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Research Paper

Confirmation of Antimicrobial Resistance by Using Resistance Genes of Isolated *Salmonella* spp. in Chicken Houses of North West, South Africa.

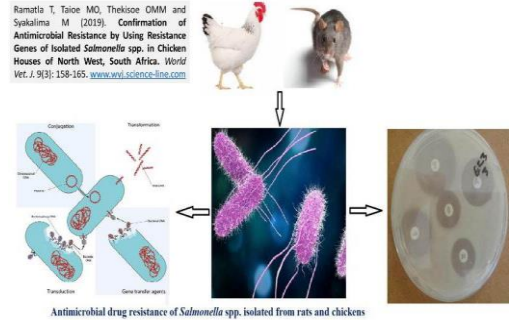
Ramatla T, Taioe MO, Thekiso OMM and Syakalima M.

World Vet. J. 9(3): 158-165, 2019; pii:S232245681900020-9
DOI: <https://dx.doi.org/10.36380/scil.2019.vwj20>

ABSTRACT: The widespread use of antibiotics for treatment of bacterial infections and growth promotion in the poultry industry has effectively increased antibiotic resistance around the world. Antibiotics resistance can be caused by different mechanisms and can be determined through phenotypic and molecular methods. The aim of the present study was to determine the occurrence of antibiotic resistance in *Salmonella* serovars isolated from layer chickens and rats in poultry houses. Phenotypic testing of antimicrobial resistance was performed using the Kirby-Bauer disc diffusion method. Furthermore, molecular evaluations and PCR assay were conducted for detecting resistance genes and class 1 integrons. A total of 144 *Salmonella* isolates (68 from rats and 46 from chickens) serovars were assessed. Evaluation of phenotypic resistance patterns demonstrated that *Salmonella* isolates have the highest antibiotic resistance for rifampicin (100%) followed by tetracycline (68%), ciprofloxacin (48%), sulphonamides (42%), chloramphenicol (39%), nalidixic acid (33%), ampicillin (28%), cephalothin (18%), streptomycin (18%), amoxicillin-clavulanic acid (6%), enrofloxacin (5%), and gentamicin (4%). Some *Salmonella* serovars revealed multi-drug resistance for up to four different antibiotics. According to PCR results, all the tested resistant gene markers (*tet*, *cat*, *bla*TEM, *sul*, *qnrA*, and *aadA*) were detected from the *Salmonella* isolates. The study further confirmed that 68% of *Salmonella* isolates were harboring class 1 integrons and the majority of the isolates (n=52) which were harboring these genes were recovered from the rats. The results of the present study provided that the *Salmonella* spp. isolated from chickens and rats in poultry houses, exhibited significant antibiotic resistance. Moreover, the current research ultimately highlights the importance of rats as carriers of antibiotic-resistant bacteria and the risk of transmission to chickens and humans.

Key words: Antibiotic resistance pattern, Class 1 integrons, Resistance genes, *Salmonella* serovars

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Research Paper

Antibacterial Effect of Aloe Vera Gel Extract on *Escherichia coli* and *Salmonella enterica* Isolated from the Gastrointestinal Tract of Guinea Fowls.

Adzitey F, Agbolosu AA and Udoka UJ.

World Vet. J. 9(3): 166-173, 2019; pii:S232245681900021-9
DOI: <https://dx.doi.org/10.36380/scil.2019.vwj21>

ABSTRACT: *Aloe vera* has a long history as a medicinal plant with diverse therapeutic applications. This study was conducted to assess the antibacterial effect of *Aloe vera* gel extract against *Escherichia coli* and *Salmonella enterica* isolated from the gastrointestinal tract (GIT) of guinea fowls. The conventional method was used for the isolation of *Escherichia coli* and *Salmonella enterica*. The antibacterial activity of *Aloe vera* gel extracts (50, 100 and 200 mg/ml) and standard antibiotics were evaluated using the disk diffusion method. The prevalence of *Escherichia coli* in the GIT of the guinea fowls was 100% (15/15). All the *Escherichia coli* were susceptible to ciprofloxacin. At 48h and 72h of incubation, all the *Escherichia coli* were susceptible to gentamicin but not at 24h. Inhibition zones using the *Aloe vera* gel extract ranged from 7.87-12.23mm (50 mg/ml), 8.53-17.23mm (100 mg/ml) and 7.43-10.67mm (200 mg/ml) for *Escherichia coli*. Also, antibacterial test for *Escherichia coli* using the *Aloe vera* gel extract revealed an inhibition zone of 9.10-12.23mm for *Escherichia coli* isolate GIT1, 7.8-8.57mm for *Escherichia coli* isolate GIT2 and 7.43-17.23mm for *Escherichia coli* isolate GIT7. The prevalence of *Salmonella enterica* in the GIT of the guinea fowls was 40% (6/15). All *Salmonella enterica* were susceptible to suphamethoxazole/trimethoprim and tetracycline but not at 24h. Inhibition zones using *Aloe vera* gel extract ranged from 7.13-12.57mm (50 mg/ml), 4.2-6.7mm (100 mg/ml) and 0-9.23mm (200 mg/ml). Furthermore, antibacterial test for *Salmonella enterica* using the *Aloe vera* gel extract revealed an inhibition zone of 5.3-12.57mm for *Salmonella enterica* isolate GIT9, 0-7.8mm for *Salmonella enterica* isolate GIT10 and 4.2-9.0mm for *Salmonella enterica* isolate GIT15. The study revealed that *Aloe vera* gel extract possessed antibacterial properties. Therefore, it can be added to the feed of guinea fowls as a prophylactic to reduce bacterial infections.

Key words: *Aloe vera*, Antibiotics, *Escherichia coli*, Gut, *Salmonella enterica*



Adzitey F, Agbolosu AA and Udoka UJ (2019). Antibacterial Effect of Aloe Vera Gel Extract on *Escherichia coli* and *Salmonella enterica* Isolated from the Gastrointestinal Tract of Guinea Fowls. *World Vet. J.* 9(3): 166-173. www.wjv.science-line.com

Review

The Effects of Grass-Based versus Grain-Based Feeding of Ruminants on the Human Hygienic Status, a Review.

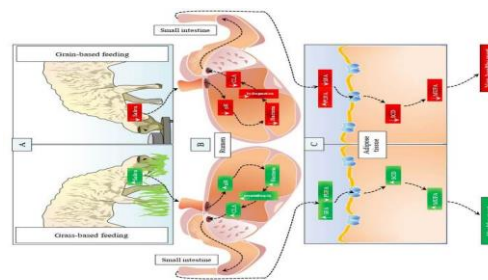
Al-Thuwaini TM and Al-Shuhaib MBS.

World Vet. J. 9(3): 174-180, 2019; pii:S232245681900022-9

DOI: <https://dx.doi.org/10.36380/scil.2019.wvj22>

ABSTRACT: Ruminant meat quality is one of the important factors contributing to the recent spreading of several diseases, such as obesity, cancer, and cardiovascular problems, which have increased predominately. Feeding regiment plays an important role in the determination of the composition of fatty acids and meat quality in ruminants. This review aims to highlight the main factors that lie behind the variability of ruminant meat quality and its effect on human being's health. The reduction in grass-feeding decreases saliva levels in the ruminants, which has several consequences on the rumen, including a reduction in pH level, along with a reduction in the microorganism activities and conjugated linoleic acid levels. In adipose tissues, the expression of the stearoyl-CoA desaturases gene is negatively affected by the decreased conjugated linoleic acid levels in the rumen, which leads to a decreased transformation of saturated fatty acids to monounsaturated fatty acids. Therefore, the lower monounsaturated fatty acids and the parallel increase in the proportion of saturated fatty acids in the consumed meat can be associated with some human diseases. Thus, the present study provided a molecular explanation for the superiority of grass-based feeding in ruminants raised at pasture in term of production of meat with a healthier quality for consumers than those raised on grains.

Key words: Grain; Grass, Human disease, Ruminant meat, SCD enzyme



Al-Thuwaini TM and Al-Shuhaib MBS (2019). The Effects of Grass-Based versus Grain-Based Feeding of Ruminants on the Human Hygienic Status, a Review. *World Vet. J.* 9(3): 174-180. www.wvj.science-line.com

Research Paper

Characterization of Pregnancy-Associated Glycoprotein as a Biomarker of Pregnancy in Etawa Crossbred Goat.

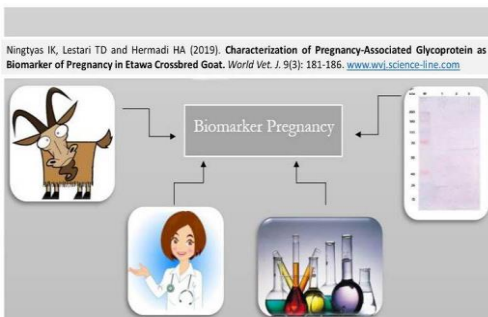
Ningtyas IK, Lestari TD and Hermadi HA.

World Vet. J. 9(3): 181-186, 2019; pii:S232245681900023-9

DOI: <https://dx.doi.org/10.36380/scil.2019.wvj23>

ABSTRACT: Pregnancy-Associated Glycoprotein (PAG) is secreted by the placenta, which is produced in mononucleate and binucleate trophoblast cells. The current research was conducted to find out a substance for diagnosing early pregnancy in Etawa crossbred goats. Six Etawa crossbred goats (not pregnant, three months pregnant and four months pregnant) were subjected in the present study from Livestock Government Institution Breeding in Singosari, Malang. The research methods consisted of sample collection, identification PAG with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the determination of concentration with Biuret method and specificity test with Western Blot assay. The obtained results showed that the molecular weight of PAG from pregnant Etawa crossbred goats was 55.85 kDa. The average concentrations of PAG in the goats of non-pregnant, three months pregnant, and four months pregnant were 1.83 ± 2.98 , 3.79 ± 2.72 and 4.36 ± 2.63 , respectively. The results of the specificity test with the Western Blot molecular revealed a molecular mass of PAG was 55 kDa. The findings of the present study demonstrated PAG in Etawa crossbred goats can be used as an indicator of pregnancy.

Key words: Biomarkers, Etawa crossbred, PAG, Pregnancy



Ningtyas IK, Lestari TD and Hermadi HA (2019). Characterization of Pregnancy-Associated Glycoprotein as a Biomarker of Pregnancy in Etawa Crossbred Goat. *World Vet. J.* 9(3): 181-186. www.wvj.science-line.com

Research Paper

Incretin Mimetics Vildagliptin and Exenatide Improve Pedicle Skin Flap Survival in Rats.

Danilenko LM, Tarasova AP, Pokrovskiy MV, Trunov KS, Stepenko YV, Artyushkova EB and Gudyrev OS.

World Vet. J. 9(3): 187-191, 2019; pii:S232245681900024-9

DOI: <https://dx.doi.org/10.36380/scil.2019.wvj24>

ABSTRACT: Hypoxia and tissue ischemia are the leading factors in the alteration of tissues in many pathological conditions. Prevention and reversion of the effects of local ischemia, which develops during various surgical interventions, is an actual problem of modern medicine. The aim of the present study was to investigate the effect of exenatide and vildagliptin on the survival rate of an isolated pedicle skin flap in sixty adults Wistar rats. Simulation of a pedicle skin graft was performed on the second day of the experiment. After anesthesia under aseptic conditions, a skin graft was cut out:



isolated in a plastic bag, the edges of the skin were stitched with interrupted sutures (nylon 3/0). Rats were divided into six groups: control group, exenatide group (10 µg/kg/day subcutaneously for nine days after surgery), vildagliptin group (0.2 mg/kg/day intraperitoneally for nine days after surgery) and pentoxifylline group (100 mg/kg/day intravenously, two hours before the surgical intervention). In the other two groups, glibenclamide (5 mg/kg) were administered before injection of incretin mimetics. On the third, seventh and tenth day, area of the surviving tissue was measured. Subsequently, the survival rate of the skin graft was calculated. The area of the surviving tissue in exenatide and vildagliptin group was 1.5 and 1.7 times more compared to the control group, respectively. Preliminary blockade of ATP-dependent potassium channels by glibenclamide eliminated the protective effect of exenatide and vildagliptin. The increase in the survival of ischemic tissues using exenatide and vildagliptin has been experimentally proved. The current study confirmed the important role of ATP-dependent potassium channels in dermatoprotective properties of incretin mimetics.

Key words: Dermatoprotective properties, Exenatide, Ischemia, Pedicle skin graft, Vildagliptin.

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Research Paper

Use of Untreated and Autoclave-Treated Wheat Germ Meal in Growing Rabbit Diets.

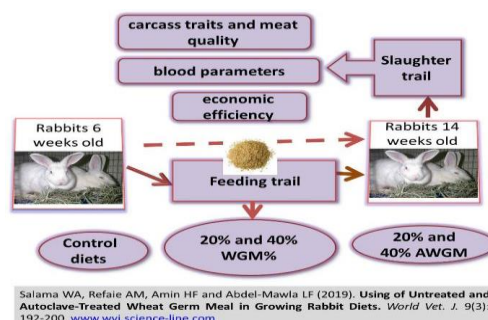
Salama WA, Refaie AM, Amin HF and Abdel-Mawla LF.

World Vet. J. 9(3): 192-200, 2019; pii:S232245681900025-9

DOI: <https://dx.doi.org/10.36380/scil.2019.wvj25>

ABSTRACT: The present study was intended to investigate the influence of using 20% and 40% treated or untreated wheat germ meal in growing New Zealand rabbit diets. A total of 75 weaned New Zealand White rabbits aged six weeks old, with an average initial weight of 659.60±18.84g were divided into five groups with five replicates in each group (three rabbits per replicate). The first group was fed on a basal diet (T₁), second and third groups received diets containing Wheat Germ Meal (WGM), as replacement of soybean meal protein, at levels of 20% and 40% and were labeled as T₂, T₃, respectively. Fourth and fifth groups were fed with 20% and 40% autoclave-treated autoclaved WGM (T₄ and T₅, respectively). The trial was continued until 14 weeks of age. The present study was evaluated growth performance, blood parameters, carcass traits, meat quality in different groups and also economic efficiency was calculated. There were insignificant differences in terms of live weight, daily weight gain, carcass weight and dressing percentages among rabbits in groups of T₁, T₂, and T₄. Rabbits in the group of T₄ achieved the best feed conversion ratio. Digestion coefficients of crude protein, crude fiber, ether extract, nitrogen-free extract, and nutritive value in terms of digestible crude protein, total digestible nutrition, and digestible energy did not significantly differ between T₁ and T₄. However, these factors significantly decreased in T₃ and T₅ compared to T₁. Plasma total protein and globulin significantly increased in rabbits of T₂ and T₄ compared to those fed in T₁ group. A significant decrease in total cholesterol and total lipid for rabbits in groups of T₄, T₅, and T₂ was observed. Moreover, rabbits fed on T₄ or T₂ diets had the highest economic efficiency. Conclusively, the untreated or autoclaved WGM can be used in growing rabbit diets up to 20% for replacing the soybean meal protein, which caused low feed costs without adverse effects on the growth performance of rabbits.

Key words: Rabbits, Soybean meal, Wheat germ meal



Salama WA, Refaie AM, Amin HF and Abdel-Mawla LF (2019). Use of Untreated and Autoclave-Treated Wheat Germ Meal in Growing Rabbit Diets. *World Vet. J.* 9(3): 192-200. www.wvj.science-line.com

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Research Paper

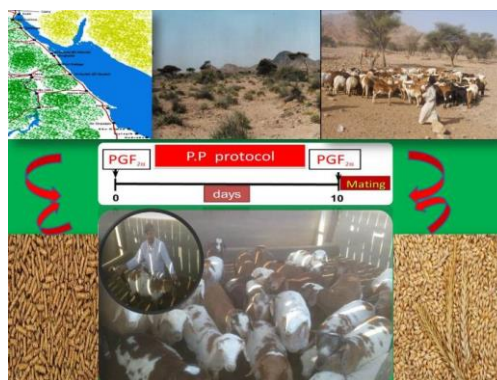
Productive Characteristics and Reproductive Responses to Estrus Synchronization and Flushing in Abou-Delik Ewes Grazing in Arid Rangelands in Halaieb - Shalateen - Abouramad Triangle of Egypt.

Farrag B.

World Vet. J. 9(3): 201-210, 2019; pii:S232245681900026-9

DOI: <https://dx.doi.org/10.36380/scil.2019.wvj26>

ABSTRACT: There are a few reports about the reproductive aspects or uses of both of flushing and estrus synchronization in Abou-Delik ewes grazing in the South Eastern zone of Egypt. Thirty-three Abou-Delik ewes were allocated to three experimental groups (n = 11 in each) to study the effects of estrus synchronization and flushing on reproductive responses and productive characteristics under arid conditions of South Eastern zone of Egypt. Group one served as control represent the system dominant in the area (without estrus synchronization and flushing ration). Ewes in group two were estrus synchronized with two doses of PGF_{2α}, 10 days apart without flushing ration. Ewes in group three were estrus synchronized just like the second group and received 300g of barley grain/head/day as flushing meal for three weeks before the start of breeding season. All ewes were grazed *Panicum turgidum* (natural vegetation dominant in the area) for eight hours daily. Results showed that, the percentage of estrus exhibition in group three reached 100%, while the lowest percentage was observed in group one (81.82 %). Estrus activity signs in synchronized groups, occurred in 70 and 81.81% during the first 48 h after the second dose of PGF_{2α}, for groups two and three respectively, compared to control group (22.22 %). The onset of estrus was earlier in synchronized groups than control group. Duration of estrus did not differ significantly. Estrus intensity in group three was higher (P < 0.05) compared to the other groups. Conception and lambing rates were 100% in group three. Third group showed the highest insignificant litter size that was 18% higher than the other groups.



The overall mean of birth weight, weaning weight and average daily gain of Abou-Delik lambs were 2.91, 16.89 and 0.116 kg, respectively. There is no significantly effect on concentrations of plasma progesterone among groups. While there were significant differences between sampling periods. In conclusion, under grazing on arid rangelands conditions in the South Eastern zone of Egypt, using flushing and/or estrus synchronization can be useful to improve reproductive and productive characteristics of Abou-Delik sheep.

Key words: Abou-Delik sheep, Estrus synchronization, Flushing, Productive performance, Rangelands, Reproduction

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Research Paper

Histopathological Alterations of Ceca in Broiler Chickens (*Gallus gallus*) Exposed to Chronic Heat Stress.

Adji AV, Plumeriastuti H, Ma'ruf A and Legowo D.

World Vet. J. 9(3): 211-217, 2019; pii:S232245681900027-9
DOI: <https://dx.doi.org/10.36380/scil.2019.wvj27>

ABSTRACT: Heat stress has been found to cause adverse effects on small intestinal microstructure, but little is known about its impact on chicken's cecum. In this research, the histopathological alterations of broiler chicken's cecum following chronic heat stress were evaluated. 20 broiler chickens were randomly divided into control group and treatment group containing 10 replicates, respectively. Both groups were reared under standard conditions until 21 days of age. From day 22 to day 42, the control group was kept at 24-28°C as well as relative humidity of 40-55%, while the treatment group was exposed to high temperature of 36-40°C and relative humidity of 45-65% for eight hours per day. At the end of the period, proximal part of each chicken's cecum was collected and made into histopathological slides with Hematoxylin and Eosin staining. Villus height, villus width, crypt depth, villus surface area, and villus height to crypt depth ratio were examined from 10 villi per replicate. Results analysis revealed that chronic heat stress profoundly ($P<0.05$) reduced the crypt depth. Insignificant ($P>0.05$) changes of the villus despite the long-term heat exposure might imply that the damage is at its early phase. In conclusion, chronic heat stress can produce morphological alterations in the ceca of broiler chickens, though requiring longer duration due to cecum's durability.

Key words: Broiler chicken, Cecum, Heat stress, Intestinal morphology



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Research Paper

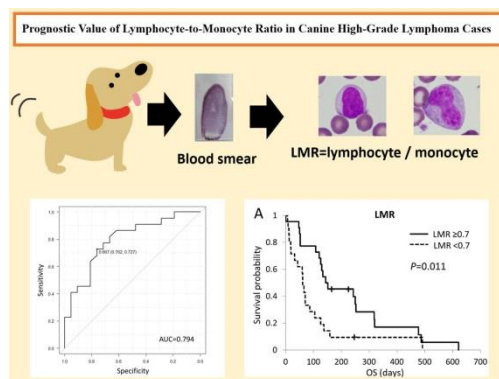
Prognostic Value of Lymphocyte-to-Monocyte Ratio in Canine High-Grade Lymphoma Cases.

Tagawa M, Shimbo G, Matsumoto K and Miyahara K.

World Vet. J. 9(3): 218-229, 2019; pii:S232245681900028-9
DOI: <https://dx.doi.org/10.36380/scil.2019.wvj28>

ABSTRACT: The Lymphocyte-to-Monocyte Ratio (LMR) has been described as a useful prognostic marker for human patients with various cancers and dogs with diffuse large B-cell lymphoma. The objective of this study was to determine whether the LMR could predict disease outcome as measured by the Time To Progression (TTP) and Overall Survival (OS) of dogs with different types of high-grade lymphoma. The medical records of 43 dogs diagnosed with high-grade lymphoma at the Veterinary Medical Center of Obihiro University of Agriculture and Veterinary Medicine between 2013 and 2018, were retrospectively analyzed. Receiver Operating Characteristic (ROC) curve analysis was used to determine the optimal LMR cutoff values. The prognostic influence of the LMR and other clinicopathological data on TTP and OS was studied by Kaplan-Meier curves. To identify the independent prognostic factors, univariate and multivariate Cox proportional analyses were used. The optimal cutoff value of the LMR was 0.7, which corresponded to the maximum sensitivity (0.727) and specificity (0.762) of the LMR for predicting the median days of OS with ROC analysis (area under the curve, 0.794). Log-rank tests showed that dogs with a high LMR had significantly longer TTP and OS than dogs with a low LMR. Moreover, immunophenotype, body weight, treatment regimen and response to treatment were significantly associated with TTP and OS. In multivariate analysis, treatment and response to treatment were independent risk factors for TTP. Moreover, the LMR, treatment regimen and response to treatment were independent predictors of OS.

Key words: Dog, Lymphocyte to monocyte ratio, Lymphoma, Prognosis



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Research Paper

Computed Tomography Scan and Polyester resin 40 Plastination Technique: Teaching Aids to Illustrate Anatomical Structure of Donkey Brain.

Mohamed ShKhA, El-Behery EI and Mahdy EAA.

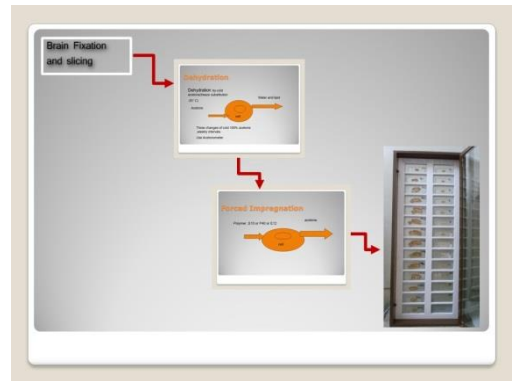
World Vet. J. 9(3): 230-240, 2019; pii:S232245681900029-9

DOI: <https://dx.doi.org/10.36380/scil.2019.wvj29>

ABSTRACT: The present study investigated the collaboration between the plastinated sagittal sections and computed tomography (CT) images of the donkey brain. Four adult healthy donkeys of both sexes from native breeds were analyzed. The animals were sedated with a combination of xylazine and ketamine. The donkeys were positioned in sternal recumbency during CT brain examination and contiguous sagittal 3 mm thick slices of the head were obtained. Polyester resin 40 (P40) technique for the brains were done through five main steps including fixation, slicing, dehydration, forced impregnation and light-curing of brain glass chambers by UVA light source. Furthermore, a survey was conducted using questionnaires involving second-year veterinary medical students of anatomy department, Zagazig University to compare the educational capacity of CT brain images and P40 plastination of brain slices. 52% of students voted that using of brain sheeted slices are much better, 38% prefer to learn both parallel to each other. The obtained results revealed that both the plastinated brain sections and the CT images portrayed a combination of many complex neuroanatomical structures that provide an excellent aid for researchers in educational and diagnostic purposes. In addition, the plastinated brain serves as a good tool for the interpretation of CT images.

Key words: Brain, CT, Donkey, Plastination, Polyester resin 40

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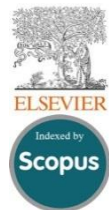
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Confirmation of Antimicrobial Resistance by Using Resistance Genes of Isolated *Salmonella* spp. in Chicken Houses of North West, South Africa

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ABSTRACT

The widespread use of antibiotics for treatment of bacterial infections and growth promotion in the poultry industry has effectively increased antibiotic resistance around the world. Antibiotics resistance can be caused by different mechanisms and can be determined through phenotypic and molecular methods. The aim of the present study was to determine the occurrence of antibiotic resistance in *Salmonella* serovars isolated from layer chickens and rats in poultry houses. Phenotypic testing of antimicrobial resistance was performed using the Kirby-Bauer disc diffusion method. Furthermore, molecular evaluations and PCR assay were conducted for detecting resistance genes and class 1 integrons. A total of 144 *Salmonella* isolates (68 from rats and 46 from chickens) serovars were assessed. Evaluation of phenotypic resistance patterns demonstrated that *Salmonella* isolates have the highest antibiotic resistance for rifampicin (100%) followed by tetracycline (68%), ciprofloxacin (48%), sulphonamides (42%), chloramphenicol (39%), nalidixic acid (33%), ampicillin (28%), cephalothin (18%), streptomycin (18%), amoxicillin-clavulanic acid (6%), enrofloxacin (5%), and gentamicin (4%). Some *Salmonella* serovars revealed multi-drug resistance for up to four different antibiotics. According to PCR results, all the tested resistant gene markers (*tet*, *cat*, *bla*TEM, *sul*, *qnrA*, and *aadA*) were detected from the *Salmonella* isolates. The study further confirmed that 68% of *Salmonella* isolates were harboring class 1 integrons and the majority of the isolates (n=52) which were harboring these genes were recovered from the rats. The results of the present study provided that the *Salmonella* spp. isolated from chickens and rats in poultry houses, exhibited significant antibiotic resistance. Moreover, the current research ultimately highlights the importance of rats as carriers of antibiotic-resistant bacteria and the risk of transmission to chickens and humans.

Key words: Antibiotic resistance pattern, Class 1 integrons, Resistance genes, *Salmonella* serovars

INTRODUCTION

Several antimicrobial agents are used in animals and humans to treat certain infections (Raissy and Ansari, 2011). Despite the obvious benefits, improper use of antibiotics can lead to the development of bacterial resistance in infectious diseases (Hong et al., 2018). Approximately 95% of antibiotics administered to livestock are excreted unchanged, therefore, people who live in an environment close to animal waste are constantly exposed to antibiotics and this condition may develop resistance (Choi, 2007).

Antibiotic resistance can be conferred by intrinsic or acquired mechanisms (Schroeder et al., 2017). Mechanisms of acquired resistance can be through Horizontal Gene Transfer (HGT) or elevated mutation rates (Schroeder et al., 2017). In general, mechanisms of antibiotic resistance are classified into three categories including modification of cell permeability, replacement or modification of the antibiotic targets and inactivation of the antibiotics via enzymatic destruction or modification (Frye and Jackson, 2013; Schroeder et al., 2017).

Recent studies have demonstrated that *Salmonella* serotypes such as *S. heidelberg*, *S. typhimurium*, *S. infantis*, *S. uganda*, *S. newport*, *S. typhi*, *S. paratyphi*, *S. agona* and *S. hadar* exhibit antibiotic resistance (Mathole et al., 2017; Zhao et al., 2017; Odoch et al., 2018; Thung et al., 2018). Furthermore, the increasing frequency of resistance of *Salmonella* spp. to antibiotics such as chloramphenicol, tetracycline, and ampicillin has been reported in many countries (Olobatoke and Mulugeta, 2015; Odoch et al., 2018; Thung et al., 2018). Antimicrobial resistance, especially in *Salmonella* serovars, has also been implicated to play a role in their virulence (Mathole et al., 2017).

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It has now been established that *Salmonella* spp. contain different Antibiotic Resistance Genes (ARGs) (Abatcha et al., 2018). Most of the resistance genes are located on the plasmids, bacterial chromosome or transposons which can be transferred by mobile genetic elements. Apart from antibiotic resistance genes detected from *Salmonella* spp. and resistance integrons included class 1, 2 and 3 has also been identified from *Salmonella* spp. (Odoch et al., 2018). The majority of the ARGs have mostly located in class 1 integrons. So that, class 1 integron-mediated antimicrobial resistance among diverse *Salmonella* serovars (Thong and Modarressi, 2011).

Although rodents are not normally treated with antibiotics, they have still been found to harbor antibiotic-resistant bacteria in the environment. Therefore, rats can be used as good indicators for the presence of antibiotic-resistant bacteria in a specific area. Currently, there is still inadequate information about the antibiotic resistance patterns of *Salmonella* spp. in chicken and rats in South Africa. Therefore, the present study sought to document the occurrence of antibiotic-resistant *Salmonella* serovars in chickens and rats found in chicken houses of North West Province, South Africa.

MATERIALS AND METHODS

Ethical Approval

Prior to the commencement of the study, the research proposal was approved by the Animal Research Ethics Committee (Ref No: NWU-00274-18-A5) following guidelines of North West University Research Ethics Regulatory Committee (NWU-RERC), North West, South Africa.

Sampling

A list of layer farms in Mafikeng, North West province of South Africa were randomly selected by using the records of Agriculture Department. A few farms in the north, south, east, and west were randomly selected. A total of 274 fecal samples were collected from chicken (n=120) and rats (n=154) in six commercial farms. Cleaning and disinfection of surfaces before sampling of rats were done with 70% ethanol to avoid cross-contamination. Dissection of the abdominal cavity was done using a surgical blade, a pair of forceps and the samples were harvested from cecum. The fecal samples of chickens were collected from three different floors in each poultry farm once a week. This sampling was done to have a good representation and distribution of the organisms. The collected samples were packed in properly labelled sterile polyethylene bags and transported under a complete aseptic condition in an icebox, then processed immediately upon arrival to the laboratory. All samples were labelled and prepared for analysis however the samples which did not analysed within 24 hours, immediately refrigerated at -4°C.

Bacteria isolates

Salmonella was isolated from feces following the International Organization for Standardization method (ISO-6579: 2002). The DNA of the isolates were amplified using Polymerase Chain Reaction (PCR) and then PCR products were sequenced. Generated sequences were aligned on the GenBank database using basic local alignment search tool from the National Center for Biotechnology Information to identify sequences with high similarity. The *16S rDNA* gene sequences of *Salmonella* isolates in the current study were deposited into the GenBank database and were given accession numbers ranging from MH352147 to MH352214 for rat and from MH356670 to MH356715 for chicken.

Phenotypic test for detecting antimicrobial resistance

The phenotypic antibiotic resistance test was performed using the Kirby-Bauer disc diffusion method (Magiorakos et al., 2012). Pure *Salmonella* isolates were sub-cultured on nutrient agar (Merck, Wadeville, South Africa) medium, incubated at 37°C for up to 24 h. Then fresh overnight cultures were used for antibiotic sensitivity tests. Aliquots of 100 µl from the suspensions were spread-plated on Mueller-Hinton agar (Biolab, supplied by Merck) using a sterile cotton swab. Single disc diffusion method was used to assess the susceptibility of *Salmonella* isolates to commonly used antimicrobial agents. A total of 12 antibiotic discs (Davies diagnostics, SA) were used in this investigation including gentamicin (GM; 10 µg), ciprofloxacin (CIP; 5 µg), rifampicin (RIF; 5 µg), chloramphenicol (C, 30 µg), nalidixic acid (NA; 30 µg), ampicillin (AMP; 10 µg), enrofloxacin (ENR; 5 µg), tetracycline (TE; 30 µg), cephalothin (KF, 30 µg), Sulphonamides (SSS; 300 µg), streptomycin (STR; 10 µg) and amoxicillin-clavulanic acid (AMC; 30 µg). The antimicrobial profile of isolated bacteria to different antibiotics was determined following recommendations of the clinical laboratory institute standards interpreted as intermediate (I), sensitive (S), and resistant (R). The *E. coli* (ATCC 25922) was used as quality control. Strains which showed resistance to at least three classes of antibiotics were considered as multi-drug-resistant (MDR) isolates (Zhao et al., 2017).

Genotypic test for detecting antimicrobial resistant

A total of six antimicrobial resistance genes (*bla*TEM, *tet*, *sul*, *cat*, *qnrA*, and *aadA*) were amplified by PCR using respective primer sets targeting the different antimicrobial resistant genes including class 1 integrons. The details of oligonucleotide sequences, their base pairs (bp) including the PCR cycling conditions are shown in table 1. Resistance to aminoglycosides is associated with carriage of *aadA* gene; resistance to quinolones is associated with carriage of *qnrA* gene; resistance to β -lactams is associated with carriage of a *bla*TEM (ESBL) gene; resistance to chloramphenicol is associated with carriage of *cat* gene; resistance to sulfonamide is associated with carriage of *sul* genes, and resistance to tetracycline is associated with *tet* gene. In addition, *intI1* is associated with class 1 integrons (Özgen, 2007; Olobatoko and Mulugeta, 2015; Kim et al., 2016).

Table 1. The list of different primers used to detect antimicrobial resistance in *Salmonella* species

Primers	Sequence (5'-3')	Amplicon size (bp)	Conditions	References
<i>Sul3</i> <i>Sul4</i>	F: TCAACATAACCTCGGACAGT R: GATGAAGTCAGCTCCACCT	707	94°C, 5 mins; 30 cyc of 94°C, 30 S; 60°C for 40 s, 72°C for 30 S, ext. at 72°C, 5 mins	(Özgen, 2007)
<i>tem1</i> <i>tem2</i>	F ATGAGTATTCAACATTTCCGTG R TTACCAATGCTTAATCAGTGAG	792	95°C, 3 min; 30 cyc. of 95°C, 1 min, 55°C, 1 min, 72°C, 1 min, ext. at 72°C, 5 mins	(Olobatoko and Mulugeta, 2015),
<i>tet</i> <i>tet</i>	F: GCACCTGTCTCCTGTTTACTCCCC R: CCTGTGGTTATGTTTTGGTTCCG	659	94°C, 2 mins; 35 cyc. of 94°C, 20 S, 53°C, 10 S, 65°C, 45 s, ext. at 65°C, 4 mins	(Olobatoko and Mulugeta, 2015)
<i>cat</i> <i>cat</i>	F: TCCCAATGGCATCGTAAAGAAC R: TCGTGGTATTCACTCGAGAGCG	310	95°C, 5 mins; 35 cyc. of 94°C, 30 S, 55°C, 30 S, 72°C, 30 s, ext. at 72°C, 10 mins	(Olobatoko and Mulugeta, 2015)
<i>aadA</i> <i>aadA</i>	F: ATCCTTCGGCGCGATTTTG R: GCAGCGCAATGACATTCTTG	282	94 °C, 3 mins; 30 cyc, 94 °C 30 s, 62 °C 30 s, 72 °C 1 mins, ext at 72 °C, 7 mins	(Aarestrup et al., 2003)
<i>qnrA</i> <i>qnrA</i>	F: TCAGCAAGAGGATTTCTCA R: GGCAGCACTATGACTCCCA	627	95°C, 5 mins, 30 cyc, 94°C 40 s, 50°C 60 s, 72°C 90 s, ext at 72°C, 10 mins	(Akiyama and Khan, 2011)
<i>intI1</i> <i>intI1</i>	F: ACATGTGATGGCGACGCACGA R: ATTCTGTCTGGCTGGCGA	568	95°C 3 mins; 30 cyc., at 95°C 30 s, 55°C 30 s, 72°C 30 s; ext 72°C for 10 mins	(Tankson et al., 2005)

RESULTS

Identification of *Salmonella* spp.

The *Salmonella* serovars detected from rats included *S. typhimurium* (n=26, 38.2%), *S. enteritidis* (n=12, 17.6%), *S. newport* (n=8, 11.8%), *S. heidelberg* (n=7, 10.3%), *S. bongori* (n=6, 8.8%), *S. enterica* serovar Paratyphi B (n=4, 5.9%), *S. tennessee* (n=3, 4.4%) and *S. pullorum* (n=2, 2.9%). The predominant *Salmonella* serovars isolated from chickens were *S. typhimurium* (n=18, 39.1%), *S. heidelberg* (n=9, 19.6%), *S. bongori* (n=7, 15.2%), *S. enteritidis* (n=6, 13.0%), *S. paratyphi B* (n=3, 6.5%) and *S. newport* (n=3, 6.5%).

Phenotypic antimicrobial resistance of *Salmonella* species

The *Salmonella* isolates revealed resistance to rifampicin (n=114, 100%), tetracycline (n=78, 68%), ciprofloxacin (n=55, 48%), sulphonamides (n=48, 42%), cephalothin (n=20, 18%), chloramphenicol (n=45, 39%), streptomycin (n=20, 18%), enrofloxacin (n=6, 5%), ampicillin (n=32, 28%), amoxicillin/clavulanic acid (n=7, 6%) and nalidixic acid 38 (33%), and gentamicin 5 (4%). All *Salmonella* isolated from rat were susceptible to gentamicin as shown in table 2.

Five *S. typhimurium* were MDR for up to six different antibiotics (rifampicin, tetracycline, enrofloxacin, cephalothin, ciprofloxacin, and sulphonamides). Some isolates like *S. enteritidis* and *S. typhimurium* exhibited resistance for up to five antibiotics (rifampicin, tetracycline, enrofloxacin, cephalothin, and ciprofloxacin) and one *S. enteritidis* for five (rifampicin, enrofloxacin, cephalothin, ciprofloxacin, and streptomycin). Furthermore, different isolates exhibited resistance for three to four antibiotics as listed in table 3.

Genotypic antimicrobial resistance of *Salmonella* isolates

The results of the analysis of *Salmonella* isolates for the occurrence of ARGs are shown in table 4. Seventy-eight (68%) isolates encoding class 1 integrons were detected from *Salmonella* spp. and most of the isolates which were harboring different resistance genes were also carrying class 1 integrons. In addition, the majority of the isolates which were harboring class 1 integrons were from the rats (n=52) while 26 isolates were from the chickens. Different resistance gene patterns were indicated by *S. typhimurium*, *S. enteritidis*, *S. heidelberg*, *S. bongori*, *S. newport*, *S. enterica* serovar Paratyphi B, *S. tennessee* and *S. pullorum*; respectively. The mentioned results are shown in table 5.

Table 2. Antimicrobial resistance of *Salmonella* serovars isolated from rats (*Rattus* spp.) and layer chickens in poultry houses, North West, South Africa

Antibiotic	Abbreviation	Breakpoint disc (µg)	Chickens (n=46)	Rats (n=68)	Total (n=114)
Ampicillin	AMP	(10 µg)	29(63%)	3(4%)	32(28%)
Sulphonamides	SSS	(300 µg)	36(78%)	12(18%)	48(42%)
Cephalothin	KF	(30 µg)	8(17%)	12(18%)	20(18%)
Tetracycline	TE	(30 µg)	46(100%)	32(47%)	78(68%)
Ciprofloxacin	CIP	(30 µg)	34(74%)	21(31%)	55(48%)
Nalidixic acid	NA	(30 µg)	37(80%)	1(2%)	38(33%)
Chloramphenicol	CA	(10 µg)	36(78%)	9(13%)	45(39%)
Gentamicin	GM	(5 µg)	5(11%)	0(0%)	5(4%)
Enrofloxacin	ENR	(5 µg)	0(0.0%)	6(9%)	6(5%)
Rifampicin	RIF	(10 µg)	46(100%)	68(100%)	114(100%)
Streptomycin	STR	(5 µg)	12(25%)	8(12%)	20(18%)
Amoxicillin/clavulanic Acid	AMC	(30 µg)	5(11%)	2(3%)	7(6%)

Table 3. Antimicrobial resistance and the prevalence of resistant strains in *Salmonella* isolates from rats (*Rattus* spp.) and layer chickens in poultry houses, North West, South Africa

MAR Phenotypes	Isolates									
	<i>S. typhimurium</i>	<i>S. enteritidis</i>	<i>S. newport</i>	<i>S. heidelberg</i>	<i>S. bongori</i>	<i>S. enterica paratyphi B</i>	<i>S. pullorum</i>	<i>S. tennessee</i>		
ENR, TE, GM, KF, STR, SSS	5	–	–	–	–	–	–	–		
ENR, TE, GM, KF, STR	1	3	–	–	–	–	–	–		
ENR, GM, KF, STR, RIF	1	1	–	1	–	–	–	–		
ENR, NA, TE, KF, STR	–	1	–	–	1	–	–	–		
ENR, AMP, SSS, NA	1	–	–	–	–	–	–	–		
ENR, AMP, TE, SSS	1	–	–	–	–	–	–	–		
ENR, NA, TE, STR	–	–	1	–	–	–	–	1		
ENR, NA, TE, SSS	1	–	–	–	2	–	–	–		
ENR, TE, GM, KF	3	–	–	–	–	–	–	–		
ENR, CA, TE, GM	–	–	–	–	1	–	–	–		
ENR, TE, STR, SSS	–	1	–	–	–	–	1	–		
ENR, AMP, KF, SSS	–	–	1	–	–	–	–	–		
ENR, TET, KF	–	–	–	1	–	–	–	–		
ENR, NA, SSS	–	–	–	1	–	–	1	–		
ENR, AMP, STR	1	–	1	–	–	–	–	–		
ENR, NA, RIF	–	1	–	–	–	1	–	–		
ENR, TE, RIF	–	–	–	–	–	–	–	1		

MAR: Multiple Antibiotic Resistance, GM: Gentamicin, RIF: Rifampicin, NA: Nalidixic Acid, AMP: Ampicillin, ENR: Enrofloxacin, TE: Tetracycline, KF: Cephalothin, SSS: Sulphonamides, STR: Streptomycin; *S.*: *Salmonella*.

Table 4. Antibiotic resistance genes among the different *Salmonella* spp. isolated from rats (*Rattus* spp.) and layer chickens in poultry houses around North West, South Africa.

Antimicrobial agent	Class of antimicrobials	Genes tested	No. of isolates	Resistant phenotype (%)	Resistance genes and integrons (%)
Chloramphenicol	Phenicol	<i>cat</i>	114	45(39%)	11(10%)
Tetracycline	Tetracycline	<i>tet</i>	114	78(68%)	21(18%)
Ampicillin	Beta-lactam	<i>blaTEM</i>	114	32(28%)	4(4%)
Sulfonamide	Sulfonamide	<i>Sul</i>	114	48(42%)	16(14%)
Streptomycin	Aminoglycoside	<i>aadA</i>	114	20(18%)	6(5%)
Ciprofloxacin	Quinolones	<i>qnr-A</i>	114	55(48%)	20(18%)
Class I integrons	–	<i>intI1</i>	114	–	78(68%)

No: number,

Table 5. The number of *Salmonella* isolates containing antimicrobial resistance genes and class 1 integrons recovered from rats (*Rattus* spp.) and layer chickens in poultry houses around North West, South Africa.

Genotype	S. typhimurium	S. enteritidis	S. newport	S. heidelberg	S. bongori	S. enterica paratyphi B	S. pullorum	S. tennessee
<i>cat</i>	17	6	6	7	7	2	1	2
<i>tet</i>	25	7	5	9	7	2	–	–
<i>blaTEM</i>	17	7	–	1	3	1	–	–
<i>sul</i>	21	6	–	7	3	3	–	–
<i>aadA</i>	1	1	–	5	2	1	–	–
<i>qnrA</i>	13	8	1	–	1	2	–	–
<i>intI1</i>	30	19	11	4	7	5	1	1

aadA gene mediates bacterial resistance to streptomycin, *blaTEM* mediate bacterial resistance to ampicillin, *cat* gene mediates bacterial resistance to chloramphenicol, *Sul3*, *Sul4* genes mediate bacterial resistance to sulfonamide, *qnrA* gene mediates bacterial resistance to quinolones, *tet* gene mediates bacterial resistance to tetracyclines, *intI1* = Class 1 Integrons, S.: *Salmonella*.

DISCUSSION

This is the first study to demonstrate antimicrobial resistance in *Salmonella* spp. isolated from rats captured from chicken houses in North West province and to our knowledge in the whole of South Africa. The 12 antimicrobial drugs were tested in the present study, are commonly used for the treatment of bacterial infections of animals and human's health. Finding resistance to these commonly used antimicrobials is very significant because rats are never treated with antibiotics thus the antimicrobial resistance observed in *Salmonella* isolates from rats represents the contamination and circulation of resistant strains in the environment which ultimately affects both animals and humans.

The current study demonstrated a very high prevalence (100%) resistance of *Salmonella* isolates to rifampicin using disc diffusion test. This result is consistent with previous studies conducted in Egypt (Mahmoud et al., 2018), in Sardinia, Italy (Piras et al., 2011), in Shandong, China (Zhao et al., 2017) and in the United Arab Emirates (Khan et al., 2010). Rifampicin is used as the first-line drug for the treatment of humans Tuberculosis (TB) in South Africa (McIntosh et al., 2018). In South Africa due to the presence of gold mines, a high number of people suffer from TB because of silica dust exposure (Phillips et al., 2014). Therefore, many people around the North West province, a province which has minings, are taking TB treatment (Chirehwa et al., 2018) and this may explain the high possibility that the drug is being discharged into the environment and as a result, environmental bacteria and rats are constantly exposed to the drug. Therefore, this finding is a significant concern for public health and further research is necessary to evaluate its effect on other bacterial species.

A high resistance rate of 78 isolates to tetracycline was observed on disc diffusion. However, when the same isolates were assessed using molecular methods targeting the *tet* gene, only 55 of the isolates were positive. Tetracycline is a low-cost medication in comparison to other antibiotics (Odoch et al., 2018) and therefore is extensively used for therapy and prophylaxis of animal and human infections (Ammons and Copié, 2013; Granados-Chinchilla and Rodríguez, 2017; Almaaytah et al., 2018). It is also commonly used at sub-therapeutic levels for growth promotion (Chopra and Roberts, 2001). This encourages selection for resistance and results in the presence of higher percentages of bacteria in the environment that cannot respond to treatment. Thus the high resistance finding did not surprising because previous studies have also reported that the most frequently detected ARG is *tet* gene (Zishiri et al., 2016).

Forty-eight (42%) *Salmonella* isolates were resistant to sulphonamides on disc diffusion compared to 40 (35%) of these isolates on molecular evaluation using the *Sul* gene. This result is not surprising because sulphonamides are a common antibiotic in chickens flocks (Bertelloni et al., 2017). Sulphonamides are used to treat some infectious diseases in chickens such as fowl typhoid, coccidiosis coryza and pullorum disease (Mehtabuddin et al., 2012) and thus are common in poultry houses. According to the results of a survey, 95.4% of sulphonamides are used as water medication in South Africa (Eagar et al., 2012). Thus, this condition makes these drugs common environmental contaminants in water spills that can be picked up by all organisms of environment, consequently exert selective pressure on ARGs.

The results of the present study indicated 55 (48%) of the *Salmonella* isolates were resistant to ciprofloxacin on disc diffusion. However, when these isolates were subjected to molecular evaluation of resistance using the *qnr-A* gene, 25 (22%) out of 114 isolates were positive for the *qnr-A* gene. Quinolones or fluoroquinolones have been used as a treatment option for salmonellosis for over 40 years (Balasundaram et al., 2017). Fluoroquinolones have actually been considered as one of the last options for the treatment of *Salmonella* spp. (Abdel-Maksoud et al., 2015). The absence of the *qnr-A* gene in isolates that had a positive phenotype indicated that the disc diffusion method may either be more sensitive and causing a few false positive. To explain this contradiction it can also be noted that the target gene for ciprofloxacin is not always the *qnrABSCD* genes (Kim et al., 2016).

A total of 20 (18%) isolates were found to be phenotypically resistant to streptomycin, of which half of these isolates were harboring *aadA* gene. This observation may be explained by a fact that this class can be encoded by different resistant genes. Chloramphenicol was used as the main treatment against *Salmonella*, since its discovery up to 1990 (Ishaleku et al., 2015). The increasing resistance of *Salmonella* spp. to chloramphenicol has previously been reported (Olobatoke and Mulugeta, 2015) from chicken samples in North West province, South Africa. The present evaluation isolated 45 (39%) *Salmonella* specimens which were phenotypically resistant to chloramphenicol and 44 (39%) of them were harboring *cat* gene. This suggests that there is an ongoing use of this antibiotic due to its broad-spectrum activity, despite awareness of resistance.

Only 32 (28%) *Salmonella* isolates in this study were phenotypically resistant to ampicillin while in the molecular evaluation of the *bla*_{TEM} gene, 29 (25%) isolates were resistant. The absence of mentioned gene from some isolates that were phenotypically positive may indicate that the target gene for this antibiotic is not always the same or the disk diffusion test has high sensitivity and but not specificity to antibiotics resistance (Dickert et al., 1981). This finding causes more concerns because antibiotic resistance for ampicillin has previously been detected from poultry products around this area (Olobatoke and Mulugeta, 2015). A high number of the isolates showed MDR to tetracycline, ciprofloxacin, and sulphonamides, which are antimicrobial agents commonly used in veterinary medicine. This is worrying because some antibiotics are recommended drugs for the treatment of salmonellosis (Hirose et al., 2001). MDR *Salmonella* has been observed in other countries such as Malaysia (Thung et al., 2018). MDR *Salmonella* isolates are considered to be highly virulent than non-MDR (Thung et al., 2018) and this finding was a major concern in the current study.

In the present research, seventy-eight (68%) *Salmonella* isolated from chickens and rats were harboring class 1 integrons and the majority of the isolates belonged to the rats (n=52). It was possible that these rats picked up *Salmonella* infections with resistance gene from the environment. Even though there were four classes of integrons associated with the resistance gene cassette, class 1 integrons had been more commonly observed than the other classes (Thong and Modarressi, 2011; Abatcha et al., 2018). According to literature, class 1 integrons are the most common integron types in MDR *Salmonella* spp. and plays a significant role in assisting the transfer of the resistance genes (Thong and Modarressi, 2011). The obtained results in the present study revealed that the *Salmonella* isolates had one or more genes that encode antibiotic resistance.

MDR genes were encountered from two *Salmonella* isolates harboring more than two resistance genes. Out of two the isolates, *S. typhimurium* was MDR regarding both disc diffusion test and gene resistance markers. MDR *Salmonella* isolates has been reported to cause illnesses in either humans and animals in different countries including; the USA and Denmark (Aarestrup et al., 2007), Italy (Graziani et al., 2008), Eastern China (Lu et al., 2014) and Vietnam (Vo et al., 2010). Moreover, the presence of MDR genes in isolated *Salmonella* spp. from rats must be taken seriously as these vertebrates can act as reservoirs and potentially can spread the bacteria to both human and animal surroundings.

CONCLUSION

The current research has pioneered antibiotic resistance investigation on *Salmonella* isolates from rats inhabiting chicken farms in North West, South Africa. The obtained results in the present study revealed that antibiotic resistance is well established in most of the *Salmonella* isolates infecting chickens and rats and the majority of isolates harbor more than two resistance genes. These findings provided a better understanding of the importance of rats in the transmission and maintenance of the antibiotic-resistant *Salmonella* spp. in poultry premises which can potentially be transferred to humans via chicken products.

DECLARATIONS

Author's contributions

Ramatla T. performed the experiments and wrote the first draft. Moeti OT and Thekisoe OMM provided the analysis tools and data analysis and reviewed the manuscript. Michelo S conceived and designed the experiments, provided reagents, materials and approved the final paper.

Consent to publish

All the authors agreed to publish the manuscript and declared that this work has not been previously published elsewhere.

Competing interests

The authors declare that they have no conflict of interest

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Antibacterial Effect of *Aloe Vera* Gel Extract on *Escherichia coli* and *Salmonella enterica* Isolated from the Gastrointestinal Tract of Guinea Fowls

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ABSTRACT

Aloe vera has a long history as a medicinal plant with diverse therapeutic applications. This study was conducted to assess the antibacterial effect of *Aloe vera* gel extract against *Escherichia coli* and *Salmonella enterica* isolated from the gastrointestinal tract (GIT) of guinea fowls. The conventional method was used for the isolation of *Escherichia coli* and *Salmonella enterica*. The antibacterial activity of *Aloe vera* gel extracts (50, 100 and 200 mg/ml) and standard antibiotics were evaluated using the disk diffusion method. The prevalence of *Escherichia coli* in the GIT of the guinea fowls was 100% (15/15). All the *Escherichia coli* were susceptible to ciprofloxacin. At 48h and 72h of incubation, all the *Escherichia coli* were susceptible to gentamicin but not at 24h. Inhibition zones using the *Aloe vera* gel extract ranged from 7.87-12.23mm (50 mg/ml), 8.53-17.23mm (100 mg/ml) and 7.43-10.67mm (200 mg/ml) for *Escherichia coli*. Also, antibacterial test for *Escherichia coli* using the *Aloe vera* gel extract revealed an inhibition zone of 9.10-12.23mm for *Escherichia coli* isolate GIT1, 7.8-8.57mm for *Escherichia coli* isolate GIT2 and 7.43-17.23mm for *Escherichia coli* isolate GIT7. The prevalence of *Salmonella enterica* in the GIT of the guinea fowls was 40% (6/15). All *Salmonella enterica* were susceptible to gentamicin. At 48h and 72h of incubation, all the *Salmonella enterica* were susceptible to sulphamethoxazole/trimethoprim and tetracycline but not at 24h. Inhibition zones using *Aloe vera* gel extract ranged from 7.13-12.57mm (50 mg/ml), 4.2-6.7mm (100 mg/ml) and 0-9.23mm (200 mg/ml). Furthermore, antibacterial test for *Salmonella enterica* using the *Aloe vera* gel extract revealed an inhibition zone of 5.3-12.57mm for *Salmonella enterica* isolate GIT9, 0-7.8mm for *Salmonella enterica* isolate GIT10 and 4.2-9.0mm for *Salmonella enterica* isolate GIT15. The study revealed that *Aloe vera* gel extract possessed antibacterial properties. Therefore, it can be added to the feed of guinea fowls as a prophylactic to reduce bacterial infections.

Key words: *Aloe vera*, Antibiotics, *Escherichia coli*, Gut, *Salmonella enterica*

INTRODUCTION

Avian pathogenic *Escherichia coli* strains can cause avian colibacillosis in guinea fowls. Colibacillosis is characterized by colisepticemia, hemorrhagic septicemia, coligranuloma, and chronic respiratory disease, which contributes to economic losses for the world's poultry industries (Nolan et al., 2013). *Salmonella enterica* causes salmonellosis in guinea fowl. *Salmonella enterica* serovars Pullorum and Gallinarum are particularly involved in poultry salmonellosis. They can cause death in chicks, poults and adult poultry. Poultry including guinea fowls infected with *Salmonella enterica* huddle near a heat source are anorectic, appear weak and have whitish diarrhoea around the vent. They also show symptoms of fever, are pale, dehydrated, have diarrhea, swollen liver, brittle and often bile-stained (Schepop, 2017).

Escherichia coli and *Salmonella enterica* infections are controlled by the use of antibiotics. Nevertheless, the use of antibiotics for prophylactics and treatment of these infections especially anarchically contribute to antimicrobial resistance. Berghichen et al. (2018) noted a massive use of antibiotics and mostly used without adhering to their principles of application in Algeria. Resistance of bacteria to multiple antimicrobials is a concern to public health and scientific studies (Raja et al., 2017; van den Honert et al., 2018; WHO, 2018; Adzitey et al., 2019; Berghiche et al., 2019; Tay et al., 2019). This is so because, the use of antimicrobial drugs in veterinary medicine and animal husbandry may compromise human health if resistant bacteria develop in animals and are transferred to humans through the food chain and environment (McNulty et al., 2016). The evolution and development of multiple drug resistant pathogenic

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microorganisms has necessitated the search for new source of antimicrobial substances, including plant metabolites (Nostro et al., 2000). Hence, the assessment of the efficacy of plant-based drugs in traditional veterinary medicine is of paramount importance because these drugs educe few side effects, affordable and easily available. Sofowora (2003) reiterated that the use of traditional veterinary medicine may be due to its low cost, availability and ease of application compared to modern veterinary medicine. Many plants have beneficial multifunctional aspects which are derived from their specific bioactive components (Mothana and Lincleqist, 2005; Kar and Bera, 2018). Many plants have also been subjected to pharmacological test, and a substantial number of new antibiotics have been developed from them (Mothana and Lincleqist, 2005).

Aloe vera is an ancient plant with its origin in African continent and has been reported to have beneficial effects on the growth performance, gut microflora, hematological characteristics, carcass characteristics and immune response of poultry (Kaithwas et al., 2008; Yadav, 2017; Kar and Bera, 2018). It can survive under a wide variety of conditions and has been shown to have many medicinal and antibiotic properties (Christaki and Florou-Paneri, 2010; Kar and Bera, 2018). Jain et al. (2016) reported that *Aloe vera* gel has been used for management of various infections since ancient times as it has anti-inflammatory, anti-microbial, and immune-boosting properties. Guinea fowls (*Numida meleagris*) in Ghana are dominated by local or traditional breeds reared mostly in Northern part of Ghana. The meat of guinea fowls is cherished and relished by many Ghanaians (Adzitey et al., 2015). Nonetheless, they are kept mainly under the extensive and semi-extensive systems, and are exposed to a variety of bacteria during scavenging (Teye et al., 2000; Adzitey et al., 2015). The gastrointestinal tracts of animals are well known to harbor bacteria such as *Campylobacter spp.*, *Clostridium spp.*, *Escherichia coli*, *Lactobacillus spp.*, *Salmonella spp.*, *Shigella spp.* and *Vibrio spp.* Guinea fowls can feed on plants which may include *Aloe vera* during scavenging.

This study was conducted to determine the antibacterial effect of *Aloe vera* extracts on *Escherichia coli* and *Salmonella enterica* isolated from the gastrointestinal tract of guinea fowl.

MATERIALS AND METHODS

Study area, sample collection and analysis

This study was conducted at the Microbiology Laboratory (in the Spanish laboratory) of University for Development Studies, Nyankpala Campus, Ghana. Fresh *Aloe vera* plants (harvested from backyard garden) and the gastrointestinal tract (collected from slaughter slab) of 15 guinea fowls (*Numida meleagris*) were obtained from the Tamale metropolis. The gastrointestinal tracts were analyzed immediately upon reaching the laboratory for the presence of *Escherichia coli* and *Salmonella enterica* following the procedures in the bacteriological analytical manual of the FDA-USA (Feng et al., 2017; Andrews et al., 2018).

Isolation and confirmation of *Escherichia coli* and *Salmonella enterica*

For the isolation of *Escherichia coli*, one g of gastrointestinal tract content was pre-enriched in 10 ml of Buffered Peptone Water (BPW) and incubated at 37°C for 18-24 h. After which, the aliquots were plated on Levine Eosin-Methylene Blue Agar and incubated again at 37°C for 24 h. For *Salmonella enterica*, aliquots from BPW were further enriched in Rappaport Vassiliadis (RV) and Selenite (SN) Broths. Samples in RV broth were incubated at 42°C for 24 h while those in SN broth were incubated at 37°C for 24-48 h. After incubation, the aliquots from RV and SN broths were plated on xylose lysine deoxycholate and brilliant green agar, and incubated at 37°C for 24 h. Presumptive *Escherichia coli* and *Salmonella enterica* were purified on trypticase soy agar (incubated at 37°C for 24 h) and confirmed using the appropriate biochemical tests as stated by Feng et al. (2017) and Andrews et al. (2018), and serological tests using *Escherichia coli* and *Salmonella* latex agglutination test kits (Oxoid, Basingstoke, UK). All incubations were done under aerobic conditions and all media used were purchased from Oxoid, Basingstoke, UK.

Extraction of *Aloe vera* gel extract

The *Aloe vera* gel extract was prepared according to Thiruppathi et al. (2010). Briefly, the *Aloe vera* gel was extracted under aseptic condition, dried in an oven at 70°C for 24 h and then milled into powder. 10g of the oven-dried *Aloe vera* gel powder was suspended in 50ml of 80% ethanol and kept on a shaker for 24 h for proper dissolution. This was then filtered through Whitman paper no.1 and allowed to evaporate in an oven at 80°C for 24 h. The dried extract was dissolved in Dimethyl Sulfoxide (DMSO) and stored in the refrigerator at 4°C for further uses.

Preparation of standard concentrations of *Aloe vera* extract and bacteria inocula

200, 100 and 50 mg of *Aloe vera* gel powder were separately dissolved in one ml of DMSO. Single colony of pure cultures of *Escherichia coli* and *Salmonella enterica* were isolated and grown in Trypticase Soy Broth (TSB) and incubated at 37°C for 24 h. Then, the concentration was adjusted to 0.5 McFarland turbidity using sterile TSB.

Antibiotic susceptibility test using standard antibiotics and *Aloe vera* gel extract

These were done using the disc diffusion method of Bauer-Kirby (1966). For the conventional standard method, the isolates (in TSB adjusted to 0.5 McFarland turbidity) were swabbed onto Mueller-Hinton (MH) agar using a sterile cotton swab. Eight different standard antibiotics, notably: Ampicillin (Amp) 10 µg, Chloramphenicol (C) 30 µg, Gentamicin (Cn) 10 µg, Ceftriaxone (Cro) 30 µg, Ciprofloxacin (Cip) 5 µg, Erythromycin (E) 15 µg, sulfamethoxazole/Trimethoprim (Sxt) 22 µg, and Tetracycline (Te) 30 µg were placed on the MH agar at a distance to prevent overlapping of the inhibition zones. The MH agar was incubated aerobically at 37°C for 24 h, 48 h and 72 h, and the inhibition zones measured in millimeters (mm). The inhibition zones were interpreted according to the Clinical Laboratory Standard Institute (2014).

For the antibacterial test using *Aloe vera* gel extracts at different concentrations, blank antibiotic discs purchased from Oxoid, Basingstoke, UK were individually impregnated with different concentrations (200mg, 100mg and 50mg) of the *Aloe vera* extract, and were placed on the surface of the MH agar which has been inoculated with the isolates. Then, it was incubated at 37°C for 24 h, 48 h and 72 h, and the inhibition zones measured in mm. All the antibiotic discs were placed at a distance to avoid overlapping of inhibition zones.

Statistical analysis

The data obtained in this study were analyzed using One-way ANOVA of the GenStat Release 12 Edition. Significant differences were determined at 95% (P<0.05)

RESULTS

Prevalence of *Escherichia coli* and *Salmonella enterica* in gastrointestinal tract of guinea fowls

The prevalence of *Escherichia coli* and *Salmonella enterica* in the gastrointestinal tract (gut) of the guinea fowl was 100% (15/15) and 40% (6/15), respectively.

***Escherichia coli* and *Salmonella enterica* sensitivity tests**

The antibiotic resistance of three randomly selected *Escherichia coli* isolates is shown in table 1. Out of the three *Escherichia coli* isolates subjected to antibiotic susceptibility test, 37.5% were susceptible, 45.8% were resistant and 16.7% were intermediate resistant. *Escherichia coli* isolates were 100% resistant to Amp and Te at 24 h but not at 48 h and 72 h. The *Escherichia coli* isolates were all susceptible to Cip (100%) at all incubation periods (24, 48 and 72 h). The *Escherichia coli* isolates also exhibited some intermediate resistances to Cro (at 24 h), G (at 24 h), Sxt (at 24 h) and E (at 24, 48 and 72 h). Resistance to C and Cro were not the same at 24, 48 and 72 h of incubation whilst it was the same for Sxt and E. The antibiotic activity of the *Salmonella enterica* isolates is presented in table 2. Overall, 70.8% of the *Salmonella enterica* isolates were susceptible, 29.2% were resistant and none exhibited intermediate resistant. All the *Salmonella enterica* isolates were susceptible to gentamicin at 24 h, 48 h and 72 h. With the exception of Sxt and Te, the results for all the antibiotics against the *Escherichia coli* isolates were the same for all the incubation periods. Results for Sxt and Te were however, the same at 48 h and 72 h of incubation.

Evaluation of antibacterial activity of *Aloe vera* extracts against *Escherichia coli* and *Salmonella enterica*

The inhibition zones for the *Escherichia coli* isolates ranged between 9.10 to 12.23 mm for *Escherichia coli* isolate GIT1, 7.8 to 8.57 mm for *Escherichia coli* isolate GIT2 and 7.43 to 17.23 mm for *Escherichia coli* isolate GIT7 (Table 3). Inhibition zones for the *Escherichia coli* isolates (GIT1, GIT2 and GIT3) did not significantly differ (P>0.05) at 50 mg/ml and 200 mg/ml but significantly differed (P<0.05) at 100 mg/ml. The inhibition zones for 50 mg/ml, 100 mg/ml and 200 mg/ml *Aloe vera* gel extract ranged between 7.87 to 12.23 mm, 8.53 to 17.23 mm and 7.43 to 10.67 mm, respectively. The results in table 3 indicated that *Aloe vera* extracts at 100 mg/ml was the most effective in controlling of *Escherichia coli* isolates. At 100 mg/ml, most of the *Escherichia coli* isolates produced higher inhibition zones compared to other concentrations (50 mg/ml and 200 mg/ml).

The inhibition zones for the *Salmonella enterica* ranged between 5.3 to 12.57 mm for *Salmonella enterica* isolate GIT9, 0 to 7.8 mm for *Salmonella enterica* isolate GIT10 and 4.2 to 9.0 mm for *Salmonella enterica* isolate GIT15 (Table 4). Also in table 4, inhibition zones for the *Salmonella enterica* isolates (GIT9, GIT10 and GIT15) differed significantly (P<0.05) at 50 mg/ml and 200 mg/ml but not at 100 mg/ml (P>0.05). The inhibition zones for 50 mg/ml, 100 mg/ml and 200 mg/ml *Aloe vera* gel extract ranged between 7.13 to 12.57 mm, 4.2 to 6.7 mm and 0 to 9.23 mm, respectively. The results in table 4 revealed that *Aloe vera* gel extracts at 50 mg/ml was effective in controlling *Escherichia coli* isolates. At 50 mg/ml, most of the *Salmonella enterica* isolates produced higher inhibition zones (P<0.05) compared to other concentrations (100 mg/ml and 200mg/ml).

Table 1. Percentage antibiotic activity of *Escherichia coli* isolated from gastrointestinal tract of guinea fowl at 24, 48 and 72 hours in the Tamale Metropolis, Ghana

Antibiotics	No. of Isolates	24 hours			48 hours			72 hours		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin (AMP)	3	0.00	0.00	100	0.00	33.3	66.7	0.00	33.3	66.7
Chloramphenicol (C)	3	33.3	0.00	66.7	33.3	33.3	33.3	33.3	33.3	33.3
Ciprofloxacin (Cip)	3	100	0.00	0.00	100	0.00	0.00	100	0.00	0.00
Ceftriaxone (Cro)	3	33.3	33.3	33.3	66.7	0.00	33.3	66.7	0.00	33.3
Gentamicin (Cn)	3	66.7	33.3	0.00	100	0.00	0.00	100	0.00	0.00
Erythromycin (E)	3	0.00	33.3	66.7	0.00	33.3	66.7	0.00	33.3	66.7
Sxt	3	66.7	33.3	0.00	66.7	33.3	0.00	66.7	33.3	0.00
Tetracycline (Te)	3	0.00	0.00	100	33.3	33.3	33.3	66.7	33.3	0.00

No: number, S: susceptible, I: Intermediate, R: resistant, Sxt: Suphamethoxazole/trimethoprim.

Table 2. Percentage antibiotic activity of *Salmonella enterica* isolated from gastrointestinal tract of guinea fowl at 24, 48 and 72 hours in the Tamale Metropolis, Ghana

Antibiotics	No. of Isolates	24 hours			48 hours			72 hours		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin (AMP)	3	66.7	0.00	33.3	66.7	0.00	33.3	66.7	0.00	33.3
Chloramphenicol (C)	3	66.7	0.00	33.3	66.7	0.00	33.3	66.7	0.00	33.3
Ciprofloxacin (Cip)	3	66.7	0.00	33.3	66.7	0.00	33.3	66.7	0.00	33.3
Ceftriaxone (Cro)	3	66.7	0.00	33.3	66.7	0.00	33.3	66.7	0.00	33.3
Gentamicin (Cn)	3	100	0.00	0.00	100	0.00	0.00	100	0.00	0.00
Erythromycin (E)	3	66.7	0.00	33.3	66.7	0.00	33.3	66.7	0.00	33.3
Sxt	3	66.7	0.00	33.3	100	0.00	0.00	100	0.00	0.00
Tetracycline (Te)	3	66.7	0.00	33.3	100	0.00	0.00	100	0.00	0.00

No: number, S: susceptible, I: Intermediate, R: resistant, Sxt: Suphamethoxazole/trimethoprim.

Table 3. Zones of inhibition by *Aloe vera* extract against the *Escherichia coli* isolated from gastrointestinal tract of guinea fowl at 24, 48 and 72 hours in the Tamale Metropolis, Ghana

<i>E.coli</i> isolates	24 hours			48 hours			72 hours		
	200 mg/ml	100 mg/ml	50 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml
GIT 1	9.13	9.10 ^b	11.23	10.3	9.10 ^b	12.23	10.67	9.33 ^b	12.33
GIT2	7.8	8.53 ^b	7.87	8.1	8.53 ^b	8.13	8.33	8.57 ^b	8.23
GIT7	7.43	14.43 ^a	9.2	9.2	17.10 ^a	10.37	9.67	17.23 ^a	10.3
SEM	3.258	1.986	3.032	5.1	3.186	2.928	4.568	3.706	3.236
P value	0.577	0.002	0.164	0.704	0.003	0.081	0.616	0.006	0.113

Escherichia coli: *Escherichia coli*, GIT: Gastrointestinal tract, The GIT 1, GIT2 and GIT7 were randomly selected from positive *Escherichia coli* isolates, SEM: Standard Error of Means. Means with different superscript along the columns are different at $P \leq 0.05$.

Table 4. Zones of inhibition by *Aloe vera* extract against the *Salmonella enterica* isolated from gastrointestinal tract of guinea fowl at 24, 48 and 72 hours in the Tamale Metropolis, Ghana

<i>E.coli</i> isolates	24 hours			48 hours			72 hours		
	200 mg/ml	100 mg/ml	50 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml
GIT 1	8.23 ^a	5.3	9.00 ^a	9.13 ^a	6.7	12.57 ^a	9.23 ^a	6.7	12.57 ^a
GIT2	0.00 ^c	4.3	7.13 ^b	0.00 ^b	4.7	7.77 ^b	0.00 ^b	4.7	7.87 ^b
GIT7	6.67 ^b	4.2	7.67 ^b	8.23 ^a	4.6	7.77 ^b	9.00 ^a	4.8	7.80 ^b
SEM	0.722	6.8	1.022	1.468	7.66	1.178	1.942	7.74	1.176
P value	0.001	0.938	0.026	0.001	0.83	0.001	0.001	0.85	0.001

GIT: Gastrointestinal tract, The GIT 9, GIT10 and GIT15 were randomly selected from positive *Salmonella enterica* isolates, SEM: Standard Error of Means. Means with different superscript along the columns are different at $P \leq 0.05$

DISCUSSION

Escherichia coli and *Salmonella enterica* were found in the gastrointestinal tract (gut) of the guinea fowls. It is common to find these bacteria in the gastrointestinal tract of animals since it is a natural habit for microorganisms. However, they can pose problems to poultry and consequently humans when they are present in large numbers. Also, pathogenic *Escherichia coli* and *Salmonella enterica* strains can cause *Escherichia coli* and *Salmonella* infections, respectively. The presence of *Escherichia coli* and *Salmonella enterica* in the gastrointestinal tract of guinea fowls present opportunity for cross contamination unto carcasses during processing. Adzitey et al. (2015) found *Escherichia coli* and *Salmonella enterica* in fresh and smoked guinea fowls and attributed them to poor processing during dressing and cross contamination from their natural habitat. Kilonzo-Nthenge et al. (2018) isolated *Salmonella enterica* from 23% of whole carcass rinses of guinea fowl.

This study revealed that the *Escherichia coli* isolates exhibited overall susceptibility (37.5%), resistant (45.8%) and intermediate resistant (16.7%). There were some changes in resistant patterns with incubation time which was observed for ampicillin, chloramphenicol, ceftriaxone, gentamicin and tetracycline. Bacteria can develop resistance to antibiotics via mutation or the acquisition of resistant genes from other bacteria (Wintersdorff et al., 2016). Furthermore, intermediate resistances were found at 24 h, 48 h, and/or 72 h for all the antibiotics except ciprofloxacin. Any isolate that exhibited intermediate resistant has the tendency to become resistant (Adzitey et al., 2016; Adzitey, 2018). Kilonzo-Nthenge et al. (2008) reported that *Escherichia coli* isolated from guinea fowls were resistant to ampicillin but susceptible to ciprofloxacin, erythromycin and tetracycline, which was similar to this present work.

In this study, most of the *Salmonella enterica* isolates were susceptible ($\geq 66.8\%$) to the eight different antibiotics tested. Intermediate resistant was not observed for the *Salmonella enterica* isolates, however, some of the *Salmonella enterica* isolates exhibited 33.3% resistance. All the *Salmonella enterica* isolates were susceptible to Cn. The reason for the development of resistant among pathogens including *Salmonella enterica* have been linked to the indiscriminate use of antibiotics for therapeutic and growth purposes in animals (Wintersdorff et al., 2016). *Salmonella enterica* species isolated from guinea fowls were resistant to Amp and Te, but susceptible to Cip and E (Kilonzo-Nthenge et al., 2008). In this study 33.3% resistance was found at 24 h for Amp and Te, but 66.7% susceptibility for Cip and E at 24 h. At 48 h and 72 h, all the *Salmonella enterica* isolates were susceptible to Sxt and Te. These results suggested that the mentioned antibiotics require more than 24 h to be bactericidal for *Salmonella enterica* isolates examined in this study.

The antibacterial activity of *Aloe vera* gel extract was investigated against *Escherichia coli* and *Salmonella enterica* isolates of guinea fowl gastrointestinal tract origin using the disc diffusion method. The results obtained revealed that *Aloe vera* gel extract had antibacterial activities. Isolates of *Escherichia coli* treated with 100 mg/ml concentration of *Aloe vera* gel extract caused the highest overall inhibition zone than those treated with 50 mg/ml and 200 mg/ml of *Aloe vera* gel extract. Irshad et al. (2011) reported that *Aloe vera* extract produced an average inhibition zone of 2 mm against *Escherichia coli*. Ferro et al. (2003) found an inhibition zone of 12.66 mm for *Escherichia coli* isolates. The inhibition zones in this study were generally higher than the findings of Irshad et al. (2011) and had some similarity with findings of Ferro et al. (2003). Irshad et al. (2011) reported that that *Aloe vera* extracted using acetone exhibited stronger activity against *Escherichia coli* as compared to aqueous or ethanol extracts. Ferro et al. (2003) reported that sap water extract was more effective than leaf extract against *Escherichia coli*. In this study, *Aloe vera* gel extract exhibited antibacterial activity against all the *Escherichia coli* isolates, and was the highest at 100 mg/ml.

The growth of *Salmonella enterica* isolates was inhibited at 50 mg/ml than those treated with 100 mg/ml and 200 mg/ml of *Aloe vera* gel extracts across 24 h, 48 h, and 72 h of incubation. Qadir et al. (2013) found that the best concentration of *Aloe vera* water extract inhibition for *Escherichia coli* was at 75%, with inhibition zone of 9.75 ± 0.25 mm. Inhibition zones lower or higher than 9.75 mm were found in this study. Jonson et al. (2011) examined 35 clinical *Salmonella enterica* isolates and reported that, 17 isolates had inhibition zone of 7 to 32 mm in 0.007 mg/ml of the extract. The highest zone of inhibition produced by the *Aloe vera* gel extract in this study against *Salmonella enterica* isolate was 12.27 mm which was lower than the 32 mm reported by Jonson et al. (2011).

Kaithwas et al. (2008) studied the antimicrobial activity of *Aloe vera* gel by using disc diffusion method and reported that, the gel was effective against *Salmonella enterica*. Kar and Bera (2018) indicated that, the *Aloe vera* gel is rich in variety of secondary metabolites, such as anthraquinones glycosides, glycoproteins, gamma-lanoline acid, prostaglandins and mucopolysaccharides, which are mainly responsible for its antimicrobial activity. In this study, there were differences in the antimicrobial activities of the *Aloe vera* gel extract. Azwanida (2015) revealed that differences in antibacterial activity of *Aloe vera* plant extracts can be attributed to the age of the plant, physical factors such as temperature, light, water, time of harvesting of plant and drying method used before the extraction process.

CONCLUSION

This study confirmed the potential of *Aloe vera* gel extract as an antibacterial agent in poultry farming. *Aloe vera* gel extracts can be used as prophylactic antibiotics to reduce over dependence on conventional antibiotics that can lead to the

development of resistant strains. It is recommended that, toxicity studies of the *Aloe vera* gel extract should be done to determine the safety indices of the extracts. Clinical trials should also be carried out to explore the potential of *Aloe vera* extracts in the treatment of bacterial infectious diseases of poultry.

DECLARATIONS

Competing interests

All authors declare that they have no conflict of interest.

Author's contribution

Adzitey F, Udoka JU and Agbolosu AA conceived and designed the experiment. Adzitey F and Udoka JU performed the experiment. Adzitey F and Agbolosu AA analyzed and wrote the manuscript. All authors read and approved this manuscript.

Concept to publish

All authors gave their informed consent prior to their inclusion in the study.

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The Effects of Grass-Based versus Grain-Based Feeding of Ruminants on the Human Hygienic Status, a Review

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ABSTRACT

Ruminant meat quality is one of the important factors contributing to the recent spreading of several diseases, such as obesity, cancer, and cardiovascular problems, which have increased predominately. Feeding regiment plays an important role in the determination of the composition of fatty acids and meat quality in ruminants. This review aims to highlight the main factors that lie behind the variability of ruminant meat quality and its effect on human being's health. The reduction in grass-feeding decreases saliva levels in the ruminants, which has several consequences on the rumen, including a reduction in pH level, along with a reduction in the microorganism activities and conjugated linoleic acid levels. In adipose tissues, the expression of the stearoyl-CoA desaturases gene is negatively affected by the decreased conjugated linoleic acid levels in the rumen, which leads to a decreased transformation of saturated fatty acids to monounsaturated fatty acids. Therefore, the lower monounsaturated fatty acids and the parallel increase in the proportion of saturated fatty acids in the consumed meat can be associated with some human diseases. Thus, the present study provided a molecular explanation for the superiority of grass-based feeding in ruminants raised at pasture in term of production of meat with a healthier quality for consumers than those raised on grains.

Key words: Grain; Grass, Human disease, Ruminant meat, SCD enzyme

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INTRODUCTION

Ruminant meat plays a key role in human nutrition, as it provides an inevitable source for high-quality protein (Quiñones et al., 2017). Moreover, it is a proven reservoir for essential micronutrients and several bioactive lipids (Shingfield et al., 2013). On the other hand, meat is a main source of fat in the diet and particularly Saturated Fatty Acids (SFA), (Coutinho et al., 2014) which have been related to the recent spreading of several diseases in humans, such as Cardiovascular Diseases (CVDs), obesity, diabetes, cancer, neurological and skin damages (Makkar and Beever, 2013; Savoini et al., 2016; Hilmi et al., 2017; Temple, 2018). Atherosclerotic complications are primarily responsible for elevated morbidity and mortality in many people around the world (Mansour and Ajeel, 2013). It is estimated that 17.5 million individuals die from CVDs annually, accounting for 31% of all deaths worldwide (WHO, 2016). Impaired insulin sensitivity has been reported due to saturated fats which pose specific lipotoxicity to pancreatic β cells. Thus, replacing SFA with Monounsaturated Fatty Acids (MUFAs) has a role in the improvement of lipoprotein and glycemic levels in individuals with type 2 diabetes (De Souza et al., 2015). However, several factors related to feeding regiment have been found to have various effects on ruminant meat quality by playing a crucial role in the determination of meat content (Priolo et al., 2001). The nutrition of cattle and sheep has a considerable impact on the deposition of intramuscular fat that has a remarkable effect on human health (Popova et al., 2008). The content of MUFA in intramuscular fat is substantially higher for grass-fed steers as compared to steers offered grass silage and/or concentrates (Nieto and Ros, 2012). The higher proportion of MUFA to SFA enhances both meat quality and feeding value of animal products (Gamarra et al., 2018). On the other hand, the Stearoyl-CoA Desaturases (*SCD*) gene encodes an enzyme that transforms SFA into MUFA in the adipose tissues of ruminants (Tian et al., 2017) and a positive correlation has been observed between MUFA and *SCD* expression in ruminants (Costa et al., 2013). Although the benefits of grass nutrition on ruminant meat quality and its positive consequences on human health has been described (Pighin et al., 2016), little information is known about ruminant nutrition through what mechanism affects the meat quality. Furthermore, there is a lack of data regarding the contributing effect of biochemical molecules on human health. Therefore, this review aims to describe the main factors that cause variability in several meat-controlling molecules in the ruminants, their consequent effect on ruminant meat, and their eventual effects on human health.

Effects of saturated fatty acids on human health

The Fatty Acid (FA) composition of dietary lipids and its consequences on meat quality have gained a lot of attention due to its effect on human health (Cho et al., 2010). It has been demonstrated that higher consumption of SFA is associated with diminished insulin sensitivity, hyperglycemia, increasing the risk of metabolic syndromes and cancers such as prostate and breast carcinomas (Khan et al., 2010; Alisson-Silva et al., 2016; Savoini et al., 2016). Furthermore, excessive consumption of ruminant meat, which is a major source of medium-chain SFA and trans FA in the human diet, is considered a risk factor for heart disease (Shingfield et al., 2013). Red meat is also characterized by a particular SFA profile that has been linked to carcinogenesis and CVD (Quiñones et al., 2017). For instance, beef contains several damaging SFAs, such as myristic and palmitic acids that are assumed to be dangerous for the heart, because it raises the serum cholesterol concentrations by four to six folds (de Lemos et al., 2017).

Impacts of polyunsaturated fatty acids and conjugated linoleic acid on human health

The recommended proportion of PUFA to SFA in ruminant meat should be 0.4 or higher (Wood et al., 2003). In this context, changing FA compositions by decreasing concentrations of PUFA and increasing levels of SFA leads to decrease the PUFA: SFA ratio in red blood cell membranes, which may be linked to lower membrane fluidity, as seen in the chronic diseases (Ristić-Medić et al., 2013). On the other hand, the consumption of meat with an imbalanced ratio of n-6:n-3 PUFA can be a risk factor for CVDs, resulting in blood clots and possibly leading to a heart attack (Wood et al., 2003). Furthermore, n-6:n-3 PUFA may also be associated with the progression of carcinogenesis (Azrad et al., 2013). Meanwhile, it has been demonstrated that ruminant meat contains a beneficial ratio of n-6:n-3 PUFA, that is below 4, especially when those have consumed grass-based diets (Pighin et al., 2016). More grazing leads to a higher percentage of omega-3 FA, CLA, vitamin E, β -carotene (Van Elswyk et al., 2014), vitamin A and also a low ratio of n-6:n-3 PUFA in ruminant meats which makes it highly desirable for consumption (Simopoulos, 2016). Beef from pasture-finished steers contains greater levels of n-3 PUFA in comparison to concentrate-finished steers (Pighin et al., 2016). High levels of omega-3 long-chain PUFA (n-3 LC-PUFA) in the diet plays a major role in preventing several diseases, including diabetes, atherosclerosis, and arthritis (Widmann et al., 2011), through inhibition of platelet aggregation, microbial growth (Desbois and Lawlor, 2013), and prevention of the blood clot formation (Phang et al., 2013). Interestingly, a lower incidence of depression, age-related memory loss, and developing Alzheimer's is linked to higher consumption of n-3 LC-PUFA (Wani et al., 2015).

CLA synthesized by microorganisms in the rumen as a byproduct (Arshad et al., 2018), holds considerable benefits for human health. It is believed that the consumption of CLA is beneficial to health because of its ability to increase lean muscle mass while decreasing body fat. It has been suggested that CLA has therapeutic potentials with regard to insulin resistance and hyperlipidemia which are key characteristics of type 2 diabetes (Molony et al., 2004). This relation has recently been explained by the ability of CLA to enhance insulin sensitivity (Cho et al., 2016). Therefore, it was observed that increased CLA has a strong association with anticancer (Peng et al., 2010), anti-adipogenic (Maleki et al., 2015), anti-atherosclerotic properties (De Hartigh, 2019), and cardioprotective effects (Parodi, 2009).

Stearoyl-CoA Desaturases effects on human health

The *SCD* gene product is a major enzyme for controlling intracellular FA composition by stimulating the desaturation of SFA, thereby resulting in the conversion of SFA to MUFA in the adipose tissues of ruminants (Tian et al., 2017). Genetic groups of ruminants have shown significant differences in the activity of the *SCD* gene product (Ivanović et al., 2016). In different organisms, there are five variants of *SCD* genes that are known as, *SCD1-SCD5*, placed at several chromosomal positions (Furqon et al., 2017). Furthermore, the expression of *SCD* in the muscle tissues can be affected by nutrition composition, especially n-3 PUFA contents. The level of *SCD* gene expression plays a key role in terminal adipocytes differentiation as adipogenesis has been found to be greatly induced by *SCD* gene expression (Madsen et al., 2005). This pattern of *SCD*-induced adipocytes differentiation leads to a consequent accumulation of intramuscular fat, which points to the link between the gene expression of *SCD* and increasing FA deposition in the ruminant muscle (Costa et al., 2013). However, hormones and several nutrients including FAs, carbohydrates, and cholesterol, have been recognized as a potent modulator of the *SCD* gene expression. Moreover, the expression of the *SCD* is positively related to elevated CLA and MUFA contents, which entails considerable impacts on human health (Barton et al., 2010).

The impacts of grass-based versus grain-based feeding on the rumen environment

In the ruminant digestive system, the composition of the FAs present in the rumen is affected by the rumen microorganisms and ruminal pH (Kashani, 2015). SFA, like palmitic (C16:0) and stearic (C18:0) acids, pass through the rumen unaltered, while the Unsaturated Fatty Acid (UFA) is subjected to biohydrogenation and converted to SFA to decrease the toxic effects of dietary UFA on the microorganism's growth and production of CLA as a byproduct (Shingfield et al., 2013). LC-PUFA has also been reported to have differential toxicity and inhibitory effects on rumen

microorganisms (Lourenco et al., 2010). Thus, the conversion of UFA to SFA, or biohydrogenation, in the ruminants represents a major human health issue. It is noteworthy that the high forage proportion in the grass-based feeding causes higher secretion of saliva (Figure 1A), ensuring higher rumen pH (Lee et al., 2016), and thus, strengthening the effect of lipolysis and/or biohydrogenation (Scollan and Enserb, 2003). Grass-feeding increases the content of CLA due to the biohydrogenation of the PUFA linolenic acid in the rumen that has a twofold higher concentration in the digestion of the pasture-fed steers, as compared to corn-fed steers (Smith et al., 2009). Regarding grain-based feeding, the biohydrogenation process was found to be inhibited by a decrease in the ruminal pH and ultimately growth inhibition of the CLA-producing bacteria (Wood et al., 2008) (Figure 1B). The reduction in the pH values is typically linked with this sort of diet, as the presence of concentrated food components reduces lipolysis, which is a fundamental step in biohydrogenation (Menezes et al., 2010). If ruminal biohydrogenation of UFA can be controlled, it may be possible to enhance the quality of ruminant meats via generally increasing UFA, CLA, and n-3 FAs in particular. Most of the alimentary FA are UFAs (oleic acid [C18:1 cis-9]; linolenic acid [C18:3n-3], and linoleic acid [C18:2n-6]) that are converted to SFA (C18:0) in the rumen by biohydrogenation, with a plethora of CLA isomers throughout the process of isomerization (Dewanckele et al., 2018). Therefore, meat products supplied by more isomers of CLA, result in higher CLA concentrations in consumed meat (Silveira et al., 2007).

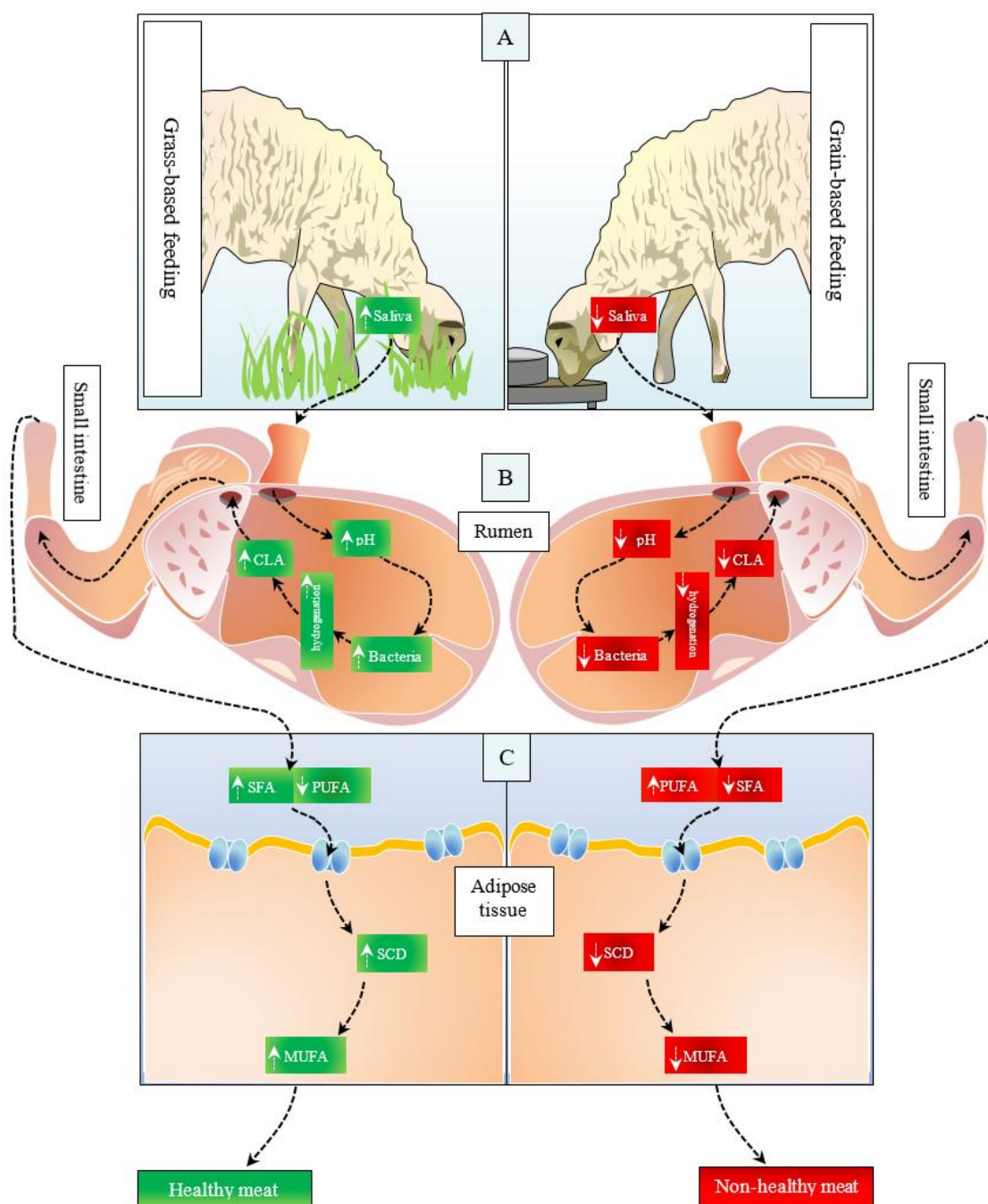


Figure 1. Comparison between grass-based versus grain-based ruminant feeding routes. A, B and C refer to impacts of consumed feed on saliva, rumen, and adipose tissues, respectively.

The impacts of grass-based versus grain-based feeding on adipose tissue

About 70–80% of FAs were accumulated in the tissues as CLA during the ruminal biohydrogenation of dietary linoleic acid (Lobo et al., 2014). The levels of rumenic acid, the most important CLA isomer, along with arachidonic (C20:4c5) and phytanic acid, are at least three times higher in the muscles of grass-fed animals in comparison to grain-fed animals (Moholisa et al., 2018). The increased concentrations of SFA and decreased concentrations of PUFA in ruminant tissues is due to a higher ruminal pH and consequently higher biohydrogenation rate in the rumen (Hughes, 2011). However, lower gene expression of the *SCD* as a result of an increased level of PUFA has been reported in different animal species and tissues (Conte et al., 2012). Thus, inhibiting the biohydrogenation pathway could increase the levels of PUFA in tissues and lead to a reduction in the gene expression of *SCD* in ruminants (Figure 1C). Moreover, PUFA suppresses the expression of lipogenic genes by inhibiting proteolysis of sterol regulatory element-binding protein 1 in the Golgi apparatus (Jacobs et al., 2013). Therefore, a nutritional regimen that increases PUFA absorption in the muscles will reduce meat quality. A parallel relationship of n-6: n-3 PUFA proportion with levels of *SCD* gene expression and in turn, CLA production in the muscle tissue, was reconfirmed (Ebrahimi et al., 2018).

The higher the expression of the *SCD1* gene in grass-feeding is associated to the elevated converting ratio of MUFAs to SFAs, increased levels of phospholipid membrane composition, fat metabolism, and adiposity (Hilmia et al., 2017). Thus, the high proportion of MUFA to SFA enhances both the quality and feeding value of animal products. The correlation analysis among oleic acid proportion and CLA percentage, with the levels of *SCD* expression in ruminants, appears to have been effective (de Castro et al., 2013). Positive correlations between *SCD* expression and FA content is observed, particularly for total FAs, MUFA, and c9,t11-CLA, while lower *SCD* expression in adipose tissues is detected in Holstein-Friesian cows and mature culled cows when fed with PUFA (Gamarra et al., 2018). Thus, imparting nutrition for animals with PUFA enriched diets results in a lower expression *SCD* gene, which appears to be due to the inhibitory role of PUFA on the promotion of the *SCD* gene in the liver and adipose tissues (Benítez et al., 2017)

CONCLUSION

In conclusion, several factors affect the meat quality of ruminants and lead to sanitation problems for human health. In the last 60 years, reduction in the pasture quality due to a lack of vegetation and the reliance of the farmer to the use of grains for ruminant nutrition caused a decrease in CLA levels, reduced *SCD* activity in tissues, and decreased MUFA (or increased SFA) levels in ruminant meat which are involved in the pathogenesis of several chronic human diseases. Conversely, high expression of the *SCD1* gene in grass-fed ruminants is effective in meat production with a highly favorable quality for the consumers' health.

DECLARATIONS

Competing interests

The authors have declared that no competing interest exists.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

Authors' contributions

TM Al-Thuwaini designed the main idea and wrote the review. MBS Al-Shuhaib revised the review and drew the schematic diagram presented in figure 1.

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Characterization of Pregnancy-Associated Glycoprotein as a Biomarker of Pregnancy in Etawa Crossbred Goat

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ABSTRACT

Pregnancy-Associated Glycoprotein (PAG) is secreted by the placenta, which is produced in mononucleate and binucleate trophoblast cells. The current research was conducted to find out a substance for diagnosing early pregnancy in Etawa crossbred goats. Six Etawa crossbred goats (not pregnant, three months pregnant and four months pregnant) were subjected in the present study from Livestock Government Institution Breeding in Singosari, Malang. The research methods consisted of sample collection, identification PAG with sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the determination of concentration with Biuret method and specificity test with Western Blot assay. The obtained results showed that the molecular weight of PAG from pregnant Etawa crossbred goats was 55.85 kDa. The average concentrations of PAG in the goats of non-pregnant, three months pregnant, and four months pregnant were 1.83±2.98, 3.79±2.72 and 4.36±2.63, respectively. The results of the specificity test with the Western Blot molecular revealed a molecular mass of PAG was 55 kDa. The findings of the present study demonstrated PAG in Etawa crossbred goats can be used as an indicator of pregnancy.

Key words: Biomarkers, Etawa crossbred, PAG, Pregnancy

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INTRODUCTION

Diagnosis of early pregnancy in goat can be done in two ways: through detection of specific substances in the peripheral circulation such as Pregnancy-Associated Glycoprotein (PAG) or non-specific substances in the blood, urine or milk such as progesterone and estrone sulfate (Hafez, 2000). PAGs are pregnancy indicators that are produced by mononucleate and binucleate trophoblastic cells (Perenyi et al., 2002; Karen et al., 2003; Sousa et al., 2006). Garbayo et al. (1998) purified three PAGs from goat placenta which differed in amino acid sequences and molecular weight (55 kDa, 59 kDa, and 62 kDa) and each of them had several isoforms with different isoelectric points. Isolation of ovine PAG was obtained at a molecular weight of 30.86 kDa from placental cotyledon (Setiati et al., 2009). In cattle, PAG isolated from the blood serum during 274-279 days of gestation was characterized in molecular weight of 67.34 kDa (Lestari and Ismudiono, 2011). In livestock reproductive management, early pregnancy diagnosis is very economically advantageous in determining pregnancy status after mating (Restall et al., 1990; Goel and Agrawal, 1992). Generally, the length of the estrous cycle of the goat is around 21 days (Jainudeen et al., 2000). The economic losses of pregnant goats can be minimized or prevented by methods of early pregnancy diagnosis (Singh et al., 2004).

Pregnancy tests have the potential to be very suitable for field practice. PAG can be measured in maternal blood circulation (Shahin et al., 2013). Pregnancy is diagnosed using PAG test on day 24 of gestation (Reese et al., 2017). The knowledge of mechanisms involved in the production and control of PAG is beneficial in livestock breeding and facilitates diagnosis of pregnancy (Santos et al., 2018). Therefore, the current study was designed to evaluate blood serums for early pregnancy diagnosis in Etawa crossbred goats managed in intensive conditions in Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Institutional Animal Ethics Committee Brawijaya University (Code No.1108-KEP-UB).

Collection of Samples

The present study was conducted on six crossbred goat aged 3 to 4 years in Livestock Breeding Institution Government in Singosari, Malang, East Java, Indonesia. The analysis of blood samples was conducted at the Department of Veterinary Reproduction Airlangga University, Surabaya and Biosains Laboratory, Brawijaya University, Malang,

East Java, Indonesia. The goats were maintained under intensive system of management in well-ventilated pens and dietary and management conditions were the same for animals. Blood samples were taken from jugular veins of non-pregnant and pregnant goats in the different gestational age (3-4 month). The serum samples were centrifuged at 3000 rpm for 10 minutes and the supernatant was transferred in a new tube and was stored at -20°C until further use.

Identification of PAGs with Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The running gel was inserted into the SDS-PAGE tool through the wall to below the top line. Then, 1 ml butanol was added and left for 25 minutes. After the gel freeze, butanol was removed and cleaned with PBS then dried with Whatman paper. The 12% stacking gel is inserted from the top of the wall until it was fully set for 25 minutes. The comb was inserted and the remaining gel was cleared with a buffer. The samples were mixed with 5 µl of liquid buffer and heated at 100°C for 5 minutes. Then samples were inserted into the mold hole with a tip of 200 µl. The mold is inserted into an electrophoresis gel device, the power supply at the start was 125 V, 40 mA for one hour. When the electrophoresis was completed, it was turned off and the plate was opened and separated, then the gel was stained with Coomassie Brilliant Blue (Merck, Germany). Molecular weight determined using standardized regression between relative migration and molecular weight markers. Then, it was tested for specificity with Western Blots test.

Examination of PAGs levels using the Biuret method

The not-colored SDS-PAGE gel was cut to the desired tape. Each gel was inserted into a nylon sack and packed in a glass block containing PBS while were mixed on a magnetic stirrer for 24 hours. PBS was replaced every 6 hours. The gel pieces were stained with silver staining to detect protein. The total protein concentration was determined using Biuret reagent by adding a standard solution of Bovine Serum Albumin (BSA) protein. The sample cuvette was prepared with a PAG and 2.5 ml of the Biuret reagent. The standard cuvette as filled with 0.05 ml BSA and 2.5 ml of the Biuret reagent. The blank cuvette was prepared by adding 2.5 ml Biuret reagent and 0.05 ml of distilled water. Three cuvettes were left for 30 minutes and color intensity was read by Bausch Lomb Spectronic Spectrophotometer at a wavelength of 540 nm.

Specificity test of PAG with Western Blot

Western blot was carried out by using fragments of PAG bands which had been run in SDS-PAGE and were transferred to the nitrocellulose membrane. The membrane was blocked with 3% BSA in 20 mM Tris-HCl at pH 7.5 and 150 mM NaCl for one hour then was incubated with the primary antibody (anti-PAG) diluted in Tris/NaCl containing 1% BSA. After washing with Tris-Cl containing 0.05% TWEEN 20, the membrane was incubated with secondary antibodies (anti-rabbit IgG labeled AP, 1:1000 dilution) and was added Western Blue Substrate (Promega, USA) (Aulanni'am, 2004).

Statistical analysis

The standard protein curve was made to obtain the molecular relative mass of the samples (Gaspersz, 1995). The relative molecular mass of each protein defined by data converted from relative migration distance (R_f) values according to following linear equation: $Y = b_0 + b_1X$; where Y is molecular weight (kDa), b_0 is a constant, b_1 is coefficient of relative migration, and X is relative migration of protein band.

The total protein concentration of PAGs by Biuret method was calculated as follows: $Y = 5.10^{-5}X$ Where Y is absorbance and X is a concentration of protein (µg/ml). The data of the Biuret method was statistically analyzed using ANOVA multivariate. Data were analyzed using SPSS version. 17.0 software (SPSS Inc, USA). A $p < 0.05$ were regarded as statistically significant.

RESULTS

The profile of the PAGs isolated from blood serum of Etawa crossbred goats using SDS-PAGE are shown in figure 1. The protein molecular weight was measured by relative migration when the protein passes through the separating gel (Figure 2). Then, based on the logarithmic equation ($y = 2.401 + -1.4752X$; $R^2 = 0.9829$) obtained by calculating the relative migration, the molecular weight was obtained as a band that appeared on electrophoresis. The molecular weight of protein bands from six samples are presented in table 1. The serum of control and pregnant goats has the same protein profile. However, in blood serums of pregnant goats, there was a protein with a molecular weight of 55.85 kDa which was expected to be a specific PAG.

To ensure that the electroelution protein was a PAG, the elution results was examined by Biuret method to determine PAG protein levels. The results of the examination using the Biuret method can be seen as isolation of PAG in table 2. The protein concentration was the lowest in non-pregnant goats (1.83 ± 2.98), then 3 months pregnant (3.79 ± 2.72) and showed the highest value in 4 months pregnant (4.36 ± 2.63). The indicated correlation in blood serum had a significant difference ($P < 0.05$).

Specificity tests were carried out to ensure that the detected protein was PAG. The results of the Western Blot test showed purplish bands on nitrocellulose membranes with a molecular weight of 55 kDa (Figure 3). This finding proved that the visible bands were PAG molecules. The molecular weight can be read using a reference marker protein with a molecular weight range of 15 to 260 kDa. Protein band with a molecular weight of 55 kDa was found in samples of pregnant goats in gestational age 3 and 4 months, whereas in samples of non-pregnant goats there was a protein band with a molecular weight of 23 kDa.

Table 1. Molecular weight of protein obtained from blood serum of Etawa crossbred goats

Serum Sample	Protein Molecular Weight (kDa)											
	15.29	20.23	24.10	31.89	39.35	48.55	55.85	62.04	82.10	120.68	165.39	197.03
Non-pregnant	+	+	+	+	+	+	-	+	+	+	+	+
3 month pregnant	+	+	+	+	+	+	+	+	+	+	+	+
4 month pregnant	+	+	+	+	+	+	+	+	+	+	+	+

(+) = positive band; (-) = Negative band

Table 2. The average concentration of PAGs isolated from blood serum of pregnant Etawa crossbred goats by using Biuret method

No.	Sample	Absorbance 1	Absorbance 2	Average concentration (µg/ml)
1	non-pregnant	1.48	2.18	^a 1.83±2.98
2	3 month	5.22	2.36	^b 3.79±2.72
3	4 month	2.45	6.23	^c 4.36±2.63

Different superscript letters indicate significant differences ($p < 0.05$); PAG: Pregnancy-Associated Glycoproteins; Absorbance was read in a spectrophotometer at a wavelength of 540 nm

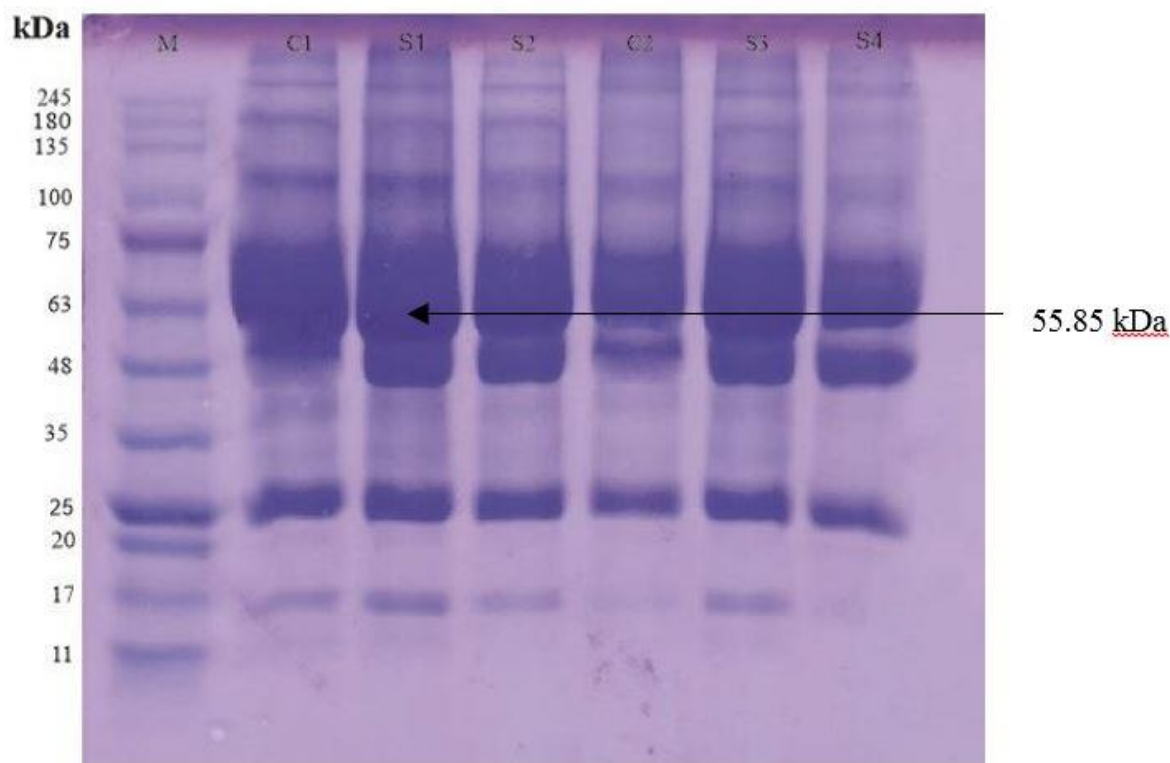


Figure 1. This is the SDS-PAGE analysis of PAGs isolated from blood serum of Etawa crossbred goats. Lane M: Tris-Glycine 4-20%, 11-245 kDa as marker; Lane C1 and C2: non-pregnant; Lane S1 and S3: 3 months pregnant; Lane S2 and S4: 4 months pregnant.

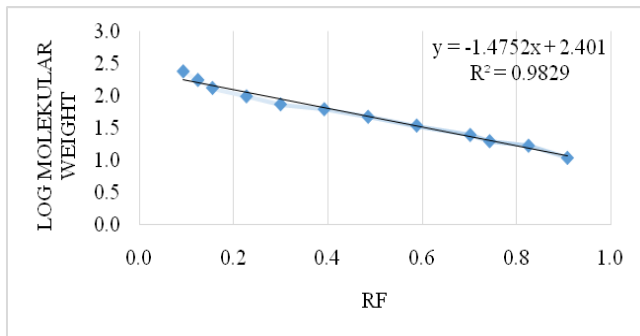


Figure 2. Determination of molecular weight by calculating the relative migration

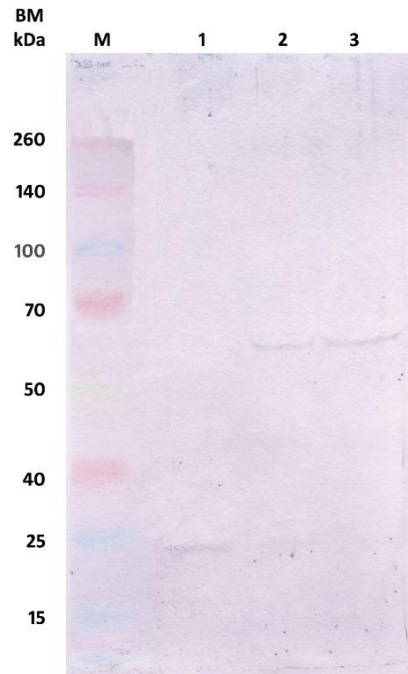


Figure 3. The Western Blot analysis for PAG isolated from blood serum of Etawa crossbred goats. Lane M: marker; Lane 1: non-pregnant; Lane 2: 3 months pregnant; Lane 3: 4 months pregnant.

DISCUSSION

According to the obtained results in the present investigation, the molecular weight of PAG in Etawa crossbred goats was 55.85 kDa. This finding is in accordance with the discovery of caprine PAG in the previous study by Amiri et al., (2004) that identification protein was performed by SDS-PAGE and found the molecular weight of caprine PAG was between 55 to 66 kDa. Moreover, Garbayo et al. (1998) reported different molecular mass (55 kDa, 59 kDa, and 62 kDa) for caprine PAG. PAGs can be detected in the maternal blood circulation from embryo implantation (Gordon, 1999). Trophoblast placental cells are present in blood circulation during implantation until parturition and are responsible for producing PAGs throughout the gestation period (Gonzales et al., 2000).

The protein concentration of PAG increases progressively at 3 and 4 months of gestation. Blood PAG levels steadily increased during early pregnancy in goats (Singh et al., 2019). Isperto et al., (2016) reported PAG concentrations were significantly higher in twins compared to single pregnancies. The current research is in agreement with one of the statements by Cavanagh (1996) who mentioned PAG was first discovered as a substance-related to pregnancy and was detectable in 6-24 hours after conception in all species such as rats, humans, pigs, and sheep. Duplants (2000) declared that PAG was detected after the implantation and remained in the pregnant goat until parturition and disappeared after the birth process. Many factors influence the concentration of PAG, such as breeding (Ranilla et al., 1994; Guilbault et al., 1991), the number of fetuses (Benitez-Ortiz, 1992) and *in vitro* culture period (Ectors et al., 1996). Therefore, differences in PAG expression observed in the present study can be related to variations in breeds, procedures, and geographical location.

The results of present study showed the protein bands on nitrocellulose membranes, indicating a specific bond between PAG antibodies and PAG antigens isolated from Etawa crossbred goats pregnant. The further production of PAG increases the bond between PAG antibodies and PAG antigens and provides thicker protein bands. This finding is supported by Aulanni'am (2004), in Western Blot method, PAG antibodies recognize PAG antigens as specific antigens and bind together thus purplish-colored protein bands become visible.

CONCLUSION

The present study characterized PAG with a molecular weight of 55.85 kDa in Etawa crossbred goats at 3 and 4 months before parturition. Moreover, application of PAG as a biomarker of pregnancy was confirmed in Etawa crossbred goats.

DECLARATIONS

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Consent to publish

All authors contributed to write and publish manuscripts in the World's Veterinary Journal.

Competing interests

The authors declare that they have no competing interests.

Author's contribution

IKN wrote the manuscript and conducted the research, TDL conceptualized the research, and HAP revised the final form of the manuscript.

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Incretin Mimetics Vildagliptin and Exenatide Improve Pedicle Skin Flap Survival in Rats

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ABSTRACT

Hypoxia and tissue ischemia are the leading factors in the alteration of tissues in many pathological conditions. Prevention and reversion of the effects of local ischemia, which develops during various surgical interventions, is an actual problem of modern medicine. The aim of the present study was to investigate the effect of exenatide and vildagliptin on the survival rate of an isolated pedicle skin flap in sixty adults Wistar rats. Simulation of a pedicle skin graft was performed on the second day of the experiment. After anesthesia under aseptic conditions, a skin graft was cut out: isolated in a plastic bag, the edges of the skin were stitched with interrupted sutures (nylon 3/0). Rats were divided into six groups: control group, exenatide group (10 µg/kg/day subcutaneously for nine days after surgery), vildagliptin group (0.2 mg/kg/day intraperitoneally for nine days after surgery) and pentoxifylline group (100 mg/kg/day intravenously, two hours before the surgical intervention). In the other two groups, glibenclamide (5 mg/kg) were administered before injection of incretin mimetics. On the third, seventh and tenth day, area of the surviving tissue was measured. Subsequently, the survival rate of the skin graft was calculated. The area of the surviving tissue in exenatide and vildagliptin group was 1.5 and 1.7 times more compared to the control group, respectively. Preliminary blockade of ATP-dependent potassium channels by glibenclamide eliminated the protective effect of exenatide and vildagliptin. The increase in the survival of ischemic tissues using exenatide and vildagliptin has been experimentally proved. The current study confirmed the important role of ATP-dependent potassium channels in dermatoprotective properties of incretin mimetics.

Key words: Dermatoprotective properties, Exenatide, Ischemia, Pedicle skin graft, Vildagliptin.

INTRODUCTION

In the modern view, ischemic-reperfusion injury is the leading cause of most of the critical situations that are somehow associated with ischemia including myocardial infarction, stroke, organ transplantation, and shock of various etiologies (Lankin et al., 2005; Sloth et al., 2014; Sharafeev and Bayazitova, 2016). The resumption of blood flow, occurring after ischemia, paradoxically causes deep tissue damage and further cell necrosis (Savas et al., 2003).

At the same time, none of the proposed methods and their combinations provide guaranteed protection against ischemia-reperfusion injury, in connection with which the search and selection of innovative and promising compounds and preparations of this orientation continue. Preclinical studies at the systemic (Korokin et al., 2014; Peresyphkina et al., 2016; Yakushev et al., 2016), cellular (Danilenko et al., 2016; Kravchenko et al., 2016; Kalmikov et al., 2018), and molecular (Bogus et al., 2018; Dzhimak et al., 2018; Soldativ et al., 2018) levels, including specific activity (Gumanova et al., 2007; Denisyuk et al., 2016; Danilenko, 2018) and toxicology studies (Kolesnichenko et al., 2018) in conjunction with bioequivalence studies (Kalmikov et al., 2018), therapeutic equivalence and effectiveness (Avdeeva et al., 2016) are an integral part of the research of innovative drugs.

Incretin hormones are secreted in response to food intake and stimulate insulin secretion (Gautier et al., 2008). Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by the small intestine to regulate glucose concentration in the blood. GLP-1 stimulates insulin release by binding to the GLP-1 receptor (GLP-1R) on the beta cells of the pancreas. Moreover, stimulation of GLP-1Rs has cytoprotective and anti-apoptosis effects on tissues cells bearing this receptor (Ban et al., 2010; Anagnostis et al., 2011).

The GLP-1R belongs to class B1 (secretin receptor-like) of the family of G-protein-coupled receptors (GPCR). The interaction of GLP-1 with its receptor is accompanied by activation of adenylate cyclase (AC) and followed by an increase in cyclic adenosine monophosphate (cAMP) level and subsequently leading to activation of protein kinase A (PKA), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (PKB, also known as Akt), thereby realizing the effect of GLP-1 on the functioning of target cells, as well as on the processes of apoptosis and regeneration (Deacon et al., 2006; Ban et al., 2010; Wei et al., 2016) because GLP-10 metabolites with respect to GLP-1R. At the same time, according to some studies, metabolites of incretins have their own physiological effects (Deacon et al., 2006; Saraiva and Sposito, 2014).

In addition to the hypoglycemic effect, there is evidence that GLP-1R agonists and DPP-4 inhibitors have pleiotropic effects (Ban et al., 2008; Vlasov et al., 2016). Due to the fact that the pancreas, brain, heart and other organs have exactly the same type of GLP-1R, it is reasonable to assume that the cytoprotective effect will extend to all types of tissues including skin tissue (Tyurenkov et al., 2017). In this regard, the present study intended to evaluate the efficacy of the incretin mimetic agents including exenatide and vildagliptin in preventing and ameliorating the consequences of ischemia-reperfusion injury.

MATERIALS AND METHODS

The present study were conducted in laboratory of Medical Institute of Belgorod State University, Belgorod, Russia. The experiments were carried out on 60 adult male rats of the Wistar line weighing 200-250 g. All rats were divided into six groups of 10 animals. Simulation of an isolated pedicle skin flap was performed on the second day of the experiment, according to the findings of Kolesnik (2010). On the abdomen of the rat, the skin was cut off and stepping back 1 cm from the xiphoid process along the white line of the abdomen. A skin graft was cut off (1 × 4 cm) while maintaining the supply vessel. It was placed in an insulated plastic bag and sewn to the skin. Operations were performed under anesthesia with chloral hydrate (300 mg/kg, intraperitoneally).

To study the cytoprotective effect of exenatide and vildagliptin on the model of an isolated skin graft on the pedicle, all experimental animals were divided into six groups. In the control group, rats did not receive any treatment. In exenatide group, exenatide (10 µg/kg/day) was injected subcutaneously for 9 days after the operation, rats in vildagliptin group received vildagliptin (0.2 mg/kg/day) intraperitoneally for 9 days after the operation. In the other group, pentoxifylline as a drug reference was injected intravenously at a dose of 100 mg/kg/day 2 hours before the experiment.

To determine the role of ATP-dependent potassium channels in the implementation of the mechanism of action of incretin mimetics, 5 mg/kg glibenclamide were injected intragastrically through a probe to animals, 30 minutes before drug administration. The areas of surviving tissue were measured on third, seventh and tenth days and eventually, the survival rate of the skin graft was calculated (the ratio of the area of the surviving tissue to the initial area of the graft × 100%).

Ethical approval

All of the experimental process was conducted in according to “modern ethical requirements for animal experiments” (Kopaladze, 1999).

RESULTS AND DISCUSSION

In the control group, the area of the surviving tissue on the fifth day was $1.62 \pm 0.02 \text{ cm}^2$, which is 41% of the original area (4 cm^2). The administration of exenatide and vildagliptin led to an increase in the area of the surviving tissue to $2.68 \pm 0.03 \text{ cm}^2$ and $2.41 \pm 0.09 \text{ cm}^2$, respectively. In the group of pentoxifylline (100 mg/kg/day), surviving graft area was $1.81 \pm 0.07 \text{ cm}^2$, which is 1.1 times more than the control group, but less than in exenatide and vildagliptin.

When simulating a skin graft on the pedicle, both studied incretin mimetics contributed to a significant increase in the area of the surviving tissue in comparison with the control group at 3, 7, and 10 days (Table 1). According to the obtained results, vildagliptin had more beneficial effects on graft survival rate compared to the control group.

In the present study, it was revealed that Preliminary blockade of ATP-dependent potassium channels by glibenclamide eliminates the protective effect of incretin mimetics when simulating a pedicle skin graft in rats. The survival of the skin graft in experimental animals with the administration of glibenclamide did not differ from that of the control group, which confirms the fact that glibenclamide at the applied dose does not affect the survival of ischemic tissues (Table 2). When using glibenclamide before the administration of exenatide and vildagliptin, the area of the surviving tissue was 1.3 and 1.4 times less than with their isolated administration, respectively.

The GLP-1R has been identified in the skin of mice; in cultured skin cells, GLP-1 activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway associated with cell proliferation, differentiation, and cytoprotection (List and He, 2006). In this regard, many recent studies have demonstrated the beneficial role of GLP-1 analogs such as exenatide and DPP-4 inhibitors such as vildagliptin in patients with diabetes (Lee and Lee, 2017), as well as promote ulcers healing on the feet (Long et al., 2018) and the healing of diabetic wounds in rodents (Roan et al., 2016). In addition, DPP-4 expression is increased in dermal fibroblasts of mouse muscle after skin damage (Schürmann et al., 2012) and is a prerequisite for fibroblast migration and proliferation, indicating the role of regional incretins in regulating fibroblast functions and, possibly, wound healing. Recent experimental findings indicated the possible role of incretins in promoting tissue regeneration and, therefore, their ability to stimulate the migration of endothelial cells (Kang et al., 2013) to inhibit apoptosis (Favaro et al., 2012) and reduce inflammation and oxidative stress during ischemia (Gurtner et al., 2008).

Most researchers talk about the connection of the GLP-1R with the most important intracellular signaling cascades - PI3K/PKB (Akt), AC/PKA/MAPK, PKB (MAPK)/NF- κ B and PKB (PKA)/eNOS. GLP-1R agonists (including liraglutide and exenatide) are able to have an endothelium protective effect in diabetes mellitus, reducing the activation of many pro-inflammatory mediators and adhesion-enhancing factors (including TNF α , PAI-1, VCAM-1, ICAM-1, MCP-1 and E-selectin) which stimulate adhesion and infiltration of the vascular wall by monocytes and macrophages and are associated with endothelial dysfunction and atherogenesis (Stewart et al., 2015; Jiang et al., 2018).

Nevertheless, there is no consensus in the literature in explaining the mechanism of the cytoprotective action of incretin mimetics. A number of studies suggest the implementation of a protective effect of incretin mimetics according to the type of ischemic preconditioning, where the mitochondrial ATP - dependent potassium channels can be considered as the final effector link, the activation of which directly leads to an increase in resistance during ischemia (Tamareille et al., 2011; Ussher and Drucker, 2012).

Table 1. Indicators of skin graft survival rates when using exenatide, vildagliptin, and pentoxifylline in male rats.

Medicaments (dose; number of members)	Necrosis area of the skin graft (%)		
	Day 3	Day 7	Day 10
Control group (saline)	30.98 \pm 3.2	65.76 \pm 2.4	84.55 \pm 3.3
Exenatide (10 μ g/kg/day; 10)	27.37 \pm 3.1*	52.9 \pm 4.4*	61.8 \pm 3.5*
Vildagliptin (0.2 mg/kg/day; 10)	24.1 \pm 2.8*	44.3 \pm 3.1*	59.1 \pm 2.4*
Pentoxifylline (100 mg/kg/day; 10)	36.9 \pm 1.9*	39.5 \pm 3.8*	67.8 \pm 2.4*

*significant different (p<0.05) compared to the control group. Values are expressed as mean \pm standard deviation

Table 2. Protective effect of exenatide and vildagliptin in the background of glibenclamide on skin graft survival rate.

Medicaments (dose; number of members)	Necrosis area of the skin graft (%)		
	Day 3	Day 7	Day 10
Control group: saline (10)	30.9 \pm 3.2	65.7 \pm 2.4	84.5 \pm 3.3
Exenatide (10 μ g/kg/day; 10)	27.3 \pm 3.1*	52.9 \pm 4.4*	61.8 \pm 3.5*
Glibenclamide (5 mg/kg; 10)	31.0 \pm 2.2	64.3 \pm 3.1	83.9 \pm 2.7
Glibenclamide (5 mg/kg) + Exenatide (10 μ g/kg/day) (10)	29.1 \pm 2.04	61.7 \pm 2.53	82.5 \pm 3.21
Vildagliptin (0.2 mg/kg/day; 10)	24.1 \pm 2.8*	44.3 \pm 3.1*	59.1 \pm 2.4*
Glibenclamide (5 mg/kg) + Vildagliptin (0.2 mg/kg) (10)	31.2 \pm 2.12	64.71 \pm 2.59	84.5 \pm 3.13

*Significant different (P<0.05) compared to the control group. Values are expressed as mean \pm standard deviation

CONCLUSION

The obtained results in the present research testify the protective effect of exenatide and vildagliptin incretins when simulating a pedicle skin graft, which was accompanied by a decrease in the severity of ischemic tissue damage. The blockade of ATP-dependent potassium channels by glibenclamide on a pedicle skin graft reduced the effects of exenatide and vildagliptin. There is a necessity for further experimental and clinical studies to explain the mechanism for implementing the protective effects of incretins on various models.

DECLARATIONS

Authors' contributions

All of authors had equal roles in writing, editing, and experimental process and finally checked and approved the last edition of article.

Competing interests

The authors have declared that no competing interest exists.

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Use of Untreated and Autoclave-Treated Wheat Germ Meal in Growing Rabbit Diets

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ABSTRACT

The present study was intended to investigate the influence of using 20% and 40% treated or untreated wheat germ meal in growing New Zealand rabbit diets. A total of 75 weaned New Zealand White rabbits aged six weeks old, with an average initial weight of 659.60 ± 18.84 g were divided into five groups with five replicates in each group (three rabbits per replicate). The first group was fed on a basal diet (T_1), second and third groups received diets containing Wheat Germ Meal (WGM), as replacement of soybean meal protein, at levels of 20% and 40% and were labeled as T_2 , T_3 , respectively. Fourth and fifth groups were fed with 20% and 40% autoclave-treated WGM (T_4 and T_5 , respectively). The trial was continued until 14 weeks of age. The present study was evaluated growth performance, blood parameters, carcass traits, meat quality in different groups and also economic efficiency was calculated. There were insignificant differences in terms of live weight, daily weight gain, carcass weight and dressing percentages among rabbits in groups of T_1 , T_2 , and T_4 . Rabbits in the group of T_4 achieved the best feed conversion ratio. Digestion coefficients of crude protein, crude fiber, ether extract, nitrogen-free extract, and nutritive value in terms of digestible crude protein, total digestible nutrition, and digestible energy did not significantly differ between T_1 and T_4 . However, these factors significantly decreased in T_3 and T_5 compared to T_1 . Plasma total protein and globulin significantly increased in rabbits of T_2 and T_4 compared to those fed in T_1 group. A significant decrease in total cholesterol and total lipid for rabbits in groups of T_4 , T_5 , and T_2 was observed. Moreover, rabbits fed on T_4 or T_2 diets had the highest economic efficiency. Conclusively, the untreated or autoclaved WGM can be used in growing rabbit diets up to 20% for replacing the soybean meal protein, which caused low feed costs without adverse effects on the growth performance of rabbits.

Key words: Rabbits, Soybean meal, Wheat germ meal

INTRODUCTION

Wheat, maize, rice, oats, barley, millets, sorghum, and rye are important cereals for human nutrition, either for cooking or as raw material for obtaining flour for baking (De Vasconcelos et al., 2013). Wheat is one of the important cereals and food ingredients around the world due to its ability to be converted to flour. Wheat flour is produced by milling wheat and extraction rate varies from 73% to 77%, depending on the variety of wheat and the production conditions (Elliott et al., 2002). Therefore, in the milling process, by-products such as wheat germ, wheat bran and parts of the endosperm comprise 23 to 27% of the production.

The wheat germ as a significant by-product of the cereal industry accounts for 2.5 to 3.8% of the total grain weight (Brandolini and Hidalgo, 2012). The wheat germ oil is the richest natural source of vitamin E (α -tocopherol) (Gerald et al., 2017). Hafez et al. (2019) found that rats received 3g/kg wheat germ oil had a non-significant increase in Malondialdehyde (MDA) levels of serum at the 15th day of study as compared to control group. Saleh (2016) found that oral administration of wheat germ oil (1400 mg/kg) to mice for eight days improved the liver and small intestine damage induced by carbon tetrachloride.

The Wheat Germ Meal (WGM) is produced when the oil is extracted from the wheat germ. WGM is high in protein content and rich in carbohydrates which could be processed further into livestock feeds. WGM as a good source of water-soluble vitamins also has a high content of lysine, threonine, and histidine (Barton and Monr, 1946). In addition, Moran and Summers (1967) reported that WGM contains 29% Crude Protein (CP), 2.1% Crude Fiber (CF), 10.3% Ether Extract (EE). Moreover, it contains gluten and antinutritional factors such as hemagglutinins and trypsin inhibitor, which inhibit enzymatic digestion of proteins (Creek and Vasaitis, 1962). Therefore, methods such as autoclaving or biological treatment are essential for improving the nutritive value of WGM. Autoclaving destroys the activity of these factors (Creek et al., 1962). Moran and Summers (1967) demonstrated the Autoclaved Wheat Germ Meal (AWGM) at 121 °C for 20 minutes, incorporated to chicken diets at the level of either 47% or 63.8% improve growth performance and feed utilization compared to groups fed WGM autoclaved for 90 min and control group. Hence, the objective of the present

study was to investigate the effects of partial replacement of soybean meal protein with autoclave treated WGM protein on feed cost and growth performance of rabbits.

MATERIALS AND METHODS

The experiments were conducted at Borg-El Arab, located in Alexandria governorate, Egypt. The autoclave treatments were performed at the laboratories of By-Products Research Department, Animal Production Research Institute, Agriculture Research Center, Giza, Egypt.

Ethical approval

The study was carried out after obtaining the ethical approval from the Animal Production Research Institute, Egypt.

Wheat germ meal processing

WGM was obtained from the processing and extraction unit of natural oils, National Research Center, Giza, Egypt. It was ground by a hammer mill and stored in an air-tight condition then kept for further processing.

Autoclave treatment

WGM was treated with an autoclave at 121 °C for 20 minutes as recommended by Moran and Summers (1967). After treatment of the WGM, the drying was done in the air for 10 min and then kept for chemical analysis before mixing to the diets. Dry gluten content was determined according to Haraszi et al. (2011) and trypsin inhibitor activity was assessed as defined by Makkar et al. (2008). These measurements were performed before and after treatment.

Animal management and feeding

Seventy-five weaned New Zealand White rabbits of both sexes (six weeks old, the average body weight of 659.60±18.84 g) were allocated to five dietary treatments of 15 rabbits per treatment. Each treatment was replicated 5 times (three rabbits per replicate). The T₁ was fed the control diet, the T₂ and T₃ received 20% and 40% untreated WGM in replacing of soybean meal protein, respectively (6.12 and 12.31% of the whole diet, respectively). The T₄ and T₅ were fed on 20% and 40% autoclave-treated WGM, which consisted of 6.20 and 12.41% the whole diet, respectively. The ingredients and chemical composition of diets are presented in table 1. All the experimental diets were formulated to be isonitrogenous and isocaloric, to meet all the essential nutrient requirements of growing rabbits according to (Egyptian Agriculture Ministry Decree, 1996). The animals were reared in metal battery cages equipped with separated feeders and automatic nipple drinkers. All animals were receiving control diet for one week before the start of the experimental period and vaccinated against diseases during the veterinary examinations. Feed and water were offered *ad libitum*. The management and hygienic conditions were identical for all groups.

Table 1. Ingredients and chemical composition of experimental diets

Ingredients (%)	T ₁	T ₂	T ₃	T ₄	T ₅
Barley	30.26	27.48	25.93	27.48	25.83
Clover hay	26.74	27.05	26.05	27.05	26.05
Wheat bran	18.40	18.40	18.40	18.40	18.40
Soybean meal (44%)	18.10	14.47	10.86	14.47	10.86
Wheat germ meal	--	6.15	12.31	6.20	12.41
Molasses	3.00	3.00	3.00	3.00	3.00
Di calcium phosphate	2.20	2.20	2.25	2.20	2.25
Sodium chloride (NaCl)	0.30	0.30	0.30	0.30	0.30
Vit.& min. mix ¹	0.30	0.30	0.30	0.30	0.30
Lime stone	0.35	0.35	0.35	0.35	0.35
DL-Methionine	0.30	0.25	0.20	0.25	0.20
Anticoccidia (Diclazuril)	0.05	0.05	0.05	0.05	0.05
Total	100	100	100	100	100
Chemical analysis²					
Crude protein (%)	17.70	17.37	17.04	17.36	17.06
Crude fiber (%)	12.87	12.64	12.08	12.65	12.08
Ether extract (%)	2.17	2.44	2.70	2.39	2.70
Nitrogen-free extract (%)	59.22	59.88	58.98	58.39	59.16
Digestible Energy (kcal/kg)	2533.35	2515.5	2512.5	2515.6	2512.4
Methionine+ cysteine (%)	0.55	0.54	0.54	0.54	0.54
Lysine (%)	0.91	1.10	1.28	1.09	1.28

T₁: control diet, T₂: 20% untreated wheat germ meal, T₃: 40% untreated wheat germ meal, T₄: 20% autoclave -treated wheat germ meal, T₅: 40% autoclave-treated wheat germ meal. ¹Vitamins (Vit.) and minerals mixture; each 3 kg contains: Vit. A 6000.000 IU, Vit.B₁ 2000mg, Vit.B₂ 4000mg, Vit.D₃ 900000 IU, Vit E 40000mg, Vit. K₃ 2000 mg, Pantothenic acid 10000mg; Nicotinic acid, 50000g; Vit. B₆ 2000 mg; Vit. B₁₂ 10 mg, Folic acid 3.0g, Biotin 50 mg, choline 250000mg, Cu 5g, Mn85g 60g, Fe 50g, , Co 0.1 g, Se 0.1 g, Zn 50 g, I 0.2 g and Antioxidant 10000mg. ²Chemical analysis of ingredients according to AOAC, 2000.

Experimental parameters

Growth performance

Feed Intake (FI, g/ rabbit/ day) and Body Weight Gain (BWG, g/ rabbit/ day) were recorded weekly. Moreover, the Feed Conversion Ratio (FCR) was calculated as FI/BWG over an experimental period of 8 weeks.

Digestion trial

Digestibility test was carried out using five male rabbits from each experimental group in the last week of the experiment (14 weeks of age). The feces were daily collected separately for six days. The feces were sprayed with 2% boric acid solution for trapping released ammonia and dried at 60° C for 48 hours (until constant weight). Feces were ground and stored for subsequent chemical analysis. Samples of WGM, diets, and feces were analyzed for moisture, ash, Nitrogen-Free Extract (NFE), EE, CF, and CP according to AOAC (2000).

Carcass traits

At 14 weeks of age, 25 rabbits (five rabbits per treatment) were slaughtered to determine carcass traits according to Biasco and Ouhayoun (1996).

Chemical meat measurement

Longissimus dorsi muscles from 25 samples were frozen at -20 °C for determination of total cholesterol, MDA and Total Antioxidant Capacity (TAOC) by colorimetric methods using analytical kits (Bio Diagnostic Company, Egypt)

Blood parameters

Blood samples were taken from the five rabbits per treatment at the time of slaughter and were collected in dry clean tubes containing heparin and centrifuged at 3000 rpm for 20 minutes. Then plasma was separated and stored in a deep freezer at approximately -20°C till the time for determining total lipids, total cholesterol, total protein, and albumin. Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) were colorimetrically determined using commercial kits, according to the manufacturer's instruction (purchased from Bio Diagnostic, Cairo, Egypt). The concentration of globulin (g/ dl) was calculated by subtracting albumin from total protein values.

Statistical analysis

The experimental data were subjected to using analysis of variance (ANOVA) in the general linear model using SAS version 9 (SAS Institute Inc.) by the following model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where μ was overall mean of Y_{ij} ; T was the effect of treatment; i was (1, 2, ..., etc.), and e_{ij} was experimental error. The Significant differences between treatments means were separated by Duncan's multiple range test (1955).

Economic efficiency

Economic efficiency was calculated using the following equation: Economic efficiency = Net revenue / total costs
Total cost was calculated in Egyptian pound according to the price at the time of the experiment.

RESULTS

Chemical composition

Chemical analysis of WGM and AWGM in comparison to soybean meal are shown in table 2. The CP was higher in soybean meal (44 %) compared to WGM (25.83%) and AWGM (25.62%). Also, the CF was higher in soybean meal (7.3 %) compared to WGM (1.28 %) and AWGM (1.22%). However, the EE of soybean meal was lower (1.50%) in comparison to WGM (6.02%) and AWGM (5.30%). On the other hand, DE of soybean meal was 3200 kcal/ kg closed with WGM (2900 kcal/kg) and AWGM (2901 kcal/ kg). WGM contained 4% dry gluten and trypsin inhibitor activity was 2.817 mg/g, while autoclave treatment reduced dry gluten by 50% and trypsin inhibitor activity by 62.4% compared to WGM.

Growth performance

The results of final body weight, daily BWG, FI, and FCR are presented in table 3. Rabbits fed either T_1 or T_4 showed significantly higher final body weight followed by group fed T_2 compared to T_3 and T_5 . While groups fed T_3 and T_5 had the lowest values. Rabbits fed control diet and T_4 recorded insignificantly higher BWG compared to T_2 and T_5 . While the lowest values were recorded for T_3 . The FI was not significantly affected by any of the tested diets. Regarding FCR, the group fed T_4 recorded the higher value without significant differences with T_1 group. Whereas, the lowest values were found in groups fed T_3 and T_5 .

Table 2. Chemical analysis of soybean meal, wheat germ meal and autoclave-treated wheat germ meal based on dry matter percentage

Items	Soybean meal*	WGM	AWGM
Organic matter (%)	93.50	93.40	93.48
Crude protein (%)	44.00	25.83	25.62
Crude fiber (%)	7.30	1.28	1.22
Ether extract (%)	1.50	6.02	5.30
Nitrogen-free extract (%)	40.7	60.27	61.34
Ash (%)	6.50	6.60	6.52
Digestible energy** (Kcal/kg)	3200	2900	2901
Anti-nutritional factors			
Dry gluten (%)	-	4.00	2.00
Trypsin inhibitor activity (mg/g)	21.00	2.817	1.058

WGM: Wheat Germ Meal, AWGM: Autoclave-treated Wheat Germ Meal. *Chemical analysis according to Feed composition for animal and poultry feedstuff used in Egypt (2001). **Digestible energy calculation was performed according to (Cheeke, 1987).

Table 3. Growth performance of New Zealand White rabbits (six weeks old) fed diets containing untreated or treated wheat germ meal

Items	T ₁	T ₂	T ₃	T ₄	T ₅	SEM
Initial body weight (g)	645.00	668.00	671.00	641.00	673.00	18.84
Final body weight (g)	2143.3 ^a	2031.7 ^{ab}	1872.0 ^c	2108.0 ^a	1965.0 ^{bc}	30.56
Daily body weight gain (g)	26.76 ^a	24.35 ^{ab}	21.44 ^b	26.20 ^a	23.07 ^{ab}	0.70
Daily feed intake (g)	103.59	94.59	94.27	99.33	95.00	3.80
Feed conversion ratio (g feed/ g gain)	3.87 ^{bc}	3.88 ^{bc}	4.39 ^a	3.79 ^c	4.11 ^{ab}	0.10

Different superscript letters within the same row mean significant differences ($P < 0.05$). T₁: control diet, T₂: 20% untreated wheat germ meal, T₃: 40% untreated wheat germ meal, T₄: 20% autoclave-treated wheat germ meal, T₅: 40% autoclave-treated wheat germ meal. SEM: Standard error of the mean.

Digestion coefficient

According to results shown in table 4, AWGM increased nutritive values for the rabbit compared with 40% untreated WGM. These results indicated that CP digestibility was significantly higher in T₁ and T₄ groups in comparison to other groups. Digestibility of CF, EE, and NFE were significantly improved in T₁, T₂ and T₄. Digestible CP values were significantly increased in T₁ and the lowest values were found with inculcation untreated or treated WGM at the level of 40%. Feeding with T₄ and T₁ diets enhanced total digestible nutrients, digestible crude protein and digestible energy compared to other treatments.

Carcass characteristics

The results of carcass characteristics are presented in table 5. Carcass and dressing percentage were significantly higher in T₁, T₂ and T₄ in comparison to other groups. While T₃ and T₅ were significantly decreased in the carcass weight and dressing percentage compared to T₁. However, the percentage of liver, kidney, heart, and giblets were not significantly affected by any treatments.

Meat quality

Table 6 shows that the T₁ and T₅ recorded the highest TAOC value values in rabbit meat followed by T₂ and the lowest values were recorded for T₃ and T₄. The highest MDA value was recorded for T₂ followed by T₅. While T₁ group was found to have the lowest value. Regarding total cholesterol in rabbit meat, T₂ recorded significantly the highest level. The lowest level was found in the groups of T₅ and T₃.

Blood constituents

The plasma concentration of total protein, albumin, globulin, total cholesterol, and lipids are shown in table 7. The obtained values of blood parameters were within the normal range. Insignificant differences were found in the concentration of plasma albumin, ALT and AST in different tested treatments. Plasma total protein increased significantly in T₂ and T₄ compared to control and T₅ groups. Globulin values were significantly higher in rabbits fed T₂ and T₄ compared to other groups. The concentration of total cholesterol and lipid in plasma were significantly higher in T₁ and T₃ compared to control group.

Economic efficiency

Effects of replacement of treated or untreated WGM on economic efficiency are shown in table 8. The results indicated that inclusion 20 or 40% treated or untreated WGM decreased total feed cost /rabbit as a result of decreasing FI compared to control group. Growing rabbits fed diets either T₄ or T₂ recorded high economic efficiency followed by T₁. Also, selling price increased in T₄ than control (71.88 and 73.40 LE, respectively). The same trend was found in relative economic, where the best values were recorded in T₄ and T₂ groups (109.19 and 105.6 %, respectively), while the poorest value was recorded in T₃ group.

Table 4. Digestion coefficient of New Zealand White rabbits (six weeks old) fed on autoclave-treated or untreated wheat germ meal.

Items	T ₁	T ₂	T ₃	T ₄	T ₅	SEM
Organic matter (%)	64.05	60.60	58.67	61.44	58.81	1.18
Crude protein (%)	74.31 ^a	65.94 ^b	60.67 ^b	71.80 ^a	61.34 ^b	1.62
Crude fiber (%)	40.85 ^a	33.99 ^{ab}	27.18 ^b	42.39 ^a	30.75 ^b	1.80
Ether extract (%)	80.77 ^a	77.09 ^{ab}	68.27 ^c	78.15 ^{ab}	73.82 ^{bc}	1.33
Nitrogen-free extract (%)	76.89 ^a	72.79 ^{ab}	68.68 ^b	75.41 ^a	71.73 ^{ab}	0.96
Digestible crude protein (%)	13.15 ^a	11.37 ^{ab}	10.33 ^b	12.46 ^a	10.46 ^b	0.41
Total digestible nutrients (%)	58.33 ^a	54.61 ^b	48.80 ^c	57.00 ^{ab}	50.66 ^c	1.03
Digestible energy*(kcal/kg)	2584.01 ^a	2419.22 ^b	2161.84 ^c	2525.1 ^{ab}	2244.23 ^c	45.59

T₁: control diet, T₂: 20% untreated wheat germ meal, T₃: 40% untreated wheat germ meal, T₄: 20% autoclave-treated wheat germ meal, T₅: 40% autoclave-treated wheat germ meal. SEM: Standard error of the mean. Different superscript letters within the same row mean significant differences (p<0.05). * Digestible energy = Total digestible nutrients* 44.3, according to Schneider and Flatt (1975).

Table 5. Carcass traits of New Zealand White rabbits (six weeks old) fed on autoclave-treated or untreated Wheat germ meal

Items	T ₁	T ₂	T ₃	T ₄	T ₅	SEM
Carcass weight (%)	56.69 ^a	55.43 ^a	53.29 ^b	55.69 ^a	53.74 ^b	0.37
Dressing (%)	60.38 ^a	58.89 ^{ab}	56.80 ^c	59.52 ^a	57.59 ^{bc}	0.39
Liver (%)	2.91	2.42	2.60	2.91	2.89	0.09
Heart (%)	0.275	0.293	0.269	0.273	0.267	0.01
Spleen (%)	0.035	0.048	0.055	0.036	0.053	0.003
Kidney (%)	0.706	0.705	0.588	0.614	0.645	0.01
Giblets (%)	3.69	3.46	3.51	3.83	3.85	0.08

T₁: control diet, T₂: 20% untreated wheat germ meal, T₃: 40% untreated wheat germ meal, T₄: 20% autoclave-treated wheat germ meal, T₅: 40% autoclave-treated wheat germ meal. SEM: Standard error of the mean. Different superscript letters within the same row mean significant differences (p<0.05)

Table 6. Meat quality of New Zealand White rabbits (six weeks old) fed on untreated or autoclave-treated wheat germ meal

Items	T ₁	T ₂	T ₃	T ₄	T ₅	SEM
TAOC (mM/g)	1.025 ^{ab}	0.3800 ^{ab}	0.465 ^c	0.9200 ^b	1.25 ^a	0.085
MDA(mg/g)	1.26 ^d	3.17 ^a	1.835 ^c	1.815 ^c	2.445 ^b	0.162
Total cholesterol(mg/g)	13.50 ^c	25.95 ^a	12.85 ^c	22.15 ^b	12.85 ^c	0.922

TAOC: Total Antioxidant Capacity MDA: Malondialdehyde. T₁: control diet, T₂: 20% untreated wheat germ meal, T₃: 40% untreated wheat germ meal, T₄: 20% autoclave-treated wheat germ meal, T₅: 40% autoclave-treated wheat germ meal. Different superscript letters within the same row mean significant differences (P<0.05). SEM: Standard error of the mean.

Table 7. Blood constitutes of New Zealand White rabbits (six weeks old) with treated or untreated wheat germ meal

Items	T ₁	T ₂	T ₃	T ₄	T ₅	SEM
Total protein (g/ dl)	5.44 ^b	6.10 ^a	5.59 ^{ab}	6.10 ^a	5.46 ^b	0.10
Albumin (g/ dl)	2.82	2.96	2.90	2.69	2.75	0.05
Globulin (g/ dl)	2.62 ^b	3.14 ^a	2.69 ^b	3.41 ^a	2.71 ^b	0.09
Total cholesterol (g/ dl)	83.75 ^a	57.90 ^b	75.17 ^a	58.16 ^b	53.56 ^b	3.46
Total lipid (g/ dl)	311.90 ^a	254.33 ^b	290.87 ^a	190.45 ^c	187.49 ^c	14.13
ALT(U/ L)	12.73	12.48	12.32	13.02	12.54	0.12
AST(U/ L)	16.22	15.01	14.70	15.44	15.02	0.25

T₁: control diet, T₂: 20% untreated wheat germ meal, T₃: 40% untreated wheat germ meal, T₄: 20% autoclave-treated wheat germ meal, T₅: 40% autoclave-treated wheat germ meal. SEM: Standard error of the mean. Different superscript letters within the same row mean significant differences (p<0.05).

Table 8. Economic efficiency of New Zealand White rabbits (six weeks old) fed on diets with treated or untreated wheat germ meal

Items	T ₁	T ₂	T ₃	T ₄	T ₅
Total weight gain (kg)	1.498	1.363	1.200	1.467	1.292
Price of 1kg body weight	49	49	49	49	49
Selling price/rabbit (LE) (A)	73.40	66.78	58.85	71.88	63.31
Total feed intake	5.801	5.297	5.279	5.562	5.320
Price/kg feed (LE)	4.44	4.27	4.16	4.28	4.17
Total feed cost/rabbit (LE) (B)	25.75	22.61	21.96	23.80	22.18
Net revenue(LE) ¹	47.65	44.17	36.89	48.08	41.13
Economic efficiency ²	1.85	1.953	1.679	2.02	1.854
Relative economic efficiency ³	100	105.60	90.80	109.19	100.23

T₁: control diet, T₂: 20% untreated wheat germ meal, T₃: 40% untreated wheat germ meal, T₄: 20% autoclave-treated wheat germ meal, T₅: 40% autoclave-treated wheat germ meal. LE: Egyptian pound. 1: Net revenue = A – B. 2: Economic efficiency = (A-B/B). 3: Relative Economic Efficiency= Economic efficiency of treatments other than the control/ Economic efficiency of the control group.

DISCUSSION

Chemical composition

According to the results obtained in the present study, AWGM did not affect chemical composition of CP, CF, ash, and DE, while EE was decreased from 6.02 to 5.30% and lowered the anti-nutritional factor dry gluten by 50% and trypsin inhibitor activity by 62.4% compared to untreated WGM. Blair (2018) reported that WGM contains 250-300g/kg CP, 70-120 g/kg crude fat and 30-60 g/kg CF. Moran and Summers (1967) reported that raw WGM contains 29% CP, 2.1% CF, and 10.3% EE, and the content did not influence antitrypsin activity. It was also found that autoclaving of raw WGM for 90 min did not influence CP, CF, EE, ash, and DE contents but antitrypsin activity was degraded. Creek et al. (1962) stated that autoclaving destroyed the activity of trypsin inhibitor and hemagglutinin factor. Also, Zhu et al. (2006) found that defatted WGM contains high amount of protein (34.9%) and has a well-balanced amino acids profiles. Parrish and Bolt (1963) declared that raw WGM contain gluten as an anti-nutritional factor.

However, Bayley et al. (1968) found that raw WGM contained 29% CP, 3.4% CF, 10.3% EE, while after autoclaving for 45 or 20 min decreased CP contain from 29% to 28.6 and 28.7%, respectively. Moreover, CF reduced from 3.4% to 2.9% and EE from 10.3% to 9.7%. While, Ceve et al. (1968) found that the pelleting process increased the nutritive value of raw WGM, particularly in ME values.

Growth performance

Values of final body weight, BWG, and FCR almost close to the values found in rabbits fed on the low level (20%) of treated or untreated WGM. However, at an increased untreated WGM in diet, significantly decreased final body weight and daily BWG and improved FCR. These results can be explained by Lawrence et al. (2002) who reported growth performance in pigs fed on diets containing wheat gluten at levels of 3, 6, 9 and 12% was not improved.

This decline in growth performance with increasing WGM level may be due to an increase in anti-proteolytic factor (trypsin inhibitor) which inhibit enzymatic digestion of proteins or other hemagglutinin factors (Creek and Vasaitis, 1962). Also, Moran and Summers (1967) found that anti trypsin activities were very high in raw WGM. Parrish and Bolt (1963) confirmed the impairment of growth rate, feed efficiency and fat absorption in chicks as a result of feeding raw WGM and the presence of gluten. In addition, Creek et al. (1961) reported that when chicks fed raw WGM led to a reduction in growth rate and feed efficiency, accompanied by hypertrophy of the liver and impairment of the absorption of fat and protein. In the present study, FI was not significantly affected by dietary treatments. Moran and Summers (1967) studied the effects of WGM autoclaved at 121 °C for 20 minutes then incorporating to chicken diets at the level of either 47% or 63.8% and found an improvement in these groups in growth performance and feed utilization compared with other groups fed WGM autoclaved for 90 min and control group. Also, Ceve et al. (1968) found that addition of AWGM (autoclaving at 118 °C for 20 or 45 min) to broiler diet at a level of 33% improved BWG and FCR. Furthermore, Rafai et al. (2011) concluded that BWG of pigs was improved by 6% when they fed diet supplemented with fermented wheat germ extract at the level of 1g/kg of diet compared with the control group. Also, Ellakany et al. (2017) documented that incorporation of fermented wheat germ extract with *Saccharomyces cerevisiae* at levels of 0.5, 1.5 and 3 g/kg of diet increased body weight significantly in broilers, especially at the dose 3g/kg of diet.

Digestibility coefficient

The effect of experimental diets on digestion coefficient and nutritive values were in agreement with Moran and Summers (1967) who found denaturation of raw WGM increased digestibility because of decreased resistance to enzymatic degradation and destruction of toxic factors. Also, Nesheim et al. (1962) stated that fat absorption of raw soybean meal in chickens was improved by heat treatment. Moreover, similar processing of raw soybean enhanced meal digestibility from 64 to 88% in the cockerel (Nitsan, 1965) and from 54 to 89% for the laying hen (Nesheim and Garlich, 1966). Contrarily, Ceve et al. (1968) found that addition of 33% AWGM at 118 °C for 20 or 45 min) to broiler diet were significantly improved metabolizable energy and fat digestibility compared to those fed on raw WGM. Suliman et al. (2015) found an insignificant decrease in terms of digestion coefficients of CP, CF, and NFE when rabbits fed diets containing chemically and biologically treated castor meal at 20% as replacement of soybean meal.

Carcass characteristics

The results obtained in the current study was consistent with Duwa et al. (2014) who reported rabbit fed with 30% roasted sunflower seed meal indicated highest average values in terms of slaughter weight, carcass weight and dressing percentage in comparison to groups received 10, 20 % roasted sunflower seed meal and control group. While, there was no significant difference in liver, kidney, lung, and heart among the treatment groups. Gasmı et al. (2007) found that liver weight was not affected when rabbits fed with rapeseed meal (canola seed meal) replaced up to 14% dietary soybean meal. On the other hand, Leeuw et al. (2009) detected no difference in carcass weight and dressing percentage when using 75 or 100% maize germ meal in steers diets. Ellakany et al. (2017) found that combination of fermented wheat

germ extract with *Saccharomyces cerevisiae* at doses of 0.5, 1.5 and 3 g/kg of diet significantly increased the percentage of intestinal weight and decreased the weight of the liver and total body fat in broilers.

Meat quality

The supplementation of natural antioxidant in feed decreases lipid peroxidation and improves stability of meat. Consistently, Gnanasampandam and Zayas (2007) found that the addition of 7% wheat germ protein flour decreased the fat content in frankfurters. On the other hand, Arshad et al. (2013) found that combination of wheat germ oil and lipoic acid improves the quality of the broiler meat. Arshed et al. (2017) reported that nuggets were made from the leg meat of chickens fed with combination of α -lipoic acid (150 mg/kg) and wheat germ oil (200 mg/kg) showed maximum antioxidant power as well as stability during storage.

Blood constituents

In the present experiment, the results of blood constituents were in agreement with Ellakany et al. (2017) who found the incorporation of fermented wheat germ extract with *Saccharomyces cerevisiae* at rates of 0.5, 1.5 and 3 g/kg diet significantly decreased triglyceride level in blood. A significant decrease in AST concentration was observed at a dose of 1.5 g/kg of diet, while both doses of 1.5 and 3 g/kg of diet decreased significantly ALT level in the blood. In this respect, Louis et al. (1991) reported that blood lipid was decreased when adding small quantities of raw and defatted wheat germ in the rat diet.

Economic efficiency

Rabbit fed on the diets contained untreated or treated WGM at level of 20 % had higher economic return than control diets. These results were in agreement with Leeuw et al. (2009) who found lower feed cost when using 100% defatted maize germ meal in steers' diets.

CONCLUSION

It is concluded that untreated or autoclave-treated WGM can be used up to 20% in growing rabbit diets as a substitution for soybean meal protein without adverse effects in rabbit performance and an improvement in economic efficiency compared to those fed with 40% untreated or autoclave-treated WGM.

DECLARATIONS

Author's contributions

Dr. Walaa A. Salama designed the work and drafted the manuscript. Dr. Amira M. Refaie performed the statistical analysis, tabulation of the experimental data and chemical analysis. Dr. Hamdy F. Amin participated in review the manuscript and Dr. Lamiaa F. Abdel Mawla performed the practical part of the experiment.

Competing interests

The authors have declared that no competing interests exist.

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Productive Characteristics and Reproductive Responses to Estrus Synchronization and Flushing in Abou-Delik Ewes Grazing in Arid Rangelands in Halaieb - Shalateen - Abouramad Triangle of Egypt

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ABSTRACT

There are a few reports about the reproductive aspects or uses of both of flushing and estrus synchronization in Abou-Delik ewes grazing in the South Eastern zone of Egypt. Thirty-three Abou-Delik ewes were allocated to three experimental groups (n = 11 in each) to study the effects of estrus synchronization and flushing on reproductive responses and productive characteristics under arid conditions of South Eastern zone of Egypt. Group one served as control represent the system dominant in the area (without estrus synchronization and flushing ration). Ewes in group two were estrous synchronized with two doses of PGF₂α, 10 days apart without flushing ration. Ewes in group three were estrous synchronized just like the second group and received 300g of barley grain/head/day as flushing meal for three weeks before the start of breeding season. All ewes were grazed *Panicum turgidum* (natural vegetation dominant in the area) for eight hours daily. Results showed that, the percentage of estrus exhibition in group three reached 100%, while the lowest percentage was observed in group one (81.82 %). Estrus activity signs in synchronized groups, occurred in 70 and 81.81% during the first 48 h after the second dose of PGF₂α, for groups two and three respectively, compared to control group (22.22 %). The onset of estrus was earlier in synchronized groups than control group. Duration of estrus did not differ significantly. Estrus intensity in group three was higher (P<0.05) compared to the other groups. Conception and lambing rates were 100% in group three. Third group showed the highest insignificant litter size that was 18% higher than the other groups. The overall mean of birth weight, weaning weight and average daily gain of Abou-Delik lambs were 2.91, 16.89 and 0.116 kg, respectively. There is no significantly effect on concentrations of plasma progesterone among groups. While there were significant differences between sampling periods. In conclusion, under grazing on arid rangelands conditions in the South Eastern zone of Egypt, using flushing and/or estrus synchronization can be useful to improve reproductive and productive characteristics of Abou-Delik sheep.

Key words: Abou-Delik sheep, Estrus synchronization, Flushing, Productive performance, Rangelands, Reproduction

INTRODUCTION

Sheep is one of the most important grazing animals that have impacts on the economic and social status of people inhabiting semi-arid and arid regions. Grazing natural vegetation provides inexpensive source of nutrients. Abou-Delik sheep herds, under rangeland conditions, are subjected to a series of environmental stresses during a dry, mainly inadequate nutrition and insufficient drinking water. Additional energy expenditure for movement to range areas and watering does exist. Feed availability markedly affected sheep reproductive, especially when mating occurs in the dry season (Idris et al., 2010). The nutritional limitations under rangelands and harsh environment such as high scarcity of feed, water and ambient temperature conditions have a negative effect on sheep reproduction in semi-arid areas (El-Hag et al., 2001).

The breeding season of Abou-Delik breed in the South Eastern zone of Egypt coincides with the summer months. Under such circumstances, protein or energy-based supplementary feeding often referred as flushing, around the time of mating improves reproductive performance (Molle et al., 1995 and El-Hag et al., 1998). Feeding ewes on balanced rations for a short period prior to breeding season has been used to improve fecundity (Leury et al., 1990). This feeding method has implications on lambing rate. Flushing has been known as management practice commonly applied in commercial sheep flock to achieve higher fecundity. Flushing before mating and during lactation also affects milk yield and reproductive performance positively (Snyman, 2010). Also, it has been well known that flushing before mating and during lactation in animals shows positive effects on body condition score, ovulation rate and reproductive traits (Ray et al. 2012).

On the other hand, estrus synchronization is a technique used for coordinating estrous cycle (Khaldari, 2008). Under semi intensive production systems, lambing rate efficiency and fecundity rate leading to high profitability of sheep holders could be enhanced using successful estrus synchronization programs (Knights et al., 2001). Estrus synchronization is a key element in all of the assisted reproductive technology protocols and has a major influence on enhancing the overall capabilities of reproductive function in ewes and does (Navanukraw et al., 2014).

Most recently, Kaya et al. (2018) noted that hormonal protocols are one of the main items to improve herds productivity. Prostaglandin F_{2α} effectively synchronize estrus in sheep resulting in fairly good fertility rates. Prostaglandin F_{2α} is produced by the uterus beginning around day 11 or 12 after estrus and causes the regression of the corpus luteum in non-pregnant ewe (Mekuriaw et al., 2016).

Since the response of sheep to synchronization appeared to be breed dependent, it was necessary to investigate whether synchronization with or without flushing could successfully increase estrus response rate and reproductive performance in Abou-Delik ewes. The experiment was designed to investigate the response of Abou-Delik breed under semi-intensive system in Halaieb - Shalateen - Abouramad triangle to estrus synchronization and flushing.

MATERIALS AND METHODS

Location

This study was carried out in Ras Hederba valley region at the Shalateen Research Station of the Desert Research Center, some 1300 km southeast of Cairo, Egypt, at latitude 22,00,720 N and longitude 36,48,955 E. The area is bordered by Sudan to the south and the red sea to the east. It is an arid region with average annual precipitation of 58.5mm/year mostly as erratic showers in November and December. The average ambient temperature in the area ranges between 41.2°C and 24.8°C, while the relative humidity is 86.8% (Central Laboratory for Agricultural Climate). Water resources are meager and available only to nomadic inhabitants and their animals from shallow wells. Therefore, settled agriculture activities are absent and grazing of sheep, goats and camels is the primary activity.

Ethical approval

This experiment was performed according to all ethics and animal rights (Desert Research Center). As much as this work had considering all rules and regulations in conformity with the European Union directive for the protection of experimental animals (2010/63/EU).

Animals and grazing

On July of 2017, 33 adult Abou-Delik ewes (2-4 years of age and 25.5 ± 0.64 kg averaged live body weight) were maintained under grazing (free range) conditions. Animals grazed natural vegetation dominant in the area (*Panicum turgidum*) for eight hours daily in the morning from 06:00 to 12:00 and in the afternoon from 15:00 to 17:00 and after return from the pasture, they were supplemented with concentrate feed mixture (43% yellow corn, 22% cotton seed meal, 20% wheat bran, 12% rice bran 1.5% limestone, 1% sodium chloride and 0.5% minerals mixture) at the rate of 250 g/head/day covering their maintenance energy according to Kearn (1982). The chemical compositions of *Panicum turgidum* and the concentrate supplement are shown in table 1. Animals were examined clinically and were found to be free from physical abnormalities or reproductive disorders.

Table 1. The chemical composition of *Panicum turgidum* and concentrate feed mixture provided for Abou-Delik ewes in Halaieb - Shalateen - Abouramad triangle, Egypt

Constituents	<i>Panicum turgidum</i>		Concentrate feed mixture
	Summer (Dry)	Winter (Wet)	(CFM)
Dry Matter	57.1	44.2	91.25
Organic matter	88.6	93.6	91.07
Crude Protein	6.65	5.50	14.13
Ether extract	1.20	1.82	2.01
Neutral detergent fiber	88.0	89.7	62.34
Acid detergent fiber	54.2	46.9	9.57

CFM: Concentrate feed mixture (% , dry matter basis)

Experimental design

Ewes and rams of Abou-Delik sheep raised at Shalateen Research Station, Egypt were maintained separately during grazing period as well as during their stay at shed under semi intensive system of rearing under identical conditions of housing. Ewes were randomly assigned to three groups (N = 11) as follows: Group 1, served as control without estrus synchronization and without flushing ration (system dominant in the area). Group 2, synchronized estrus with two PGF_{2α} at a rate of 1 ml for each injection (Estrumate, 250 µg Cloprostenol/ ml, Schering-Plough Animal Health, Germany) intramuscularly injections given 10 days apart without flushing ration before mating period. Group 3,

received flushing ration (300g of barley grain/ head/ day) three weeks before introducing rams. Estrus was synchronized with two PGF₂α, 1 ml intramuscularly injections given 10 days apart.

Estrus detection and mating

During the mating season (July, 2017), ewes were presented to three fertile Abou-Delik rams (1 ram / 11 ewes) and natural mating was allowed. The duration of the mating period was 17 days (equal to one estrus cycle length) in the control group. Synchronized groups (2 and 3) had four days (= 96 h) for mating after second PGF₂α injection. Painted-breast fertile rams were introduced to ewes and left with them for estrus detection and natural mating. Rams were allowed to rotate among ewes in different groups (to avoid mixing effect for rams within groups).

Estrus detection was checked through the observation of the paint marks on the ewe's rumps. Ewes were considered in estrus and mating when the raddle mark was heavy and evenly distributed or when copulation was observed. Commencement of the estrus was defined as the time when the ewe first stood to be mounted by the ram. Duration of estrus was defined as the interval between the onset and end of estrus signs. The end of estrus was the time when the ewe refused to be further mounted.

The estrus intensity was measured as per the indications of Homeida et al. (2009) and Kumar et al. (2016) with a score card of degree of expression of restlessness (0-3), standing to be mounted (0-3), vocalization (0-3) and swelling of vulva and mucus discharges (0-3) and these parameters can reveal quantitatively weak estrus (0-4), intermediate estrus (5-8) and intense estrus (9-12) of animals. Reproductive and productive parameters were calculated according to Landais and Cissoko (1986), as follows:

Estrus response (%) = number of ewes showing estrus/total ewes treated in each group×100.

Conception rate (%) = number of pregnant ewes/number of ewes showing estrus and mated in each group×100.

Lambing rate (%) = (number of ewes lambing /number of ewes mated) ×100.

Abortion rate (%) = (number of ewes aborted / number of ewes mated) ×100.

Litter size = number of lambs born / number of ewes lambed.

Mortality rate up to weaning (%) = lambs born alive - lambs weaned / lambs born alive ×100.

Blood collection and hormonal assay

Blood samples (5 ml) were withdrawn, in the morning before access to feed and water, from the jugular vein into EDTA tubes. The samples were collected during four periods as follows: first sampling, zero time (before ram introduction in control group and at first injection of PGF₂α in groups 2 and 3), second sampling, four days after ram introduction in control group and four days after first injection of PGF₂α in groups 2 and 3, third sampling, at day 10 in control group and at second injection of PGF₂α in groups 2 and 3, fourth sampling at end mating period (on day 17 in control group and 96 h after second injection of PGF₂α in groups 2 and 3). The samples were used to monitor the changes in plasma concentrations of progesterone (P₄). Plasma was harvested after centrifugation at 3000 rpm for 15 minutes, and then stored at -20°C for later analysis.

Progesterone hormone assay

Progesterone hormone (P₄) was quantified by ELISA method using BIOS kit (Chemux Bioscience Corporation South San Francisco, USA) according to Abraham (1981). The standard curve ranged between 0-50 ng/ml. The sensitivity of the curve was 0.2 ng/ml.

Statistical analysis

The statistical analyses were conducted using GLM procedure of SAS (SAS, 2004). Chi-square (χ²) analysis was used to evaluate reproductive parameters differences. Differences (P ≤ 0.05) between means were tested for significance using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Abou-Delik sheep is one of the dominant breeds in the South Eastern zone of Egypt. The reports are rare regarding the reproductive aspects or use of flushing and synchronization on estrus activity and reproductive parameters of Abou-Delik ewes which grazed in this region.

Estrus activity

Onset and duration of estrus and intensity of estrus are presented in table 2. The onset of estrus was significantly (P<0.05) shorter in groups 2 and 3 than group 1. The interval between the time of second PGF₂α injection and the onset of estrus between group 2 and group 3 was not significant (P ≥ 0.05) whereas the onset of estrus was earlier in group 3 as compared with group 2. In the control group, estrus behavior was less concentrated and onset happened 159 hours after

introduction of the rams. There was a significant difference ($P < 0.05$) between unsynchronized and synchronized ewes. Considering that ewes in group 1 were mated within 17 days. The delay in G1 is attributed to the absence of estrus synchronization treatment in this group. The interval from the time of administration of second $\text{PGF}_2\alpha$ injection to the time of first appearance of estrus behavior in G2 and G3 ewes was akin to the observations of [Homeida et al. \(2009\)](#) in Naeimi ewes (41.3 h) and [Ashmawy \(2012\)](#) in Rahmani ewes (42 to 49 h). [Contreras-Solis et al. \(2009\)](#) in West African ewes and [Letelier et al. \(2011\)](#) in Manchega ewes observed lower values of 32.49 h and 36 h, respectively. While [Zohara et al. \(2014\)](#) in indigenous ewes observed longer interval from the time of the second $\text{PGF}_2\alpha$ injection to the time of first appearance of behavioral estrus (55 - 58 h) which was comparable to the present results.

Table 2. Estrus response, onset, duration and intensity of estrus in unsynchronized and synchronized Abou-Delik ewes which grazing on arid rangelands in Halaieb - Shalateen - Abouramad triangle, Egypt

Variable	G1	G2	G3	Overall mean
Number of ewes	11	11	11	
Number of ewes in estrus	9	10	11	
Onset of estrus (h)	159.55± 20.21 ^a	44.30± 19.18 ^b	40.54± 18.28 ^b	77.50±14.62
Duration of estrus (h)	27.33± 1.69 ^a	29.80± 1.60 ^a	31.27± 1.53 ^a	29.60± 0.94
Intensity of estrus	5.77± 0.69 ^b	6.60± 0.75 ^b	7.45± 0.72 ^a	6.66± 0.44
Weak estrus % (N)	22.22 (2/9)	10 (1/10)	0 (0/11)	
Intermediate estrus % (N)	66.66 (6/9)	70.0 (7/10)	72.72 (8/11)	
Intense estrus % (N)	11.11 (1/9)	20 (2/10)	27.27 (3/11)	

G1: Unsynchronized and not flushed; G2: Synchronized and not flushed and G3: Synchronized and flushed; N: number; ^{a, b, c} means in the same row bearing different superscripts are significantly different ($P < 0.05$)

The duration of estrus was found to be 27.33, 29.80 and 31.27 h in G1, G2 and G3, respectively with differences being insignificant ($P < 0.05$), although G3 showed the longer duration of estrus (Table1). Further, slightly longer duration of estrus observed in the present investigation in group 3 might be due to flushing before mating. The overall mean of estrus duration of Abou-Delik ewes was 29.60 h. Results of the present study agreed with that recorded by [Naqvi et al. \(1997\)](#) in Kheri ewes (28.5 to 30.6 h) and [Zohara et al. \(2014\)](#) in indigenous ewes (30.5 to 31.6 h). [Daghash et al. \(2017\)](#) found that the duration of estrus in Egyptian ewe lambs were 32.5 h in control group, 29 h in group treated with $\text{PGF}_2\alpha$ and 31.62 h in flushed group. However, [Homeida et al. \(2009\)](#) found that the duration of estrus in Naeimi ewes after second injection of $\text{PGF}_2\alpha$ was earlier (16.2 h) compared to the present experiment.

Intensity of estrus measured in ewes was 5.77, 6.60 and 7.45 in G1, G2 and G3, respectively. The intensity of estrus score was significantly ($P < 0.05$) higher in group 3 compared to other groups. Estrus intensity of ewes synchronized in this study (6.60 and 7.45) higher than estrus intensity in Naeimi ewes synchronized with $\text{PGF}_2\alpha$ (6.0). The overall mean of estrus intensity was close with that of in Nellore Jodipi ewes synchronized with 250 μg cloprostenol, $\text{PGF}_2\alpha$ (6.83). Present results are similar to those of [Kumar et al. \(2016\)](#) reported that intensity of estrus was 7.38 and 7.47 in ewe lambs synchronized by progesterone intra vaginal sponge for 12 days along with Pregnant Mare Serum Gonadotropin (PMSG) 200 and 300 IU. Among the groups only the percentages of weak estrus were 22.22%, 10% and 0% in groups 1, 2 and 3, respectively. Percentages of intermediate estrus were shown by 66.66%, 70.0% and 72.72 % in groups 1, 2 and 3, respectively. The intense estrus values were indicated by 11.11%, 20% and 27.27 % in groups 1, 2 and 3, respectively. The most intensity of estrus symptoms was in intermediate estrus and consisted of 66.66%, 70% and 72.72% in groups 1, 2 and 3, respectively, these results conform to those of some earlier studies on the intensity of estrus symptoms, [Kumar \(2015\)](#) reported that intensity of estrus symptoms were intermediate in 94.22% and 88.89% ewes. [Kumar et al. \(2016\)](#) found that intermediate estrus was 75% and 64.71% in ewe lambs. Differences in the various studies might also be due to breed variation, nutrition, season and geographical location ([Zonturlu et al., 2008](#); [Zohara et al., 2014](#)).

The number of ewes exhibiting estrus was 9, 10 and 11 out of 11 ewes in groups 1, 2 and 3, respectively. Estrus detection during 408 h (17 days) after ram introduction in group 1 (Figure 1) and during 96 h (4 days) after second ejection of $\text{PGF}_2\alpha$ and ram introduction in groups 2 and 3 presented in (Figure 2). In group 1 estrus activity signs were detected in 22.22% of ewes at 24, 96 and 264 h, while 11.11% of ewes were detected in 168, 192 and 312 h during estrus cycle. Whereas they were observed within 24 h (20% and 27.27%) and were detected within 48 h in the vast majority (50% and 54.54%) and within 72h (20% and 18.18%) and within 96 h (10% and none) in groups 2 and 3, respectively. Overall, in synchronized groups, estrus activity signs occurred at a narrow range (70% and 81.81% for G2 and G3, respectively) of total ewes showed estrus during the first 48 h after the second dose of $\text{PGF}_2\alpha$ than unsynchronized group (G1) which was 22.22% during same period, this due to no using protocol of estrus synchronization in G1. According to [Yildiz et al. \(2004\)](#), the nutritional status of sheep also influences luteinizing hormone secretion. In this study, estrus expression was detected within 48 h following the second injection of $\text{PGF}_2\alpha$ in the vast majority (50% and 54.54%) of ewes in synchronized groups (G2 and G3). [Sozabilir et al. \(2006\)](#) found that the signs of estrus were detected within 48 h

in the vast majority (92.3% and 60%) of ewes following the second injection of PGF₂ α in two groups (with 10 vs. 14 day intervals, respectively). [Menchaca et al. \(2004\)](#) reported that estrus behavior was observed within 72 hours after the second dose of PGF₂ α in 93.9% and 82.4% of multiparous and nulliparous ewes, respectively. Similarly, [Godfrey et al. \(1997\)](#) observed that within 72 h, 71.4% of ewes came into estrus after the second injection of PGF₂ α . This result is in agreement with study of [Menchaca et al. \(2004\)](#) that observed 80% of ewes with estrus expression between 25 and 48h after treatment with double injections of PGF₂ α analogs within seven days interval. Which confirmed present results, [Wildeus et al. \(1989\)](#) reported that hair ewes that were flushed for four weeks prior to breeding exhibited estrus earlier in the breeding season than ewes that were not flushed.

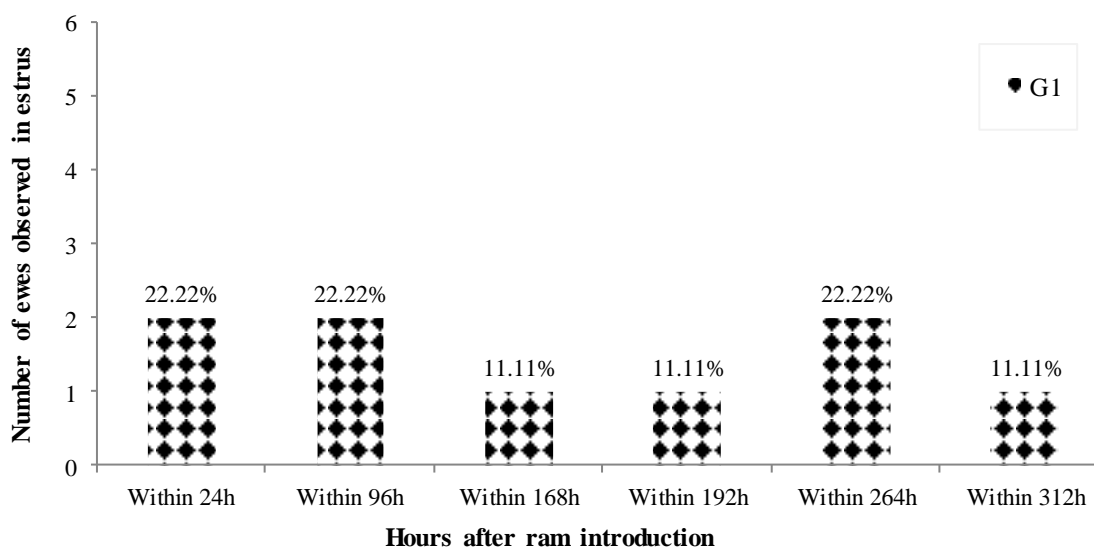


Figure 1. Distribution of estrus activity during 17 days (=408h) after ram introduction of Abou-Delik ewes unsynchronized (G1: Un-synchronized and un-flushed)

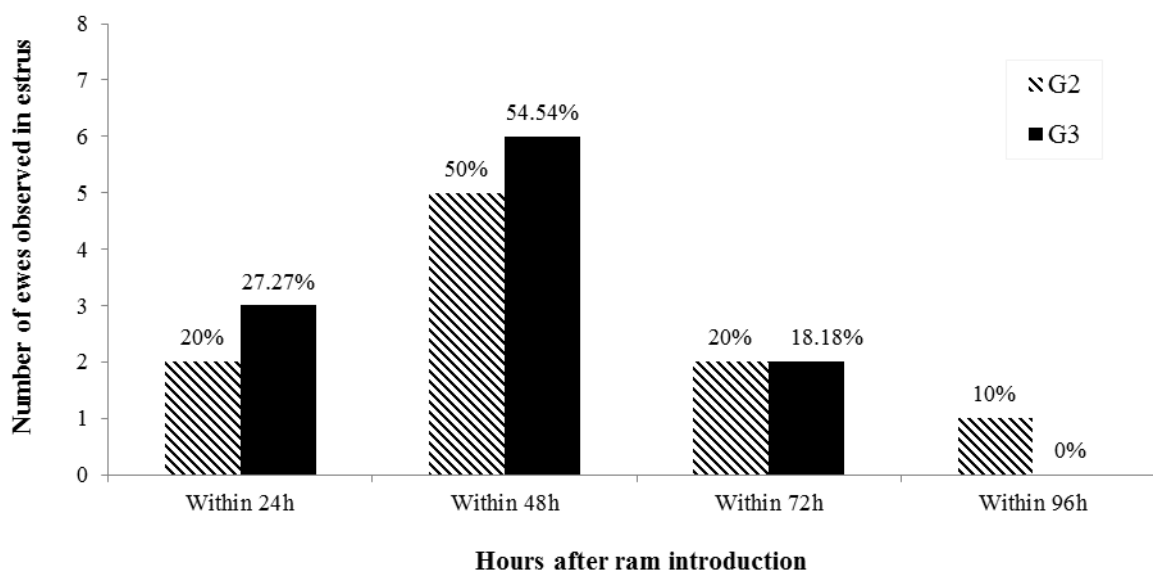


Figure 2. Distribution of estrus activity during 96 hours after ram introduction of Abou-Delik ewes synchronized with double injections of PGF₂ α (G2: Synchronized and un-flushed and G3: Synchronized and flushed)

Reproductive responses

Estrus response, conception, abortion, lambing rates, litter size and sex of lambs of Abou-Delik ewes are presented in table 3. Estrus response was 81.82, 90.91 and 100% in the groups 1, 2 and 3, respectively. Conception rate was 100% in all groups. Abortion rates were 11.11, 10 and 0%, lambing rates were 88.89, 90 and 100% and litter size was 100, 100 and 118% in groups 1, 2 and 3, respectively. While, sex of lambs was 50% males and 50% females, 55.56% males and 44.44 % females, 61.54 % males and 38.46% females in the groups 1, 2 and 3, respectively. There were no statistically significant differences ($P>0.05$) for the estrus response, conception, abortion, lambing rates and sex of lambs between these groups.

Table 3. Some reproductive responses of Abou-Delik ewes which grazing on arid rangelands in Halaieb - Shalateen - Abouramad triangle, Egypt

Variable	G1	G2	G3	Chi value
Number of ewes	11	11	11	
Estrus response % (N)	81.82 (9)	90.91 (10)	100.00 (11)	2.04
Conception rate % (N)	100.00 (9)	100.00 (10)	100.00 (11)	2.25
Abortion rate % (N)	11.11 (1)	10.00 (1)	0.00 (0)	1.98
Number of barren ewes	3	2	0	
Lambing rate % (N)	88.89 (8)	90.00 (9)	100.00 (11)	1.98
Litter size (%)	100	100	118	1.98
Sex of lambs				
Male % (N)	50.00 (4)	55.56 (5)	61.54 (8)	0.63
Female % (N)	50.00 (4)	44.44 (4)	38.46 (5)	0.63

G1: Unsynchronized and not flushed; G2: Synchronized and not flushed and G3: Synchronized and flushed; N: number

In the present study, use of estrus synchronization program allowed to induce the estrus response in 90.91 and 100% of the ewes in the groups 2 and 3, respectively, which were higher than control group (G1, 81.82%). Similar result was reported by [Waheeb et al. \(2017\)](#) that reported 90% estrus induction rate using two injections of $\text{PGF}_2\alpha$ in Barki ewes compared to control group (60%). The present results were similar to those reported by [Ozturkler et al. \(2003\)](#) and [Sozibilir et al. \(2006\)](#) in Tuj ewes (100%) and [Zohara et al. \(2014\)](#) indigenous ewes (100%). In contrast, [Godfrey et al. \(1997\)](#) observed that 71.4% of females came into estrus after the second application of $\text{PGF}_2\alpha$. [Kumar \(2015\)](#) found that estrus response observed by using apronized ram in synchronized ewes and treated with 250 μg $\text{PGF}_2\alpha$ was 85.71%. [Homeida et al. \(2009\)](#) reported estrus response to be 80% in Naeimi ewes treated with $\text{PGF}_2\alpha$. Further, the lower percentage of estrus response observed in control group could be due to not using estrus synchronization program. This higher rate of estrus detection in group 3 might be due to use of flushing regime before mating. Variations in estrus response rate and the discrepancies between the results in the literature could be attributed to the difference in the breed variations, nutrition, seasonality effect, climatic and environment factors ([Das et al., 1999](#); [Dogan et al., 2006](#)).

Conception rate was 100% in all groups with no significant difference among each group ($P > 0.05$). These results were in agreement with results gained by [Yavuzer et al. \(2010\)](#) who used double injection of $\text{PGF}_2\alpha$, 10 days apart with or without injection of follicle stimulating hormone (FSH) and obtained 100 and 95% conception rates, respectively. In contrast to present investigation, [Zohara et al. \(2014\)](#) observed conception rate was 75% in Bangladesh indigenous ewes synchronized by two doses of $\text{PGF}_2\alpha$ (175 μg). [Kumar \(2015\)](#) found that conception rate 80.95% in Nellore Jodipi ewes treated with 250 μg $\text{PGF}_2\alpha$. Present findings recorded in conception rate were higher than the findings of [Homeida et al. \(2009\)](#) in Naeimi ewes treated with $\text{PGF}_2\alpha$ (81.25 %). Furthermore, variations in conception rate observed among different studies might be due to difference in the breed ([Karagiannidis et al., 2001](#)) and breeding season ([Ataman et al., 2006](#)).

Abortion rates were 11.11, 10 and 0% in the groups 1, 2 and 3, respectively. The effects of pregnancy stress on ewes are manifested in increased abortions, weight loss and mortality. It was observed that un-flushed ewes were higher in abortion percentage compared to flushed ewes, which may be ascribed to the effect of flushing before mating ([Yoder et al., 1990](#)).

The highest lambing rate (100%) was in group 3 when compared to other groups (88.89% in group 1 and 90% in group 2) but there was no significant difference ($P \geq 0.05$) between the groups. Present findings recorded in synchronized groups were higher than the findings of [Sozibilir et al. \(2006\)](#) who have reported lambing rate in Tuj ewes 60% and 73% with double injections of $\text{PGF}_2\alpha$ at 10 and 14 day intervals respectively. [Ozturkler et al. \(2003\)](#) reported that lambing rate in Tuj ewes double injected with 0.075mg of $\text{PGF}_2\alpha$ at 11 day intervals was 53.3%. The present results were not consistent with these mentioned reports, which may be due to the high lambing rate in Abou-Delik sheep (ranged from 89 -100%).

Litter size in ewes is shown in table 3. Increasing sheep productivity by increasing lambing rate, litter size and fertility rates was considered as an important factor in the development of sheep production. In the present study, there was an increase in litter size in third group as compared to the other groups. The mean litter size in groups 1, 2, and 3 were 100, 100 and 118%, respectively. Third group showed the best results, with litter size that was 18% higher than the other groups. Although there was no difference in the rates of litter size among the treatment groups ($P > 0.05$), it is interesting to note that twin births only occurred among the nutritionally flushed ewes. The highest litter size was obtained in third group, and this can be ascribed to the increase energy-based supplementary feeding (flushing) before the mating season. These results agree well with those reported by [Ribeiro et al. \(2002\)](#) for ewes supplemented with 0.5 kg per animal of ground corn. Similar benefits on litter size and lambing rates after flushing were described by [El-Hag et al. \(1998\)](#). In line with these results [Marzouk et al. \(2018\)](#) reported that enhancing ration during breeding period is enough to improve the reproductive performance of Ossimi ewes.

Gestation period

The average gestation length period was 157.37, 154.88 and 153.45 days for G1, G2 and G3, respectively (Table 4). Gestation length was significantly ($P < 0.05$) shorter in group 3 compared with other groups. Sex of lamb had no significant ($P > 0.05$) effect on gestation period which was 155 days in ewes carrying males and 154.84 days in ewes carrying females. Likewise, type of birth had no effects on gestation period (155.11±0.37 days in ewes carrying singles vs. 154.00±1.36 days in ewes carrying twins).

Variations were found in gestation period as a result of treatment (Table 4). The average gestation period was 155 days, ranging from shorter average of 153.45 days for ewes on G3 to longer gestation period of 157.37 days for ewes on G1. Furthermore, sex of lamb and lamb type of birth exerted no main effects on gestation period. Moreover, *Aboul-Ella (2006)* reported that length of gestation period was shorter in ewes treated with PG-600 and flushing in Barki ewes. As well *Safranski et al. (1992)* reported that pregnancy period in control ewes and ewes treated with melengestrol acetate + PG-600 was 163.8 and 157.2 days, respectively. In contrast, *Zarkawi (2000)* reported that the treatment had no effect on the duration of pregnancy, which averaged 150.3 days in control and 150.4, and 150.8 days in synchronized groups with 10mg and 15mg $PGF_{2\alpha}$, respectively. This difference can be likely due to the different breed of sheep and the environment. The long gestation period in the groups that did not flushed because of the breeding and gestation periods are thus in the dry season when the rangelands are at their lowest nutritional quality, this is reflected in high gestation length period and high abortion rates.

Table 4. Effect of treatment, sex and birth type on gestation length (mean ± S.E) of Abou-Delik ewes which grazing on arid rangelands in Halaieb - Shalateen - Abouramad triangle, Egypt

Items	Factors	N	Gestation Period, days
Treatment	G1	8	157.37±0.37 ^a
	G2	9	154.88±0.34 ^b
	G3	13	153.45±0.31 ^c
Sex of lamb	Male	17	155.00±0.46 ^a
	Female	13	154.84±0.53 ^a
Lamb type of birth	Single	26	155.11±0.37 ^a
	Twin	4	154.00±1.36 ^a
Overall Mean		30	154.99±0.35

^{a, b, c} means in the same column bearing different superscripts are significantly different ($P < 0.05$); N: number

Productive characteristics

The effect of experimental groups on birth weight, weaning weight, average daily gain and mortality rate are presented in table 5. There were no significant ($P > 0.05$) differences among the experimental groups in productive characteristics of Abou-Delik ewes. The overall mean of birth weight, weaning weight and average daily gain of Abou-Delik ewes were 2.91, 16.89 and 0.116 kg respectively. Higher birth weight was observed in lambs born in G3 (3.02 kg) than the other groups. However, there was no significant difference observed ($P > 0.05$). The birth weight in lambs of G3 was higher than other groups which could be attributed to flushing of ewes. Flushing significantly affected litter weight at birth and weaning (*Sormunen-Cristian and Jauhiainen, 2002*). This finding is in agreement with *El-Hag et al. (1998)* who reported higher birth weights in ewes supplemented with flushing ration during breeding time as compared to ewes which were not supplemented. Average weaning weights of lambs at three months of age was found as 16.89 kg. Weaning weight of lambs in G3 (17.19 kg) was higher than those in other groups (16.27 kg for group 1 and 16.98 kg for group 2). *Zhang et al. (2008)* reported that weaning weight was affected by birth weight. The current results are in line with this imposition. Average daily gain in groups was estimated to 118, 125 and 128g in groups 1, 2, and 3, respectively. Mortality rate from birth until weaning in groups 1, 2, and 3 were estimated to 12.5, 0 and 7.69%, respectively.

Table 5. Least squares means (± SE) for productive characteristics of Abou-Delik ewes which grazing on arid rangelands in Halaieb - Shalateen - Abouramad triangle, Egypt

Traits	G1	G2	G3	Overall mean
Birth weight (kg)	2.91 ± 0.11	2.93 ± 0.13	3.02 ± 0.08	2.91±0.06
Weaning weight (Kg)	16.27 ± 0.79	16.98 ± 0.93	17.19 ± 0.87	16.89±0.50
Average daily gain (g)	118.0 ± 6.83	125.0 ± 7.57	128.0 ± 6.84	116.0±4.04
Mortality rate (%)	12.50±9.12	0.0±8.60	7.69±7.15	6.66±4.63

G1: Unsynchronized and not flushed; G2: Synchronized and not flushed and G3: Synchronized and flushed

Progesterone concentration

There were no significant ($P>0.05$) effects of treatments on progesterone concentrations but there were significant ($P<0.05$) effects of sampling periods on the concentrations of plasma progesterone (Table 6). The mean progesterone concentrations were 2.80, 2.48 and 2.09 ng/ml in groups 1, 2 and 3, respectively. While, the overall mean progesterone concentrations were 3.55, 1.28, 3.79 and 1.20 ng/ml on first sampling, second sampling, third sampling and fourth sampling, respectively. The obtained results from this experiment indicated that the mean progesterone concentrations in second sampling (1.56 and 1.18 ng/ml) and fourth sampling (0.89 and 0.71 ng/ml) decreased after the first and second injection of $\text{PGF}_2\alpha$ as compared to first sampling (3.34 and 2.78 ng/ml) and third sampling (4.14 and 3.71 ng/ml) before first and second injection of $\text{PGF}_2\alpha$. This result was similar to the presented results by Demiral et al. (2008) in Akkaraman ewes treated with $\text{PGF}_2\alpha$, in nine days interval. The mean plasma progesterone concentration in $\text{PGF}_2\alpha$ synchronized ewes estimated in first sampling (at prostaglandin first dose) was 3.55 ng/ml. Similar progesterone concentration was observed by Demiral et al. (2008) in Akkaraman ewes (2.67 ng/ml). Progesterone concentration decreased in second sampling (four days after first dose of prostaglandin) was 1.28 ng/ml. This result was in accordance with that observed by Homeida et al. (2009) in Naeimi ewes (1.1 ng/ml). The P_4 concentration on third sampling (at day of the second $\text{PGF}_2\alpha$ injection) was 3.79 ng/ml, this result was accordance with observed progesterone concentration by Naderipour et al. (2012) in Kalkuhi ewes (3.76 ng/ml) and Sozbilir et al. (2006) in Tuj ewes 4.29 ng/ml. The progesterone concentration in day four after mating was 1.20 ng/ml, the average progesterone concentrations decreased significantly on day four after the second injection of $\text{PGF}_2\alpha$ as compared to the prior of the second injection. Plasma progesterone concentrations on day four after the second injection of $\text{PGF}_2\alpha$ indicated the regression of corpus luteum. The progesterone concentration values of present study are in line with Sozbilir et al. (2006) and Naderipour et al. (2012).

Table 6. Mean (\pm SE) plasma progesterone concentrations of control and synchronized Abou-Delik ewes which grazing on arid rangelands in Halaieb - Shalateen - Abouramad triangle, Egypt

Item	Sampling periods	Groups			Overall mean
		G1	G2	G3	
P_4 (ng/ml)	First	4.54 \pm 0.88	3.34 \pm 0.83	2.78 \pm 0.65	3.55 ^a \pm 0.46
	Second	1.12 \pm 0.30	1.56 \pm 0.46	1.18 \pm 0.27	1.28 ^b \pm 0.19
	Third	3.54 \pm 0.71	4.14 \pm 0.51	3.71 \pm 0.97	3.79 ^a \pm 0.41
	Fourth	2.02 \pm 0.45	0.89 \pm 0.06	0.71 \pm 0.10	1.20 ^b \pm 0.21
	Overall mean	2.80 \pm 0.41	2.48 \pm 0.39	2.09 \pm 0.39	---

G1: Unsynchronized and not flushed; G2: Synchronized and not flushed and G3: Synchronized and flushed; ^{a, b} Means of period with different superscripts are significant differences ($P<0.05$); P_4 : progesterone concentrations

CONCLUSION

Under grazing conditions in arid rangelands in the South Eastern zone of Egypt, using flushing and estrus synchronization can be useful to improve reproductive and productive responses of Abou-Delik sheep. The present study indicates that both flushing and estrus synchronization improved reproductive responses of Abou-Delik ewes particularly estrus response, lambing rate and litter size. It is clear during the results of the present experiment that the Abou-Delik sheep breed is a good breed originally under grazing conditions in arid zones and we need further studies and bigger numbers of sheep to confirm this.

DECLARATIONS

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Competing interests

The author declares that he has no conflict of interest with respect to the research, authorship, and/or publication of this article, the author declares that he has no competing interests.

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Histopathological Alterations of Ceca in Broiler Chickens (*Gallus gallus*) Exposed to Chronic Heat Stress

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ABSTRACT

Heat stress has been found to cause adverse effects on small intestinal microstructure, but little is known about its impact on chicken's cecum. In this research, the histopathological alterations of broiler chicken's cecum following chronic heat stress were evaluated. 20 broiler chickens were randomly divided into control group and treatment group containing 10 replicates, respectively. Both groups were reared under standard conditions until 21 days of age. From day 22 to day 42, the control group was kept at 24-28°C as well as relative humidity of 40-55%, while the treatment group was exposed to high temperature of 36-40°C and relative humidity of 45-65% for eight hours per day. At the end of the period, proximal part of each chicken's cecum was collected and made into histopathological slides with Hematoxylin and Eosin staining. Villus height, villus width, crypt depth, villus surface area, and villus height to crypt depth ratio were examined from 10 villi per replicate. Results analysis revealed that chronic heat stress profoundly ($P < 0.05$) reduced the crypt depth. Insignificant ($P > 0.05$) changes of the villus despite the long-term heat exposure might imply that the damage is at its early phase. In conclusion, chronic heat stress can produce morphological alterations in the ceca of broiler chickens, though requiring longer duration due to cecum's durability.

Key words: Broiler chicken, Cecum, Heat stress, Intestinal morphology

INTRODUCTION

Heat Stress (HS) is defined as biological response to high ambient temperature which disrupts the heat exchange equilibrium (Lara and Rostagno, 2013). Over the past decades, the Earth's surface temperature has gone beyond the former baseline, going as far as nominating 2016 as the warmest year since the recording was initiated in 1880. Consequently, the risk of HS' emergence in livestock projects to a higher level (Thornton et al., 2009). Broiler chickens are particularly more prone to contract heat stress because their cardiovascular and respiratory systems, which are vital for heat loss mechanisms, are unable to cope with their heavier body weight and high metabolism rate resulting from genetic selection (Sozcu, 2019).

HS has generally been found to be detrimental to small intestine's morphology in broiler chickens (Al-Fataftah and Abdelqader, 2014; Yi et al., 2016). The underlying mechanisms involve hypothalamic-pituitary-adrenal (HPA) axis activation, ischemia, oxidative stress, or perturbation of intestinal microbiota (Bolek, 2013; Song et al., 2014; Yi et al., 2016). The activated HPA axis subsequently elevates corticosterone level which in turn undermines immunity (Mishra and Jha, 2019). It has also been discovered that HS can suppress the number of goblet cells and the mRNA expression of tight junctions and adherence junctions in broiler chickens subjected to 33°C, 10 hours/day for 21 consecutive days (Zhang et al., 2017). These occurrences along with the impaired immune system facilitate the penetration of both toxins and pathogenic bacteria into the circulation afflicting various organs. Meanwhile, cecum is a part of chicken's intestine with the most abundant and diverse microorganisms colonizing its lumen (Ijaz et al., 2018). Furthermore, cecum regularly receives the backflow of urine rendering potentially more severe damage by the pathogens trespassing (Kum et al., 2015).

The available research on the influence of HS on cecal morphology produced conflicting results. Reduction in villus length and crypt size in the ceca of Japanese quails (*Coturnix coturnix japonica*) exposed to chronic HS was demonstrated by El-Daly et al. (2014). In contrast, negative results were obtained in Jaafar (2013)'s research. Other experiments, for the most part, are concerned with the microbiome in cecum, for instance HS was proven to be able to restrain the growth of normal flora colonies, like *Lactobacillus* sp., while enhancing the colonies of *Escherichia* sp., *Salmonella* sp., and aerobic bacteria in the cecum of broiler chickens (Park et al., 2013).

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It was proposed that HS influenced the intestine variably in compliance with susceptibility level of each segment and the duration of HS occurrence (acute or chronic) (Loyau et al., 2015; Varasteh et al., 2015). This was borne out by analogous findings in Cherry-Valley ducks exhibiting no substantial crypt depth discrepancy in the cecum despite pronounced changes in the jejunum and ileum (He et al., 2019). Taking into account the lack of consistent and adequate number of results, the current research had an objective to evaluate the impact of chronic HS for 21 days on the microstructure of broiler chicken's cecum.

MATERIALS AND METHODS

Ethical approval

This experiment was performed on the basis of approval by the laboratory animals use research ethics committee of faculty of veterinary medicine, Airlangga University, Indonesia.

Experimental animals

In this research, 20 one day old Cobb broiler chicks were housed at the cage of laboratory animals, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia. The chicks marketed as Wonchick originated from Wonokoyo Jaya Corporindo Ltd. (Limited Company, Surabaya, Indonesia). The rearing temperature followed the guidelines provided by the referred company, namely the leaflet containing Wonchick broiler chicken rearing guide. The chicks were allowed *ad libitum* access to feed and water. This went on up to 21 days.

Experimental design

The chickens were randomly allocated to two groups (n= 10, respectively), the control (C) group and the treatment (T) group, from day 22 onwards. This starting point was chosen because broilers in the later stage of life have greater sensitivity to high surrounding temperature than the younger birds (He et al., 2018). In that, true experimental design, specifically the post-test-only control group design, was applied. The C group was maintained on thermoneutral conditions (24-28°C and relative humidity (RH) 40-55%), while the T group was exposed to high ambient temperature and humidity (36-40°C and RH 45-65%) for eight hours/d then returned to 24-28°C and RH 40-55% for the rest of the day. On day 42, all the chickens were humanely slaughtered as affirmed by the research ethics committee and organ collection was carried out.

Cecal histopathology

The proximal part of cecum was dissected from 23.59% - 23.65% proximal of its length for all chickens. About one cm of the segment was fixed in neutral buffered formalin 10% and then routinely processed into slides with Hematoxylin and Eosin (H&E) stain. The sections were analyzed under light microscope with computer-assisted digital image analyzer (NIS Elements) for Villus Height (VH), Villus Width (VW), and Crypt Depth (CD) (Figure 1). Measurements of VH, VW, and CD were based on the methods used by Abdelqader and Al-Fataftah (2016); Godwin et al. (2016) and Shokryazdan et al. (2017). The Villus Surface Area (VSA) was calculated from the formula $= \pi \times VW \times VH$. The Villus Height to Crypt Depth (VH:CD) ratio is a comparison of VH to CD (Santos et al., 2015). Collection of all the variables was done on 10 intact and the longest villi with their associated crypts in one cecal cross section per chicken. These values were averaged to obtain mean value of each variable for each chicken.

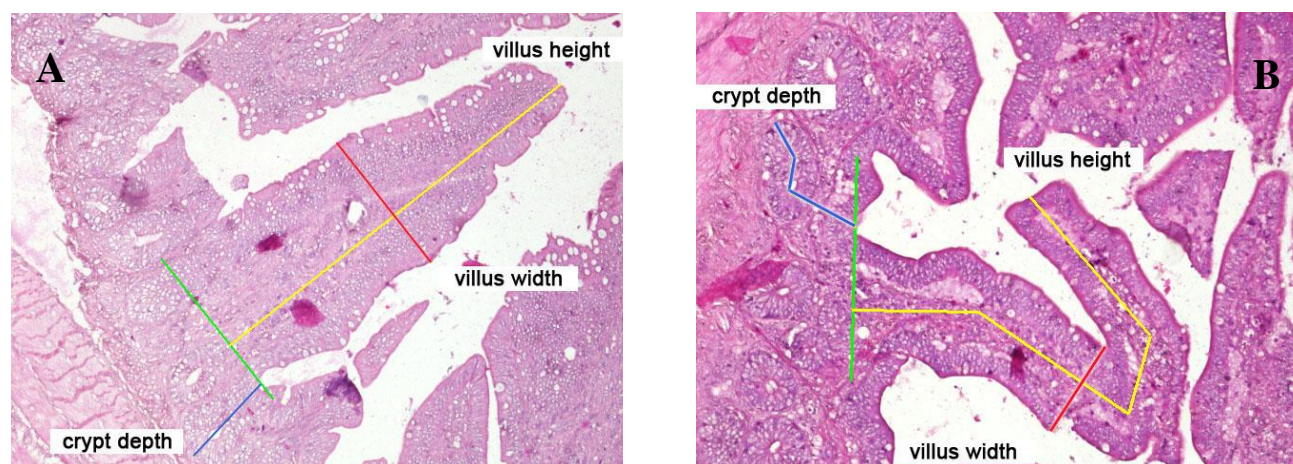


Figure 1. Methodology for morphometric assessment of villus-crypt unit of cecum in broiler chickens, **A:** in straight unit and **B:** in irregularly-oriented unit (H&E, 200×), Villus height (yellow line) was measured from the tip of the villi to the villus-crypt junction (green line), Villus width (red line) was measured from side to side of the villi at its half height, Crypt depth (blue line) measurement was based on the depth of invagination between adjacent villi.

Statistical analysis

Data analysis used the two-tailed T Test for independent samples, and $p < 0.05$ was the accepted significant value. It was conducted with SPSS (Version, 24) for Windows OS (SPSS, Chicago, IL, USA). The results are presented in mean \pm standard error.

RESULTS AND DISCUSSION

From the five dependent variables examined, profound decline of CD ($p < 0.05$) was observed in the chickens of T group compared to C group (Figure 2 and figure 3). The remaining variables did not express significant difference between the two groups ($p > 0.05$) (Table 1).

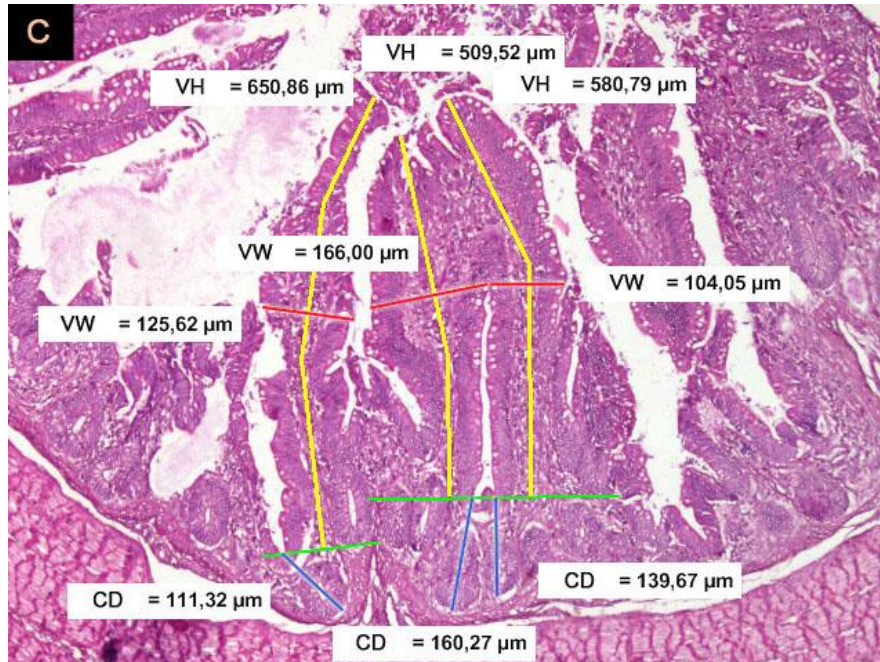


Figure 2. Cecal morphology measurements of broiler chickens of control group from the study in Surabaya, Indonesia between August and September 2017. The yellow lines, red lines, and blue lines represent villus height, villus width, and crypt depth respectively (H&E, $\times 100$). VH: villus height; VW: villus width; CD: crypt depth

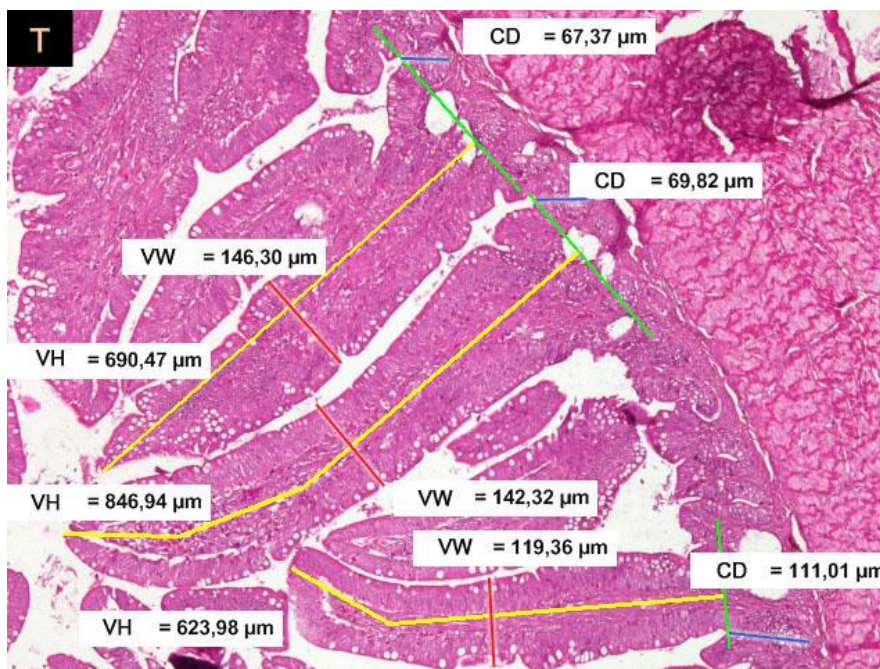


Figure 3. Cecal morphology measurements of broiler chickens from treatment group which were exposed to high ambient temperature and humidity from 22 to 42 days of age during the study in Surabaya, Indonesia between August and September 2017. The yellow lines, red lines, and blue lines represent villus height, villus width, and crypt depth respectively (H&E, $\times 100$). VH: villus height; VW: villus width; CD: crypt depth.

Table 1. The effect of heat stress on histopathology of ceca in broiler chickens in Surabaya, Indonesia between August and September 2017

Variable	Group C	Group T
VH (μm)	518.66 \pm 31.44	493.22 \pm 45.01
VW (μm)	115.23 \pm 2.86	122.92 \pm 5.25
CD (μm)	116.67 \pm 4.65 ^a	98.84 \pm 5.83 ^b
VSA (μm^2)	189,980.84 \pm 14,538.90	195,195.07 \pm 22,117.80
VH:CD	4.75 \pm 0.28	5.38 \pm 0.56

C: control; T: chickens were exposed to high ambient temperature and humidity from 22 to 42 days of age; VH: villus height; VW: villus width; CD: crypt depth; VSA: villus surface area; Values are provided with mean \pm standard error; ^{a,b} Different superscripts within the same row mark significantly different means ($p < 0.05$).

HS is able to delay epithelial turnover by means of decreasing feed intake as a result of HPA axis activation (Hu and Guo, 2008). Elevated plasma corticosterone in broiler chickens is indirectly held responsible for the decrease in VH and CD in duodenum and jejunum, but this only occurs in the acute phase instead of chronic (Aggarwal and Upadhyay, 2013). Another proposed mechanism is ischemia in the event of blood diversion to heat-releasing organs, such as the turbinates and the respiratory muscles (Song et al., 2014). Ischemia will lead to vacuolation, rapid sloughing, and loss of microvilli in the intestinal epithelium (Al-Fataftah and Abdelqader, 2014). Moreover, ischemia is also reported to cause oxidative and nitrosative stress which implicated damaged cell membrane, disrupted cellular ion homeostasis, opened tight junctions, and reduced integrity of intestinal mucosa (Al-Fataftah and Abdelqader, 2014). This may proceed to shorter villi but deeper crypts to compensate (Song et al., 2014). The injured mucosa also disables the attachment of commensal bacteria. In addition, the expression of Heat Shock Protein (HSP) may act as receptor for pathogens, while lack of goblet cells which equals falling mucin production promotes the adhesion of pathogens to mucosa (Burkholder et al., 2008; Shah et al., 2019).

The VH, VW, VSA, and VH:CD ratio in present research were barely affected by the heat exposure (36-40°C and RH 45-65%) for eight hours/day along 21 days. Similar findings were attributed by Quinteiro-Filho et al. (2010) to the rapid periodical re-epithelization of intestinal mucosa in the absence of structural adaptation. This explanation is however inappropriate for this case given that it normally occurs in acute or shorter period of cyclic chronic HS, i.e. (seven days). Chronic HS for over one week has been recorded to consistently diminish VH in the absence of adaptation or compensation, although temperature exceeding 36 \pm 1°C could accelerate this process into less than a week (Santos et al., 2015; Abdelqader and Al-Fataftah, 2016; Yi et al., 2016). Meanwhile, CD showed variable responses to HS (decreased, increased, or no difference) regardless of the HS duration. This denoted that there are different sequences of alterations, and their progression is dependent on the level of temperature and internal variation of the organisms subjected to HS. Within the earlier period of HS when the CD of cecum and small intestine have recovered, their VH often did not reach to a similar state, in other words changes in villi are initiated by the crypts (Deng et al., 2012; Abdelqader and Al-Fataftah, 2016).

Crypt of Lieberkühn, being the location of stem cells, is the center for proliferation of intestinal epithelium. The newly generated enterocytes then migrate up and are ejected at the villus tip. Because crypts merely grow in length, the activity level of crypts is proportional to CD (Cummins et al., 2011). Significantly ($p < 0.05$) shallower CD in the T group thus indicated less proliferation rate. The crypts due to proliferative property, were the first part of intestine observed to suffer from stress as remarked by Burkholder et al. (2008). By combining this fact with current results, it can be interpreted that the cecum of chickens in T group is on early phase of damage despite the prolonged HS. The possibility that injury on the cecum of T group was a start can also explain the insignificant ($p > 0.05$) alterations of other variables besides CD. This idea is reinforced by the inference that colon (including cecum) is one of the segments more resistant to HS characterized by lower expression of HSP70 and HSP90 mRNA (Varasteh et al., 2015). If the order of segment susceptibility is to be associated with epithelial migration time, they may lay the basics for cecum's resistance against HS. The proximal cecum of Leghorns had demonstrated longer turnover time than duodenum (four days compared with 72 hours) in the research performed by Takeuchi et al. (1998). Similarly, epithelial migration time in the ceca of Japanese quails was much higher than in their small intestine, and it was second only to rectum, as noted by Starck (1998).

The development of adaptations by the chickens subjected to present study can be considered as another reason. It takes longer period to impair the intestinal compensatory mechanisms in adaptive birds according to Santos et al. (2015). This was reflected by the zero mortality of the Cobb broiler chickens taking part in comparison to 12.5% death of Hubbard chickens following a shorter period of HS with slightly lower temperature applied in Al-Fataftah and Abdelqader (2014)'s research. The adaptive trait might have originated from the Cobb strain itself or from inherited

adaptation subsequent to breeding attempts in the warm climate of Indonesia. Warm ambience could unknowingly perform an act termed as thermal manipulation on chickens prior to hatching which is capable of assisting thermotolerance acquisition (Al-Zghoul et al., 2019). Likewise, it is known that poultry are very likely to develop acclimatization when kept under tropical and subtropical climates with over two months of high temperature (He et al., 2018). Therefore, it can be deduced that the chickens in this study did not need deep crypts in their ceca to preserve their villi from potential casualties because they had created such an effective mechanism enabling them to withstand the lengthy HS (Biasato et al., 2018).

These results contradict those obtained by Deng et al. (2012) which found that the CD of layer hens' ceca was initially reduced by chronic HS on day six but returned to normal on day 12 (last day) while VH remained low throughout the experiment. This difference may be linked to breed, implying that broilers are more resistant than layers, or age of the chicken at the time of HS.

The retarded crypt activity might bring about decreased VH later as the HS progresses (Al-Fataftah and Abdelqader, 2014). CD, VW, and VSA can be enhanced in order to compensate the increased destruction, but still providing sufficient absorption area for nutrients (Santos et al., 2015). The VH:CD ratio inversely portrays the rate of epithelial cell turnover in the intestine, its value is smaller in heat stressed chickens due to induced inflammatory reaction (Laudadio et al., 2012; Shah et al., 2019). When the value is constant, the number of villi per unit area is required to be taken into account. It is recommended to eliminate the possibility that HS has reduced the number of villi instead of the villus morphometry (Marchini et al., 2016).

CONCLUSION

The resilience of broiler chickens' ceca to chronic HS was confirmed in this research. This feature can be credited to intestinal segment-specific resistance which may be explained by the slower epithelial turnover rate in cecum than in small intestine. Additionally, the current findings also suggest that broiler chickens in Indonesia have undergone an adaptation process to the high daily temperature. Nevertheless, it did not remove the possibility of damage progression if the heat exposure is extended.

DECLARATIONS

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Competing interests

The authors have declared that no competing interests exist.

Consent to publish

All the authors approved and agreed to publish the manuscript and declared that this work has not been previously published elsewhere.

Authors' contributions

Hani Plumeriastuti drafted and revised the manuscript while monitoring the course of the study. Anwar Ma'ruf was involved in revising the manuscript, also data analysis, presentation, and interpretation. Djoko Legowo devised the study and participated in its design and coordination, as well as guided the examination and analysis of the histopathological slides. Antonia Vania Adji participated in the execution of the study, data analysis, composition and revision of the manuscript. All authors read and approved the final manuscript.

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Prognostic Value of Lymphocyte-to-Monocyte Ratio in Canine High-Grade Lymphoma Cases

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ABSTRACT

The Lymphocyte-to-Monocyte Ratio (LMR) has been described as a useful prognostic marker for human patients with various cancers and dogs with diffuse large B-cell lymphoma. The objective of this study was to determine whether the LMR could predict disease outcome as measured by the Time To Progression (TTP) and Overall Survival (OS) of dogs with different types of high-grade lymphoma. The medical records of 43 dogs diagnosed with high-grade lymphoma at the Veterinary Medical Center of Obihiro University of Agriculture and Veterinary Medicine between 2013 and 2018, were retrospectively analyzed. Receiver Operating Characteristic (ROC) curve analysis was used to determine the optimal LMR cutoff values. The prognostic influence of the LMR and other clinicopathological data on TTP and OS was studied by Kaplan-Meier curves. To identify the independent prognostic factors, univariate and multivariate Cox proportional analyses were used. The optimal cutoff value of the LMR was 0.7, which corresponded to the maximum sensitivity (0.727) and specificity (0.762) of the LMR for predicting the median days of OS with ROC analysis (area under the curve, 0.794). Log-rank tests showed that dogs with a high LMR had significantly longer TTP and OS than dogs with a low LMR. Moreover, immunophenotype, body weight, treatment regimen and response to treatment were significantly associated with TTP and OS. In multivariate analysis, treatment and response to treatment were independent risk factors for TTP. Moreover, the LMR, treatment regimen and response to treatment were independent predictors of OS.

Key words: Dog, Lymphocyte to monocyte ratio, Lymphoma, Prognosis

INTRODUCTION

Canine lymphoma is a prevalent malignancy in dogs, representing almost 80% of all hematopoietic cancers (Argyle and Pecceu, 2016). Combination chemotherapy regimen consisting of Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone (known as CHOP), is often recommended as the standard therapy for dogs with lymphoma. Initially, the disease is highly responsive to CHOP-based protocols with first remission rates of 85% or greater. However, drug resistance occurs in most cases, resulted in disease recurrence and overall median survival times are limited to 10–12 months (Vail et al., 2013). Several factors assessed at diagnosis have been proposed as predictors of clinical outcome in dogs with high-grade lymphoma, including anatomical form, immunophenotype, clinical stage and substage (Teske et al., 1994; Vail et al., 2013). The gastrointestinal form of lymphoma has a poorer prognosis compared to the multicentric form (Vail et al., 2013). The two prognostic factors, T-cell immunophenotype and substage b are most consistently identified as poor prognostic factors (Childress et al., 2018).

Lymphocytes mediate humoral and cellular antitumor immune responses, whereas monocytes promote cancer progression through local immune suppression and angiogenesis (Parihar et al., 2010; Whiteside, 2005). In human medicine, low lymphocyte and high monocyte counts have been observed in patients with advanced cancer which are associated with poor prognosis in patients with various malignancies (Ray-Coquard et al., 2009; Aoki et al., 2013). Recently, the prognostic value of the Lymphocyte-to-Monocyte Ratio (LMR), which is calculated by dividing the absolute lymphocyte count by the absolute monocyte count, has been reported for a number of different malignancies, including hematopoietic tumors (Failing et al., 2017). Moreover, it has been reported that a low LMR may indicate an unfavorable prognosis in dogs with diffuse large B-cell lymphoma (Marconato et al., 2015; Davies et al., 2018).

However, there are many different canine lymphoma types, such as multicentric, gastrointestinal, skin forms and B- or T-cell immunophenotypes. It is unclear whether the LMR behaves as a prognostic factor for these different types of lymphomas or not. The present study retrospectively analyzed the medical records of dogs with high-grade lymphoma to investigate the prognostic value of the LMR at diagnosis with respect to the Time To Progression (TTP) and Overall Survival (OS).

MATERIALS AND METHODS

Ethical approval

This study was subjected to ethical examination at the Veterinary Medical Center, Obihiro University of Agriculture and Veterinary Medicine (VMC-OUAVM) and was approved.

Study population and diagnosis

This retrospective study reviewed Medical records of dogs with high-grade lymphomas, classified according to the updated Kiel classification (Fournel-Fleury et al., 1997), which were cytologically or histologically confirmed at the VMC-OUAVM, Japan between 2013 and 2018. The dogs that had not received lymphoma specific treatment regimens, including prednisolone or any other cytotoxic agents, were excluded from the study. Dogs were clinically staged at the time of diagnosis according to the WHO staging system (Owen, 1980), based on findings of three-view thoracic radiography, abdominal ultrasound, and hematological examinations. The immunophenotype of tumor samples, obtained by biopsy or fine-needle aspiration, was determined by immunohistochemistry, flow cytometry, or Polymerase chain reaction for Antigen Receptor Rearrangement (PARR). Briefly, the lymphoma immunophenotyping evaluated histomorphological features as well as CD3 and CD20 expression by tumor cells through immunohistochemical analysis. These procedures had been conducted at the OUAVM Pathological Laboratory or a commercial laboratory. Flow cytometric analysis, which was previously described by Tagawa et al. (2018), was performed by analyzing morphological scattergrams and CD4/CD8 (clone YKIX302.9/YCATE55.9; AbD Serotec, Raleigh, NC, USA) and CD21 (clone CA2.1D6; AbD Serotec, Kidlington, United Kingdom) expression using BD FACS Cant and FACS Diva software (BD Biosciences, San Jose, CA, USA). PARR was performed as previously described (Lana et al., 2006).

Data sampling

All the clinicopathological data including age, breed, sex, body weight, complete blood count (CBC) and serum biochemistry profiles, for each case, were retrieved from the medical records of VMC-OUAVM. The CBC was measured using automated hematology analyzer (Celltac α , MEK-6458; Nihon Kohden, Tokyo, Japan), and serum biochemistry analysis was performed using VETTEST analyzer (IDEXX Laboratories, Tokyo, Japan). Blood smears were reviewed by trained medical technologists and, in some cases, a veterinarian. Leukocyte differential counts were performed manually by counting 100 cells. The LMR was calculated as the ratio of the absolute count of lymphocytes to monocytes in peripheral blood samples obtained on the day of diagnosis. Neoplastic cells that were larger than a neutrophil and had a nucleus that was more than 1.5 times the size of a red blood cell, with loose chromatin and evident nucleoli were excluded from the calculation (Graff et al., 2014).

Response assessment

The definitions for the response to treatment and relapse criteria used at each visit were described by Vail et al. (2010) based on physical examination, radiography, abdominal ultrasound, or hematologic examination at the clinician's discretion. Complete Remission (CR) was defined as the resolution of all measurable parameters of the disease. Partial Remission (PR) was defined as a 30% or more reduction in all measurable lesions. Dogs did not achieve CR or PR were categorized as No Remission (NR). TTP was defined as the elapsed time from the day of diagnosis to the day of stage progression. OS was defined as the time interval between the date of diagnosis and death, caused by any reason, or euthanasia. Dogs that remained alive at the end of the follow-up period or that were lost to follow-up were censored from the OS analysis. In case of relapse, rescue treatment was offered to the owners.

Statistical analysis

Receiver Operating Characteristic (ROC) curve analysis was used to determine the optimal LMR cut-off values for the prediction of a survival time exceeding median days. A minimum Area Under the Curve (AUC) of 0.7 was required to consider for the ROC model. The variables assessed for prognostic significance for the TTP and OS were as follows: age (\geq or $<$ 10 years), sex (male or female), weight (\geq or $<$ 10 kg), WHO stage (I–V), substage (a or b), anatomical form (multicentric, alimentary, cutaneous, and other form), immunophenotype (B-cell, T-cell, or null type), hypercalcemia (presence or absence at presentation), anemia (presence or absence at presentation), lymphopenia (\geq or $<$ 1000 cells/ μ l), monocytosis (\geq or $<$ 1400 cells/ μ l), treatment regimen (CHOP-based, single agent, or prednisolone alone), response to treatment (CR, PR, or NR), prior prednisolone administration (yes or no) and the LMR (high or low). A Kaplan-Meier analysis with log-rank test was performed to estimate the TTP and OS. Univariate and multivariate Cox proportional analyses were used to identify independent prognostic factors. Variables with $p \leq 0.1$ in the univariate analysis were then entered into the multivariate analysis. All analyses were performed using JMP 13 (SAS Institute Inc., Cary, NC, USA). Differences were considered statistically significant at a p -value $<$ 0.05.

RESULTS

Patient characteristics and cut-off point determination

A total of 62 cases were identified for initial review and 19 dogs were excluded from the study because of the lack of clinical data or not meeting the eligibility criteria. Finally, 43 dogs with high-grade lymphomas were included in the present study. The Breeds included miniature Dachshund (7 dogs), mixed breed (7 dogs), Golden Retriever (4 dogs), Miniature Schnauzer (3 dogs), Shiba (3 dogs), Labrador Retriever (2 dogs), Border Collie (2 dogs), Toy Poodle (2 dogs), and Shih Tzu (2 dogs). In addition, American Cocker Spaniel, English Cocker Spaniel, Flat-coated retriever, miniature Pinscher, Maltese, French Bulldog, Beagle, Papillon, Chihuahua, Shetland Sheepdog, and Pembroke Welsh Corgi included one dog each. Patient characteristics are summarized in table 1.

Table 1. Clinical characteristics and patient variables of 43 dogs diagnosed with high-grade lymphoma at Veterinary Medical Center of Obihiro University of Agriculture and Veterinary Medicine between 2013 and 2018.

Characteristics	Case	Percentage (%)
Age, years; median (range)	9 (1-15)	
≥ 10 years	21	48.8
< 10 years	22	51.2
Body weight, kg; median (range)	8.02 (2.56-45.50)	
≥ 10 kg	19	44.2
< 10 kg	24	55.8
Sex		
Male (Castrated)	26 (16)	60.5 (37.2)
Female (Spayed)	17 (13)	39.5 (30.2)
Stage		
I	3	7.0
II	5	11.6
III	10	23.3
IV	16	37.2
V	9	20.9
Substage		
a	17	39.5
b	26	60.5
Immunophenotype*		
B cell	20	48.8
T cell	17	41.5
Null type	4	9.8
Anatomical form		
Multicentric	24	55.8
Alimentary	11	25.6
Cutaneous	5	11.6
Others (renal, CNS, mediastinal)	3	7.0
Hypercalcemia		
Yes	4	9.3
No	39	90.7
Presence of anemia		
Yes	16	37.2
No	27	62.8
Lymphopenia		
≥ 1000 cells/ μ l	25	58.1
< 1000 cells/ μ l	18	41.9
Monocytosis		
≥ 1400 cells/ μ l	26	60.5
< 1400 cells/ μ l	17	39.5
Treatment		
CHOP-based	27	62.8
Single-agent	10	23.3
Prednisolone	6	14.0
Prior administration of prednisolone		
Yes	8	18.6
No	35	81.4

*: 2 dogs were excluded because of inadequate data; TTP: Time to Progression; OS: Overall Survival; CNS: Central Nervous System; CHOP: Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone

The median age was 9 years (ranged between 1–15 years) and median body weight was 8.02 kg (ranged between 2.56–45.50 kg). There were 26 male dogs (16 were castrated) and 17 female dogs (13 were spayed). According to the WHO staging criteria, three dogs were classified as stage I, five as stage II, 10 as stage III, 16 as stage IV and nine as stage V. Moreover, 17 and 26 dogs were classified as substage ‘a’ and ‘b’, respectively. Immunophenotyping was performed in 41 dogs, accomplished by immunohistochemistry of biopsy samples in 10 dogs and using flow cytometry and PARR of lymph node aspirate samples in 19 and 12 dogs, respectively. According to the obtained results from immunophenotyping of 41 dogs, 20 and 17 cases had B- and T-cell lymphoma, respectively. The remaining four cases were null type. Regarding the anatomical forms, 24 dogs were classified as multicentric, 11 dogs as alimentary (gastrointestinal and hepatosplenic) and five dogs as cutaneous. In addition, the renal, central nervous system, and mediastinal form were each found in one case. Hypercalcemia and anemia were observed in 4 and 16 dogs, respectively. Regarding treatment, 27 dogs were managed with a CHOP-based protocol, whereas six dogs had prednisolone monotherapy. The remaining 10 dogs were treated with other single agents (including L-asparaginase, doxorubicin, mitoxantrone, and lomustine). Only eight dogs were treated with prednisolone prior to sampling.

The median absolute lymphocyte concentration was 1176 cells/ μ l (ranged between 0–12376 cells/ μ l; reference range: 1000–4800 cells/ μ l; Mutz et al., 2015). Eighteen dogs had a lymphocyte count < 1000 cells/ μ l. The median absolute monocyte concentration was 1547 cells/ μ l (ranged between 274–6200 cells/ μ l; reference range: 100–1400 cells/ μ l; Mutz et al., 2015). Twenty-six dogs had a monocyte count > 1400 cells/ μ l. The optimal LMR cut-off value was 0.7, which corresponded to the maximum sensitivity (0.727) and specificity (0.762) of the LMR for predicting the median days of OS with ROC analysis. The AUC of ROC for LMR was 0.794 ($p = 0.002$; Figure 1). An LMR ≥ 0.7 (high LMR) was found in 22 dogs, and an LMR < 0.7 (low LMR) was found in 21 dogs.

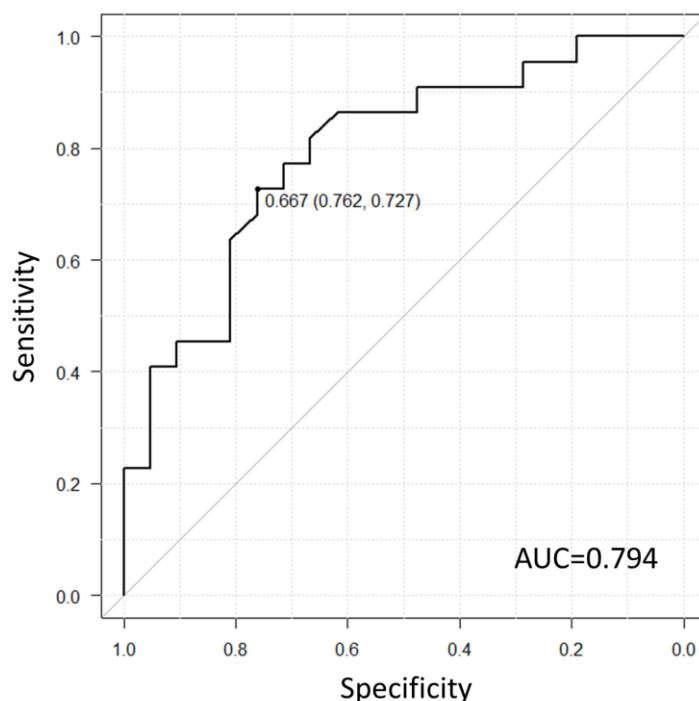


Figure 1. Receiver operating characteristic and area under the curve (AUC) of the lymphocyte-to-monocyte ratio at the time of diagnosis for all dogs. The AUC was 0.794 (95% confidence interval, ranged between 0.659–0.930, $p = 0.002$).

Analysis of outcomes

The overall median TTP and OS were 46 days (ranged between 1–289 days) and 108 days (ranged between 1–621 days), respectively. At the end of data analysis, three dogs were still alive and in CR status, and survival times were 168, 226, and 248 days. Survival analysis with Kaplan-Meier and log-rank tests showed that dogs with a high LMR had significantly longer TTP ($p = 0.048$) and OS ($p = 0.011$) compared to dogs with a low LMR (Table 2, Figures 2 and 3). Moreover, immunophenotype (TTP, $p = 0.017$; OS, $p = 0.034$), body weight (TTP, $p = 0.049$), treatment (TTP, $p < 0.001$; OS, $p < 0.001$), and response to treatment (TTP, $p < 0.001$; OS, $p < 0.001$) were associated with TTP and OS (Table 2, Figures 2 and 3). There were no significant differences in the TTP and OS in relation to anatomical form, clinical stage, substage, age, sex, hypercalcemia, anemia, lymphopenia, monocytosis, and prednisolone treatment prior to sampling (Table 2).

Table 2. Analysis of prognostic factors for time to progression and overall survival in 43 dogs with high-grade lymphoma at Veterinary Medical Center of Obihiro University of Agriculture and Veterinary Medicine between 2013 and 2018.

Parameters	Case (Number)	TTP*	p-value	OS*	p-value
Age			0.103		0.439
≥ 10 years	21	40 (5-267)		71 (9-621)	
< 10 years	22	64 (1-289)		123 (1-487))	
Sex			0.438		0.219
Male (Castrated)	19	47.5 (1-238)		97.5 (1-492)	
Female (Spayed)	24	55 (1-289)		121 (19-621)	
Body weight			0.049		0.064
≥ 10 kg	19	93 (1-289)		152 (7-621)	
< 10 kg	24	43.5 (1-248)		68.5 (1-492)	
Stage			0.808		0.769
I	3	28 (10-267)		50 (47-621)	
II	5	38 (19-248)		61 (119-248)	
III	10	54 (8-171)		119 (13-320)	
IV	16	45 (1-289)		79 (1-492)	
V	9	93 (5-226)		159 (9-253)	
Substage			0.812		0.949
a	17	72 (1-171)		130 (13-478)	
b	26	45 (1-289)		68.5 (1-621)	
Immunophenotype†			0.017		0.034
B cell	20	73.5 (1-289)		136 (1-621)	
T cell	17	43 (1-248)		66 (9-478)	
Null type	4	25 (1-44)		46 (7-105)	
Anatomical form			0.123		0.186
Multicentric	24	73.5 (1-289)		136 (1-621)	
Alimentary	11	43 (1-135)		66 (7-253)	
Cutaneous	5	44 (1-107)		105 (32-478)	
Others (renal, CNS, mediastinal)	3	10 (5-248)		47 (9-248)	
Hypercalcemia			0.838		0.959
Yes	4	42 (19-238)		60.5 (19-492)	
No	39	49 (1-289)		121 (1-621)	
Presence of anemia			0.287		0.670
Yes	16	50.5 (1-289)		104 (1-492)	
No	27	49 (1-267)		108 (13-621)	
Lymphopenia			0.480		0.428
≥ 1000 cells/μl	25	63 (1-289)		130 (1-492)	
< 1000 cells/μl	18	45 (1-264)		68.5 (7-621)	
Monocytosis			0.201		0.076
≥ 1400 cells/μl	26	43.5 (1-289)		66 (1-492)	
< 1400 cells/μl	17	83 (1-267)		134 (19-621)	
Treatment			<0.001		<0.001
CHOP-based	27	63 (1-289)		134 (1-621)	
Single agent	10	49 (1-135)		106.5 (47-478)	
Prednisolonealone	6	15 (1-38)		22 (7-61)	
Response to treatment			<0.001		<0.001
CR	16	147 (28-289)		250 (43-621)	
PR	18	47.5 (13-132)		96 (13-478)	
NR	9	8 (1-52)		18 (1-66)	
Prior administration of prednisolone			0.271		0.126
Yes	8	47.5 (10-107)		68.5 (47-138)	
No	35	52 (0-289)		121 (1-621)	
LMR			0.048		0.011
≥ 0.7	22	79 (1-289)		148 (1-621)	
< 0.7	21	43 (1-248)		61 (9-492)	

*: Median (range); †: 2 dogs were excluded because of inadequate data. TTP: Time To Progression; OS: Overall Survival; LMR: Lymphocyte-to-Monocyte Ratio; CHOP: Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone; CNS: Central Nervous System; CR: Complete Remission; PR: Partial Remission; NR: No Remission.

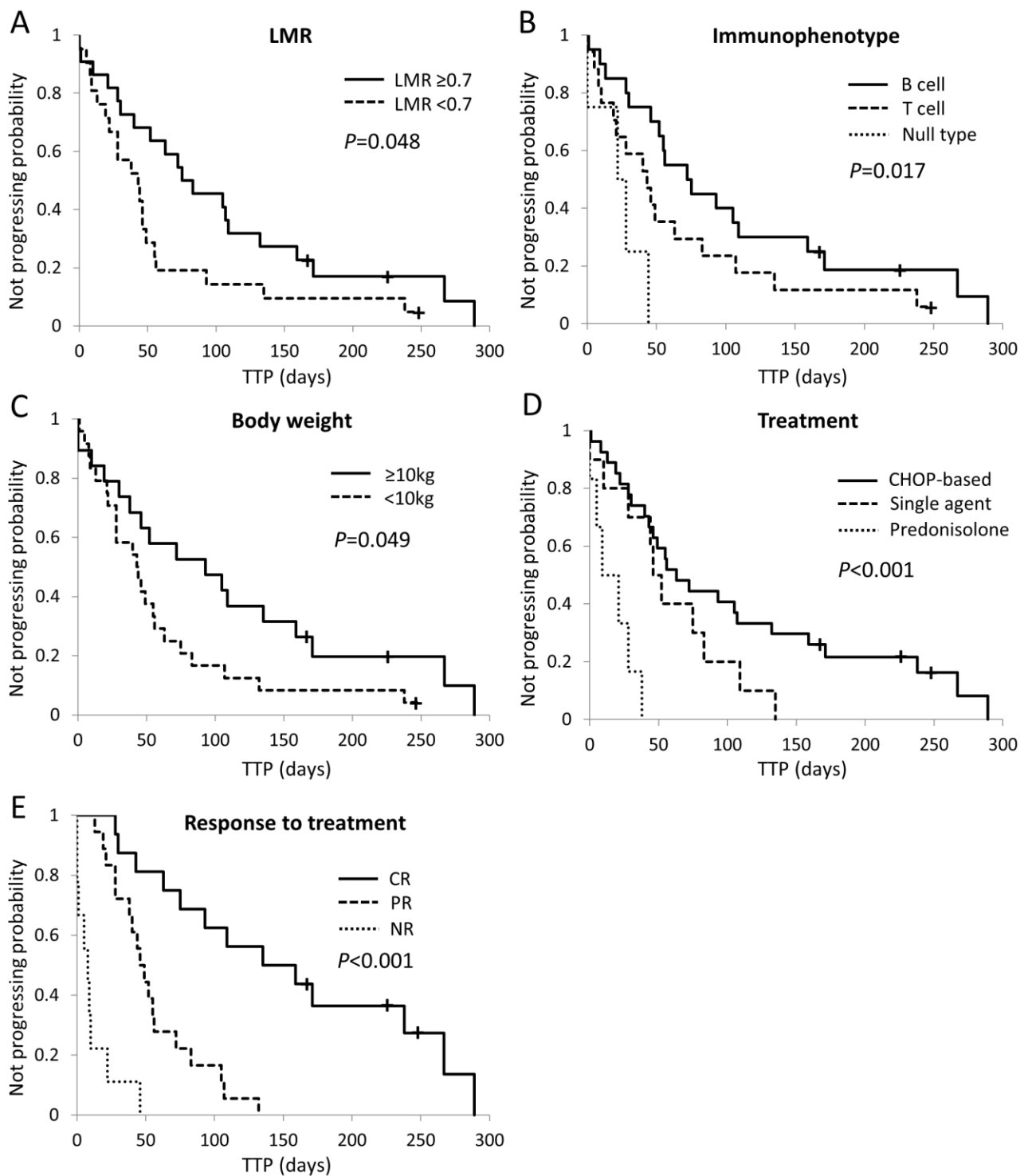


Figure 2. Kaplan-Meier curves of Time To Progression (TTP) for 43 dogs with high-grade lymphoma according to Lymphocyte-to- Monocyte Ratio (LMR) (A), immunophenotype (B), body weight (C), treatment (D), and response to treatment (E). +: censored case; CHOP: Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone.

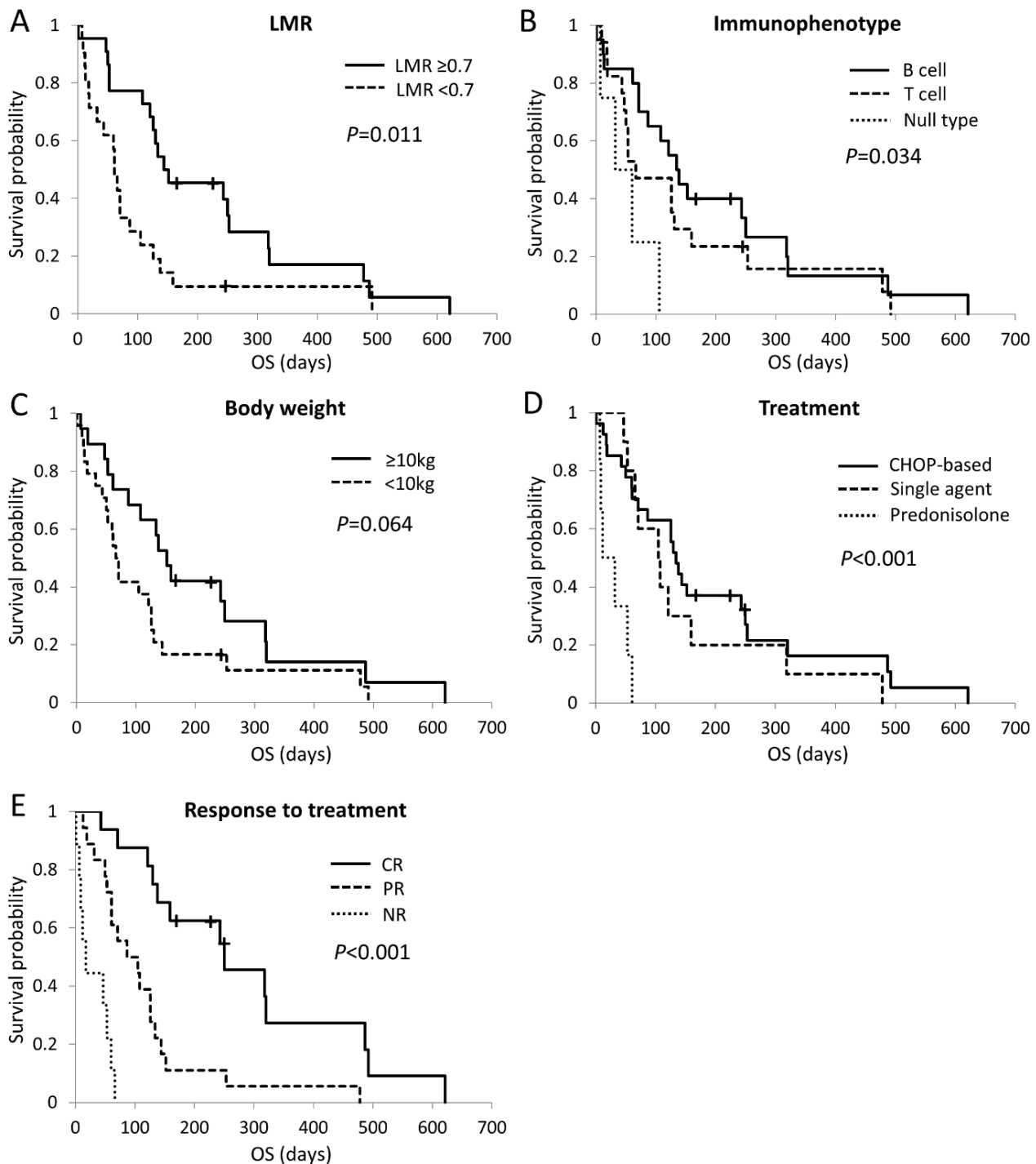


Figure 3. Kaplan-Meier curves of Overall Survival (OS) for 43 dogs with high-grade lymphoma according to Lymphocyte-to- Monocyte Ratio (LMR) (A), immunophenotype (B), body weight (C), treatment (D), and response to treatment (E). +: censored case; CHOP: Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone.

To develop a combined model for the prognosis of patients with high-grade lymphoma, the associations between patient prognosis and LMR (\geq or $<$ 0.7), immunophenotype (B-cell or other types), anatomical form (multicentric or other types), body weight (\geq or $<$ 10 kg), monocytes (\geq or $<$ 1400 cells/ μ l), treatment (CHOP and single agent or prednisolone alone), and response to treatment (CR or other), which were all $p \leq 0.1$ parameters in the univariate analysis, were evaluated using multivariate Cox analysis. The multivariate analysis showed that treatment ($p = 0.048$) and response to treatment ($p < 0.001$) were independent risk factors for the TTP (Table 3). Moreover, the LMR ($p = 0.020$), treatment ($p = 0.029$) and response to treatment ($p < 0.001$) were independent predictors of OS (Table 4).

Table 3. Univariate and multivariate analysis of prognostic factors for time to progression of 43 dogs with high-grade lymphoma at Veterinary Medical Center of Obihiro University of Agriculture and Veterinary Medicine between 2013 and 2018.

Parameters	Univariate analysis		Multivariate analysis	
	p-value	HR (95% CI)	p-value	HR (95% CI)
LMR				
≥ 0.7	0.053		0.118	
< 0.7		1.900 (0.991-3.672)		1.887 (0.851-4.259)
Immunophenotype				
B cell	0.058		0.982	
Other*		1.867 (0.979-3.639)		0.990 (0.411-2.439)
Anatomic form				
Multicentric	0.051		0.834	
Other**		1.930 (0.997-3.714)		0.920 (0.421-2.037)
Body weight				
≥ 10 kg	0.051		0.984	
< 10 kg		1.924 (0.998-3.823)		0.991 (0.404-2.329)
Treatment				
CHOP and single agent	0.001		0.048	
Prednisolone alone		6.633 (2.215-18.279)		3.277 (1.013-9.915)
Response to treatment				
CR	<0.001		<0.001	
PR, NR		6.352 (2.780-16.545)		6.331 (2.430-18.406)

*: T cell and null type; **: Alimentary, cutaneous, renal, central nervous system, and mediastinal form.; HR: Hazard Ratio; 95% CI: 95% Confidential Interval; LMR: Lymphocyte-to- Monocyte Ratio; CHOP: Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone; CR: Complete Remission; PR: Partial Remission; NR: No Remission.

Table 4. Univariate and multivariate analyses of prognostic factors for overall survival of 43 dogs with high-grade lymphoma at Veterinary Medical Center of Obihiro University of Agriculture and Veterinary Medicine between 2013 and 2018.

Parameters	Univariate analysis		Multivariate analysis	
	p-value	HR (95% CI)	p-value	HR (95% CI)
LMR				
≥ 0.7	0.014		0.020	
< 0.7		2.264 (1.179-4.363)		2.551 (1.161-5.753)
Anatomical form				
Multicentric	0.056		0.360	
Other*		1.909 (0.983-3.694)		0.703 (0.330-1.501)
Body weight				
≥ 10 kg	0.069		0.837	
< 10 kg		1.824 (0.962-3.545)		0.926 (0.446-1.946)
Monocytosis				
≥ 1400 cells/μl	0.076		0.479	
< 1400 cells/μl		0.557 (0.280-1.061)		0.766 (0.359-1.602)
Treatment				
CHOP and single agent	<0.001		0.029	
Prednisolone alone		7.712 (2.565-21.330)		3.635 (1.149-10.754)
Response to treatment				
CR	<0.001		<0.001	
PR, NR		4.421 (2.137-9.867)		5.858 (2.259-16.172)

*: Alimentary, cutaneous, renal, central nervous system, and mediastinal form. HR: Hazard Ratio; 95% CI: 95% Confidential Interval; LMR: Lymphocyte-to- Monocyte Ratio; CHOP: Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone; CR: Complete Remission; PR: Partial Remission; NR: No Remission.

DISCUSSION

Prognostic factors for patients with cancer provide information regarding possible clinical outcomes and help to classify patients into different risk groups. In dogs, several consistent prognostic factors for lymphoma, including WHO clinical stage and substage, immunophenotype, anatomical form, hypercalcemia and anemia, have been reported (Rassnick et al., 2009; Parihar et al., 2010; Marconato et al., 2011). A low LMR has been reported to be a prognostic factor in human patients with hematologic malignancies (Failing et al., 2017). Although recent findings indicated that the LMR might predict the clinical outcome of canine diffuse large B-cell lymphoma (Marconato et al., 2015; Davies et al., 2018), there are limited data available on the prognostic value of the LMR for various types of high-grade lymphoma. The current study retrospectively investigated the association between clinicopathological variables, including the LMR and patient outcome of 43 dogs with high-grade lymphoma.

In the present study, log-rank tests revealed that dogs with a low LMR (≤ 0.7) had significantly shorter TTP and OS than those had a high LMR (> 0.7). The actual mechanisms concerning the relationship between low LMR and poor patient outcome are unclear. There are several possible explanations accounting for this correlation. Lymphocytes are the basic components of antitumor immunity and tumor-infiltrating lymphocytes have a vital effect on tumor development (Rahir and Moser, 2012). Several studies have shown that lymphopenia was associated with poor prognosis (Taulikar et al., 2008; Castillo et al., 2010). Monocytes play a vital role in tumor progression. These cells are recruited to the tumor stroma and differentiated into Tumor-associated macrophages (TAMs) (Richards et al., 2013). TAMs have also been implicated in promoting tumor invasion and angiogenesis as well as having immunosuppressive effects on the anti-tumor response of lymphocytes by producing various cytokines and chemokines (Nielsen and Schmid, 2017). TAM infiltration has also been associated with vascular endothelial growth factor expression and linked to poor prognosis in canine breast carcinoma (Raposo et al., 2014; Raposo et al., 2015). It is suggested that the peripheral monocyte level correlates with TAM density of the tumor microenvironment (Shibutani et al., 2017). Briefly, the LMR is a simple biomarker that reflects the status of immune homeostasis and the tumor microenvironment. In the present study, an optimal cut-off value of LMR (0.7) was used for further analysis. The cut-off value of this study was lower than the LMR cut-off values reported in two previous studies on canine lymphoma (Marconato et al., 2015; Davies et al., 2018). These divergent results may have arisen for a number of reasons. The methods for calculating the percentages of lymphocytes and monocytes were different between present study and one of the aforementioned studies. In addition, the mentioned studies only analyzed diffuse large B-cell multicentric lymphoma, whereas this study included various lymphoma types. Moreover, in this study, dogs that were pretreated with prednisolone were not excluded from the leukocyte count.

In animals, lymphopenia occurs in response to endogenous or exogenous glucocorticoids. It has been reported that lymphocyte counts were significantly decreased compared to those of healthy control dogs after two weeks of anti-inflammatory doses of prednisone therapy (Moore et al., 1992). However, it is reported that prednisone administration to healthy dogs with allergic dermatitis caused no significant changes in lymphocyte concentration (Masters et al., 2018). In this study, eight dogs treated with prednisolone prior to sampling had lower lymphocyte concentrations (median 555 cells/ μ l; ranged between 0–1944 cells/ μ l) compared to the remaining dogs (median 1395 / μ l; ranged between 0–12376 cells/ μ l). Due to the small sample size, it is difficult to assess whether the lymphocyte concentrations of these eight dogs were affected by prednisone administration or not. In the studied cohort of dogs, lymphopenia at diagnosis was not found to be a significant prognostic factor for the TTP and OS. In addition, the prognostic significance of lymphopenia has not been documented in veterinary medicine. For further prospective studies of assessing lymphopenia as a prognostic factor, it is suggested that a history of prior glucocorticoid should be an exclusion criterion.

The log-rank tests also indicated that immunophenotype, body weight, treatment, and response to treatment were associated with patient outcome. Dogs with B-cell lymphoma were found to have longer TTP and OS compared to those with T-cell lymphoma or null type. In general, T-cell lymphomas have shorter remission and survival times than B-cell lymphomas (Zandvliet, 2016). It has been reported that some subsets of T-cells have 40 times more P-glycoprotein gene expression than B-cells (Klimecki et al., 1994). The lack of response to chemotherapy in T-cell lymphomas may be partially explained by their multidrug resistance. In fact, intrinsic drug resistance was more common in T-cell than B-cell lymphoma (Zandvliet, 2016). Moreover, extranodal lymphomas, including gastrointestinal, hepatosplenic, mediastinal, cutaneous, and renal lymphoma, have poorer prognoses than the multicentric form (Zandvliet, 2016). However, in the present study, no significant differences in the TTP and OS in relation to anatomical form were found. This contradiction can be attributed to small sample size. In addition, 28 % of multicentric lymphomas had T-cell immunophenotype which is a negative prognostic factor. The log-rank test showed that dogs with body weights ≥ 10 kg were found to have significantly longer TTP and a longer OS tendency. Marconato et al. (2011) studied the predictors of long-term survival in Rottweilers, Pointer, Boxer, Yorkshire Terrier, Bernese Mountain Dog, and Doberman Pinscher dogs with high-grade multicentric lymphoma. The mentioned study found that 11/13 dogs that were long-term survivors had body weights ≥ 10 kg. In addition, excessive body weight in dogs has been associated with positive outcomes (Romano et al., 2016). More research is needed to elucidate the relationship between body weight and prognosis in dogs with lymphoma. In

veterinary medicine, various chemotherapies have been described, and multi-agent therapy protocols including CHOP have the highest response rate and longest response durations for treating high-grade lymphoma (Castillo et al., 2010; Valli et al., 2013). It is thought that single-agent therapies are less effective than a doxorubicin-based multi-agent protocol and should be reserved for palliative therapy (Sauerbrey et al., 2007; Higginbotham et al., 2013). However, several single-agent protocols have shown no significant differences in remission or survival time compared to multidrug treatments (Simon et al., 2008; Deravi et al., 2017). In this study, the TTP and OS were substantially longer in dogs treated with chemotherapy protocols versus prednisolone alone. Response to treatment has been a strong prognostic factor in several case studies of canine lymphoma (Curran and Thamm, 2016; Goodman et al., 2016). The present study also revealed that dogs reaching CR as best response to therapy had significantly longer survival times compared to the other dogs. According to multivariate analysis of data from dogs with high-grade lymphoma, treatment regimen and response to treatment remained significant for the TTP. Moreover, the LMR, treatment, and response to treatment were independent prognostic factors. In particular, the LMR may be useful in clinical practice as a simple and readily available prognostic marker since it can only predict the outcome in pre-treatment dogs with high-grade lymphoma.

CONCLUSION

A low LMR was significantly associated with a poor prognosis in dogs with different types of high-grade lymphoma. The current study had several limitations. First, the small sample size for each analysis may limit the detection of differences between groups. Second, the present study was a retrospective analysis and first-line and rescue treatments were not standardized. The choice of treatment mainly depended on the owner's opinion and the patient's condition. Thus, future prospective studies which using standardized treatment are necessary for adequate evaluation. Further prospective studies and analysis of the LMR levels obtained from routine blood tests may provide additional information to assist in the management of dogs with high-grade lymphoma.

DECLARATIONS

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Authors' contributions

MT designed the study and drafted the manuscript, GS and KM performed the practical part of the experiment. KM reviewed the manuscript. All the authors approved the final manuscript.

Consent to publish

All the authors approved and agreed to publish the manuscript.

Competing interests

The authors clarify that they have no competing interest, and with respect to this search, all the authors are in agreement with each other and have no conflict with authorship or article publication, all authors approved the publishing of the paper.

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Computed Tomography Scan and Polyester resin 40 Plastination Technique: Teaching Aids to Illustrate Anatomical Structure of Donkey Brain

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ABSTRACT

The present study investigated the collaboration between the plastinated sagittal sections and computed tomography (CT) images of the donkey brain. Four adult healthy donkeys of both sexes from native breeds were analyzed. The animals were sedated with a combination of xylazine and ketamine. The donkeys were positioned in sternal recumbency during CT brain examination and contiguous sagittal 3 mm thick slices of the head were obtained. Polyester resin 40 (P40) technique for the brains were done through five main steps including fixation, slicing, dehydration, forced impregnation and light-curing of brain glass chambers by UVA light source. Furthermore, a survey was conducted using questionnaires involving second-year veterinary medical students of anatomy department, Zagazig University to compare the educational capacity of CT brain images and P40 plastination of brain slices. 52% of students voted that using of brain sheeted slices are much better, 38% prefer to learn both parallel to each other. The obtained results revealed that both the plastinated brain sections and the CT images portrayed a combination of many complex neuroanatomical structures that provide an excellent aid for researchers in educational and diagnostic purposes. In addition, the plastinated brain serves as a good tool for the interpretation of CT images.

Key words: Brain, CT, Donkey, Plastination, Polyester resin 40

INTRODUCTION

Plastination is one of the standard methods for preservation of biological tissues and it is used in many fields such as anatomy, art, biology, clinics, and medicine (Henry and Latorre 2007). Plastinated slices require low storage space and are stable, inodorous and non-toxic, thus they are applied as a good teaching tool in veterinary anatomy. The plastinated slices are well preserved and excellent for demonstration compared to the harmful formalinized specimens. However, they could not completely replace the traditional method as students learning will be best by hands-on dissection. Shrinkage, discoloration, high-price and need to a well-trained person are the major problems associated with plastination technique (Reda and Roger, 2018 and Renukaradhya et al., 2018).

The plastinated specimens are useful in self-learning and research, thus facilitate the teaching of neuroanatomy, the lifelike visualization of specimens of the central and peripheral nervous system. P40 plastination technology is a specific module for the tissue preservation and production of polyester plastinated brain slices which have been used in teaching and research (Latorre et al., 2002 and Sivrev et al., 2013). The Biodur™ P35 resin was used in 1980s for the preparation of brain sections with a thickness of 4-8mm (Von Hagens et al., 1987). The P40 resin was introduced in the mid-1990s as a less complicated process to produce thinner brain slices giving better white and gray matter differentiation (Von Hagens, 1994).

Computed Tomography (CT) is an important tool for the investigation of intracranial disease in humans. Recently, this technique has become more available to veterinarians and has used for the diagnosis of brain disorders in horses, ruminants, and carnivores. The neurodegenerative diseases, tumors, cysts, hemorrhage, brain infarction, congenital anomalies, and hydrocephalus could be detected and diagnosed by using CT scans (Lieve et al., 2005).

Radiographic assessment of the equine skull is difficult due to its complex anatomy. The location and extent of lesions in the head region are often difficult to define by clinical and radiographic examination. CT scanning is particularly useful for studying the complex anatomical structures due to the ability to obtain transverse and midsagittal images and manipulate its contrast and latitude (Karen et al., 2000).

The use of CT scan in the anatomical investigation and clinical examination of Equidae is still very limited due to unsuitable design of the CT machine for big-sized horses and its high expense. The CT scanners used in veterinary

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medicine are designed for human patients. Hence, these machines are only suitable for small-sized animals such as donkeys (Cabreráa et al., 2015). P40 and CT carrels are available for the learning of neuroanatomy and describing the most complex parts of the nervous system such as the brain.

A survey of both teaching modules was done on the second-year veterinary students. The purpose of the present study was to reveal the benefits of applying the P40 plastinated brain sections in matching with CT images. To be more combination of P40 and CT techniques can be used for better understanding and imagination to the most complex anatomy parts of the donkey brain which also confers accurate interpretation of CT images. Both these tools can also be used in the clinical training of medical students in the neurosurgery and radiological anatomy.

MATERIALS AND METHODS

Ethical approval

Prior approval from Institutional Animal Care and the Research Ethics Committee of the Zagazig (approval no. ZU-IACUC/2/F/92/2018) was obtained.

Animals

Four adult healthy donkeys (more than two-years-old) of both sexes from native breeds were selected from different farms in Sharkia Governorate, Egypt. Food was withheld for 24 hours prior to sedation. Donkeys were sedated with a combination of xylazine (1.1 mg/kg) and ketamine (0.05-0.1 mg/kg) via the intravenous route (Nora and Tex, 2002). First, animals were exposed to CT radiation then the brains were used for the P40 plastination technique.

Computed tomography technique

The CT scanning was performed at the Emergency Hospital in Zagazig, Sharkia Governorate, Egypt. The donkeys were placed in sternal recumbency, with the head and neck was extended and supported on a sandbag. Contiguous sagittal 3 mm slices of the brain were obtained using standard CT protocols. CT images were taken without contrast medium by applying a couch index 10.00, RVP 140, MA 108, MAS 225, imaging time 2.2 sec, using CT scanner capable of acquiring up to 32 slices per second with a fast whole-body scan time of 0.5 seconds (third-generation equipment, TOSHIBA 600 HQ, Japan) (Jonathon et al., 2016).

Polyester resin 40 technique

Polyester resin 40 (P40) technique was performed in P40 room of Zagazig Plastination Laboratory in five steps as described by Henry and Latorre (2007)

Fixation

After scanning, the donkeys were injected with 10% formalin solution through the common carotid artery. The skulls were crushed to observe the brains after 4 weeks. To good fixation, the obtained brains were kept in 5% formalin solution for another 1-2 weeks. A well-fixed firm brain was desirable for slicing on a rotatory meat slicer.

Slicing

The well-fixed brains were kept at -20 °C for 36-48h for better slicing of the brain using a rotary meat slicer to produce 4-6 mm thick sections for P40 technique of plastination (Figures 1A and B).

The prepared brain sections were rinsed in distilled water at 3-5°C for 24-48 hours.

Dehydration (Freeze Substitution)

The brain slices were placed in a closed bath containing cold pure acetone at -20 °C for 2-4 days to remove the water and lipid content. The ratio of specimen weight to volume of acetone was 1:10 (Figure 1C).

Forced impregnation

The dehydrated sections were imbedded into the liquid polyester P40 under vacuum and cool conditions. The vacuum drew out the acetone from the tissue and was replaced by the polymer. Equipment needed for this step included explosive proof freezers, vacuum chamber, vacuum pump, vacuum control unit, and wire boxes. This step took 5-7 days and included the following three procedures:

1. Low pressure immersion. After the dehydration process, the brain sliced sections were quickly immersed in a polymer solution (20 ml of hardener A9 added to one-liter of polyester resins "P40 ") at 4°C. First, low pressure (2-3mm Hg) of vacuum was used for 30 minutes to remove the excessive air-bubbles in the specimens then samples were kept overnight in the first polymer mixture at 4°C. Polymer solution must be kept in dark bottles (Figures 1D and 2A).

2. High pressure immersion. The polymer solution was replaced three times at 24 h intervals under a low vacuum level. After replacing the third polymer mixture, the pressure was increased up to 12 mm Hg overnight in the dark vacuum chamber at room temperature. The vacuum chamber should be kept in a dark place (Figures 1D and 2A).

3. Preparation of the plastinated sheets. a) Two glass plates were used to prepare a flat chamber suitable for 4 mm brain slices. The two plates were separated by a flexible, elastic gasket with 6mm thickness and then compressed by

large fold-back clamps. The slices were carefully placed into the chamber after completion of immersion in the polymer solution (Figure 2B); b) The flat glass chamber was filled with a freshly prepared polymer mixture, then due to air bubbles interfere with the curing of the P40 resin, 1 mm wire was used to remove them (Figure 2B).

Light curing

To start the process of polymerization, a 200 Watt Ultraviolet A (UVA) light source was used for 45 minutes to be exposed on both sides of the glass chamber which act as the catalyst. During UVA light exposure, the flat chamber should be cool as the temperature can damage the slices. After that, the flat chamber was placed in an oven at 40 °C for six days or under sun rays for two weeks to complete the polymerization process. When hardening of the slices was completed, the flat chamber was dismantled, and a scalpel blade was used to score along the length of the junction of the glass with the resin on one or more sides. The brain plastinated slices were covered with plastic foil after trimming any excessive polymer with a band saw or sandpapers (Figure 2C). Eventually, the obtained brain P40 slices were photographed and correlated with the CT images to identify the normal anatomical structures of the brain. The nomenclatures were taken as a basis for the denomination on the brain (NAV, 2017).

Survey of dichotomous questions

A questionnaire survey was conducted among second-year undergraduate veterinary medical students at the Faculty of Veterinary Medicine, Zagazig University. The plastinated brain slices along with printed labeled CT images of corresponding levels were distributed among 100 students in practical sessions at anatomy departments. Students were asked to write their opinions regarding the preferable method for brain teaching aid.



Figure 1. P40 room in Plastination Laboratory, Zagazig University. **A:** Rotary meat slicer used in sheet brain plastination for cutting the latter fixed brain during P40 technique, blade diameter: 210mm: rotate speed: 8000 round/min (adjustable). Slice thickness adjustment: 4 mm; **B:** Stainless steel grids as a carrier of brain slices; **C:** Freezer containing two dehydration acetone panels and an acetometer using in the step of dehydration of the latter fixed slices and **D:** Freezer containing P40 filled kettle which connects with vacuum pump and a monitor using in the step of forced impregnation of the latter dehydrated slices.



Figure 2. P40 room in Plastination Laboratory, Zagazig University. **A:** Forced impregnation step of the dehydrated sections under vacuum (glass covering); **B:** Glass brain chambers of 6 mm space, silicon gasket and fixation clips filled with the polymer imbedded brain sections and **C:** Glass brain chambers after curing and removing the silicon gasket and fixation clips.

RESULTS

CT brain images

The present study revealed that some cranial structures could be seen obviously in the plastinated slices while not be seen on its corresponding CT images, and vice versa which may be due to CT artifacts. The widest area of the donkey brain measured from the right to the left cerebral hemispheres was about 5.5-6.5 cm. Fourteen sagittal sections of the formalinized donkey brains were sliced (seven sections for each right and left hemisphere) using the meat rotatory slicer and correlated with the corresponding obtaining CT images. The thickness of each section was about 4 mm. CT scans and the corresponding P40 longitudinal sections were labeled anatomically.

The white and gray matter differentiation was clear in CT scanning while optical quality was better in P40 sections (Figures 3A, 3B, 3C, 3D, 4A and 4B). All four ventricles and the mesencephalic aqueduct were more clearly visible in CT images due to the presence of radiolucent CSF compared to plastinated sections. In contrast, the choroid plexuses within these ventricles and the central canal were not detected in CT images. Ventriculus lateralis with a width measuring 1.6 cm, was only apparent in CT images of the first six sagittal sections (Figures 4C, 5A and 5C) but Ventriculus lateralis could be found in both CT and P40 images of the last seven sagittal section of the brain (Figure 6A and 6B). Also, ventriculus tertius was extended around the interthalamic adhesion and aqueductus cerebri was located dorsal to crus cerebri (Figures 5C, 6A and 6B). The width of both structures was about 0.8 mm.

Ventriculus quartus, measuring 0.8 mm in width, appeared along the ventral aspect of the cerebellum in two images of CT scanning (Figures 5C and 6A), also it was detected in last paramedian P40 slice (Figure 6B). The width of the brain ventricles was approximately 1.6 mm extended along four CT scans (Figures 4C, 5A, 5C and 6A) that could be easily identified in CT scanning rather than plastinated sections. Cerebrospinal fluid was visualized inside ventriculus lateralis, tertius, quartus, and aqueductus cerebri as black color. On the other hand, not all cranial nerves could be seen in CT images or P40 slices, except nervus olfactorii, nervus opticus, nervus oculomotorius (Figures 4, 5 and 6).

Hemispherium cerebri was detected in all CT and P40 levels that included the whole diameter of donkey brain which was 5.6 mm, while cerebellum was only extended along the thickness of four sections (Figures 4, 5 and 6). Some anatomic features of the cerebrum and cerebellum were not well visualized. In addition, most of the bony boundaries seen on the CT images were also identified and labeled in figure 3A.

P40 brain sections

Sheet plastinated donkey brain sections (4-6 mm thickness, longitudinal cut) by P40 technique provided a teaching module which gives durable, semi-transparent, flexible and less susceptible to damage and can easily be correlated with CT images. Fourteen semi-transparent P40 brain sections were sliced to clarify more macroscopic details such as some nuclei and nerve bundles which appeared as darker than white matter. These details enhance the teaching method of neuroanatomy to medical students, anatomists, radiologists, and physiotherapists.

Brain P40 sections provided good differentiation between the darker zones of prosencephalon (commissura grisea and basal nuclei) and the lighter one (commissura alba) of hemispherium cerebelli (Figures 3, 4, 5 and 6). Fissura sylvii were only observable in P40 sections (Figure 3B). One of the darker areas which can be easily seen in P40 sections in comparison to CT images was nucleus caudatus that formed the rostral part of the bottom of ventriculus lateralis and accompanied it for four slices. This nucleus consists of two parts; a very large rostral head that related to ventriculus lateralis and corpus callosum and a small caudal tail part situated dorsally to the thalamus (Figures 4, 5 and 6). Another dark nucleus is putamen which is situated lateral to the rostral head part and just lateral to putamen could find capsula

externa followed by Claustrum (Figure 4B). Capsula interna was found lateral to nucleus caudatus and ventral to thalamus and also separated them. Thalamus was located ventromedial to the two parts of nucleus caudatus (Figures 4, 5 and 6). Thalamus was the large nervous band of diencephalon which fused sagittally with thalamus in the opposite side forming adhesio interthalamica (Figures 4, 5 and 6). Just lateral to thalamus, nucleus geniculatus lateralis was located which is convex or semicircular and surrounded by thalamus (Figure 3D). Ventriculus tertius extended and accompanied corpus pinealis for shorter distance (Figure 6A and 6B). The dorsal structure of mesencephalon is corpora quadrigemina which consists of four large colliculi, two rostral and two posterior colliculi (Figures 4 and 5). The ventral part of mesencephalon is Crus cerebri (Figure 6A and 6B). Ventriculus quartus and Plexus choroideus ventriculi quarti were located dorsal to medulla oblongata and ventral to cerebellum which connected rostrally with aqueductus cerebri and posteriorly with canalis centralis medullae spinalis (Figures 5C, 6A and 6B). Broken sections were one of the defects during the slicing technique of the brain. This may be due to excessive freezing of brain which led to a distortion of the brain tissue by the rapid slicer knife. Immersion of the brain slices in acetone should not exceed 10-14 days, because it may lead to shrinkage if kept for a longer period. Minimal or normal shrinkage was obtained within 7-10 days and reached 8% in fixed and plastinated specimens (Figure 6C and 6D).

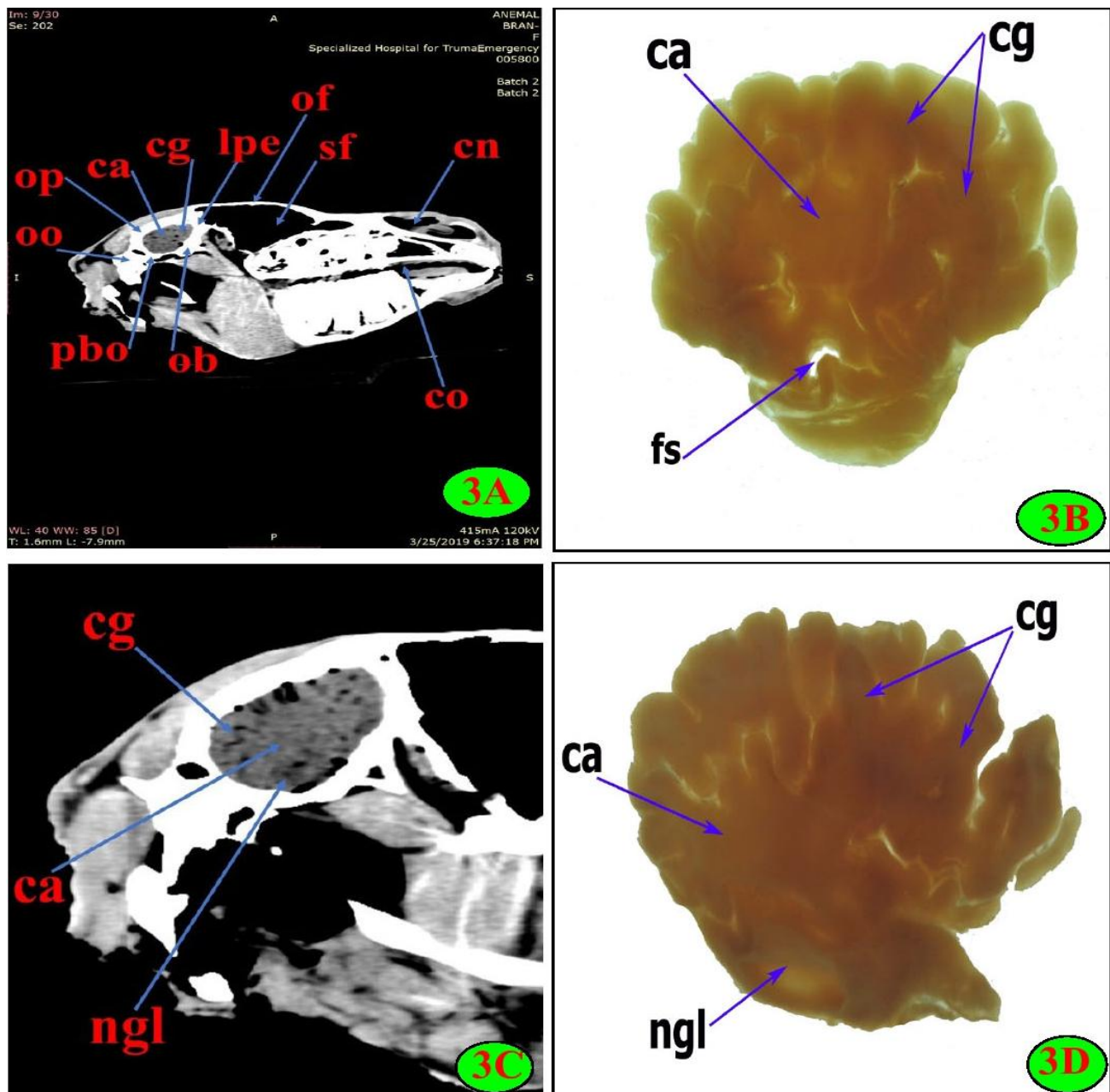


Figure 3. The computed tomographic images of donkey head (cranium region) (A,C) in corresponding to the same levels of plastinated longitudinal brain sections (P40) (Left half of brain) (Medial view) (B,D). Fissura sylvia (fs), Commissura alba (ca), Commissura grisea (cg), Os occipitale (oo), Pars basilaris occipitale (pbo), Os basisphenoidale (ob), Cavum nasi (cn), Cavum oris (co), Os frontale (of), Sinus frontalis (sf), Os parietale (op), Lamina perpendicularis ethmoidale (lpe) and Nucleus geniculatus lateralis (ngl).

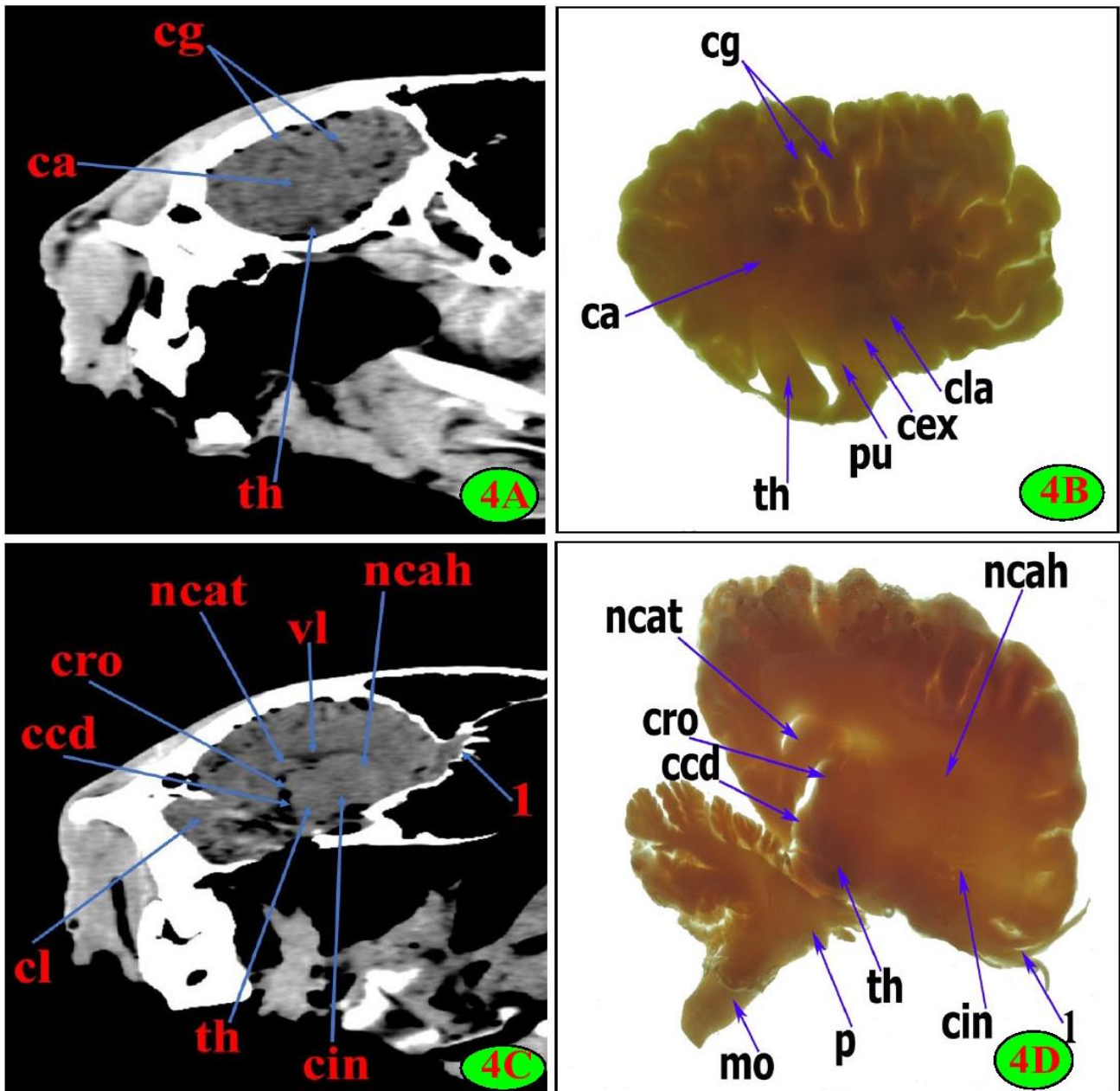


Figure 4. The computed tomographic images of donkey head (cranium region) (A,C) in corresponding to the same levels of plastinated longitudinal brain sections (P40) (Left half of brain) (Medial view) (B,D). Commissura alba (ca), Commissura grisea (cg), Thalamus (th), Putamen (pu), Capsula externa (cex), Clastrum (cla), Nucleus caudatus (tail) (ncat), Colliculus rostralis (cro), Colliculus caudalis (ccd), Nucleus caudatus (head) (ncah), Capsula interna (cin), Nervi olfactorii (1), Pons (p), Medulla obligate (mo), Cerebellum (cl) and Ventriculus lateralis (vl).

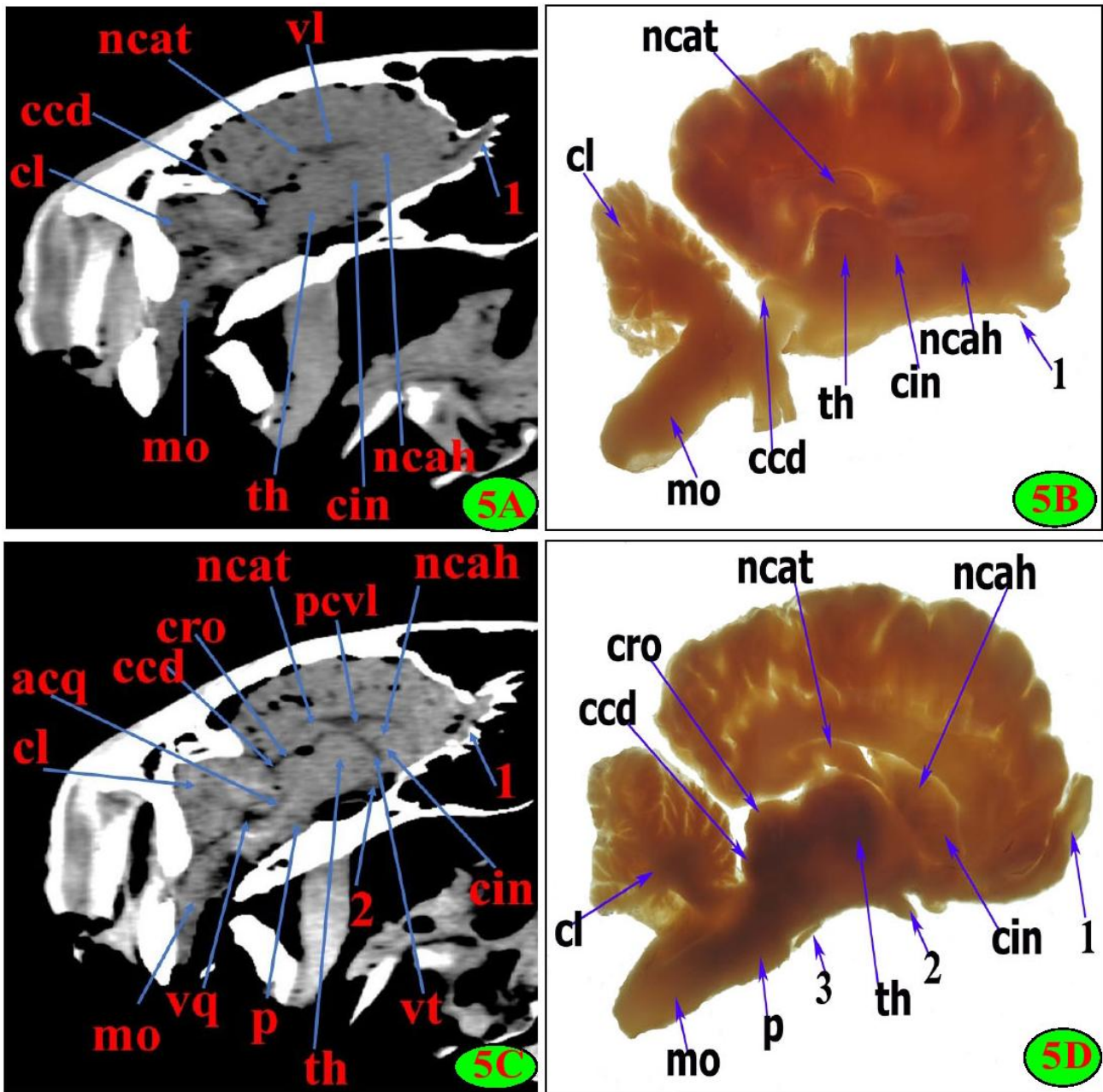


Figure 5. The computed tomographic images of donkey head (cranium region) (A,C) in corresponding to the same levels of plastinated longitudinal brain sections (P40) (Left half of brain) (Medial view) (B,D). Thalamus (th), Nucleus caudatus (tail) (ncat), Colliculus rostralis (cro), Colliculus caudalis (ccd), Nucleus caudatus (head) (ncah), Capsula interna (cin), Nervi olfactorii (1), Pons (p), Medulla obligate (mo), Cerebellum (cl), Ventriculus lateralis (vl), Nervus opticus (2), Nervus oculomotorius (3), Plexus choroidea ventriculus quartus (pcvl), Aqueductus cerebri (aqc) and Ventriculus tertius (vt).

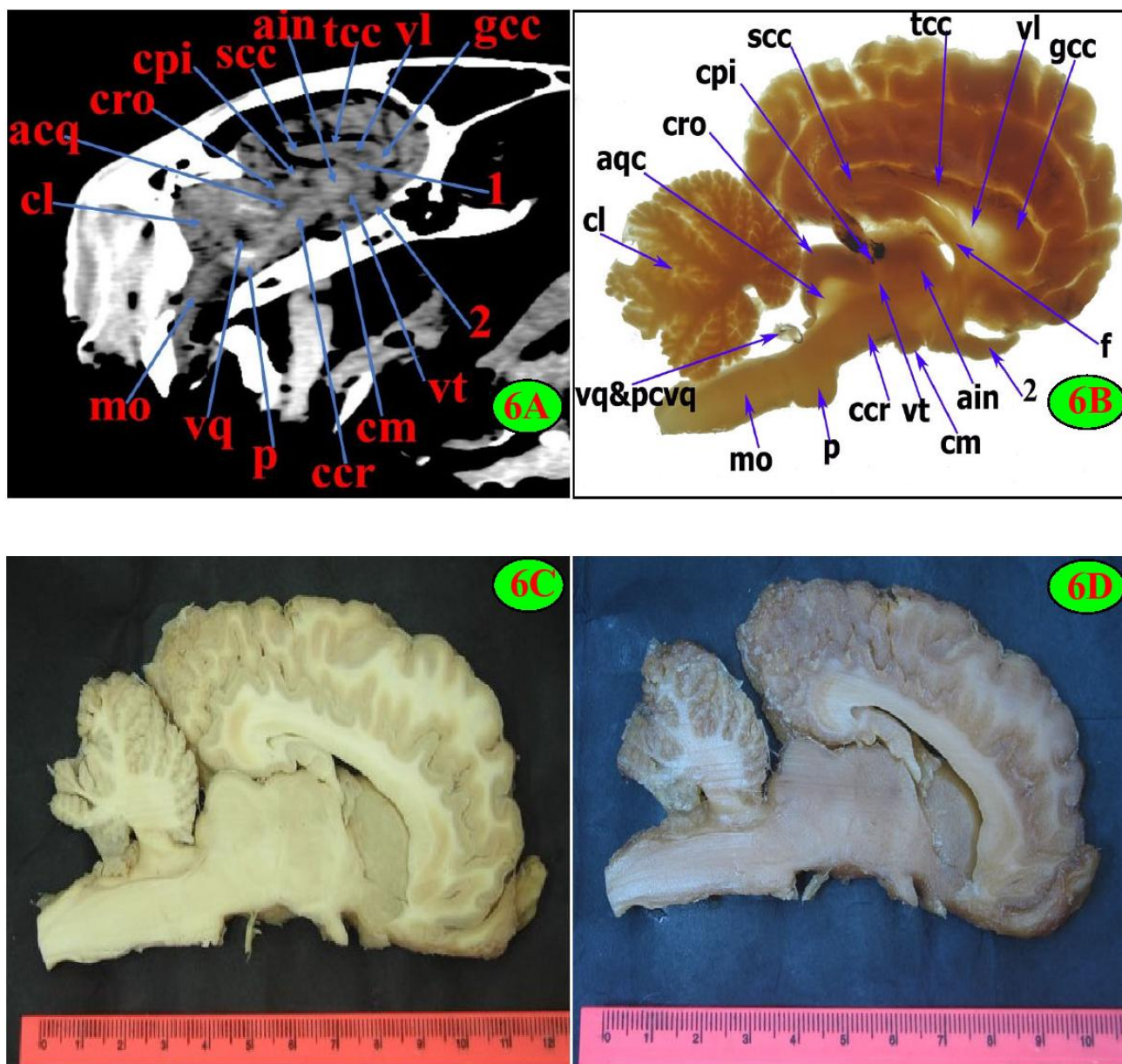


Figure 6. The computed tomographic image of donkey head (cranium region) (A) in corresponding to the same level of plastinated longitudinal brain section (P40) (Left half of brain) (Medial view) (B). Colliculus rostralis (cro), Nervi olfactorii (1), Pons (p), Medulla obligate (mo), Cerebellum (cl), Ventriculus lateralis (vl), Nervus opticus (2), Aqueductus cerebri (aqc), Adhesio interthalamica (ain), Corpus pinealis (cpi), Crus cerebri (ccr), Genu corporis callosi (gcc), , Ventriculus tertius (vt), Truncus corporis callosi (tcc), Splenium corporis callosi (scc), Fornix (f), Corpus mamillare (cm) and Ventriculus quartus and Plexus choroidea ventriculus quartus (vq&pcvl); Formalinized 4mm paramedian section of the left half of the donkey brain before immersion stage; total length: 12.3 cm (Section 7) (Medial view) (C) and after immersion stage; total length: 10 cm (D).

Survey

The survey described the opinion of 100 second-year veterinary medical students from Anatomy and Embryology department about using different self-learning anatomical tools including CT brain images and P40 brain slices. A majority of students (52%) expressed that using plastinated slices are better teaching aid as it is sensible, handable and depicts most of the brain anatomical structures. In this survey, 38% of students preferred to learn through both the techniques to compensate the invisible structures in one technique. Only 5% of students opined that both the techniques are equal for learning. Five percent mentioned that the CT images create more interest and motivation as well as availability at any time and easy storage makes them a preferable method. All the students strongly agreed that these methods were supporting and very useful in self-studying at home.

DISCUSSION

Long exposure to formaldehyde gas is hazardous to health (carcinogenic, mutagenic and toxic effects). Formaldehyde discolors the tissues and emits vapors which are irritant to the eyes and respiratory tract. Repeated and long exposure to formalin can lead to cancer of the respiratory system, asthma, and bronchitis (Sivrev et al., 2013; Alnagar et al., 2018; Jones et al., 2019). It is needed to apply new teaching aids which are safer, more economical and more effective in visualizing the anatomical structure and its surrounding relations to improve the quality of anatomy learning.

P40 plastination technique and CT scanning confer highly qualified teaching aids for sectional anatomy (Latorre et al., 2002). The current study introduced two recent teaching carrels of the donkey brain including sheet plastination using P40 and its corresponding CT images to describe the brain anatomical structures for the benefits of radiologists, veterinary clinicians and also for accurate detection of small lesions by surgeons. CT images of the donkey cranium served as a reference for different anatomical structures of the brain and interpreted well with corresponding sagittal plastinated sections, except some structures could not be seen on the corresponding CT images. Lieve et al. (2005) identified numerous structures of the anatomic sections of canine brain using the CT images. CT imaging of the brain provided an amazing view and a good method in the diagnosis of the cranium disorders in the donkey. Lieve et al. (2005) used CT scanning for diagnosis of neuropathological conditions of the dog brains. The current study clarified the normal neuroanatomical structures to detect the pathological one. CT imaging in veterinary medicine is limited due to its expensive cost, less availability and the size of animals such as foals and adult horses (MacKay 2005; Dickey et al. 2011).

Most of the anatomic atlases for CT imaging obtained from comparing between *in vivo* images and the corresponding formalized anatomical slices obtained *ex vivo* (Solano and Brawer 2004; Probst et al 2005). The use of plastinated P40 slices accompanied with CT brain scanning allows to better understand complex anatomical structures and their adjacent tissues. Moreover, plastinated specimens facilitate the study of three-dimensional characteristics in certain structures such as deep nuclei, the origin of the cranial nerves and extension of the ventricular system. Legrand and Carlier (1999) identified the choroid plexus of the lateral ventricles by CT evaluation of the dog head.

Parallel with Cabrera et al. (2015), the CT images of the brain and associated structures of normal neonatal foals provided excellent detail of clinical anatomy. Previous studies reported the normal anatomy of the equine head showed similar results with CT images (Arencibia et al., 2000; Smallwood et al., 2002; De Zani et al., 2010). Although, the findings of the present study and Karen et al. (2000) noticed that most of the cranial nerves (VII, IX, X, XI, and XII) surrounding the pouches could not be identified on CT images. In agreement with Assheuer and Sageer (1997), the presence of CSF in the ventricles and subarachnoid space facilitated the accurate identification of the surrounding structures and tissues in CT images obtained in the present investigation.

The present study found a precise correlation between sectional CT images and sheet P40 plastination. Some morphological parts observable in plastinated slices were more accurate and clearer than in CT images. Thus, studying of plastinated specimens improve the diagnosis, understanding of clinical signs and pathology of some central nervous system lesions (Reina et al., 2015). In present study revealed that both thin plastinated P40 slices and CT brain images were helpful in differentiating between white and gray matter. Morphological characteristics of the ventricular system, as well as the basal ganglia, were observed in P40 slices. In addition, plastinated slices conferred detailed knowledge about very small anatomical structures that cannot be distinguished on CT images, for example, some brainstem nuclei. The present study agreed with a study by Pauline et al. (2015) who reported the obtained brain P40 slices were clean, dry, odorless, light, non-toxic, non-fragile, not sticky to touch and durable, however, CT imaging showed better anatomical details of the organs than plastinated slices that were inconsistent with findings from the current study.

The P40 plastination sections of donkey brain have enough rigidity compared to formalized specimens, due to enough curing. Plastination sections were more economical and easier to use for teaching purposes. The weight of plastinated sheets with P40 is about one-tenth of the weight of formalized one (O'Sullivan and Mitchell, 1995 and Weiglein, 1997). Five steps followed during the plastination of P40 technique were in accordance with Sivrev et al. (2013). Although Sivrev et al. (2013) and Henry and Latorre (2007) had used 10 % formalin solution (4-6 weeks) for fixation of the brain, this study added that, the well-fixed brains may need more time (1-2 weeks) in 5% formalized solution. The slicing procedure followed in the present study was similar to that mentioned by Mircea-Constantin et al. (1999). The present study agreed with Henry and Latorre (2007) that flushing of the brain slices should be performed in cool running tap water for one or two days to complete removal of debris and formalin.

Although dehydration leads to shrinkage, the P40 technique is much better than the S10 standard one for plastination of brain slices. Shrinkage is the main problem in plastination processes, which in turn depends on the technique used (S10, E12 or P40). A notable shrinkage will decrease the value of the specimens due to change its shape and size (Sora et al., 2002). Mircea-Constantin et al. (2015) suggested that shrinkage rates under 10% is normal and indicate correct plastination procedure. In the present study shrinkage rate of 8% was observed.

The time and concentrations of specimen dehydration in the present study were in agreement with Sivrev et al. (2013). The ratio of specimen weight to volume of acetone was 1:10 in accordance with Henry and Latorre (2007). Henry and Latorre (2007) and Sivrev et al. (2013) used polyester P40 with hardener A4 in ratio 100:2 whereas activator A9 was used in the present study.

In the study by Sivrev et al. (2013), curing of P40 resin was done by exposure to artificial UVA and sunlight. Although the mentioned study used 40 watts UVA light tubes in hardening while 200 watts light was used in the present study. Henry and Latorre (2007) stated that P40 resin must be kept in dark as UVA light works as the catalyst for polymerization. Arencibia et al. (2003) turned on the UV lights for 15 minutes then turned off for 30 minutes to avoid excess heat release during the curing step and used a ventilator to cool the chambers.

In the present study, using semi-transparent P40 slices from the imaged specimens of the same animal allowed accurate assessment of CT images in many anatomic structures. Though using brains of different animals in both techniques may provide asymmetric levels of P40 sections and CT images. Most features of the brain identified on anatomic sections could be identified on the corresponding CT scans despite the low contrast between anatomic structures and adjacent bony and soft tissues. The rapid diagnosis of diseases by using imaging techniques requires a deep detailed knowledge of normal anatomy, three-dimensional morphology and the topographical relationships with adjacent structures which were achieved by sheet P40 brain slices. Most the veterinary medical students have the same opinion that using P40 sheet plastination of the brain will greatly facilitate the understanding of the neuroanatomical structure especially in collaboration with labeled CT images.

CONCLUSION

P40 plastination technique was developed as a quick, less expensive and less complex method for preparing of brain slices which are suitable for teaching purposes. This technique provided translucent brain slices which is a new innovative method in sectional anatomy. These unique slices are excellent teaching aids especially when are correlated parallel with modern diagnostic images such as CT. Both techniques allow an accurate assessment of many complex anatomical three-dimensional structures and relationships which has wide application in neurology, neurosurgery, radiology, and neuroradiology. In addition, the interpretation of CT images is easier in the presence of the corresponding plastinated brain sections.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

Authors' contributions

Dr. Sherif Khayri Abdelmoati Mohamed collected the samples, designed the P40 experiment and sent animal for CT imaging, also was responsible for writing, revising and submission of the manuscript. Dr. Eman Ismail El-behery and Dr. Eman Abdelrahman Ahmed Mahdy obtained the data, critically revised the manuscript for important intellectual contents. All authors approved the final version of manuscript before publication.

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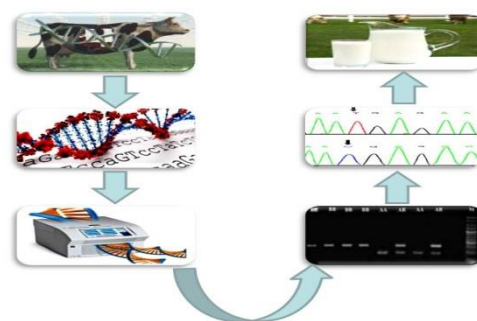
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Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; Chukwura, 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001).

--Examples (at References section)

a) For journal:

Lucy MC (2000). Regulation of ovarian follicular growth by somatotropin and insulin- like growth factors in cattle. *Journal of Dairy Science*, 83: 1635-1647. DOI: XXX

Kareem SK (2001). Response of albino rats to dietary level of mango cake. *Journal of Agricultural Research and Development*. pp 31-38. DOI: XXX

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. *African Journal of Biotechnology*. 7: 3535-3539. DOI: XX

b) For symposia reports and abstracts:

Cruz EM, Almatar S, Aludul EK and Al-Yaqaout A (2000). Preliminary Studies on the Performance and Feeding Behaviour of Silver Pomfret (*Pampus argentens euphrasen*) Fingerlings fed with Commercial Feed and Reared in Fibreglass Tanks. *Asian Fisheries Society Manila, Philippine* 13: 191-199. [Link](#)

c) For edited symposia, special issues, etc., published in a journal:

Korevaar H (1992). The nitrogen balance on intensive Dutch dairy farms: a review. In: A. A. Jongebreur et al. (Editors), *Effects of Cattle and Pig Production Systems on the Environment: Livestock Production Science*, 31: 17-27. [Link](#)

d) For books:

AOAC (1990). *Association of Official Analytical Chemists. Official Methods of Analysis*, 15th Edition. Washington D.C. pp. 69-88. [Link](#)

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications*. McGraw-Hill Inc., New York, pp. 591-603. [Link](#)

e) Books, containing sections written by different authors:

Kuney M (1979). Pig Fattening. In: A. Alexiev (Editor), *Farm Animal Feeding*. Vol. III. Feeding of Different Animal Species, Zemizdat, Sofia, p. 233-243 (Bg). [Link](#)

In referring to a personal communication the two words are followed by the year, e.g. (Brown, J. M., personal communication, 1982). In this case initials are given in the text.

Nomenclature and Abbreviations

Nomenclature should follow that given in NCBI web page and Chemical Abstracts. Standard abbreviations are preferable. If a new abbreviation is used, it should be defined at its first usage. Abbreviations should be presented in one paragraph, in the format: "term: definition". Please separate the items by ";".

E.g. ANN: artificial neural network; CFS: closed form solution...

Abbreviations of units should conform to those shown below:

Decilitre	dl	Kilogram	kg
Milligram	mg	hours	h
Micrometer	mm	Minutes	min
Molar	mol/L	Mililitre	ml
Percent	%		

Other abbreviations and symbols should follow the recommendations on units, symbols and abbreviations: in "A guide for Biological and Medical Editors and Authors (The Royal Society of Medicine London 1977).

Papers that have not been published should be cited as "unpublished". Papers that have been accepted for publication, but not yet specified for an issue should be cited as "to be published". Papers that have been submitted for publication should be cited as "submitted for publication".

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5. Levels of statistical significance which can be used without further explanations are *P < 0.05, **P < 0.01, and ***P < 0.001
6. In the English articles, a decimal point should be used instead of a decimal comma.
7. In chemical formulae, valence of ions should be given, e.g. Ca²⁺ and CO₃²⁻, not as Ca⁺⁺ or CO₃.
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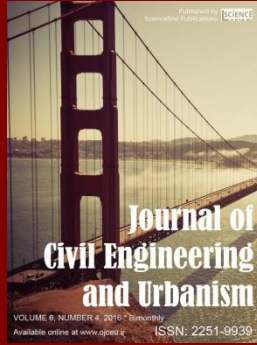
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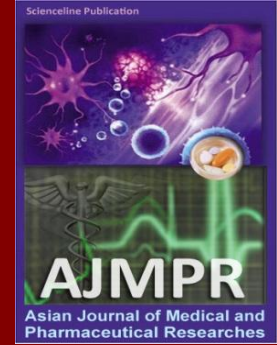
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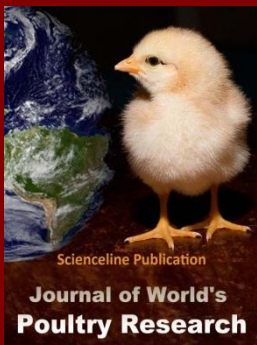
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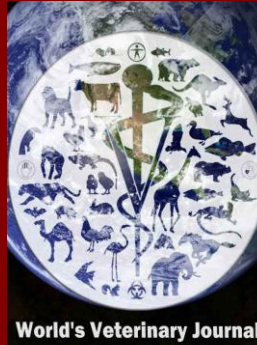
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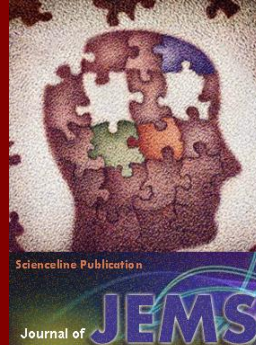
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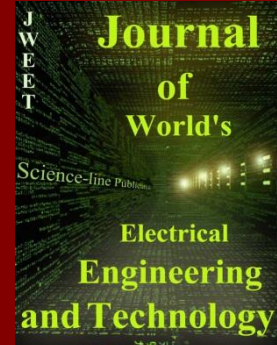
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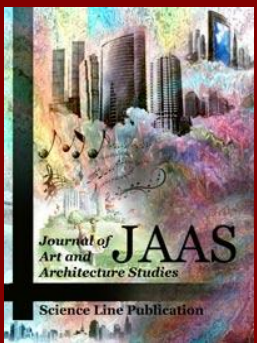
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