicles from primordial to primary, secondary, tertiary and preovulatory follicle. The direct role of P4 in the regulation of folliculogenesis remains controversial. One school of thought considers that PR is not needed for follicle growth, differentiation of granulosa cells or luteinisation but is specifically and absolutely required for the rupture of follicles and release of oocytes at ovulation (Robker et al., 2009). This notion is based on the studies on PRKO mice ovaries with normal follicular growth until the preovulatory stage. The other school of thought believes that the coordinated series of folliculogenesis is regulated by hormones and local intraovarian factors. This notion is based on the fact that reduced P4 during follicle growth altered follicular dynamics and follicular fluid composition in dairy cows (Cerri et al., 2011). In the last decade, there has been a growing interest in the contribution of non-genomic actions of P4 in the regulation of folliculogenesis. The involvement of exact receptor protein is debatable however the identification of PGRMC1 in the rat granulosa and mPRs in the ovine ovary cells make them a potential candidate for P4 action.

Cumulus cell expansion

The relationship between cumulus cells and oocytes is believed to play an important role in oocyte cytoplasmic maturation and subsequent developmental competence (Fair and Lonergan, 2012). Though not expressed in mice cumulus cells (Lydon et al., 1995; Robker et al., 2000), there are reports of PR expression in porcine and bovine cumulus cells along with the expression of multiple non-genomic P4 receptors in bovine oocytes and cumulus cells (Aparicio et al., 2011). IVM studies showed an impaired cumulus cell expansion after inhibition of P4 synthesis or blocking of PR (bovine: Aparicio et al., 2011; porcine: Shimada et al., 2004a) and mPRβ (porcine: Qiu et al., 2008). In the bitch, though mucification of cumulus cells is clearly apparent several hours after the LH peak, few innermost layers of granulosa cells remain unmucified for several days (Reynaud et al., 2006). This cumulus-oocyte linkage is believed to block the oocyte meiotic resumption (Luvoni et al., 2001). Furthermore, the mucified complex of granulosa cells disappears following ovulation as soon as the oocyte reaches the oviduct, the site of oocyte maturation (Reynaud et al., 2012). Like other mammals, though the mucification occurs shortly after the LH peak, the canine COC's acquire mucification ability only after reaching follicular diameter of 4–4.5mm (Reynaud et al., 2009).

Ovulation

The evidence of P4 participation in regulation of ovulation has emerged from several studies in the past few years. Administration of P4 antagonists (mifepristone: RU486 and onapristone: ZK28299) in rat and mice prevented or reduced follicular rupture and subsequent rate in mice (Akison and Robker, 2012). This stance was further strengthened by the inability of PR null mice to ovulate despite exogenous stimulation to gonadotrophins (Lydon et al., 1995). These mutant mice exhibit a normal follicular development/ differentiation with the oocytes capable of normal maturation and fertilization *in vitro*. This indicates that PR has a precise role in the LH-dependent rupture of follicules (ovulation) but not in the differentiation of granulosa cells (luteinisation) (Robker et al., 2000). Contrary to this, intrafollicular injection of trilostane, to suppress P4 synthesis, did not block ovulation or subsequent corpus luteum function in cattle (Li et al., 2007). Recently, microarray comparison of wild-type (WT) and PR-null (PRKO) mice revealed that expression of about 300 genes, encoding diverse molecules, was significantly altered in ovaries of PRKO compared with WT mice, shortly before follicular rupture (Kim et al., 2009).

Oocyte maturation

Oocyte maturation is a process which is first initiated during embryonic development, arrested at

birth and later evoked shortly before ovulation in response to preovulatory LH surge. The role of P4 and PR signalling in mammalian oocyte maturation has been controversially debated. Steroidal involvement in meiotic maturation remains to be a major puzzle in mammalian reproductive biology because ovarian steroids appear to not serve as obligatory mediators of this process. Despite the critical role of P4 to initiate oocyte maturation in oocytes of frog and fish (Zhu et al., 2003a), PRKO mice oocytes remain capable of resuming meiosis (Robker et al., 2000). Nonetheless, evidences such as elevated P4 during development of ovulatory follicle, switch between E2 and P4 dominance between LH surge and ovulation support the notion of a role for P4 in oocyte maturation (Fair and Lonergan, 2012). Furthermore, the recent discovery of various P4 receptors in bovine oocytes and variation in their expression at the time of oocyte maturation suggests P4 involvement in this process (Akison and Robker, 2012). In the bitch, the mechanisms controlling meiotic resumption of oocyte remain to be determined. An earlier report failed to demonstrate the gene expression of nuclear (PR) P4 receptors in canine oocytes at different stages (proestrus, oestrus, dioestrus and anoestrus) of the estrus cycle (Gonçalves et al., 2009). Moreover, supplementation of IVM media with P4 did not induce any dramatic increase in meiotic completion rates (Hewitt and England, 1997; Vannucchi et al., 2009). It should, however, be noted that the doses of P4 added in culture medium might not correspond to the P4 concentrations to which the COC's are exposed within the oviduct. P4 has been reported to be 1000 fold higher in the follicular fluid than the circulating blood, but there is no information on P4 concentrations in the oviductal fluid. These significantly higher concentrations of P4 at the time of meiotic resumption point to a possible role of this steroid in oocyte maturation.

Oocyte developmental competence

The developmental competence is the ability of an oocyte to produce a viable embryo once fertilization has been achieved. The functional relevance of P4 and PR signalling has primarily been reported in acquisition of developmental competence. Addition of P4 in culture medium improved blastocyst rates in rhesus monkey (Zheng et al., 2003) while use of trilostane (inhibitor of P4 synthesis) or RU486 (PR antagonist) reduced it dramatically (bovine: Aparicio et al., 2011; porcine: Shimada et al., 2004b). Similarly, progression of bovine oocytes through early cleavage stages was affected after using antibodies against PGRMC1 (Luciano et al., 2010) or mPRα (Aparicio et al., 2011). Elevated P4 during the growth of Graffian follicle increased the quality of embryos in stimulated cows (Rivera et al., 2011). Lower rates of cumulus cells apoptosis have been correlated with developmental potential in both human (Lee et al., 2001) and bovine (Salhab et al., 2011) oocytes. P4 anti-apoptotic effects are mediated via the PR and PGRMC1 which point to the role of P4 in oocyte developmental competence (Fair and Lonergan, 2012).

Regulation of oviductal functions

In mammals, the oviduct is the site of gamete maturation and transport, fertilization and early embryo development. However, much of the research in the past has been focused on ovarian and uterine functions. The successful *in vitro* fertilization and embryo transfer techniques, while bypassing the oviduct, create an impression that the study of oviductal physiology is somewhat redundant. At present, the oviduct is considered as a highly specialized secretory organ that maintains and regulates the dynamic fluid-filled environment to orchestrate few of the most fundamental reproductive processes.

Gamete and embryo transport

The oviduct is lined by a transporting epithelium similar to those that line the other internal and external surfaces of the body. The ampulla transports the oocytes mainly via the beating activity of ciliated cells while the isthmus transports the sperms and the embryos through the contractile activity of myosalpinx. The combined action of ciliary beating and muscular contractions induce a current of oviductal fluid that moves the oocytes/sperms and embryos through the oviduct (Croxatto, 2002). Plasma P4 levels as well as the expression of P4 receptors in the oviduct have been reported to vary during the course of estrous cycle (Tahir et al., 2013) suggesting a regulatory role for P4 in the transport of gametes and embryos. This idea was confirmed by the early studies demonstrating that oviductal transport was speeded up by E2 and slowed down by P4 in a dose and time-dependant manner (Chang, 1966; Wessel et al., 2004). Similarly, P4 was shown to decelerate the transport of ova and embryos through the oviduct in the guinea pig (Nakahari et al., 2011). In the same way, post-coital administration of RU486 in rats accelerated the transport of ova and embryos through the oviduct resulting in their premature entry into the uterus (Psychoyos and Prapas, 1987). Similarly, sperm motility and their timely arrival at the fertilization site is supposed to be regulated by preovulatory or ovulatory follicles which would locally act on the oviductal epithelium via the steroid hormones. Thus, the lumen of the isthmus would change from extremely narrow to wide allowing synchronization between flagellar movements and myosalpingeal contractions (Hunter, 2005).

Gamete and embryo selection and storage

The oviduct is capable of selecting viable oocytes by letting the mature or immature COC's to adhere with its epithelium while degenerated or denuded COC's fail to do so. The sperms also bind the oviductal epithelium of caudal portion of the isthmus which actually serves as a functional sperm reservoir. Indeed, the isthmus imposes a gradient of sperms to the fertilization site and minimises the

chances of polyspermy (Hunter and Leglise, 1971a and b). The specific adhesion between the sperm head and the microvilli of oviductal epithelium is also believed to prolong the life of sperm until ovulation takes place. The changes in ovarian steroid secretion during the estrous cycle are critical to determine the dynamics of storage and release of sperm from the oviduct. In rats, E2 increased the number of sperm entering the oviduct while E2 combined with P4 induced sperm binding to oviductal epithelium (Orihuela et al., 1999). In pigs, large quantities of P4 are locally transferred by a vascular counter-current system to the walls of the isthmus shortly before ovulation and play an important role in the storage and release of sperm (Hunter et al., 1983). Moreover, P4 microinjections in the wall of the isthmus resulted in the release of huge number of sperms leading to a high rate of polyspermy (33%) in sows (Hunter, 1972). In short, the oocytes are selected by interactions with the cells of ampulla while the fertilizing sperms are selected and retained in the isthmus and get released when the oocyte is ready to be fertilized. Likewise, a regulatory role of the oviduct, to delay the passage of embryos until the uterus is ready for implantation, was confirmed by observation of synchronization between uterine proliferation and embryonic stage of development in rabbits (Chang, 1950).

Tubal fluid secretions

Fluid accumulation in the oviduct occurs during the follicular phase and is believed to follow the same fashion as in other secretory epithelia. Together with a selective transudate of serum and follicular contents released at ovulation, these secretions form the oviductal fluid. Oviductal fluid contains steroid hormones with P4 concentrations significantly higher in the oviductal fluid as compared to circulating blood e.g. 500 $ng.ml^{-1}$ vs 4-6 $ng.ml^{-1}$ in cow (Wijayagunawardane et al., 1998) and around 15,000 $ng.ml^{-1}$ vs 10 $ng.ml^{-1}$ in dogs (Fahiminiya et al., 2010). Though no data exists for P4 concentrations in the canine oviductal fluid but owing to the preovulatory luteinisation, a P4 rich microenvironment could be speculated. Steroid hormones could influence the movement of Cl^- ions, which normally occur from the basal pole toward the apical surface of oviduct epithelial cells, either directly by modulating basal Cl-absorption or indirectly by modulating the intracellular Ca^{2+} concentrations via presence of extracellular ATP thus altering the rate of fluid secretion (Cox and Leese, 1995).

Conclusions

For a long time, P4 has been mainly been considered a "pro-gestational" hormone and the primary focus of P4 actions has been uterine receptivity and pregnancy maintenance. However, recent studies have highlighted the role of P4 in gonadotrophin secretion, ovulation, oocyte maturation, early embryonic development, implantation, maintenance of pregnancy, mammary gland development and sexual behaviour. P4 mediates its actions through genomic as well as non-genomic pathways. The expression of its receptors shows a tissue- and stage-dependant variation in various reproductive tissues. Interestingly, some of these variations coincide with the timing of variety of key reproductive processes, particularly oocyte meiotic resumption and fertilization in the bitch which points to the importance of P4 during the periovulatory period in this species. Further studies are needed to explore the exact mechanisms of P4 action, determination of P4 concentrations in the oviductal microenvironment and use of similar concentrations to achieve improved rates of canine *in vitro* maturation.

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Review Article

In Vitro Culture of Feline Embryos: A Review

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Abstract

In vitro embryo culture is regarded as a crucial step of *in vitro* embryo production. Among several factors affecting cultured feline embryos, optimal culture environment is considered exceptionally vital to their developmental competence. Practically, the optimal culture system is assessed by embryo quality in terms of morphology, developmental potential, cryotolerance and ability to establish a pregnancy. Though these methods can directly evaluate the embryo equality, there are inevitable variations in transferring outcomes of embryos with the same quality. This simply implies the need of more sensitive tool to assess embryo quality. Recently, gene expression analysis has been recognized as an additional tool for quality assessment of feline embryos. However, the representative genes implying the embryo quality for this specie is still limitedly known. This short review aimed to summarize the effect of post fertilization culture environment on feline embryos and the use of gene expression evaluation to imply their quality.

Keywords: *in vitro* culture, feline, embryos

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Introduction

For decades, domestic cat has been used as an excellent model to develop *in vitro* embryo production (IVP) system for non-domestic cat (Thongphakdee et al., 2006; Thongphakdee et al. 2010). Despite its establishment in several species, the success rate of feline IVP is still markedly limited (Pope, 2014), and the quality of *in vitro* produced feline embryos is always inferior to *in vivo* derived embryos (Farstad, 2000). These differences are demonstrated by their developmental competences, morphologies, chromosomal integrities and alterations in gene expression patterns (Sananmuang et al., 2011; Sananmuang et al. 2013). Several studies have suggested the incompatibility between *in vivo* and *in vitro* culture environments as a major contributor to this phenomenon.

History of in vitro embryo production in feline species

The ovarian stimulation and artificial insemination were initially developed in rare felids as a tool of assisted reproductive technologies (ARTs). However, the success to produce their offspring is still limited due to mating and conception failure. To overcome the problem, *in vitro* fertilization (IVF) and embryo culture were subsequently developed using domestic cat as a model (Pope, 2014). *In vitro* embryo production has been developed in feline for more than 40 years. The two initial reports of IVF-derived embryos were published by Bowen et al. (1977) and Hamner et al. (1970) and the first kittens from IVF-derived embryos was firstly reported in 1988 (Goodrowe et al., 1988).

Indicators of embryo quality

Embryo morphology and the kinetics of development (timing of cleavage and blastocyst formation) are the common methods for embryo assessment and selection prior to transfer (Van Soom et al., 2003). Blastocyst quality can be assessed using many methods including counting the total number of blastomeres, counting the fragmentation of the chromosome and determining the ratio between inner cell mass (ICM) and trophectoderm after 7 days of *in vitro* culture (Gomez et al. 2010). By this means, the acquired blastocysts are graded and selected with an aim to increase the success rate of subsequent embryo transfer.

Interestingly, some studies have indicated the discrepancy between the conventional embryo features and the embryo developmental competences (Sananmuang et al., 2011; Sananmuang et al. 2013). The evidences thus imply the insufficiency of morphology and developmental kinetics to evaluate feline embryo quality. Due to this reason, more sensitive tool like determination of changes in gene expressions is additionally applied to fulfill this inadequacy. Correlation between feline embryo quality and its gene expression is demonstrated in several studies, and suggests the efficiency to decipher hidden effects of culture environments on embryos (Hribal et al., 2012; Sananmuang et al., 2011; Sananmuang et al. 2013). For example, the underlined stress and apoptosis are recovered in feline cultured embryos with poor quality (Sananmuang et al., 2011; Sananmuang et al. 2013). Since these abnormal embryos could not be ruled out by conventional assessments based solely on morphology determination, the use of these embryos should greatly affect the success rate of their subsequent transfers.

Effect of post-fertilization culture environment on developmental competence

As mentioned previously, the quality of *in vitro* produced embryos are usually inferior to *in vivo* ones. This contradiction is believed to be majorly due to the difference between culture environment and *in vivo* environment. *In vitro* embryo production consists of three main steps, *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). During IVM and IVF, approximately 60-80% of immature oocytes reaches nuclear maturation and undergoes fertilization. However, only 40-50% of such fertilized oocytes could achieve blastocyst stage during IVC (Sananmuang et al., 2010; Sananmuang et al., 2011; Sananmuang et al. 2013). These evidences remark the promising fall-off in embryo development during post-fertilization, and several factors in culture environment are regarded as contributors.

Though the pre-implanted embryos can exhibit the plasticity and tolerance to changes in environments, their future development is surprisingly sensitive to these fluctuations. The post-fertilization culture environment is encompassed by several factors, such as culture medium (Herrick et al., 2007; Sananmuang et al. 2011), embryo culture density (Sananmuang et al., 2011; Sananmuang et al. 2013), and temperature and gas atmosphere (Johnston et al., 1991). Although there are many protocols of IVP have been developed and result in high blastocyst rate, the success of kittens born from this technique is still very limited. This suggests the hidden effect of culture environment on embryo development.

Effect of post-fertilization culture environment on gene expression

In addition to variation in morphological and developmental outcomes, aberrant gene expressions associated with *in vitro* cultured embryos have been denoted in several species (Lonergan et al., 2003; Rizos et al., 2002; Sananmuang et al., 2011; Sananmuang et al. 2013). During embryo development, dynamic changes of several gene regulations have been implicated in several important events from the first cleavage division, embryonic genome activation, morula compaction, to blastocyst formation. Observation of these changes can thus provide useful information for implying the quality and abnormality of the produced embryos. In feline

embryos, the culture environment is shown to perturb gene expressions in developing feline embryos (Hribal et al., 2013; Sananmuang et al. 2011). Decreasing levels of NANOG and OCT-4, the transcription factors regulating during embryo development, are reported in *in vitro* derived feline embryos compared to *in vivo* derived ones (Nestle et al. 2012). Beside these transcription factors, cat embryos cultured in high density also demonstrated increasing levels of apoptotic-related gene (BAX) and stress-response gene (HSP70) compared with low density cultured and *in vivo* derived embryos (Sananmuang et al., 2011; Sananmuang et al. 2013).

Future direction of in vitro culture of feline embryos

Gene expression analysis is successfully applied as a sensitive tool to optimize embryo production methods in several species (Corcoran et al., 2006; Nowak-Imialek et al., 2008; Wrenzycki et al., 2006). In feline species, this attempt is however a challenging issue due to great variations of gene expressions among individual feline embryos (Hribal et al. 2012). This notice provokes us the immediate need to clarify more reliable genes correlated with early embryo development in this specie. During the past 5 years, cDNA microarray has been widely applied to determine and compare gene expressions between *in vivo* and *in vitro* derived embryos in human, bovine and murine embryos. Interestingly, several reliable biological markers implying embryo competency have been continuously updated based on microarray studies (Vallee et al., 2008; Wang et al., 2004). By use of the same approach, we proposed cDNA microarray as a future tool for identifying better biological markers that can effectively imply embryo development in feline species. Moreover, such global analysis of gene expression could profitably relate the differentially expressed genes with complex embryo metabolisms (Vallee et al., 2005; Wang et al., 2004). Implements of such characteristics should provide us hidden keys to modify culture system both in domestic cats and lesser-known felid species, and thus will hopefully improve the success of *in vitro* embryo production (IVP) for wildlife conservation.

Conclusions

Although IVP technique has been remarkably progressed in domestic cat, the successful rate of embryo production outcome is still limited. This is usually due to failure of *in vitro* fertilized oocytes to develop to blastocyst stage *in vitro*. While embryos can exhibit plasticity in their ability to adapt to suboptimal *in vitro* conditions, their sensitivity to the culture environment can lead to long-term alteration in the characteristics of the fetal and postnatal growth and development. Since such effects cannot be evaluated by conventional grading of pre-implanted embryos according to their morphology, sensitive tool like gene expression has become an interesting option. However, more reliable markers for embryo competency determination are still required in feline species. According to this reason, identification of core gene regulation during embryo development should be considered as an important direction for future innovation of ARTs in feline species.

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Review Article

Artificial Insemination in Goats: An Update

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Abstract

The success of the artificial insemination in goats is associated with the sperm collection, storage and use. Furthermore the technique of insemination dictate the fertility rate, the vaginal insemination with the fresh semen provide high fertility rate when using the frozen thawed semen the fertility rate is low. Therefore the deeper semen is deposited increase the fertility. The cervical insemination increases the fertility and the insemination intrauterine by laparoscopy has the highest fertility. Due to the expensive equipment and requiring skilled inseminator of laparoscopic insemination, the trans-cervical insemination has been developed to replace its. The trans-cervical insemination is performed by passage the inseminating pipette through the cervical canal and deposit semen intrauterine. The complexity of cervical anatomy limits the success rate of the trans-cervical insemination. Therefore the cervical relaxation is required to achieve this technique.

Keywords: Artificial insemination, goat, semen, buck

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Introduction

Artificial insemination (AI) has an important role in goat breeding especially in the intensive systems of production. It is to control the reproduction which conjunction to an accurate progeny test. The progeny test with AI speed up the identification of superior buck at a younger age. Then it allows extensive use of the available superior buck results in faster genetic progress then increases the rate of genetic improvement. At the farm level the control of reproduction in particular population of goats allow kidding at a precise season of the year taking advantage of oestrous synchronization with its precise control of ovulation and parturition and furthermore allows the advantage of out of season breeding. Other benefits of AI include more efficient genetic selection schemes and the manipulation and storage of the genetic material by the production and cryopreservation of goat semen for the artificial insemination with frozen thawed semen. In addition AI is associated with other animal health benefits. This technique helps avoid disease transmission and allows the transport of semen and AI reduces the risk of spreading sexually genital infections by natural mating. AI allows rapid widespread diffusion of improved genotypes and exchange of genotypes without transmitting diseases.

The success of an AI program depends of the proper management of semen collection, storage and use, the proper oestrous synchronization and the insemination technique which also relate to the time of insemination. The storage of semen especially in the frozen state causes the intrastructual, biochemical and functional damage to the spermatozoa resulting of motility, viability, impaired transport and fertility (Leboeuf et al., 2000). The fertility of stored semen is generally lower than that of fresh semen. Furthermore the site within the genital tract where the semen is deposited also relates to the fertilization rate of does. The artificial insemination techniques utilized in goats are the vaginal AI, cervical AI, Trans cervical AI and Laparoscopic AI.

Artificial insemination techniques

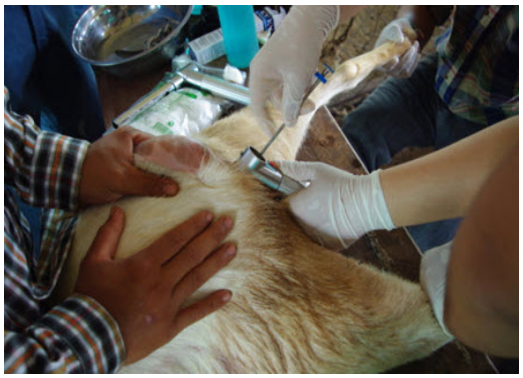
Artificial insemination techniques and semen deposited site affect the fertility in goats (Arrebola et al., 2012). There are four AI techniques that have been used in goat breeding, vaginal insemination, cervical insemination, trans-cervical AI and laparoscopic intrauterine insemination. The laparoscopic AI technique allows the semen deposit intrauterine which ease the spermatozoa transportation toward the site of fertilization resulting the higher pregnancy rate than those the insemination techniques such as cervical or vaginal insemination where the semen are deposited at the cervical canal or at the deep end of the vagina.

Vaginal Insemination (VAI)

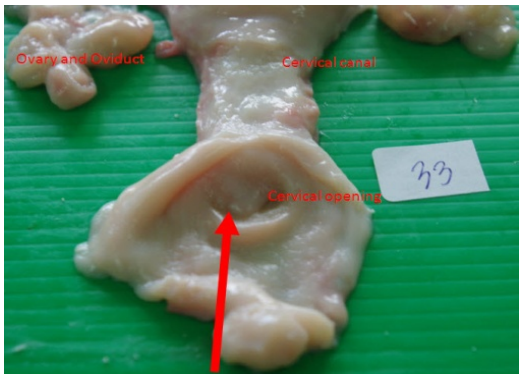
The vaginal insemination method involves depositing semen deep in the vagina without any attempt to locate the cervix then the semen is deposited at an anterior end of vagina (Fig. 1). Vaginal insemination using fresh diluted semen is the simplest and quickest method but requires a large semen dose (150-400 x 10^6 spermatozoa per insemination). The study in the Norwegian Dairy goats illustrates that the vaginal insemination with 200x 10^6 liquid spermatozoa resulted in 25-day non-return and kidding rates of 85.5 and 74.3%, respectively (Paulenz et al., 2005). While, the vaginal insemination with the frozen thawed semen gave the lower fertilization, The field trial in the Norewegian dairy goats show that non return rate and kidding rate after the vaginal deposition of 400 x 10^6 frozen-thawed spermatozoa were 64% and 58.3% (Nordstoga et al., 2010). This information suggests that the vaginal insemination with the fresh diluted spermatozoa gave higher fertility rate than those with frozen-thawed spermatozoa.

a) The doe is put on the rail helping allow the hind quarter of her body elevated. The vaginal speculum is utilized to locate an anterior of vagina.

b) The frozen semen is thawed prior deposited into the vagina.



c) The inseminating pipette is used for the deposition the frozen thawed semen



d) The red arrow presents the semen deposition at an anterior of vagina.

Figure 1 The vaginal artificial insemination in goat.

The number of semen deposition does not affect the fertility rate when inseminations vaginally as long as the total number of inseminated spermatozoa are equal. Nordstoga et. Al. (2010) showed that fertility rates earned from the single insemination with 400 x 10^6 frozen-thawed spermatozoa and the double insemination with 200 x 10^6 frozen-thawed spermatozoa had not different statistically. In fact, when the number of spermatozoa per insemination reduces the fertility rate is lower. The further investigation on reducing the number of spermatozoa is presented. The data suggest that non return rate at 25 day after the insemination and kidding rate follow the vaginal insemination with 200 x 10^6 frozen-thawed spermatozoa were 37.3% and 24% respectively (Nordstoga et al., 2011). Even though the vaginal insemination technique is a simple and less cost but the fertility follow the use of frozen thawed semen is poor that prevents the extensive utilize of the best available buck lead to delay the rate of genetic improvement in goat breeding. Therefore the investigation for increase the fertility rate follow the insemination with frozen thawed semen is required.

Intra-cervical insemination (CAI)

Intra-cervical insemination using fresh diluted semen is commonly used in AI of goat at the farm level. When performed properly, cervical insemination with fresh diluted or undiluted semen results in high fertility, whereas the fertility obtained following intra-cervical insemination with frozen-thawed (F-T) semen is lower. The cervical insemination with 200 × 10^6 liquid spermatozoa resulted in 25-day non-return and kidding rates of 87.0 and 78.0%, respectively (Paulenz et al., 2005).When the cervical insemination with the frozen thawed semen gave the lower fertility rate, the experiment in mixed bred goat in Thailand illustrate that pregnancy rate after the cervical insemination with 150 x 10^6 frozen thawed spermatozoa were 15.789 % when performed single insemination and 38.70% respectively (p<0.05) when performed double insemination (Leethongdee et al., 2013). The semen deposition affects the pregnancy rate, the deeper the semen was deposited in the genital tract, the higher is the rate of pregnancy obtained, being greater when the catheter reach the uterus. The experiment during non breeding season in Murciano-Granadina goats in Spain shows that post-cervical insemination with the frozen-thawed semen had the pregnancy rate 57% (Salvador et al., 2005). The depth of cervical penetrability is associated with the cervix anatomy. The study in Thai bred goat shows that multiparous does have longer cervices than the nulliparous does (4.2±0.2 cm vs 3.5±0.2 cm; p<0.05) without any difference in the number of internal rings (p>0.05) (Intrakamhaeng et al., 2011). The depth of penetration in multiparous does was greater than in nulliparous does (3.8±0.2 cm vs 2.3±0.2 cm; p<0.05) (Intrakamhaeng et al., 2011). The deeper cervical penetration in multiparous does suggests a potential of deeper semen deposition which lead to the higher pregnancy rate.

Trans-cervical intrauterine insemination (TCAI)

Trans-cervical AI (TCAI) is a method of insemination where semen is deposited deep in the cervix or even into the uterus via the cervix (Fig. 2). This method involves depositing semen as deeply as possible; it aims to reach the uterus. The greater the depth of insemination, the higher the expected pregnancy and lambing rates. The success of the trans-cervical insemination is related with the cervical penetrability. However the complexity of the cervix prevents the passage of inseminating pipette through the cervix. Goat cervix has long tubular shape and the internal cervical ring. These factors limit the cervical penetration. The study of Intrakamhaeng et. al. (2011) illustrates that an average length of multiparous does is 4.2±0.2 cm. The local cervical administration of Follicle stimulating hormone or Prostaglandin E2 increase the cervical penetration in cervix of Thai goats (Chatsumal et al., 2011). The expression of Prostaglandin receptor (EP) such as EP2 and EP4 indicating the relaxation mechanism in the cervix (Leethongdee et al., 2011). The cervical relaxation allows the passage of the inseminating pipette into the uterus. The trans-cervical intrauterine insemination

has been reported that the kidding rate follow the insemination with the frozen semen was 71% and the litter size was 1.76 (Sohnrey and Holtz, 2005). The passing of the inseminating pipette may cause the injury of cervical epithelium which may affect the fertility. The trans-cervical artificial insemination provides and acceptable kidding rate in goats. However the further investigation to increase the depth of cervical penetration without the trauma of cervical canal is warranted.

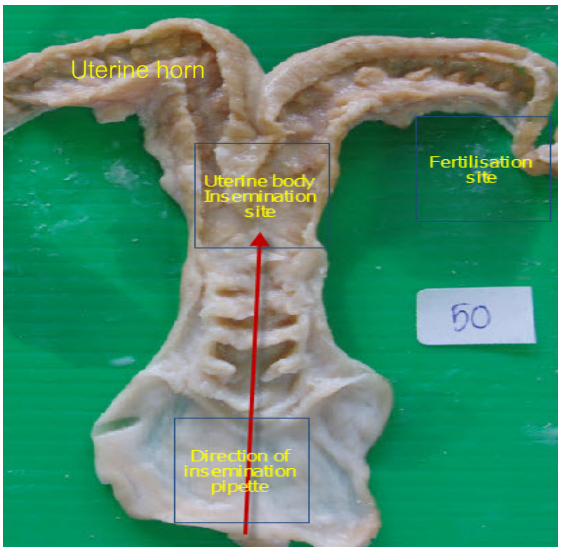
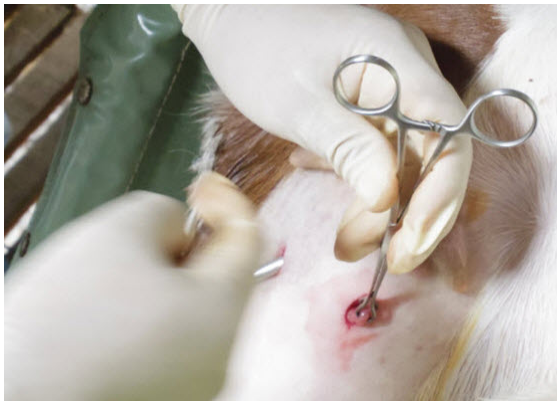


Figure 2 The semen deposition by the trans-cervical intra artificial insemination using the inseminating pipette passage through the cervical canal into the uterus. The arrow represents the direction of the inseminating pipette.

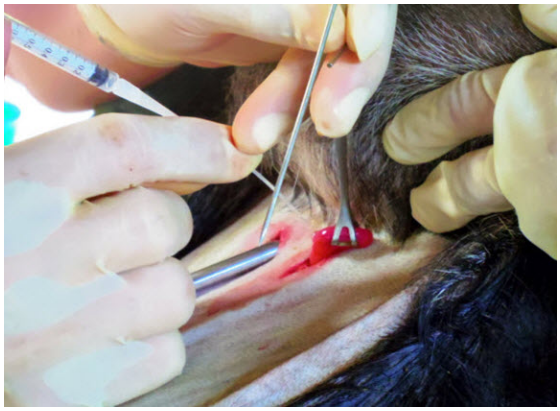
Laparoscopic intrauterine artificial insemination

Semen is deposited directly into the uterus through the uterine wall with the aid of a laparoscope (Fig. 3). Sedation and local anesthesia are required. Fertility and pregnancy rates are high with either fresh or frozen thawed semen. A lower number of spermatozoa can be used, typically 40 to 80 x 10^6 spermatozoa per insemination.

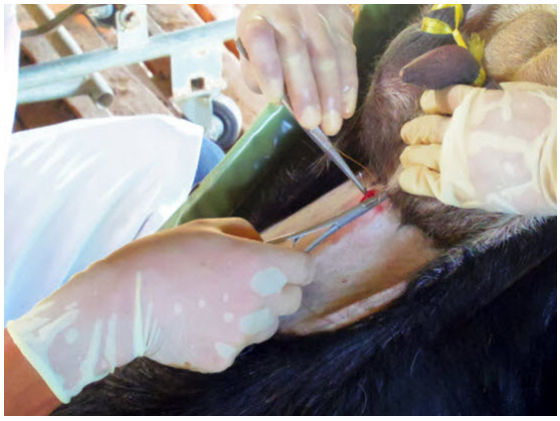
a) Laparoscopic technique.



b) Uterine horn.



c) Frozen thawed semen is deposited.



d) The skin is closed and sutured.

Figure 3 The laparoscopic intrauterine artificial insemination in goat.

The pregnancy rate follows the laparoscopic insemination with frozen thawed semen in cashmere does was 64.5% when does were oestrous synchronization by CIDR and 62.7 % when oestrus was induced using progestagen sponge (Ritar et al., 1990). It seem like the method of oestrous synchronization does not affected the pregnancy rate after the laparoscopic insemination. Whereas the time of insemination had affected the pregnancy rate, the insemination later than 65 hour after hormonal treatment tends to reduce the pregnancy rate (Ritar et

al., 1990). The laparoscopic insemination earns the benefit of spermatozoa is deposited close to the site of fertilization in the genital tract. The fertility rate when insemination with 10 million spermatozoa has not differed from the insemination with 20 million sperma-tozoa, 6.15% vs. 63.7% respectively (Ritar et al., 1990). Even though, laparoscopic insemination is requiring high skillful inseminator using the laparoscope which is expensive tool and consider not animal friendly welfare. The technique provides the high fertility rate which it may be considered use to inseminate the goat with superior genetic value.

Conclusions

The review has addressed mainly the insemination techniques that have provided in the goat breeding. The insemination technique gave the different fertility rate according to the site of insemination and the storage method of semen. The fresh semen gave the higher fertility rate compare to the frozen thawed semen which has been stored in liquid nitrogen which may damage the spermatozoa. The site of semen deposition affects the fertility, the deeper the semen deposition into the genital tract the increase of the fertility. Therefore the insemination intrauterine earns the highest fertility rate comparing to others techniques. The reducing of spermatozoa per insemination has been investigated. For the laparoscopic insemination, the reducing of the spermatozoa did not affect the fertility rate. This technique has the benefit of high fertility rate and uses lesser number of spermatozoa per insemination. However laparoscopic insemination is limited because of its expensive protocol and equipment. Alternatively the trans-cervical insemination provide the high fertility rate either and it is simpler technique as deposition of semen in uterine via cervical canal. But the complexity of the cervical anatomy limits the success of the cervical penetrability. The depth of cervical penetration has been investigated to improve this technique aims to replace the laparoscopic insemination and make it available at the farm level.

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Review Article

Artificial Fertilization in Nile Tilapia *Oreochromis niloticus*

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Abstract

Among cultured freshwater fish, Nile tilapia *Oreochromis niloticus* is considered as one of the most important species. With the current intensive culture, reproduction of Nile tilapia takes place in captivity where numbers of male and female broodstocks spawn independently in the mating tank. The artificial fertilization, as well as collection of milt and eggs from Nile tilapia broodstocks are applied for the purpose of genetic manipulation and transportation of genetic materials. Milt and eggs collection are performed by applying pressure on an abdomen of the anesthetized fish in the direction from anterior to posterior. The artificial fertilization in Nile tilapia can be achieved from using fresh and preserved milt, chilled and frozen. Preservation of Nile tilapia milt using cool storage techniques is a suitable choice, regarding its practicalness for farm management and cost effectiveness. Freshly collected Nile tilapia milt can be preserved in extender(s) at 4^{o}C for 8-16 hours and remains adequate sperm viability and motility. It is recommended to wash the freshly collected sperm with 0.85% NSS, and the extender should be supplemented with antibiotic to decontaminate the milt before preservation. The development of Nile tilapia embryo in the *in vitro* incubation system following an artificial fertilization using chilled sperm is comparable to the development of naturally fertilized egg. The successful artificial fertilization can promote selective breeding, and thus, induce the desirable broodstock colony for intensive aquaculture.

Keywords: artificial fertilization, eggs collection, milt collection, Nile tilapia

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Introduction

Nile tilapia *Oreochromis niloticus* is a major freshwater fish cultured worldwide, due to its ability to grow rapidly, tolerate a wide range of environment and diseases (Liang and Chien 2013). In 2011, the total world production of Nile tilapia was approximately 2.8 million metric tons which over two-third of this volume came from Asia (FAO, 2014). The majority of tilapia products are from China while Thailand ranks in the first five, producing more than 200,000 tons per year (Tveteras, 2013).

Nile Tilapia was first introduced into Thailand in 1965 by the Emperor Akihito, as His Royal Highness Crown Prince of Japan. Fifty fish were sent as the royal tribute to His Majesty the King Bhumibol Adulyadej. With the farsighted vision of His Majesty the king, the royal projects were initiated to reproduce the complimented Nile tilapia colony. Consequently, the fish were distributed to Thai agriculturists all over the country because His Majesty the king intended to provide his people protein food from Nile tilapia. Nowadays, Nile tilapia has been genetically improved for domestic culture in Thailand (Department of Fisheries, 2014). The production is sufficient for local consumption and global trade. Exported products from Nile tilapia include whole frozen fish, frozen fillets and fresh fillets.

The accomplishment of reproduction in captivity is a major factor for sustainable aquaculture. Many reproductive techniques such as milt collection, artificial fertilization have been widely applied in fish (Cabrita et al., 2010). Artificial fertilization in Nile tilapia is used mainly for the purpose of selective breeding because paired mating of the selected broodfish can be disadvantageous due to aggressive behavior of males. Artificial fertilization is also beneficial for transportation of genetic materials as moving broodfish between farm to farm for genetic selection is not practical. This review summarized information viewing the artificial fertilization in Nile tilapia and our study in the domesticated Nile tilapia broodstocks.

Artificial fertilization

Mode of reproduction in Nile tilapia is an external fertilization. Males build the breeding nest, and after a display of courtship behavior of the pair, sperm and eggs are released into nesting area. Nile tilapia is known as "mouthbrooders" for a reason that female incubates fertilized eggs in her buccal cavity (El-Sayed, 2006; Kullander, 1988). Males are aggressive in nature; consequently, paired mating sometimes leads to injuries of high value broodstock. Removing of premaxilla (upper lip) of the male was used to eliminate this offensive behavior (Thodesen et al., 2011). Artificial fertilization is applied to overcome this problem and induce fertilization of the selected the eggs and sperm from the captive fish. Artificial fertilization in Nile tilapia is not complicated and required minimum equipments. Eggs and sperm are manually collected and gently stirred together for few minutes (approximately 2-3 min) in the clean container added with some water for spermatozoa activation. The fertilized eggs are then incubated in the indoor tank-based hatchery and supplied with flow-through recirculating water system (Fig. 1).

Collection of Nile tilapia eggs and sperm

Eggs and sperm of Nile tilapia can be collected by hand, applying gentle pressure on the fish abdomen from anterior to posterior direction. In culture condition of Thailand, we found the proper stage of broodstocks for eggs or sperm collection is when they reach the size of approximately 300 g body weight or 30-40 cm body length. Generally the broodstocks are reproductive effectiveness during 1 year - 3years of age. To minimize stress and possible injuries to the fish, fish should be anesthetized prior the striping. The anesthetized broodstock should be handled with enough moisture during the removal of fish from water for eggs or sperm collection. Contamination of urine and feces must be avoided during sperm collection by stripping the bladder of each male. Collection of eggs is more challenging, as female Nile tilapia are asynchronous spawners. Each female contains different stages of egg development, causing non uniformity of the striped eggs which are not all suitable for artificial fertilization (De Lapeyre et al., 2009). Several methods have been used for spawning induction in Nile tilapia, such as photoperiod (Biswas et al., 2005), temperature (De Lapeyre et al., 2009) and hormonal treatment (El-Gamal and El-Greisy, 2005). Fernandes et al. (2013) successfully developed protocol to induce final oocyte maturation for artificial fertilization in Nile tilapia using human chorionic gonadotropin (hCG) treatment (1000-5000 IU/kg). Our current study found that the intramuscular administration of GnRH analog (Suprefact®, Sanofi Aventis, Canada), at the dosage of 10 µg/kg body weight of fish, twice at 6 hours interval was efficient to synchronize the maturation of eggs for collection at 24 hours following the second dose of Suprefact® administration. Reproductively active females can be observed from their physical appearances, the suitable female broodstock for egg collection usually carries tenderly enlarged abdomen and reddish prominent, hyperemic genital papilla (Khaw et al., 2008).

Egg quality can be roughly evaluated from morphological appearances, unusual size and color of egg may represent the off-quality. In addition, physico-chemical parameters of coelomic or ovarian fluid were used for egg quality prediction in rainbow trout *Oncorhynchus mykiss* (Aegerter and Jalabert, 2004; Dietrich et al., 2007) and turbot *Scophthalmus maximus* (Fauvel et al., 1993). However, evaluation of egg quality is not well study in Nile tilapia and it is not likely to be practical for farm management. Progressive motility and duration of movement are commonly used for sperm evaluation in reproductive researches on several fish species (Kime et al., 2001). Sperm motility is an indicative of the integrated functions actively performed by many cellular

components of spermatozoa, such as mitochondria, plasma membrane, axoneme structure and composition (Bobe and Labbé, 2010). Unlike terrestrial animals, movement of fish sperm is needed to be activated. Sperm of freshwater fish and marine fish are immotile at ejaculation, and then the movement of sperm is initiated with the osmotic shock from hypotonic or hypertonic environments (Morita et al., 2003). Semi-quantitative evaluation of the collected sperm can be carried out by an activation of the sperm movement. The collected milt is dropped in activating medium (such as water), on a glass side, and sperm motility can be observed under a dark field or phase contrast microscope. However, the result of sperm quality evaluation can be subjective because different grading criteria have been reported by several authors. For example; McMaster et al. (1992) used score from 1 to 5 for poor motility (0-20% slow movement) to excellent motility (80-100% rapid movement), Utsugi (1993) used (-) for non motile sperm, (±) for vibratory, (+) for not very active and (+ +) for rapid movement. Computer-assisted sperm analysis (CASA) was also applied for sperm quality evaluation for researches on reproduction in several some species (Kime et al., 2001), for example red seabream *Pagrus major* (Liu et al., 2007) and African catfish *Clarias gariepinus* (Rurangwa et al., 2001).

Cool storage of Nile tilapia milt

Fertilization was reported to be successful, by using fresh and frozen milt in Blue tilapia and Nile tilapia (Khaw et al., 2008; Zak et al., 2012). However, cool- short term storage of Nile tilapia milt is not well documented while it was studies in several fish species, such as Mozambique tilapia *Oreochromis mossambicus* (Harvey and Kelley, 1984), Indian major carp *Labeo calbasu* (Hassan et al., 2014), common carp *Cyprinus carpio* (Saad et al., 1988), salmonid *Oncorhynchus spp.* (Scott and Baynes, 1980), milk fish *Chanos chanos* (Hara et al., 1982), Japanese eel *Anguillu japonica* (Ohta and Izawa, 1996), striped bass *Morone saxatilis* (Jenkins-Keeran et al., 2001), Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* (Dorsey et al., 2011) and piracanjuba *Brycon orbignyanus* (Viveiros et al., 2010). Milt preservation by cool storage is simple to perform, required less special skill and expensive equipments. Chilled fish milt is prepared using dilute fresh milt with extender (with or without antibiotics) or undiluted, and maintained in a common refrigerator or ice box at 0-4 ºC (Hassan et al., 2014; Jenkins and Tiersch, 1997). It allows sperm to be preserved for period of time which assists the shipment of genetic materials for hybridization of selective breeding programs and facilitates hatchery management (Vuthiphandcha et al., 2009). Our preliminary study showed that washing Nile tilapia milt with 0.85% normal saline and diluting with extender; Freshwater fish saline or Modified Ringer solution (Chao et al., 1987; Rana and McAndrew, 1989) added with penicillin and streptomycin (8 IU/ml and 1 mg/ml extender, respectively) could maintain the sperm quality for successful artificial fertilization for at least 8 hours. Different semen extenders; Freshwater fish saline, Hank's balance salt, Ringer solution, Ringer with milk and Ringer with honey, were reported their use on Nile tilapia milt (Muchlisin, 2005). Extenders are essential for the preservation of fish milt, tilapia milt is concentrated in small amount; hence, extender is needed for increasing the volume of milt and maintaining the osmolarity (Alavi and Cosson, 2006). Cool storage of Nile tilapia milt appears to be more suitable than cryopreservation techniques for Thai aquaculture, because the cool storage technique is practicable for any farm and it preserves milt quality for an enough period for the transportation of milt within the country.

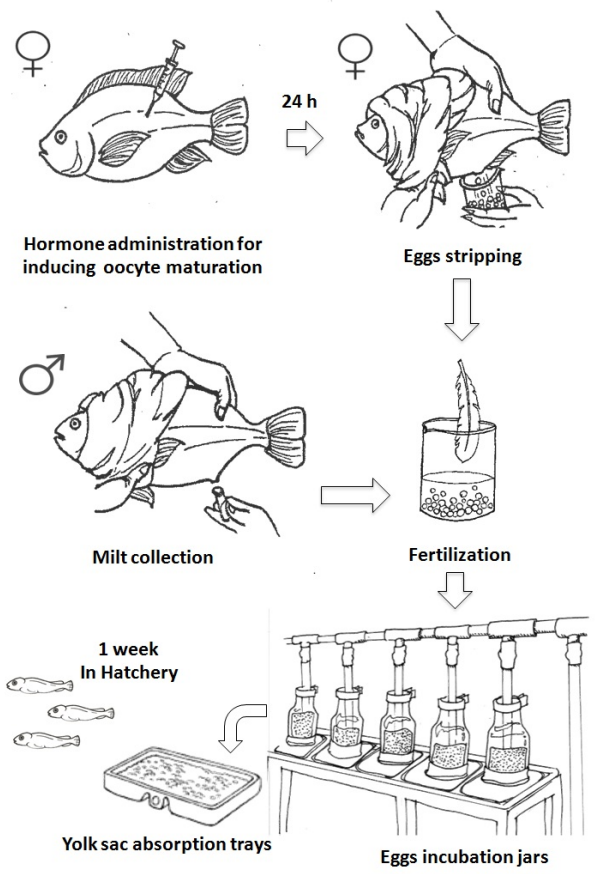

Figure 1 Artificial fertilization in Nile tilapia *Oreochromis niloticus*.

Incubation of the fertilized egg

In vitro incubation of the fertilized egg is influential for the success of artificial fertilization. The incubation system usually contains 2 parts; cylindrical, round bottom or conical container for egg incubation and yolk sac absorption tray. Round bottom container is preferable to the conical one. Survival of tilapia fry was reported higher in the round bottom compared to the conical container (Rana, 1988). Continuous flow of water is necessary for egg incubation, and flow-through, closed-recirculating water system is commonly applied. The

optimal condition of temperature/salinity/pH for hatching of Nile tilapia fertilized egg reported by Hui et al. (2014) was 27.1°C/9.2 ppt/7.4. Similary, the optimal condition for Nile tilapia hatchery in Thailand is 25-26°C/> 3 ppt/7-7.5. Development of fertilized eggs to hatching stage took approximately 90-120 hours at temperature 28±1 °C (Fujimura and Okada, 2007). During summer in Thailand, the water temperature may reach 30±1 °C, embryo development may take only 65-80 hours post fertilization (hpf) to hatch. Figure 2 presents developmental stages of Nile tilapia embryo observed during the *in vitro* incubation which is regularly exercised in Thai's commercial Nile tilapia management.

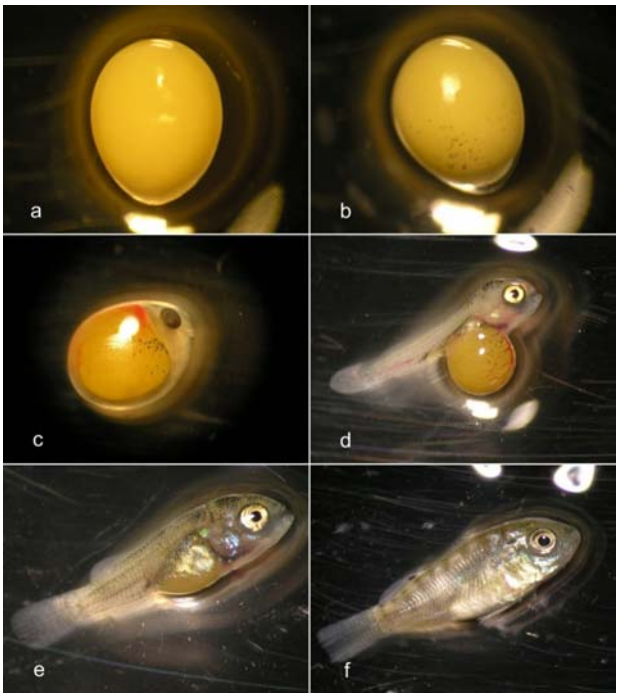

Figure 2 Post fertilization developments of Nile tilapia *Oreochromis niloticus*, observed under 25±1 °C incubation temperature: (a) stage 1: no development visible, fertilized egg, (b) stage 2: eyed egg, yellowish color egg with black eye spots (24 hpf), (c) stage 3: pre-hatched, dark brown-yellowish color egg with clearly black eye spots on the head part (56-64 hpf), (d) and (e) stage 4: yolk-sac larvae, the early stage fry with yolk (72-96 hpf) and (f) stage 5: free-swimming fry, yolk-sac is completely absorbed (200 hpf). hpf = hour(s) post fertilization

Conclusions

Production of Nile tilapia is a good example of sustainable aquaculture. The fundamental factors supporting Nile tilapia production in Thailand are the capability of reproductive manipulation and the efficient *in vitro* hatching system. Breeding program of broodstock are well established which enable to serve increasing demand of Nile tilapia seeds. Reproductive techniques such as artificial fertilization and sperm preservation can facilitate pair mating of the selected broodstocks, as well as distribution of genetic materials. Nevertheless, protocols of reproductive techniques being used in Nile tilapia culture are adapted from other fish species which may not be the most suitable for Nile tilapia, particularly for the culture condition in Thailand. Together with the progress in Nile tilapia production of Thailand, forthcoming studies in reproductive techniques are necessary for the use in genetic improvement of our captive broodstocks and the culture patterns operated in Thailand.

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Review Article

Reproduction in Sea Turtle

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Abstract

The studies provide the information on reproduction of sea turtles in Thailand, which were olive ridley and Hawksbill turtles. Semen of sea turtle was the first time successfully collected using electroejaculation with 2-6 volts electrical stimuli in 4 cycles. Seminal fluid was turbidity, opalescence, viscosity and mucoid appearance. Semen volume was low and sperm had narrow head and moved in spiral movement. The suitable extender to dilute and preserve semen was refrigeration medium test yolk buffer and Tyrode medium supplemented with albumin, lactate and pyruvate. The effective sperm staining for study on sperm morphology were Eosin stain and Diff-Quik stain. Hormones studies found that testosterone and thyroxcin in male sea turtles were elevated in March-April. However, elevated E2 and P4 were found at 2 times of a year which was in March-April and August-October.

Keywords: sea turtle, semen, electroejaculation, hormone

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Introduction

There are 5 species of sea turtles in Thai Gulf and Andaman sea coast. They are leatherback turtle *(Dermochelys coriacea*), the green turtle *(Chelonia mydas),* the hawksbill turtle *(Eretmochelys imbricate)*, the loggerhead turtle *(Caretta caretta)* and the olive ridley turtle *(Lepidochelys olivacea)*. In each year, numbers of sea turtles were declined, especially olive ridley turtle. The reasons of decreasing numbers of sea turtles in Thailand may come from environmental and temperature changes, decrease in natural food, or live threatening from human. At the present time, there was no data available on reproductive study of sea turtle in Thailand. The objectives of the studies were to find the method to collect turtle's semen, studies on semen, evaluation, preservation and reproductive hormones of sea turtles in Thailand. The experiments were conducted during in 2007-2010.

Genital system of sea turtles

The urogenital system is composed of kidneys, ureter, gonads and their ducts, urinary bladder, and derivatives of the genital papilla (penis and clitoris). The gonads of sea turtles are located dorsally in the body cavity, posterior to the lungs, and ventral to the kidneys and peritoneal wall (Fig. 1). Female reproductive tract consists of paired ovaries, oviducts (Mullerian ducts) and suspensory ligaments (mesovarium, mesosalpinx, and mesotubarium). The cranial pole of the ovary is located posterior to the lung, and extends posteromedially toward the cloaca. The ovarian surface is attached between ovaries and kidneys by mesovarium. There are no tubules connecting the ovary directly to the oviduct. However, the oviduct lines lateral to the ovary and oviduct ending is a funnel shaped opening. With the age change, and between breeding and nonbreeding seasons, the ovaries and oviducts will be changed in size and composition. Male reproductive tracts consist of 2 testes, epididymis, vas deferens, suspensory ligaments (mesorchium from the body wall to the testis) and one penis. The testis is fusiform shaped and it cranial pole is located posterior to the lung. It extends posteromedially toward the cloaca. Like female sea turtle, age and between breeding and nonbreeding seasons effect on the size of testes. The penis is retracted, except during mating, trauma, or death. Penis lies in the ventral floor of cloaca and composed of a paired called "urethral groove". When erection, the walls of the urethral groove meet dorsally to form a tube which allow sperm and fluid pass through. Adult male sea turtles characterized by a long tail with the cloaca opening near the tip, and strongly curved claws on the second digit. During breeding season, the midventral plastron becomes soft (Wyneken, 2007).

Study Places

The studies were performed at Phuket Marine Biological Center (PMBC), Phuket province and Eastern Marine and Coastal Resources Research Center (EMCOR) Rayong Province, Thailand.

Male turtle studies

Animals: Six male olive ridley turtles aging approximately 30 years old and weighed between 28-35 kilograms from PMBC, and 3 male Hawksbill turtles aging 16 years old and weighed between 40-49 kilograms from EMCOR were included in the studied for semen collection and evaluation.

Semen collection

Electroejaculation and semen evaluation was the first time successfully performed in the olive ridley turtles (*Lepidochelys olivacea*) and following on hawksbill turtles (*Eretmochelys imbricata*). The study in the Olive Ridley turtle semen was performed in May 2007 (Summer season) and in the Hawksbill turtle semen was performed in October 2007 (Rainy season). Before electroejaculation, each turtle was sedated with 25 mg/kg ketamine HCL intramuscularly, and electrical rectal probe was applied into its cloaca and stimulated with 2-6 volts electrical stimuli in 4 cycles. After electrical stimulation, manual stimulation was continued for complete penile erection (Sahatrakul et al., 2007, Tanasanti et al., 2007, Sahatrakul et al., 2008).

Semen quality

An average semen volume was 1 ml (range 0.01 to 2.2 ml) for Olive Ridley turtles (n=6), and 4.4 ml for Hawksbill turtle (n=1). Seminal fluid was turbidity, opalescence, viscosity and mucoid appearance. An average sperm motility was 28.25 % (range 0 to 98 %) for Olive Ridley turtle, and 60 % for Hawksbill turtle. An average sperm concentration was 67.3 million/ml (range 11.5 to 150 million/ml) for Olive Ridley turtle, and 512 million/ml for Hawksbill turtle, respectively (Table 1). The sperm had narrow head and moved in spiral movement (Fig. 2). There were also some motile lobulated appearances in seminal fluid which was unknown function. Sperm viability in external milieu was approximately 90 minutes (Sahatrakul et al., 2007, Tanasanti et al., 2007, and Sahatrakul et al., 2008).

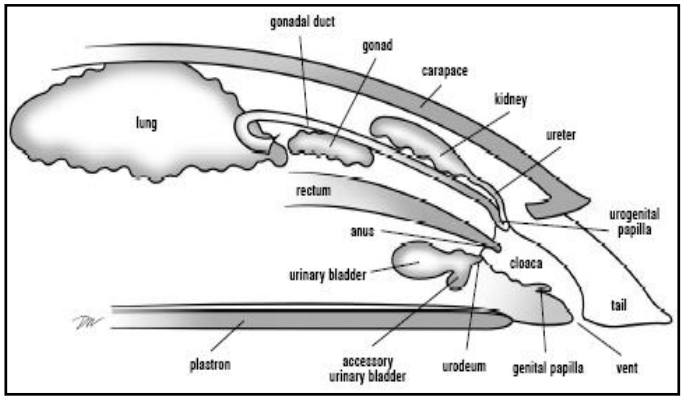

Figure 1 Urogenital anatomy of sea turtles.

Table 1 The data showed the results of semen evaluations from six male olive ridley turtles (from 8 ejaculations), and from one hawksbill turtle. The data was presented as average and range.

Parameters	Olive ridley turtle (n=6)	Hawksbill turtle (n=1)
Appearance	Turbid, mucous opalescence, viscosity	Turbid, mucous opalescence, viscosity
Volume (ml)	1 (0.01-2.22)	4.4
pH	6 (5-7)	5.5
% Motility	28.25 (0-98)	60
% Progressive motility	17.75 (0-60)	30.5
Concentration of sperm ($x10^6$)/ml	67.3 (11.5-150)	512
Total number sperm ($x10^6$)/ejaculation	40.9 (1.2-82.4)	1510.4

Sperm staining

Sea turtle sperm was experimentally stained with 3 stainings; Eosin stain, Wright's stain (Diff-Quik) and Toluidine Blue stain for study on sperm morphology. The results of sperm staining technique found that the effective sperm staining for study on sperm morphology in Olive Ridley Turtles and Hawksbill Turtles was Eosin stain (90.57%), Diff-Quik stain (86.11%), and Toluidine Blue stain (22.58%), respectively (Woonwong et al., 2009).

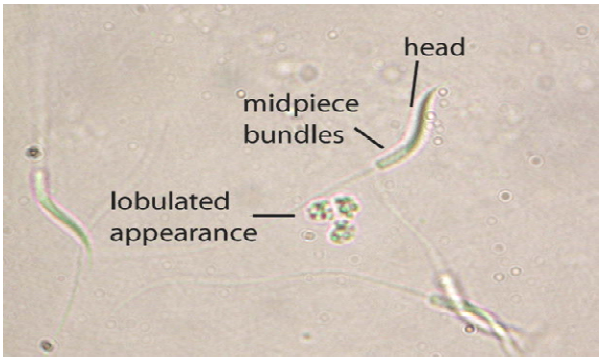

Figure 2 Olive ridley turtle sperm had narrow head and moved in spiral movement. The sperms were unstained.

Extender for sea turtle sperm dilution and preservation

Eight different extenders were studied in order to find the suitable extender to dilute and preserve sea turtle semen. There were 1) refrigeration medium test yolk buffer, 2) Tyrode medium supplemented with albumin, lactate and pyruvate, 3) Beltsville poultry semen extender, 4) 3% Sodium citrate buffer, 5) Phosphate-buffered solution, 6) EEL extender, 7) 1% bovine serum albumin, and 8) HAM F-10 extender. Diluted semen was kept at 4˚C for 48 h. The results found that 28% olive ridley turtles, and 25% hawksbill turtles that their spermatozoa diluted in extender1, and 14% both sea turtles that their spermatozoa diluted in extender 2 could be survived for 24 hours. However, the motility from both sea turtles semen in both extenders was decreased by 50-80 %. Most sperm died after diluted in the last 6 extenders. By conclusion, extender1 and 2 were suitable extenders for sea turtle semen viability, however, adding other ingredients should be considered to enhance in viability (Sirinarumitr et al., 2009).

Blood profiles and hormones in male and female sea turtle studies

The objective of the study was to monitor health status of sea turtles by measurements pack cell volume (PCV), plasma protein (PP), testosterone (T), progesterone (P4) estrogen (E2) and thyroxin hormone (T4) in Olive Ridley Turtles (*Lepidochelys olivacea*) and in Hawksbill Turtles (*Eretmochelys imbricata*) during 2 seasons of the year in January and May 2008. Blood samples were collected from 8 male and 19 female Olive Ridley turtles. Hormones were measured by chemiluminescence method. Blood samples from male turtles were measured for PCV, PP, T and T4. Blood samples from female turtles were measured for PCV, PP, E2, P4, T, and T4. The results found that, there was no difference between averages PCV during these 2 seasons, Average PP of turtles in January was higher than in May. Testosterone and T4 from male turtles were higher in May than in January. Semen collections by electroejaculation were successful collected in male turtles with high T. In female Olive Ridley Turtles (n=9), average E2, P4 and T4 in May were higher than in January, but there was no difference in T. In Hawksbill turtles (n=2), there was no difference between average E2, P4 and T, except average T4 in May was higher than in January.

Hormone profiles in sea turtles were repeated in 6 male and 8 female Olive Ridley turtles in PMBC during October 2008-August 2009. Blood from each turtle was collected every month for one year. The results found that both T and T4 in male sea turtles were high in March and April. However, elevated E2 and P4 were found at 2 times a year which was in March to April and August to October.

Conclusions

Data from these studies may provide some information on reproductive system in sea turtles. In order to conserve the threatened and endangered sea turtle species and to increase numbers of sea turtles in Thailand in the future, further studies on artificial insemination or frozen semen should be considered.

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Review Article

Snake Reproductive System

Taksa Vasaruchapong

Abstract

Like other animals, the reproductive system of snake is one of the important systems that allow the living organisms to reproduce their offspring. Snake reproductive system has been studied for more than 50 years. In this review article, the basic anatomy, biology, physiology, common problem and biotechnology of the snake reproductive system will be discussed and demonstrated some differences from the mammals.

Keywords: reproductive, anatomy, physiology, medicine, snake

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Introduction

The reproductive system is one of the important systems that allow the living organisms to reproduce their offspring. Snake reproductive system has been studied for more than 50 years that cover almost every aspect including basic biology, anatomy, physiology, medicine, surgery and biotechnology. Snake has the reproductive system that alike the mammals but there are some different on its biology, anatomy and physiology.

Snake reproductive anatomy

Male reproductive tract consists of yellowish or cream, oval shape testes located at two third of body coelom of which the right testis is located cranial to the left testis. Each testis is connected to the spermatic duct that line caudally along the intestine and pass the cloaca to hemipenis at the base of the tail. Snake has two hemipenis, the right and the left, which are kept separately inverted in hemipenis sac in the base of the tail (Jacobson, 2007; Denardo, 2006). The hemipenis of each species has particular size and shape which can be used for species identification (Jintakune, 2000).

Female reproductive tract consists of long and slender ovaries located at two third of body coelom which is the same location of male testes. The cranial part of oviduct is dilated to form infundibulum then line caudally along the intestine. The oviducts are opened to the cloaca separately as the vagina without forming the uterus. The vagina is opened into the cloaca through urogential opening or separate opening into the cloaca (Jacobson, 2007).

Sex differentiation in snake is performed by inserting the blunted-tip sexing probe into the hemipenis sac. Therefore, sexing probe can be inserted in male much deeper than in female (Fitch, 1960). Some species have sexual dimorphism, such as pythons and boas, which male has a pair of pelvic spur beside the cloaca larger than female (Denardo, 2006).

Snake reproductive biology

Snake has both seasonal and non seasonal breeding. Most of snakes in Thailand are seasonal bred once a year during the beginning of winter to summer season. Female releases pheromone as a chemical signal from anal scent gland. Male will track the pheromone by tongue flick to search for female (Carpenter, 1980). If there are more than one male presented, they will compete for female by trying to push the other down which is called combat dance. Male will copulate female by using only one hemipenis depended on the suitable position of particular side. The ejaculation can take place for several hours. For some instance, female can store sperms in the seminal receptacle on the oviduct wall that lead to the delayed fertilization. Snake has both oviparous and ovoviviparous which has different time of gravidity. The oviparous snakes will lay eggs after two months of gravidity. The eggs will be incubated for the other two or three months to hatch depends on species. The ovoviviparous snakes will give birth to their offspring after four or five months of gravidity depends on species too. The snake eggs, as in the other reptiles, have different biology from avian eggs. Snake eggs need to be steady in the same position with proper temperature and humidity until hatching. Rotation of the eggs, like avian eggs, can lead to embryonic death. The sex determination of offspring is based on the chromosome that differed from Crocodilia and Chelonia which are temperature-depended sex determination (Denardo, 2006).

Snake reproductive physiology

The physiological study of snake reproductive system has been limited when compared with the mammals. In many literatures revealed that it is controlled by seasonal and hormonal changes.

Male reproductive physiology: Testes consist of seminiferous tubules that used for spermatogenesis. Spermatozoa have long and spindle-liked head with flagellum. Sperm maturation takes place in the epididymis before entering the spermatic duct and pass to the hemipenis without the evidence of any accessory gland. Then the sperm enter the spermatic sulcus in the middle of hemipenis into female vagina. The follicular stimulating hormone stimulates the spermatogenesis while the luteinizing hormone stimulates the Leydig's cell to produce testosterone in the same fashion as mammals (Licht, 1974). In monocled cobra (*Naja kaouthia*), the serum testosterone level has an annual cycle pattern. The concentration is started to increase from January to the highest concentration in September and then decline to the base level in December (Meesook, 2008). That is correlated with the present of spermatozoa in cloaca and the copulatory plug at the opening of hemipenis sac.

Female reproductive physiology: In the beginning of breeding season, the multiple follicles develop to graffian follicles along with vitellogenesis to store yolk in ova. After ovulation, the ova pass infundibulum into the oviduct which all ova are fertilized. In oviparous snakes, the shell formation occurs in the oviduct and the eggs will be laid after two months of gravidity. In ovoviviparous snakes, the embryo will develop in their individual fetal membrane within the oviduct. The embryo derived the nutrients from yolk while oxygen and water are derived from the diffusion in the oviduct. The follicular stimulating hormone stimulates the follicle growth and lead to vitellogenesis. The luteinizing hormone stimulates ovulation and change follicles to corpus luteum that produce progesterone (Licht, 1974; Edwards and Jones, 2001). In monocled cobra, the serum estrogen has rapid increased in September to the highest level during October to November and decrease to the base level in December (Meesook, 2008).

Common problem in snake reproduction

Snake is ectothermal animal which the ambient environment plays an important role for controlling its physiological condition. Therefore, the ambient temperature has influence in almost entire process of reproduction especially breeding cycle and egg-laying. If the snakes in captivity are failed to match their optimum environment, they will not proceed to breed, fertilization, abortion or egg retention. Moreover, there are some methods of sex determination, such as improper sexing probe or pushing the hemipenis to evert out of hemipenis sac or popping technique, can injure the hemipenis and sometimes lead to hemipenile prolapse.

Hemipenile prolapsed: The most common problem of male reproductive tract in snake farm, Queen Saovabha Memorial Institute is hemipenile prolapse. It is mostly occurred after copulation and snake cannot reduce its hemipenis back into the sac due to stenosis of the opening or traumatic injury after copulation. The correction of hemipenile prolapse depended on the period and the degree of infection. If the hemipenis has only swelling without infection, the hypertonic substance such as sugar or salt can help to reduce the edema and allow pushing hemipenis back into the sac. Then, the pressure bandage is recommended for preventing the protusion of hemipenis. But if the prolapse is left with the unnoticed period until being dried and infected, the surgical amputation of hemipenis is considered.

Egg retention: Egg retention is found in the gravid snakes that were captured and donated to snake farm, Queen Saovabha Memorial Institute. Despite of the stress during capture and fail to adapt to the new environment, the egg retention is the consequence. If only a few number of eggs retained near the cloaca, the gently manipulation can be performed to remove the eggs through cloaca. Another method is bathing the snake in tap water for half an hour to stimulate the contraction that may lead to lay the egg. But if there are a large number of eggs or eggs retained in a far distance from cloaca, the general anesthesia is recommended. Under anesthesia, the muscle is more relaxed that allow gently manipulation through cloaca can be possible. The surgical removal by opening the oviduct should be performed after the failure to correct as the previous methods described.

The biotechnology in snakes

There have been a number of studies on snake biotechnology such as semen collection by ventral massage from the testes area to the cloaca (Mengden et al., 1980, Zacariotti et al., 2007), semen storage Mengden et al., 1980, Fahrig et al., 2007) and artificial insemination (Mengden et al., 1980; Mattson et al., 2007). Each study has the different purposes such as the conservation on rare species or the improvement of genetic variation on the captive snakes. But most of these techniques still need more studies prior to adapt for the practical use.

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Review Article

Luteinizing Hormone and Follicle Stimulating Hormone Receptors: Functions and Clinical Implications in Extra-gonadal Tissues

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Abstract

The pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) that elicit their biological responses by binding to the specific receptors are essential in controlling biosynthesis of the gonadal steroid hormones and gamete development in both males and females. In addition, the role of LH and FSH in extra-gonadal tissues has been proposed because the receptor for LH (LHR) and FSH (FSHR) are present in various tissues outside the gonads, especially the reproductive tract. A common theme in LH and FSH action in extra-gonadal tissues across all species is the up-regulation of PG synthesis. The functional importance of PGs, especially in the reproductive physiology, is well recognized as potent mediators. The major clinical implication of LHR and FSHR has been focusing on the development of an effective treatment regimen for cancer that targeted ablation of neoplastic cells based on the evidence that cancerous tissues express LHR and/or FSHR to a great extent. However, targeted ablation of carcinoma cells has not yet generally implicated in clinical practice of human and veterinary medicine. The objective of the present review is to provide an overview of the role of LHR and FSHR in extra-gonadal reproductive tissues, i.e. oviduct, uterus, cervix, prostate and the clinical implications of LHR and FSHR.

Keywords: clinical implication, gonadotropins, receptor

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Introduction

The fact that hormones act through their specific receptors on target organs has been well recognized. Once in circulation, LH and FSH finalize the communication by binding to their specific receptors that mainly reside on the surface of somatic cells and transmitting signals to the target cells (George et al., 2011). The tissue specificity of hormone action depends on both the hormone and its receptor being present; hormonal activity can be regulated either by controlling the availability of the hormone or by controlling the expression of the receptor. The latter control is extremely important in reproduction (Martin and Barry, 2003). Because the measurement of variation in hormone levels alone may not provide an adequate basis for understanding physiological function of the target organ (Martin and Barry, 2003), the receptor profile must be taken into account in order to better understand the physiology of hormone and its clinical implications. The objective of the present review is to provide an overview of the role of LHR and FSHR in the extra-gonadal reproductive tissue, i.e. oviduct, uterus, cervix, prostate and the clinical implications of LHR and FSHR.

Extra-gonadal LHR and FSHR

The main physiological functions of gonadotrophins in the gonads (ovary and testis) are well documented. They together control biosynthesis of the gonadal steroid hormones and gamete development in both males and females. Previously, it was assumed that gonadotrophins, i.e. luteinizing hormone (LH) and follicle-stimulating hormone (FSH), regulate only gonadal tissues because only these tissues were primarily thought to contain gonadotrophin receptors. However, over the last decade it has become more apparent that the gonads are not the only target site for gonadotrophin actions. Non-gonadal tissues of domestic animals including the gastrointestinal tract (Sand et al., 2013) and the urogenital tract, i.e. oviduct, endometrium, myometrium, cervix and uterine vessels, the feto-placental unit, mammary gland (Ziecik et al., 2005), prostate gland (Mariani et al., 2006; Ponglowhapan et al., 2012), urinary bladder and urethra (Ponglowhapan et al., 2007; Ponglowhapan et al., 2008) also contain receptors for LH (LHR) and/or FSH (FSHR). Surprisingly, LHR is present in human sperm, mostly confined to the sperm head (Eblen et al., 2001). Although the precise role of LHR in sperm function that possibly involves in fertility has yet to be elucidated, the receptors are functional as indicated by cellular response to LH or hCG challenge (Eblen et al., 2001).

All tissues of the reproductive tract are reported to be able to respond to gonadotrophin LH and FSH (Rao, 1996). In addition, it is interesting to note that changes in mRNA and protein expression for LHR and FSHR in non-gonadal tissues are oestrous cycle dependent, thus indicating that the two receptors are dynamically regulated (Shemesh, 2001). In non-gonadal tissues, it seems that most studies emphasized on physiological role of LHR rather than FSHR and in the reproductive tract rather than other tissues. It is assumed that the role of LHR and FSHR in non-gonadal tissues is not different as evident by an indistinguishable response of LHR and FSHR that regulates bovine cervix coupling to the prostaglandin (PG) synthesis (Mizrachi and Shemesh, 1999).

The presence of LHR and FSHR in the extra-gonadal tissue raises the question concerning their functional importance and the essential role of LH and FSH along with their corresponding receptors and remains a subject for further studies in many species. Nonetheless, a common theme in LH and FSH action outside the gonads across all species is the up-regulation of PG synthesis. Prostaglandins are the most common prostanoids that have been implicated as important regulators of many physiological and pathological processes. Specific actions of PGs depend on a particular tissue layer where the receptor is present. However, the functional importance of PGs, especially in the reproductive physiology, is well recognized as potent mediators.

The oviduct

The presence of LHR (Lei et al., 1993) and FSHR (Zheng et al., 1996) has been demonstrated in human fallopian tube epithelium. In domestic animals, the expression of LHR have been reported in the oviduct tissue of bovine (Sun et al., 1997) and porcine (Gawronska et al., 2000). The fact that the quantity of LHR expressed in the oviduct varied with the hormonal status indicates that LH coupling to its receptors might directly regulate tubal function during different stages of oestrous cycle. It is postulated that LHR is responsible for (i) controlling oviductal contractions, (ii) opening the ampulla-isthmic junction for spermatozoa and (iii) facilitating the passage of embryos through the isthmus toward the uterus via its relaxing action (Ziecik et al., 2005).

The uterus

In 1986, LHR expression in non-gonadal tissues was first reported in the porcine uterus (Ziecik et al. 1986). There is evidence that LHRs are found throughout the reproductive tract in many species and the degree of expression is dependent on tissue structure and hormonal status (Fields and Shemesh, 2004; Ziecik et al., 2005). The physiological action of receptor-mediated LH activity is different depending on tissue layers that LHR is being present (epithelium, subepithelial stroma or muscle). Gene expression for LHR in *the endometrium* has been investigated at the mRNA and protein levels. A common theme for LH action in the endometrium is the LH induction of cyclooxygenase-2 (COX-2), the rate-limiting enzyme in PG production (Stepien et al., 1999; Shemesh et al., 1997). However, the role of LHR in the endometrium is considered to regulate glandular and luminal epithelial cell function viz cAMP mediation or by increasing the local synthesis of steroid hormones (Bonnamy et al., 1989). A summary of the

physiological role of LHR or FSHR in *the myometrium* has been proposed that these receptors play a role in the hyperplastic hypertrophy of the uterus and uterine motility. This is consistent with a decrease of intracellular calcium concentrations observed in human myometrium smooth muscle cells after hCG treatment (Kornyei et al., 1993). It was also shown that high concentrations of LHR allow a relaxing effect on the pig myometrium, which was not seen in the absence of LHR (Flowers et al., 1991). Prostaglandin E_2, a product of LH stimulation, can also cause the contraction or relaxation of cervical smooth muscle, depending on the subtype of its receptor and the biochemical and structural changes in the connective tissue (Fields and Shemesh, 2004). It is proposed that gonadotropins may play a role in regulation of contractive activity during oestrous period (Ziecik et al., 1992).

The cervix

The cervix is mainly composed of connective tissue and smooth muscle, supported by extracellular matrix (ECM). It undergoes constriction and relaxation depending on the reproductive stage (oestrous cycle, pregnancy or parturition) associated with the underlying biochemical and structural changes in the cervical connective tissue and ECM. The receptor for LH has been shown in the epithelium of the cervix (Stepien et al., 2000). The LH is strongly involved to regulate collagen turnover via the collagenases in the extracellular matrix (Luck et al., 1996). Cervical relaxation and opening is in part induced by PGE_2, a product of LH stimulation (Duchens et al., 1993). Nevertheless, both stimulatory (EP1, EP3) and inhibitory subtypes (EP2, EP4) of PGE_2 receptor are found in the female reproductive tract, PGE_2 could cause both the contraction or relaxation of cervical muscle, depending on the subtype of the receptor being activated (Coleman et al., 1989). Similarly, bovine cervix at the time of the circulating plasma FSH peak also contains high levels of FSHR and responds to FSH by increasing the biosynthesis of PGE_2 responsible for cervical relaxation at oestrus (Mizrachi and Shemesh, 1999). Recently, it has been shown that the degree of LHR, but not FSHR, and the content of hyaluronan ECM in the sheep cervix changes after intra-cervical application of misoprostol (PGE_1 analogue) to soften the cervix (Leethongdee et al., 2010). The findings substantiate the notion that there is a functional relationship between LHR/FSHR and PGs in the cervical tissue.

The prostate gland

It is widely accepted that sex steroid hormones, i.e. androgens and estrogens, are imperative in the control of cellular growth and differentiation of the prostate. Both hormones are of great significance in the biology of the prostate because they play a crucial role in the pathogenesis of benign prostatic hyperplasia (BPH), a common condition of the ageing human and dog. However, other hormonal factors could be involved in the regulation of both the physiology and pathology of the prostate. Previous observations have shown that LHR was first reported in the rat prostate (Reiter et al., 1995; Tao et al., 1995) and FSHR in the human prostate (Dirnhofer et al., 1998). Recently, the co-expression of the two receptors has been first reported in the dog prostate, suggesting a potential role of gonadotropin LH and/or FSH in direct regulation of the canine prostate particularly the glandular epithelium where the expression is most abundant (Ponglowhapan et al., 2012). The fact that the enzyme aromatase catalyzes the conversion of androgens to oestrogens in the prostate and aromatase activity in the gonads at least is regulated by FSHR activation, had led Dirnhofer et al. (1998) to propose that the phenomenon by which FSH regulates aromatase expression via FSHR and subsequent oestrogen production potentially exists in the prostate gland. The functional significance of LHR in cell proliferation (Sriraman et al., 2001) and regulation of secretory activity of the epithelial cells (Reiter et al., 1995) has been shown in the rat prostate. It is possible that such effects of LH and FSH might be common in other mammals including the dog as gonadotrophins' function in the reproductive system appear to be similar or conserved among species (Ponglowhapan et al., 2012).

Clinical implications

In humans, the major clinical implication of LHR and FSHR has been focusing on the development of an effective treatment regimen for cancer that targeted ablation of neoplastic cells based on the evidence that cancerous tissues, e.g. prostate, mammary gland, highly express LHR and/or FSHR (Leuschner and Hansel, 2005; Mariani et al, 2006). A targeted treatment that effectively destroys cancerous cells that express LHR has been developed for research purposes and it is revealed that cells die by necrosis rather than apoptosis after treatment (Bodek et al., 2005). To our knowledge targeted ablation of carcinoma cells has not yet generally implicated in clinical practice of both human and veterinary medicine.

Conclusions

It is becoming increasingly evident that LHR and FSHR are confined in various non-gonadal tissues and their dynamic pattern of receptor expression either under physiological or pathological condition of the reproductive tract in domestic animals indicates that gonadotropin LH and FSH have a role in the extra-gonadal reproductive tissue. However, the impact of both receptors in tissues outside gonads is not yet fully understood. It is most likely that interplay of sex steroid hormones and gonadotropins to target tissues of the reproductive tract exists; the action of sex steroids may have a major and significant influence in comparison with gonadotropins.

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Review Article

Recent Progress on Pig Genetic Resources: Cryopreservation of Gametes and Gonadal Tissues

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Abstract

Cryopreservation of gametes or gonadal tissue has been expected as an effective method for the preservation of genetic resources in mammals including pigs. We have conducted two studies; 1) cryopreservation of porcine female gametes and 2) xenografting to produce male gametes from cryopreserved testicular tissues. Even though piglets have been produced, the efficacy is still very low. For the advanced gene banking, this issue should be focused towards improving the efficacy and thus further research will be necessary.

Keywords: vitrification, immature oocyte, xenografting, testicular tissue

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Introduction

One of the *ex situ* preservation methods for mammalian genetic resources is the cryopreservation of gametes, embryos and gonadal tissues. In pigs, freezing of sperm is the most reliable and well established method for this purpose. On the other hand, cryopreservation of female gametes (oocytes) and gonadal tissues – usually by vitrification – has been associated with very low efficacies. Recently, in our laboratory, we have conducted some research themes for this issue. In this symposium, we are introducing recent data on the 1) vitrification of porcine immature oocytes and 2) vitrification of porcine testicular tissues followed by their xenografting into nude mice to produce male gametes.

Cryopreservation of unfertilized oocytes

Oocyte cryopreservation combined with assisted reproductive technologies is a basic strategy for gene banking. In recent years, we have tested the efficacy of vitrification using 35% (v/v) ethylene glycol (EG) in immature (at germinal vesicle stage; cumulus-oocyte complexes (COC)) and mature oocytes (at metaphase-II stage) oocytes (Somfai et al., 2012). The survival rate for mature oocytes was relatively high; however, their fertilization and developmental abilities after in vitro fertilization were greatly impaired. On the other hand, vitrified immature oocytes could maintain or resume their normal fertilization and embryo development, however, survival rate remained very low (mature oocyte; 71.4% vs immature oocytes 27.7%).

In order to improve embryo production from vitrified oocytes for applicable gene banking, we decided to put effort towards the improvement of survival rates for vitrification of immature oocytes. The low permeability of the oocyte membrane at the GV stage has been suspected as the prime reason for the low survival rates. Therefore, to improve the survival rates, we have investigated the feasibility of propylene glycol (PG), a cryoprotectant showing an excellent permeating speed comparing with the others (e.g. EG or dimethyl sulfoxide), and we also checked the fertilization and developmental abilities of vitrified oocytes after in vitro fertilization (Somfai et al., 2013a). Immature COCs were vitrified in 2 µl microdroplets either in 35% EG or in 35% PG or in the mixture of 17.5% EG and 17.5% PG. Vitrified droplets were warmed by transfer into a warming solution (0.4 M trehalose in base solution) at 38.5 ºC. One to two minutes later, oocytes were consecutively transferred for 1-min periods into 500-µl droplets of medium supplemented with 0.2, 0.1 and 0.05 M trehalose. After vilification and warming, they were subjected to in vitro maturation (IVM), and surviving oocytes were in vitro fertilized (IVF) and cultured. The mean survival rate of oocytes vitrified in the PG was higher than that in the EG. Oocyte maturation rates did not differ among vitrified and non-vitrified control groups. Blastocyst formation in the EG group was higher than that in the PG group but was lower than that in the control group. On the other hand, treatment without vitrification revealed a higher toxicity of PG on subsequent blastocyst development compared with EG. The combination of EG and PG resulted in reasonable survival (42.6%) after vitrification. The maturation and fertilization rates of the surviving oocytes were similar in the vitrified, control and toxicity control (TC; treated with EG+PG combination without cooling) groups. Blastocyst development in the vitrified group was lower than that in the control and TC groups which, however, had similar development rates. In conclusion, PG alone enabled a higher oocyte survival rate after vitrification compared with EG. However, PG showed great toxicity to oocytes. The combination of EG and PG yielded reasonable survival rates without toxic effects on embryo development. The application of this cryoprotectant combination lead to the production of normal piglets after transfer of embryos produced from cryopreserved oocytes to recipients for the first time (Somfai et al., 2013b).

Xenografting of testicular tissues

A series of studies have been conducted to evaluate if boar spermatogonia can develop to sperm in testicular tissues grafted into immundeficient nude mice and if live piglets can be produced from this sperm by intracytoplasmic sperm injection (ICSI) into oocytes.

Porcine testicular tissues from male piglets were minced into small samples measuring 1.5 × 1.5 × 1.5 mm, and transplanted under the skin of the backs of castrated nude mice. Spermatids and spermatozoa were obtained after at least 4 months Spermatozoa showed only faint motility; therefore, ICSI was used to fertilize oocytes. After culture, blastocyst rate and their total mean number of cells were similar to those of the in vitro fertilized and developed blastocysts (52.7 cells, (Nakai et al., 2009; Kikuchi et al., 2002). When the oocytes at the pronuclear stages after ICSI were transferred to oviducts of estrous synchronized recipients, we could obtain piglets (Nakai et al., 2010).

For the more advanced utilization of this technique, we have checked the possibility of vitrification and cryopreservation of testicular tissue fragments before xenografting, which enables the long-time storage of the tissue and the production of sperm at any time we want. Tissue fragments were incubated in vitrification solution with 35% EG for 10 or 20 minutes (10- or 20-min group) at room temperature. Then, they were dropped in liquid nitrogen (LN) with a small volume (2 µl) of vitrification solution. After storage in LN, microdroplets, each containing a tissue fragment were transferred into warming solution (0.4 M trehalose in base solution) and then consecutively transferred stepwise to the basic solution as described above. A total of 30 tissue fragments were xenografted into a nude mice. After 120 days, sperm were recovered from the grafts with the same efficacy in both 10-min and 20-min group. The sperm recovery rate increased

with time after grafting from 180 to 350 days. One out of 4 gilts that had received oocytes fertilized using sperm from the 10-min immersion group delivered 2 live piglets, and one of another 4 gilts from the 20-min group delivered 4 live piglets (Kaneko et al., 2013).

Conclusions

Recent studies showed a remarkable progress in the fields of cryopreservation and utilization of genetic resources in pigs. Technologies which seemed inconceivable several decades ago could be applied; however, the efficacy has been remaining very low. For further advantages in gene banking, this issue should be focused towards improving efficacies and thus further research will be necessary.

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Review Article

Efficient Production of Sexed Embryos by *In Vitro* Fertilization of *In Vivo*- Matured Oocytes with X-sorted Sperm in Dairy Cows

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Abstract

Since *in vivo* matured oocytes show higher developmental competence compared with in vitro matured oocytes, their use might improve embryo production of in vitro fertilization systems in which sex-sorted sperm is used. We have established an efficient system for the production of female embryos in dairy cows by in vitro fertilization using X-sorted sperm and in vivo-matured oocytes aspirated by trans-vaginal oocyte collection after superstimulation combined with induction ovulation by GnRH administration.

Keywords: in vivo-matured oocyte, sex-sorted sperm, *in vitro* fertilization, superstimulation

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Introduction

The ability to produce calves of the desired sex is an attractive reproductive technology for dairy farmers wishing to breed replacement heifers. Recently, sexed frozen semen is commercially available for artificial insemination (AI) and the accuracy of the sexing procedure has been reported to be close to 90%. However, the potential use of this technology, fertility rates after AI with sex-sorted semen are lower than those achieved with conventional unsorted semen. Ovum pick up (OPU) combined with in vitro fertilization (IVF) has been known to be an efficient method of embryo production compared with AI. To maximize the production female embryos, IVF of in vivo-matured oocytes may be a potent way. In the present report, we introduce an efficient program for the production of female embryos in dairy cattle by IVF using in vivo-matured oocytes collected by OPU and sex-sorted sperm.

Optimum timing of in vivo matured oocytes collection and IVF

Superstimulation of follicles in non-lactating Holstein cows was achieved by CIDR insertion (Day 0), dominant follicle removal on Day 5 and administration of FSH twice a day, starting on the evening of Day 6 until morning of Day 9 in decreasing doses (6, 6, 4, 4, 3, 3, 2, 2 mg; 30 Armor Unit in total). On the evening of Day 8, CIDR removal and the administration of 0.225 mg d-croprostenol was performed. For ovulation induction, 200 μg GnRH were injected on the morning of Day 10 (0 h). Ovulation observation was performed in every 3 h from 20 h to 41 h after GnRH by an ultrasound scanner to fix the optimal timing for aspiration of in vivo matured oocytes and IVF after GnRH on Day 10.

Ovulatory follicles were occurred from 26 h to 38 h after GnRH. The peak of ovulated oocytes was found from 29 h to 32 h after GnRH administration. Based on these results, 25 h to 26 h after GnRH administration was selected for the timing of OPU for the harvest of in vivo-matured oocytes and 30 h (average of 29 h and 32 h) after GnRH administration was selected for the IVF.

Development of an efficient sexed-embryo production system

Embryo production from in vivo and in vitro matured oocytes using commercially available X-sorted frozen-thawed semen was assessed. After dominant follicle ablation (DFA) on Day 5 (DFA group) or 100 μg GnRH on Day 5 (GnRH group), in vivo matured oocyte were collected by OPU at 25-26 h and inseminated at 30 h after GnRH on Day 10. Immature oocytes obtained by OPU of non-stimulated cows (Non-stimulated OPU group).

The highest oocyte recovery (recovered oocytes /observed follicles with more than 5 mm in diameter) was recorded for the in vivo-matured oocyte collection group compared with the non-stimulated immature oocyte collection group ($P<0.05$, 83.5 % vs. 65.8 %, respectively). There was no difference between the DFA group and the GnRH group in numbers of recovered oocytes with expanded cumulus (14.6 and 18.5, respectively). Blastocyst rates among the DFA, GnRH and non-stimulated OPU groups did not differ (58.0 %, 52.8 % and 49.9 %, respectively). However, numbers and rates of high quality blastocysts (Code 1, according to International Embryo Transfer Society) were higher in the DFA group compared with the GnRH group ($P<0.05$, 4.1 and 54.9% vs. 0.9 and 21.5%, respectively). Following sex determination by the LAMP method, 92.1% of embryos were female.

Conclusions

The results of these studies demonstrate that high numbers of produced female embryos and high rates of good-quality blastocysts can be obtained by in vitro fertilization with X-sorted sperm using in vivo matured oocytes collected OPU from dairy cows.

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Review Article

Cutting Edge of Bovine Reproductive Technology: Production of Prion Gene KO Cow to Prevent Spontaneous BSE

Noboru Manabe[1*] Ichiro Onoyama[1] Yutaka Sendai[2] Yoshito Aoyagi[2]

Abstract

In 1986, bovine spongiform encephalopathy (BSE) was found in United Kingdom (UK type BSE, typical BSE, variant type Creutzfeldt-Jakob disease: vCJD). More than 182,000 cattle were sacrificed. In UK type BSE, cattle were given abnormal prion protein (Pr: pathogen of BSE) in the brain of sheep with Scrapie. Orally administered abnormal Pr of BSE cattle causes vCJD infection in human. Cattle derived products are widely used as foods (beef, entrails, milk, tallow fat etc.), drugs and cosmetics (gelatin, collages, bile essence, placenta essence, spleen essence, heparin, hormones, steroids, trypsin, plasmin, protroporphyrin, thrombin, ligamentum, casein, lactpherin, lipids, pentaglycan, elastin, mocopolysaccarides etc.), and medical equipments (seam binding string, artificial blood vessel, artificial heart valvu, dental filler etc.). In 2003, spontaneous BSE (atypical BSE) was found in many countries. Spontaneous BSE is transmissible from cattle to human (monkey). In 2012, United States Department of Agriculture (USDA) reported that spontaneous BSE cow was found in California. Thus, BSE continues to be critical problem in animal science and veterinary science area. To make prion gene homo-knockout (KO) cattle is only solution against BSE problem.The aim of the research was to make prion gene homo-KO cows (Japanese Black cow) and to reveal their characteristics. Firstly, prion gene was knockouted in fibroblast cells prepared from embryo. The somatic cell nucleus of prion gene-hetero KO fibroblast was transferred into oocyte with removal of nucleus (somatic cell nuclear cloning: SCNC). After activation and *in vitro* culture, the blastocysts were transplanted into the uterus. Secondary, prion gene was knockouted in the embryo fibroblast cells. SCNC was performed. Finally, eleven prion gene-homo KO calves were born. Their characteristics were discussed. In conclusion, to produce the prion gene-homo KO cattle is useful and the only way to ensure the safety of bovine derived products (foods, drugs and medical materials).

Keywords: BSE, prion gene, food product, bovine

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Review Article

The Roles of Estrogen Receptors on Follicle Development and Artificial Insemination

Sayamon Srisuwatanasagul

Abstract

Oestrogen regulates reproductive functions through its specific receptor protein which expressed differently in all reproductive tissues. There are two subtypes of oestrogen receptor, the classical one was named oestrogen receptor alpha (ERα) while the newer one was called oestrogen receptor beta (ERβ). Though the two receptor proteins mediated different reproductive regulatory mechanisms, these two oestrogen receptors have some common reproductive functions. In this review, the role oestrogen receptors on reproductive organs were discussed mainly on the ovary and uterus where the dominant subtypes were ERβ and ERα respectively. The correlation of ER in the uterus and different artificial insemination methods were also studied.

Keywords: follicle development, oestrogen receptor, artificial insemination

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Introduction

Reproductive functions in female mammals, including the pig, involve a complexity of interactions regulated by the endocrine system. Under the control of pituitary gonadotropin hormones, the ovarian sex steroid hormones oestradiol (E_2) and progesterone (P_4) are released primarily from the follicle and corpus luteum respectively. During the oestrous cycle, the levels of oestrogen increase and reach a peak approximately at the start of standing oestrus. The high level of oestradiol stimulates the preovulatory surge of the luteinizing hormone (LH) which in turn stimulates the final maturation of the follicle and ovulation. Though, the role of estrogen in reproductive biology is complex, one of the major functions of oestrogens is regulation of cellular proliferation and growth of reproductive tissues. In the ovary, estrogens stimulate granulosa cell proliferation and facilitate the actions of FSH and LH (Richards et al., 1980). Estrogens pass through the target cell membrane by normal diffusion. Within the target cells, it can bind to specific receptors in the nucleus (Tsai and O'Malley, 1994). After binding to the target gene, the estrogen-receptor complex initiates synthesis of specific messenger ribonucleic acid (mRNA) molecules from deoxyribonucleic acids (DNA) in the chromatin. The mRNA is then translocated to the cytoplasm where synthesis of new proteins occurs. Consequently, these synthesized proteins are thereafter actively transported to the nucleus (Guiochon-Mantel et al., 1996) where they are responsible for the biological activities of estrogens in its target tissues. Therefore, the effects of estrogens also depend upon its receptors in the target cells as shown in several earlier studies (Britt and Findlay, 2002; Baker, 2011).

Oestrogen receptor (ER) subtypes: ERa and ERβ

Biochemical characterization has shown that oestrogen receptors are intracellular and belong to the nuclear superfamily of ligand-activated transcription factors. The nuclear receptor proteins are composed of multiple functional domains which are a DNA-binding domain, a steroid binding domain and an N-terminal domain (for review see Lee et al. 2012, Kumar et al., 2011) . In general, two subtypes of ER have been described, ERα and ERβ. The latter was first reported in the rat prostate and ovary (Kuiper et al., 1996). These two ER subtypes have been shown to have structural dissimilarities in the ligand binding domain and the N-terminal transactivation domain (Kuiper et al., 1997) as well as different biological activities related to tissue distribution (Kuiper et al., 1997; Shughrue et al., 1998; Wang et al., 1999; Pelletier et al., 2000; Pavao and Traish, 2001; Kumar et al., 2011). However, in a review article by Muramatsu and Inoue (2000), it was suggested that ERβ may recognize and bind with the oestrogen responsive element at the same site as ERα since the both subtypes have an amino acid identity of 96% in the DNA-binding domain. Moreover, homologies of ERα and ERβ have been identified in species such as human (Mosselman et al., 1996; Enmark et al., 1997; Cowley and Parker, 1999), mice (Tremblay et al., 1997) and fish (Karels and Brouwer, 2003). Several studies have reported organ and/or tissue specific differences in the localization of ERα and ERβ. In the ovary, both ERα and ERβ are expressed in granulosa cells of preantral and antral follicles (Kuiper et al., 1996; Drummond et al., 1999; Drummond et al., 2002). However, the information on the expression of the respective ER proteins in granulosa cells of different follicle sizes especially in pigs is still lacking

Oestrogen receptors and follicle development

As mentioned above that there are two subtypes of ER in the ovary, ERα and ERβ in which ERβ being the dominant form in the ovary (Byers et al., 1997; Brandenberger et al., 1998; Drummond et al., 1999; Drummond and Findlay, 1999; Hiroi et al., 1999; Sar and Welsch, 1999; Slomczynska et al., 2001; Slomczynska and Wozniak, 2001). Since, the level of ERβ mRNA is higher than ERα mRNA in the ovary, ERβ will mainly regulate signaling in the ovary, and the two ERs have some common reproductive functions (Byers et al., 1997; Drummond et al., 1999; Drummond and Findlay, 1999). In order to investigate the specific functions of these oestrogen receptor subtypes, the oestrogen receptor knockout mice have been generated which are called the ERα knockout (ERKO) and ERβ knockout (BERKO) mice. It was shown that the female ERKOs are acyclic, infertile and showed hyperemic ovaries which devoid of corpora lutea.

Folliculogenesis is arrested at the antral stage with large follicles becoming cystic and haemorrhagic (Lubahn et al., 1993; Schomberg et al., 1999; Couse and Korach, 2001; Emmen and Korach, 2003). However, folliculogenesis, ovulation and copora lutea formation can occur in these mice after gonadotrophin treatment (Rosenfeld et al., 2000). Thus, it would appear that some of the ovarian functions could occur in the absence of ER α in the ovary. Regarding the female BERKOs, the fertility was compromised with reduced numbers of offspring/litter, consistent with the reduced numbers of corpora lutea observed (Krege et al., 1998). Moreover, fewer large antral follicles could be observed from the ovaries of BERKOs with the higher apoptosis in large follicles which indicate that ERβ appeared to facilitate follicle maturation from the early antral to the preovulatory stage and that ERβ is essential for normal ovulation but is not essential for female or male sexual differentiation. (Emmen et al., 2005). Therefore, it was shown that ERα and ERβ play the different role in folliculogenesis and that it could be hypothesize that the proliferative actions of oestrogen require ERα, whereas ERβ is important for follicle maturation from the antral stage of development and follicle rupture to ovulation but not luteinisation (Britt and Findlay,

2002; Couse et al., 2005).

Oestrogen receptor and the artificial insemination

Only little was known about the artificial insemination effects on the expression of oestrogen receptor in the reproductive organs. In pig, the pattern of ERα-staining in the uterus after insemination was markedly different to that of the cyclic sows at the comparable stages (Sukjumlong et al., 2004) as a lower level of ERα was observed as early as 20-25 h after ovulation in inseminated sows. The lower levels of ERα in the inseminated sows compared with cyclic could be effects from insemination and pregnancy, i.e. semen and viable embryos. Even though the general view is that oestrogens upregulate ERα, it has also been shown that oestradiol can down-regulate the expression of its own receptor, ERα-mRNA, in the endometrium of ovariectomized gilts (Sahlin et al., 1990) and the ERα protein in the uterus of immature ewes (Meikle et al., 2000). Therefore, it may be the high oestrogen levels in boar semen (Claus et al., 1987) together with embryonic oestrogens at later stages (Claus et al., 1987) that cause a decrease of ERα in the uterus.

When comparing different insemination methods which were conventional artificial insemination (AI), Intrauterine insemination (IUI) and deep intrauterine insemination (DIUI), our earlier study showed higher ERα immunostaining in the glandular epithelium in AI group compared to the others (Tummaruk et al., 2010; Srisuwatanasagul et al., 2013). This demonstrates that different insemination techniques may have the influence on the expression of ERα. It has been demonstrated that oestrogen (E_2) in boar semen can up-regulates steroid receptors in the pig reproductive organs, a small volume of semen used for IUI and DIUI groups might also influence the lower expression of ERα due to the lower amount of E_2. However, the influence of E_2 in boar semen should be different in different reproductive organs and tissues. In the uterus, marked positive staining for ERα was found in the glandular epithelium in AI groups. The glandular epithelium was known to have secretory activities in order to facilitate conceptus survival and development in pigs (Bazer and Roberts, 1983; Gray et al., 2001; Mathew et al., 2011). Therefore, the higher volume of spermatozoa and semen in AI technique may involve in the mechanism of high expression of ERα. On the other hand, in the uterine surface epithelium, high expression of ERα was found in DIUI group compared to AI group. The downregulation of ERα in the surface epithelium observed as soon as 5-6 h after AI might be the results from insemination as described before by sukjumlong et al.(2004). In 1993, ott et al. suggested that the decrease in oestrogen receptor protein was the way the endometrium was able to remain responsive to progesterone and thereby maintain pregnancy. Therefore, the low presence of ERα may be the results of the increasing progesterone levels after ovulation. However, this low presence of ERα in the surface epithelium occurred already at 12 h after insemination. Therefore the amount of semen could also responsible for this as it is shown that oestradiol can down-regulate its own receptor in the endometrium of ovariectomized gilts (Sahlin et al., 1990). Taking together, the artificial insemination showed some influence on the expression of ERα in the pig reproductive organs especially the uterus but with different response in different cell types as shown by our earlier studies.

Conclusions

Oestrogen can mediate varieties of reproductive functions via its receptors, which depends on the different receptor subtypes, different level of expression, and tissue/cell type specific. In the ovary, ERα and ERβ have different roles for follicle development. In addition, other mechanisms such as insemination, volume of semen may have some effects on the expression of oestrogen receptors and which consequently may involve with the successful fertilization.

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Review Article

Artificial Insemination in Horse: A Review

Theerawat Tharasanit

Abstract

Artificial insemination (AI) has become a major tool for accelerating genetic improvement in domestic animals. Development of artificial insemination in horse has gained much interest for breeders, and the technology has now well adapted to equine industry with relatively high success. However, this AI in horse is somewhat prohibited in some breeding registries. This article reviews anatomical and physiological aspects of spermatogenesis, semen collection and processing, semen preservation, techniques for artificial insemination and post-AI management.

Keywords: artificial insemination, sperm, stallion

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Introduction

Horse has strongly linked to humans and also to the wealthiness of animal-based industry. Despite a long history of artificial insemination in horses, the AI technology is being developed and has become the most frequently used for genetic improvement of desired animals. The attempt on AI was first documented in Arabian mare since 1322. To date, artificial insemination has gained public acceptance and also increased levels of interest for horse breeders because this technology provides several advantages over natural breeding. The AI can be used as male-related genetic improvement of the foals. This technology also allows to increase stallion reproductive performance (*i.e.* increase number of foal per year) and it is logistically acceptant if cooled and frozen semen is used. However, the artificial insemination in Thoroughbred is prohibited for some specific reasons by some breeding societies (*e.g.* Jockey Club). Indeed, high susceptibility of stallion sperm to cold stress renders the widespread use of chilled and cryopreserved semen. Mares also have unique reproductive physiology and thus controlling ovarian cycle and ovulation time causes limited applications of AI technology.

Spermatogenesis: anatomical and physiological aspects

Reproductive organs of stallions are composed of several structures including testes, epididymis, vas deferens, accessory glands (seminal vesicles, prostate gland and bulbourethral glands) and other supporting structures. These structures play a central role in spermatogenesis and also quality of the ejaculated sperm. Stallion becomes mature around 18-24 months of age. During this time, gonadotropin releasing hormone (GnRH) drives the spermatogenesis via hypothalamic-pituitary-gonadal axis. This process takes place with the convoluted structure of seminiferous tubules. As similar to other species, spermatogenesis simultaneously occurs in a wave-like fashion as referred to spermatogenic cycle. The complete cycle of spermatogenesis in stallion requires around 57 days and comprises three different phases: mitosis, meiosis and spermiogenesis. The mitosis typifies by dividing of diploid spermatogonial stem cells to undifferentiated and differentiated spermatogonia (type A, intermediate and type B). The type B spermatogonia thereafter divide to primary spermatocytes where the meiotic phase is initiated. The preleptotine spermatocytes escape from blood testis barrier which is formed by two adjacent Sertoli cells. Following meiotic division, haploid spermatids are formed and the spermiogenesis thereafter takes place. This process involves Golgi, capping and acrosomal phases, tail formation and maturation phase and thus elongated/mature sperm are then released/transported to the epididymis via rete testis. Epididymis functions as sperm transport and storage whilst secrets several molecules essentially for final maturation of the sperm. This maturation of functional sperm acquires the ability of the sperm to motile, fertilize mature oocytes and also contribute to fully embryo development. The transit of epididymal sperm takes around 5 to 14 days in stallion (França et al., 2005). The modification of the sperm during epididymal transit involves predominantly the changes of the compositions of sperm membrane. It is worth nothing that the sperm membrane cannot be newly synthesized at this stage, so the sperm membrane has become quiescent (Flesch and Gadella, 2000). Although the mechanism and molecules that integrate to the sperm membrane are still poorly understood, cholesterol/phospholipid enriched small membrane vesicles so called 'epididymosomes' have been identified in stallion epididymis (Sostaric et al., 2008). As a result, the incorporation of the epidymosomes increases cholesterol and is believed to facilitate the sperm membrane stabilization (Saether et al., 2003). Indeed, compositions of sperm plasma membrane are different among species such as human sperm have high amount of cholesterols compared with stallion and boar sperm. The variations in sperm membrane cholesterol/phospholipid contents determine the fluidity of sperm plasma membrane and also the cryopreservability.

Semen collection and evaluation

Sperm to be used for insemination can be collected from ejaculate and also from sperm flush of the involute tubules of the caudal epididymis. However, epididymal sperm have different sperm physiology such that the epididymal sperm are more difficult to artificially induce sperm hyperactivation (capacitation) and acrosome reaction essentially required for fertilization. Only a small proportion of sperm collected from cauda epididymis can undergo acrosome reacted sperm when compared with ejaculated sperm (Rathi et al., 2003). This is likely to involve an inappropriate exposure of progesterone receptors (Cheng et al., 1998) or sperm plasma membrane would need further modifications during uterine and oviductal transits. It is therefore not surprising that the pregnancy rates after insemination via endoscopic-guided technique of epididymal sperm was lesser than that obtained from fresh ejaculated sperm (46% versus 80%) (Morris et al., 2002). Thus, semen to be used for routine insemination will be collected from ejaculate unless this semen collection from live donor would be impossible. In case that the stallion would unexpectedly die, obtaining sperm from caudal epididymis and vas deferens would only be the technique of choice for sperm recovery. Semen collection from live donors can be done by several methods including condom, pharmacologic stimulation, manual stimulation and artificial vagina techniques. Indeed, semen collection by artificial vagina is frequently used worldwide due to its simplicity and cost-effective. Several models of artificial vagina are commercially available such as Colorado, Niskikawa, Missouri and Hannover types. These artificial vaginas share the common physical properties. The ejaculation of the stallion involves

neuromuscular mechanisms and stimulation of this mechanism would need appropriate temperature and pressure during semen collection (McDonnell, 1992). To achieve this condition, the water jacket is filled with 45-50 °C water and the pressure of the AV can be adjusted using increased amount of water or air. The failure of semen collection is often associated with malposition and too low temperature and pressure of the AV. In case with difficult semen collection, the internal temperature can be further increased but this high temperature may result in extensive sperm death. After semen collection, the ejaculate must be filtered through sterile gel filter or gauze. The gel fraction of the semen is predominantly produced from seminal vesicle glands. The gel-free fraction is subsequently examined for gross appearance (*e.g.* volume and color) and basic semen evaluation such as concentration, viability and motility should be performed. Depending on laboratory equipment available, more advance semen evaluation, we have to date, can also be done. However, this advance semen technology requires expensive equipment, experience scientists and thus it may not well suit to the field conditions. The advance technology for semen evaluation includes, for instance, sperm motion analysis, plasma membrane properties and functions, acrosomal status, DNA integrity, mitochondria functionality, proteomic and metabolic assessments (Jasko 1992, Colenbrander et al., 2003). As aforementioned discuss, these sperm parameters are fairly sophisticated and only conventional semen evaluation is commonly used. Although the basic semen evaluation is quite subjective, it is easily assessable and less time consuming. In fact, there is no single sperm parameter can be used to predict the fertility of particular semen and thus combination of several semen parameters is needed (Colenbrander et al., 2003). The semen with poor sperm quality frequently result in decreasing pregnancy rate. By contrast, semen with superior quality cannot be used for guaranteeing the successful fertility of particular stallion since other factors, such as insemination time and post-insemination management are also involved.

Semen preservation

As similar to other domestic species, stallion semen can also be preserved by means of cold storage and cryopreservation. However, such a way of breeding selection by genetic background and fertility trails (progeny test) frequently used for bulls and boars is not totally be used for selection of desired stallions. Breeding of particular stallion is selected primarily based on their genetic background, athletic performance and even personal desirability. Stallions therefore have a wide range in semen characteristics and also cold stress susceptibility. Stallion sperm are very susceptible to cold stress when they expose to temperature between 8°C and 19°C (Amann and Pickett, 1987) because of transition of plasma membrane status from highly liquid (as membrane fluidity) to gel state. In general, semen obtained from only 50% of whole population of the stallions worldwide can be cooled storage. It is therefore suggested that the semen should be tested for cold susceptibility prior to be used for artificial insemination. After semen collection, the semen should be centrifuged to remove excessive seminal plasma and the semen extender is then added to the semen. This semen extender minimizes the cold stress as it provides several advantages such as energy source, membrane stabilization and prevention of microbial overgrowth. To date, several types of semen extender can be purchased or prepared in-house. The common semen extenders used for cold storage are milk- and egg yolk-based extenders. The extended semen would also require slow cooling especially during critical temperature in order to reduce cold shock. If the semen is appropriately cooled, the semen can be stored for at least 24-48 h without remarkable effects on fertility (Jasko et al., 1992). Indeed, approximate 60-70% pregnancy rate can be expected for good semen cooler stallions (Aurich 2008; Battelier et al., 2001). In contrast to the high pregnancy rates obtained from chilled semen, the pregnancy rates after insemination with frozen-thawed semen are generally low when compared with fresh and chilled semen. This is due to the fact that stallion sperm are very sensitive to cold temperature especially during freezing and thawing processes. During cryopreservation, sperm undergo rapid changes in thermal and osmotic stresses and the sperm will be injured at several levels including plasma membrane, mitochondria and DNA. The sperm become supercooled at subzero temperature. As a result, lethally intracellular ice formation is extensively formed. Intracellular concentration of electrolytes is also gradually increased along with decreased temperature (Amann and Pickett, 1987). It is therefore essential that optimal cooling rate during cryopreservation must be employed in order to maintain/maximize the sperm viability and function. Until recently, several freezing protocols and freezing extenders can effectively be used. However, pregnancy rates of mares inseminated with frozen-thawed semen are still highly variable (Samper and Morris, 1998). Due to high susceptibility of stallion sperm to cryopreservation, it has been estimated that only 25% of stallion population are classified to be suitable freezer stallions and the remaining population is classified as either moderate or poor freezers (Amann and Pickett, 1987). It is therefore important to note that stallions with great athletic performance may provide sperm with insufficient quality for semen freezing and genetic back-up using cryopreservation technology seems likely unsuccessful. Frozen-thawed semen to be used for insemination should contain more than 30% progressive motility. Apart from per se quality of stallion sperm after freezing and thawing, insemination regimen is very import to achieve high pregnancy rate since the longevity of these sperm is very poor. Thus, timing of artificial insemination and also insemination technique are critical.

Artificial insemination: technical aspects

Depending on types of preserved sperm to be used for artificial insemination, fresh semen (raw semen) must be inseminated into mare's reproductive tracts within 30-60 min after semen collection as seminal plasma has very little capacity to buffer and provide nutrients for sperm. When the raw semen is supplemented with appropriate semen extender, the sperm longevity can be further extended for about 24-48 h, especially if the semen is kept at low temperature (around 4-5 °C). The ideal dilution of semen for artificial insemination is 25-50 million sperm/ml and it is globally accepted to use 250-500 million progressive motile sperm per insemination dose. However, this insemination dose can also be variable among insemination technique. Mares will need to carefully be checked for reproductive status and diseases. Vaccination and deworming programs should also be rechecked. Mares should be daily examined for the presence of large follicles using rectal palpation and ultrasonography. The ovulation is then estimated using combined parameters including size, shape and contents of preovulatory follicle, degree of uterine edema, and also mare behaviors. The presence of two or more large follicles should also be noted for further twin management. The insemination is then performed one day prior to the expected ovulation time as the sperm can be viable for approximately 24-48 h within the oviduct. However, if poor quality sperm are used, it is advisable to inseminate as close as possible to the ovulation time. For frozen-thawed semen, control of ovulation time is a critical factor in determining the success of AI because of poor sperm viability with extremely short longevity. In practice, it is generally accept to insemination mare within 6 h before or after ovulation. However, ovulation time of mare is highly variable and thus ovulation induction is necessary. Two commercially available agents frequently used for timing the ovulation in mares are human chorionic gonadotropin and gonadotropin releasing hormone agonist (deslorelin). Insemination techniques in mare can be divided into 3 techniques including uterine body insemination, deep uterine horn insemination and hysteroscopic or endoscopic guided deep horn insemination. The deposition of semen through insemination pipette at uterine body is the most common technique for artificial insemination in mare because it is easy to perform, less expensive and also the sperm can be transported to both sides of the uterus. However, this site of insemination is far from the opening of uterotubule junction (UTJ) and a large amount of sperm will be lost during sperm transport. Semen can also be loaded into flexible AI catheter. This catheter can be guided rectally to deeply insert to the tip of uterine horn ipsilaterally to the ovulation site. This technique can bypass the sperm to the UTJ but insertion of this flexible AI pipette may damage the endometrium. The endoscopic guided intrauterine insemination offer a great potential to lower the insemination dose especially for sex sorted sperm. This technique involves the use of flexible endoscope to visualize the UTJ. The small amount (200-500 µl) of semen is then deposited directly onto the UTJ. This technique has proven to obtain pregnancy around 50% if inseminated with at least 1 million sperm (Morris et al., 2000). This technique is rather labor intensive and should be used only for extremely valuable stallion or for sex sorted sperm

Post-breeding management

Post-breeding management is also a critical step to increase the pregnancy rate after artificial insemination. Mares prone to have malformation of vulva lips should be checked for pneumovagina. If this condition is present, Claslick's operation should be performed. The mare must be checked for ovulation and re-insemination may be required 24 to 48 after the first insemination. Small amount of uterine fluid can frequently observed after insemination. This uterine fluid indicates the uterine defense mechanism but large amount of uterine fluid indicate inflammatory reactions or infection. Poor uterine tone and abnormal relaxation of the cervix can also be the causes of accumulation of uterine fluid. As the embryo enters the uterus around day 6 to 6.5 after ovulation (Freeman et al., 1991), it is therefore essential to evacuate this uterine fluid as soon as possible. Severe accumulation of uterine fluid should be treated by flushing the uterus with appropriate antimicrobial agents. Bacterial culture and drug sensitivity should also be performed. Pregnancy can be detected as early as 10 days after fertilization using ultrasonography but embryonic sac is too small and is difficult to visualize (Leith and Ginther, 1984). Routinely, embryonic vesicle can be easily determined by day 13-14 post-ovulation. Twin reduction should be performed prior to the day of embryo fixation (day 16).

Conclusions

Artificial insemination is one of the most importance techniques. This artificial insemination technique involves several steps including collection, evaluation, handling and processing of semen and insemination procedure. Equine sperm are very sensitive to cold stress and pregnancy obtained may be highly viable. Several factors such as individual effect, preservation techniques and synchronization between ovulation and insemination time and post-insemination management are the major factors controlling the pregnancy. Further study to modify semen technology and its management is still required in order to improve reproductive efficiency after artificial insemination.

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Review Article

Cryopreservation of Buffalo Oocytes

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Abstract

The cryopreservation of the female livestock genetics has become an international priority. The buffalo is the major milk and meat producing farm animal in many developing countries. Cryopreservation of buffalo oocytes is very important in conserving the superior genetics for future use. This review presents the cryopreservation of buffalo oocytes including associated problems, source of oocytes cryopreservation, and the future of buffalo oocytes vitrification.

Keywords: buffalo oocytes, cryopreservation, vitrification, slow freezing

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Introduction

Nowadays, buffalo is a source of draft power, transportation, meat, milk and livelihood of the farmers in the world population especially in the developing countries. Successful buffalo breeding highly, depends on the genetic improvement that can be achieved by the application of assisted reproductive technologies (ART). Buffalo oocytes obtained from slaughterhouse derived ovaries are a useful sources for reproductive biotechnology such as somatic cell nuclear transfer (SCNT), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Yet the limited number of buffalo oocytes made it difficult to assess the success rate of these reproduction technologies. From this problem, the topic of "How to store the oocytes for further used?" has been raised several years ago.

Two main methods for cryopreservation of oocytes are slow freezing and vitrification. With the exception of cryoprotectant (CPA) concentration and cooling rate, these two methods differ slightly with regards to equilibration, vitrification and warming, (Vajta and Kuwayama, 2006). However, buffalo and porcine oocytes are more sensitive to chilling and yield poor results following slow cooling (Ledda et al. 2001). In vitrification, several protocols have been applied on buffalo oocytes. In those protocols, however, the basic concept is similar, and the differences between the protocols are related to the vitrification container, the type and concentration of CPA and duration of exposure of CPA In this paper, we reviewed the problems associated with cryopreservation of buffalo oocytes, source of oocytes cryopreservation, and the future of buffalo oocytes vitrification.

Related problems of buffalo oocytes cryopreservation

The major problem with buffalo oocyte cryopreservation is low survival and/or poor developmental competence. Although, the biotechnologies of reproduction have been used in this species, most of them are not as efficient as in bovine. Because of the high intracytoplasmic lipid content, buffalo oocytes are supposed to be particularly sensitive to chilling injuries (Boni et al. 1992). Slow freezing results of immature and matured buffalo oocytes demonstrated that slow freezing is not suitable for immature buffalo oocytes, as proven by both poor maturation rates (18-35%) and development to morulae (4.5%; Gautam et al. 2008a). Vitrification is more effective than slow freezing for the cryopreservation of *in vitro*-matured buffalo oocytes (Gautam et al. 2008b).In order to overcome this problem, several devices have been used for buffalo oocytes vitrification by using very small amounts of solution including French straw (Dhali et al., 1999; Dhali et al., 2000; Wani et al., 2004a; Wani et al., 2004b; Sharma et al., 2007; Boonkusol et al., 2007; Mahmou et al., 2008; Gautam et al., 2008b), solid surface vitrification (SSV, Gasparrini et al. 2007; Boonkusol et al. 2007; Liang et al. 2012a), cryoloop (Gasparrini et al. 2007), Cryotop (Muenthaisong et al. 2007; Attanasio et al., 2010a; Liang et al. 2012a; Liang et al. 2012b), open pulled straw (OPS, Mahmou et al. 2008), microdrop (Liang et al. 2011; Liang et al. 2012b). Several protocols have been applied on buffalo oocytes vitrification as shown in Table 1. In those protocols, however, the basic concept is similar, and the differences between the protocols are related to the vitrification devices, temperature, volum of vitrification solution, the type and concentration of CPA and duration of exposure of CPA.

The main source of oocytes for vitrification is from abattoir ovaries. Follicles aspiration derived oocytes are at the Germinal Vesicle (GV) stage of maturation, which the genetic material is contained at early prophase within the contours of a nucleus and no spindle is present. The GV stage oocytes are assumed to be less liable to microtubular and chromosomal damage. However, efficiency in the vitrification of GV stage buffalo oocytes presented the low ability of embryo development (Dhali et al. 1999; Liang et al., 2012a). The other stage is matured oocyte at the metaphase II (MII) stage. Although vitrification of MII buffalo oocytes worked better than GV (Sharma and Loganathasamy 2007), the following embryo development competence were still lower than the fresh oocytes. In bovine oocytes, CPA and exposure of matured oocytes to low temperatures were reported to cause chromosome dispersal, microtubule depolymerisation (Aman and Parks 1994).

Vitrification of buffalo GV stage oocytes

Oocytes at the GV stage are characterized by extended chromosomes held within a nuclear membrane, the absence of a spindle, cortical granules that are not ready to be released, and a compact cumulus. Buffalo GV stage oocytes were cryopreserved by vitrification in traditional French straws with high post-warming survival but poor maturation rates, which have been described by Dhali et al. (1999,2000) and Wani et al. (2004a). Subsequently, the first successful production of buffalo blastocyst derived from IVM and IVF of vitrified GV stage oocytes was reported by Wani et al. (2004b), although the efficiency remained low. Insufficient cooling rates of oocytes were considered one of the principal obstacles in vitrification. In 2007, Sharma and Loganathasamy proved that the meiotic stage affects survival rates of buffalo cumulus oocyte complexes (COCs) submitted to vitrification / warming, with higher values for those with 24 h maturation compared to those with a shorter one. Later on, Mahmoud et al., (2007) showed that the CPA combination of EG+DMSO gave the highest maturation rate of buffalo GV stage oocytes using either straw or OPS vitrification.

Our previous research showed that Cryotop was better than SSV method in terms of survival rate (82-86% and 71-80%, respectively) after vitrification of GV stage of buffalo oocytes. Nevertheless, both

methods perform equally in terms of *in vitro* nuclear and cytoplasmic maturation of vitrified oocytes. We also revealed that CB does not effectively improve survival, maturation or developmental rates of vitrified GV stage buffalo oocytes (Liang et al., 2012a). The low efficiency of vitrified GV stage buffalo oocytes may be related to the lower penetrability of GV stage membrane when compared with MII stage.

Vitrification of buffalo MII stage oocytes

Because of their higher membrane stability during freezing, MII stage oocytes are still preferred to GV stage oocytes for vitrification. Recently, *in vitro* matured buffalo oocytes have been successfully cryopreserved by SSV and Cryoloop vitrification, as indicated by their capability to cleave and develop into blastocyst stage after IVF (Gasparrini et al. 2007). In another study, parthenote blastocysts were obtained from *in vitro* matured buffalo oocytes vitrified by SSV and French straw vitrification, and the SSV procedure resulted in less damage and better blastocyst development (Boonkusol et al., 2007). One of the most successful vitrification devices is Cryotop that has resulted in excellent survival and developmental rates with human and bovine oocytes (Kuwayama et al. 2005). Cryotop (Muenthaisong et al. 2007; Attanasio et al., 2010a; Liang et al. 2012b) has been successfully utilized to vitrify buffalo MII stage oocytes, which retain the capability to develop into blastocyst following parthenogenetic activation, nuclear transfer, and IVF. Muenthaisong et al. (2007), the first reported successful production of cloned buffalo blastocysts using MII stage oocytes vitrified byCryotop. Recently, vitrified denuded *in vitro* matured buffalo oocytes co-cultured with intact cumulus-oocyte complexes (COCs) during IVF could increase fertilization capability without improving blastocyst development (Attanasio et al., 2010a). In another study, blastocyst production after IVF of vitrified matured oocytes, proved the feasibility of Cryotop in buffalo (Attanasio et al., 2010b). The microdrop method is the containerless and simplest way of vitrification by dropping oocyte containing solutions directly into LN. More than 90% of survival rate and 15% of blastocyst rate have been obtained from microdrop vitrified MII oocytes (Liang et al., 2011). In other research, microdrop and Cryotop methods were equally effective in terms of oocyte recovery, survival, and embryo developmental rates (Liang et al., 2012b).

CPA mixtures may have some advantages over solutions containing only one permeable CPA (Sharma et al., 2007; Gautam et al., 2008b), and a mixture of EG and DMSO has been widely used for buffalo oocyte vitrification (Dhali et al., 1999; Dhali et al., 2000; Muenthaisong et al. 2007; Mahmou et al. 2008; Gautam et a;., 2008b; Attanasio et al., 2010; Liang et al., 2011; Liang et al., 2012b). In other report, bovine (Vieira et al., 2007) blastocysts vitrified in 20% EG + 20% DMSO with glass micropipettes showed better hatching rates than those vitrified in 40% EG with straw. Recent progress in the establishment and improvement of oocyte vitrification methods in buffaloes shown that mixture of EG and DMSO has gained popular in this species.

Cryopreservation is known to induce changes in the zona pellucida (ZP) resulting in reduced fertility rates; however, these structural changes may be overcome by ICSI. Our previous research proven that MII stage buffalo oocytes can be vitrified, fertilized (43%) by ICSI and developed to blastocysts stage (15%) by microdrop (Liang et al., 2011). In the following ICSI study, normal activation has been compromised by vitrification according to the decreased second PB formation, cleavage and blastocyst rates when compared with fresh oocytes (Liang et al., 2012b).

Conclusions

Vitrification technologies applied on buffalo oocytes has become more successful as an alternative to slow cooling method, and MII stage oocytes are still preferred to GV stage for their higher membrane stability during freezing. Protocols applied on buffalo oocytes vitrification are related to the device, the type and concentration of CPA and duration of exposure of CPA. The future research must overcome the low maturation rate of GV stage oocytes, low fertilization and blastocyst rate following oocytes vitrification.

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Table 1 Protocols: container, concentration, time and properties of the buffalo oocytes vitrification.

Author and reference	**Type of container**	**Oocytes stage**	**Equilibration step**	**Vitification step**	**Warming step**
Dhali et al., 1999	straw	GV	2.25M EG+1.7M DMSO 1 or 3 min	4.5M EG + 3.4M DMSO 2 min	0.5M S. 5min
Dhali et al., 2000	straw	GV	I: 2.25M EG+1.7M DMSO 1 or 3 min II: 1.75 M EG 1 or 3 min	I: 4.5M EG + 3.4M DMSO 2 min II: 3.5 M EG 2 min	0.5M S. 5min
Wani et al., 2004a,b	straw	GV	1.5 M of DMSO, EG, PROH and glycerol, respectively, 5 min	3.5,4,5,6,and 7 M of DMSO, EG, PROH and glycerol, respectively, 5 min	0.5M S. 5min+ 0.25M S. 5min+ 0.1M S. 5min
Gasparrini et al., 2007	SSV, CLV	MII	SSV: 4%EG 12-15min CLV:7.5%EG+7.5% DMSO 3min	SSV:35%EG+5%PVP+0.4M trehalose 25-30s CLV:16.5%EG+16.5%DMSO 25s	SSV: 0.3M trehalose 3min CLV: 1.25M S. 1min+0.62M S. 30s+0.42M S. 30s+0.31M S. 30s
Sharma et al., 2007	straw	MII, GV, 6hIVM, 12hIVM, 18h IVM		40%PROH+0.2M trehalose 3 min	1 M S. 15min
Boonkusol et al., 2007	SSV, straw	MII	SSV: 4%EG 5-10min Straw: 4%EG 5-10min	SSV: 35% EG+ 5%PVP+ 0.4M trehalose 25-30 s Straw:40%EG+5% PVP+0.4M trehalose 1 min+ LN_2 vapor 3min	SSV: 0.3M trehalose 1min+0.15M trehalose 2min+0.075M trehalose 2min Straw: 0.3M trehalose 1min
Muenthaisong et al., 2007	cryotop	MII, enucleated MII	7.5% EG+7.5%DMSO 4, 7 or 10 min	15%EG+15%DMSO +S. 1 min	0.5M S. 5min
Mahmou et al., 2008	Straw, open pull straw	GV	1: 3M EG 2: 1.5M EG+1.5M DMSO 3: 1.5M EG+1.5M glycerol 4: 1.5M DMSO+1.5M glycerol 45 s each	1: 6M EG 2: 3M EG+3M DMSO 3: 3M EG+3M glycerol 4: 3M DMSO+3M glycerol 25 s each	0.5M galactose 5 min
Gautam et al., 2008b	straw	MII	1: 10%, 25%, 40% EG each 1min 2:10%,25%,40% DMSO each 1min 3:10%EG+10%DMSO 1min 4: 10%EG+10%PROH 1min	1: 40%EG 1min 2: 40%DMSO 1min 3:20%EG+20%DMSO 1min 4:20%EG+20%PROH 1min	0.5M S. 1min+ 0.33M S. 1min+ 0.17M S. 1min
Attanasio et al., 2010a	cryotop	MII	10%EG+10%DMSO 3 min	20%EG+20%DMSO 20-25 s	1.25M S. 1min+ (0.62, 0.42, 0.31) M S. 30s each
Attanasio et al., 2010b	cryotop	MII	1: 7.5%EG+7.5%DMSO 3 min 2: 10%EG+10%DMSO 3 min	1:16.5%EG+16.5% DMSO 20-25 sec 2:20%EG+20% DMSO 20-25 sec	1.1.25M S. 1min +(0.62, 0.42, 0.31) M S. 30s each 2.0.25M S. 1min+0.15M S. 5min
Liang et al., 2011	microdrop	MII	10%EG+10%DMSO 1 min	20%EG+20%DMSO 30 s or 45 s	0.5 M S. 5 min
Liang et al., 2012a	cryotop, SSV	GV	1: 7.5 µg/mL CB 15 min +10%EG+10% DMSO 1 min 2: 10%EG+10% DMSO 1 min	20%EG+20%DMSO 30 s	0.5 M S. 5 min
Liang et al., 2012b	cryotop microdrop	MII	10%EG+10%DMSO 1 min	20%EG+20%DMSO 30 s	0.5 M S. 5 min

GV: germinal vesicle; MII: metaphase II; S.: sucrose; CLV: cryoloop vitrification;

Optimization of Pre-processing Procedures for Canine Mammary Tumor Oligonucleotide Microarray Meta-analysis

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Keyword: mammary tumor, gene expression profiling data, gene expression microarray meta-analysis, dog

Introduction

Canine mammary tumor (CMT) is common cancer of female dog that can lead to fatal outcome similar to the breast cancer in human. By this means, CMT has recently become the outstanding model for human breast cancer research (1). Many advances in genetic technologies for CMT are greatly innovated including the performance of gene expression microarray on CMT cell. With the increasing availability of publicly accessible CMT canine gene expression profiling data, there is a growing need for integrative computational methods to evaluate these multiple independent microarray datasets. Gene expression microarray meta-analysis is the technique that allows simultaneous analysis of collective data from varied microarray experiments. Nonetheless, collective data obtained from different experiments are inevitably affected by several non-biological sources of variations (2). This addresses the vital need of suitable pre-processing procedure to handle these variations. Since the operation is still limitedly evaluated with collective CMT expression profiles, the current study aimed to optimize the system for CMT microarray gene expression meta-analysis research.

Materials and Methods

Computer system: Bio-Linux-7 operating systems (OS) (3) with 10 GB hard-drive space, 4GB RAM, and Intel Pentium V processor.

Software packages and R script: R suite is freely available at (http://www.r-project.org/). Bio-conductor open source software packages (http://www.bioconductor.org/) used for the study are 'affy' (4), "arrayQualityMetrics" (5), "sva" (6), and "vsn" (7). Global loess normalization script was from Freudenberg (8).

Canine mammary tumor (CMT) gene expression profiling datasets: Three canine gene expression datasets of Affymetrix Canine Genome 2.0 Array platform (Affymetrix, Santa Clara, CA) were retrieved from Gene Expression Onimbus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) to put together total 51 arrays (24 arrays from GSE20718, 23 arrays from GSE22516 and 4 arrays from GSE25586). These arrays were from four cell sources as follows: (i) normal mammary cells (n=12), (ii) breast cancer cells (n=35), (iii) breast cancer cell line (n=2), and peripheral blood leukocytes (n=2).

Pre-processing performance: Pre-processing performance was categorized into 5 steps as following, (i) Background correction, (ii) Normalization, (iii) PM correction, (iv) Batch effect correction and (v) Summarization. The methods implemented in each processing step were described in Table 1.

Table 1 Methods used in pre-processing performance.

Step	Methods[a]
Background correction	None; RMA; MAS
Normalization	None; Quantile; VSN; Global Loess
PM correction	None; MAS
Batch correction	None; COMBAT
Summarization	TBI, MP

[a]*COMBAT - Combating Batch Effects When Combining Batches of Gene Expression Microarray Data, Global Loess – Global Loess normalization, MAS - MAS 5.0, MP – Medianpolish, None – No treatment applied, Quantile - Quantile normalization, RMA - Robust Multi-array Average correction, TBI - Tukey Bi-Wight, VSN - Variance Stabilization and Normalization*

Data quality assessment: The raw and pre-processed data's qualities were assessed by the use of quality metrics for diagnosis and outliner detection (Table 2)

Table 2 Diagnostic methods and statistics for outliner detection.

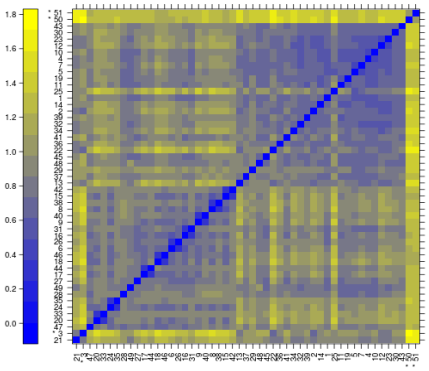
Section	Diagnostic methods	Statistics for outliner detection
Between array comparison	Distances between arrays Principal Component Analysis	Sum of the distances Dissimilarity between arrays
Array intensity distributions	Boxplots Density plots	Kolmogorov-Smirnov statistic Shifting of distribution
Variance mean dependence	Standard deviation versus rank of the mean	Trade-off from zero standard deviation
Individual array quality	MA plots	Hoeffing's D-statistic

Results and Discussion

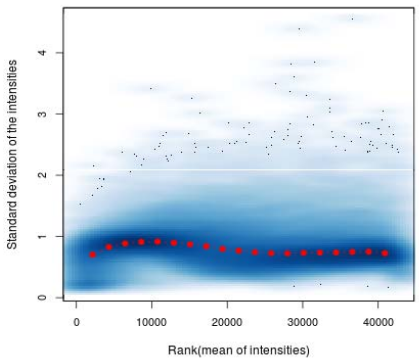
With the proposed pre-processing procedures, 18 pre-processed datasets were prepared for quality assessment and comparison. The raw datasets regarded as non-processed controls received no treatments but summarized for each single gene expression value using both Tukey Bi-Wight and Medianpolish approaches (resulted in 2 raw datasets). All raw and pre-processed datasets acquired from all treatment protocols were determined for their qualities using quality metrics (5). Numbers of array samples considered as outliners were reported and summarized in Table 3. The three combined treatments generated lowest numbers of outliners as following: (i) Global loess normalization without background correction summarized by Medianpolish method; (ii) VSN normalization without background correction summarized by Medianpolish method; and (iii) VSN normalization MAS5.0 background correction summarized by Medianpolish method. To identify the best treatment, heatmap of distances between arrays and Standard deviation versus rank of the mean plot were determined. According to the result, the (i) Global loess normalization implemented methods provided the most biologically reasonable clusters of arrays (Fig. 1a) and the most constant running median of standard deviation (Fig. 1b).

Table 3 Number of outliner samples identified by quality metrics.

Normalization method	No Background correction		RMA Background correction		MAS 5.0 Background correction	
	TBI	MP	TBI	MP	TBI	MP
Global	34	3	40	7	30	4
Quantiles	4	4	5	5	4	5
VSN	6	3	27	4	7	3
None	37	33	-	-	-	-



1a)



1b)

Figure 1 False color heatmap of the distances between arrays (1a) and Standard deviation versus rank of the mean plot (1b) of global loess treated data. Color scale of the heatmap is chosen to cover the range of distances encountered in the dataset. Clustering of the arrays among arrays of normal cells (blue), tumor cells (yellow) was noticeable (1a). For Standard deviation versus rank of the mean plot, the running median of standard deviation (red line) was also approximately horizontal in (1b). This indicated the good data quality due to no obvious variance-mean dependence of the expression intensity.

Pre-processing performance in expression microarray analysis was determined to deal with non-biological variations among arrays with the major purpose to establish a single, potentially background corrected, and normalized expression value per gene for subsequent analysis. The current study implied Global loess normalization, without background correction, summarized by Medianpolish method most suitable combination for the collective CMT meta-analysis. It should be noted that these assumptions only based on analytical environment provided in this study. Individual optimization of pre-processing procedure is still strongly recommended for every other meta-analysis experiment.

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Cholesterol Loaded Cyclodextrin Improves Sperm Survival in Tris-Based Extender with Less Egg Yolk Used in Buffalo Bulls Semen

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Keywords: cholesterol, sperm, egg yolk, cryopreservation, buffalo

Introduction

Cryopreservation of gametes allows the long-term availability of genetic resources and hence plays a key role in reproductive biotechnology, e.g., artificial insemination (AI). However, the process of cryopreservation (e.g. cooling, freezing and thawing) compromises the quality of processed semen (1) in term of reduced sperm motility and viability, deterioration of plasma membrane, acrosomal and DNA integrity of cattle bull (2, 3) and buffalo bull (4). Sperm cells face osmotic and thermal shocks that occur during the dilution, cooling, equilibration, freezing and thawing procedure (5). Moreover, equilibration results in a considerable loss in motility of buffalo sperm (4). Toxicity due to cryoprotectants (6) and amino acid oxidase activity of dead sperm (7) has also been reported. Although egg yolk (EY) has protective effect against cold shock but also stimulates enzyme (amino oxidase) activity of spermatozoa (7). Higher level of egg yolk possesses greater risk of bacterial contamination and endotoxins production (8). Additionally, components of egg yolk cause hindrance in sperm respiration thus it is needed to exclude those components from egg yolk or replace the cryoprotective component with other sources (9).

Cholesterol is an integral part of sperm plasma membrane which plays key roles in various sperm functions, including effects on stabilization of membranes at low temperatrue. It is reported that cholesterol efflux occurs during cryopreservation, phenomenon known as cryo-capacitation (10), affects the integrity of sperm membrane. Indeed, increasing the cholesterol content of sperm membrane by treating it with cholesterol-loaded cyclodextrin (CLC) prior to cryopreservation resulted in improved sperm cryo-survival rate in stallion (11), bull (12), ram (13), buck (14) and bison (15) sperm. Therefore, the objective of present study was to replace fraction of egg yolk using CLC and observe its effect on sperm quality parameters before and after freezing and thawing in buffalo bulls.

Materials and Methods

CLC was prepared as described previously by (16). Semen was collected from three mature buffalo (Nili-Ravi) bulls maintained at semen production unti Qadirabad-Pakistan. Two ejaculates per bull, twice a week (replicates=3) were collected by an artificial vagina at 42°C. Each ejaculate was transferred within a minute to laboratory for evaluation and kept at 37°C until processed. The ejaculates from each bull possessing >70% motility and >350x10^6/ml sperm were pooled. Sperm concentration was determined by spectrophotometer (IMV, France). The pooled semen sample was divided into two aliquots (control & CLC group). Both aliquots were diluted to 120 × 10^6 cells in 1 ml of Tris-citrate buffer (prepared as described by (17). The control group remained untreated and CLC group was treated with 4mg CLC and incubated for 15 minutes as described by (Purdy and Graham 2004). Control group was then diluted with Tris-citrate buffer containing 40% EY and 14% glycerol to make final dilution with 20% EY and 7% glycerol shown as 20/0. The CLC treated sample was diluted 1:1 (v:v) with Tris-citrate buffer containing 20% EY and 14% glycerol to make final dilution with 10% EY and 7% glycerol shown as 10/4. Samples were cooled to 4°C in 2 hours and equilibrated at 4°C for further 2 hours. Before freezing the samples were analyzed for sperm assays (described later). Semen was packaged was done into 0.5 ml French straws, frozen in liquid nitrogen

vapor 4cm above liquid surface for 10min, and plunged into liquid nitrogen for storage. Straws were thawed in a 37 °C water bath for 40 s, prior to post thaw analysis. After thawing, sperm assays were performed as described below. The subjective motility % was assessed under phase contrast microscope (400×) by taking semen sample on pre warmed (37°C) glass slide. Sperm viability was assessed via eosin-nigrosin staining method. Plasma membrane integrity (PI) was assessed by hypo-osmotic swelling (HOS) test and normal apical ridge (NAR) were assessed with 1% formal citrate solution (method described by (Rasul et al. 2000). The data were analyzed using PROC GLM procedure of SAS Enterprise Guide (version 4.2)

Results and Discussion

The data on visual motility, viability, plasma membrane integrity and NAR before freezing showed no difference (p>0.05) between groups (Fig. 1).

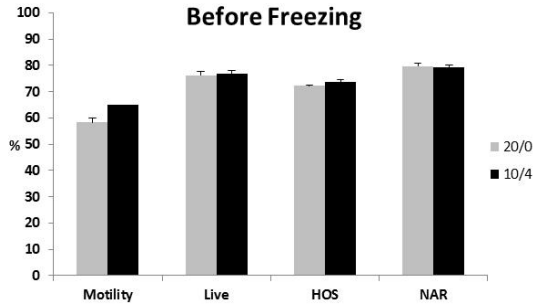

Figure 1 Sperm motility, viability, plasma membrane integrity (HOS) and normal apical ridge (NAR) before freezing in control (20/0) and CLC (10/4) group.

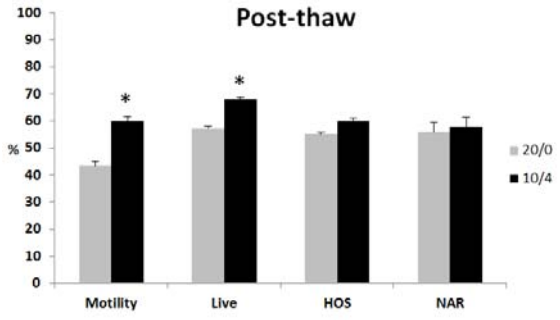

Figure 2 Sperm motility, viability, plasma membrane integrity (HOS) and normal apical ridge (NAR) after freezing and thawing in control (20/0) and CLC (10/4) group.

However, the CLC group showed higher percentage (65% ± 0.66%) of motile sperm as compared to control (58.34% ± 0.66%). Similar trend was seen in viability and plasma membrane integrity. Upon freezing and thawing, the CLC pretreatment significantly (p<0.05) increased the motility (60±0.00 and 43.33±1.66%) and live sperm (68± 1.73 and 57.33± 0.66%) in CLC and control group respectively (Fig. 2).

The CLC group also resulted in high percentage of plasma membrane integrity (60% ± 3.05%) and NAR (57.66% ± 1.15%) as compared to control group (PI = 55% ± 1.00% and NAR = 56% ± 3.51). Sperm pretreated with CLC resulted in improved cryo-survival rate as described in stallion (11), bull (12), ram (13), buck (14), and bison (15). As the buffalo sperm are more sensitive to cryopreservation as compared to cattle (19), so there is need to modify the cryopreservation protocols. Our results showed that addition of CLC in egg yolk extender can reduce the inclusion rate of egg yolk. It protects the sperm before and after freezing and thawing. Sperm motility and membrane integrity parameters were maintained in CLC group. The exact role of EY for protecting sperm is unknown (18). EY in higher concentration (20%), is responsible for deleterious effects (7). Our study demonstrated the useful effect of CLC when used with less egg yolk in the extender. This effect is due to incorporation of cholesterol in membrane of sperm which maintained the membrane integrity which resulted in higher survival rate before and after freezing and thawing. It is concluded that CLC can be a substitute of egg yolk in Tris-based extender. Further studies are needed to observe interactive effect of CLC and egg yolk for better protection of sperm during cryopreservation.

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PCR Detection of Porcine Circovirus Type 2 DNA in the Ovarian and Uterine Tissues of Gilts with Reproductive Disturbances

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Keywords: PCV2, reproduction, ovary, uterus, gilt

Introduction

Porcine circovirus type 2 (PCV2) is the main causative agent of porcine circovirus-associated disease (PCVAD) (1). The PCVAD involves many clinical symptoms in pigs including reproductive failure. The common features of PCV2 associated reproductive failure consists of abortions, stillbirth and mummification (2). Furthermore, PCV2 has also been detected in the oocyte, oviduct, follicular fluid and uterus of the sows under experimental conditions (3). However, under filed conditions, the PCV2 has often been detected in swine breeding herds without any clinical symptoms (4). To our knowledge, no comprehensively study on the association between reproductive disorders and PCV2 DNA detection in replacement gilts has been done. The objective of the present study was to determine the association between reproductive disorders in replacement gilts and the detection of PCV2 DNA in their ovarian and uterine tissues.

Materials and Methods

In total, 172 paraffin-embedded tissues from 70 ovaries and 102 uteri of slaughtered gilts obtained from our previous study (5) were included. The gilts were culled due to anoestrus (n=28), abortion (n=7), repeated service (n=7), abnormal vaginal discharge (n=40), and non-reproductive causes (n=20). The age and body weight at culling was 280±37 days and 144±18 kg, respectively. The paraffin embed tissues were cut by sterile blade and submitted for DNA extraction processes. The viral DNA was extracted by using a commercial extraction kit (NucleoSpin® RNA virus, MACHEREY-NAGEL, Germany). PCV2 detection was carried out by PCR technique. The forward primer sequence was ATG CCC AGC AAG AAG AAT GGA AGA AG and reverse primer sequence was AGG TCA CTC CGT TGT TGT CCT TGA GAT C. The PCR condition consisted of denaturing at 95 ^{o}C for 20 sec, annealing at 55 ^{o}C for 30 sec and extension at 72 ^{o}C for 45 sec. The procedure was repeated for 35 cycles to amplify 350 bp product. The statistical analyses were carried out by using SAS. Categorical data were expressed as percentage and were compared by using Chi-square test. The values of $p<0.05$ were considered statistically significant.

Results and Discussion

PCV2 was detected in 45% (46/102) of the uterus and in 30% (21/70) of the ovary. The percentages of PCV2 DNA detection in the ovarian and uterine tissues of the gilts classified by a noticed clinical symptom of the gilts before culling (i.e., the reason for culling) are presented in Table 1 and 2, respectively.

Table 1 Percentage of PCV2 detection in uterine tissues of gilts (n=102) by clinical symptom.

Clinical symptom	n	PCV2 positive uterus
Anestrus	28	15 (53%) [a]
Abortion	7	6 (85%) [c]
Repeated service	7	2 (28%) [ab]
Vaginal discharge	40	19 (47%) [ac]
Non-reproductive	20	4 (20%) [b]

Different superscript within column differ significantly ($p<0.05$).

Table 2 Percentage of PCV2 detection in ovarian tissues of gilts (n=70) by clinical symptom.

Clinical symptom	n	PCV2 positive ovary
Anestrus	28	9 (32%) [a]
Abortion	7	4 (57%) [a]
Repeated service	7	1 (14%) [a]
Vaginal discharge	8	2 (25%) [a]
Non-reproductive	20	5 (25%) [a]

Different superscript within column differ significantly ($p<0.05$).

PCV2 was detected in both normal (17/46) uteri and in uteri with congenital abnormality (22/41), endometritis (6/9), and miscellaneous (1/6). Likewise, the PCV2 DNA was also found both the normal ovaries (14/53) and in the ovary with, single cyst (1/5), multiple cyst (4/9), and miscellaneous (2/3). This wis in agreement with PCV2 DNA detection in the fresh ovaries and uteri in sows (3). Thus, the detection of PCV2 in both the uteri and ovaries was not surprised. Nevertheless, this finding confirmed a strong association between PCV2 and reproductive disturbances in gilts. A previous study found that PCV2 caused acute and chronic vasculitis of ovarian arteries (6). In the present study, PCV2 DNA was detected in gilts with various reproductive problems. This indicated the risk of PCV2 associated reproductive failure in the replacement gilts under filed conditions. Additional studies should be carried out to carefully determine the influence of the PCV2 DNA on the ovarian and uterine function of replacement gilts.

In conclusion, PCV2 DNA was detected in 45% of the uterus and in 30% of the ovary of gilts culled due to reproductive disturbances under field conditions.

Acknowledgement

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Dynamic Changes of GFRα-1 Expressing Testicular cells in Pre- and Post- Pubertal Cats

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Keywords: spermatogonial stem cells, gonocytes, testis, cat

Introduction

Spermatogonial stem cells (SSCs) are derived from primitive male germ cells or gonocytes. They reside at the basement membrane of seminiferous tubules within the testis. Spermatogonia are the progenitor cells for the spermatogenesis. The activity of these cells is controlled by SSC microenvironment which is formed by Sertoli cells and other supporting testicular cells. Our recent study demonstrated that, the SSCs from pubertal cat could be successfully isolated and cultured for only 57 days (1). In addition, age of cat has been postulated to influences on the self-renewal capability of SSC by unknown mechanisms (2). Until recently, glial cell-derived neurotrophic factor (GDNF) is an essential factor that promotes self-renewal of SSCs. The GDNF family receptor α-1 (GFRα-1) is the co-receptor with RET tyrosine kinase for GDNF. The objective of this study was to examine transitional phase of testicular germ cell development between pre-and post-pubertal age of cat.

Materials and Methods

The testes of domestic cat (*Felis catus*) were collected and categorized into 2 groups: group A (0 to 6 months (n=3)) and group B (>6 months (n=3)). All testes were performed with conventional histology (H&E staining) and immunohistochemistry (IHC) with rabbit polyclonal GFRα-1 (1:50, Abcam, MA, USA) and Dako REAL™ EnVision™ Detection System (Dako, Glostrup, Denmark).

For quantitative analysis of SSC phenotype, the testes were digested, fixed and stained with 2-step immunofluorescence (GFRα-1/Alexa Fluor® 488 goat anti-rabbit IgG (H+L) antibody (Molecular Probes, CA, USA). Consequently, the cell suspension was introduced and quantitatively analyzed using BD FACSCalibur™ (Becton Dickinson, NJ, USA). The results were demonstrated using descriptive analysis.

Results and Discussion

Testicular histology in group A showed no different spermatogonia in the seminiferous tubules. Seminiferous tubules of group A testes presented only 2 types of germ testicular cells (gonocytes and undifferentiated spermatogonia or SSCs) with primitive Sertoli cells. However, the number of gonocytes was decreased by increasing the age. This coincided with the presence of SSCs which had condensed chromatin and darken cytoplasm. For group B, gradual development of spermatogonia were observed. The differentiating spermatogonia entering to the meiosis transformed to spermatocytes, spermatids and spermatozoa, respectively. Complete spermatogenesis was observed at 7 months of age. This is in an accordance to the duration (46.8 days) of cat spermatogenesis (3). GFRα-1 was detected in seminiferous tubules of group A and B. This GFRα-1 typically expressed at plasma membrane and cytoplasm of spermatogonia.

However, the percentages of GFRα-1 positive cell in group A (14.41±7.18%) was significantly greater than group B (0.66±0.56%). Therefore, the results indicated the pre-pubertal age may be suitable for successfully SSC isolation and *in vitro* culture. Alike in mouse, this transition timing between gonocyte and SSC in testis is critical for establishing mouse SSC culture (4,5). This transition phase is important to eliminate undefined factors from testicular somatic cells of pre- and post-pubertal ages. The analysis of germ cell development in pre- and post-

pubertal cats is essential for enrichment of the SSCs in domestic cats.

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Effect of Immunization Against Gonadotropin Releasing Hormone (Improvac®) on Reproductive Functions in Rusa Deer Stags (*Cervus timorensis*)

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Keyword: GnRH vaccine, GnRH antibody titer, serum testosterone concentration, semen quality

Introduction

In Thailand, overpopulation of some wildlife species, particular cervidae family occurs in some zoo and wildlife breeding center. It is effected to available living area, food supply and natural behaviour. In breeding season, the stags are aggressive. They fight together and leaded to a serious injury. These stags, sometime, attack to other deer and also keeper too. In some population, the founder has few numbers. The un-wanted breeding continues. Traditional animal sterilization is surgical method (1). However, deer castration is difficult caused by anesthesia limitation. In some species, this procedure makes some disadvantage such as truma, production loss and potential death (2). The application of vaccination against natural gonadotropin-releasing hormone (GnRH) has been proposed as an alternative to surgical castration of various species including of companion animal (1), domestic species such as ram, stallions, bull, swine (3) and wildlife (4, 5). The function of GnRH vaccine is to create an immunological barrier between the hypothalamus and the anterior pituitary gland. The antibody binds to GnRH in the hypothalamo-hypophyseal portal circulation, which blocks GnRH from binding receptors on pituitary gonadotropes and subsequent to suppress gonadotropin releasing (5). The purpose of this study was to investigate the efficacy of GnRH vaccine (Improvac®) in the rusa deer on antibody concentrations, plasma testosterone concentrations and semen quality.

Materials and Methods

Ten rusa deer stags were used for this study, which comprised two experiments containing a control (n=3) and vaccinated group (n=7). Seven stags were vaccinated subcutaneously with 2 ml of commercial GnRH vaccine (Improvac®, Pfizer, Australia). Three times injection in every 4 weeks (at week 0th, 4th and 8th) were done. Then, the booster after remarkable decreased of GnRH antibody level (at week 29th) was done. Blood samples were collected from each stag prior to initial vaccination for 2 weeks. Then, every two weeks collection from week 0th to week 12th, and then at week 20 th, 29 th, 31 th, 34 th, 40 th, 42 th, 47 th and 52 th were done. Serum was separated by centrifugation and frozen at -20 ^{O}C until analysis.

The semen collections were conducted during 2 weeks before vaccinations and again during after vaccination (at week 8 th, 42 th and 52 th). In non-breeding season, during week 8 th to week 42 th, semen was not collected. Semen collection and evaluation were performed as described by Monfort et al. (1993) (6). Values of the sperm motility and sperm concentrations of control group and vaccinated group were analyzed by using *t*-test.

To evaluate the GnRH antibody, indirect ELISA was used. Briefly, 96 well microtitre plates (Maxisorb, Nunc, Denmark) were coated overnight at 4 °C with 1.18233 µg/ml LHRH human acetate salt (Sigma-Aldrich®) in bicarbonate coating buffer pH 9.6. Plates were washed twice with washing buffer and blocked with 1% BSA for 90 min at 37 °C. Then, blocking solution was poured out. The Rusa deer serum samples were assayed in duplicate. The serum was diluted 1:100 in blocking solution and incubated for 60 min at 37°C. Two negative

controls were run on each plate: one negative control was buffer without rusa deer serum and other was non-vaccinated rusa deer serum. The high-titer rusa deer serum was served as positive control. Plates were washed and diluted with protein A which was labeled with horseradish peroxidase (KPL, USA) (1:5,000) in blocking solution and incubated for 30 min at 37°C. Plates were washed again and the reaction color was developed with tetramethylbenzidine (SureBlue™ ,KPL,USA). Optical density was determined at 650 nm. Absorbance values of GnRH antibody titer were reported. The samples had higher absorbance than the cut-off values were classified as positive sample.

The testosterone concentrations in serum were determined by using competitive enzyme immunoassay according to Brown et al (2004) (7). Antibody for testosterone analysis (polyclonal anti-testosterone R156/7, 1:16,700 dilutions) was obtained from Jo Corbin (Department of population Health and Reproduction Clinical Endocrinology Laboratory, Ca, USA). Standard ranged from 23 pg to 6,000 pg/ml. Serum samples were diluted 1:5 -1:16 in assay buffer. The intra- and inter assay coefficients of variation were 8.35 % and 14.20 % (n=8 plates).

GnRH antibody titer and plasma testosterone concentrations were analyzed as repeated measures analysis of variance. The correlation of GnRH antibody titer and testosterone concentrations from all time point was determined by correlation test.

Results and Discussion

Our research started the experiment in breeding season. Vaccinated stags were seronegative for GnRH antibody prior to the first vaccination. Then, stags in vaccinated group were immunized with the first vaccination in April and boost in May, June and October. After first vaccination, seropositive for GnRH antibody in vaccinated stags were detected (Fig 1b). Then, after second vaccination, the antibody titers reached peak (during June to July or week 8th to 12th). After fourth vaccination, in November, the highest level of antibody was detected again (week 31th to 34th). Two peaks of antibody titer of vaccinated group were significantly higher than control group (p<0.05). Low level of GnRH antibody in control group was found throughout the experiment (Fig. 1a).

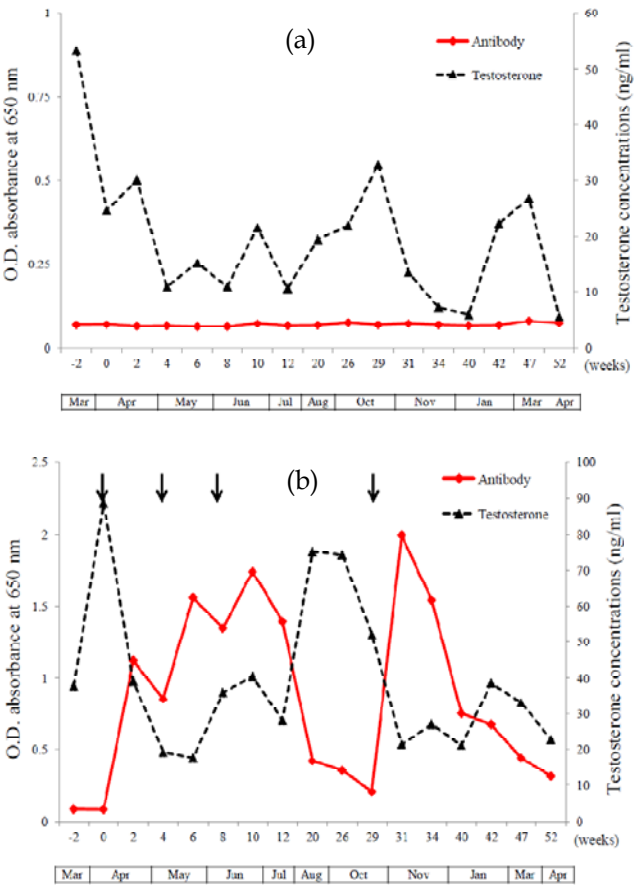

Figure 1 Mean of the optical density (O.D.) of GnRH antibody titer and testosterone concentrations of control stags (a) and vaccinated stags (b).

Serum testosterone concentrations were not significantly difference by using group and time comparison. In vaccinated group, testosterone concentration was negatively correlated with GnRH antibody titer (r = -0.25). Vaccinated group showed a temporary decrease in serum testosterone during week 2th to week 12th after the first vaccination and during week 31th to 40th after the fourth vaccination. For semen quality, none of vaccinated stags produced sperm after the third vaccination (at week 42th) (p<0.05). At week 52th, vaccinated group also showed low percentage of progressive motility and had lower sperm concentrations (p<0.05) compared with control animal. In control stags, they had normal semen quality.

In this study, GnRH vaccine (Improvac®) produced the high antibody titer in rusa deer stags. This characteristic was similar to a variety of species including of goat (8), rams (9), White-tailed deer [4], Black-tailed deer (10) and stallion (11). In this study, the presence of antibody titer

caused a reduction of gonadotrophic hormones and probably subsequent to neutralizes antibody of GnRH from hypothalamus (12). Antibody response in this study lasted at least 3 months after the third vaccination. Similarly, previous study in rams immunized with Improvac® vaccine twice 3 weeks apart revealed the prolong antibody titer until 3 months after the second vaccination (9). Serum testosterone concentrations of vaccinated group were suppressed after the first vaccination for 4 weeks. Similarly, the previous study of testosterone concentration in vaccinated stallions after the second vaccination was reported (11). High GnRH antibody titer resulted in the subsequent reduction of testosterone (13). The transient suppression of testosterone concentrations resulting in effects on sperm concentrations and sperm motility. These results indicated that testosterone necessary to spermatogenesis and sperm maturation in testis (13).

In conclusion, the application of GnRH vaccine in rusa deer stags was raised GnRH antibody titer, decreased testosterone concentration and reduced sperm concentration and motility.

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Canine Prostatic Specific Esterase (CPSE) in Association with Age and Prostatic Volume

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Keywords: canine prostatic specific esterase, hyperplasia, prostate, serum marker

Introduction

The presence of the prostate is universal in mammals but it is more important in humans and dogs because prostatic disorders observed in these two species spontaneously develop and the incidence markedly increased with age. Canine benign prostatic hyperplasia (BPH) is correlated to age and is commonly diagnosed in middle to advanced age (>5 years old) (1). In veterinary practice, presumptive diagnosis of BPH includes the clinical sign, digital rectal plapation and prostate imaging (radiology and ultrasonography). Although prostate biopsy is a definitive diagnosis providing detailed evaluation of tissue architecture (2), the technique is invasive and required general anesthesia. For many years, prostate-specific antigen (PSA) testing has been routinely used to identify the prostate changes in humans and men over the age of 50 are recommended to be annually screened with a PSA test. Measurement of PSA is a non-invasive, sensitive and specific biomarker for detecting and monitoring prostatic diseases and cancer (3). The canine prostatic specific esterase (CPSE), similar to PSA, is a protein synthesized and secreted by the prostatic epithelial cells (4). Recently, an enzyme-linked immunosorbent assay (ELISA) test for CPSE (Odelis®, Virbac Animal Health, Suffolk, UK) has been developed and available in small animal practice. Although the manufacture suggests the cut-off point of CPSE values in normal and BPH dogs (61 ng/mL), there remain important areas of uncertainty regarding the effects of age and prostatic volume on CPSE as human PSA is correlated with ageing (5) Crawford, 1993) and prostatic volume (3, 6). The objective of this study was to investigate if, in clinically healthy dogs, the CPSE level is affected by age and prostatic volume.

Materials and Methods

Intact male Beagles with known birth dates were included in the study. Physical examination, digital rectal palpation, and ultrasonography were performed on each dog. Dogs showing any congenital anomalies or clinical symptoms of the urogenital system including those diagnosed as having prostatic cysts, abscess, prostatitis or prostatic cancer were excluded. Blood samples were collected and serum concentrations of CPSE were measured in duplicate using an ELISA test kit (Odelis®) with the sensitivity of 97.1% and specificity of 92.7%. The prostate sizes were measured by transabdominal ultrasound scan. The length (L), width (W) and dorsoventral diameter (D) of the prostates were recorded after blood collection. The prostatic volume was calculated from the formula previous reported (6); prostatic volume (cm^3) = [(L×W×D)/2.6] +1.8. Data were statistically analyzed using Pearson correlation coefficients.

Results and Discussion

The study population consisted of 28 Beagle dogs with a mean age (±SD) of 5.7±1.6 years (range, 1.7 to 8.2) and a mean body weight of 11.2±2.2 kg (range, 7.2 to 17.8). The linear relationship between age and CPSE concentrations (r=0.46, $p=0.013$) as well as prostatic volume and CPSE concentrations (r=0.49, $p<0.01$) were observed in this study. In addition, a positive correlation between age and prostatic volume was shown; prostatic volume increased with age (r=0.59, p=0.0008). Our findings are in line with previous reports in human showing the relationship between age and serum PSA as well as between prostatic volume and serum PSA (3, 5, 7, 8).

Table 1 Mean (±SD) and range of age, prostatic volume and CPSE in clinically healthy intact male dogs (n = 28).

Variables	Mean±SD	Range
Age (year)	5.7±1.6	1.7-8.2
Prostatic volume (cm^3)	13.5±4.0	6.2-24.7
CPSE (ng/mL)	103.5±56.4	10.0-197.6

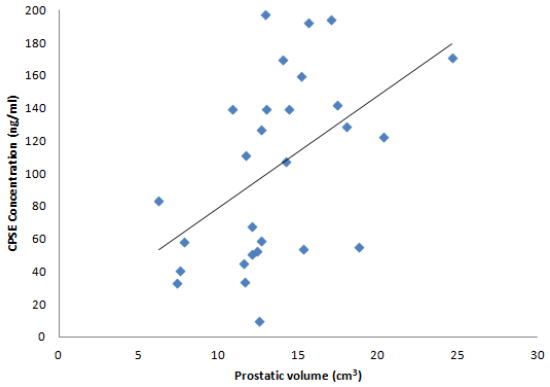

Figure 1 The positive correlation between CPSE concentrations and prostatic volume (r=0.49, *p<0.01*)

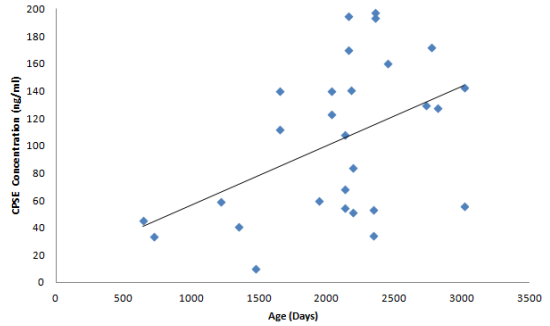

Figure 2 The positive correlation between CPSE concentrations and age (r=0.46, *p=0.013*)

The results observed in this study suggested that, in clinically healthy dogs, serum CPSE level is affected by age and prostatic volume. Although the manufacture's recommendation suggests that the cut-off point of CPSE for BPH dogs is >61 ng/mL, an average of 103.5 ng/mL was detected in this study involving 28 healthy dogs free of any clinical signs of BPH. This suggests that evaluation of CPSE in asymptomatic (subclinical) and symptomatic (clinical) BPH in a clinical trial should be established. However, because the mean age of animal used in our preliminary study was in the middle age (5.7±1.6), a certain number of them may have developed subclinical BPH. Further study on prostatic volume in relation to CPSE in normal prostates will provide better knowledge of the canine prostate physiology regarding the serum CPSE concentration. A positive correlation of serum CPSE and prostatic volume observed in this study indicates that CPSE is one of the valuable tools in diagnosing BPH and monitoring the prostate size during ongoing treatment of BPH in the dog.

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Relationship among Average Daily Gain, Age at First Boar Contact, and Age at First Observed Estrus in Replacement Gilts Raised under Tropical Climate

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Keywords: average daily gain, boar contact, puberty, gilt

Introduction

The major productivity of the swine breeding herds results from the replacement gilts since they occupy the highest proportion in the farm. Approximately 50% of the sow removal takes place annually. The substitution by the replacement gilts has to be conducted to maintain the number of sow on production in the herd. Accordingly, reproductive performance of them dramatically effects on the overall herd productivity (1). Puberty attainment is the starting point of the complete reproductive capability. It is defined only when the first standing estrus and first ovulation happen (2). However, an exact age at puberty is difficult to determine, instead, age at first observed estrus is utilized to identify puberty in pigs (3). Under tropical region, average daily gain (ADG) affects swine reproductive performance; those having low ADG tend to have low conception rate and be culled from the herd finally (3). In addition, boars are crucial since they were always taken to examine whether the pigs are in estrus. The present study aims to study the influence of ADG and age at first boar contact on age at first observed estrus of the gilts.

Materials and Methods

In total, 766 replacement gilts, consisting of 351 Landrace (L), 228 Yorkshire (Y), and 187 LY gilts, were included. All the gilts were accommodated in an evaporative cooling system of a commercial swine herd in the northeastern Thailand. They were fed by 3 kg/day/gilt. The gilts were categorized on the basis of ADG and age at first boar contact (Fig 1). ADG was calculated from ADG (g/d) = $\frac{body\ weight\ (kg)-1.5}{age\ (d)}$ x 1000. To record age at first observed estrus, all the gilts were performed back pressure test with the existence of mature boars with high libido. Statistical analyses were performed by SAS version 9.0. Pearson's correlation was conducted to analyze the relationship among variables. Values with $p<0.05$ were regarded as statistical significance.

Results and Discussion

Reproductive data, including ADG, age at first observed estrus, and age at first boar contact, of 766 replacement gilts categorized by breed difference are shown in Table 1. ADG of the gilts, in this study, was 615.5±57.6 g/d (Fig. 1). Most of them had 500-600 g/d. They first contacted with the boar at 22.5±2.9 weeks of age. Majority did at 20-25 weeks (Fig 2). They expressed first observed estrus at 28.9±4.9 weeks of age. Most of them did at 25-31 weeks (Fig 3). Age at first observed estrus negatively correlated with ADG (r=-0.11, $p=0.085$) and positively correlated with age at first boar contact (r=0.27, $p<0.001$).

Table 1 Descriptive statistics (Mean±SD) of the 766 replacement gilts classified by breed.

Variables	L (n=351)	Y (n=228)	LY (n=187)
ADG (g/d)	624.0±62.0	620.5±61.6	602.2±46.1
[a]AFE (wk)	27.9±5.1	29.9±5.0	30.0±2.6
[b]AFB (wk)	22.6±2.9	23.0±2.8	21.9±2.8

[a]AFE – age at first observed estrus.
[b]AFB – age at first boar contact.

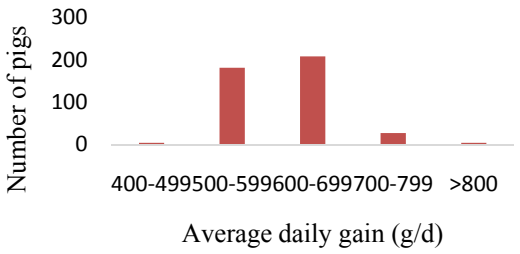

Figure 1 Average daily gain of the gilts.

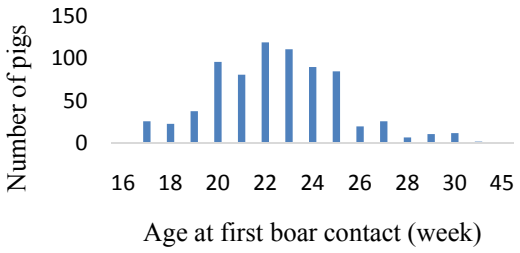

Figure 2 Age at first boar contact of the gilts.

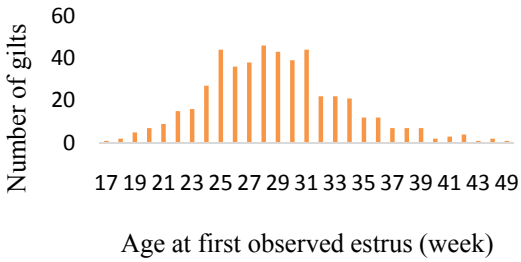

Figure 3 Age at first observed estrus of the gilts.

According to ADG, the gilts, in the current study, having high ADG tended to expressed first observed estrus faster than those with low ADG. This corresponded with the previous study (4). Moreover, ADG not only affected age at first observed estrus, but it also dominated any other reproductive indices of the female pigs, such as age at first mating, age at first farrowing (4), uterine weight, and follicular growth (5). The former study reported that the gilts with high ADG possessed the mentioned indices decently (4). Owing to age at first boar contact, the gilts first contacting with the boar faster, in the current study, showed first standing estrus earlier than those contacting the boar late. This was supported by the previous study demonstrating that younger gilts first contacting with the boars had younger age at puberty than those did at older age (6). Basically, the gilts were suggested to contact with boars at approximately 24-25 weeks of age in order to stimulate reproductive capability. A preceding study reported that the gilts could contact with boars at age 20 weeks; it was found that 75% of them showed estrus signs within 40 days after the boar stimulation (7). Moreover, the gilts with high ADG (726-830 g/d) could be in estrus within 20 days after first boar stimulation.

In conclusion, ADG and age at first boar contact should be the important factors to focus on in the breeding herds since it contributed to a declined non-productive days, entailing the magnificent reproductive productivity of the herds.

Acknowledgments

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$PGF_{2\alpha}$ Shortens Weaning-to-service Interval in Primiparous Sows

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Keywords: $PGF_{2\alpha}$, weaning-to-service interval, primiparous sows,

Introduction

The replacement gilts are considered the largest population in the swine breeding herd since the removal from the herds with planned and planned reasons occupied approximately 50% per annum (1). Accordingly, their reproductive performances considerably affect the overall productivity of the breeding herds. Considering non-productive day (NPD), weaning-to-service interval (WSI) is one of the reproductive indices to be focused on since it will cause economic loss if the pig cannot return to estrus in the appropriate time. Normally, the female pigs should be in estrus after weaning within 7 days; the sows in higher parity number should return to estrus faster than the gilts. As a result, the faster the pigs can return to estrus, the more the NPD can be deducted. Prostaglandins F2α ($PGF_{2\alpha}$) is regarded as the crucial regulator of corpus luteum (CL) functions, uterine contractility, ovulation, and embryo attachment (2). One of the extended WSI results from the maintenance of CL after weaning. Post-farrowing treatment of $PGF_{2\alpha}$ effects on an induction of luteolysis and a stimulation of uterine involution (3). The comprehensive investigation between $PGF_{2\alpha}$ predomination and WSI in the first parity gilts was scanty. The present study aims to examine the effect of $PGF_{2\alpha}$ on WSI of the primiparous gilts.

Materials and Methods

One hundred and forty nine gilts from a swine commercial farm in eastern part of Thailand were included in the study. All gilts were accommodated in open-housing system with the same rearing system and routine managements. They were fed by 3 kg/head on a daily basis. Water could be accessed all times via water nipples equipped above the crates.

Prior to imsemination, heat detection by back pressure test and boar exposure were performed two times on a daily basis to ensure that those were in estrus. Backfat thickness measurement was conducted by an A-mode ultrasonography (Renco lean meter®, MN, USA) at P2 position (4). All the gilts were first mated with conventional artificial insemination at approximately 36 weeks of age. After farrowing, the piglets were allowed to inquire milk for 21 days. After weaning, 10 mg PGF2α (Enzaprost®, CEVA Animal Health, Thailand) was intramuscularly administered to the individuals at 36 h postpartum.

The primiparous sows were categorized into two groups according to the administration of $PGF_{2\alpha}$: Control (no $PGF_{2\alpha}$, n=74) and Treatment (with $PGF_{2\alpha}$, n=75). Reproductive data, e.g., age at first farrowing, litter size at birth, and WSI, were recorded. After farrowing, those expressed standing reflex while testing with back pressure and boar contact were considered they were ready to service.

All data were presented as mean±SD. The comparison between control and treatment groups was undertaken by student *t*-test (SAS 9.3, SAS®, *SAS* Institute Inc., Cary, NC, USA). Values with $p<0.05$ were regarded as statistical significance.

Results and Discussion

Reproductive data between the gilts in control and treatment groups are displayed in Table 1. They went to the first insemination with backfat depth of 23.2±0.2 mm. The gilts in control and treatment groups farrowed 10.5±0.1 and 10.5±0.2 TB at 254.4±1.01 and 254.9±1.01 days of age, respectively. The sows in treatment group took shorter WSI than those in control group significantly (2.6±0.1 vs 4.5±0.2 d, $p<0.001$).

The present study demonstrated that the primiparous sows treated with $PGF_{2\alpha}$ at 36 h after weaning apparently showed a shorter time to service than those weaned without the injection of $PGF_{2\alpha}$. It was well documented that

one of the major reponsibilities of $PGF_{2\alpha}$ was to regress CLs on the ovaries in order to start the new estrus cycle (2). In some cases, the sows might have had active CLs and/ or semi-active CLs on the ovaries after farrowing. This contributed to the presence of progesterone (P_4) the the swine blood circulation. P_4 from such CLs was regarded as an immunosuppressor to the endometrium (5). It might cause an abnormal endometrial environment, affecting the readiness for the next estrus cycle of the sows. Postfarrowing adminstration of $PGF_{2\alpha}$ aimed not only to enhance uterine involution, but also to eliminate the immunosupressive effect of P_4 from such CLs (5). In summary, $PGF_{2\alpha}$ possessed an ability to devastate the remaining CLs and immunosuppressive effects of P_4 to endometrium after farrowing and weaning in the primiparous sows.

Table 1 Reproductive data (Mean±SEM) of the primaparous sow between control and treatment groups according to PGF2α administration from 149 pigs

Parameters	Control (n=74)	Treatment (n=75)
Age at farrowing	254.4±8.7[a]	254.9±8.8[a]
(d)	10.6±1.2[a]	10.5±1.5[a]
TB (piglets)	4.5±2.1[a]	2.6±1.1[b]
WSI (d)		

[a,b] Different superscripts defined statistical difference (p<0.05)
TB – total number of piglets born per litter
WSI – weaning-to-service interval

Consequently, the primiparous sows weaned, together with the injection of $PGF_{2\alpha}$ had shorter WSI than those weaned without $PGF_{2\alpha}$ administration.

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Localization of Estrogen Receptor β in the Porcine Ovary

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Keywords: estrogen receptor, immunohistochemistry, ovary, gilt

Introduction

Our previous study has demonstrated that a certain number of gilts were culled from the herd due to reproductive failure, especially anestrus and/or abnormal estrus behavior (1). The mechanism associated with anestrus or poor estrus behavior in gilts is needed to be explored. It is well established that estrogen plays an important roles in the growth and differentiation of reproductive system (2). In general, estradiol-17β is the most active form of estrogen. This hormone is secreted by antral follicles of the ovarian tissue (3). Furthermore, corpus luteum (CL) in pig also produces this hormone that performs as a paracrine/autocrine regulator (4). Estrogen receptor beta (ERβ) is the remarkable subtype of ERs in the porcine ovary (5). The ERβ plays a vital role in follicular maturation and ovulation (3). Therefore, the present study aims to explore ERβ expression in different locations of the porcine ovary using immunohistochemical method.

Materials and Methods

Ovarian tissues were obtained from 25 Landrace x Yorkshire pubertal gilts (1). The tissue sections were deparaffinized in xylene, rehydrated through graded ethanol dilutions. Thereafter, the tissue sections were placed in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 750 watts for 15 min to retrieve antigenicity. Endogenous peroxidase was blocked by freshly prepared 3.0% hydrogen peroxide at room temperature (RT) for 10 min. Nonspecific staining was blocked with normal horse serum at RT for 30 min. The slides were incubated with primary antibody, rabbit polyclonal anti-ERβ (Santa Cruz Biotechnology, Inc., Texas, USA) at a dilution 1:100, overnight at 4°C. Thereafter, secondary antibody, biotinylated-horse anti-mouse anti-rabbit IgG (Vector Laboratories, CA, USA) was applied to the samples at RT for 30 min, followed with avidin-biotin-peroxidase complex at RT for 30 min. Staining was developed with DAB substrate. The sections were counterstained with haematoxylin stain and mounted with mounting medium. Negative control section was subjected to the same immuno-histochemical procedure replacing primary antibody by using PBS. Immuno-staining of ERβ in follicles and corpus luteum were determined by Image-Pro® plus software. The percentage of ERβ positive cells was calculated as: ERβ positive cells (%) = [(number of ERβ positive cells/ total number of cells counted) x 100]. For each tissue section, 5 microscopic areas were randomly evaluated. The results were expressed as mean±SD. The percentages of ERβ positive cells were compared among different region of the ovaries including follicles and corpus luteum by using ANOVA. The value of $p<0.05$ was regarded to be statistically significance.

Results and Discussions

The immuno-staining of ERβ in the porcine ovaries are demonstrated in Figure 1 and 2. The immuno-staining of ERβ in the porcine corpus luteum are demonstrated in Figure 3.

The ERβ immuno-expression was found in both follicles and corpus luteum of the porcine ovary. The ERβ immuno-staining was mainly detected in the theca cells of the follicles (Fig. 2) and in the luteal cells in the CL (Fig. 3). In the CL, ERβ immuno-staining located in the cytoplasm of the luteal cells (Fig. 3).

Table 1 ERβ immuno-expression in follicles and corpus luteum (CL) in the porcine ovary (mean±SD)

Part of ovary	Number of observation	ERβ expression (%)
Follicles	25	22.6±19.0[a]
CL	25	87.2±9.0[b]

[a,b]different letters within column differ significantly ($p<0.05$)

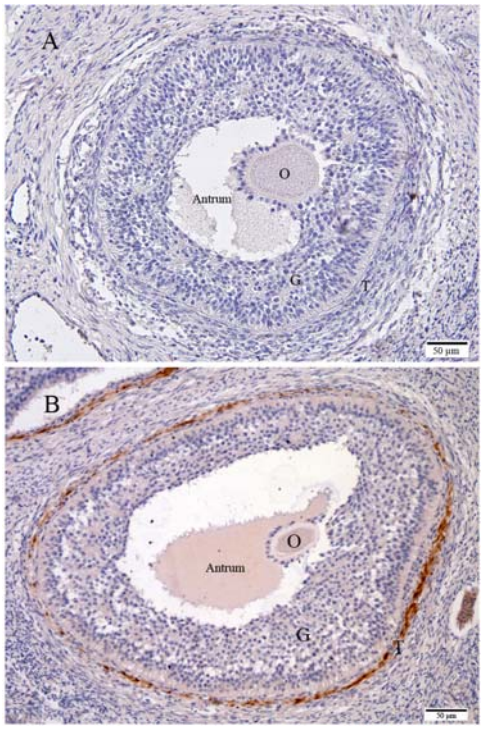

Figure 1 The ERβ immuno-staining in the ovary of pubertal gilts (B) compared with negative control (A). 100× magnification.

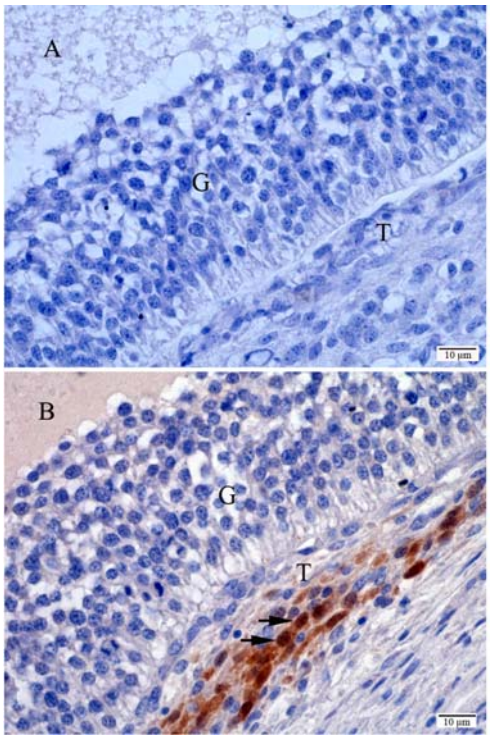

Figure 2 Immuno-staining was found in nuclei and cytoplasm (arrow) of theca cells in follicles (B). Negative control (A). 400× magnification.

In the ovarian follicles, most of the ERβ immunoreaction was found in the cellular nuclei and cytoplasm of the theca cells. This is in accordance with a previous that ERβ protein was exhibited mostly in theca interna cells of the follicle (3). However, the ERβ immunoreaction was rarely expressed in granulosa cells of the follicle (Fig. 1). In the CL, the ERβ immunoreaction was detected in the cytoplasm but not in the nuclei of the luteal cells (Fig. 3). The positive staining in CL indicated that CL could be a source of estradiol production in the porcine species and also in early luteal phase of bovine species (4).

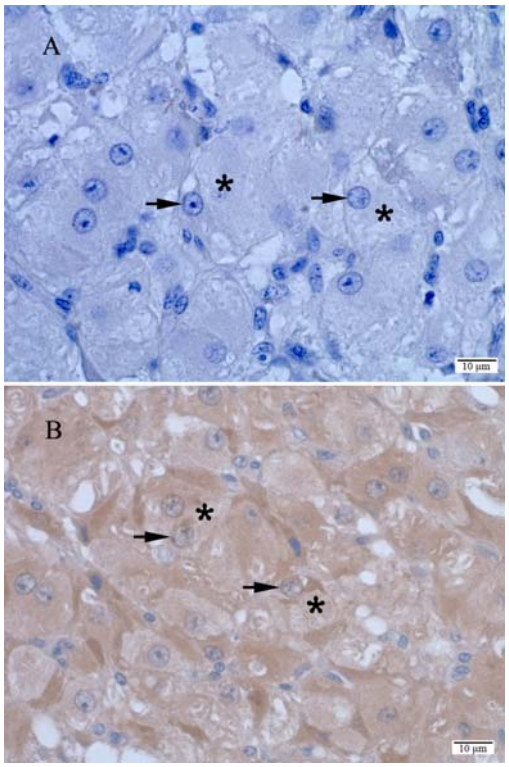

Figure 3 ERβ immunostained was detected in cytoplasm (star) of luteal cells (B) compared with negative control (A) in CL. 400× magnification

The proportion of ERβ expression in the follicles and CL are presented in Table 1. On average, ERβ immunoreaction was detected in 22.6% of the theca cells and in 87.2% of the luteal cells. In conclusion, ERβ immuno-staining was detected in the theca cells and luteal cells in the porcine ovary. This infers that ERβ might play an important role in the porcine ovarian function. Additional study on the variation of ERβ immunoreaction associated with reproductive status will be further investigated.

Acknowledgement

Financial support for the present study was provided by Ratchadaphiseksomphot Endowment Fund 2014, Chulalongkorn University. D. Phoophitphong is a grantee of the Royal Golden Jubilee (RGJ) Ph.D. Program, the Thailand Research Fund.

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Return Rate and Interestrus Interval in Thai Swine Commercial Herds Associate with Parity Number

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Keywords: return to estrus, regular return, irregular return, repeated breeding, parity

Introduction

The proportion of gilts and sows returning to estrus after mating (so called "return rate") is one of the most common reproductive parameters indicating infertility problems in pigs (1). Pregnancy failure includes failure to fertilization, embryonic loss and abortion. These often result in return to estrus of the mated pigs. The proportion of return-to-estrus female after mating is generally categorized as "regular return" and "irregular return". Regular return is characterized by 18-24 days of interestrus interval, whereas irregular or delayed return is sentenced by over 24 days of that interval (2). In general, the regular return indicates fertilization failure which is affected by many factors, e.g., poor semen quality and poor mating behaviors (3). The irregular return often depicts embryonic loss and abortion (4). If embryonic mortality occurs before implantation (10-16 days of gestation), it will be resorbed, resulting in regular return to estrus. If embryonic mortality occurs between 17-35 days of gestation, the pigs will show irregular return to estrus (2).

Although the female returning to estrus will be remated, their subsequent fertility traits decrease (1, 5). In addition, the sows with repeated breeding had a significantly higher subsequent return rate and a lower subsequent farrowing rate than normal sows (1, 5). The return rate has been reported in 8.6% to 16.9% in several countries in Europe [5]. However, no comprehensive study has been investigated on return rate in swine commercial herds in Thailand. The present study aimed to investigate the relationship among return rate, interestrus interval and parity number of gilts and sows in swine commercial herds in Thailand.

Materials and Methods

The retrospective reproductive data of gilts and sows during 2008-2009 were collected from 15 swine commercial breeding herds (A to O) in the central, eastern and northeastern parts of Thailand. The data consisted of 99,620 mating from 44,344 gilts and sows. Those with recorded interestrus interval less than 18 days or longer than 100 days were excluded from the dataset, leaving 99,255 mating from 44,271 gilts and sows for analyses. Return rate was calculated by number of pigs returning to estrus after mating divided by number of mated pigs and multiplied by 100.

According to interestrus interval, they were classified into 6 groups: 18 to 24 days (regular return), 25 to 31 days, 32 to 38 days, 39 to 45 days, 46 to 52 days and >52 days. Return rate was analyzed using generalized linear-mixed models (GLIMMIX) with Tukey-Kramer adjustment for multiple comparisons. The statistical models included the effect of herd (A to O), parity number at mating (0, 1, 2-3, 4-5, ≥6) and mating month. The percentage of sows returning to estrus in each group was compared using *Chi*-square test. Values with $p<0.05$ were considered statistically significant.

Results and Discussion

On average, the return rate was 5.0%, which was varied among herds from 0.5% to 10.6% ($p<0.001$) (Fig. 1). The return rate also varied among parity numbers ($p<0.001$) (Fig. 2).

The highest return rate was found in gilts (7.3%) and the lowest return rate was found in old sows (parity ≥6, 2.4%).

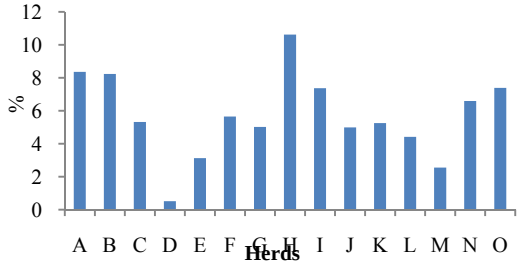

Figure 1 Return rate of gilts and sows by herd

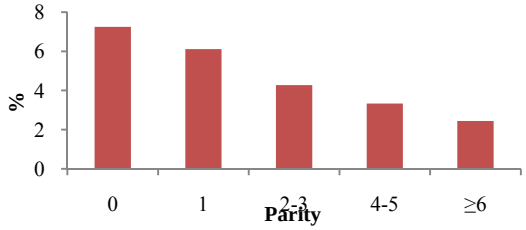

Figure 2 The return rate of gilts and sows by parity.

Table 1 presents the percentage of gilts and sows with return to estrus after breeding classified by parity and interestrus interval. The highest percentage (39.4%) was found 18-24 days after breeding (regular return). The percentage of gilts and sows with irregular return to estrus (>24 days after breeding) was 18.8%, 7.7%, 7.4%, 5.1% and 21.6% in gilts and sows with interestrus interval 25-31 days, 32-38 days, 39-45 days, 46-52 days and 53-100 days, respectively. The regular return to estrus was highest in gilts (46.2%) and the irregular return to estrus was highest in sows' parity number 4-5 (66.6%).

Table 1 The percentage of gilts and sows with return to estrus after breeding by parity and interestrus interval

Parity	% female with return to estrus					
	18-24	25-31	32-38	39-45	46-52	53-100
0	46.2	16.7	6.9	6.9	3.9	19.5
1	34.8	16.3	9.0	8.0	4.9	27.0
2-3	37.7	20.5	7.7	7.2	5.9	21.0
4-5	33.4	23.6	7.6	7.8	7.0	20.6
≥ 6	34.8	22.0	7.3	6.7	5.5	23.8
Total	39.4[a]	18.8[b]	7.7[c]	7.4[c]	5.1[d]	21.6[e]

[a,b]Different superscripts within rows indicate statistically significant differences (p<0.05)

The present study demonstrated the return rate in Thai swine commercial herds. The average of return rate of Thai swine commercial herds was relatively lower than that reported in Europe (5). The return rate was highest in gilts. Moreover, the higher the parity number of sows, the higher the return rate was found. In addition, gilts had highest percentage of regular return to estrus, whereas sows parity number 4-5 had highest percentage of irregular return to estrus. Two peaks of irregular return to estrus were found at 25-31 and 53-100 days after mating. The first peak might be associated with embryonic death after implantation, meanwhile the second peak might be relevant to abortion (2). Since irregular return to estrus was high during 25-31 and 53-100 days after mating, the estrus detection should be carefully performed during these periods.

From the present study, it could be concluded that regular return to estrus was likely to be found in gilts, whereas irregular return to estrus seemed to be noticed in sows' parity 4-5. In addition, it was often found during 25-31 days and 53-100 days after mating.

Acknowledgments

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Inclusions of Equex and Glycerol in Freezing Extenders and the Number of Sperm Dilution Steps during Freezing Affect *In Vitro* Characteristics of Frozen-thawed Epididymal Cat Sperm

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Keywords: epididymal sperm, cryopreservation protocol, Equex STM paste, domestic cat

Introduction

Equex STM paste (Equex; Nova Chemical Sales Inc., Scituate, MA, USA) was incorporated in freezing extenders used for cat sperm cryopreservation, and after thawing the improved *in vitro* sperm quality was verified (1). Although the advantages of Equex have already been recognized, there may be some other co-factors influencing its action and efficacy. Therefore, the present study was conducted partly to investigate whether there are any interactions between this compound and freezing methods utilized herein, with the ultimate goal of determining the cryopreservation conditions under which effect of Equex could be optimized.

Cat sperm are commonly diluted with freezing extenders in two steps, at room temperature and 4-5°C, respectively, prior to be frozen (2). However, one-step dilution is generally performed in sperm cryopreservation protocols for bulls, stallions and dogs (3), with acceptable post thawed sperm quality acquired. This thereby raises the question of whether it is possible for cat sperm to be frozen with the one-step dilution protocols, which in my opinion are more convenient than two-step dilution. In cat sperm cryopreservation, 4-5% glycerol was commonly incorporated in freezing extenders as a permeating cryoprotectant (CPA) (1, 4). To achieve 5% in final concentration, some researchers added glycerol to both extenders I (3% glycerol) and II (7% glycerol) (1), whereas another group put this CPA into freezing extenders used for the second step dilution only (i.e. extenders I and II contained 0% and 10% glycerol, respectively) (4). The present study was undertaken to clarify if there are any differences between these two freezing protocols in the aspect of *in vitro* quality of frozen-thawed epididymal cat sperm.

Materials and Methods

The epididymides separated from the testicles of 126 castrated male cats were re-suspended in a Tris buffer (3.0% (w/v) Tris (hydroxymethyl) aminomethane, 1.4% (w/v) citric acid, 0.8% (w/v) glucose, 0.06% Na-benzylpenicillin and 0.1% (w/v) streptomycin sulphate in distilled water) and cut into small pieces. After 15 min of 37°C incubation, remained fluid was allocated to six aliquots (I to VI) and centrifuged (500xg, 10 min). The sperm pellets were re-suspended in egg yolk-Tris based extenders using one (III and IV) or two (I, II, V and VI) steps dilution. For one step dilution, the pellets were re-suspended at room temperature in plain egg yolk-Tris medium (EYT; Tris buffer plus 20% (v/v) egg yolk) plus 5% glycerol with (IV) / without (III) 0.5% Equex and then were cooled to 4°C for 1 h. For two steps dilution, the pellets were re-suspended at room temperature in EYT (I and V) and in EYT plus 3% glycerol (II and VI) to achieve $50x10^6$ sperm/ml. After 1 h of 4°C incubation, the processed sperm were further diluted 1:1 (v/v) with EYT plus 10% glycerol with (I) / without (V) 1% Equex and with EYT plus 7% glycerol with (II) / without (VI) 1% Equex. The final concentrations of sperm and glycerol in all six groups were $25x10^6$ /ml and 5%, respectively. In Equex added samples, the final concentration of Equex was 0.5%. The processed sperm were loaded in 0.25 ml straws and frozen. The thawed sperm were diluted 1:1 (v/v) with Tris buffer and incubated at 37°C for sperm evaluation. The total sperm motility was evaluated subjectively with a light microscope (400x). The sperm viability was studied in smears stained with an eosin-nigrosin dye. The integrity of the sperm plasma membrane was appraised through the hypo-osmotic swelling test (HOST). The acrosomal status of sperm was assessed with a dual fluorescent staining

technique (FITC-PNA/PI) using epifluorescent microscopy.

Results and Discussion

The present study found that egg yolk-Tris based extenders containing Equex added to the processed sperm just for 10 min before freezing (groups I and II) were beneficial for the acrosome intact sperm both 0 and 2 h after thawing, compared to the extenders without Equex (groups V and VI) (Table 1). These results are in agreement with (1). The present study additionally found that among Equex added samples (groups I, II and IV), positive effects of this compound on the thawed sperm were obvious in the samples processed with two-step dilution (i.e. shorter exposure to Equex before freezing); this is similar to what has been observed in dog sperm (5). It has been suggested that the prolong exposure to Equex rendered an excessive fluidity to the sperm membranes, which would indicate that the beneficial effect of Equex is partly depended on an exposure period (5). Comparing between one- and two-step dilutions, the advantages of two-step dilution on the frozen-thawed sperm quality were discovered both in the Equex added and the Equex-free samples and both at 0 and 2 h of incubation. The present observations, however, did not totally agree with the first report in cat sperm (6) where the percentage of intact acrosomes evaluated after thawing was not significantly affected by temperature (i.e. room temperature vs 5°C) of glycerol addition during the cryopreservation process. The difference between this and previous findings might be partly related to differences in the semen freezing protocol, which a comparison among different protocols is very complicated and offers less reliability (4). The present study found that none of the sperm characteristics were significantly different between the samples diluted with EYT plus 0% and 10% glycerol, respectively (preparation 1) and with EYT plus 3% and 7% glycerol, respectively (preparation 2), regardless of an Equex inclusion and post thawed incubation period. These findings suggest that low glycerol concentration (3% glycerol) is not an essential component of egg yolk-Tris based extenders used to re-suspend a sperm pellet at room temperature in the first step of dilution during freezing, as it gives no advantages/drawbacks to *in vitro* quality of thawed sperm compared to egg yolk-Tris based extenders without glycerol (0% glycerol). However, prior to draw a conclusion on this particular topic, additional *in vitro* tests such as analyses of sperm motion characteristics using a computerized instrument (so called "CASA"), DNA integrity and *in vitro* fertilization (IVF) should be conducted.

On the basis of all results of the present study, it can be inferred and suggested that: 1) egg yolk-Tris based extenders used as freezing media should be supplemented with Equex; 2) during freezing process, the sperm dilution should be performed in two steps at room temperature and 4°C, respectively, rather than only one step at room temperature; and 3) egg yolk-Tris based extenders prepared for the first step dilution can be with/without adding 3% of glycerol. The above suggestions should be followed in order to improve the quality of frozen-thawed edididymal cat sperm.

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Table 1 The *in vitro* quality of post thawed epididymal cat sperm, at 0 and 2 h of 37°C incubation, frozen in egg yolk-Tris based extenders using six different freezing protocols

Time	group	Sperm parameters (%)			
		Motility	Viability	HOST[1]	FITC[2]
0 h (15 min)	I	20.0±3.1 ^{A}	62.6±2.2	67.7±3.1 ^{a}	43.6±3.0 ^{a,A}
	II	17.0±3.5	61.0±2.8	70.0±1.7 ^{a}	42.0±1.5 ^{a,b,A}
	III	15.5±3.5	58.4±3.1 ^{A}	67.7±2.8 ^{a}	25.1±2.2 ^{d,A}
	IV	15.5±4.4	58.8±2.2 ^{A}	59.9±2.5 ^{b}	35.5±3.6 ^{b,c,A}
	V	16.5±3.2	58.9±3.0	70.9±3.3 ^{a}	32.0±2.0 ^{c,d,A}
	VI	12.5±3.7	56.3±3.0	69.3±2.3 ^{a}	33.9±1.5 ^{c,A}
2 h	I	1.5±0.8 ^{B}	56.4±3.2 ^{a}	60.8±3.1 ^{a,b}	30.3±1.6 ^{a,B}
	II	2.0±1.1	54.0±3.3 ^{a}	63.1±2.7 ^{a,b}	29.1±2.4 ^{a,b,B}
	III	4.0±1.5	41.1±5.5 ^{b,B}	64.6±2.6 ^{a}	17.5±1.4 ^{d,B}
	IV	7.5±2.3	47.2±4.4 ^{a,b,B}	55.6±2.2 ^{b}	23.3±1.6 ^{c,B}
	V	2.5±1.3	53.9±3.2 ^{a}	67.2±3.4 ^{a}	23.3±1.6 ^{c,B}
	VI	3.5±1.3	50.0±3.5 ^{a,b}	65.9±2.8 ^{a}	25.1±1.4 ^{b,c,B}

[1]The plasma membrane integrity of sperm detected by the hypo-osmotic swelling test (HOST), [2]The acrosome integrity of sperm assessed with FITC-PNA/PI staining (FITC), Values with different letters indicate significant difference within columns [a, b, c, d comparing among groups (I to VI) within each incubation times; A, B comparing between incubation times (0 and 2 h) within each groups, $p<0.05$].

Effect of Blood Glucose Concentration on Colostrum Intake in Newborn Piglets

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Keywords: glucose, colostrum, preweaning mortality, piglet

Introduction

Blood glucose concentration is an indicator for postnatal survival (1). It has been demonstrated that both too low (24-30 mg/dl) and too high (45-162 mg/dl) blood glucose concentration at birth are associated with greater chance of mortality up to 3 to 7 days after birth (2). In general, the neonatal piglets should consume at least 250 grams of colostrum to ensure an optimal growth and passive immunity (3). Data on blood glucose concentration in newborn piglets associated with individual colostrum intake has not been elucidated. The present study aims to determine the effect of blood glucose concentration on individual colostrum intake in newborn piglets.

Materials and Methods

The present study was carried out in a commercial swine herd in the western part of Thailand between June and August 2013. In total, 1,160 piglets born from 85 sows were included. All sows were kept in a conventional open housing system. The sows were kept in individual farrowing pens during lactation. Pregnant sows were moved to the farrowing pens about one week before the expected farrowing date. The farrowing process was supervised. The sows were allowed to farrow naturally. Blood samples were collected from umbilical cord. Blood glucose concentration was evaluated by a portable human glucometer (Accu-Check® Performa, Roche Diagnotis (Thailand) Co. Ltd., Bangkok, Thailand) within 5 min after birth. All piglets were not cross-fostered until weaning. Survival rate of the piglets was determined at 1 and 7 days of age. Individual birth weight of the piglets was measured immediately after birth and again at 22.4±1.2 h after the first piglet was born by using an electronic balance. An individual colostrum consumption of the piglets was estimated: colostrum consumption (g) = -217.4 + (0.217*t) + (1,861,019*BW2/t) + BW*(54.8 - 1,861,019/t)* ((0.9985-3.7*10^{-4}*tFS) + (6.1*10^{-7}*tFS^2)); where BW=birth weight (kg), BW2=body weight at the second weighing (kg), t= time elapsed between the first and the second weighting (min), and tFS = the interval between birth and first sucking (min) (4). Colostrum intake more than 250 grams were defined as "adequate colostrum intake" otherwise it was defined as "inadequate colostrum intake". Data was analyzed by Chi-square test.

Results and Discussion

On average, the blood glucose concentration was 51.0±19.3 mg/dl (range 10-216 mg/dl) and the individual colostrum intake was 237.2±160.1 g (range 0-901.0 g). The proportion of individual colostrum intake of piglets is presented in Figure 1. The proportion piglets that had adequate colostrum intake were 53.9%.

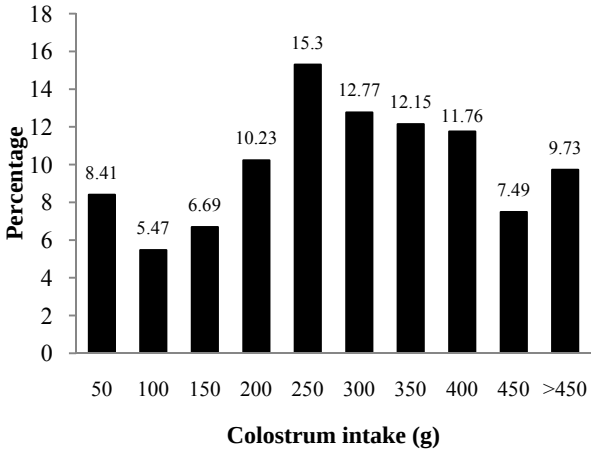

Figure 1 Frequency distribution of individual colostrum intake (grams) in neonatal piglets (n=1,160).

Table 1 Proportion of piglets received adequate colostrum intake (%) by blood glucose concentration classes

Glucose, mg/dl	n	Piglets received adequate colostrum intake n (%)
<25	50	7 (14.0)[a]
26-40	244	115 (47.1)[b]
41-50	308	156 (50.7)[b]
51-60	251	134 (53.4)[b]
>61	251	117 (46.6)[b]

[a,b]different superscript within column differ significantly (p<0.001)

Low blood glucose concentration related with low viability due to increase first sucking time influencing colostrum consumption [2, 3]. It can be concluded that blood glucose concentration was significantly influenced the colostrum intake of the piglet.

Acknowledgements

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Neonatal Piglet Survival Associated with Blood Glucose Concentration

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Keywords: glucose, survival, piglet

Introduction

Physiological parameters such as birth weight, rectal temperature, heart rate, blood oxygen saturation, birth order, skin color, integrity of the umbilical cord, and blood glucose concentration are associated with neonatal piglet survival (10. Blood glucose concentration of the piglets is related to health status and energy reserve of newborn piglet (2). It has been demonstrated that too low (24-30 mg/dl) or too high (45-162 mg/dl) blood glucose concentration in the newborn piglets increase the risk of piglet's pre-weaning mortality (3). However, data on blood glucose concentration in newborn piglets under tropical climates associated with their survival has not been elucidated. The present study aims to determine the association between blood glucose concentration and survival rate of the neonatal piglets under field conditions.

Materials and Methods

The present study was carried out in a commercial swine herd in the western part of Thailand between June and August 2013. In total, 1,160 piglets born from 85 sows were included. All sows were kept in a conventional open housing system. The sows were kept in individual farrowing pens during lactation. Pregnant sows were moved to the farrowing pens about one week before the expected farrowing date. The farrowing process was supervised. The sows were allowed to farrow naturally. Blood samples were collected from umbilical cord. Blood glucose concentration was determined by a portable human glucometer (Accu-Check® Performa, Roche Diagnotis (Thailand) Co. Ltd., Bangkok, Thailand) within 5 min after birth. All piglets were not cross-fostered until weaning. Survival rate of the piglets was determined at Days 1 and 7 after birth. Data was analyzed by Chi-square test.

Results and Discussion

On average, the blood glucose concentration was 51.0±19.3 mg/dl (range 10-216 mg/dl). The blood glucose concentration of the piglets is presented in Figure 1. Cumulative mortality rate at Day 1 and Day 7 after birth were 2.7% and 8.5%, respectively. Blood glucose concentration of the newborn piglets significantly influenced the survival rate at Day 1 and Day 7 (Table 1).

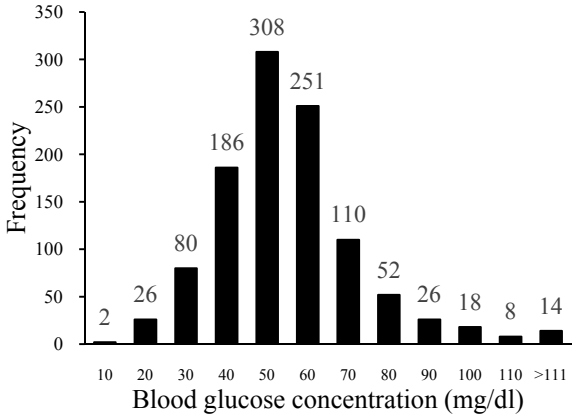

Figure 1 Frequency distribution of blood glucose concentration (mg/dl) in neonatal piglets (n=1,114)

Table 1 Survival rate (%) of piglet at 1 and 7 days after birth by blood glucose concentration

Glucose mg/dl	n	Survival rate (%)	
		Day 1 n (%)	Day 7 n (%)
<25	50	22 (44.0)[a]	19 (38.0)[a]
26-40	244	220 (90.2)[b]	208 (86.3)[b]
41-50	308	284 (92.2)[b]	267 (86.7)[b]
51-60	251	233 (92.8)[b]	217 (86.5)[b]
>61	251	224 (89.2)[b]	214 (85.3)[b]

[a,b]different superscript within column differ significantly (p<0.001)

Table 2 Odds ratio of the survival rate of the piglets at Days 1 and 7 after birth by blood glucose concentration (blood glucose <25 mg/dl was set as control)

Glucose	Survival rate (%)	
mg/dl	Day 1	Day 7
	Odd ratio (CI 95%)	Odd ratio (CI 95%)
<25	1.0 (NA)	1.0 (NA)
26-40	11.7 (5.8-23.5)*	9.8 (4.8-19.5)*
41-50	15.1 (7.5-30.1)*	10.6 (5.5-20.5)*
51-60	16.5 (7.9-34.4)*	10.4 (5.3-20.5)*
>61	10.6 (5.3-21.0)*	9.4 (4.8-18.4)*

CI=Confidence interval; NA= not applicable.
*differ significantly (p<0.001).

Table 2 presented odd ratio of the survival rate of the piglets by blood glucose groups. Piglets with blood glucose below 25 mg/dl were set as control. The survival rate of the piglets increases 16 times when blood glucose at birth increase from <25 mg/dl to 51-60 mg/dl (p<0.001). It can be concluded that blood glucose concentration was significantly influenced the survival rate of the piglet.

Acknowledgements

Financial support for the present study was provided by The National Research Council of Thailand. M. Nuntapaitoon is a grantee of the Research and Researchers for Industries (RRI) Ph.D. program, the Thailand Research Fund.

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Expression of Estrogen Receptor Alpha in the Endometrium of Gilts Infected with Porcine Reproductive and Respiratory Syndrome Virus

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Keywords: ERα, immunohistochemistry, PRRS, uterus, gilt

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease in pig industry (1). The target cells of PRRS virus (i.e., macrophage) are presented in the endometrium of pig (2). Karniychuk et al. (3) found that PRRS virus can replicate and induce apoptosis in the fetal implantation sites at the last stage of gestation. These findings indicate that PRRS virus infections may cause pathological changes in the endometrium and lead to infertility. However, to our knowledge, the impact of PRRS virus infection on the endometrial function has not been fully elucidated. Thus, the objective of the present study was to determine the effect of PRRS virus infection on the expression of estrogen receptor α (ERα) in the endometrium of gilts using immunohistochemistry.

Materials and Methods

Uterine tissues were collected from 56 Landrace × Yorkshire crossbred gilts (4). The uterus were fixed in 10% neutral-buffered formalin and processed histologically. The expression of ERα was determined using immunohistochemistry on paraffin sections using polyclonal antibodies against ERα (5). ERα staining was evaluated in epithelium, subepithelium, and glandular layers of the uterus. Five objective fields of each uterine section were randomly selected to evaluate using Image-Pro® Plus software under a light microscope. The percentage of ERα positive cells was calculated as: ERα positive cells (%) = [(number of ERα positive cells/ total number of cells counted) × 100]. The gilts were classified according to PRRS virus detection as positive (n=26) and negative (n=30). The reproductive status of the gilts was defined according to the ovarian appearance into three groups, i.e., prepubertal (n=12), luteal phase (n=26) and follicular phase (n=16) (4). The statistical analyses were carried out using SAS. Multiple ANOVA was used to analyze the data. The statistical model included the effect of reproductive status (prepubertal, luteal and follicular phases), tissue layers (epithelium, subepithelium, and glandular layers), PRRS virus detection (positive and negative), and the interaction between reproductive status and PRRS virus detection. Least-square means were calculated and compared using Tukey-Kramer test. $p<0.05$ was regarded as statistically significant.

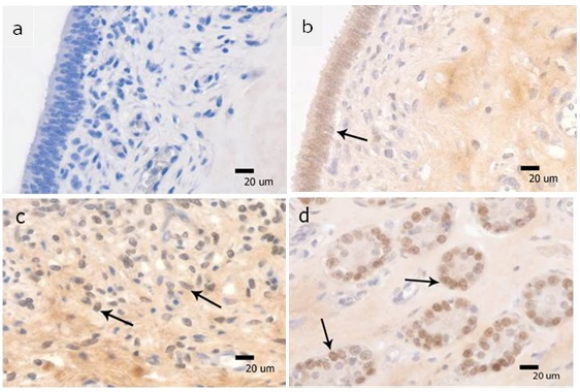

Figure 1 Expression of ERα in endometrium of PRRSV infected gilts: a) negative control; b) positive control; c, d) immunostaining of ERα in subepithelial and glandular layers. Black arrows indicate positive staining cell, 200× magnification.

Results and Discussion

The result showed that the immuno-staining of ERα in the endometrium of gilts are presented in epithelial, sub-epithelial and glandular layers of the uterus (Fig. 1). The percentage of ERα immuno-staining did not differ significantly between the PRRS virus detection and those without PRRS virus detection in the endometrium of gilts (Table 1). The difference of ERα immuno-staining was found between prepubertal and follicular

phase gilts in subepithelial layer (17.9 and 30.4%, respectively, p=0.014) and glandular layer (15.1 and 46.1%, respectively, p<0.001) of the gilt's endometrium. The difference in ERα immuno-staining between prepubertal and follicular phase gilts indicated that the ovarian steroid hormones in gilts influenced the expression of ERα rather than the presence of PRRS virus. In conclusions, PRRS virus did not affect the expression of the ERα in endometrium.

Table 1 The percentage of ERα positive cells in the epithelium, subepithelial and glandular tissues layers of the endometrium in gilts with and without PRRS virus detection in the uterus (LS means±SEM.)

Tissue layer	PRRS virus detection	
	Negative	Positive
Epithelium	22.8±2.6[a]	25.2±2.8[a]
Subepithelium	12.4±1.8[a]	15.3±1.9[a]
Glandular	32.8±2.9[a]	31.2±3.1[a]

[a]Common superscript within row did not differ significantly (p>0.05)

Acknowledgements

Financial support for the present study was provided by Ratchadaphiseksomphot Endowment Fund.

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Impact of Porcine Reproductive and Respiratory Syndrome Virus Infection on the Expression of Estrogen Receptor Beta (ERβ) in the Ovary of Gilts

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Keywords: ERβ, estradiol, ovary, PRRS, gilt

Introduction

Estrogens play the important roles in growth and differentiation of the reproductive system in gilts and sows. The most active estrogen synthesized by antral follicles is estradiol-17β. Theca interna and granulosa cells are major component of the antral follicles that synthesize estrogen. Estrogen regulates reproductive function by binding to specific receptor protein, estrogen receptor (ER). In general, ER is expressed as two isoforms, i.e., ERα and ERβ (1). ERα is the classical ER that was identified since 1986, while ERβ has been identified later (3). Although ERα and ERβ are encoded on different chromosome, they have a considerable sequence homology in their domain (3). Therefore, it can be speculated that the presence of any of the ER subtypes indicate the tissue action of estrogen. The objective of the study was to determine the effect of porcine reproductive and respiratory syndrome (PRRS) virus detection on the expression of ERβ in ovarian follicles of gilt.

Materials and Methods

Ovarian tissues were collected from 62 Landrace x Yorkshire crossbred gilts (4). The ovaries were fixed in 10% neutral-buffered formalin and processed histologically. PRRS virus detection in the gilt ovaries was carried out by immunohistochemistry (2). The ovarian tissues sections were incubated with rabbit polyclonal anti-ERβ as a primary antibody, biotinylated secondary antibody-horse anti-mouse anti-rabbit IgG and avidin-biotin-peroxidase complex. Five follicles per ovarian tissue sections were evaluated using Image-Pro® Plus software. The percentage of ERβ positive cells was calculated. The gilts were classified according to PRRS virus detection as positive (n=38) and negative (n=24).

The statistical analyses were carried out using SAS (SAS Cary, NC, USA). Multiple ANOVA was used to analyse the ERβ positive cells using the general linear model procedure of SAS. The statistical model included the effect of reproductive status (prepubertal and cycling gilts), PRRS virus detection (positive and negative) and the interaction between reproductive status and PRRS virus detection. Least-square means were compared using Tukey-Kramer test. $p<0.05$ was regarded as statistically significant.

Results and Discussion

The result showed that ERβ immunoreaction was found in both pre-antral and antral follicles (Fig. 1).

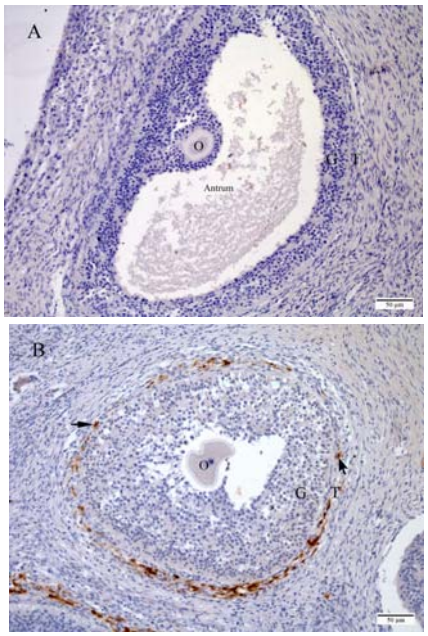

Figure 1 Localization of ERβ protein in the representative paraffin sections of gilt ovarian follicles. Positive immunostaining in the ovarian theca interna cells (B) compared with negative

control (A). Bar = 50 µm. theca interna cells (B) compared with negative control (A). Bar = 50 µm.

On average, ERβ was detected in 23.7% of the theca interna cells surrounding the follicles. The ERβ expression was not detected in the granulosa cells of the porcine ovarian follicles (Fig. 2).

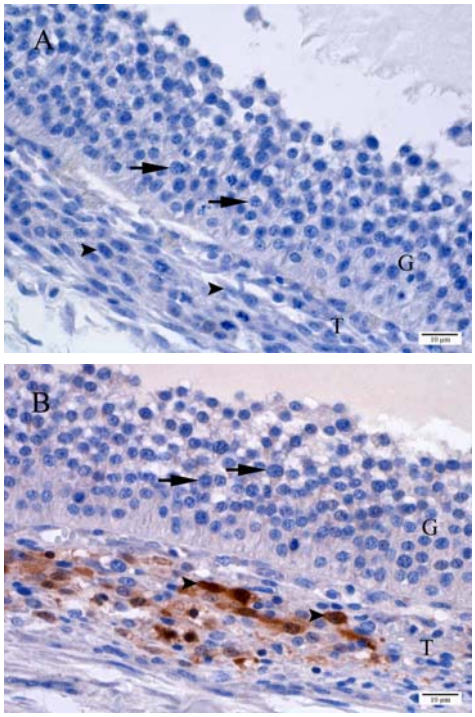

Figure 2 ERβ immunostaining expressed mainly in the nucleus and cytoplasm of theca cells (arrow head, B) compared with negative control (A). No ERβ immunostaing was found in granulosa cells (arrow). Bar = 10 µm.

The percentage of ERβ immuno-staining did not differ significantly between the ovarian tissues detected PRRS virus and those without PRRS virus detection (p>0.05). This indicated that although PRRS virus has an effect on reproductive system, the mechanism may not be mediated through the expression of ERβ in the ovary. In conclusions, PRRS virus detection did not significantly influence the ERβ expression in the ovary of gilt.

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Financial support for the present study was provided by Ratchadaphiseksomphot Endowment Fund.

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Serum Leptin Concentration in Relation to Breed, Body Weight, Backfat Thickness and Age at First Observed Estrus in Gilts

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Keywords: leptin, puberty, reproduction, performance, gilt

Introduction

Leptin is a protein hormone mainly produced from adipose tissue and playing an important role in feed intake regulation and energy balance (1, 2). This hormone has been suggested to control various aspect of reproduction (1, 2, 3). The objective of the present study was to investigate the concentration of serum leptin in Landrace and Yorkshire replacement gilts in relation to their breed, bodyweight, backfat thickness and age at first observed estrus.

Materials and Methods

In total, 80 serum samples from 48 Landrace and 32 Yorkshire gilts were included. The gilts were categorized by bodyweight into 4 groups: <121 kg (n=20), 121-130 kg (n=20), 131-140 kg (n=18), >140 kg (n=9) and grouping by backfat thickness into 4 groups: 10.0-13.0 mm (n=7), 13.5-16.0 mm (n=24), 16.5-19.0 mm (n=14), 19.5-26.0 mm (n=22) and by age of first estrus into 3 groups: ≤180 days of age (n=18), 181-200 days of age (n=17), >200 days of ages (n=32). Serum leptin was investigated by an enzyme immunoassay (Porcine Leptin ELISA kit, MyBioSource Ltd., CA., USA.). The data were analyzed by using Pearson's correlation, multiple ANOVA and least-significant difference test. Values with $p<0.05$ were considered statistically significant.

Results and Discussion

On average, serum leptin concentration of the gilts was 0.9±0.8 ng/ml. The gilts exhibited first standing estrus at 201.5±28.9 days of age and were mated at 253.6±19.2 days of age. Body weight and backfat thickness of the replacement gilts were 136.6±12.3 kg and 16.9±3.5 mm, respectively. The serum leptin concentration did not differ significantly between Landrace and Yorkshire gilts (Table 1). Likewise, the serum leptin concentration did not correlate with the bodyweight, backfat thickness and age at first observed estrus of the gilts (Table 2). The serum leptin concentration did not differ significantly among different body weight classes of the gilts (Table 3). The gilts with backfat thickness (Table 4, 5) of 16.5-19.0 mm had a higher serum leptin concentration than those with backfat thickness of 10.0-13.0 mm (1.31±0.2 vs 0.51±0.3 ng/ml, $p=0.034$) and 13.5-16.0 mm (0.70±0.2 ng/ml, $p=0.028$).

Table 1 Serum leptin concentration in Landrace and Yorkshire gilts

Breed	N	Leptin (ng/ml)
Landrace	41	0.96 ± 0.11[a]
Yorkshire	26	0.60 ± 0.19[a]

Table 2 Pearson's correlation between serum leptin concentration and gilt's reproductive data

Parameters	Correlation
Bodyweight (kg)	r=-0.08, *P*=0.53, n=67
Backfat thickness (mm)	r=0.09, *P*=0.42, n=79
Age at first estrus (d)	r=0.21, *P*=0.07, n=76
Age at first mating (d)	r=-0.08, *P*=0.49, n=79
Non-productive days	r=-0.10, *P*=0.37, n=79

Table 3 Serum leptin concentration by body weight classes

Bodyweight (kg)	N	Leptin (ng/ml)
≤120	20	0.86±0.18[a]
121-130	20	0.87±0.18[a]
131-140	18	0.62±0.20[a]
>140	9	0.76±0.27[a]

Table 4 Serum leptin concentration by backfat thickness classes

Backfat (mm)	N	Leptin (ng/ml)
10.0-13.0	7	0.51±0.3[a]
13.5-16.0	24	0.70±0.2[a]
16.5-19.0	14	1.31±0.2[b]
19.5-26.0	22	0.82±0.2[ab]

Table 5 Serum leptin concentration by age at first observed estrus classes

Age at estrus (d)	N	Leptin (ng/ml)
≤180	18	0.58±0.21[a]
181-200	17	0.85±0.21[a]
>200	32	0.91±0.14[a]

This is the first report on the serum leptin concentration of replacement gilts in Thailand. Furthermore, it was found that the gilts with a backfat thickness of 16.5-19.0 mm had a higher level of serum leptin concentration than those with a lower backfat thickness. This implies that the gilts with high level of backfat thickness might have had a better energy balance and feed intake than those with a lower backfat thickness. This may subsequently contributed to a better reproductive function in the gilts with high backfat and high leptin level.

In conclusion, the serum leptin concentrations did not differ between Landrace and Yorkshire and did not correlate with bodyweight and age at first observed estrus. However, gilts with a backfat thickness of 16.5-19.0 mm had a significantly higher serum leptin concentration than gilts with a backfat thickness of below 16.0 mm.

Acknowledgements

Financial support for the present study was provided by Ratchadaphiseksomphot Endowment Fund.

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Effect of GnRH Vaccine (Improvac™) on Gross Morphology of the Genital Organs in Boars

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Keywords: reproduction, testis, sperm, vaccine, boar

Introduction

The principle of GnRH vaccine (Improvac™) is to activate immunization against gonadotropin-releasing hormone (GnRH) for disrupting the function of the Leydig cells that affect to testicular hormones synthesis and reproductive organs development (1-3). The objective of the present study was to evaluate the effectiveness of Improvac™ vaccine on the development of the genital organs in boars.

Materials and Methods

A total of 53 non-castrated male pigs were investigated. The boars were divided in to four groups, i.e., group I: non-castrated male pigs aged 23-26 weeks (n=11), group II: intact boars aged 2-3 years old (n=10), group III, vaccinated boars with single dose of Improvac™ (n=10), and group IV: vaccinated boars with 2 doses (4 weeks apart) of Improvac™ (n=22). The vaccinations (2 ml/pig, 200 µg/ml, Improvac™, Zoetis) were administered subcutaneously at 13 and 17 weeks of age and the pigs were slaughtered between 4 and 8 weeks after the second vaccination. Reproductive organs including testes, epididymis, bulbourethral glands, seminal vesicle, prostate glands and penis were collected from slaughter house. Data were compared among groups by ANOVA.

Results and Discussion

The gross morphological examination of reproductive organs revealed that testicular weight (95.5 versus 311, 388, 262 grams, $p<0.001$) and testicular circumference (13.6 versus 20.7, 21.3, 19.6 cm, $p<0.001$) of the boars in group IV was lower than that in groups I, II and III, respectively (Figure 1). This is in agreement with a previous study (3). Additionally, the length (21 versus 26 and 19 cm, $p>0.05$) and weight (22 versus 65 and 57 grams, $p>0.05$) of the epididymis did not differ significantly between group IV and groups I and III, respectively. Nevertheless, both the length (36 cm) and the weight (142 grams) of the epididymis of the boars in group II was significantly higher than that in the other groups ($p<0.05$).

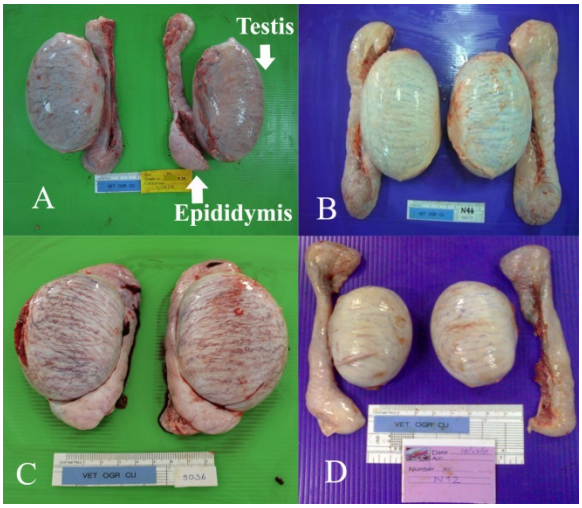

Figure 1 Testes and epididymides of the boars: (A) group I, non-castrated boar aged 23 weeks, (B) group II, non-castrated boars aged 2 years, (C) group III, single dose of Improvac™, (D) group IV, boars vaccinated with two doses of Improvac™.

In conclusion, two doses of Improvac™ vaccines significantly reduced the testicular size of the boars but did not alter the size of the epididymis.

Acknowledgement

Zoetis (Thailand).

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Increase Efficiency of Artificial Insemination in Pigs with Post-Cervical Artificial Insemination: Decrease Perio of Insemination Time and Boar Semen Volume

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Keywords: intrauterine insemination, fresh semen, farrowing rate, litter size, pig

Introduction

Swine breeding (reproductive) technologies have developed continuously, especially artificial insemination (AI). Swine breeding technologies can decrease risk of disease transmission, reduce semen volume and increase superior genetic distribution, which enhance genetic improvement. Generally, AI process consists of disposable artificial insemination catheter (rod) insertion through the cervix and semen deposition inside reproductive tract. In this process, the position depend on technical specification, for example, cranial cervix (cervical insemination or conventional artificial insemination: CAI), uterine body (post-cervical artificial insemination: PCAI) and at the proximal end of uterine horn (deep intrauterine insemination: DIUI)(1). In addition, each technic require appropriate spermatic concentrations and volumes. For CAI, fresh semen 80-100 ml doses that contain 2-5 × 10^9 spermatozoa were used. For PCAI, both frozen- thawed semen and fresh semen were used with concentration of 2×10^9 spermatozoa in 20 ml doses and $1\text{-}4\times10^9$ spermatozoa in 50-85 ml doses (2) respectively. For DIUI, frozen- thawed semen with concentration of 1×10^9 spermatozoa in 10 ml doses (3) were used. In this study 20 multiparous sows after weaning were selected and inseminate with fresh semen from boar that usually use in farm was diluted to the concentration of 4×10^9 spermatozoa in 20 and 80 ml per doses. The objective of this study was to emphasize the efficiency improvement of artificial insemination in pigs with PCAI by decrease both period of insemination time and boar semen volume.

Materials and Methods

Semen extension

Semen was collected from boars which reside at the farm. After semen quality evaluation (motility ≥ 70%, and sperm with normal morphology ≥85% (4)), semen doses were prepared with concentration of 4×10^9 spermatozoa in total volume of 20 ml and 80 ml, for use in treatment group and control group, respectively. All of doses were stored at 16°C (5) and use within 48h (6).

Sows

A total of 20 hybrid multiparous, Landrace × Yorkshire crossbred are use in the experiment with parity ranging from 1 to 9. All of them were inseminated at first estrus after weaning (weaning to estrus interval from 4 to 7 day and weaning day considered day 0). The sow population was divided into two groups: one was used for PCAI with 20 ml of semen volume (n=10) and the remaining one group using the PCAI with 80 ml of semen volume (n=10).

Detection of estrus and post-cervical artificial insemination (PCAI)

After weaning 3 days, a backpressure technique was tested during direct mature boar exposure to determine the onset of standing estrus every 6h. Time to inseminate were indicated that consist of 12h after estrus detection (6-8h before ovulation), and then repeatedly at 24h and 36h later with same semen boar**.** The sows, which inseminated by using PCAI method with both 4×10^9 spermatozoa in 20 ml and 80 ml dilutions, were observed bleeding or the semen backflow during insemination and up to 30 min after insemination were also evaluated . The volume of semen backflow was measured if occur.

Data collection

After breeding, heat detection should repeat at 18-24 days. Moreover, the conception rate was determined by transcutaneous real-time

ultrasono-graphy at 30 days after breeding. Finally, both farrowing rate and litter size were recorded.

Statistical analysis

Data are expressed as the mean±SD. The farrowing rate was analysed by chi-square test and reproductive performance including total born per litter and total born alive per litter were compared by *t*-test (SAS 9.3, SAS®, Cary, NC, USA.). The differences of data were considered statistically significant at $p<0.05$.

Results and Discussion

The result of this present study is presented in Table 1. Insemination duration of both 20 ml and 80 ml semen doses are 1-2 min and 4-5 min, respectively. The farrowing rate, total born piglet and total born alive piglet have no difference between treatment and control group.

Table 1 Reproductive performance in sows by PCAI with semen volume 20 ml vs 80 ml of 4×10^9 spermatozoa per dose.

Reproductive performance	PCAI		*p*-value
	20 ml (treatment group)	80 ml (control group)	
N	10	10	
Farrowing rate (%)	70	80	0.3483
Total born per litter	12.43±3.74	9.25±3.20	0.0989
Born alive per litter	10.86±3.72	8.5±4.07	0.2652

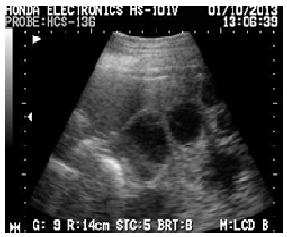

Figure 1 Transcutaneous real-time ultrasono graphy at 30 days after breeding.

With the deposition of 1×10^9 spermatozoa or less, there is a higher risk of the success of the IUI technique being compromised (7). The use of 1.5×10^9 spermatozoa was considered safe for the PCAI of primiparous and multiparous sows (8) therefore in the present study use 4×10^9 spermatozoa. The sows that were inseminated by PCAI technique with different semen volume can reduce the period of insemination time. In addition, none-return rate of treatment group (semen volume 20 ml) were 10% higher than control group (semen volume 80 ml). On the other hand, there were 2 cases of abortion at 25 day of gestation in treatment group therefore the farrowing rate of this group equal to 70% that was lower than control group (80%) but without difference ($p>0.05$). In treatment group, total born and total born alive per litter are equal to 12.43±3.74 and 10.86±3.72, respectively that were higher than control group.

In conclusion, research results shows that PCAI in field condition can be totally recommended. This concentration and volume of semen are useful for commercial farms that import semen because this method can reduce cost of production. Moreover, it might benefit for the farm that lack of boars. Reduce semen volume for insemination can increase quantity of semen dose per each semen collection.

Acknowledgements

We would like to thank Sang-worn farm owner and staffs (Pig farm in Chon Buri Province) that provide sows and facility for this study.

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Immunolocalization of Estrogen Receptor Alpha in the Endometrium of Gilts with Multiple Ovarian Cysts

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Keywords: estrogen receptor, reproductive failure, ovarian cyst, uterus, gilt

Introduction

The reproductive disturbances are the major problem for culling gilts in the swine farm industry and these failures are frequently composed of repeat breeding, anestrus, vaginal discharge, not being pregnant and abortion (1, 2). Reproductive function of sows and gilts is intricate to clinically investigate under field conditions as a result the reproductive organs from the abattoirs are the potential source of important data considering the fertility problems of the female pigs (3).

The reproductive organs, particularly the uterus, are influenced on the complicated actions of steroid hormones produced by the ovaries and the suitable hormonal levels construct an appropriate milieu of endometium (4). Undoubtedly, the disorders of the ovaries, e.g. ovarian cysts, might interrupt the uterine function and lead to infertility problem (5). Among the abnormalities of the ovary in gilts culled due to reproductive failure in Thailand, the incidence of ovarian cysts was approximately 15% and the multiple ovarian cysts were detected up to 6% of these gilts (6). Since the ovarian cysts are the endocrinological disease and the steroid hormones achieve their functions by binding through specific receptors, the expression of these receptors in the target tissues might reflect the functional efficiency of hormonal control (7).

The aim of the present study was, therefore, to determine the influence of the multiple ovarian cysts on the localization of estrogen receptor in the culling gilt endometrium.

Materials and Methods

Twelve reproductive tracts of crossbred Landrace × York Shire culling gilts (n=12) were collected from the abattoir. The ovaries and uterine horns were dissected out of the tracts and both ovaries were classified into the multiple ovarian cysts (n=7) and normal ovary at follicular phase (control group, n=5). The uterine tissues were immersed in 4% paraformaldehyde, embedded in paraffin and cut at 5 µm thickness. The procedure of Avidin-Biotin Peroxidase immunohistochemical method (Vector Laboratories Inc.) was used and the primary antibody applied was mouse monoclonal antibody to estrogen receptor α (ERα, sc-787, Santa Cruz) at a dilution of 1:100. Positive staining was detected using DAB substrate and counterstained by haematoxylin. Control slides were carried out by replacing primary antibody with normal mouse IgG. The ERα immunolocalization in epithelium, subepithelial connective tissue (CNT) layer, and glandular epithelium was evaluated under a light microscope.

The intensity of positive labeling in each tissue compartments was scored as three different levels, i.e.weak (1), moderate (2) or strong (3) and all data was statistically analyzed using the SAS statistical package (version 9.0).

Results and Discussion

The positive localization of ERα was detected as a clearly brown nulear staining of the uterine epithelial cells, stromal cells in the subepithelial CNT and glandular epithelial cells in the control group (Fig. 1A).

In contrast, very weak ERα immunolabeling was present in all endometrial compartments of the gilts with multiple ovarian cysts (Fig. 1B). The scores of ERα immunostaining results were summarized in Table 1. The greater ERα intensity score was significantly (p<0.05) found in the nuclei of all endometrial compartments in the control group compared with the group of multiple ovarian cysts.

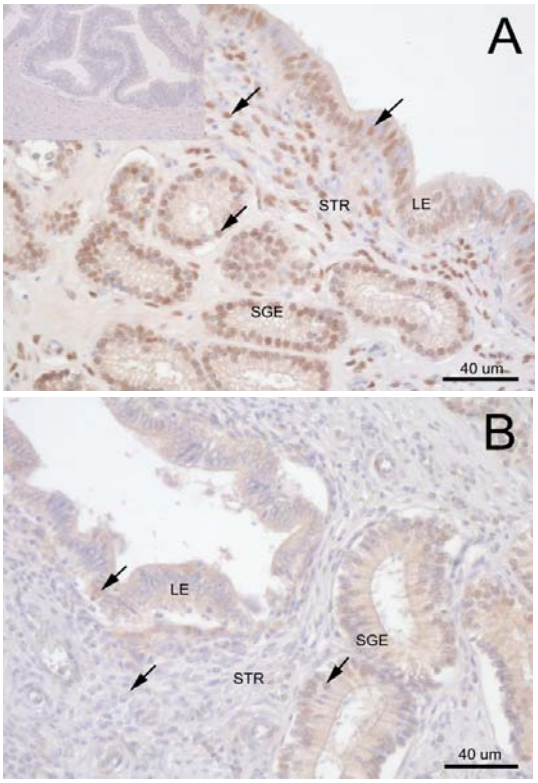

Figure 1 Immunohistochemical positive staining of ERα (black arrows) in nuclei of luminal epithelium (LE), stromal cells in stromal CNT layer (STR) and superficial glandular epithelium (SGE) of control group (A) and multiple ovarian cysts' group. Negative control was shown in A (inset).

Table 1 The immunohistochemical expression intensity scores of ERα in each compartment of culled gilt endometrium.

Groups	Luminal epithelium	Stromal CNT layer	Glandular epithelium
Control	2.40±0.38[a]	2.60±0.55[a]	2.60±0.55[a]
Multiple cysts	0.80±1.03[b]	1.50±0.70[b]	1.00±1.15[b]

*Different letters within the same column represent significant differences (p<0.05).

As it is known that ERα and its receptor were important for diverse regulatory of the uterus, e.g. proliferation of luminal epithelium, uterine secretory activity and growth of endometrium glands (8). The results in the present study were corresponded with the previous research (9) which suggested that the expression of female steroid receptors in the uterus involved in the pathological conditions took place in the gilts culled due to reproductive failure. In addition, the multiple ovarian cysts demonstrated real endocrinological outcome and caused the morphological changes in the porcine endometrium (10). Our present information also showed that the inconsistency of ERα level and the decrease in its receptor might ascribe to the multiple ovarian cysts that impacted to the target tissues of the ERα. In conclusion, the low expression of ERα in the endometrium may involve in the multiple ovarian cysts and also influence on the normal functions of endometrium of these gilts.

Acknowledgements

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Relationship between Hyaluronan Binding Assay and Standardized Methods for Semen Evaluation in Boar

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Keywords: hyaluronic acid, sperm, assessment, boar

Introduction

Artificial Insemination **(**AI**)** has now started to dominate the reproductive process on almost pig farms in Thailand. One of the advantages of AI is that it's permit you to assess semen quality before insemination such as sperm motility, viability, morphology (1), acrosome and DNA integrity, and *in vitro* fertilization rate (2, 3), resulting in using high semen quality for AI then. However those techniques are not suitable for general farms especially for smallholder farms as they are costly, technically challenging, time consuming, and a large number of advanced and expensive instruments are needed, for instance fluorescent microscopy and incubator.

The hyaluronan binding assay (HBA) evaluates the maturity of sperm in a fresh semen sample (4). Naturally fertilization, only mature sperm bind to hyaluronan, the main component of cumulus matrix, contrast, immature sperm do not bind (5, 6). With this fundamental principle, the HBA test, simple, short and less costly technique, was developed for replacing those complicated, long and expensive techniques in infertility clinics for human (4). However it has no report in animals. The objective was therefore to assess the relationship between HBA test and former standardized methods for semen evaluation in boar whether it is possibly to use easily and practice instead of the others.

Materials and Methods

Twelve semen samples were collected by gloved-hand method from three boars. Then sperm were evaluated and recorded with routine analysis including sperm motility (1), sperm morphology (7), sperm viability (8), and acrosome integrity (9).

Semen samples were also tested with HBA (Biocoat, INC, Ft. Washington, PA, USA), following the manufacturer's instructions. Then the numbers of bound and unbound motile sperm were counted under a microscope. The HBA score (%) was calculated as # bound motile sperm/ # total motile sperm. Whether a low level of sperm binding to hyaluronan is calculated, there is a low proportion of mature sperm in the semen sample (4). The relationship between HBA score and conventional semen analysis methods were analyzed by Pearson's correlation and linear regression method.

Results and Discussion

The relationship between HBA score and conventional semen analysis methods were demonstrated in Table 1. The data obtained in this study showed no statistically significant relationship between the HBA score and sperm motility, viability and acrosome integrity. These finding were contrast with the previous in human (10), they found that HBA was highly correlated with sperm motility and inferred that HBA is likely to reflect the semen quality revealed by the routine semen analysis. For this contrast we suggest that sperm motility, viability and acrosome integrity compared with sperm maturity should be statistically independent as demonstrating in Table 1.

Due to not only mature sperm are able to motile but even immature sperm are also motile as well, for instance. Therefore mature sperm does not affect the probability of others properties, vice versa. Interestingly, however HBA score was significantly correlated with normal sperm ($r = 0.696$, $P = 0.011$) and the retention of sperm droplets ($r = -0.614$, $P = 0.033$). Also, the predicted equation between percentage of HBA and droplets retention was demonstrated in Figure 1. The coefficient of determination was 0.3769.

Table 1 Relationship between HBA score and conventional semen analysis methods

	HBA	Motility	Viability	Acrosome	Normal	Droplets
HBA	1.000	-0.248[1] (0.455)[2]	0.296 (0.347)	-0.294 (0.353)	0.696 (0.011)	-0.614 (0.033)
Motility			-0.325 (0.301)	0.368 (0.239)	0.102 (0.752)	-0.167 (0.602)
Viability				-0.359 (0.251)	0.215 (0.500)	0.534 (0.073)
Acrosome					-0.960 (0.001)	-0.579 (0.048)
Normal						0.339 (0.280)

[1]correlation(r); [2] significant difference
Normal = normal morphology, Droplets = cytoplasmic droplets

Cytoplasmic droplets are remnant of the germ cell cytoplasm which remain adherent at the neck region of the elongating spermatid while spermatid when it is shed as a testicular spermatozoon during normal spermatogenesis. In most species, fewer sperm have droplets in the ejaculates of semen as they are removed around time of ejaculation (11). Therefore the retention of cytoplasmic droplets indicates a failure of normal epididymal maturation. Highly percentage of sperm with proximal and distal cytoplasmic droplets had a negative correlation to infertility characterized by reduced pregnancy rate and litter size in pigs (12). Normally in boar semen, less than 10-15% prevalence of cytoplasmic droplets is therefore accepted.

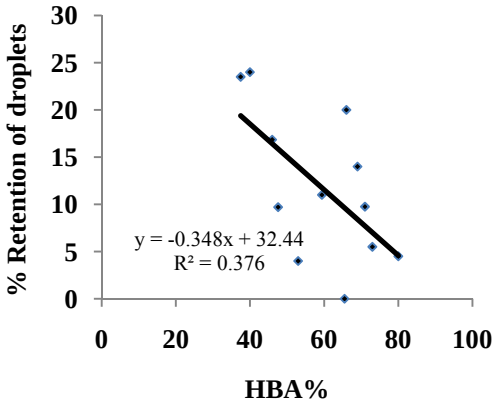

Figure 1 Linear regression analysis of HBA score compared with retention of droplets.

In conclusions, the HBA is not a representative of all conventional semen analysis methods in pigs. However the HBA test could replace the morphology especially the retention of droplets. If the HBA score is decrease, the morphologically abnormal sperm cells in terms of cytoplasmic droplets will increase followed as regression equation (Fig. 1). The using of semen then should be reminded. Therefore the HBA test, simple, short and less costly technique would be a screening prior to insemination at farms. Further study is needed to investigate the relationship between the HBA test and fertility rate after insemination.

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Morphological Changes of the Testicular Tissues and Seminiferous Tubule after GnRH Vaccination (Improvac™) in Boars

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Keywords: puberty, sperm, testis, vaccine, boar

Introduction

Boar attains puberty at approximately 6 months of age. The puberty attainment accelerates reproductive development, increase spermatogenesis and develop sexual behavior. Seminiferous tubule is an important part of the boar's testicular tissue consisting of germ cells and Sertoli cells. The immunocastration (i.e., vaccination against GnRH, Improvac™) has been successfully developed since 2001 in Australia (1). The main objectives of the GnRH vaccination are to minimize the boar taint and to reduce the aggressive behavior of the mature boars by disrupting the Leydig cells function and reduce the androgens synthesis (2). The objective of the present study was to determine the morphological changes of the testicular tissues and seminiferous tubules after GnRH vaccination in boars.

Materials and Methods

A total of 33 non-castrated male pigs were investigated. The boars were divided in to 2 groups, i.e., group I: non-castrated boars aged 23-26 weeks (n=11) and group II: immunocastration boars boars (n=22). The immunocastrtation was carried out by vaccinating the boars with 2 doses of Improvac™ (400 µg/dose, Zoetis) subcutaneously at 13 and 17 weeks of age. The boars were slaughtered between 4 and 8 weeks after the second vaccination. Reproductive organs were collected from slaughter house. The samples from testis were cut into 1x1cm^2 and fixed in 4% (w/v) paraformaldehyde. Thereafter, the tissue sections were dehydrated and embedded in paraffin. Four µm thick sections were cut from each block, processes histologically and stained with H&E. All of the tissue sections were photographed by Nikon ECLIPSE TE2000-U and were evaluated by image analysis (Image-Pro PLUS 6.0 Programming software, Media Cybernetics, Inc.). The germ cells were counted in 10 randomly selected fields in each compartment (seminiferous tubules and interstitial area). The results were presented as mean±SD and were compared by Wilcoxon's rank sum test. $p<0.05$ was considered to be statistically significance.

Results and Discussion

Morphological changes of the testicular tissues and seminiferous tubules in immunocastrated boars compared with normal intact boar are demonstrated in Figure 1. As can be seen from the figure, a dramatically changes was found in both the interstitial compartments and the seminiferous tubules.

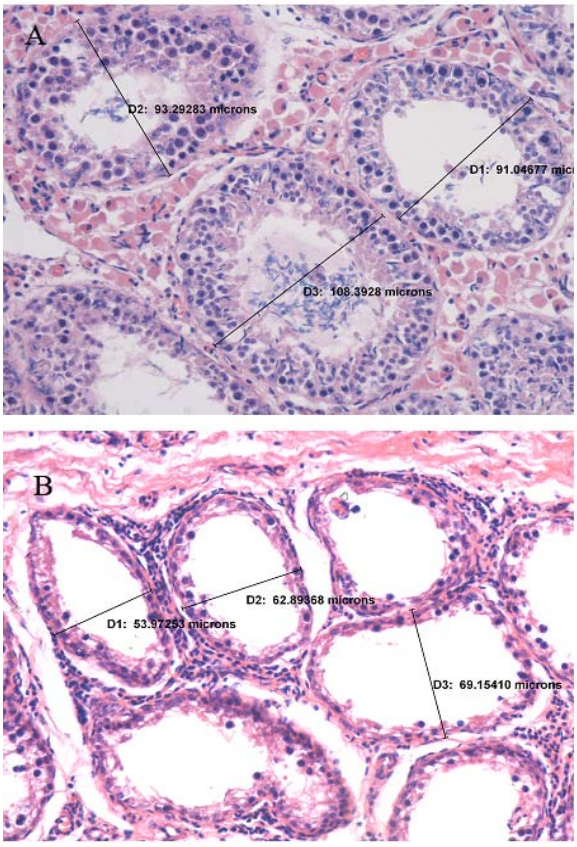

Figure 1 Histology and diameter of seminiferous tubule of the boars (A) Group I, non-castrated boar and (B) group II, boar vaccinated with two dose of Improvac™.

Table 1 Diameter of seminiferous tubule and number of germ cells in the seminiferous tubule in intact boar and in immunocastrated boar (Improvac™).

Parameters	Intact boar (n=11)	Improvac™ (n=22)
Diameter of seminiferous tubule	68.2±31.9[a]	60.5±16.6[a]
Spermatogonia	18.0±14.8[a]	15.4±14.8[b]
Primary spermatocyte	21.7±21.9[a]	11.4±11.0[b]
Secondary spermatocyte	47.7±45.3[a]	17.6±20.8[b]

[a,b] different superscripts within column differ significantly (p<0.05).

The numbers of germ cells in the seminiferous tubules in immunocastrated boars compared with intact boars are presented in Table 1. The total number of spermatogonia, primary spermatocyte and secondary spermatocyte in the seminiferous tubule of the immunocastrated boars were significantly lower than that in the non-castrated boars (p<0.05). The results indicated that the immunocastration using Improvac™ caused a dramatically changes in the histological structure of the testicular tissues and significantly reduced number of all types of germ cells in the seminiferous tubules. In addition, a previous study has demonstrated that the effect of the immunocastration persisted for at least 22 weeks after the second vaccination (2).

In conclusion, two doses of Improvac™ vaccine significantly cause dramatically changes in the testicular tissues and significantly reduced the total number of germ cells in the seminiferous tubules of the boars.

Acknowledgement

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Long Term Antibody Response after Vaccination with PCV2 ORF2 Subunit Vaccine under Field Conditions in Gilts and Sows

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Keywords: immunity, PCV2, reproduction, vaccine, pig

Introduction

Porcine circovirus type 2 (PCV2) is a non-enveloped single stranded DNA virus containing 3 open reading frames (ORF1, ORF2 and ORF3) and can be divided at least five subtypes a, b, c, d and e (1). PCV2 is the primary causative agent of porcine circovirus–associated disease (PCVAD). In Thailand, PCV2b is predominated field isolate of PCVAD (2). Under field conditions, strategies to control PCVAD include either management or vaccination or both. In Thailand, PCV2 vaccine is commonly used in nursery pigs (3). A previous study found that piglet mortality in the litter that comes from sows with high PCV2 antibody titer was lower than those from sows with low antibody titer (4). This implies that maternal antibody may play an important role in PCVAD. To fulfill knowledge concerning the gilts and sows immunity against PCV2 under field conditions, information on a long term serological response of PCV2 vaccination in gilts and sows is required. The objective of the present study was to investigate a long term serological response after PCV2 ORF2 subunit vaccination under field conditions in gilts and sows.

Materials and Methods

The present study was conducted in a commercial swine herd in the eastern part of Thailand between November 2012 and June 2013. PCV2 vaccination was used for the first time in gilts and sows by using PCV2 ORF2 subunit vaccine (Porcilis® PCV, Intervet International BV, The Netherlands). The gilts were vaccinated at 19 and 21 week of age and the sows were vaccinated at 84 days of gestation. Serum samples from 125 gilts and 127 sows were collected at 0, 2, 4, 6, 12, 16 and 20 weeks after vaccination. All serums samples were tested for PCV2 antibody titers by an indirect enzyme-linked immunosorbent assay using a recombinant truncated capsid protein of PCV2 (5). Data were presented as mean±SD. Data analyses were carried out by multiple ANOVA. The statistical models included effects of group of animals (gilts and sow), weeks after vaccination (0, 2, 4, 6 12, 16, and 20) and interaction. Least square means was calculated and was compared among groups by LSD test. The values of $p<0.05$ were considered statistically significant.

Results and Discussion

Antibody titer against PCV2 at 0, 2, 4, 6, 12, 16 and 20 weeks after vaccination in gilts and sows are presented in Table 1.

Table 1 Antibody titer against PCV2 at 0, 2, 4, 6, 12, 16 and 20 weeks of PCV2 vaccination in gilts and sows (LSmeans±SEM)

Weeks after vaccination	Gilt (n=125)	Sow (n=127)
0	1.24±0.62^{ae}	1.03±0.28^{b}
2	1.32±0.33^{ac}	1.54±0.30^{ac}
4	1.59±0.38^{b}	1.67±0.25^{ac}
6	2.06±0.41^{g}	1.95±0.28^{ad}
12	0.96±0.03^{ad}	1.09±0.13^{b}
16	1.17±0.38^{a}	1.67±0.26^{ace}
20	1.38±0.26^{af}	1.74±0.24^{a}

^{a,b,c,d,e,f,g} different super script within column differed significantly ($p<0.05$)

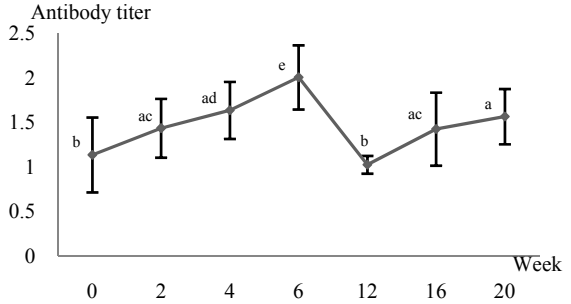

Figure 1 Antibody titer against PCV2 at 0, 2, 4, 6, 12, 16 and 20 weeks of PCV2 vaccination in females.

A recent study has found that PCV2-seropositive gilts can be infected with PCV2 after intrauterine exposure of the virus via artificial insemination (6). Furthermore, PCV2 positive fetuses were found in the animal with low anti-PCV2 titer before insemination (6). Under filed conditions, a huge variation of anti-PCV2 titer was observed in both gilts and sows. This might be a risk factor causing a high variation on the passive immunity against PCV2 of the piglets. The present study demonstrated that vaccination PCV2 in both gilts and sows significantly increased the antibody titers of the animals within 2-4 weeks of vaccination then increase to the highest level at the sixth week of vaccination. Enhancing the gilts and sow immunity against PCV2 may, at least in part, reduced the risk of transplacental infection and hence minimize the number of PCV2 infected fetuses. In conclusion, PCV2 vaccination in gilts and sows significantly increased antibody titer against PCV2 within 2-4 weeks after vaccination.

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Factors Associated with Stillbirth in Swine Commercial Herds in Thailand

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Keywords: stillborn, total born, parity, preweaning mortality

Introduction

Major goal of swine production in pig industry aims to maximize the number of piglets weaned per sow per year. Stillbirths are one of the main problems causing economic loss. The percentage of stillbirths per litter (SB) is generally 3-8% (1). Several studies has been reported the factors influencing SB, such as litter size, sow's parity, gestation length, sow's body condition, piglet's birth weight, farrowing duration, birth interval, environment and diseases (1-4). However, the studies investigating stillbirth and its affecting factors in Thai swine herds has been still limited and usually performed in few herds. The present study aimed to investigate the influence of SB-relating factors, i.e., number of total piglets born per litter (TB), average of piglet's birth weight (PBW) and sow's parity number, on SB in Thai swine commercial herds.

Materials and Methods

The reproductive data of sows during 2008-2009 were collected from 15 swine commercial breeding herds (A to O) in the central, eastern and northeastern parts of Thailand. The data consisted of 93,053 farrowing from 41,081 sows. Those with recorded parity more than 10 or no piglets born or TB more than 25 were excluded from the dataset, leaving 92,972 farrowing from 41,053 sows for analyses. SB was calculated by number of stillbirths per litter divided by TB and multiplied by 100. PBW was calculated by the litter birth weight divided by number of piglets born alive per litter. The relationship among SB, TB and PBW were analyzed by using Spearman's correlation. SB were compared among groups of TB (<10, 10-13 and >13 piglets/litter), PBW (<1.4, 1.4-1.7 and >1.7 kg), parity number (1, 2-3, 4-5 and 6-10) and season (November-Febuary: cool, March-June: hot, July-October: rainy) using general linear model with Tukey-Kramer adjustment for multiple comparisons. The statistical models included the effect of herd, parity, season, TB classes and PBW classes. Values with $p<0.05$ were considered statistically significant.

Results and Discussion

On average, SB was 6.0%, which was varied among herds from 3.8% to 10.5% ($p<0.001$) (Figure 1). SB positively correlated with TB ($r=0.25$, $p<0.001$). In addition, TB negatively correlated with PBW ($r=-0.22$, $p<0.001$). SB by TB classes, PBW, parity and season are presented in Table 1. SB was highest in sows with TB more than 13 piglets/litter (8.8%), PBW less than 1.4 kg (7.5%) and parity 6-10 (7.3%), nonetheless; it was lowest in sows with TB 10-13 piglets/litter (5.1%), PBW 1.4-1.7 kg (5.2%) and parity 2-3 (5.2%). Seasonal differences did not dominate SB ($p=0.594$).

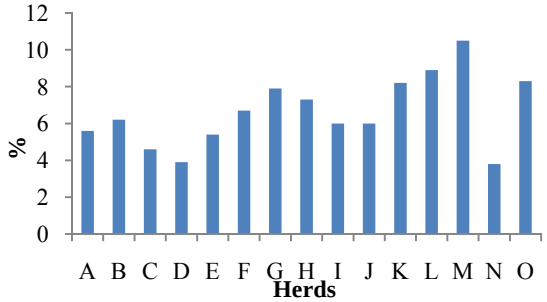

Figure 1 The percentage of stillbirths per litter by herds.

The present study demonstrated the percentage of stillbirths per litter in Thai swine commercial herds. This is in agreement with previous studies (3, 4). It has been reported that larger litter size was associated with prolonged farrowing duration and resulted in higher risk of fetal hypoxia (1, 5). Moreover, since TB was negatively related to piglet's birth weight, the larger litter size might be associated with lower

piglet's birth weight, causing the higher risk of stillbirth. In addition, piglets with low birth weight had smaller umbilical cords, resulting in higher risks of umbilical rupture and hypoxia during parturition (1, 6). In contrast, the smaller litter size might be associated with higher piglet's birth weight, entailing the higher risk of dystocia caused by fetal oversize and hypoxia caused by prolonged farrowing duration (1). In agreement with previous studies, the percentage of stillbirths per litter was highest in old sows (parity ≥6) (1, 2, 4). In conclusion, the percentage of stillbirths per litter was lowest in sows with TB 10-13 piglets/litter, PBW 1.4-1.7 kg and in sows parity numbers 2-3.

Table 1 The percentage of stillbirths per litter (SB) by the total number of piglets born per litter (TB), average piglet's birth weight (PBW), sow's parity number and seasons.

Variables	Level	n	SB (%)
TB	<10	19,093	5.4[a]
	10-13	54,747	5.1[b]
	>13	19,132	8.8[c]
PBW	<1.4	24,162	7.5[a]
	1.4-1.7	46,299	5.2[b]
	>1.7	22,511	5.8[c]
Parity	1	21,573	6.2[a]
	2-3	32,934	5.2[b]
	4-5	23,833	6.0[a]
	6-10	14,632	7.3[c]
Season	Cool	29,100	5.9[a]
	Hot	34,749	6.0[a]
	Rainy	29,123	5.9[a]

[a,b,c] Different superscripts within column of each variable indicate statistically significant differences ($p<0.05$).

Acknowledgments

E. Olanratmanee is a grantee of the RGJ Ph.D. Program, the Thailand Research Fund.

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Bilateral Alopecia due to Ovarian Disorders: A Case Report

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Keywords: ovarian cyst, non-pruritic symmetrical alopecia, bilateral alopecia, papillary cystadenoma

Introduction

There are many diseases and conditions which can cause alopecia in the dog. Skin infections from bacteria or external parasites such as lice, tick, flea or fungi and metabolic abnormalities should be included in the differential diagnosis for dogs with clinical signs and specific dermatologic test of alopecia (1). Bilaterally symmetric nonpruritic alopecia often caused by endocrinopathies (2,3) associated with hyperadernocorticisim, hypo- thyroidism, imbalances of estrogens, androgens and progesterone (4). The alopecia is effect of hormones on the hair follicle, inhibiting the normal cyclic pattern of hair growth (5). Compared with these endocrine disorders, hyperestrogenism is a rare dermatosis which may be produced by ovarian imbalance such as cystic ovaries, granulosa cell tumor, testicular tumors or by prolonged estrogen therapy. There is no breed predisposing. Because estrogen is a known inhibitor of anagen initiation, the clinical sign most commonly seen with hyperestrogenism is bilaterally symmetrical alopecia sparing the head and extremities (6,7). In addition, comedones, hyperpigmentation, nipples and vulva enlargement, abnormalities of the heat cycle such as persistent or irregular estrus may be present. In both sex dogs, aplastic anemia may be present (8). The present report describes dermatological manifestation and pathology of bilateral alopecia due to ovarian cyst in dog.

History and physical examination

A 6 year-old intact female poodle dog was presented at Kasetsart University Veterinary Teaching Hospital with clinical signs of non-pruritic symmetrical alopecia with scales on lateral flank and black comedone at some nipples for 2 years. These signs had begun since she was 4 years old. The owner did not recognized about estrus cycle of the dog. The physical examination found non-pruritic symmetrical alopecia, erythema, thin skin, prominent superficial vessels, comedones and rat-tailed appearance (Fig. 1).

Clinical examination

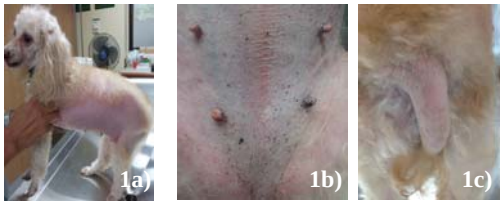

Figure 1 Clinical signs in the presented case.
a) Symmetrical alopecia with scales,
b) Comedone at last nipple, c) Rat tail

Dermatological examination with Wood's lamp test, pinna pedal reflex test, superficial and deep skin scraping test were negative. Vaginal cytological examination revealed non-cornification epithelial cells; parabasal and small intermediate cells; and few erythrocytes (Fig. 2a). Abdominal ultrasonography detected hypoechoic cystic lesion at left ovary position and both adrenal glands were normal. The first result of hematology and blood chemistry were shown in Table1.

Table 1 Hematology and blood chemistry profiles.

Parameters	1 st	2 nd	References
HGB	19.8	20.50	10-18 g/dl
PCV	61.7	61.4	35-55%
RBC	9.02	8.75	5-9 x10^6/cumm
MCV	88.4	70.17	60-77 fl
MCHC	32.1	33.39	32-36 gm%
WBC	9.580	7.490	6-17 x10^3/cumm
Segs	8.334	5.243	3-11.4 x10^3/cumm
lymph	1.245	1.947	1-4.8x10^3/cumm
Platelet	217	219	200-500 x10^3/ul
Protein (ref.)	7.2	7	6.0-7.5 gm%
BUN	7	-	10 – 26 mg%

Creatinine	0.87	-	0.5 - 1.3 mg%
ALT	31	29	6-70 37°C IU/L
ALP	28	12	8-76 IU/L
Estradiol	16	16	8-19 pg/ml (anestrus)
Testosterone	-	< 0.13	< 1 ng/ml
Progesterone	-	0.8	< 1 ng/ml
Thyroxine (T4)	-	2.4	1.5-4.0 µg/dl
GH	-	< 0.5	1-4.5 ng/ml

Treatment

Ovariohysterectomy was performed in 2 weeks later. On the operating day, the dog had serosanguneous discharge, and vaginal cytology revealed numerous small intermediate cells, large intermediate cells and red blood cells (Fig. 2b). Tissue samples of ovaries and uterus were submitted for histopathological examination. Results of histopathological finding were papillary cystadenoma (Fig. 3) and multiple follicular cysts in the left ovary (Fig. 4). Right ovary was normal. Cystic endometrial hyperplasia was found in uterus (Fig. 5). One month after ovariohysterectomy, the alopecia was improved and hair was regrew. Dog was checked blood profiles and hormones as shown in Table1.

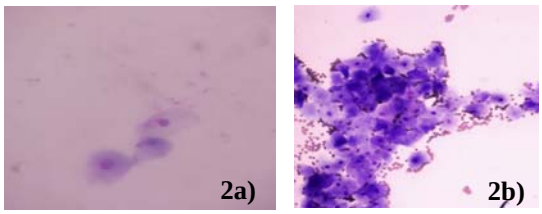

Figure 2 Result of vaginal cytology (H&E). 20x
a.) On first day present non-cornification epithelial cells and few red blood cells.
b.) On operating day present numerous small and large intermediate cells and red blood cells.

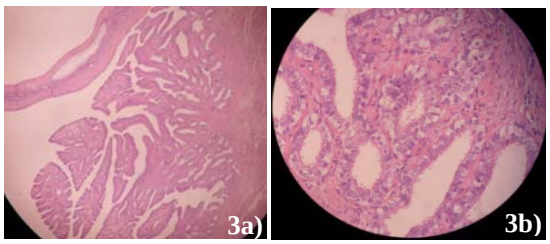

Figure 3 Papillary cystadenoma of left ovary on histopathological examination.
a.) There were finger-liked projections from the surface of ovary. (4x)
b.) These finger-liked projections were lined by simple layer of epithelial cells. These cells were slightly pleomorphic. (10x)

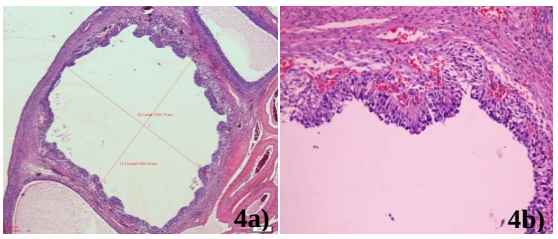

Figure 4 Result of histopathological examination.
a.) The size of cysts in left ovary. There was eosinophilic proteinacious substance in the lumen of cyst. There was no ova in the lumen of the cyst. (4x)
b.) Cysts were lined by granulosa cell. (20x)

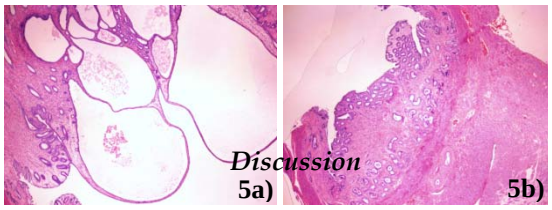

Figure 5 Result of histopathological examination.
a.) Cysts in endometrium. (4x)
b.) Endometrial gland hypertrophy. (4x)

Discussion

Normally, to diagnose follicular cyst, there should be confirmed by a sample of fluid from a cyst of the ovary to evaluate estrogen level (9). In alopecic dogs due to follicular cyst, hyperestrogenemia may not be detected because number of cutaneous estrogen receptors was increased. The cutaneous hyperestrogenism may be foun. (10) Characteristic of papillary cystadenoma can be found in dogs that recieved estrogen from outside the body (exogenous estrogen) for a long time, however, there was unclear whether or not caused by endogenous estrogen (11).

In addition, the detection of cystic endometrial hyperplasia was associated with obtaining estrogen for a long time, which supports the results of the histopathological characteristics of ovarian follicular cyst on left ovary. Treatment of choice for canine ovarian tumor and follicular cysts is ovariohysterectomy. For dogs that have valuable reproductive potential, induction of gonadotropin releasing hormone, human Chorionic Gonadotropin or pituitary LH may be attempted (12, 13, 14). The pathogenesis of follicular cyst was not clearly understood. It may be the result of not enough secretion of luteinizing hormone level from anterior pituitary gland to lutinization, which was the cause of follicular cysts (15, 16).

Acknowledgements

This study was kindly helped by Dr. Theerapol Sirinarumitr, Dr. Chalermpol Lekcharoensuk, Dr. Phudit Maneesaay, Dr. Wallaya Phongphaew, Dr. Benjang Hakhen, and the autors would like to thank Dr. Prasert Tunganurakpongsa, owner of presented case.

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Chronic Pseudomenbranous Cystitis and Prostatitis in Dog: A Case Report

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Keyword: pseudomembrane, cystitis, prostatitis, male Bulldog

Introduction

Pseudomembranous cystitis occurs from chronic infection or inflammation of urinary bladder. Most common causative bacteria is *Proteus* spp. The necrotizing pseudomembrane covering mucosal surface of urinary bladder occur from long-term irritation or inflammation especially from uroliths and chronic infection.Thedistinctive sign of cystitis is pollakiuria, stranguria or hematuria. The urine may appear cloudy and abnormal odor (2). The diagnosis for common cystitis is confirmed by urinalysis but in pseudomembranous cystitis can demonstrated the pseudomembrane in ultrasonographic finding. Only medical treatment for this case almost ineffective because the causative bacteria also covered under the pseudomembrane. Surgical treatment is the most effective treatment to remove pseudomembrane and combined with antibiotic treatment selectedbased on the results of urine culture and drug sensitivity test. (1,3).

Prostatitis is an inflammation of prostate gland, normally from bacterial infection. Benign prostatic hypertrophy is common predisposing factor to develop prostatitis in intact old dogs (4). The mechanism is likely to occur following cystitis. In sexually intact males, there may be a preexisting prostatitis. Dogs which go long periods of time without eliminating have a greater risk of developable bladder infection too (2). This case report studied about the clinical signs and also pathologically of pseudo-membranous cystitis with urolithiasis and prostatitis of intact male bull dog.

History and physical examination

A 9 years old, intact male bulldog was referred to Kasetsart University Veterinary Teaching Hospital with problems of hematuria, pollakiuria and stranguria and weight loss for 1-2 years. He had been treated with Enrofloxacin, Amoxicillin clavulanic acid and Finesterid from private clinic. On the 1st visiting day, he showed clinical sign of hematuria and trenesmus. The physical examination found two masses at caudal abdomen.

Clinical examination

Blood profile demonstrated mild anemia leukocytosis and hypoproteinemia (Table 1). From ultrasonographic examination found that both renal pelvesdilatated. Urinary bladder wall was thickening and some uroliths were found in the urinary bladder. There were hypoechogenic cavities in both prostatic lobes and at the center of prostate gland showed hyperechogenicity (Fig. 1). Red color and opaque urine, alkaliuria, proteinuria, hematuria, crystalluria, pyuria and bacteriuria were observed from urinalysis (Table 2).

Table 1 Result of complete blood count on 7th June 2013.

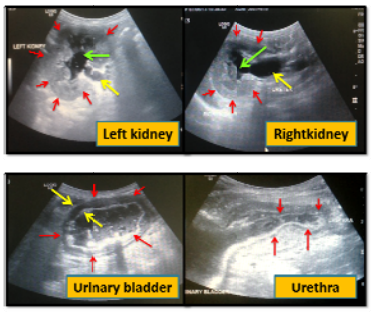
Hematology	7 Jun 13	Reference	
HGB	10.4	10-18	(gm%)
PCV	31.4	35-55	(%)
RBC	4,120	5,000-9,000	(/cumm)
MCV	76.4	60-77	(fl)
MCHC	33.2	32-36	(gm%)
WBC	58,200	6-17	(/cumm)
BAND		0-300	
SEGS	52,962	3,000-11,400	
LYMPH	4,656	1,000-4,800	
MONO	582	150-1350	
EOS		100-750	
PLATELETS	327	200-500	($\times 10^3/\mu l$)
PROTEIN (REFRACT)	5	6-7.5	gm%

Table 2 Result of complete urinalysis on 7th June 2013.

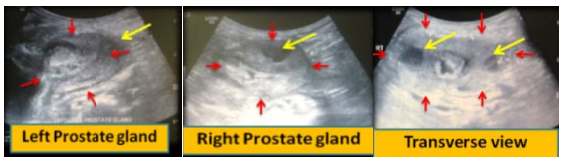
Urinalysis		7 Jun 13
Collection technique		UB catheterization
Color		red
Transparency		Cloudy
Sp.Gr.		1.030
Protein		4+
pH		9
Blood		4+
Glucose		Negative
RBC	/HPF	5-10
WBC	/HPF	>20
Crystals	/HPF	Triple Phos.2+
Bacteria		

Treatment

Tentative diagnostic was prostatitis or prostate tumor, chronic cystitis, renal hydronephrosis and urinary tract infection. The dog was not responded to antimicrobial drug treatment selected from bacterial culture. The dog was designed to have surgical treatment. The surgical treatment was performed on the dog in order to remove pseudomembrane and uroliths from urinary bladder including marsupialization and castration. Urinary bladder wall and prostate tissue were collected and submitted for histopathology examination. Calcium phosphate carbonate, severe diffuse purulent cystitis with ulcer and subacute multifocal lymphoplasmocytic prostatitis were diagnosed (Fig. 2).



A)



B)

Figure 1 Ultrasonographic examination of kidney, urinary bladder and prostate gland the 1st visiting day (7th June 2013). **A):** Both renal pelveskidney dilated with proximal ureter dilated (absent of obstruction site) and urinary bladder had hyperechoic band surrounding inside urinary bladder wall. **B):** Prostate gland has cavitary lesion in both sites. At left lobe hadhyperechoiccenter. Prostate volume = 31.68 ml.

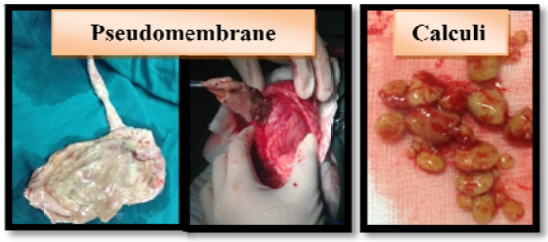


Figure 2 Pseudomembrane and calculi were removed from surgery.

After operation, the dog was ongoing antibiotic based on bacterial culture and drug sensitivity test. One month after marsupialization, he had normal urine color. The second operation was performed in order to correct marsupialization and then the dog had urinated through hisurethra.One month after marsupialization closure, the urine has turned to dark brown, viscous, and the dog has shown urinary incontinence. Therefore, urine sample was collected for bacterial culture and drugs sensitivity test. *Klebsiella pneumoniae* was isolated from urine sample and it was resistance to all antimicrobial drugs tested. Ultrasonographic examination show that the urinary bladder wall was thickening and dilated renal pelvis. The Veterinarian decided to use imipenem, which was not tested, for cystitis treatment on the dog. He had normal urine color.

Discussion

Old intact male dogs with chronic prostatitis always are found with chronic cystitis. The suitable way to treat was using antimicrobial drug based on bacterial culture and drug sensitivity test from both semen and urine samples. Castration combined with long term antibiotic treatment was recommended for this case because it may help involution of prostate gland and decreases factors for bacterial growth. Anemia from this case might has been from chronic blood loss for 1-2 years. However, it was improved after the disease had been treated combined with hematonic and antifibrinolytic drug. Pseudomembrane will cut off antimicrobial effect, so medicine is unable to

penetrate to the mucosa of bladder wall. For successful treatment, pseudomembrane is necessary to removed and followed with antimicrobial drug based on urine culture and drug sensitivity test. Imipenem, which is not widely used, was selected for treatment because the last urine bacterial culture test found resistance to all drug, even though it had not been tested. It is not convenience to use this drug because it needs to be given intravenously, three times a day. The unsuccessful treatment in this dog may from less care from the owner and the dog was unable to be controlled restrict diet.

Acknowledgements

This study was kindly helped by Dr. Theerapol Sirinarumitr for pathological results, and thank you Mr. Wipol Preeda, owner of the presented case.

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A Preliminary Study on the Delay of Puberty in Female Cats Following GnRH Agonist (4.7 mg Deslorelin) Implantation at Early Prepuberty Age

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Keywords: GnRH agonist, deslorelin, female cat

Introduction

Overpopulation of cats has been a problem in big cities for the pass years. Contraception is one of the most successful methods for population control in many animal species. Traditional ways of contraception by ovariohysterectomy is presently used but with many risks in the anesthetic procedure. Risk in anesthetics in young animals is a major problems in early-age surgical neutering (1). Meanwhile puberty in cats may occur in very young ages (4-12 months). As soon as puberty occurs unexpected mating could happen. Therefore, nonsurgical neutering could be an alternative to surgical methods. A new contraceptive method by GnRH-agonist implantation (Suprelorin®; Peptech Animal Health) was proven to be used in female domestic cats (2-4) for long-term reversible contraceptive effects without negative effects to the animals. Therefore, this study will concentrate on the effects from the usage of GnRH agonist implantation (4.7 mg Deslorelin) in early prepubertal aged female cats.

Materials and Methods

The objective of this study was to investigate the effect of GnRH agonist (4.7mg Deslorelin) implantation in early prepuberty female cats. Five domestic short hair female cats at the age of 3 months were kept together in an airflow-ventilated room. An intact male cat at the same age was kept together in additional to monitor sexual behavior of the female cats. After one month of acclimatization in the new environment one of the female cats was randomly selected to be a control cat without hormonal implantation. Four other female cats were subcutaneously implanted with GnRH agonist (4.7mg Deslorelin) at the interscapular area after an aseptic technique (4). Then, sexual behaviors was monitored for estrous detection for 30 minutes every day for three months, and at least 30 minutes weekly. After 30 weeks of implantation. Estrus in cats with estrous behaviors was confirmed by the vaginal cytology (5). Pregnancy was also observed, as one of the indicators of female reproductive function. After delivery of their first litters of offspring, all queens (control vs implanted) were ovariohysterectomiezed. The uterus and ovary of each queen were fixed in 4%paraformaldehyed (48 hrs) and 70%alcohol (after paraformaldehyde) for further investigation.

Results and Discussion

After 2 months of implantation, the control female cat showed a complete estrus behavior at the age of 6 months old. Mating between the control female and the intact male was first observed at about 4 months after the implantation. Whereas before the mating occurred the control female was detected and confirmed to be in estrus for at least 4 times. After the first mating the control female became pregnant and delivered 2 healthy offspring at the age of 10 months old. The control female also delivered 2 more litters of offspring before the implanted queen started to show sexual behavior and started to mate with the intact tomcat. The amount of offspring and litters of each queen are shown in table 1. The female cats that were implanted with GnRH agonist (4.7mg Deslorelin) became in heat at about 18 months after the hormonal implantation (age of 22 months old) without any sexual behavior until the first heat occurred at about 24 months after the hormonal implantation at the age of 28 months old.

The H&E staining for the ovaries and the uteri are shown in figure 1 and 2. The histological morphology of the ovaries and the uteri had no pathological lesion and moreover the histology showed that both implanted female and control

female had ovaries with all stages of follicles, which could be consider as functioned ovaries.

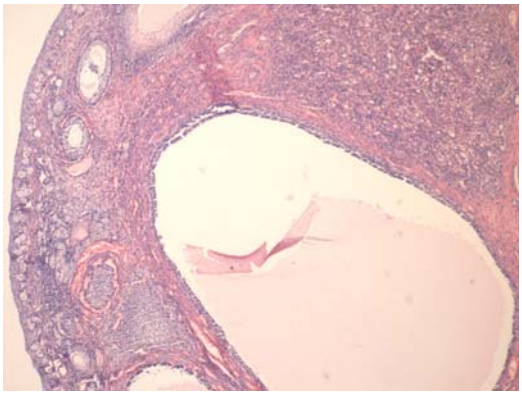

Figure 1 The histology of the ovary of treated female cat.

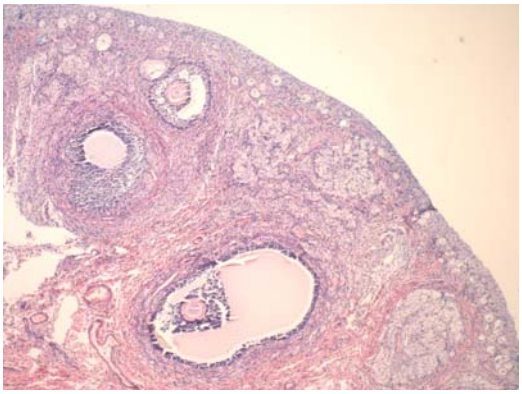

Figure 2 The histology of the ovary of untreated female cat.

Table 1 The number of offspring and litters of each queen (CC = control queen, C1, C2, C3 and C4 = implanted queens) from the day of hormonal implantation until 30 months after hormonal implantation.

Female cats	Total litters	Total offspring
C	3	10
C1	1	2
C2	1	4
C3	1	3
C4	1	2

From this recent study we can conclude that GnRH agonist (4.7 mg Deslorelin) implantation in early prepubertal female cats could delay the puberty of female cats without adverse effects to the cats for at least 22 months with a complete reversible of reproductive tract function. In the past studies of GnRH agonist implantation in pubertal aged female cats, an up regulation of reproductive function is found in the first period after implantation (3, 4, 6), which is a disadvantage point for the contraceptive purpose. An earlier study in dogs showed that dogs implanted with GnRH agonist (Deslorelin) at prepubertal age (7 months old) also showed the up-regulation estrus signs while those implanted at early prepubertal age (4 months old) had no up-regulation effect (7). While in early prepubertal implantation, no up-regulation of the reproductive function is observed as well. Furthermore, studies of the effect of GnRH agonist (4.7 mg Deslorelin) on the reproductive tract tissue are still in further investigating process.

Acknowledgments

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Expression of Multidrug Resistance Associated Proteins in Canine Transmissible Venereal Tumor during Vincristine Sulfate Treatment

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Keywords: transmissible venereal tumor, multidrug resistance associated proteins, vincristine, dog

Introduction

Chemotherapeutic resistance is the most important obstacle for cancer treatment. Canine transmissible venereal tumor (cTVT) is the disease responded to various treatment modalities especially chemotherapy. The most effective chemotherapeutic agent for cTVT is vincristine sulfate given once weekly for 6 to 8 times yielding 80 to 90% of remission rate but resistant cases have been reported (1). The survived tumor cells may develop some structures manipulating intracellular drug concentration and the expression of certain proteins in cTVT might be involved in cellular response to vincristine treatment (2). Multidrug resistance associated proteins are families of ATP-binding cassette (ABC) proteins that function as intracellular drug manipulation in many tissues such as liver, kidney and brain. These proteins do not excrete only Vinca alkaloids (vincristine sulfate and vinblastine) but also blood-pressure lowering statins or cyclosporine (3). Srisilapakorn et al. (2008) reported the expression of multidrug resistance associated protein-1 (MRP1) expressed in paraffin-embedded cTVT while Gaspar et al. (2010) studied the expression of P-glycoprotein (P-gp) in cTVT with cellular type and treatment response. These two proteins might relate to the treatment response of vincristine sulfate. Therefore, the present study aimed to study the expression level of P-gp and MRP-1 in cTVT during vincristine treatment. More information of the expression of these drug-related resistance proteins may explain the mechanism of vincristine resistance in cTVT.

Materials and Methods

Twelve dogs diagnosed as cTVT via cytological method using impression smear stained with modified Giemsa staining (Diff-Quick®, SE Supply, Bangkok, Thailand). Tissue collections were carried out on week0, week1, week2 and week3 of vincristine sulfate treatment for evaluation the expression of P-gp and MRP-1 immunostaining. Tissue sections were incubated with mouse monoclonal antibody (MAb) for each protein (C494 MAb for P-gb and MRPm6 MAb for MRP-1) (2, 4). Positive staining was evaluated by using image analysis program (Image Pro PLUS Version 6.0). Intensity score was classified into 4 grades as 0 = negative staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. Percentage of staining was used for evaluation the expression. Ten microscopic areas in 400X magnification were randomized from each section (5). The expression of P-gp and MRP-1 was evaluated using an expression index derived by a percentage expression and intensity score (Expression index = [%expression × intensity score]/100). Paired t-test was used for determined the difference of the expression index between week of vincristine sulfate treatment.

Results and Discussion

Expression of P-gp and MRP1 was observed in cytoplasm and cell membrane of cTVT cells (Fig. 1 and 2).

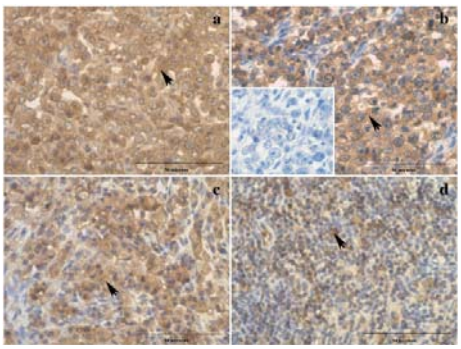

Figure 1 P-gp immunostaining of cTVT in week0 (a), week1 (b), week2 (c) and week3 (d) during vincristine treatment. The negative control was showed in (b; inset), arrow heads

showed positive staining in cytoplasm of cTVT cells.

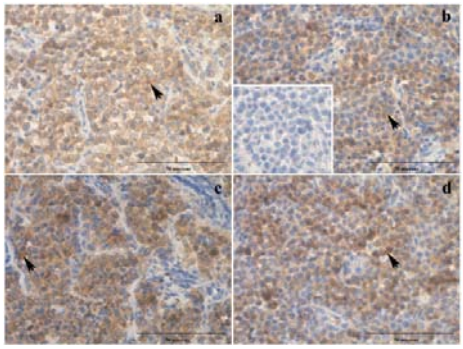

Figure 2 the MRP1 immunostaining of cTVT in week0 (a), week1 (b), week2 (c) and week3 (d) during vincristine sulfate treatment. The negative control was showed in (b; inset), arrow heads showed positive staining in cytoplasm of cTVT cells.

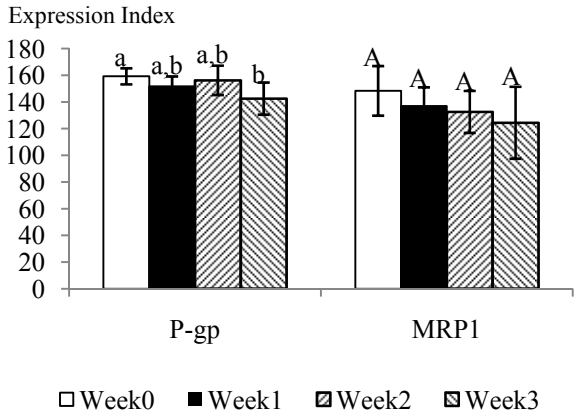

Figure 3 Expression indexes (mean ±SEM) of P-gp and MRP1 in cTVT in week0, week1, week2 and week3 of treatment. Different superscripts indicate significantly differences among weeks (p<0.05). The small letters are used for P-gp comparisons while the capital letter used for MRP1 comparisons.

The mean expression index of P-gp differed between week0 and week3 (p=0.02) but the mean expression index of MRP1 showed no significant difference among weeks of treatment (p>0.05) (figure 3). The highest mean expression index of P-gp was found in week 0 and the lowest was found in week 3 of treatment. The expression of P-gp and MRP1in cTVT before vincristine treatment showed in this study suggested that the two proteins have roles in cTVT cells and cellular exposure to vincristine affected P-gp expression as observed between week0 and week 3 of treatment. Vincristine sulfate treatment might be a selective condition for the survived cells. A resistance process might occur during treatment similar to other tumor such as mast cell tumor (6) or transitional cell carcinoma (7) that expressed ABC-proteins.

Further investigation on P-gp and MRP1 expression in resistant cTVT to vincristine sulfate will provide better understanding in the role of these proteins and will provide more information on the resistance process.

Acknowledgement

This research was financially supported by The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

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Fibroblast Growth Factor 2 Supplementation during *In Vitro* Maturation Improved Cytoplasmic Maturation of Cat Oocytes

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Keywords: FGF2, oocyte maturation, domestic cat

Introduction

Fibroblast growth factor-2 (FGF2) or Basic fibroblast growth factor is a member of the fibroblast growth factor family which involves in several biological processes including angiogenesis, wound healing and embryonic development. It has been shown to enhance the effect of follicular stimulating hormone (FSH) on growth of primodial and preantral follicle in cow and buffalo (1, 2). FGF2 supplementation in maturation medium can promote *in vitro* oocyte maturation and blastocyst formation rate in pig and bovine (3, 4). However, there is no report of FGF2 on developmental competence of cat oocyte matured *in vitro*. The objective of this study was to investigate the effects of FGF2 supplementation on nuclear and cytoplasmic maturation of cat oocytes.

Materials and Methods

The cumulus oocyte complexes (COCs) were obtained from the ovaries of female cats after routine ovariohysterectomy. They were cultured (6 replicates) in *in vitro* maturation (IVM) medium supplemented with 0.1 IU/ml FSH and FGF2 at different concentrations (10, 20, 40 and 80 ng/ml). After 24-h culture, the nuclear maturation of the oocytes was assessed by 4', 6-diamidino-2-phenylindole (DAPI) staining. Stained oocytes were categorized to 4 groups including degeneration, germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages according to their chromatin configurations. The oocytes were also *in vitro* fertilized (7 replicates) to examine cytoplasmic maturation in terms of *in vitro* embryonic development during 7-day culture. Cleavage, morula and blastocyst rates were observed on day 2, 5 and 7 after fertilization, respectively. All blastocysts were stained by DAPI to examine the blastomere number.

Results and Discussion

Our result demonstrated that FGF2 supplementation enhanced cytoplasmic maturation but did not influence nuclear maturation of cat oocytes, as indicated by the number of MII- stage (matured) oocytes (Table 1). Oocytes cultured with 20 ng/ml FGF2 during *in vitro* maturation yielded higher embryonic development than the control group (Table 2; $p<0.05$). It may be due to FGF2 stimulated the mitogen-activated protein (MAP) kinase activity (5) resulted in expedited meiosis resumption and germinal vesicle breakdown (6). Although FGF2 did not affect blastomere numbers, it was lower in the groups of 10 and 80 ng/ml FGF2 treatments when compared with the other two concentrations ($p<0.05$). The improvement of cytoplasmic maturation by FGF2 was not dose dependent. The finding that embryo development trended to decrease when FGF2 concentration was increased more than 20 ng/ml may be associated with the suppression of FSH activation on its receptor (7).

In conclusion, FGF2 supplementation at a proper concentration could enhance cytoplasmic maturation of cat oocytes. However, high FGF2 concentration may negatively affect on embryo development *in vitro*.

Acknowledgements

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Table1 Nuclear maturation of oocytes matured in IVM medium with different FGF2 concentrations (ng/ml).

Con.	No.	GV (%)	MI (%)	MII (%)	Degen.
0	128	10 (7.8)[a]	13 (10.1)	99 (76.7)	6 (4.6)
10	131	2 (2)[b]	15 (11.0)	100 (75.8)	14 (10.6)
20	130	8 (6.2)[a]	16 (12.3)	96 (73.8)	10 (7.7)
40	128	7 (5.4)[a,b]	19 (14.6)	96 (73.8)	6 (4.6)
80	129	6 (4.7)[a,b]	22 (17.1)	92 (71.3)	9 (7.0)

Different lower case letters within a column denote values that significantly differ between FGF2 concentrations. (p<0.05). Conc. = concentration; No. = number of COCs; Degen. = degeneration.

Table2 Cytoplasmic maturation of oocytes matured in IVM medium with different FGF2 concentrations (ng/ml).

Conc.	No.	Stages of development n (%)			Blast. no.
		Cleavage	Morula	Blastocyst	
0	115	49 (42.6)[a]	34 (29.6)[a]	28 (24.3)[a,c]	191.1±18.6[a,b]
10	115	67 (58.3)[b]	52 (45.2)[b]	37 (32.2)[a,b]	143.3±13.9[b]
20	118	73 (61.9)[b]	51 (43.2)[b]	46 (39.0)[b]	206.5±19.4[a]
40	115	60 (52.2)[a,b]	38 (33.0)[a]	34 (29.6)[a,b,c]	233.1±23.3[a]
80	114	56 (49.1)[a]	30 (26.3)[a]	23 (20.2)[c]	147.6±19.9[b]

Different lower case letters within a column denote values that significantly differ between FGF2 concentrations. (p<0.05). No.* = number of COCs.

Effect of Preservation Time on Cat Sperm Quality from Cauda Epididymis

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Keywords: testicle, cauda epididymis, sperm, storage time, domestic cat

Introduction

At the present time most wildlife feline species are at risk of extinction (1). The studies on the techniques and processes of sperm storage may be important in order to preserve the genetic materials. Especially, in the case of sudden dead animal or in some reasons animals have to be castrated. Collected sperm from epididymis may be the suitable way to keep the valuable genetics (2, 3). In this study, domestic cats were used as the model for wildlife felids sperm preservation. The objectives of the study were 1) to study the effect of storage time (within 6 and 24 hr) at 4°C of testes and epididymides in normal saline solution (NSS) on sperm quality and 2) to study the effect of immersed time (0, 0.5, 1 or 2 hr) in the extender of cauda epididymides on sperm quality.

Materials and Methods

Testes and epididymides were obtained from a total of 17 healthy sexually mature domestic male cats of various aged from 8 months to 1 year after routine castration with closed prescrotal castration. Testes and epididymides were immediately placed in a plastic bottle filled with NSS 25 ml then processed as in Figures 1 and 2. Each testicle was randomed to be kept in NSS either within 6 or for 24 hr at 4°C. The cauda epididymis was removed and immersed in extender for 0, 0.5, 1 or 2 hr, and after that it had been squeezed to recover the sperm (4, 5). The quality of sperm was evaluated using computer-assisted semen analyzer (CASA). Sperm quality data was analyzed using two-ways repeated measures ANOVA (the mixed model) to investigate the effect of storage and immersed time.

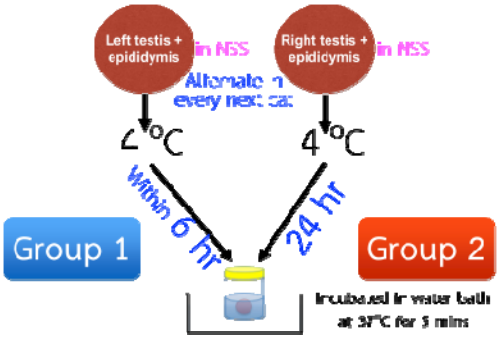

Figure 1 Diagram shows the sample collection and classification.

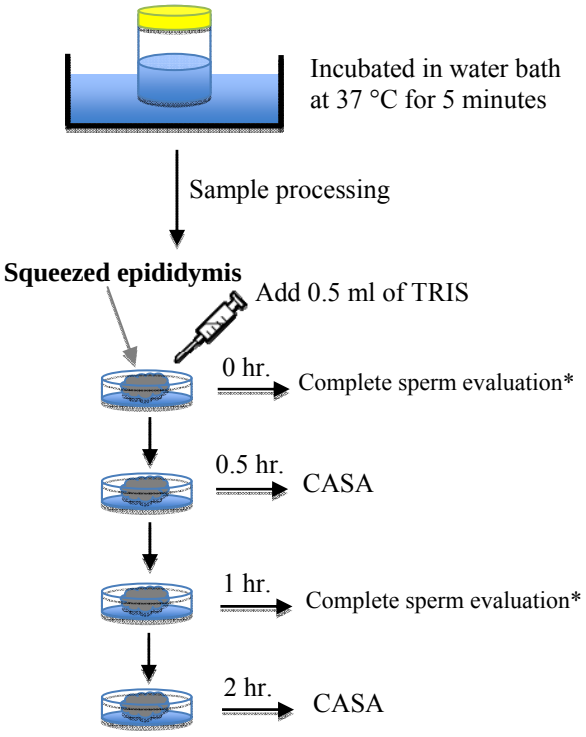

Figure 2 Epididymal sperm processing protocol. *Complete sperm evaluation = sperm concentration, sperm motility, sperm viability, plasma membrane integrity, acrosome integrity and sperm morphology.

Results and Discussion

The results found that there were significant differences (p<0.05) in the percentage of progressively motile sperm (Fig. 3) and normal morphological sperm between within 6 and 24 hr storage times, in which those of within 6 hr were better. For the effect of immersed time of cauda epididymis in extender, the significant differences (p<0.05) were observed in the percentage of total motile sperm, progressively motile sperm and plasma membrane integrity (6, 7). The 0 and 0.5 hr gave better results than 1 and 2 hr immersed times. For epididymal cells effect on sperm, cut and squeeze methods can make the cells let into spermatic content and when the immersed time was longer, sperm will attach to these cells (Fig. 4). This will effect on sperm concentration count (retrieved from the fluid), percentage of progressively motile sperm, so the overall, decreased sperm quality, moreover it will effect on sperm preservation (8). In conclusion, in order to obtain epididymal sperm with good quality, the sperm should be collected from the epididymis and kept at 4° C in NSS no longer than 6 hr and the immersed time of epididymis in the extender should not exceed 0.5 hr. The present results might be taken into account for the recovery of epididymal sperm of endangered felids.

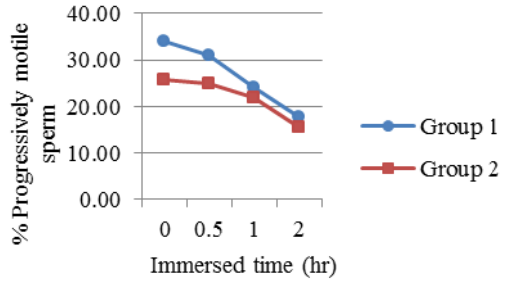

Figure 3 The percentage of progressively motile sperm determined by computer-assisted sperm analysis of cat epididymal sperm.

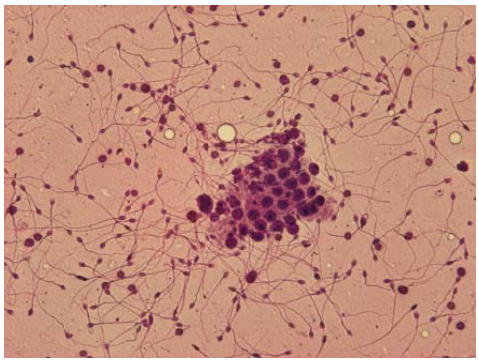

Figure 4 Sperm attach to epididymal cells.

Acknowledgements

Financial support was received from the Faculty of Veterinary Medicine, Kasetsart University. We thank Miss Piyawan Suthunmapinanta and Mr. Kornchai Kornkaewrat (technicians at Laboratory of Animal Reproductive Biotechnology and Cellular Biology) for helpful advice and technical assistance. We are really grateful to Dr. Supatee Kitkha for the statistical analyses.

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The Relationship between Pedometric Activity and Estrus Behavior in Dairy Cattle

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Keywords: pedometer, estrus detection, dairy cattle

Introduction

The method normally used for estrus detection in dairy cattle is visual observation of mounting behavior. However, the method requires frequently long-period observing time for efficient detection. Also, any estrus occur during nighttime is tend to be undetected. The observation of activity such as the number of steps might be alternatively used as a tool for estrus detection. The increasing number of step during estrus was previously documented (1, 2) and ensured this method as a promising tool for accurate detection of estrus. Since there is individual variation among herds in terms of farm size and farm management, the objective of this study was to study the relationship between the activity measured by pedometer and estrus behavior in dairy cattle in a medium holder farm model.

Materials and Methods

The study was carried out at research animal unit, faculty of veterinary medicine, Kasetsart university, Kamphaeng Saen campus, Nakhon Pathom. Data were collected from 56 Holstein Friesian cows equipped with the pedometer for a total period of 100 days. The calculation of activity during study period was performed by the software (Afimilk Ltd., Israel). The data of activity were analyzed with farm data to find the relationship among the activity change and estrus behavior. Heat detection was done 3 times daily as a routinely basis.

Results and Discussion

The average activity of cow not in estrus from this study was 17.83±51 steps/cow while the significantly higher activity (281.29±67 steps/cow) was observed during estrus ($p<0.05$). The average activity during estrus was 1.43 fold higher when compare to normal period and was 1.61 fold higher when focused only in the cows inseminated and detected pregnant. These finding might explained by the fact that cow tended to have more steps in the early stage of estrus when the mounting activity happened to be resisted than that happened in the later stage of standing heat.

The earlier the cow detected in estrus provided more changes to inseminate in the appropriate time. This result was also in agreement with the previous study which demonstrated that the insemination should be performed 7- 24 h pre-ovulation (3) and under 19 h after highest activity was observed (4). The effective of pedometer on estrus detection also observed. From the total of 331 estrus prediction by the farm management program, the pedometer could detected more estrus using the analysis of activity than visual observation of estrus (295 and 226 detections, respectively; $p<0.05$).

In dairy practice, the undetected estrus happened when observations were under performed, absent of standing heat, occurrence of behavioral estrus in the evening and night, illness and short estrus (5, 6). The use of pedometer estrus could be overcome the limitation of confounding factor mentioned above. The ability to detected silent estrus, with no expression of estrus behavior, was argued since pedometer estrus detection were based on the expression of estrus behavior. However, the of 100% detection of estrus including silent estrus was reported (7) in agreement to the results found in this study. The pedometer could be used as a tool for estrus detection in the reproductive manage- ment of dairy cattle as shown here in this study.

The study on factors influencing expression of activity (housing system, number of animals in estrus, parity, etc.) could further performed to optimize the use of pedometers as a promising tool for estrus detection.

Acknowledgements

This study was supported by the Faculty of Veterinary Medicine, Kasetsart University.

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Evidence of Sex Steroid Hormone Receptors in the Preoptic Area and Arcuate Hypothalamic Nuclei in Cycling Buffaloes

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Keywords: distribution, estrogen receptor alpha, progesterone receptor, hypothalamus, buffalo

Introduction

Studies have been done on the relationship between sex steroid hormones (such as estrogen and progesterone) and hypothalamic kisspeptin and GnRH releasing in many species (1-3), but never in buffalo: a ruminant with unique reproductive characteristics (4). Research in other ruminants has determined that the preoptic area (POA) and arcuate (ARC) are the main hypothalamic nuclei through which kisspeptin acts on hypothalamic- pituitary-ovarian (HPO) axis (5, 6). The aims of this study, therefore, were to determine the localization and the distribution of estrogen receptor alpha (ERα) and progesterone receptor (PR) in the POA and ARC hypothalamic nuclei of cycling buffalo cows.

Materials and Methods

Brains were collected from 6 buffaloes (3 brains in the follicular phase and 3 brains in the luteal phase) and processed for paraffin blocks. Four-micron paraffin sections of the POA and ARC hypothalamic nuclei were prepared for standard single label immunohistochemistry using a rabbit anti- human ERα polyclonal antibody and a rabbit anti- human PR polyclonal antibody.

Results and Discussion

Both ERα (Fig. 1) and PR (Fig. 2) immuno-reactions (ir) were found in the nucleus of the POA and ARC neurons. The populations of ERα and PR-ir neurons (mean±SEM) distributed in the POA (ERα; 76.17±4.06%, PR; 42.83±10.61%) were greater than in the ARC area (ERα; 51±6.85%, PR; 25.33±5.46%) by a statistically significant percentage for ERα (p<0.01) but not for PR.

However, there was no difference in the intensity of ERα and PR reactions between the POA and ARC hypothalamic nuclei which showed similar moderate reactions (grade 2) in both follicular and luteal phase cows (p>0.5).

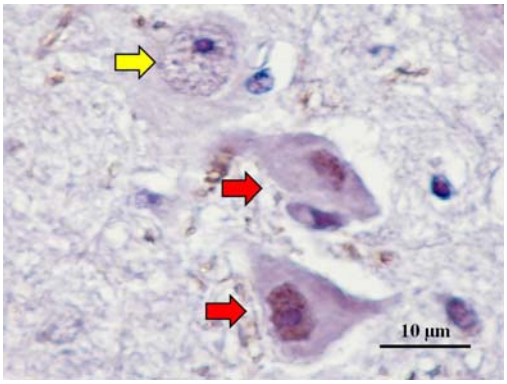

Figure 1 The ERα-ir appears as a granular formation in the nucleus of the POA neurons (red arrows) but is not present in every neuron (yellow arrow).

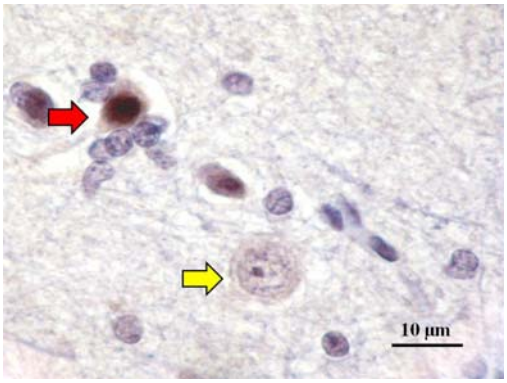

Figure 2 The PR-ir expresses in the nucleus of the POA hypothalamic nucleus (red arrow) but does not appear in another neuron (yellow arrows).

This research points to the possibility of a relationship between sex steroid hormones (estrogen and progesterone) and some neurons in the POA and ARC hypothalamic nuclei and suggests that they may be involved in kisspeptin and GnRH regulation in the HPO axis in buffalo.

Acknowledgements

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Effect of Estrous Cycle on Plasma Fibrinogen Levels and the Development of 'Homemade' Sheep-Derived Fibrin Glue

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Keywords: diestrus, estrus, fibrinogen concentration, sheep-derived fibrin glue, ewe

Introduction

Fibrinogen is a glycoprotein that plays a major role in blood clotting, as well as in maintaining blood viscosity and blood flow. Until recently, fibrinogen is primarily prepared for topical hemostatic agents, fibrin glue. Two different types of fibrin glues have widely been used in surgery, commercial and homemade fibrin glues (1). The fibrinogen concentration is indeed influent by various factors, including genetic, age, exercise, infection and hormonal status (2). Several reports also indicated that fibrinogen concentrations were high at estrus and diestrus (3, 4, 5, 6). The proposes of this study were 1) to extract and compare the levels of plasma fibrinogen derived from either estrus or diestrus ewes and 2) to study the efficacy of homemade fibrinogen by means of clotting time following fibrinogen-thrombin binding.

Materials and Methods

Healthy Merino ewes, age ranging from 1-4 years old were used in this study. Ewes (n=10) were estrus synchronized using medroxyprogesterone sponge (Sincrogest sponges, 65 mg) for 12-14 days. They were subsequently received 250 IU equine chorionic gonadotropin (eCG) to stimulate follicle development. At 36 to 48 h after eCG treatment, ewes were checked for estrus signs, and their ovaries were visualized for the presence of dominant follicle (s) via laparoscopy. The presence of estrus signs with large dominant follicle (s) typifies the estrus phase (day 0). Ewes were considered to be in diestrus by 1-2 weeks thereafter. At particular reproductive cycle, blood collection was performed using a 18 G needle connected to a 10 ml syringe pre-coated with anticoagulant (3.7% sodium citrate). The blood was immediately mixed with 3.7% (w/v) sodium citrate at a ratio of 9:1 and then kept at 4 °C prior to centrifugation. To obtain fibrinogen-rich fraction, plasma was centrifuged at 4 °C (2000 rpm) for 10 minutes. The plasma was heated for 3 minutes in a water bath (58±1°C) and then centrifuged for 3 min (12,000 x *g*) at room temperature. Total plasma protein concentration was measured by a refractometer. Fibrinogen concentration (mg/ml) was calculated as fibrinogen free plasma protein subtracted with total plasma protein and then multiplied by 1000. Fibrinogen was extracted from plasma using ammonium sulphate precipitation method (7). The clotting time of extracted fibrinogen was performed after binding with 1000 IU/ml bovine thrombin. The clotting time of fibrinogen-thrombin complex was recorded. Pair-t test was used to evaluate statistical significance of fibrinogen concentrations between estrus and diestrus ewes. Clotting time of extracted fibrinogen was descriptive analysis.

Results and Discussion

The results revealed that the mean of fibrinogen levels in estrus and diestrus phases was 620 mg/ml and 600 mg/ml, respectively (Table 1). Plasma fibrinogen did not significantly differ between estrus and diestrus ewes ($p>0.05$). This finding is in an agreement with other reports demonstrating that the fibrinogen concentrations during estrus phase tended to be higher than diestrus phase (3, 4). Following fibrinogen precipitation, plasma derived from diestrus ewes had significantly greater fibrinogen levels compared with estrus ewes (Table1, $p<0.05$).

The efficacy of extracted sheep-derived fibrinogen was tested. The fibrinogen-thrombin complex started to clot within 2-4 seconds (mean 3.2± 0.84), and the clots were completed by 5-9 seconds (mean 7.2±1.48).

Table 1 Mean fibrinogen concentration (mean± SD) in plasma of estrus and diestrus ewes**.**

Phases of estrus cycle	Mean fibrinogen concentration (mg/ml)			
	Before precipitation	range	After precipitation	range
Estrus	620±382.39	200-1200	3900±2152.52	2800-9200
Diestrus	600±400	200-1400	6160±1388.20	3200-8000

The result in this study demonstrated that fibrinogen can be extracted from plasma of ewes, irrespective estrous cycle. Ammonium sulphate precipitation can be used to concentrated blood derived fibrinogen. Further study on improving clotting efficiency of sheep-derived fibrinogen is required in order to use these plasma by-products as fibrin glue for controlling hemorrhage.

Acknowledgement

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Developmental Competence of Eld's Deer Embryos Derived by *In Vitro* Fertilization and Inter-species Nuclear Transfer

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Keywords: AI, IVF, ET, nuclear transfer, conservation, Eld's deer

Introduction

Eld's deer or brow-antlered deer is a medium sized deer localized to small areas in Southeast Asia. The Indian subspecies, Sangai (*Rucervus eldii eldii*) only exist in Manipur of Northeast India. Two subspecies of Eld's deer, Thamin (*Rucervus eldii thamin)* and Siamensis (*Rucervus eldii siamensis*), exists in Southeast Asia. Thamin is only found in Myanmar's dry forest and became extinct in the wild in Thailand. The Siamensis only exists in Cambodia and Lao PDR and became extinct in the wild in Thailand and Viet Nam. The species is considered endangered (EN) due to IUCN Redlist based on estimated rates of decline which primarily due to hunting (for local consumption of meat and traditional medicinal products). In Thailand, the species is extinct in the wild but 50 individuals of Siamensis and 1,000 Thamin remain in captivity. The species is listed as reserved species under the Thai Wildlife Preservation and Protection Act (1992).

Due to small population and few founders, inbreeding is a major risk of extinction. The use of reproductive technologies such as artificial insemination (AI), *in vitro* fertilization (IVF), nuclear transfer (cloning) and embryo transfer (ET) would serve a tool to maintain genetic diversity of these small populations. Over a decade, semen cryopreservation has been conducted routinely during breeding season (December-April) as a long-term genetic storage for future offspring propagation. Frozen semen from selected sire in remote captivity could be distributed to another Thamin herd to improve genetic diversity through intrauterine AI in 2009. Today, 4-year-old AI Eld's deer has been proved for successful natural breeding and delivered healthy fawn after reintroduced to Salakpra Wildlife Sanctuary. This reveals the possibility and importance of AI for transfer valuable male genetic from long–term frozen stored sperm to the next generation. This technique is also possible be used for sustaining global genetic diversity in the future. For more advance technology, IVF and cloning have been studied in several species but not yet in endangered Eld's deers. The application of the techniques is to produce multiple genetically valuable embryos which can be transferred to many recipients or can be frozen for future propagation.

The objectives of the study were to investigate 1) the development of Eld's deer embryos derived by IVF and nuclear transfer and 2) the developmental competence of IVF embryo after transferred to recipients.

Materials and Methods

Over two breeding seasons (2010-2011), oocyte donors were synchronized for IVF/ET by inserting intravaginal progesterone sponges (CIDR-G) and administering GnRH (Receptal®). In 8 oocyte donors, FSH was additionally administered (Follitropin®), at 12 h intervals before oocyte collection. On day 21, oocytes were collected after ovariectomy or laparoscopically aspirated. The recovered ova were *in vitro* matured and fertilized with frozen-thawed Thamin sperm in IVF DOF medium. Presumptive zygotes were *in vitro* cultured at 38.5°C in 5% CO_2, 5% O_2 in DSOF medium for either 8 days or transferred into synchronized recipients at the 2 to 8-cell stage.
For interspecies somatic cell nuclear transfer has been developed to observe the feasibility

to produce Siamensis embryos. Because collection of amount number of Eld's deer oocytes is a limitation for research, bovine and swamp buffalo oocyte collected from slaughterhouse was used instead as a recipient cytoplasm. The oocytes were *in vitro* matured in M199 supplemented with 10% fetal bovine serum, hCG (Chorulon®) and FSH for 20 h and subsequence enucleated. Female Siamensis skin tissue was cultured in DMEM supplemented with 10% fetal bovine serum and fibroblasts were used as donor cells. Siamensis cell -enucleated bovine/swamp buffalo oocyte couplets were fused by electrical induction. The couplets were activated by calcium ionophoreA23187 and incubated in SOFaa medium supplemented with cycloheximide and cytochalasin D for 5 h. Embryos were cultured at 38.5°C in 5% CO_2 in SOFaa medium for either 8 days.

Results and Discussion

Percentage of cleaved IVF embryos was 63% (51/81) at 36 hours post insemination (hpi), percentage of morula was 6.2% at 144 hpi and percentage of blastocyst was 5% at 192 hpi. Pregnancies (5/11, 45.5%) were diagnosed by EIA analysis of progesterone metabolite in feces (>1,000 ng/g). Two pregnancies failed prior to 90 days of gestation and two fawns died shortly after spontaneous preterm births at days 215 and 224 of gestation. A healthy female fawn was born unassistedwith no complications at day 234 of gestation.This study demonstrated the first successful *in vitro* embryo production and subsequent birth of a live Eld's deer fawn. However, the development and adaptation of reproductive technologies compatible with the easily stressed Eld's deer remains a challenge.

For cloning, the quality of swamp buffalo oocytes was poor compared to those of bovine (maturation success was 46 vs. 80%, respectively). The fusion successes of Siamensis cell- bovine and swamp buffalo oocyte couplets were 91 and 77%. The couplets derived from bovine and swamp buffalo oocytes could developed to cleavage 80 vs. 36%, morula 23 vs. 9% and blastocyst 13 vs. 2%, respectively. The results show the possibility to produce Siamensis embryos from bovine and swamp buffalo oocytes. However, the competence of the embryo derived from interspecies cloning after transferred to recipients need to be more fully study because many factors i.e. epigenetic abnormality may limit the development of embryos to term.

In conclusions, the present study indicates that innovative reproductive technologies are useful tools for fundamental science investigation and offspring propagation. Application of these technologies with intensive population management would bring hope for sustain genetic diversity of endangered Eld's deers.

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Semen Characteristics and Sperm Morphometry of a Malayan Sun Bear (*Helarctos malayanus*)

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Keywords: semen, sperm, morphometry, Malayan sun bear

Introduction

Malayan sun bear (*Helarctos malayanus*) is the smallest of the bears, native to Southeast Asia and classified as IUCN vulnerable (1). In Thailand, for the last ten years, only one cub was born in captivity at Khao Kheow Open Zoo, therefore the breeding project was initiated and started in 2013. Information of male and female fertility of this species is scarce. To our knowledge, there is no report of semen characteristics in this species. The semen quality status could provide a certain approach to the success of either natural breeding or other means of reproduction e.g. artificial insemination. Therefore attempt to collect semen by electro-ejaculation (EE) was carried out in a male in captivity. Collectable semen was also evaluated for its characteristics.

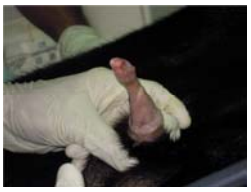
Materials and Methods

A male, aged 12 yr old, weight 74.6 kg, was rearing at Khao Kheow Open Zoo. After general anesthesia, attempts of EE were carried out on 6th February and 17th June 2013. In the first trial, the low electrical stimulating series (1-7 V) (2) were done. In the second trial, high voltages (6-10 V) series were applied.

Semen was collected using a micropipette following each stimulating series. Collectable semen was evaluated for its volume, the percentages of sperm motility (light microscope), live sperm (eosin-nigrosin staining; n=200) normal morphology (Williams staining; n=200), and sperm with normal acrosomal integrity (Coomassie blue staining; n=200). Moreover, sperm morphometry (n=50) was also measured using ImageJ version 1.47 (http://rsbweb.nih.gov/ij/).

Results and Discussion

The bear was safely recovered from the anesthesia in both trials. In the first trial using low voltages series, only the erection of the penis was observed (Fig. 1a). No semen could be collected. In the second trial, using high voltages series resulted in an ejaculation, but with little volume (273 µl), and 70% sperm motility, 81.0% live sperm, 85.5% normal sperm, 83.5% sperm with normal acrosomal integrity (Figure 1 b) and sperm concentration was 323 x 10^6 sperm/ml. Sperm morphometry was presented in Table 1.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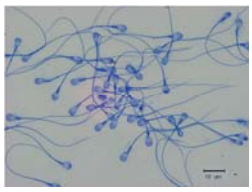
b

Figure 1 a) the erection of the Malayan sun bear penis following electrical stimulation b) Malayan sun bear sperm (Coomassie blue staining).

Table 1 Sperm morphometry of a Malayan Sun Bear.

Sperm parameters	Mean ± SEM	Min	Max
Head length (µm)	4.40±0.04	3.61	5.22
Head width (µm)	3.22±0.04	2.73	3.60
Head perimeter (µm)	13.30±0.11	11.12	15.63
Head area (µm²)	14.48±0.21	10.98	17.70
Acrosomal length (µm)	2.30±0.03	1.82	2.94
Midpiece length (µm)	7.90±0.03	7.15	8.45
Tail length (µm)	44.05±0.16	41.81	46.98

The semen of a Malayan sun bear in this study could be considered as good quality, compared to those of Asiatic black bear (*Ursus thibetanus*) (2). Furthermore, its sperm morphometry deemed to be smaller than those of American black bear (*Ursus americanus*), brown bear (*Ursus arctos*), and Asiatic black bear (3-5). The success rate of EE in Asiatic black bear was 96% (2), whereas the appropriate EE protocol for the Malayan sun bear still needs more investigation.

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Use of Ketamine and Xylazine to Achieve Intraperitoneal Anesthesia in BALB/c Mice

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Keywords: intraperitoneal, ketamine, xylazine, atropine sulfate, mice

Introduction

The mouse (*Mus musculus*) is one of the most common specie of the genus *Mus*. They have small body size, shorter generation time and commonly used as a mammalian research model. Experimental trials in biomedical study on mouse often need anesthesia (1). The selection of any anesthetic protocol depends upon several factors such as objective of study, species and health status of the animal, time duration, safety and recovery period of anesthesia. Technique for induction and maintenance of any anesthetic protocol comprises of injectable and gaseous anesthetics. Though gaseous anesthesia offers many benefits such as full operator control over anesthesia depth, less effect on cardiac and pulmonary system of animal but injectable anesthesia is preferred because minimum equipment and training is required (2).

Anesthetic procedures used in mice are based on the combination of two drugs, which are generally injected intraperitoneally (3). The most common combination is of dissociative drugs (Ketamine) and α2 agonists (Xylazine). However, anticholinergic drugs (atropine sulfate) are sometimes added to reduce the side effects of Ketamine (4). Recommended doses of Ketamine and Xylazine range from 80-200 and 5-16 mg/kg, respectively. However, some reports recommend that these doses do not produce surgical tolerance in mice (2). Hence, our objective was to optimize the concentrations of ketamine and xylazine in order to perform vasectomy and embryo transfer in mice

Materials and Methods

Male BALB/c mice (n=36), weighing 30-40 g, at the age of 8-10 weeks, were selected from the mice colony established in University of Veterinary and Animal Sciences, Lahore, Pakistan. The mice were kept on a dark : light cycle of 12:12 h (6 pm to 6 am) with the room temperature of 20±3°C and relative humidity of 55±10%. Feed and sterile water were *ad libitum*.

Mice were weighed and injected (intraperitoneally) with different concentrations of Ketamine and Xylazine as shown in Table 1. Atropine sulphate (0.05 mg/kg) was added in all groups. All drugs were diluted in saline and administered in a final volume of 100 µl per 10 g body weight.

Table 1 Experimental design.

Gr.	Species	N	Drug Combination	Dose (mg/kg)
1	BALB/c	6	Ketamine/Xylazine	100/10
2	BALB/c	6	Ketamine/Xylazine	100/15
3	BALB/c	6	Ketamine/Xylazine	150/10
4	BALB/c	6	Ketamine/Xylazine	150/15
5	BALB/c	6	Ketamine/Xylazine	200/10
6	BALB/c	6	Ketamine/Xylazine	200/15

Table 2 Time-related parameters.

Time	Definition	Interval
T0	Time of injection	Induction time T0-T1
T1	Loss of rightening Reflex	Immobilization T1-T3
T2	Loss of motion	Sleeping time T1-T4
T3	Spontaneous movements	Recovery period T3-T4
T4	Regain of Rightening Reflex	Surgical tolerance from negative PWR to positive PWR*
T5	Complete Recovery	

*PWR= Pedal withdrawal reflex

To evaluate the depth and duration of anesthesia, various reflexes and parameters were recorded. The reflexes monitored were cutaneous reflex, tail pinch reflex, pedal withdrawal reflex and corneal reflex. All these reflexes were monitored at the interval of five minutes after the loss of rightening reflex till animal showed spontaneous movement. Reflexes scoring (0-3) was done as previously described (2) and graded as no response (0);

weak response (1); moderate response (2) and strong response (3). On the basis of these reflexes time related parameters were recorded. Time related parameters were induction time, sleeping time, immobilization, recovery period and surgical tolerance demonstrated in Table 2. All measurements were carried out by the same operator to reduce variations.

Results and Discussion

The comparison of number of mice achieving surgical tolerance for different concentrations of both drugs is shown in Table 3. All the time-related parameters such as induction time, surgical tolerance, sleeping time and recovery period are presented as mean±SEM.

Table 3 Time-related parameters (mean±SEM)

Gr.	ST	IT	Duration of ST	Sl.T	R.P
1	4/6	2.4 ± 0.6	18.3±7.8	49.5±6.3	6.6± 1.8
2	4/6	1.5 ± 1	15.8±6.5	55± 2.1	7.5± 3.2
3	3/6	1.6± 0.2	11.7±5.8	69.7±7.3	11.3±2.4
4	4/6	1.3± 0.06	22.5±9.5	63.1±3.4	7± 1.3
5	5/6	1.5± 0.1	23±8.4	93± 5.7	21± 5.2
6	4/6	1.3± 0.1	30±10.1	89.5±1.3	18± 4.2

(ST=Surgical Tolerance, IT = Induction Time, Sl.T = Sleeping Time, R.P = Recovery Period)

The most important parameter in anesthesia is surgical tolerance as it represents the time duration when animal is totally unconscious of its surroundings. In order to calculate this parameter, most reliable reflex is pedal withdrawal reflex (1) which disappears later and reappears earlier than all other reflexes. Our results showed better surgical tolerance of 30 minutes and induction time of 1.3 minutes under the protocol 6, making it better than all other protocols. Ketamine induces excellent analgesia but not enough hypnosis and muscle relaxation (5). To achieve this, α2 agonists (xylazine) was added. To reduce the potential respiratory and cardiac side effects, an anticholinergic drug (atropine sulfate) was added. In the literature, a combination of Ketamine, Xylazine and Acepromazine has been recommended in mice (2) but due to the unavailability of Acepromazine in Pakistan, we added atropine sulfate in ketamine/xylazine. In another study, the combination of ketamine and xylazine produced a surgical tolerance of 25 minutes (1). The differences observed in our study are possibly due to the variations in environment, health status and strain. Further studies are needed to improve our understanding of achieving the most suited intraperitoneal anesthesia in mice.

Acknowledgements

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Hemipenes Infection in Common King Snake: The Case Report

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Keywords: snake, hemipenes, bacterial infection

Introduction

Snake and lizard share the same unique male reproductive anatomy which is much different from general mammal species. With a pair of hemipenes located inside the base of its tail (4), hemipenes are well-protected from all opportunistic pathogen. Bacterial contamination in hemipenes can be found during the copulatory or mating when male snake inserts their hemipenes into the cloacal opening of female (4), which contains numerous amount of these microorganism. Without hemipenes, female have this pair of pockets, but more shallow (4). The measurement of the depth of these sacs is a standard protocol to determine the sex of snake called 'probing technique' (4). Improper use of probing instrument, technique and hygienic consideration can cause injury and infection.

Materials and Methods

In December 2013, the male common king snake (*Lampropeltis getula*) was observed to have an enlargement at the tail base (Fig. 1). Without a clear information obtained from history taking, the lesion was seen for a month since the snake was purchased from the pet market. Physical examination showed a turgid consistency which becomes more rigid after palpation for a while. Radiological examination revealed an abnormality of the intercostal gap at the tail base (Fig. 2). The aspiration biopsy was performed with needle (18G) and syringe (3 ml) to collect the accumulated pus sample from the lesion. The sample was sent for bacterial identification and drug sensitivity test for 8 antibiotics available on our clinic, which were amoxicillin clavulanic acid, bacitracin, cephalexin, enrofloxacin, erythromycin, gentamicin, metronidazole, and penicillin G.

Results and Discussion

The manually pressing on the sac was done to release the exudate accumulated inside, but only few came out. Intravascular catheter (21G) was inserted into the site of infection via the opening of the sac, only little whitish pus could be collected. Even if the normal saline solution (0.9% NaCl, NSS) was flushed, it seemed impossible to get any fluid back from the sac, so the aspiration drainage with needle (18G) and syringe (3 ml) was performed and acquired 2 ml (right sac) and 1.5 ml (left sac) of pus.

Enrofloxacin (ACME®, Samutprakarn, Thailand, 5 mg/kg) was given intramuscularly once a day at the first third of the back muscle. However, the recurrent lesion was observed within the next two days. Surgical treatment was considered in this case. Local anaesthesia with lidocaine hydrochloride (Xylocaine®, Bangkok, Thailand) was done before the longitudinal cut at the third sub-caudal scale posterior to the cloacal opening for drainage, and then flushed with normal saline. After that, 10 ml of diluted gentamicin (Gentamicin Sulfate GPO®, Bangkok, Thailand, 0.04 mg/ml) was filled into the pockets.

Figure 1 External inspection from the top view (above) and bottom view (below) showed enlargement of the tail base.

The incision wound (approximately 1 cm) closed without suture needed. Iodine solution

was applied externally with nitrofurazone (BACTACIN®, Chonburi, Thailand) for wound dressing. Enrofloxacin was administrated for 10 days, concurrent with sun exposure to increasing the body temperature of the snake. *Enterobacter* sp. was identified from pus sample, which was susceptible to enrofloxacin and gentamicin that was previously selected for the treatment (Table 1).

Generally, hemipenes infection leads to the hemipenes prolapse which cleaning and controlling of infection can be done from the outside (3). In this case, however, the sacs are closed, hemipenes and exudate could not be released from the pockets. Insertion of IV catheter via the opening was difficult, even the lubricant has been applied.

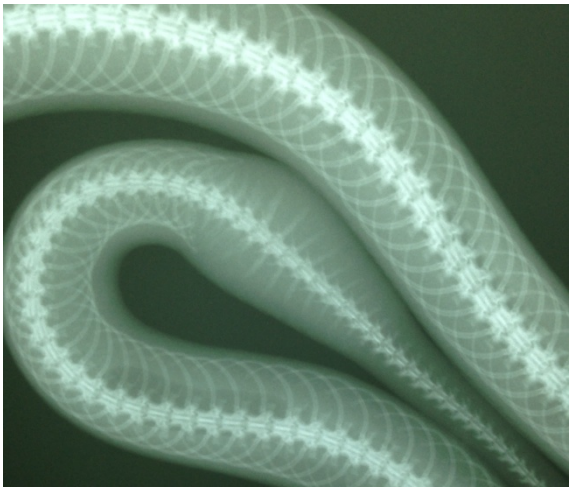

Figure 2 The radiological examination showed the enlargement of structure at the tail base.

Table 1 Drug sensitivity test result.

Drug	Susceptibility
Amoxicillin clavulanic acid	-
Bacitracin	-
Cephalexin	
Enrofloxacin	+
Erythromycin	-
Gentamicin	+
Metronidazole	-
Penicillin G	-

The cause of infection may not be clearly investigated. Poor hygienic probing may involve by introducing the pathogenic bacteria to the sacs, which were used as the multiplication site. Traumatic or forced probing could also be one of the causes of this infection. This was supported by the impossibility to draw the NSS flushing back which indicates that the anatomical structure of the sac may be damaged (3). Another route of infection may relate to mating behaviour. *Enterobacter* sp. is a common zoonotic bacteria in reptile which related to diarrhoea and urogenital infection (3). It is gram-negative aerobic or facultative anaerobic bacteria. One of the most common bacteria in this genus is *Enterobacter cloacae* (1). However, we did not test for the species identification in this case.

Drug sensitivity test showed the resistance on various group of antibiotics. Thus, for quality of service/treatment protocol improvement, bacterial identification and drug sensitivity test should be done in all cases. The treatment plan is also be an important factor for successful curative treatment. Generally, exudate accumulated in the sac is the source of pathogenic bacteria. Surgical intervention must be applied to remove all exudate and necrotic tissue (3). One of the remarkable characters for snake is the quick wound healing process. In this case, the incision wound closed and attached to each other almost after the incision was made. Therefore, no suture material was used. The healing processes in snake have been widely studied (2). Wound care can be simple as iodine solution administration and bandage. The wound was completely healed within 3 days, with some scar on animal scale remains. Environmental management, especially for temperature, is important for ectothermic animal. Without maintaining the optimum ambient temperature, snake may lose their normal physiology regulation, and lead to anorexia from lethargy, and death (3). It is recommended to keep the snake in the optimum temperature (75-85^{o}F) to reduce animal stress and improve overall homeostasis which is beneficial for the treatment (3). Providing exposure to the morning sunlight is additional option for this treatment.

In conclusion, Sex determination by probing technique should be done with care to minimize the opportunity of urogenital damaged and infection. In case of infection occurred, treatment plan should rely on medical and surgical techniques, together with general management to meet an optimal requirement of the animal. Laboratory results must not be overlooked because some drug resistant microbes may be presented in the case.

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Result of Progesterone 17-ß Estradiol Testosterone and Cortisol to Breeding Black-necked Stork (*Ephippiorhynchus asiaticus asiaticus*)

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Keywords: progesterone, 17-ß estradiol, testosterone, cortisol, Black-necked storks

Introduction

The Black-necked stork (*Ephippiorhynchus asiaticus asiaticus*) is one of the tallest extant species of storks. To date the Black-necked stork is threatened in the wild, **especially in several country of** Southeast Asia**.** It is important that a viable population is maintained in captivity. However breeding in captivity is relatively erratic. There are many factors that involving with unsuccessful breeding in captivity such as stress, infertility and non-paired breeding. In the present study describes a project initialed to establish basic reproductive parameters in this species, the diagnosis and monitoring of hormonal are two of the most important reproductive evaluation that can be made for the management of animals in breeding programs, with the ultimate aim of achieving better breeding success. By using the hormonal metabolite analysis, it's one suitable way for captive wild animals (1).

Materials and Methods

Animals in this study included 4 males **and 3 females** black necked stork at storks captive breeding zone in Khao Kheow Open Zoo, Thailand. Fecal or dropping were collected and analyze steroid hormones in feces. Hormonal assay is performed by Competitive ELISA (Enzyme Linked Immuno Sorbent Assay) according to single antibody assay methods from Smithsonian Endocrine laboratory (2). The hormonal EIA relied on a Monoclonal and Polyclonal Antibody (Provided by Quidel Corporation and California-Davis)

Results and Discussion

The study investigates the progesterone, estradiol, testosterone and cortisol profiles of these seven individuals of birds. All study in Black-necked Stork females showed at least some evidence of ovarian activity. There was seasonality in ovarian activity because cycles were not observed every month of years, with observed only in Breeding season (October-November or January-February). There was no age difference (p>0.05) between paired and non-paired females in this study population. The progesterone concentrations in breeding season of all females were significantly different between paired female and non-paired females. Paired female had higher concentrations than two non-paired females (p<0.05) but concentrations were non-significant different during the non-breed season during a year period (p>0.05). For all females had baseline concentrations of fecal progesterone averaged (Mean±SEM) 77.8±1.91 ng/g of dry feces, with peak concentrations ranging from approximately 12 to 180 ng/g.

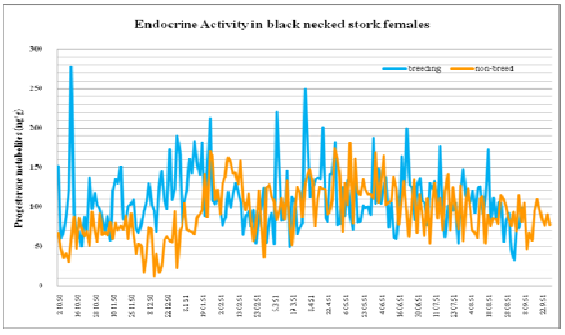

Figure 1 Fecal progesterone profiles in an individual Black-necked Stork female throughout average one year round.

The 17-ß Estradiol metabolite profiles from this study are fluctuated markedly but can indicate a difference on hormonal profiles between laying and non-laying females black-necked stork. The laying female had significantly higher 17-ß Estradiol concentration during the breeding season (January) than the non-laying females in non-breeding group (p<0.05). Baseline concentrations of fecal 17-ß Estradiol averaged (Mean±SEM) 21.87±0.87 ng/g of dry feces. Hormonal levels of the testosterone in Black-necked stork males were detected, In contrast to females, there was difference (p<0.05) between paired male and three non-paired males in this study population. The average age of paired

male was greater (p<0.05) than that for the non-paired males. There was no evidence of seasonality in testicular activity. Overall mean fecal testosterone concentrations were higher (p<0.05) in the paired male (205.60±13.10 ng/g of dry feces); range, 21-877 ng/g) than in non-paired males (128.71±4.76 ng/g of dry feces); range, 8-1,042 ng/g). All this when compared in the same non-breeding group, there was no difference (p>0.05) in average fecal testosterone concentrations.

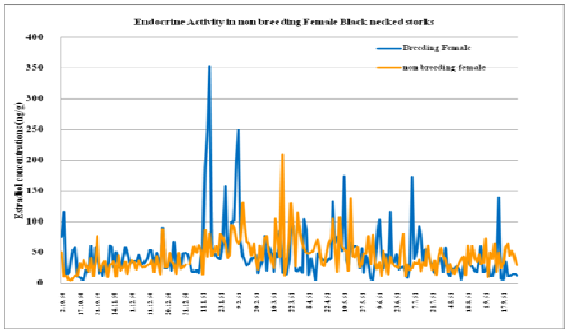

Figure 2 Fecal progesterone profiles in an individual Black-necked Stork female throughout average one year round.

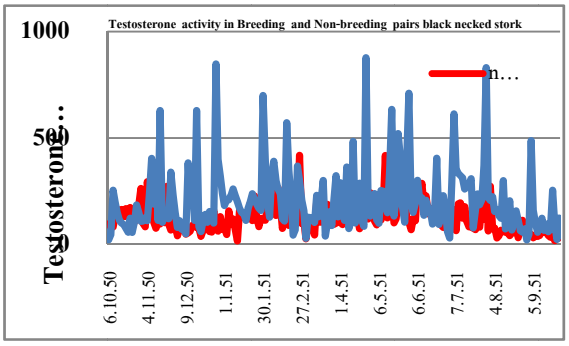

Figure 3 Fecal testosterone profiles in an individual black-necked Stork male throughout average one year round**.**

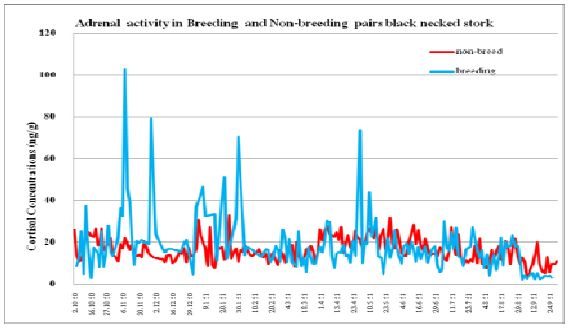

Figure 4 Comparison of cortisol hormone profiles between breeding and non-breeding birds group.

The study also found that cortisol level was higher in summer (p<0.05). Moreover, In male breeding black necked storks had significant highest cortisol concentration during the breeding season. However, an adrenocorticotropin hormone (ACTH) challenge in males and females demonstrated that clearance rate of cortisol metabolites did not show erratic stress level.

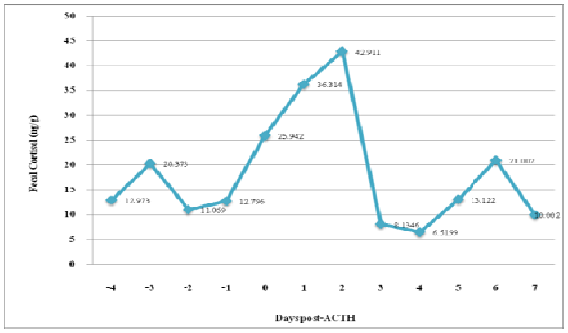

Figure 5 Mean fecal cortisol response to ACTH injection in four black necked stork.

Moreover, in paired female at Khao Kheow Open Zoo can bred 2 times and lay several of eggs in single season from cage management. Fecal hormonal assay has been used as noninvasive mean for assessing ovarian cyclicity in several species. However, Endocrine pattern in black necked stork (*Ephippiorhynchus asiaticus asiaticus*) has never been reported.

In conclusion, this study demonstrated the first report of Black necked stork endocrine pattern from fecal hormone metabolite analysis Finally, knowledge of the hormonal study of individual black-necked storks may be useful to improve the success of captive breeding management program to be appropriate for ensure sustainable conservation of this endangered species.

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Survey of Influenza A Viruses in Free-grazing Ducks in Lower Northern Part of Thailand

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Keywords: survey, influenza A virus, free-grazing duck

Introduction

Influenza A viruses can cause influenza in multiple species of avian and mammals, including human. Duck and waterfowl are the natural reservoirs for this virus without any clinical sign (1). In Thailand, free-grazing ducks were suspected to be the reservoir of H5N1 influenza viruses in the previous HPAI outbreak. Due to the frequently movement and sharing the same habitat with backyard poultry, free-grazing ducks have potential to spread the viruses from one area to the others. The high density of free-grazing ducks in the lower northern part of Thailand correlates with repeated, confirmed of HPAI outbreak (2, 3). In this study, a longitudinal survey of influenza A viruses in free-grazing ducks raised in lower northern part of Thailand was conducted.

Materials and Methods

Two free-grazing duck flocks (flock A and B) raised in the influenza high risk areas (Phichit and Phitsanulok) were selected. One hundred of 2-weeks old sentinel ducks were added into both flocks as the representative for sample collection. All sentinel ducks were marked individually and were negative for influenza A virus and antibodies before mingling. Oro-pharyngeal (OP) swabs, cloacal swabs and blood samples were collected from sentinel ducks every 2 weeks since the start of grazing at 3 weeks old until 15 weeks of age. All swab samples were isolated for influenza A viruses by egg inoculation. The virus isolates were identified for influenza A viruses by Hemagglutination (HA) test and realtime RT-PCR (M gene) then subjected for subtype identification and genetic characterization. For serological analysis, serum samples were tested for antibody against influenza A virus by using blocking ELISA (FlockChek® AI MultiS-Screen diagnostic kit, IDEXX).

Results and Discussion

Total 1,809 and 1,509 samples were collected from sentinel ducks of flock A and B respectively. The virological results showed that six swab samples of flock B were positive for influenza A virus by realtime RT-PCR (M gene). One virus was isolated from oro-pharyngeal swab collected from 13-weeks old duck. The other five viruses were isolated from oro-pharyngeal swabs (n=2) and cloacal swabs (n=3) collected from 15-weeks old duck. The virus isolates from 13-weeks old duck was identified as H4N6 subtype. The other isolates from 15-weeks old were identified as H3N8 subtype. It noted that at the age of 13 weeks, some sentinel ducks of flock B were showed clinical sign of depressed and ocular discharge. This observation was not correlated with the previous study that ducks were infected with no clinical sign (1). Furthermore, none of samples from flock A was isolated for influenza A virus. However, the serological result showed that 22.6% (19/84) of serum samples from flock A were found positive for influenza A antibody by blocking ELISA at the age of 9 weeks, but none of serum samples from flock B was positive. This study showed that the multiple subtypes (H3N8 and H4N6) of influenza A viruses circulated in the same free-grazing flock. This finding supported that free-grazing ducks are potential source of influenza A viruses reservoir in Thailand.

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Population Control of Free-ranging Long-tailed Macaque by the Non-affected Sexual Behavior Contraception and the Reintroduction and Continuing Evaluation after Contraception

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Keywords: population control, sexual behavior contraception, no-scalpel vasectomy, laparoscopy tubal ligation

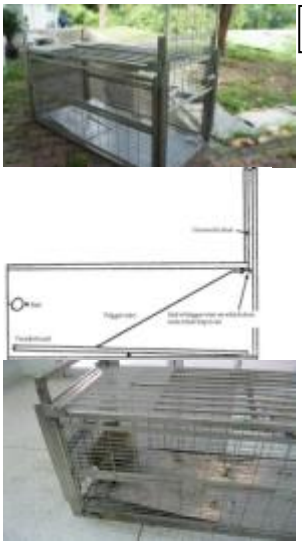
Introduction

Free-ranging Long-tailed Macaques is the one of Human-Wildlife conflict in many country of their habitat. Causes of human population expand impact to wildlife habitat lead to the conflict in overlapping area. Most of wildlife impact trend to threaten population decrease but the Long-tailed Macaques trend to over population. The solution for this problem or conflict is the four strategies to manage multi-interaction between human and macaques, i.e. 1) Involving human understanding and participating, 2) Protective and preventive systems, 3) Macaques' population control, and 4) Ecosystems rehabilitation. Population control in free-ranging Long-tailed Macaques need the method of contraception which no affected on sexual behavior in both sex and less complication after operation. No-scalpel vasectomy is preceded for contraception in male and Laparoscopy tubal ligation in female. Trapping and operation until reintroduction process on each case should not be over 24 hours cause that may effected to ranking and social interaction in the troops.

Materials and Methods

1. Population survey and census: 1,200 acres of Khao Kheow Open Zoo is the area of research there are at least 3 troops of free-ranging Long-tailed Macaques living around, population about 700 macaques.

2. Trapping and Restraint:



2.3 Automatic box trap

3. Physical examination and Identification marking:

Face tattoo for identification marking number each

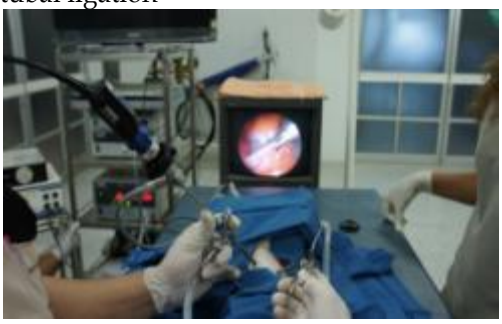
4. Non-affected sexual behavior contraception.

4.1 Males contraception: no-scalpel

Located and fix vas deferens with ring-clip forceps, skin cutting with sharp edge of 18G hypodermic needle above vas.

Retract vas deferens about 2 cm., cutting end of each with electrocautery blade, do on both side, suturing with absorbable

4.2 Females contraception: laparoscopy tubal ligation



Use 6 inches alligator forceps modified with electrocautery for cutting oviduct in females

5. Reintroduction.

After recovery macaque be reintroduction to the same troop by transferring cage (within 24 hours beyond trapping)

Results and Discussion

Totally 80 adult and sub-adult males were vasectomy. Only one female was tubal ligation. All macaques trapped and operation were not found side effect, normal sexual behavior and stay with old troop without affected of social ranking after reintroduce. The population control result may not affected during 2 years of research cause of technical constraint for contraception female didn't get to target and result from contraception on males may need evaluation more than 2 years.

Acknowledgement

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The Detrimental Effect of the Combination between Taurine and Pyruvate on Fresh Boar Semen Characteristics during Storage at 18 °C for 7 Days

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Keywords: antioxidant, fresh boar semen, pyruvate, taurine

Introduction

In pig industry, boar semen is diluted with semen extender and stored at 17-19 °C until insemination. During cold storage, boar sperm structure especially sperm plasma membrane is altered due to the high susceptibility to low temperature and high level of polyunsaturated fatty acids (PUFAs) of sperm plasma membrane. It has been shown that PUFAs level content plays an important role in the sperm membrane fluidity and causes sperm susceptible to lipid peroxidation (LPO) (1). Owing to LPO, sperm is attacked by reactive oxygen species (ROS) which leading to sperm damage and a decrease in sperm functions such as sperm motility, viability and eventually fertilizing ability (2, 3). ROS are generated as a result of oxygen metabolism including superoxide anion (O_2-), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO−) and hydroxyl radicals (OH−). Normally, small amount of free radicals can be found in semen which is balanced by antioxidants, and the residue amount is effective in stimulating the sperm capacitation, acrosome reaction, and sperm-oocyte fusion (4). Taurine (2-aminoethanesulfonic acid) is a major intracellular free-amino acid which is abundant in mammalian tissues. This intracellular antioxidant has been supplemented in boar semen extender (Modena solution extender) and revealed that the sperm characteristics such motility, viability and membrane integrity was improve after incubation with H_2O_2 at 37 °C for 12 h (5).

Besides intracellular antioxidant, there is also a pyruvate (2-oxopropanoic acid), an extracellular antioxidant, which has been added to *in vitro* culture medium to prevents the effects of ROS on human spermatozoa (6). In boar semen, pyruvate showed an ability to improve boar sperm characteristics and increase in vitro development of the porcine embryo after incubation with H_2O_2 at 37°C for 12 h (7). Considering the positive effect of taurine and pyruvate in term of their effect as intracellular and extracellular antioxidants, there is a possibility to supplement a combination of taurine and pyruvate into the boar semen extender in order to improve the sperm quality during cold storage at 18°C (8). However, no study has been reported about the combination effect of taurine and pyruvate as antioxidant in the boar semen extender. Hence, the aim of present study was to investigate the effect of the combination between taurine and pyruvate on fresh boar semen qualities.

Materials and Methods

Semen samples from 7 duroc boar were collected and divided into 4 groups according to the antioxidant supplement into Beltsville Thawing Solution extender (BTS; Minitübe Abfüllund Labortechnik GmbH & CO. KG, Tiefenach, Germany). The semen was diluted with BTS extender (T I), BTS + taurine 0.5 mM (T II), BTS + pyruvate 2 mM (T III) and BTS + taurine 0.5 mM + pyruvate 2 mM (T IV). All diluted semen samples were stored at 18°C and evaluated for sperm qualities on day 0, 1, 3, 5 and 7. After storage, diluted semen samples were incubated at 37°C until evaluation. Each thawed semen sample was evaluated for progressive motility, sperm viability and acrosome integrity. Progressive motility was assessed under phase contrast microscope at 100× and 400× magnification. Sperm viability was evaluated by using SYBR-14 and Ethidiumhomodimer I (EthD-1) staining. Acrosome integrity was performed by using fluorescein isothiocyanate labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) and EthD-1 staining. Both sperm viability and acrosome integrity were assessed under fluorescent microscope at 400× and 1000× magnification, respectively. At least 200 sperm

were counted and expressed as the percentage of viable sperm and viable sperm with intact acrosome.

Data were analyzed by using performed using Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA) and expressed as mean±SD. The specific sperm treatment were modeled according to the repeated measurements and analyzed with the GLM. When GLM revealed a significant effect, values were compared by the Least Squares test. Differences were considered statistically significant at $p<0.05$.

Results and Discussion

Osmolarity and pH are in a normal range throughout the experiment. All semen parameters on days 0 and 1 in groups T1-3 are in a normal range; however on day 1 a lower percentage (<70%) of progressive motility was found in group T4 (BTS+Taurine+Pyruvate) than in other groups. On day 3, only the percentage of progressive motility in group T2 is acceptable (>70%) for artificial insemination in pig industry. For the percentage of acrosome integrity, there was no significant difference among treatment groups. However, the percentages of acrosome integrity in all groups were higher than 80%. On days 5 and 7, no significant difference in all sperm parameters was found among groups. The percentage of progressive motility and viability were lower than 70% in all groups. However, the percentages of acrosome integrity in all groups were higher than 80%.

The present results demonstrated that the supplement of taurine at a concentration of 0.5 mM was able to maintain all sperm parameters on day 0 and 1. This is in agreement with the previous study in that taurine improve the sperm motility during incubation sperm with hydrogen peroxide (i.e. ROS) for 12 hr (5). However, on day 3, the supplement of taurine was only able to maintain the progressive motility of higher than 70% which is a standard level of diluted semen for AI in pig industry. The possible explanation might be that taurine, can pass into sperm plasma membrane as an intracellular free amino acid, and act as osmotic pressure control and also prevention of oxidant-induced tissues damage by its antioxidant capacity (9). In addition, it has also been reported that taurine are able to stabilize plasma membranes, scavenged the reactive oxygen species and reduced the production of lipid peroxidation (10) .Besides its effect as antioxidant, taurine has been reported to have a significant effect on energy metabolism such as glycolysis and aerobic metabolism (11). In bovine semen cryopreservation, it has been shown that TALP medium, containing pyruvate, in which not only its antioxidant properties, could protect sperm from oxidative damage and lipid peroxidation (12, 13), but also its ability to regenerate ATP levels, yielded better sperm motility and velocity (14) In the present study, the supplement of pyruvate alone at a concentration of 2 mM could not improve the boar semen qualities during storage at 18 °C which is contrast to the study by (7) who reported that supplementation of pyruvate during incubation boar sperm with hydrogen peroxide for 12 hr. could improve boar semen qualities when compared with control.

The combination of taurine and pyruvate as antioxidants in the boar semen extender in the present study failed to improve the semen qualities during storage at 18°C. Normally, pyruvate dehydrogenase located in the matrix space of the mitochondria which converts pyruvate to acetyl CoA. The presence of taurine may inhibit the phosphorylation of pyruvate dehydrogenase, consequently affect to intracellular ATP level biosynthesis and has negative effect on metabolism in the sperm cells (11).

In conclusion, the combination of taurine and pyruvate were not recommended to be used as antioxidants in boar semen extender.

Acknowledgements

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Selection of Morphologically Normal Epididymal Cat Spermatozoa and Sperm Loss after Cell Separation through Single-layer Centrifugation

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Keywords: colloid, feline, morphology, sperm selection

Introduction

The recovery and freezing of spermatozoa from the cauda epididymides is beneficial in preserving male gametes and maintaining the variety of genetic materials. However, epididymal sperm samples harvested either from dead animals or from orchidectomy are unavoidably contaminated with epithelial cells, red blood cells, leukocytes, bacteria and cell debris; these contaminants including abnormal and dead sperm cells produce reactive oxygen species (ROS) that impair sperm fertilizing ability (4). Cell separation and selection of morphologically normal, viable spermatozoa is an essential technique to improve sperm quality in the domestic cat (2). Additionally, this technique could be applied to other felids because seventy percents of felids are classified as teratospermia, the production of numerous morphologically abnormal sperm (≥ 60%) (5).

A novel sperm selection technique using single-layer centrifugation (SLC) through a species-specific silane coated silica colloids (Androcoll®, Swedish University of Agricultural Science, Uppsala, Sweden) has been reported in many species. Single-layer centrifugation has been shown to select a subpopulation of good quality sperm from frozen-thawed stallion semen (3) and good fertilizing capacity of frozen-thawed bull semen (6). In domestic cats, both swim-up technique and cell separation by SLC prior to sperm cryopreservation (SLC-PC) result in better sperm morphology, plasma membrane and DNA integrity and removal of cellular contamination but SLC-PC is better in recovery rate than swim up method (1). This study aimed to investigate (i) recovery rate of morphologically normal epididymal sperm and sperm loss after SLC and (ii) the efficacy of SLC in selecting sperm with normal head and tail morphology.

Materials and Methods

Epididymal sperm samples (n=12) were collected after routine castration. Sperm concentration, total sperm count, total motility (TM, %) and progressive motility (PM, score 1-5) were evaluated. Thereafter, samples were processed through cell separation using single-layer centrifugation (SLC) through silane coated silica colloids (1). Briefly, fresh sperm samples were slowly released to top on colloid layer and be carefully mixed with colloid. After centrifugation at 300g for 20 min, a sperm sample was divided into 3 parts; the first or top layer composed of morphologically abnormal sperm, dead sperm, RBCs and cellular debris, the second layer was colloid and the bottom layer was a sperm pellet which was morphologically normal sperm (1). After SLC, sperm concentration, total sperm count, TM, PM, head and tail morphology, plasma membrane integrity (Sybr14/Edthidium) acrosome (FITC/PI) and DNA integrity (Acredine Orange) were evaluated in the two populations of morphologically abnormal (top layer) and normal (bottom layer) sperm. Sperm loss (%) was calculated by subtracting the total sperm number prior to SLC from the number of sperm obtained from the top and bottom layers after SLC.

Results and Discussion

After SLC, recovery rate (mean ± SD, %) of the morphologically normal and abnormal sperm were 36.8±20.9 and 31.0±23.0, respectively. Sperm loss was found to be 35.2±26.3 compared with the total number of sperm before SLC. This finding clearly demonstrated that, after SLC and sperm harvesting, approximately one third of sperm lost. This observation suggested that relative centrifugal force (RCF) and centrifugation time should be optimized in the future study to reduce such a sperm loss.

In agreement with a previous study (1), total motility (TM) of morphologically normal sperm, not PM, was significantly improved (p<0.005) compared with fresh semen samples (73.3±13.7 and 64.2±11.7). After SLC, morphologically normal sperm had greater TM, PM and intact plasma membrane (%) than morphologically abnormal sperm (p<0.05); however, there were no significant differences in the percentage of sperm with intact acrosome and intact DNA (Table 1). The results indicated that SLC can separate epididymal cat sperm into different subpopulation and is beneficial in selecting normal, viable sperm. Thus, colloid centrifugation could be practically useful when dealing with teratospermic cats.

Overall, sperm with morphologically normal heads did not differ between top and bottom layers except that sperm with abnormal contoured and lose head (%) were higher (p<0.05) in the top layer (Table 2). In contrast, SLC could separate sperm with normal and abnormal tail morphology. Percentages of abnormal sperm with proximal and distal droplet, simple bent tail and coiled tail were greater in the top layer (p<0.05) (Table 3). These findings showed that SLC was more efficient in selecting a sperm subpopulation that had normal tail morphology, compared to head morphology selection.

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Table 1 Total motility (TM), progressive motility (PM), intact sperm membrane integrity, intact acrosome and intact DNA of epididymal cat sperm harvested from the top and bottom layers after SLC. Superscripts a and b in the same column indicate significant differences (p<0.05).

	TM (%)	PM (score 1-5)	Intact membrane (%)	Intact acrosome (%)	Intact DNA (%)
Top	22.50±11.38[a]	2.92±0.51[a]	42.58±9.94[a]	41.13±25.17[a]	43.04±19.16[a]
Bottom	73.33±13.71[b]	3.25±0.45[b]	55.42±15.70[b]	49.29±24.23[a]	53.38±22.74[a]

Table 2 Percentages of head morphology of epididymal cat sperm harvested from the top and bottom lavers

	TM (%)	PM (score 1-5)	Intact membrane (%)	Intact acrosome (%)	Intact DNA (%)
Top	22.50±11.38[a]	2.92±0.51[a]	42.58±9.94[a]	41.13±25.17[a]	43.04±19.16[a]
Bottom	73.33±13.71[b]	3.25±0.45[b]	55.42±15.70[b]	49.29±24.23[a]	53.38±22.74[a]

Table 3 Percentages of tail morphology of epididymal cat sperm harvested from the top and bottom layers after SLC. Superscripts a and b in the same column indicate significant differences (p<0.05).

	Tail morphology							
	Proximal droplet	Distal droplet	Simple bent tail	Coil tail	Midpiece defect	Acrosome defect	Loose	Normal
Top	13.97 ± 12.59[a]	15.50 ± 9.16[a]	72.33 ± 31.90[a]	13.08 ± 5.66[a]	8.17 ± 3.04[a]	0.00 ± 0.00[a]	0.50 ± 1.17[a]	76.50 ± 28.30[a]
Bottom	3.67 ± 4.52[b]	6.42 ± 4.34[b]	16.17 ± 16.44[b]	1.17 ± 1.47[a]	5.67 ± 5.47[a]	0.92 ± 3.18[a]	0.75 ± 1.22[a]	165.25 ± 18.37[b]

Pharmacologically-induced Ejaculation in Domestic Cats: A Comparison between Dexmedetomidine and Xylazine

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Keywords: α2-adrenergic agonist, chemical ejaculation, sperm, feline

Introduction

In felids, semen collection can be performed by electro-ejaculation (EE), artificial vagina (AV) and urethral catheterization (CT) after medetomidine injection. The latter has recently been reported in domestic cats (1, 2). For wild and domestic felids, EE is the most common semen collection method in both clinical and research purposes because it allows semen collection from healthy animals that can be safely anesthetized without previous training. However, EE requires relatively expensive equipment, skill and specific authorization form Ethical Committees (3). The use of AV is not practical in the cat because the tomcat has to be trained and in most cases the presence of a queen in heat is needed.

Semen collection after pharmacologically-induced ejaculation, so-called chemical ejaculation, has been practically used in stallions (4, 5) and is considered an alternative method for collecting semen in the cat (2). After α2-adrenergic agonist (medetomidine; 130-140 μg/kg) was injected and pharmacological effect was acquired, semen that released into the proximal urethra could be collected by CT (2). However, such a high dose of medetomidine (140 μg/kg; recommended dose in domestic cats is 10-90 μg/kg) reported in a previous study (2) resulted in respiratory distress and cyanosis in all cats (n=3) performed in our preliminary study and semen could be collected by CT in only 2 cats.

The present study aimed (i) at investigating if reduced dose of medetomidine is safe and sufficiently effective to collect semen by CT and (ii) to compare the success rate of semen collection by CT and semen quality (if obtainable) between the two α2-adrenergic agonists, e.g. dexmedetomidine and xylazine. Xylazine was chosen to test its efficacy because (a) xylazine is commonly used for sedation in domestic cats (b) it is generally cheaper than medetomidine, and (c) it is recommended for chemical ejaculation in other species, i.e. horse.

Materials and Methods

Sexually-intact healthy tomcats aged between 1.5-3 years old presenting for routine castration were received either dexmedetomidine (70 μg/kg) (n=16; Group I) or xylazine (3 mg/kg) (n=10; Group II) plus ketamine (5 mg/kg) intramuscularly. After injection (15 min), CT was performed and heart rate versus respiratory rate were monitored every 5 min until CT was finished. The urethral catheter (1.0×130mm) (Buster Cat Catheters, Sterile with stylet, Jørgen Kruuse, Langeskov, Denmark) was inserted approximately 8 cm into the urethra, and draw back. Semen volume, sperm motility, sperm concentration, total number of sperm, sperm morphology and viability were evaluated. If semen could not be obtained via CT, caudal epididymides were collected after castration to see if they contained sperm cells. Data were statistically analyzed using Fisher's Exact Test (success rate) and Paired *t*-test (semen quality and sperm morphology).

Results and Discussion

Twenty six healthy male cats were included in the study. No significant differences in heart rate and respiratory rate between cats received dexmedetomidine and xylazine were found (Table 1) and these two vital signs were within reference normal range during CT. The result showed that success rate of semen collection after dexmedetomidine-treated group (n=16) was higher compared with xylazine-treated group (68.8% vs 20%) ($p<0.05$) (Table 2). There were no significant differences in all parameters of the semen quality and sperm morphology between the 2 treatment groups (Table 3).

Of the cats that semen were unable to be obtained via CT, caudal epididymides were cut, smeared onto glass slides and stained using modified Wright-Giemsa stain. It was revealed

that all the epididymides contained normal sperm cells (Fig. 1). This indicated that all cats included in our study were sexually mature and their testicles were functional.

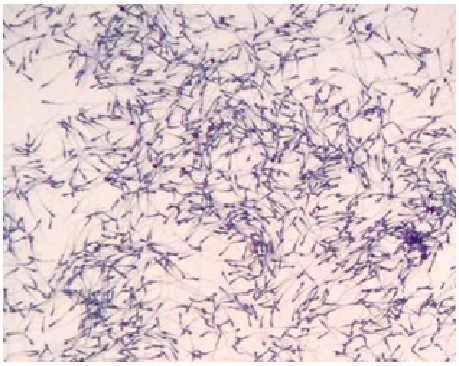

Figure 1 Impression smear of caudal epididymal tissue of a cat that semen were not obtainable via CT. (magnification 10×)

The results clearly demonstrated that both dexmedetomidine (70 µg/kg) and xylazine (3 mg/kg) in combination with ketamine (5 mg/kg) were safely used in healthy tomcats without any side effects as indicated by normal heart rates as well as respiratory rates maintained within the normal range during CT. The success rate of chemical ejaculation induced by dexmedetomidine was greater than by xylazine. The efficacy of dexmedetomidine was approximately 3.5 times higher than xylazine. This could be explained by the affinity and specificity of dexmedetomidine to α2-adrenergic receptor that is 10 times higher than xylazine (6). However, once semen was obtained, the quality of the two treated group did not differ, suggesting that both chemicals had no effects on semen quality and sperm morphology collected by CT.

Table 1 Mean (±SD) and reference normal range of heart rate and respiratory rate of dexmedetomidine-treated (n=16; Group I) and xylazine-treated tomcats (n=10; Group II)

	Heart rate	Respiratory rate
Group I	140.7 ± 25.4	22.3 ± 3.9
Group II	130.8 ± 21.3	24.9 ± 3.4
Normal range	103.5±56.4	10.0 - 197.6

Table 2 Success rate of semen collection in dexmedetomidine-treated (n=16; Group I) and xylazine-treated tomcats (n=10; Group II)

	Semen collected	Success rate (%)
Group I	11/16	68.8
Group II	2/10	20

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Table 3 Mean (±SD) semen quality and sperm morphology of dexmedetomidine-treated (n=16; Group I) and xylazine-treated tomcats (n=10; Group II)

Group	Volume (µL)	Concentration (10^6 spz/mL)	Total motility (%)	Progressive motility (score 1-5)	Viability (%)	Head abnormalities (%)	Tail abnormalities (%)	Total sperm number (10^6 spz)
I	44.1±31.2	204.7±243.4	44.1±22.7	2.3±1.1	59.5±21.7	15.6 ±6.3	43.7±15.5	8.±20.7
II	32.5±38.9	88.7±112.1	50.0±42.4	2.0±1.4	88.2±6.0	22.7±17.3	33.5±38.8	1.0±3.0

Does the *Curcuma longa* Extracted Influence the Boar Sperm Characteristic after Cryopreservation?

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Keywords: antioxidant, boar semen, cryopreservation, curcumin

Introduction

During cryopreservation processes, reactive oxygen species (ROS) occurred and are able to damage membrane lipids, proteins and DNA which lead to an impairment of sperm plasma membrane, decreased motility and viability (1-4). Curcumin is a natural antioxidant which acts as anti-inflammatory, antitoxic and anticancer agent for medical treatment (5, 6). The previous research (7) proved that curcumin exhibits protective effects against cold shock and oxidative damage by inhibits lipid peroxidation. In addition, curcumin improved frozen-thawed semen qualities for cryopreservation of goat (8) and bovine (9) semen. However, no previous study was performed for investigating the roles of curcumin on sperm qualities of post-thawed boar semen. Thus, the aim of this study was to investigate the effects of additional curcumin at different concentrations on the qualities of frozen-thawed boar semen.

Materials and Methods

Semen samples were collected from 4 Duroc boars which having proven fertility and used for routine artificial insemination. Only ejaculates with individual motility of ≥70% and ≥80 % morphologically normal were used for cryopreservation. After collection and evaluation, semen was divided into six groups (group A-F) according to the varying concentrations of curcumin in freezing extender (varied 0-1.0 mM). The semen was frozen by traditional liquid nitrogen vapor method and stored at -196˚C in the liquid nitrogen tank. After storage, frozen semen samples were thawed at 50°C for 12 sec and incubated at 37°C until evaluation. Each thawed semen sample was evaluated for progressive motility, sperm viability and acrosome integrity. Progressive motility was assessed under phase contrast microscope at 100× and 400× magnification. Sperm viability was evaluated by using SYBR-14 and Ethidiumhomodimer I (EthD-1) staining. Acrosome integrity was performed by using fluorescein isothiocyanate labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) and EthD-1 staining. Both sperm viability and acrosome integrity were assessed under fluorescent microscope at 400× and 1000× magnification, respectively. At least 200 sperm were counted and expressed as the percentage of viable sperm and viable sperm with intact acrosome. Data were analyzed using SPSS version 19.0 (SPSS 19.0; SPSS Inc, Chicago, IL, USA). All parameter was tested for normal distribution with Shapiro-Wilk Test. Sperm parameters were analyzed using one-way ANOVA according to Randomized Complete Block Design (RCBD) and comparison of parameters among treatment groups were performed by Duncan's new multiple range test. The statistical significant difference was considered as $p<0.05$.

Results and Discussion

Data of sperm parameters after frozen-thawed process are presented in Table 1. Our results showed that the percentage of progressive motility and acrosome integrity in group C was significantly higher than in group A and F ($p<0.05$). In addition, it is found that the addition of 1 mM of curcumin (group F) resulted in the lowest percentage of sperm motility and acrosome integrity. However, there was no significant difference in percentage of viability among treatment groups.

The addition of curcumin at 0.25 mMol in the freezing extender improved sperm progressive motility and acrosome integrity of frozen-thawed boar sperm. The results in the present study are in accordance with the earlier study (8) in that an addition of curcumin could increase progressive motility and acrosome integrity of frozen-thawed Angora goat semen and also improved the sperm quality of frozen bull semen (9).

Table 1 The mean (±SD) percentages of progressive motility, viability and acrosome integrity of frozen-thawed semen in the different groups from Duroc boars (n=4)

Groups	Progressive motility	Sperm viability	Acrosome integrity
A	32.5 ± 7.2^{ac}	26.3 ± 7.6^{a}	22.5 ± 3.2^{ac}
B	36.3 ± 6.3^{abc}	33.0 ± 6.4^{a}	29.0 ± 3.1^{ab}
C	43.7 ± 6.3^{b}	40.4 ± 3.5^{a}	34.8 ± 1.6^{b}
D	38.8 ± 8.5^{ab}	36.6 ± 6.7^{a}	31.6 ± 3.3^{ab}
E	33.7 ± 5.5^{abc}	34.0 ± 5.0^{a}	24.3 ± 3.7^{ac}
F	26.3 ± 4.7^{c}	33.9 ± 4.3^{a}	18.9 ± 3.9^{c}

^{a,b,c} values with different letters within the same column were significantly different (p<0.05)

The improvement in the qualities of frozen-thawed boar semen found in the present study might be explained by curcumin, a polyphenolic compound, a natural antioxidant, has the protective effects against cold shock and oxidative damage by inhibition of ROS formation (10) and lipid peroxidation (7), as a result of its scavenging property against free radicals, such as superoxide anion and hydroxyl radicals (11). With regard to the concentration of curcumin in the present study, it is in agreement with the previous reports in that the qualities of frozen-thawed boar semen depend on the concentration of antioxidant (12, 13) and its too high concentration may not be useful due to the restriction in antioxidant uptake of and may have toxic effects on sperm quality leading to decreasing the sperm motility and reduced fertility (14). In conclusion, addition to the freezing extender of curcumin during cryopreservation at a concentration of 0.25 mM is the optimum concentration of curcumin for improving the quality of frozen-thawed boar semen.

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The Use of GnRH Agonist (Deslorelin Acetate) Implantation for Influencing Testosterone Hormone and Male Behavior in a Thai Native Stallion: Preliminary Study

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Keywords: GnRH agonist, deslorelin acetate, temporary contraception, stallion

Introduction

Testosterone is one of the most important sex hormone in mammals that affect to male sexual behavior and characteristics. In stallion, this hormone is involved in both of sexual performance and sporting performance. Moreover, the sport horse should be concentrated to training program more than others, but if the stallion was finish the competition, he can still as a stallion to produce a performance foal. The aim of this study demonstrated the effect of subcutaneously GnRH agonist (4.7 mg Deslorelin acetate) implantation in mature stallions for 120 days by evaluated alteration of sexual behavior, serum testosterone (T4), testicular volume and histological characteristic of both testes.

Materials and Methods

Sixth mature stallions (Thai native pony, 5-9 year-old) received a 4.7 mg deslorelin acetate implantation subcutaneously on day 0. Blood sample and stallion behavior were collected and recorded on day 0, 3, 7, 14, 21, 30, 60, 90 and 120 for observe serum testosterone level and stallion behavior changes. Testicular volume was measured by ultrasonography on day 0 and 120. Histopathological analysis of both testes was done after surgically castrated.

Results and Discussion

After implantation, the stallion behavior was increased on day 3 to 14, and T4 was increased on day 14 to 21. Following 30 day after deslorelin acetate implantation, 5 in 6 stallions (83.3%) showed decreased in both male behavior and T4 level. On day 60 of observation, all of stallions were noted as a 'gelding-like' behavior and T4 level was lower than 1.0 ng/ml. On day 90 after treatment, one horse was surgically castrated at the owner's request. Testicular volume decreased after treatment and histological analysis revealed a greatly reduce spermatogenesis. Especially in the seminiferous tubule alteration which disappear secondary spermatocyte, spermatid and spermatozoa, likewise decrease quantity primary spermatocytes too.

According to this pilot study, deslorelin implant could be used as a contraceptive method in stallion. We preliminary concluded that subcutaneously GnRH agonist (4.7 mg. Deslorelin acetate) implantation could be effected on temporary contraception in stallion during 90 days in term of decrease male behavior, testosterone level, testicular volume and spermatogenesis, which useful tool to change the stallion behavior that initiated easily practice sporting horses. However, the complete result of the effect is to be determined in an upcoming study.

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Blastocysts Hatchability Following Oocytes and Blastocyst Vitrification

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Keywords: oocyte, blastocyst, hatchability, vitrification, cow

Introduction

'Biotechnology for better life', for that, many researchers is searching easily applicable and thumb way to its application. The reproductive biotechnology can uniquely contribute to innovation for future human life, hopefully wealthy one, through the efficient production of farm animals for human nutrition (Nagai, 2006). It will also be insisted to apply for invention of new protocol which might be employed for making or keeping the genetics. For the successful acceptance of innovation, it has not necessary to be a good idea, but needed an idea which is not only at laboratory or theoretical level it should be easily applicable for farmer's level with strong worth and high impact merit.

Oocytes cryopreservation combined with assisted reproductive technology (ART) such as *in vitro* fertilization (IVF) is the fundamental strategic technology for gene banking of female germplasm (Ledda et al., 2007; Somfai et al., 2013). In recent studies, there are various attempts for oocytes and embryo vitrification. However, to our knowledge, there is no study regarding the double vitrification. As it is challenging task to collect oocytes to produce embryos, it is promising fact to cryopreserve oocytes as well as embryos.

We can storage the surplus oocytes by using standard protocol whereas the successful vitrified matured oocytes could be used for multidimensional purposes whatever it might be used for IVF or ICSI or cloning. Simultaneously, the surplus blastocyst, derived from IVF or ICSI or cloning, could be stored for future use when needed or demand is created. Therefore, the aim of present study was to assess the embryonic development after double vitrification in MII and blastocyst stage by using paper device.

Materials and Methods

Chemicals and media

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. The medium was used for *in vitro* maturation (IVM) was TCM199 supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 0.02 AU/mL FSH (Antrin, Denka Pharmaceutical, Tokyo, Japan), 50 iu/mL hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1 μg/mL estradiol-17β. The medium for embryo culture was modified by synthetic oviduct fluid supplemented with amino acids and 0.3% fatty acid-free BSA (mSOF; Gardner et al., 1994).

Oocyte aspiration and IVM

Bovine ovaries were collected from slaughterhouses and kept in 0.9% NaCl during transport to the laboratory within 4 h at room temperature. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2 to 8 mm in diameter using an 18-gauge needle attached to a 5 mL syringe. Each of 20 COCs were cultured in 100 μL droplets of Tissue Culture medium-199 (TCM-199) supplemented with 2.5 mM Hepes (Gibco, USA) and 10% Fetal bovine serum (FBS), FSH (0.05μg/ml), LH (50μg/ml), estradiol (10μg/mL), Penicillin, and Steptomycin (1μL/mL) covered with mineral oil in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 22-23 h.

Oocytes and embryos vitrification and warming

A group of 4-5 oocytes or 2-3 embryos was washed in base medium (TCM199-Hepes + 20% FBS, BM) before being placed in BM containing 5% dimethylsulfoxide (DMSO) and 5% ethylene glycol (EG) for 30s, then exposed in BM containing 12% DMSO, 12% EG and 0.25 M sucrose for 30s after that finally exposed in BM containing 20% DMSO, 20% EG and 0.5 M

sucrose (VS) for 30s at room temperature (24-25 °C). The oocytes or embryos were placed with about 2-5 µL VS into the paper device and then plunged into liquid nitrogen. The vitrified paper devices were warmed by immersing directly into 2 mL of 0.5 M sucrose in TCM199-Hepes + 20% FBS for 2 min then transferred to 0.25 M sucrose in TCM199- Hepes + 20% FBS for 2 min then 0.125 M sucrose in TCM199- Hepes + 20% FBS for 2 min, finally washed with TCM199- Hepes + 20% FBS.

Evaluation of oocytes viability

Oocytes viability was evaluated by Fluorescein diacetate (FDA) staining according to the method previously described by Mohr and Trouson (1980).

In vitro fertilization (IVF) and culture (IVC)

After IVM, cumulus cells were partially removed by gentle pipetting with 0.1% hyarulonidase. After that a group of 10 oocytes was washed 3-4 times in 50µL Tyrode's Albumin Lactate Pyruvate (TALP) medium. The sperm was prepared by swim up technique. One straw (0.25-ml straws, 25 $\times 10^6$ sperm/ straw) was thawed at 39°C water bath for 30 seconds. The thawed semen was transferred into bottom of two snapped tube containing 2mL of TALP. After 30 minutes, the supernatum was collected in a 15 ml conical tube and centrifuged at 500×g for 5 minutes. After centrifugation, supernatum was discarded and sperm concentration was calculated by using a hem-cytometer and adjusted to 2×10^6 /mL (Seneda et al., 2001). Finally the sperm-oocytes were co-incubated for 13-14 h. Then the presumptive zygotes were further cultured in mSOF medium (20 embryos/100µL) under a humidified atmosphere of 5% CO_2, 5% O_2 and 90 % N_2 at 38.5 °C for 2 days. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviduct cells in mSOF medium under a humidified atmosphere of 5% CO_2 in air at 38.5 °C up to day 8-10. Half of the medium was replaced with fresh medium every day and the embryo development was recorded at the same time as medium changing. It is indicated that the cleavage rates were recorded on Day 2 (the day of IVF will be considered as Day 0), and the development of embryos to blastocyst stage was recorded on Day 8.

Experimental design

We divided our experiment into four groups: (A) fresh MII and blastocysts, (B) fresh MII and vitrified blastocysts, (C) vitrified MII and fresh blastocysts and (D) vitrified both MII and blastocysts.

Statistical analysis

The data of oocytes survivability, embryo development after different treatment were expressed as percentage (%), and analyzed covariance by ANOVA, using SPSS® Software (version 16.0). Differences were considered significant at a level of $P < 0.01$ and $P < 0.05$.

Results and Discussion

We found that the vitrified MII oocytes showed no significant different variation for FDA survival rate (Table 1). The embryo developmental rate to 8 cells, morula and blastocyst stages in different groups was not significantly different among the experimental groups. The rates of blastocyst production in group A, B, C and D were 15.7%, 17.9%, 9.1% and 10.4%, respectively. In case of hatchability of blastocysts in group A, B, C and D were 73.3, 58.8, 75.0 and 11.1%, respectively. The hatched rate of group C and D were not significantly different when compared with group A. The hatched rate in group D was significantly lower than that of other 3 groups.

The aim of this study was to find out the hatchability of blastocysts subsequent double vitrification at MII and blastocysts stage. The collection of oocytes from live animals through ultrasound guided ovum pick up (OPU) or slaughter house to *in vitro* embryo production is a series of complicated and sophisticated steps which implies the high cost of embryo production. It is indicated that each oocyte and embryo is very costly. Ghetler et al. (2005) mentioned that the ability to cryopreserve oocytes and embryos would have beneficial impact with overcoming the moral, ethical and legal issues. On the other hand, according to Phongnimitr et al. (2013), Liang et al. (2012), IVEP and cryopreservation of oocytes and embryos have worldwide research interest to preserve the good genetics. So it is very clear that vitrification of oocytes has the both economic and genetic values.

In this study, we found that the oocytes vitrificantion and FDA survival rate has no significant difference with fresh oocytes. Our result was agreed with Sripunya et al. (2010), Phongnimitr et al. (2013), who studied the bovine oocytes vitrification. However, the

technique, CPAs, concentration of CPAs and device or container for vitrification were obviously different.

We also found that the embryo survival and hatched rate were not significantly different among the groups A, B and C. Without the double vitrification group (group D), the survival rate of blastocyst in our study were similar with the study of Inaba et al. (2011) who used in-straw vitrification technique. Bruyere et al. (2012) used different synthetic substitution (CRY03) of animal derived serum and found that it increased the embryo survival rate through inhibit the fragility of embryo. However, double vitrification at different stage might influence the embryonic development and lead to low hatch rate. Our putative hypothesis is in agreement with the declaration of Ghetler et al. (2005) that, the chilling is injurious for cell membrane integrity and intracellular damage of ultramicroscopic structure.

Conclusions

Certain changes in membrane composition of oocytes and embryos might occur during vitrification which influences the further embryonic development after double vitrification. We can speculate that zona harden may weaken the hatchability of blastocyst.

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Table 1: Comparisons of blastocysts developmental rate after double vitrifications

Group	No. of oocytes IVM	FDA Survival n (%)	No. of oocytes IVF	Cleavage n (%)	No. (%) developed to			No. of BL	BL Survival at 24 h n (%)	Hatched BL at 48h n (%)
					8-C n (%)	Mo n (%)	D7 BL n (%)			
A	100	95 (95.0)[a]	95	59 (62.1)[a]	33 (34.7)[a]	23 (24.2)[a]	15 (15.7)[a]	15	15 (100.0)[a]	11 (73.3)[a]
B	100	94 (93.1)[a]	94	59 (62.7)[a]	37 (39.4)[a]	26 (27.6)[a]	17 (17.9)[a]	17	13 (76.5)[a]	10 (58.8)[a]
C	101	88 (87.1)[a]	88	42 (47.7)[a]	22 (25.0)[a]	14 (15.9)[a]	8 (9.1)[a]	8	8 (100.0)[a]	6 (75.0)[a]
D	101	86 (85.2)[a]	86	41 (47.6)[a]	24 (27.9)[a]	15 (17.4)[a]	9 (10.4)[a]	9	5 (56.7)[b]	1 (11.1)[b]

Four replications. Different letters ([a, b]) within a column represent significant differences ($P < 0.05$). n=number, CL= Cleavage, 8-C = 8 cells, Mo = Morula, D = day, BL = Blastocyst

Effect of Thawing Solutions on Survival of Bovine Blastocyst Using In-straw Vitrification Method

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Keywords: IVF-derived bovine embryo, Thawing solution, In-straw vitrification.

Introduction

Vitrification procedures appear to be more efficient for the cryopreservation of *in vitro* produced (IVP) bovine embryos than other freezing methods (Agca et al., 1998; Vieira et al., 2007). Although many vitrification methods have been developed for cattle IVP embryos (Massip et al., 1987; Ishimori et al., 1993; Akiyama et al., 2010), vitrification has not been widely adopted by embryo transfer for commercial use in cattle (van Wagtendonk-de Leeuw et al., 1997). Therefore, vitrification requires several steps for cryoprotectant dilution in laboratory setting (Ishimori et al., 1993) because it uses a high concentration of cryoprotectants. Previously, several reports shown that the addition of sugar in thawing solution improve survival rate of vitrified embryos (Saito et al., 1994; Ohnoshi et al., 1997; Vajta et al., 1999). This reduced the effect of cryoprotectant after warming or sugar solution as an osmotic counterforce to restrict water permeation into the embryos and to prevent overswelling of the embryos when the cryoprotectant moves out of the cell (Mahmoudzadeh et al., 1995; Inaba et al., 2011). However, Mahmoudzadeh et al. (1995) and Inaba et al. (2011) suggested that sugar solution did not affect survival rate of bovine vitrified-warmed embryos. So the objective of this study was to investigate whether the sugar solution is still required for dilution cryoprotectant in bovine embryos by using In-straw vitrification method.

Materials and Methods

In vitro bovine embryos production

The bovine ovaries were collected from slaughterhouses. Cumulus oocyte complexes (COCs) were aspirated from follicle in diameter 2-8 mm using a 10 ml syringe connected to18G needle. The COCs were washed with modified Dulbecco's phosphate buffer saline (mDPBS) supplemented with 0.1% polyvinyl pyrolidone (PVP) then were cultured in 100 µl droplet of *in vitro* maturation (IVM) medium covered with mineral oil under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 22 hours (Laowtammathron et al., 2005).

In vitro fertilization (IVF) was carried out as previously reported (Imai et al., 2006). Briefly, frozen semen of fertile bull were thawed in a 37 °C water bath for 30 s and then centrifuged in 3 ml of a 90% Percoll solution at 2,100 rpm for 10 min. Then the pellet was re-suspended and centrifuged at 1,800 rpm for 5 min in 6 ml of sperm washing medium which is BO medium (Brackett and Oliphant, 1975) supplemented with 10 mM hypotaurine and 4 U/ml heparin. Then the pellet were re-suspended with BO medium supplemented with 20 mg/ml BSA, to achieve the final concentrations of 3×10^6 spermatozoa/ml, 5 mM hypotaurine, 2 U/ml heparin and 10 mg/mL BSA. The oocyte were removed out of the maturation medium, then washed in BO medium supplemented with 10 mg/ml bovine serum albumin (BSA) and transferred into fertilization droplets (20 oocytes/drop) and cultured for 10-12 h at 38.5°C in humidified atmosphere of 5% CO_2 in air.

In vitro embryo culture (IVC) was performed in 100 µl droplets of mSOFaa medium (Parnpai et al., 1999) supplemented with 3 mg/ml BSA covered with paraffin oil. At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gentle pipetting. Twenty to twenty five zygotes were placed in each culture drop and then cultured at 38.5°C in humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 2 days. Half volume of mSOFaa was replaced daily and the development of embryos were recorded.

Vitrified and warmed of bovine embryos with In-straw vitrificaton method

Grade 1 blastocyst (IETS code 6 and 7; n = 288) were randomly separated into 4 groups, in 7 replicates. Fresh embryos as a control group were remained in culture medium until hatched stage. The selected embryos were randomly exposed to the following solution: VS1: 7.5% ethylene glycol (EG; Wako, Japan) and 7.5% dimethyl sulfoxide (DMSO) in holding medium (mDPBS + 20 fetal bovine serum; FBS) for 3 min and then transferred into holding medium containing 16.5% EG, 16.5% DMSO and 0.5 M sucrose for 30 sec. After that, embryo were loaded into 0.25 ml plastic straws containing thawing solution in each groups which are mDPBS supplemented with 20% FBS and added with sucrose at different concentration groups (0, 0.2 and 0.5 M groups). The straws were immediately heat-sealed, held vertically in liquid nitrogen (LN) for 30 sec and then immersed in LN for storage. Vitrified embryos were warmed by exposed in air for 10 sec and then plunged into 20°C water bath for 20 sec. Straw were then removed from the water and shaken to mix the columns of the straws. After that, the straw was held vertically in water bath for 5 min. The embryos were washed and cultured for 72 h in TCM-199 (GIBCO BRL) supplemented with 20% FBS and 0.1 mM β-merceptoethanol at 38.5°C in humidified atmosphere of 5% CO_2 to evaluate the survival of embryo after thawed.

Statistical analysis

The percentages of hatched rates were subjected to arc sine transformation and then analysed by ANOVA and P-value <0.05 was defined as the significant level.

Results and Discussion

In the present studies, the addition of sugar in thawing solution did not affect survival rates of vitrified-warmed embryos. As shown in Table 1, there was no differences in the rates of embryo developing to the hatched blastocyst stage after 24, 48 and 72 h, respectively, among vitrified group (0, 0.2 and 0.5 M sucrose). However, the hatched rates in all the vitrified groups were significantly lower than that of control group. The addition of sucrose in embryo thawing solution is recommended as an osmotic buffer to restrict water permeation into the embryos and prevent excessive swelling of embryos when the cryoprotetant moves out of the cells (Mahmoudzadeh et al., 1995; Inaba et al., 2011). Previously studied in bovine supported the requirement of sugar solution in vitrified embryos (Saito et al., 1994; Ohnoshi et al., 1997; Vajta et al., 1999). Sucrose concentration used in thawing solution in many reports is ranging from 0.25 to 1 M and 5-10 min for exposed times (Mahmoudzadeh et al., 1995). Therefore, 0.2 M, 0.5 M sucrose solutions were used in our studies to compared with no sucrose in solution and exposed times for 5 min. Our result is similar with report of Mahmoudzadeh et al. (1995) which compared the different concentrations of sucrose (0 M, 0.25 M, 0.5 M Sucrose) in thawing solution, and also report of Inaba et al (2011) by using 0 and 0.3 M sucrose. These above results supported our study that sucrose had no effect on embryo survival after vitrified-warmed in bovine species.

In conclusion, this study indicated that sucrose in thawing solution had no effect on bovine embryo survival following In-straw vitrification and the addition of sucrose in thawing solution is not necessary for In-straw vitrification.

Table 1. Effect of thawing solution on survival rates of vitrified-warmed bovine embryos.

Thawing solution	No. of embryos	No. of embryos developed to hatched blastocyst stage		
		24 h	48 h	72 h
0 M	70	21^{b} (30.00%)	37^{b} (52.86%)	49^{b} (70.00%)
0.2 M	72	25^{b} (34.72%)	38^{b} (52.77%)	48^{b} (66.66%)
0.5 M	74	26^{b} (35.13%)	39^{b} (52.70%)	51^{b} (68.91%)
Control	72	45^{a} (62.50%)	68^{a} (94.44%)	70^{a} (97.22%)

Means within the same column with different superscript are significantly different, $p<0.05$.

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Evaluation of Effect of Capacitaion Media on Post-Thaw Hog Deer (Axis porcinus) Spermatozoa Using Computer Assisted Sperm Analysis (CASA): A Case Report

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Keywords: Hog deer (Axis porcinus), capacitation, hyperactivation, CASA

Introduction

Hog deer *(Axis porcinus)* is classified as a protected species according to the National Wildlife Protection and Preservation Act of 1992, as well as another endangered species according to IUCN, 2011. It is a member of the Cervidae family. Habitat loss and poaching in wild population and low number and genetic diversity in captivity are leading Hog deer to extinction.

Sperm cryopreservation is one of Assisted Reproductive Technologies (ARTs) that have been used to increase animal number and genetic diversity in wildlife species (1). However, freezing and thawing protocols were effected to post-thaw sperm quality. Thus, basic sperm function of new species is important. Sperm capacitation and hyperactivation are important steps before fertilization (2). They have been described as a process of acrosome reaction (AR) induction. These processes are essential for sperm binding and penetrating the zona pellucida. Capacitation changes a spermatozoon to undergo, reorganize of membrane protein, metabolize of membrane phospholipid and reduce membrane cholesterol levels. When sperm hyperactivated, the increasing of amplitude of flagellar bends on one side of the flagellum. This produces a highly asymmetrical beat pattern (3). Thus, CASA has been used to detect hyperactivation and percentage of sperm motility. Previous study reported that hyperactivated motility is a movement pattern, which observed in spermatozoa at time of fertilization in mammals (4). Previous study (5) showed that thimerosal enhances hyperactivated motility of cryopreserved mouse sperm by using Computer Assisted Sperm Analysis (CASA), the hyperactivate motility parameter of curvilinear velocity (VCL), Amplitude of Lateral Head Displacement (ALH) and beat/cross frequency (BCF) were analyzed. In Eld's deer fresh semen, modified tyrode's medium (TALP) that was added more $CaCl_2$ had lower intact acrosomes than the TALP control, dibutyryl (dbcAMP) and fetal calf serum (FCS) treatments (6). However, there are no studies of sperm capacitation and hyperactivation in Hog deer. The aim of this study was to examine the effect of capacitation media on the motility and movement patterns of Hog deer frozen-thawed semen that incubated in different temperatures.

Materials and methods

Animals and Semen Collection

Two Hog deer bulls housed at Faculty of Veterinary Medicine Kamphaengsaen Campus, Kasetsart University were used in study. Animals were anesthesia by combination of ketamine HCl (2 mg/kg) and xylazine HCl (0.25 mg/kg) by IM route (7). Ejaculates were collected by electro-ejaculation. Semen sample with ≥ 60% progressive motility, ≥ $50x10^6$ sperm/ml and 6-8 pH were used for cryopreservation study (8).

Extender Preparation and Semen Cryopreservation

TRIS with 20% egg yolk and 5% glycerol (final concentration) were used for cryopreservation (9). The semen was diluted with TRIS by 2-step technique and equilibrated at 5 °C according to the earlier studies (8, 9). The mixture was packed in the 0.5 ml labeled plastic straw container (Krusse, Ltd., Leeds, UK) by manual technique and sealed with the sealing powder. The straw were held at 4 cm above liquid nitrogen level for 10 min (11) and then plunged into liquid nitrogen and kept in the canister of liquid nitrogen tank at least 7 days before thawing (8).

Thawing and sperm treatments

Six straws from 2 bulls were thawed for 30 sec in a 37 °C water bath. Three samples from both deer passed the criteria (total motility > 20 %) for subjected to capacitation media testing. All aliquots were diluted with equal volume of TALP and centrifuged (300 x g, 8 min). The pellets were resuspended in TALP and divided to five treatment including of 1) TALP without BSA (control); 2) TALP + BSA; 3) TALP + 10 mM $CaCl_2$; 4) TALP + 20% FCS; 5) TALP + 20% deer serum. All treatments were incubated at 37 °C and 4°C for 30 min. The percentage of motility was evaluated by CASA at 30 min. Hyperactivated motility was evaluated by curvilinear velocity (VCL), Amplitude of Lateral Head Displacement (ALH) and beat/cross frequency (BCF) using CASA (5). An analysis of hyperactivate motility were performed by NPSS. The data were presented as mean ± standard errors (S.E.M.). ANOVA was used for comparing data between additives that added into TALP and incubation temperature.

Results and Discussion

Semen were collected from two Hog deer bulls by using electro-ejaculation and immediately evaluated by light-microscope before freezing. Percentage of pre-freeze sperm motility is 91±4.58 (N=3). Post-thaw semen samples (3 straws from 2 bulls) added with capacitation media, were evaluated until sperm motility decreased to <10%. The results were shown in Table 1. The sperm hyperactivation based on VCL, ALH and BCF were analyzed. As showed in the Table 1, capacitation media with additives increased VCL, ALH and BCF. However, there was only significant difference in BCF. This parameter was higher than non-treated spermatozoa ($p < 0.05$) in both of incubation temperatures. The percentage of motility of post-thaw sperm that induced with capacitation media was lower than non-treated spermatozoa ($p < 0.05$) at 37 °C.

The results demonstrated the effect of capacitation media on post-thaw Hog deer sperm which incubated in different temperatures by using CASA evaluation. Hyperactive mammalian spermatozoa have been characterized by vigorous and non-linear movement cause by increased amplitude of flagellar beats (whiplash movement).

Table 1. CASA measurements of post-thaw spermatozoa before induced capacitation and treated with capacitation media*

Parameter	Post-thaw Sperm motility (%) [N=3]	Incubation time at 4 °C				
		TALP (control)	TALP + BSA	TALP + $CaCl_2$	TALP + FCS	TALP + DC
Motile (%)	40±6.34	18±6.34	19.67±6.34	22.67±6.34	22.5±7.77	16.67±6.34
Prog.motile (%)	13.33±2.49	3.33±2.49	4±2.49	7.3±2.49	7.5±3.05	4±2.49
VCL (μm/s)	145.47±22.65	177.67±22.65	159.73±22.65	159.07±22.65	190.6±27.74	178.37±22.65
ALH (μm/s)	5.87±0.68	5.1±0.68	4.03±0.68	5.43±0.68	4.2±0.83	4.03±0.68
BCF (Hz)	24.53±2.36[a]	34.5±2.36[b]	36.83±2.36[b]	34.43±2.36[b]	33.6±2.89[b]	35.37±2.36[b]
VAP (μm/s)	86.27±10.89	96.7±10.89	93.1±10.89	81.93±10.89	106.55±13.34	105.23±10.89
VSL (μm/s)	62.23±8.81	60.07±8.81	55.77±8.81	56.5±8.81	55.95±10.79	57.23±8.81
STR (%)	74±13.77	48.33±13.77	56.67±13.77	57.33±13.77	56.5±16.86	59.76±13.77
LIN (%)	47.33±7.10	40.67±7.10	46.67±7.10	41.67±7.10	36±8.70	41±7.10
Parameter	Post-thaw Sperm motility (%) [N=3]	Incubation time at 37 °C				
		TALP (control)	TALP + BSA	TALP + $CaCl_2$	TALP + FCS	TALP + DC
Motile (%)	40±6.09[a]	11±6.09[b]	16.5±7.45[b]	2±10.54[b]	15±6.09[b]	11.67±6.09[b]
Prog.motile (%)	13.33±2.90	5.33±2.90	8.5±3.54	0±5.02	9.67±2.90	4±2.90
VCL (μm/s)	145.47±31.27	197.43±31.27	142.7±38.30	107.9±54.16	164.57±31.27	163.83±31.27
ALH (μm/s)	5.87±0.85	5.6±0.85	5.15±1.04	0±1.47	5.03±0.85	4.73±0.85
BCF (Hz)	24.53±2.34[a]	43.07±2.34[b]	41.85±2.87[b]	60±4.05[c]	42.27±2.34[b]	36.97±2.34[b]
VAP (μm/s)	86.27±20.90	110.5±20.90	90.35±25.59	33.7±36.20	98.53±20.90	98.93±20.90
VSL (μm/s)	62.23±11.23	58.6±11.23	74.4±13.76	7.5±19.46	67.63±11.23	64.87±11.23
STR (%)	74±7.48	61.33±7.48	80±9.16	22±12.95	75±7.48	71.66±7.48
LIN (%)	47.33±6.01	37±6.01	50.5±7.36	7±10.40	47±6.01	46.33±6.01

VCL : Critical velocity or track speed, ALH : Amplitude of lateral head displacement, BCF : Beat cross frequency, VAP : Average path velocity, VSL : Straight line velocity, STR : Straightness (Average value of the ratio VSL/VAP), LIN : linearity (Average value of the ratio VSL/VCL)

*Values are the mean ± SD.

Different superscripts between means within rows indicate significant differences (P<0.05)

We used VCL, ALH and BCF for indicate sperm hyperactivation because movement of the sperm head is depended on flagellar activity (5). Increased VCL and ALH are indicative of hyperactivation in human spermatozoa (12). For boar spermatozoa a decrease in VSL and an increase in VCL, resulting in a reduced linearity, were reported upon hyperactivation (13). From the result at 4 °C, all additives that added into TALP increased BCF and VCL whereas it decreased VSL, STR and LIN. But the BCF value of post-thaw spermatozoa in TALP with additives was higher than spermatozoa without capacitation media. The BCF is a value of vigorous flagellar action (14). At 37 °C, TALP with all additives also increased BCF of post-thaw spermatozoa. TALP (control) and TALP with $CaCl_2$, FCS and DS decreased LIN. Thus, we inferred these additives effected to flagellar activity that related to hyperactive spermatozoa.

Previous study, serum albumin is a source of cholesterol-binding protein that related to increase permeability of sperm membrane (15). BSA, FCS and DS are a serum albumin that added into TALP in this study. Increases in Ca^{2+}concentration often are associated with sperm hyperactivated motility (2). From the results, capacitation media with $CaCl_2$ at 37 °C are significant higher than other additives. Probability that Ca^{2+} effected to hyperactivated motility of Hog deer spermatozoa more than serum albumin. Even though, the additives increased the movement pattern values, however, incubation times and incubation temperatures in each sample were effected to motility and movement pattern of post-thaw

spermatozoa. Sperm incubation at 4 °C might be keep sperm viability for longer than 37 °C. However, this study is a first report about the response after capacitation media adding in Hog deer sperm for used as baseline data. Therefore, in the future, further study need to be done.

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