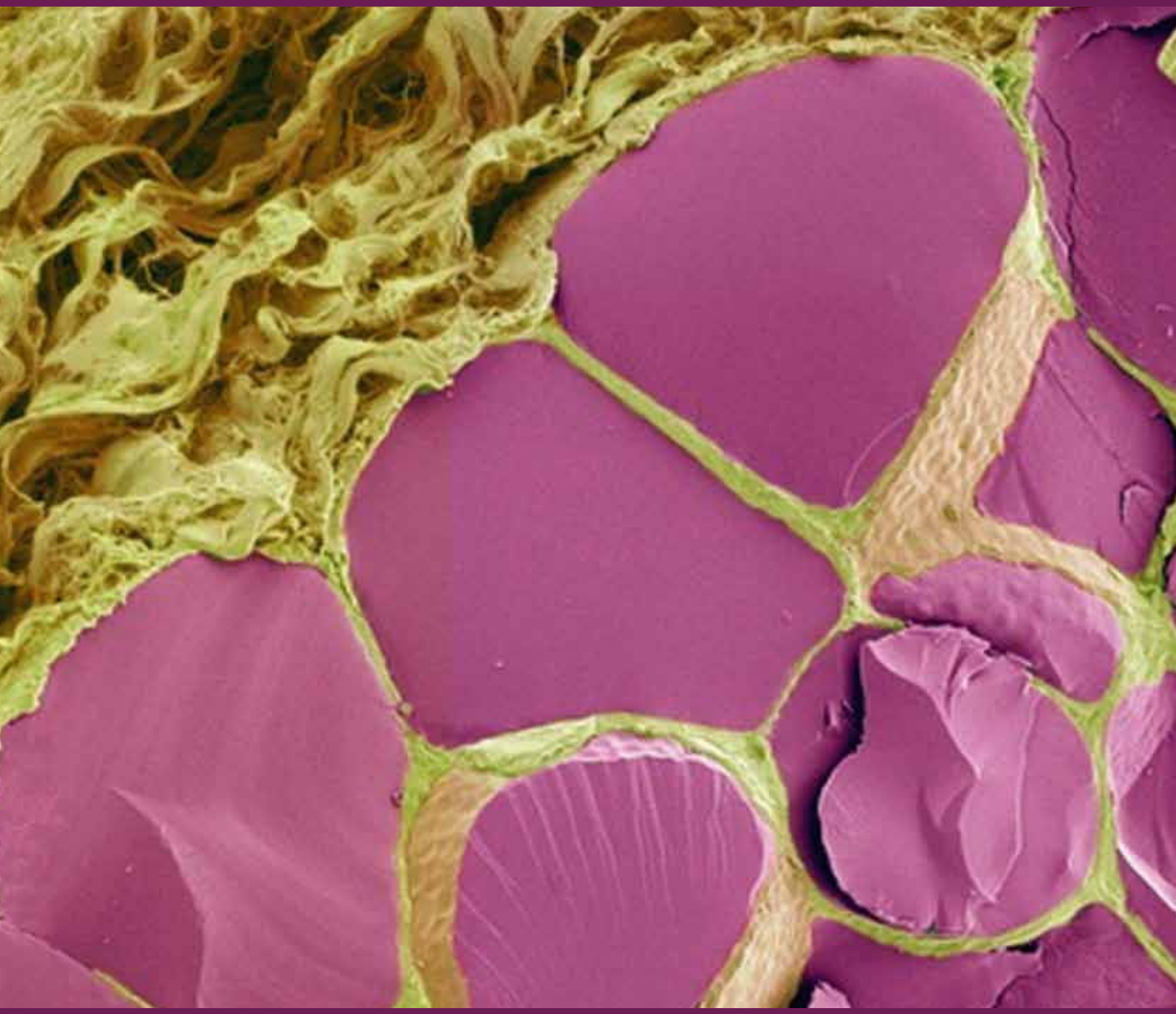
International Journal of Endocrinology

Oxidative Stress and Inflammation in Diabetic Complications

Guest Editors: Ajit Vikram, Durga Nand Tripathi, Ashutosh Kumar, and Sandeep Singh



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Editorial Board

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Editorial

Oxidative Stress and Inflammation in Diabetic Complications

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The guest editors of this issue are pleased to present this compendium of research and review articles focusing on the role of oxidative stress and inflammation in diabetes and diabetes-associated complications. Increased prevalence of insulin-resistance, a prediabetic condition, and type 2 diabetes is a major health concern all over the world. As per WHO estimates, it is expected that by 2030 the number of patients with diabetes will be more than double. Progressive deterioration in metabolic control with existing therapeutic modalities necessitates better understanding and newer therapeutic interventions for the effective management of diabetes.

Oxi-flammation (oxidative stress and inflammation) affects a multitude of cellular responses in various organ systems, and progression of insulin-resistance is known to be associated with chronic systemic inflammation and increased oxidative stress. The positive feedback cycle involving chronic systemic inflammation, oxidative stress, and progression of insulin-resistance contributes to several diabetes-associated complications, including cardiovascular diseases, nephropathy, neuropathy, retinopathy, urological diseases, and cancer [1–3]. Impairment in insulin synthesis, release, and/or action (insulin-resistance), a hallmark of diabetic condition, results in several secondary conditions. Decreased insulin-sensitivity is often accompanied by compensatory rise in the insulin level, posing an extra burden on pancreatic β-cells. In addition to maintaining plasma glucose level, insulin has a growth-stimulating effect. The IRS/PI3-Kinase dependent downstream signaling of insulin is primarily concerned with glucose uptake and metabolic effects, whereas MEK/ERK dependent signaling is responsible for its mitogenic action. Resistance does not develop equally to metabolic and mitogenic signaling of insulin [4], and therefore hyperinsulinemia results in overactivation of mitogenic signaling and has adverse effects on different tissue systems. Increased inflammation, oxidative stress, dyslipidemia, and glucotoxicity with an extra workload on β-cells eventually meet a point where β-cells are no longer able to meet the ever increasing demand of insulin, resulting in the development of frank diabetes (Figure 1). In this special issue, K. Batumalaie et al. report that gelam honey regulates the expression and/or activation of insulin-receptor signaling mediators and improves cellular insulin content in HIT-T15 cells (pancreatic β-cells) exposed to hyperglycemic condition.

Insulin-resistance promotes endothelial dysfunction and it is an independent risk factor for the development of coronary artery diseases. The review article embodied in this issue by S.I.Q.S. Ikmal et al. describes potential biomarkers of insulin-resistance and atherosclerosis in type 2 diabetic patients with coronary artery disease. J. Fuentes-Antrás et al. believe that hyperglycemia, dyslipidemia, and insulin-resistance are among the most important factors which contribute to low-grade inflammation in the diabetic heart. In this issue, their review article "*Activation of toll-like receptors and inflammasome complexes in the diabetic cardiomyopathy-associated inflammation*" suggests that toll-like receptors and inflammasome-complexes may be key inducers for inflammation probably through nuclear factor-kappa B (NF-κB)

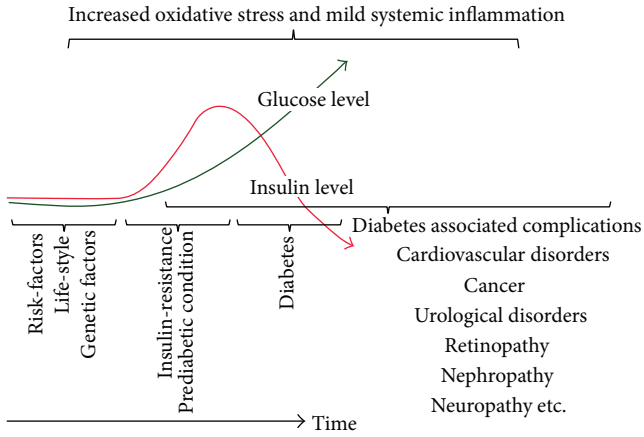

Figure 1: Life-style and genetic factors contribute to the development of oxidative stress and mild systemic inflammation resulting in decreased insulin-sensitivity which is accompanied by compensatory hyperinsulinemia and mild hyperglycemia, a prediabetic state. As β-cells fail to meet increased demand for insulin, frank diabetes develops with subnormal level of insulin and even higher glucose level. Hyperinsulinaemic condition in prediabetic state and profound dysregulation of glucose and lipid metabolism in diabetic condition further aggravate oxidative stress and inflammation. All these factors contribute to the development of diabetes associated complications.

activation and oxidative stress. However, the peroxisome proliferator-activated receptors and mammalian life-span regulator sirtuins may be the potential therapeutic targets in mitigating both toll-like receptors and inflammasome signaling. The review article by R. Sandireddy et al. outlined futuristic strategies targeting oxidative stress and neuroinflammation in diabetic neuropathy. The authors are of opinion that a combinatorial approach targeting multiple signaling pathways might be of practical use in combating diabetic neuropathy.

Heatstroke is a medical emergency condition and can have profound deleterious effect on brain and other tissue systems in body. As diabetes is characterized by deregulation of metabolic control, the patients are more likely to fall short in handling stress conditions, including heat stress. Although, the association between diabetes and heatstroke is not very clear and further studies are required, animal studies by C.-C. Hsu et al. suggest that diabetic condition augments the deleterious effects of heat stress on body temperature regulation and cerebral blood flow and is associated with neuronal death in hypothalamus. The authors have further reported that these conditions are at least partially ameliorated with the intervention of Chinese herb Honokiol.

M. Roehrs et al. reported improved glucose and lipid profile in response to Bixin, a annotto carotenoid, in streptozotocin (STZ) induced diabetic model. Moreover, Bixin treatment restored antioxidant enzyme super oxide dismutase activity and reduced oxidative stress as evident from decreased protein oxidation and nitric oxide production. On the other hand, the Norbixin, a relatively less lipophilic analogue of Bixin, was less or not effective in inducing these effects.

An increase in body fat is generally associated with increased risk of metabolic diseases such as type 2 diabetes mellitus. Body mass index (BMI) criteria are currently the primary focus in metabolic disorder treatment recommendations. S. Kaštelan et al. reported a parallel association between BMI and diabetic retinopathy.

Taurine, one of the most abundant amino acids in the mammalian organs, is known to have beneficial effects on experimental diabetic nephropathy. Here, authors have shown that taurine-mediated improvement in diabetic nephropathy could be attributed to its antioxidant property and amelioration of diabetes-induced increase or decrease in VEGF or Nephrin expression. Taurine-treated group also showed reduced reactive oxygen species levels indicating that it possibly inhibits the progression of diabetic nephropathy by strengthening antioxidant defense.

In conclusion, the natural products hold high promise to provide lead for the development of therapeutically relevant molecules. Perhaps, in contrast to the usual analytical approach, a synthetic approach where a complex mixture of molecules, which is often the case with natural products, may help us to answer complex disease conditions such as diabetes and diabetes-associated complications. Understanding of early and effective biomarkers and use of suitable protective/preventive strategies might open new therapeutic avenues for patients suffering from diabetes and its associated complication. We believe that the present issue with reviews and research articles has well summarized current development in the field and will be of interest to the readers.

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References

[1] B. E. Harcourt, S. A. Penfold, and J. M. Forbes, "Coming full circle in diabetes mellitus: from complications to initiation," *Nature Reviews Endocrinology*, vol. 9, no. 2, pp. 113–123, 2013.

[2] M. Malek, R. Aghili, Z. Emami, and M. E. Khamseh, "Risk of cancer in diabetes: the effect of metformin," *ISRN Endocrinology*, vol. 2013, Article ID 636927, 9 pages, 2013.

[3] A. Vikram, G. Jena, and P. Ramarao, "Insulin-resistance and benign prostatic hyperplasia: the connection," *European Journal of Pharmacology*, vol. 641, no. 2-3, pp. 75–81, 2010.

[4] Z. Y. Jiang, Y. W. Lin, A. Clemont et al., "Characterization of selective resistance to insulin signaling in the vasculature of obese Zucker (fa/fa) rats," *Journal of Clinical Investigation*, vol. 104, no. 4, pp. 447–457, 1999.

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Review Article

Neuroinflammation and Oxidative Stress in Diabetic Neuropathy: Futuristic Strategies Based on These Targets

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In Diabetes, the chronic hyperglycemia and associated complications affecting peripheral nerves are one of the most commonly occurring microvascular complications with an overall prevalence of 50–60%. Among the vascular complications of diabetes, diabetic neuropathy is the most painful and disabling, fatal complication affecting the quality of life in patients. Several theories of etiologies surfaced down the lane, amongst which the oxidative stress mediated damage in neurons and surrounding glial cell has gained attention as one of the vital mechanisms in the pathogenesis of neuropathy. Mitochondria induced ROS and other oxidants are responsible for altering the balance between oxidants and innate antioxidant defence of the body. Oxidative-nitrosative stress not only activates the major pathways namely, polyol pathway flux, advanced glycation end products formation, activation of protein kinase C, and overactivity of the hexosamine pathway, but also initiates and amplifies neuroinflammation. The cross talk between oxidative stress and inflammation is due to the activation of NF-κB and AP-1 and inhibition of Nrf2, peroxynitrite mediate endothelial dysfunction, altered NO levels, and macrophage migration. These all culminate in the production of proinflammatory cytokines which are responsible for nerve tissue damage and debilitating neuropathies. This review focuses on the relationship between oxidative stress and neuroinflammation in the development and progression of diabetic neuropathy.

1. Introduction

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia associated with symptoms like polydipsia, polyphagia, polyuria, blindness, weight loss or gain, sore heals, burning and tingling sensation, and so forth. Diabetic population of the world in 2013 was 382 million and it has been projected to rise to 592 million by the year 2035 [1]. Diabetes has become a challenging health problem affecting the global population and the prevalence is higher in developing countries. Among the top 10 countries having highest number of people with diabetes, 8 are middle-income rapidly developing countries. There will be a 42% increase in the developed countries and a 170% increase in the developing countries in diabetic cases by 2030 [2].

Diabetes is associated with both macrovascular and microvascular complications, in which the major microvascular complication is diabetic neuropathy (DN) with a prevalence of 50–60% [3]. The neuropathy progresses with decreasing nerve functionality and nerve blood perfusion which may result in malnourished nerve and leads to permanent nerve damage. The clinical manifestations of diabetic neuropathy include numbness, burning and tingling sensation, and intractable pain [4].

Although hyperglycemia is considered to be a major pathophysiological factor in the development of diabetic neuropathy, the associated mechanisms are not fully understood yet. Some of the major pathways like polyol pathway [5], advanced glycation end products [6], hexosamine flux [7], mitogen-activated protein kinases [8], altered activity of Na^+/K^+-ATPase [9], poly-ADP ribose polymerase (PARP) over activation [10], and cyclooxygenase-2 (COX-2) activation [11] have been reported to play a crucial role in development and progression of diabetic neuropathy (Figure 1). Nerve cells are prone to hyperglycemic injury as the neuronal glucose uptake is based on external glucose concentration

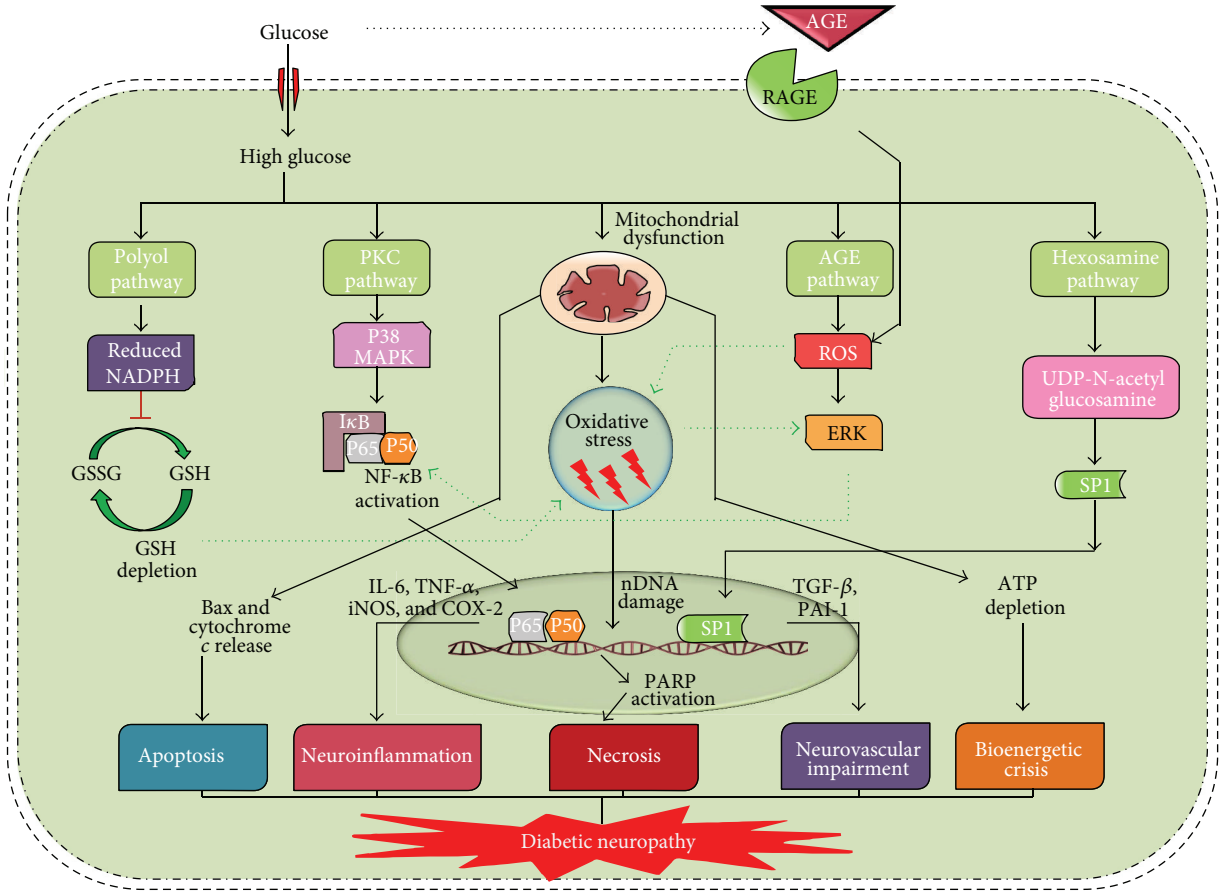

Figure 1: Pathophysiology of diabetic neuropathy. Hyperglycemia activates numerous metabolic pathways like polyol pathway, protein kinase c (PKC) pathway, advanced glycation end products (AGE) pathway, and hexosamine pathway. All these pathways are known to integrate through hyperglycemia mediated mitochondrial ROS production. Oxidative stress and these classical pathways in combination activate transcription factors such as nuclear factor kappa enhancer of B cells (NF-κB) and speciality protein-1 (SP-1), resulting in neuroinflammation and vascular impairment. Further, these pathways combined with dysfunctional mitochondria mediated apoptosis or bioenergetic depletion can lead to neuronal damage lading to DN. Poly-ADP ribose polymerase (PARP) mediated NADH/ATP depletion can lead to neuronal dysfunction due to failure of various energy dependent processes in neurons. (ERK: extracellular related kinase, IL-6: interleukin-6, iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenase-2, TGF-β: transforming growth factor-β, and PAI-1: plasminogen activator inhibitor-1.)

which is 4-5-fold higher in diabetic subjects. It has been noted in experimental diabetes that the levels of neurotrophic support, including nerve growth factor and insulin like growth factor are reduced [12], which also contribute to malnourishment of nerves. All these pathways form a common platform with end result as neuronal dysfunction and nerve damage and this translates in the development of various clinical deficits seen in patients suffering from diabetic neuropathy.

Treatment of painful diabetic neuropathy presents a great challenge to clinicians due to poor diagnostic criteria and the limited treatment options available. Currently, drugs in clinical use for diabetic neuropathic pain include tricyclic antidepressants, selective serotonin and noradrenaline reuptake inhibitors, anticonvulsants, and opioids [13]. Although there is a range of pharmacological agents available for treating the pain associated with diabetic neuropathy, only duloxetine and pregabalin are approved by US Food and Drug Administration (US FDA) for the treatment of diabetic neuropathic pain. However, as single agents, they are limited by incomplete efficacy, high cost, and dose limiting adverse effects [14–16].

In spite of the voluminous research done in the field of diabetic neuropathy, clear understanding of the pathophysiology and the interwoven mechanisms are still lacking. There is a need to investigate futuristic, combinational, and other nonpharmacological approaches for alleviating DN and associated neuronal deficits.

2. Classical Pathways of Hyperglycemic Vascular Damage

Diabetic neuropathy is a syndrome which can affect both the somatic and autonomic divisions of the peripheral nervous system. In severe diabetic conditions, longer nerve fibres show an earlier loss of nerve conduction velocity with loss of their nerve terminals. The damaged nerve terminals are the reason for tingling and loss of sensation and reflexes

are often first observed in the feet and then they ascend to affect other areas [4]. One of the major causes for all these complications is reactive oxygen species (ROS) produced from processes initiated and amplified under chronic hyperglycemic conditions. Further, classical hyperglycemic pathways like polyol pathway, protein kinase C (PKC) pathway, formation of advanced glycation end products (AGE pathway), and hexosamine pathway activation leads to aggravation of oxidative damage leading to vascular complications [17]. In polyol pathway, aldose reductase enzyme converts glucose into sorbitol, which is then oxidized into fructose by sorbitol dehydrogenase (SDH) with NAD^+ as a cofactor. In case of hyperglycemic conditions, increase in oxidative stress directly results from the accumulation of sorbitol and indirectly through consumption of NADPH, a cofactor for the regeneration of reduced glutathione (GSH) [18]. Increased flux through polyol pathway can decrease (Na^+/K^+) ATPase activity and studies suggested that decreased activity of this enzyme may activate PKC pathway [19]. Activation of PKC increases cytosolic phospholipase A2 activity and produces arachidonate and prostaglandin E_2 (PGE_2) which effectively inhibits cellular (Na^+/K^+) ATPase [20]. Persistent and excessive activation of several PKC isoforms initiates tissue injury by diabetes-induced ROS [21], which leads to enhanced de novo synthesis of DAG from glucose via triose phosphate. Increased levels of triose phosphate concentrations can increase the formation of both methylglyoxal, a precursor of AGEs, and diacylglycerol (DAG), an activator of PKC [22]. Evidence suggests that the enhanced activity of PKC isoforms results in activation of various signalling mechanisms like mitogen-activated protein kinases (MAPK), nuclear factor kappa light chain enhancer of B cells (NF-κB), and thus leads to initiation of inflammation as depicted in Figure 1. Overactivation of PKC has been also implicated in the decreased nitric oxide (NO) production in smooth muscle cells and increased expression of fibrinolytic factor, plasminogen activator inhibitor (PAI-1), tumor growth factor-β (TGF-β), and NF-κB activation in cultured endothelial cells and in case of vascular smooth muscle cells [23]. AGE pathway activation results in production of many advanced glycation end products, which act on specific receptors like receptor for advance glycation end products (RAGE) present on monocytes and endothelial cells to increase the production of cytokines and adhesion molecules and also causes the alteration of protein structure. AGEs have been shown to have an effect on matrix metalloproteinases, which might damage nerve fibres [24]. AGE receptor ligation can activate transcription of pleiotropic factor NF-κB and thus enhances the production of various proinflammatory mediators (Figure 1) [25]. Hyperglycemia and insulin resistance induced excess fatty acid oxidation also appears to be reason for pathogenesis of diabetic complications [26]. In hexosamine pathway, fructose-6-phosphate converts to glucosamine-6-phosphate by glutamine fructose 6-phosphate amidotransferase (GFAT). Glucosamine-6-phosphate then converts into UDP-N acetyl glucosamine with the help of specific O-GlcNAc transferases. Evidence suggest that inhibition of GFAT blocks hyperglycemia-induced transcription of both TGF-α and TGF-β1 [27]. The mechanism behind the hyperglycemia-induced expression of genes such as PAI-1, tumor growth factor-α (TGF-α), and TGF-β1 is not clear. However, it has been observed that hyperglycemia causes a fourfold increase in O-GlcN acylation of the transcription factor specificity protein 1 (Sp1), which mediates hyperglycemia-induced activation of the plasminogen activator inhibitor-1 (PAI-1) promoter in vascular smooth muscle cells and of TGF-β1 and PAI-1 in arterial endothelial cells (Figure 1) [28]. Activation of PAI-1, TGF-α, and TGF-β1 causes accumulation of extracellular matrix which may lead to neuroinflammation associated with diabetic neuropathy [29].

3. Oxidative Stress and Mitotoxicity: Role in Neuronal Dysfunction

Hyperglycemia induces activation of classical pathways like AGE, PKC, hexosamine, and polyol pathways to mediate cellular damage [17]. However, the hyperglycemic cell injury is the result of cumulative occurrence of this cascade of pathways discussed in the previous section [22].

Generation of superoxide from mitochondrial electron transport chain is known to contribute towards hyperglycemia initiated various etiological pathways. Hyperglycemia enhances the reducing equivalents to electron transport chain (ETC) and the electrochemical potential across the inner mitochondrial membrane and hence increases superoxide production [22]. Superoxide inhibits glyceraldehyde phosphate dehydrogenase (GAPDH) either directly or indirectly through PARP mediated $NADH^+$ depletion [30, 31]. Inhibition of GAPDH by ROS leads to accumulation of glycolytic intermediates upstream of this enzyme and redirected to initiate cellular pathways like AGE formation. Once the AGEs are formed, they bind to RAGE and activate many other crucial pathways like NF-κB and PARP. PKC pathway is activated through dihydroxy acetone phosphate mediated diacylglycerol (DAG) activation. Hexosamine pathway which is activated through enhanced flux of fructose-6-phosphate and polyol pathway by elevated glucose levels [17]. This, in turn, leads to osmotic stress in the cells which further takes the cell towards necrotic cell death. Enhanced activity of Mn-SOD, a mitochondrial form of superoxide dismutase (SOD) or overexpression of uncoupling proteins (UCP-1) in experimental diabetic animals, prevents the development of vascular complications in the animals and also reduced oxidative stress mediated neuronal damage [31, 32]. The mechanism for this neuroprotective effect can be the reduction of mitochondrial ROS generation and the clearance of the notorious ROS from the cells.

In addition to the above theory, mitochondrial abnormalities and mitochondria associated oxidative stress stands at a central position in the pathogenesis of diabetic neuropathy [33]. It has been noticed that defects in functioning of ETC chain components compromises ATP production and enhances the generation of free radicals. The free radicals generated causes damage to mitochondrial DNA (mt DNA) and nuclear DNA (n DNA) which in turn aggravates mitochondrial damage [34]. This vicious cycle developed inside mitochondria produces intense oxidative stress and drives the

cell towards apoptotic/necrotic death [35]. It is an established fact that diabetes is known to affect the respiratory capacity of ETC functional complexes and thus alters ATP production (Figure 1). Mainly complex I and complex III are known to be affected, which turn out to be electron leakage centres and thus inflates ROS production [34].

In addition to disturbed mitochondrial functionality, its dynamics (size, shape, and number) is also known to be affected in diabetic neuropathy [36]. Changes in mitochondrial morphology, movement characteristics can affect the transfer in axons which can lead to various sensorimotor changes. The glove and stocking pattern of thermal sensitivity is due to impairment in the anterograde axonal transport in sensory neurons [37]. Dysfunctional mitochondria can also mediate cell death through execution of apoptotic pathways, by releasing pro apoptotic factors from mitochondria into the cytosol [35]. Various experimental observations point towards the critical role of mitotoxicity in the pathophysiology of diabetic neuropathy.

4. Neuroinflammation and Role in Peripheral Nerve Damage

Diabetic peripheral neuropathy is characterized by debilitating pain and sensory loss which leads to diminished quality of life. Persistent hyperglycemia is believed to be underpinning for the neuroinflammation and nerve damage leading to the neuropathic pain. All the characterized classical pathways like polyol pathway, PKC pathway, MAPK pathway, and increased production of AGEs could directly or indirectly initiate and progress the production of inflammatory mediators [13]. Especially the accumulation of AGE products of proteins and lipids stimulate the generation of inflammatory mediators and activation of transcription factor NF-κB, a potent inducer of inflammatory processes [38]. These AGEs act on various receptors present on microglia and macrophages stimulate production of cytokines like IL-1, IL-6, IL-17, TNF-α, chemo attractant protein-1, C-reactive protein and chemokines like CCL-2, CXC, and so forth (Figure 1) [39, 40]. Activation of RAGE can induce inflammatory cascade through the activation of NF-κB pathway. NF-κB is a transcription factor that upregulates the gene expression of proinflammatory cytokines and also is responsible for the induction of neuronal apoptosis. Activation of NF-κB also suppresses the expression of antioxidant genes by downregulating Nrf-2 pathway and thus indirectly weakening the innate antioxidant defense (Figure 2) [41]. Persistent hyperglycemia induced inflammation also affects the structural features of neuron as the glycosylation of myelin protein alters its antigenicity causing infiltration of monocytes, macrophages, neutrophils from the blood circulation, and activation of glial cells of the nervous system [24, 42]. These immune cells in turn secrete inflammatory cytokines which further damages myelin sheath and increases nerve excitability, thus leading to edema and neuroinflammation. The stimulated monocytes and immune cells have a vicious positive feedback loop for increasing the production of inflammatory mediators thus potentiating nerve catastrophe. The cytokines like IL-1, IL-6, and IL-17 can sensitize the peripheral receptors causing neuropathic pain [43]. Additionally, neuroinflammation leads to nerve damage due to apoptosis induced by MAPK signalling [44]. TNF-α also promotes the expression of cell adhesion molecules which are capable of decreasing the blood perfusion rate and thus decreases neurotrophic support [42]. The released chemokines have been shown to produce hyperalgesia through the activation of chemokines receptors present on the nerves. Hypoxia and ischemia created in diabetes also aggravate the neuroinflammation through the induction of inducible nitric oxide synthase (iNOS), which releases NO, a physiological mediator of inflammation [45]. In large, activation of inflammatory cascade, proinflammatory cytokine upregulation, and neuroimmune communication pathways plays vital role in structural and functional damage of the peripheral nerves leading to the diabetic peripheral neuropathy.

5. Cross Talk between Inflammation and Oxidative Stress

Hyperglycemic condition is known to activate both oxidative stress and inflammatory pathways. The interaction of these two pathways complicates the hyperglycemia mediated neuronal damage. The oxidative stress induced ROS and various constitutional inflammatory pathways are known to interact at multiple levels to produce plethora of pathophysiological outcomes (Figure 2) [46].

Hyperglycemia is known to increase metabolic flux through mitochondrial electron transport chain, leading to inefficient electron transfer through redox centres and hence generating superoxide anion [22]. Excessive superoxide generation leads to production of other ROS such as H_2O_2, OH^-. Superoxide can also combine with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), a potent reactant which causes nitration of several important proteins and leads to structural and functional damage [47]. Peroxynitrite mediated DNA damage leads to activation of PARP, a nuclear enzyme which causes transfer of poly-ADP ribose units to DNA by utilising NADH energy pool. Depletion of NADH leads to bioenergetic crisis and thus drives the cell towards necrosis [48]. Necrotic cell death is known to release cellular debris, which further drives the inflammatory cells to the damaged spot and hence activates a local inflammatory episode (Figure 2).

Hyperglycemia mediated oxidative stress also activates other cellular pathways like Nrf2 and NF-κB. Activation of Nrf2 pathway enhances the production of several antioxidant and cytoprotective enzymes through transcriptional facilitation of antioxidant response element (ARE) of genome. These enzymes include SOD, GSH, HO-1, and glutathione s-transferase (GST) [49]. Activation of this Nrf2 pathway stands as one of the cellular homeostatic mechanisms to protect cells from enhanced oxidative stress. However, persistent Nrf2 activation is subdued through hyperglycemia mediated ERK activation, and hence redox homeostasis is failed in diabetic state as depicted in Figure 2 [50].

Oxidative stress mediated inflammation is known to execute NF-κB, activator protein-1 (AP-1), and MAPK pathways. ROS are known to activate inhibitory kappa –B kinase

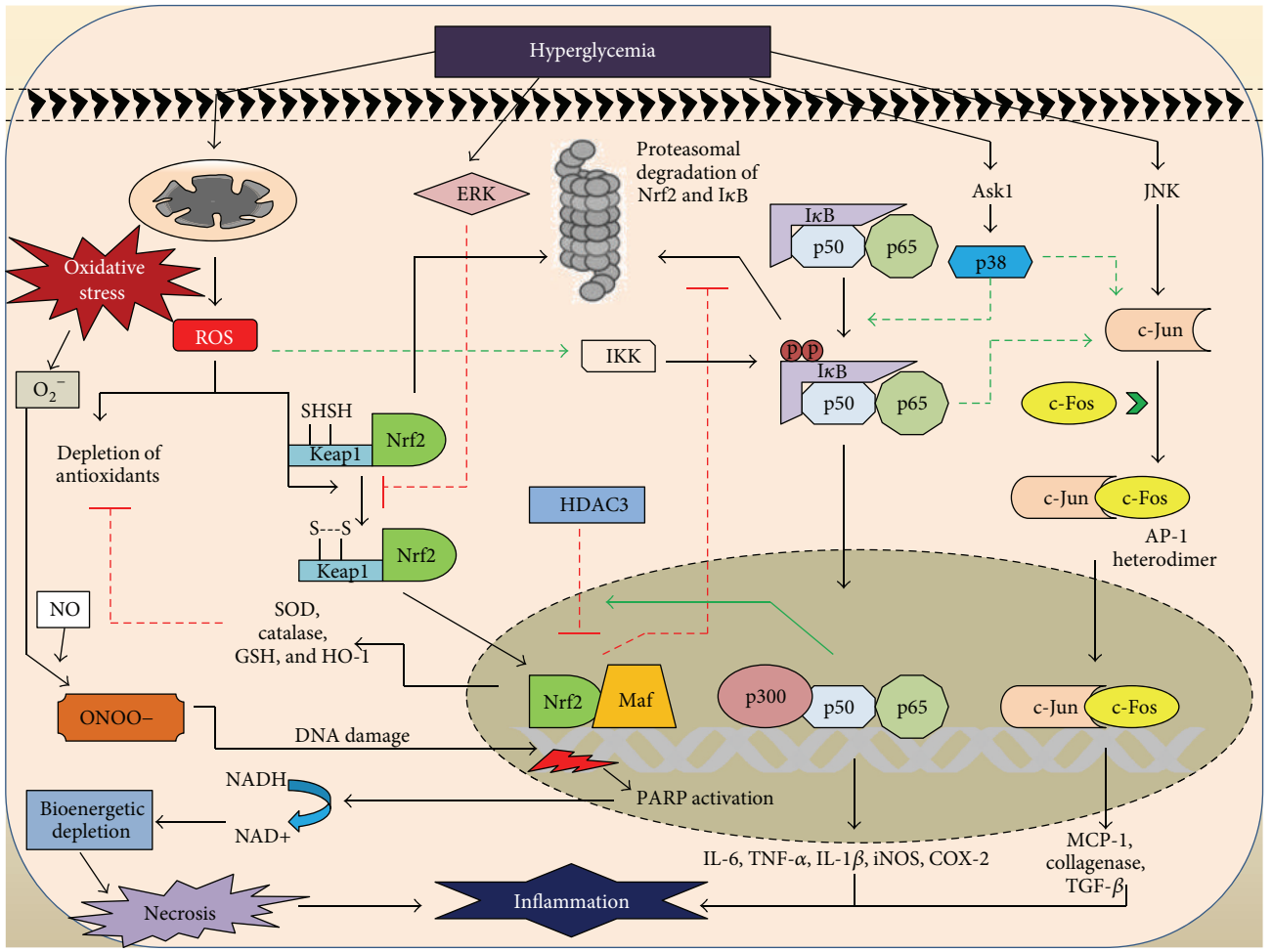

Figure 2: Crosstalk between oxidative stress and inflammation. Hyperglycemia mediated oxidative stress and inflammatory pathways are known to interact with each other at various levels. ROS activates nuclear factor (erythroid-1) related factor (Nrf2) by directly oxidising the thiol residues on kelch-like ECF associated protein (Keap-1). Nrf2 then migrates into the nucleus to activate antioxidant response elements (ARE) of genome. However, this Nrf2 activation by hyperglycemia is inhibited through extracellular related kinase activation (ERK). ROS also activates inhibitory kappa B kinase (IKK), which then phosphorylates the inhibitory kappa B protein (IκB); the latter combines with cytosolic NF-κB complex ant thus preventing its transcription. Phosphorylation of IκB labels it for ubiquitination and proteasomal degradation and, hence, releases NF-κB complex to enter into nucleus, which then expresses several proinflammatory mediators. Similarly, oxidative stress mediated c-JUN N terminal kinases (JNK) activation mediates the c-JUN component of activator protein-1 (AP-1) activation, which then combines with c-FOS subunit. The resulting AP-1 heterodimer binds with genome and increases production of various vascular inflammatory mediators. Oxidative stress mediated PARP activation also leads to inflammation through necrotic cell death. Nrf2 inhibits IκB degradation and thus prevents NF-κB mediated inflammation. NF-κB also prevents the Nrf2 signalling through histone deacetylases (HDAC3) recruitment (ASK-1-apoptosis signalling related kinase-1 and MCP-1-monocyte chemoattractant protein-1).

(IKK), which causes phosphorylation of IκB, labeling it for ubiquitination mediated proteasomal cell death [51]. Release of free NF-κB heterodimer from IκB allows it to cross nuclear membrane and bind with kappa region of genome. Transcriptional facilitation of this kappa region of genome enhances the production of inflammatory cytokines such as TNF-α, IL-6, COX-2, and iNOS as shown in Figure 2 [52]. Upstream of this pathway, NF-κB activation at cytosolic level is also facilitated by p38 MAPK. This p38 MAPK is known to be activated directly through hyperglycemia mediated apoptosis signal regulating kinase1 (ASK1) or indirectly through oxidative stress. Oxidative stress is also known to activate stress activated protein kinases, that is, c-Jun N-terminal kinases (JNK), which further activates JUN subunit of AP-1 and hence facilitate AP-1 mediated collagenase, TGF-1β, and other cytokines production (Figure 2) [53]. Although AP-1 involvement in the pathogenesis of DN needs to be explored, its activation can produce a local sequela of vascular inflammation and thus support the rationale for its participation in neuroinflammation.

Among the above mentioned pathways, the crosstalk between Nrf2 and NF-κB is critical both physiologically and pharmacologically [41]. Activation of Nrf2 pathway is known to inhibit NF-κB activation through reduced ROS mediated IKK activation and by inhibiting the degradation of IκB. Further activation of NF-κB competes with Nrf2

for binding to antioxidant response element (ARE), either directly or indirectly through recruiting histone deacetylase 3 (HDAC3) to the ARE region (represented in Figure 2) [54]. Interaction between these two pathways maintains the cellular homeostasis. However, diseases associated with excessive oxidative stress generation can cause imbalance in Nrf2-NF-κB axis and thus produce damaging consequences [41].

There is much scientific evidence supporting the involvement of inflammatory pathways in direct peripheral nerve damage and neuroinflammation. However, a growing body of researchers suggests that neuroglial cells act as connecting link between oxidative stress and neuroinflammation [55]. According to this theory, oxidative damage to glia produces excessive proinflammatory cytokines, which in turn acts on membrane receptors of neuronal cells and thus activates inflammatory pathways, causing neuroinflammation [56]. There is also evidence supporting the role of vascular inflammation in the pathogenesis of diabetic neuropathy. Accumulation of all these evidence suggests that neuroinflammation is not the sole episode underlying peripheral nerve damage but it is accompanied by inflammation and oxidative, nitrosative stress in the vasa nervorum and neuroglial cells.

6. Futuristic Strategies for Diabetic Neuropathy

Identification of pathomechanisms underlying disease pathogenesis is important to not only devise new treatment strategies but also be useful in discovering new disease biomarkers. Biomarker identification can be useful to identify the extent of disease progression and thus can amplify the scope of better drug targeting. Currently available diagnostic methods for DN include assessment of vibration perception threshold (VPT) and calculation of neuropathy disability score (NDS) based on ankle reflexes and perception changes to variety of stimuli [57]. Newer techniques with minimal invasion or noninvasive operation include corneal confocal microscopy (CCM) and skin biopsy techniques. CCM allows the identification of corneal nerve fibre length and nerve density and thus can be used as diagnostic aid to quantify peripheral neuropathy [58]. Skin biopsy and consequent immunohistochemistry allow the quantification of the number of nerve fibres per unit area [59]. However, these diagnostic procedures can be combined with examination of biochemical changes to accurately monitor the disease progression and response to treatment.

Based on the compelling evidence put forth by many research groups, it is being clear that oxidative stress mediated neurodegeneration and the accompanied inflammatory reactions play a prominent role in the pathogenesis of diabetic neuropathy [13]. Modulation of these pathways by pharmacological agents can prevent the functional and pathophysiological disturbances associated with peripheral neuropathy and can accelerate the discovery of new treatment strategies for diabetic neuropathy. Some of the important categories of drugs which have potential to affect the oxidative stress and inflammatory pathways in relevance to peripheral neuropathy will be discussed in the following section.

Oxidative stress is also known to enhance the endoplasmic stress through accumulation of misfolded proteins. Recently, the role of ER stress in the pathogenesis of diabetic neuropathy has been well observed . ER plays an important role in the proper folding and processing of proteins. Oxidative damage to the ER causes dysfunctional protein processing system and enhances the accumulation of nonfunctional proteins [60]. Chaperons are the ER proteins which help in processing newly synthesized proteins. Administration of chemical chaperons such as trimethylamine oxide and 4-phenyl butyric acid was found to inhibit the diabetes associated oxidative stress in spinal cord and dorsal horn, reduce intraepidermal nerve fibre loss, and ameliorate peripheral nerve damage and thus it can be used as a therapeutic strategy for diabetic neuropathy [61].

Nitrosative stress is also considered to be equally contributing in the pathogenesis of diabetic neuropathy as similar to oxidative stress [62]. Primarily, peroxynitrite is the toxicant of this pathway which causes biomolecular damage and PARP activation. PARP activation further depletes cellular energy pool and causes necrotic cell death [47]. Use of peroxynitrite decomposition catalysts and PARP inhibitors prevent the neuronal damage associated with diabetic neuropathy. Several trails done with these agents could alleviate the biochemical and functional impairment produced due to diabetes in sciatic nerves and dorsal root ganglion (DRG) neurons [63, 64].

Due to massive involvement of oxidative stress in the pathogenesis of diabetic neuropathy, several antioxidants have been tried in patients with diabetic neuropathy. Alpha-lipoic acid, vitamin E, and acetyl-L-carnitine were studied clinically in several controlled prospective clinical trials [65–67]. Among them alpha-lipoic acid was shown to relieve sensory and functional deficits of DN and has been approved by FDA for therapeutic use [68].

Impaired synthesis of vasoactive prostanoids and associated endothelial dysfunction is one of the pathological factors contributing to DN, which is initiated by both oxidative stress and inflammation. Diabetic neuropathy is associated with compromised blood flow which results in lack of endonutritive blood supply to neurons which may be directly or indirectly related to oxidative stress directed endothelial damage in vasa nervorum [69]. Several vasodilators like angiotensin receptor antagonists, endothelin antagonists, phosphodiesterase inhibitors, calcium channel antagonists, nitro vasodilators, and prostanoid analogues have been tested in animal models of diabetes and among them angiotensin II receptor antagonists (e.g., ZD7155) and ET_B receptor antagonists (e.g., BMS 182874) found to alleviate neurovascular deficits in STZ induced diabetes model [70, 71]. However, their clinical success needs to be explored to evaluate their therapeutic use in diabetic neuropathy. Enhanced oxygen delivery to peripheral nerves result in increased nerve regeneration through counteraction of ischemic, hypoxic, inflammatory, and necrotic episodes associated with diabetic neuropathy [72].

Neuroinflammation occurs when there is a persistent release of proinflammatory mediators and the pathways are activated through corresponding cytokines in neuronal cells.

The proinflammatory mediators include TNF-α, IL-6, IL-1β, COX-2, and iNOS as well as several chemokines [44]. Antibodies or chemical agents against these cytokines and chemokines could alleviate the proinflammatory episode associated with diabetic neuropathy [42, 73]. These agents are known to inhibit the consequences of inflammatory changes associated with neuroglial activation. Transcriptional modulators of NF-κB and MAPK can provide a two-tier targeting approach for the prevention of neuroinflammatory changes in DN.

NF-κB and Nrf2 pathways are two important pathways mediating cellular homeostasis through controlling oxidative stress and inflammation. As discussed in the above section, deregulation in the balance of Nrf2–NF-κB axis may lead to several pathophysiological consequences and hence modulators of these pathways can be used to prevent such results [41]. NF-κB pathway involvement in the pathogenesis of diabetic neuropathy was well documented. Several natural inhibitors of NF-κB like curcumin, resveratrol, and melatonin and small molecule modulators of this pathway (BAY 117082, JSH23) were used in experimental diabetic animals [41, 74–76]. These drugs were shown to be promising by modifying the sensorimotor functional and proteomic changes associated with neuropathy. The use of NF-κB inhibitors can prevent the AGE mediated proinflammatory cytokine production and thus halts the events associated with neuroinflammation. Similarly, it is being observed that overt oxidative stress in neuronal cells is a pivotal pathogenetic mechanism in nerve damage, which can be prevented by enhancing Nrf2 mediated ARE gene expression. Nrf2 enhances the production of antioxidant and cytoprotective enzymes which counteract oxidative stress. Several pharmacological antioxidants have been known to enhance Nrf2 mediated antioxidant expression in experimental models of diabetic neuropathy and found to improve behavioural, functional, and biochemical characteristics associated with diabetes [77, 78]. Rather than individually targeting Nrf2 and NF-κB, pharmacological modulators of both transcription factors can produce a better therapeutic response by simultaneously enhancing Nrf2 and inhibiting NF-κB [41].

Since mitochondria are the primary source of super oxide, mitochondria targeted antioxidants can reduce the corresponding oxidative damage. Several antioxidants like α-lipoic acid and N-acetyl cysteine are shown to have therapeutic efficacy in animal and human diabetic neuropathy [68, 79]. However, targeting the antioxidant molecules directly to the mitochondria not only reduces oxidative stress but also inhibits the other pathophysiological pathways associated with mitochondrial dysfunction. Antioxidant molecules can be effectively conjugated to lipophilic cationic molecules like triphenyl phosphonium (TPP+) and hence accumulate in the mitochondria based on the large negative potential inside the mitochondrial membrane [80]. Mito Q, Mito vitamin E, Mito PBN, and so forth are the examples of drugs that were delivered to mitochondria in various experimental setups. Other strategies like Szeto-schiller (SS) peptides can also be conjugated to antioxidants to attain maximum concentration of drug inside the mitochondrial matrix. These peptides comprise four alternative aromatic/basic amino acid back bones and direct the targeted antioxidants to inner mitochondrial membrane. These SS peptides scavenge hydrogen peroxide and peroxynitrite radicals and are known to inhibit lipid peroxidation reactions effectively [81]. These drugs have shown beneficial effect in the preclinical models of diabetic neuropathy and need to be further assessed clinically [82]. Along with mitochondrial antioxidants, drugs which can increase the mitochondrial function can alleviate bioenergetic crisis and ETC dysfunction associated with DN. One such example of drugs includes PGC-1α modulators, which can rescue the mitochondrial dysfunction by enhancing the production of mitochondrial enzymes and mtDNA transcription through nuclear respiratory factor 1 (Nrf1) activation [83]. PGC-1α activation is also known to reduce the oxidative damage through enhanced Nrf2 activation [84].

7. Summary

Oxidative stress and neuroinflammation are identified to be pivotal pathophysiological triggers in various diabetes associated microvascular complications including diabetic neuropathy. The use of drugs targeting oxidative stress-inflammatory pathways was found to improve the sensorimotor and functional deficits associated with diabetic neuropathy. But their clinical success remained inferior due to complexity in cellular redox signalling pathways and its further interaction with cellular kinome, genome, and epigenome. Since, redox imbalance produced in one pathway can elicit another pathway, combinational use of several strategies mentioned above could produce more beneficial effects than monotherapy. Mitochondrial dysfunction is known to initiate the hyperglycemic cellular injury; the use of drugs targeting mitochondria will find greater attention in the near future for the treatment of diabetic neuropathy. Still, a lot of work is warranted to further elucidate the cross talk of oxidative stress, mitochondrial dysfunction, and inflammation in the pathophysiology of diabetic neuropathy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] *IDF Diabetes Atlas*, International Diabetes Federation, 6th edition, 2013.

[2] *IDF Diabetes Atlas*, International Diabetes Federation, 5th edition, 2012.

[3] M. M. Huizinga and A. Peltier, "Painful diabetic neuropathy: a management-centered review," *Clinical Diabetes*, vol. 25, no. 1, pp. 6–15, 2007.

[4] A. I. Vinik and A. Mehrabyan, "Diabetic neuropathies," *Medical Clinics of North America*, vol. 88, no. 4, pp. 947–999, 2004.

[5] P. J. Oates, "Aldose reductase, still a compelling target for diabetic neuropathy," *Current Drug Targets*, vol. 9, no. 1, pp. 14–36, 2008.

[6] U. Schmid, H. Stopper, A. Heidland, and N. Schupp, "Benfotiamine exhibits direct antioxidative capacity and prevents induction of DNA damage in vitro," *Diabetes/Metabolism Research and Reviews*, vol. 24, no. 5, pp. 371–377, 2008.

[7] X.-L. Du, D. Edelstein, L. Rossetti et al., "Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 22, pp. 12222–12226, 2000.

[8] T. Purves, A. Middlemas, S. Agthong et al., "A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy," *The FASEB Journal*, vol. 15, no. 13, pp. 2508–2514, 2001.

[9] J. M. Forbes and M. E. Cooper, "Mechanisms of diabetic complications," *Physiological Reviews*, vol. 93, pp. 137–188, 2013.

[10] G. Negi, A. Kumar, R. K. Kaundal, A. Gulati, and S. S. Sharma, "Functional and biochemical evidence indicating beneficial effect of Melatonin and Nicotinamide alone and in combination in experimental diabetic neuropathy," *Neuropharmacology*, vol. 58, no. 3, pp. 585–592, 2010.

[11] R. Pop-Busui, V. Marinescu, C. van Huysen et al., "Dissection of metabolic, vascular, and nerve conduction interrelationships in experimental diabetic neuropathy by cyclooxygenase inhibition and acetyl-L-carnitine administration," *Diabetes*, vol. 51, no. 8, pp. 2619–2628, 2002.

[12] K. Ekberg and B.-L. Johansson, "Effect of C-peptide on diabetic neuropathy in patients with type 1 diabetes," *Experimental Diabetes Research*, vol. 2008, p. 457912, 2008.

[13] R. Singh, L. Kishore, and N. Kaur, "Diabetic peripheral neuropathy: current perspective and future directions," *Pharmacological Research*, vol. 80, pp. 21–35, 2014.

[14] J. J. Duby, R. K. Campbell, S. M. Setter, J. R. White, and K. A. Rasmussen, "Diabetic neuropathy: an intensive review," *American Journal of Health-System Pharmacy*, vol. 61, no. 2, pp. 160–176, 2004.

[15] T. S. Jensen, M.-M. Backonja, S. Hernández Jiménez, S. Tesfaye, P. Valensi, and D. Ziegler, "New perspectives on the management of diabetic peripheral neuropathic pain," *Diabetes and Vascular Disease Research*, vol. 3, no. 2, pp. 108–119, 2006.

[16] D. Ziegler, "Painful diabetic neuropathy: treatment and future aspects," *Diabetes/Metabolism Research and Reviews*, vol. 24, no. 1, pp. S52–S57, 2008.

[17] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[18] S. S. M. Chung, E. C. M. Ho, K. S. L. Lam, and S. K. Chung, "Contribution of polyol pathway to diabetes-induced oxidative stress," *Journal of the American Society of Nephrology*, vol. 14, no. 3, pp. S233–S236, 2003.

[19] N. E. Cameron, M. A. Cotter, A. M. Jack, M. D. Basso, and T. C. Hohman, "Protein kinase C effects on nerve function, perfusion, Na+,K+-ATPase activity and glutathione content in diabetic rats," *Diabetologia*, vol. 42, no. 9, pp. 1120–1130, 1999.

[20] D. Koya and G. L. King, "Protein kinase C activation and the development of diabetic complications," *Diabetes*, vol. 47, no. 6, pp. 859–866, 1998.

[21] H. B. Lee, M. R. Yu, J. S. Song, and H. Ha, "Reactive oxygen species amplify protein kinase C signaling in high glucose-induced fibronectin expression by human peritoneal mesothelial cells," *Kidney International*, vol. 65, no. 4, pp. 1170–1179, 2004.

[22] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.

[23] K.-I. Hirata, R. Kuroda, T. Sakoda et al., "Inhibition of endothelial nitric oxide synthase activity by protein kinase C," *Hypertension*, vol. 25, no. 2, pp. 180–185, 1995.

[24] R. H. M. King, "The role of glycation in the pathogenesis of diabetic polyneuropathy," *Molecular Pathology*, vol. 54, no. 6, pp. 400–408, 2001.

[25] M. Takeuchi, J.-I. Takino, and S.-I. Yamagishi, "Involvement of the toxic AGEs (TAGE)-RAGE system in the pathogenesis of diabetic vascular complications: a novel therapeutic strategy," *Current Drug Targets*, vol. 11, no. 11, pp. 1468–1482, 2010.

[26] G. Bardini, C. M. Rotella, and S. Giannini, "Dyslipidemia and diabetes: reciprocal impact of impaired lipid metabolism and Beta-cell dysfunction on micro-and macrovascular complications," *The Review of Diabetic Studies*, vol. 9, pp. 82–93, 2011.

[27] V. Kolm-Litty, U. Sauer, A. Nerlich, R. Lehmann, and E. D. Schleicher, "High glucose-induced transforming growth factor β1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells," *Journal of Clinical Investigation*, vol. 101, no. 1, pp. 160–169, 1998.

[28] Y.-Q. Chen, M. Su, R. R. Walia, Q. Hao, J. W. Covington, and D. E. Vaughan, "Sp1 sites mediate activation of the plasminogen activator inhibitor-1 promoter by glucose in vascular smooth muscle cells," *Journal of Biological Chemistry*, vol. 273, no. 14, pp. 8225–8231, 1998.

[29] F. Giacco and M. Brownlee, "Oxidative stress and diabetic complications," *Circulation Research*, vol. 107, no. 9, pp. 1058–1070, 2010.

[30] A. Rodacka, E. Serafin, and M. Puchala, "Efficiency of superoxide anions in the inactivation of selected dehydrogenases," *Radiation Physics and Chemistry*, vol. 79, no. 9, pp. 960–965, 2010.

[31] X. Du, T. Matsumura, D. Edelstein et al., "Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells," *Journal of Clinical Investigation*, vol. 112, no. 7, pp. 1049–1057, 2003.

[32] X. Shen, S. Zheng, N. S. Metreveli, and P. N. Epstein, "Protection of cardiac mitochondria by overexpression of MnSOD reduces diabetic cardiomyopathy," *Diabetes*, vol. 55, no. 3, pp. 798–805, 2006.

[33] A. P. Rolo and C. M. Palmeira, "Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress," *Toxicology and Applied Pharmacology*, vol. 212, no. 2, pp. 167–178, 2006.

[34] P. Fernyhough, T.-J. Huang, and A. Verkhratsky, "Mechanism of mitochondrial dysfunction in diabetic sensory neuropathy," *Journal of the Peripheral Nervous System*, vol. 8, no. 4, pp. 227–235, 2003.

[35] S. Srinivasan, M. Stevens, and J. W. Wiley, "Diabetic peripheral neuropathy: evidence for apoptosis associated mitochondrial dysfunction," *Diabetes*, vol. 49, no. 11, pp. 1932–1938, 2000.

[36] A. M. Vincent, J. L. Edwards, L. L. McLean et al., "Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy," *Acta Neuropathologica*, vol. 120, no. 4, pp. 477–489, 2010.

[37] M. Sajic, "Mitochondrial dynamics in peripheral neuropathies," *Antioxidants & Redox Signaling*, 2014.

[38] S. D. Yan, A. M. Schmidt, G. M. Anderson et al., "Enhanced cellular oxidant stress by the interaction of advanced glycation

end products with their receptors/binding proteins," *Journal of Biological Chemistry*, vol. 269, no. 13, pp. 9889–9897, 1994.

[39] R. Wada and S. Yagihashi, "Role of advanced glycation end products and their receptors in development of diabetic neuropathy," *Annals of the New York Academy of Sciences*, vol. 1043, pp. 598–604, 2005.

[40] H. Vlassara, W. Cai, J. Crandall et al., "Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15596–15601, 2002.

[41] V. Ganesh Yerra, G. Negi, S. S. Sharma, and A. Kumar, "Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-κB pathways in diabetic neuropathy," *Redox Biology*, vol. 1, pp. 394–397, 2013.

[42] X. Shi, Y. Chen, L. Nadeem, and G. Xu, "Beneficial effect of TNF-α inhibition on diabetic peripheral neuropathy," *Journal of Neuroinflammation*, vol. 10, article 69, 2013.

[43] X.-M. Wang, T. J. Lehky, J. M. Brell, and S. G. Dorsey, "Discovering cytokines as targets for chemotherapy-induced painful peripheral neuropathy," *Cytokine*, vol. 59, no. 1, pp. 3–9, 2012.

[44] G. Ramesh, A. G. MacLean, and M. T. Philipp, "Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain," *Mediators of Inflammation*, vol. 2013, Article ID 480739, 20 pages, 2013.

[45] S. S. Soskić, B. D. Dobutović, E. M. Sudar et al., "Regulation of inducible Nitric Oxide synthase (iNOS) and its potential role in insulin resistance, diabetes and heart failure," *Open Cardiovascular Medicine Journal*, vol. 5, no. 1, pp. 153–163, 2011.

[46] M. Maccarrone and B. Brüne, "Redox regulation in acute and chronic inflammation," *Cell Death and Differentiation*, vol. 16, no. 8, pp. 1184–1186, 2009.

[47] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.

[48] C. Szabó, "Multiple pathways of peroxynitrite cytotoxicity," *Toxicology Letters*, vol. 140-141, pp. 105–112, 2003.

[49] N. Wakabayashi, S. L. Slocum, J. J. Skoko, S. Shin, and T. W. Kensler, "When NRF2 talks, who's listening?" *Antioxidants and Redox Signaling*, vol. 13, no. 11, pp. 1649–1663, 2010.

[50] Y. Tan, T. Ichikawa, J. Li et al., "Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells *in vitro* and *in vivo*," *Diabetes*, vol. 60, no. 2, pp. 625–633, 2011.

[51] G. Poli, G. Leonarduzzi, F. Biasi, and E. Chiarpotto, "Oxidative stress and cell signalling," *Current Medicinal Chemistry*, vol. 11, no. 9, pp. 1163–1182, 2004.

[52] N. E. Cameron and M. A. Cotter, "Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway," *Current Drug Targets*, vol. 9, no. 1, pp. 60–67, 2008.

[53] M. Karin, "The regulation of AP-1 activity by mitogen-activated protein kinases," *Journal of Biological Chemistry*, vol. 270, no. 28, pp. 16483–16486, 1995.

[54] G.-H. Liu, J. Qu, and X. Shen, "NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK," *Biochimica et Biophysica Acta-Molecular Cell Research*, vol. 1783, no. 5, pp. 713–727, 2008.

[55] J. Scholz and C. J. Woolf, "The neuropathic pain triad: neurons, immune cells and glia," *Nature Neuroscience*, vol. 10, no. 11, pp. 1361–1368, 2007.

[56] R. R. Myers, W. M. Campana, and V. I. Shubayev, "The role of neuroinflammation in neuropathic pain: mechanisms and therapeutic targets," *Drug Discovery Today*, vol. 11, no. 1-2, pp. 8–20, 2006.

[57] A. Veves, "Diagnosis of diabetic neuropathy," in *Clinical Management of Diabetic Neuropathy*, vol. 7, pp. 61–75, Springer, 1998.

[58] R. A. Malik, P. Kallinikos, C. A. Abbott et al., "Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients," *Diabetologia*, vol. 46, no. 5, pp. 683–688, 2003.

[59] M. Polydefkis, P. Hauer, J. W. Griffin, and J. C. McArthur, "Skin biopsy as a tool to assess distal small fiber innervation in diabetic neuropathy," *Diabetes Technology and Therapeutics*, vol. 3, no. 1, pp. 23–28, 2001.

[60] K. L. Farmer, C. Li, and R. T. Dobrowsky, "Diabetic peripheral neuropathy: should a chaperone accompany our therapeutic approach?" *Pharmacological Reviews*, vol. 64, pp. 880–900, 2012.

[61] S. Lupachyk, P. Watcho, R. Stavniichuk, H. Shevalye, and I. G. Obrosova, "Endoplasmic reticulum stress plays a key role in the pathogenesis of diabetic peripheral neuropathy," *Diabetes*, vol. 62, pp. 944–952, 2013.

[62] P. Pacher, I. G. Obrosova, J. G. Mabley, and C. Szabó, "Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies," *Current Medicinal Chemistry*, vol. 12, no. 3, pp. 267–275, 2005.

[63] I. G. Obrosova, J. G. Mabley, Z. Zsengellér et al., "Role for nitrosative stress in diabetic neuropathy: evidence from studies with a peroxynitrite decomposition catalyst," *The FASEB Journal*, vol. 19, no. 3, pp. 401–403, 2005.

[64] I. G. Obrosova, V. R. Drel, P. Pacher et al., "Oxidative-nitrosative stress and poly(ADP-ribose) polymerase (PARP) activation in experimental diabetic neuropathy: the relation is revisited," *Diabetes*, vol. 54, no. 12, pp. 3435–3441, 2005.

[65] D. Ziegler, M. Hanefeld, K.-J. Ruhnau et al., "Treatment of symptomatic diabetic polyneuropathy with the antioxidant α-lipoic acid: a 7-month multicenter randomized controlled trial (ALADIN III study)," *Diabetes Care*, vol. 22, no. 8, pp. 1296–1301, 1999.

[66] A. A. F. Sima, M. Calvani, M. Mehra, and A. Amato, "Acetyl-L-carnitine improves pain, nerve regeneration, and vibratory perception in patients with chronic diabetic neuropathy: an analysis of two randomized placebo-controlled trials," *Diabetes Care*, vol. 28, no. 1, pp. 89–94, 2005.

[67] N. B. Tütüncü, M. Bayraktar, and K. Varli, "Reversal of defective nerve conduction with vitamin E supplementation in type 2 diabetes: a preliminary study," *Diabetes Care*, vol. 21, no. 11, pp. 1915–1918, 1998.

[68] N. Vallianou, A. Evangelopoulos, and P. Koutalas, "Alpha-lipoic acid and diabetic neuropathy," *Review of Diabetic Studies*, vol. 6, no. 4, pp. 230–236, 2009.

[69] N. E. Cameron and M. A. Cotter, "Effects of antioxidants on nerve and vascular dysfunction in experimental diabetes," *Diabetes Research and Clinical Practice*, vol. 45, no. 2-3, pp. 137–146, 1999.

[70] E. K. Maxfield, A. Love, M. A. Cotter, and N. E. Cameron, "Nerve function and regeneration in diabetic rats: effects of ZD-7155, an AT1 receptor antagonist," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 269, no. 3, pp. E530–E537, 1995.

[71] N. E. Cameron, K. C. Dines, and M. A. Cotter, "The potential contribution of endothelin-1 to neurovascular abnormalities in

streptozotocin-diabetic rats," *Diabetologia*, vol. 37, no. 12, pp. 1209–1215, 1994.

[72] A. Aydin, B. Ç. Özden, S. Karamürsel, S. Solakoğlu, Ş. Aktaş, and M. Erer, "Effect of hyperbaric oxygen therapy on nerve regeneration in early diabetes," *Microsurgery*, vol. 24, no. 3, pp. 255–261, 2004.

[73] A. P. Kellogg, T. D. Wiggin, D. D. Larkin, J. M. Hayes, M. J. Stevens, and R. Pop-Busui, "Protective effects of cyclooxygenase-2 gene inactivation against peripheral nerve dysfunction and intraepidermal nerve fiber loss in experimental diabetes," *Diabetes*, vol. 56, no. 12, pp. 2997–3005, 2007.

[74] A. Kumar and S. S. Sharma, "NF-κB inhibitory action of resveratrol: a probable mechanism of neuroprotection in experimental diabetic neuropathy," *Biochemical and Biophysical Research Communications*, vol. 394, no. 2, pp. 360–365, 2010.

[75] A. Kumar, G. Negi, and S. S. Sharma, "Suppression of NF-κB and NF-κB regulated oxidative stress and neuroinflammation by BAY 11-7082 (IκB phosphorylation inhibitor) in experimental diabetic neuropathy," *Biochimie*, vol. 94, no. 5, pp. 1158–1165, 2012.

[76] A. Kumar, G. Negi, and S. S. Sharma, "JSH-23 targets nuclear factor-kappa B and reverses various deficits in experimental diabetic neuropathy: effect on neuroinflammation and antioxidant defence," *Diabetes, Obesity and Metabolism*, vol. 13, no. 8, pp. 750–758, 2011.

[77] G. Negi, A. Kumar, and S. S. Sharma, "Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-κB and Nrf2 cascades," *Journal of Pineal Research*, vol. 50, no. 2, pp. 124–131, 2011.

[78] G. Negi, A. Kumar, and S. S. Sharma, "Nrf2 and NF-κB modulation by sulforaphane counteracts multiple manifestations of diabetic neuropathy in rats and high glucose-induced changes," *Current Neurovascular Research*, vol. 8, no. 4, pp. 294–304, 2011.

[79] S. S. Kamboj, R. K. Vasishta, and R. Sandhir, "N-acetylcysteine inhibits hyperglycemia-induced oxidative stress and apoptosis markers in diabetic neuropathy," *Journal of Neurochemistry*, vol. 112, no. 1, pp. 77–91, 2010.

[80] R. A. J. Smith and M. P. Murphy, "Mitochondria-targeted antioxidants as therapies," *Discovery medicine*, vol. 11, no. 57, pp. 106–114, 2011.

[81] H. H. Szeto, "Mitochondria-targeted peptide antioxidants: novel neuroprotective agents," *The AAPS Journal*, vol. 8, no. 3, pp. 521–531, 2006.

[82] B. K. Chacko, C. Reily, A. Srivastava et al., "Prevention of diabetic nephropathy in Ins2+/-AkitaJ mice by the mitochondria-targeted therapy MitoQ," *Biochemical Journal*, vol. 432, no. 1, pp. 9–19, 2010.

[83] J. Choi, K. Chandrasekaran, T. Inoue, A. Muragundla, and J. W. Russell, "PGC-1α regulation of mitochondrial degeneration in experimental diabetic neuropathy," *Neurobiology of Disease*, vol. 64, pp. 118–130, 2014.

[84] I. Valle, A. Álvarez-Barrientos, E. Arza, S. Lamas, and M. Monsalve, "PGC-1α regulates the mitochondrial antioxidant defense system in vascular endothelial cells," *Cardiovascular Research*, vol. 66, no. 3, pp. 562–573, 2005.

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Review Article

Activation of Toll-Like Receptors and Inflammasome Complexes in the Diabetic Cardiomyopathy-Associated Inflammation

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Diabetic cardiomyopathy is defined as a ventricular dysfunction initiated by alterations in cardiac energy substrates in the absence of coronary artery disease and hypertension. Hyperglycemia, hyperlipidemia, and insulin resistance are major inducers of the chronic low-grade inflammatory state that characterizes the diabetic heart. Cardiac Toll-like receptors and inflammasome complexes may be key inducers for inflammation probably through NF-κB activation and ROS overproduction. However, metabolic dysregulated factors such as peroxisome proliferator-activated receptors and sirtuins may serve as therapeutic targets to control this response by mitigating both Toll-like receptors and inflammasome signaling.

1. Introduction

Cardiac complications are the leading cause of morbidity and mortality in diabetic patients [1]. First introduced by Rubler et al. in 1971 [2], diabetic cardiomyopathy (DCM) is characterized by the direct effect of diabetes on cardiac structure and function in the absence of coronary artery disease, hypertension, or other cardiac pathologies. DCM entails the damage of the myocardium through fibrosis, steatosis, apoptosis, and hypertrophy [3] and results from the switch of substrate supply to free fatty acids (FFA) that follows the reduced levels of insulin, glucose transporters, and glucose consumption [4, 5]. Subsequent disruption of calcium homeostasis and myocardial remodeling leads to a progressive impairment of ventricular myocyte contractility that may result in heart failure [6–8]. In addition, an increasing body of evidence suggests a potential link between oxidative energy metabolism dysregulation and chronic low-grade inflammation [4, 9].

Inflammatory signaling in cardiomyocytes usually occurs as an early response to myocardial injury and entails cytosolic and mainly mitochondrial reactive oxygen species (ROS) overproduction [10, 11]. Classical following steps mainly involve increased activation of the proinflammatory nuclear transcription factor-κB (NF-κB), and the related expression of cytokines (i.e., tumour necrosis factor-α (TNFα), interleukins (IL-1β, IL-6), and chemokines (i.e., MCP-1)), adhesion molecules (i.e., selectins and adhesion molecules (ICAM-1, VCAM-1)), and successive migration of leukocytes into the myocardium [12, 13]. Migrated monocytes can further develop into tissue macrophages, which can then be polarized into two main groups, M1 and M2, accounting for their trend towards inflammation or healing, respectively. We and others have reported that myocardial inflammation develops in human patients and experimental models of type 1 (T1DM) and type 2 (T2DM) diabetes mellitus [8, 14, 15]. There is evidence that chronic progression of hypertrophy, fibrosis, and ventricular dysfunction is correlated with a local increase in cytokines [16] and activation of NF-κB [17, 18]. General inflammatory stimuli in the diabetic heart include hyperglycaemia, hyperlipidemia, ROS, angiotensin II, and endothelin-1 [4, 19]. Activation of Toll-like receptors (TLRs)

and the inflammasome complex has recently been proposed to be central in cardiac inflammation and likely in the pathogenesis of DCM.

2. Toll-Like Receptors and Cardiac Inflammation

TLRs are membrane-anchored proteins present in several cell types ranging from macrophages and T and B cells to nonimmune cells such as cardiomyocytes [20, 21]. They work as pattern recognition receptors (PRRs) implicated in tailoring innate immune signaling [22]. TLRs elicit conserved inflammatory pathways culminating in the activation of NF-κB and activating protein-1 (AP-1). TLR ligands include high-mobility group B1 (HMGB1), heat shock proteins (HSP60, HSP70), endotoxins, and extracellular matrix components [23]. Also ROS can modify membrane components and cause the release of factors that interact with and activate TLRs. In this sense, it has been shown that TLR2 participated importantly in the mechanism of ROS-induced activation of NF-κB and AP-1 [24]. The proximal events of TLR-mediated intracellular signaling are initiated by interactions with cytosolic adapters, mainly myeloid differentiation primary response protein 88 (MyD88) [25]. MyD88 recruits the IL-1R-associated kinase (IRAK) and TNFR-associated factor (TRAF) to induce ubiquitination and proteasomal degradation of the inhibitors of NF-κB (IκBs), thus enabling NF-κB translocation into the nuclei and further ROS generation [25] (Figure 1(a)). The isoforms predominantly expressed in cardiomyocytes are TLR2 and TLR4, although up to ten cardiac TLR mRNAs have been identified in several clinical contexts including obesity and T2DM [26–29]. TLR2 and TLR4 have a central role in the pathogenesis of diverse heart disorders. Both are strongly upregulated in chronic dilated cardiomyopathy and heart failure [30], serving as upstream inducers of a large variety of proinflammatory molecules including ICAM-1, chemokines, TNFα, interleukins, HSPs, interferon-γ (IFNγ), and inducible nitric oxide synthase (iNOS) [21, 28, 31]. Activation of TLR2 and TLR4 eventually leads to reduction of ejection fraction through NF-κB-dependent mechanisms [31, 32]. However, the specific distinction of the mechanisms and targets between TLR2 and TLR4 in cardiac inflammation is a rapidly evolving knowledge that presents some divergence. Boyd et al. reported that stimulation of TLR2 and TLR4 in HL-1 cardiomyocytes decreased contractility and initiated NF-κB-dependent inflammatory response, involving upregulation of ICAM-1, chemokines, and macrophage inflammatory protein-2 (MIP-2). However, only TLR4 activation induced the proinflammatory cytokine IL-6 [31]. More recently, Ma et al. [30] uncovered the differential effects of TLR2 and TLR4 in a doxorubicin-induced mice model of chronic dilated cardiomyopathy. TLR2 blockade reduced myocardial expression of a variety of proinflammatory factors including IFNγ and MCP-1. Conversely, TLR4 blockade increased secretion of MCP-1, IL-13, and transforming growth factor-β_1 (TGFβ_1). Besides structural cardiomyopathies, TLR2 and TLR4 have progressively gained credit as important contributors to entities of metabolic nature such as cardiac lipotoxicity. In this line, TLR4 knockdown abrogated NF-κB-dependent inflammatory response and lowered insulin resistance in high-fat fed mice [33].

2.1. Activation of TLRs in DCM. Several studies have addressed the role of TLRs in cardiac inflammation using models of T1DM, T2DM, and obesity, which share an environment characterized by high circulating levels of glucose and FFA and elevated tissue levels of ceramides. Although no direct interaction between glucose and FFA with TLRs has been described [34], high levels of glucose and lipids have been shown to stimulate TLR2 and TLR4 [33, 35, 36], thus suggesting the existence of unknown intermediates. High-fat diet-induced obese mice exhibited myocardial macrophage infiltration as well as higher expression levels of TLR4, MyD88, and IL-6 [37]. Consistent with this, both diabetic TLR2 and TLR4-deficient mouse hearts showed lower triglyceride accumulation during the early stages of diabetes, as well as restricted leukocyte infiltration and a marked decrease of NF-κB and MyD88 and phosphorylation of IRAK1 [20, 38]. Different studies in T1DM mice models show that TLR4 silencing prevents cardiac lipid accumulation, hyperglycemia-induced myocardial apoptosis, and ventricular remodeling and dysfunction. It also suppresses the diabetic upregulation of NADPH oxidase activity and thus ROS production [20, 39]. Furthermore, genetic analysis of patients has pointed an association between TLR4 polymorphisms, diabetes prevalence, and the severity of chronic cardiomyopathy [40, 41]. However, besides the focus on the alterations of cardiomyocytes, leukocyte activation and transmigration into the diabetic myocardium constitute a pivotal process in the inflammatory response. Hyperglycemia has been shown to upregulate TLR2, TLR4, MyD88, and IRAK-1 phosphorylation and TLR-mediated transactivation of NF-κB in human monocytes from T2DM patients [28, 42]. Concurrently, TLR2 increased in mononuclear cells from long-standing T1DM patients [43]. In macrophages from a model of nonobese T2DM mice, Mohammad et al. described a ten-fold increase of TLR4 and higher levels of cytokines, while anti-inflammatory IL-10 was downregulated [21]. Consistent with this view, monocytes from T2DM patients also exhibited significant increment in proinflammatory cytokines and TLR2 and TLR4 ligands (HMGB1 and HSPs) [28]. Similar to what occurred in cardiomyocytes, siRNA knockdown of TLR2 and TLR4 led to decreased NF-κB activity and IL-1β release in monocytes [42]. Therefore, it seems that TLRs may be activated in both cardiomyocytes and leukocytes, in DCM-associated cardiac inflammation ten-fold.

3. Inflammasomes and Cardiac Inflammation

The inflammasome is a group of multimeric protein complexes composed of a cytoplasmic receptor of the Nod-Like Receptor (NLR) family, an adaptor protein termed ASC (Apoptosis-associated Speck-like protein containing an N-terminal caspase recruitment domain CARD), and

procaspase-1 [44]. The best characterized complex is the NLRP3 inflammasome, which has been identified in a wide range of cells including macrophages, cardiofibroblasts, and cardiomyocytes [45–48]. NLRP3 has been reported to be held in an inactive state by cytoplasmic chaperones. Once NLRP3 is freed, subsequent oligomerization leads to the recruitment of procaspase-1, thus promoting autocleavage and activation [44]. Active caspase-1 can eventually process IL-1β and IL-18 precursors, serving as enhancer of multiple proinflammatory pathways including NF-κB, mitogen-activated protein kinase (MAPK), IFNγ, chemokines, and ROS and also promoting insulin resistance [49] (Figure 1(b)). NLRP3 can be activated by long-chain saturated FA (i.e., palmitate), ceramides, modified LDL, and hyperglycemia [50–52]. However, NLRP3 does not have a known direct ligand and it requires two-checkpoint activation process including a priming step and a second activation step [53]. NF-κB is the traditional priming signal for the transcription of the NLRP3 gene [54], whereas novel mechanisms have recently emerged as a second step. These are based on posttranslational activation of NLRP3 by deubiquitination [55], oxidized mitochondrial DNA [56], and potential ligands such us thioredoxin-interacting protein (TXNIP) [57]. In addition, a recent study by Bauernfeind et al. has revealed that NLRP3 expression is critically regulated by myeloid specific microRNA-223 [58]. Nevertheless, to date, most data about NLRP3-inflammasome implication in heart disease and inflammation come from murine models of ischemic damage and dilated cardiomyopathy [48, 59, 60]. In a model of dilated cardiomyopathy, NLRP3 ablation was related to a general reduction in proinflammatory cytokines maturation, reduced mononuclear infiltrate, maintained myocyte organization and structure, and preserved systolic performance [48]. In addition, these hearts increased phosphorylation of IκBα, what is consistent with NF-κB regulated NLRP3 gene expression. Further evidence shows upregulation of the NLRP3-inflammasome effector caspase-1 in murine and human myocardial infarction [61]. In this study, deletion of endogenous caspase-1 consistently ameliorated the ventricular function of the postinfarcted heart. However, many aspects are in need of further clarification. NLRP3 mRNA levels have been found to be markedly diminished in heart samples from the right atrium of patients undergoing coronary bypass surgery [60]. Moreover, Zuurbier et al. have recently reported that deletion of NLRP3 resulted in decreased myocardial IL-18 and IL-6 levels, but this effect was not observed for IL-1β and TNFα levels. Also, deletion of the ASC component did not downregulate IL-6, IL-1β, or TNFα [59]. Despite this, gene polymorphisms and mutations in the human NLRP3-inflammasome have been shown to be associated with an increase of IL-1β and IL-18, higher levels of C-reactive protein (CRP), and severe inflammation [62–64].

3.1. Activation of NLRP3-Inflammasomes in DCM. Not much research has been done to assess the plausible implication of inflammasomes in experimental models of DCM. However, as for TLRs, several recent studies have emphasized that NLRP3 inflammasomes might represent the link between inflammation and metabolic disorders such in the diabetic heart. It is known that NLRP3 signaling affects glycolysis and insulin sensitivity and simultaneously enhances both local myocardial cytokine levels and infiltration by macrophages [51, 65]. Recent data also suggest that NLRP3 is responsible for sensing obesity-associated host-derived inducers of caspase-1, such as ROS and lipotoxic ceramides and palmitate [66]. In fact, NLRP3 inflammasomes have been proposed to sense and mediate downstream inflammatory events of glycotoxicity and lipotoxicity during the pathogenesis of T2DM [45, 57]. Cardiac NLRP3, caspase-1, and IL-1β expression was substantially increased in obese mice and human subjects [45]. Moreover, caloric restriction and exercise-mediated weight loss in obese individuals with T2DM were shown to effectively reduce the expression levels of NLRP3 [67]. In contrast to the scarce contributions in cardiomyocytes, research on NLRP3 inflammasomes has intensively focused on inflammatory cells. NLRP3 has been reported to increase effector T-cell number, thus eliciting macrophage transmigration. Further, NLRP3 upregulates the pool of proinflammatory cytokines such as IL-1β, IL-18, and IFNγ and promotes insulin resistance in M1 macrophages [45]. In addition, both ceramides and palmitate require an intact NLRP3 signaling to induce caspase-1 activation and IL-1β and IL-18 release from macrophages [45, 66]. Thus, NLRP3 inflammasome may also participate in the cardiomyocyte and monocyte response in DCM-associated inflammation.

4. Potential Crosstalk between TLRs, Inflammasomes, and Metabolic Dysregulation in DCM

Interestingly, TLR2 and TLR4-mediated ROS generation and NF-κB transactivation upregulate NLRP3 pathway through multiple direct and indirect mechanisms, which account for both NLRP3 priming and the secondary steps of activation (Figure 2). First, ROS/NF-κB has been reported to enhance the expression of NLRP3 and caspase-1 target pro-IL-1β, and NF-κB sites in *NLRP3* promoter have been identified [36, 54, 67]. Second, ROS/NF-κB facilitates NLR posttranslational deubiquitination [55]. And third, ROS/NF-κB increases the amount of TXNIP and oxidized mitochondrial DNA, which might serve as ligands of NLRP3 [56, 57]. Thus, NLRP inflammasome activation is likely to be a key outcome of TLR stimulation in DCM. Moreover, another connection between TLRs and inflammasomes may be through metabolic dysregulated factors, such as peroxisome proliferator-activated receptors (PPARs) and sirtuins (Sirts) (Figure 2). Activation of PPARs is a key process in the myocardial switch of substrates in DCM and has recently emerged as a link between metabolism disturbance and pathological inflammatory/oxidative phenomena [68]. The PPAR transcription factor family is extensively known to regulate cardiac metabolism, mainly through PPARα and PPARβ/δ isoforms together with PGC-1α coactivator [69]. PPARα/PGC-1α leads to transcriptional induction of pyruvate dehydrogenase kinase-4 (PDK4), FAT/CD36 transporter, and FFA oxidation enzymes [70], thereby facilitating mitochondrial FFA import and β-oxidation-dependent metabolism in expenses

of glucose assimilation. Thus, NF-κB and p38-mediated PPARα/PGC-1α inhibition has been described as an important pathological mechanism in DCM progression [71]. In addition, a wide body of evidence indicates that PPARs mitigate inflammation. PPARs lower nuclear factor of activated T-cells (NFAT) signaling and prevent the expression of NADPH oxidase subunits, resulting in ROS amelioration [72, 73]. PPARs also downregulate TLR2 and TLR4 signaling by either blocking TLR expression or its NF-κB and AP-1-dependent pathways [74–76]. The effects on NF-κB seem to be mediated through direct physical interactions, sequestration of NF-κB coactivators, and transcriptional control of NF-κB-related proinflammatory genes [77–79]. Other DNA-independent mechanisms to inhibit NF-κB include activation of ERK-MAPK pathway, mainly by impairing phosphorylation of factors such as p38- and JNK-MAPK [4]. Notably, these are also targets of TLR-Myd88/IRAK signaling in the heart [18, 80]. Further direct evidence from obese T2DM mice has demonstrated that PPARβ/δ and PPARγ downregulate both TLR2 and TLR4 signaling [75, 81]. Moreover, TNFα and IL-1β have been reported to be clearly decreased upon activation of PPARα [82, 83], which can be linked with impaired NF-κB-dependent induction of NLRP3 inflammasome. In a model of chronic high-fructose-induced diabetic mice, Collino et al. described that PPARβ/δ stimulation attenuated NLRP3-dependent caspase-1 activation and IL-1β production [74]. Also, the NLR family promoter harbours binding sites for PPARγ [84]. More complex evidence regards the interference of the inflammasome assembly by phospholipase C, cyclic AMP, and protein kinase C, which are known regulators and targets of PPARs [85–87].

In addition to PPARs, Sirts may constitute another alleged nodal connection between metabolism and TLR and/or inflammasome-dependent inflammation [88] (Figure 2). Moreover, Sirts have been largely reported to interfere with the molecular pathogenic substrate of heart failure and thereby ameliorate cardiac outcome [89]. Epigenetic modulation by this class III deacetylase limits oxidative stress and inflammatory responses by targeting a relevant set of transcription factors including NF-κB, PPARs, and PGC-1α [90]. Sirtuin-1 (Sirt1), the most studied Sirt in the heart, works as an energy sensor and supports oxidative energy metabolism through PPARα/PGC-1α and AMP-activated protein kinase (AMPK) signaling, which also contribute to inhibit NF-κB and inflammation [91]. Mice overexpressing Sirt1 and exposed to high-fat diet show attenuated lipid-induced inflammatory responses [92]. Also, Sirt1 was reported to stimulate antioxidants manganese superoxide dismutase (MnSOD) and nuclear respiratory factor-1 (Nrf1) in the heart, downregulating NF-κB targets TNFα and IL-6 [93]. Several mechanisms for the modulation of NF-κB signaling by Sirt1 have been described. First, Sirt1 has been associated with PPARα-dependent inhibition of p65 subunit of NF-κB [94]. Second, Sirt1 may directly modulate NF-κB-dependent immune responses and coupled ROS production by deacetylating p65 [95]. And third, Sirt1 can negatively regulate the expression of TNFα and IL-1β by binding to specific sites in their promoters [96]. Further connections between Sirt1, TLRs and inflammasomes include Sirt1 downregulation by palmitate-induced miR-195 and Sirt1 cleavage by caspase-1 [92, 97]. Taken together, activation of both PPARs and Sirt1 may control the TLR and inflammasome-dependent pathways of inflammation in DCM, which may be useful for a therapeutic target.

5. Prospective Therapeutic Targets for DCM

Despite the prolific area of research linking inflammation, diabetes and metabolic heart disease, the drugs currently employed in the care of diabetic patients have not generally been based on an anti-inflammatory strategy. Pharmacological modulation of TLRs undoubtedly arises as a highly attractive therapeutic strategy for DCM. In this regard, several TLR antagonists have been assessed in diverse models of myocardial contractile dysfunction. Selective inhibition of TLR2 by immunoglobulin G (IgG) has been successfully attempted to ameliorate NF-κB and leukocyte infiltration in ischemic murine hearts [98]. TLR4 antagonists, eritoran, and geldanamycin resulted in attenuated myocardial inflammatory responses including reduced p-JNK and NF-κB nuclear translocation and decreased gene transcripts of TNFα, IL-1β, IL-6, MCP-1, MIP-1α, and MIP-2 [99, 100]. These data underscore the potential benefit of blocking TLR signaling for DCM. However, an increasing number of TLR inhibitors are not being proportionally tracked by studies measuring their impact in animal models of cardiac disease [101, 102]. The discovery of novel mechanisms for common drugs also paves the way for potential therapeutic strategies. For example, statins attenuated the upregulation of TLR4 and TLR2, inhibited NF-κB, and decreased the circulating levels of TNFα, MCP-1, and CRP in a mice model of dilated cardiomyopathy [103, 104]. Also, angiotensin II receptor blocker valsartan decreased TLR4-mediated NF-κB activity and subsequent cytokine release in a rat model of ischemic heart [105]. In addition, cumulative evidence on NF-κB and TNFα targeting suggests the therapeutic value of specific modulation of TLR downstream effectors. Triptolide, a potent NF-κB immunomodulator and TNFα monoclonal antibody treatment significantly decreased TNFα, IL-1β, ICAM-1, VCAM-1, and subsequent myocardial infiltration by macrophages and T-cells in diabetic hearts [106, 107].

No NLR antagonist has been identified yet, and increasing efforts are being invested as a result of successful blockade of downstream effectors IL-1β and caspase-1 in DCM. Very recent evidence reported that intravenous IgG therapy protected neurons in an experimental model of stroke through a mechanism involving suppression of the NLRP3 inflammasome activity [108]. Moreover, the anti-IL-1β Anakinra [109] and Gevokizumab [110] clinical trials resulted in reduced TNFα, IL-6, IL-1β, and CRP. However, despite reducing biomarkers of heart disease, they did not restore hyperglycemia. Nonimmune antagonists of the inflammasome machinery are equally compelling. Pralnacasan, a caspase-1 blocker, has been reported to attenuate inflammation in a model of DCM by reducing IL-1β, IL-18, TNFα, and IFNγ levels, intracardiac macrophage, and lymphocyte infiltrates and also to improve insulin sensitivity

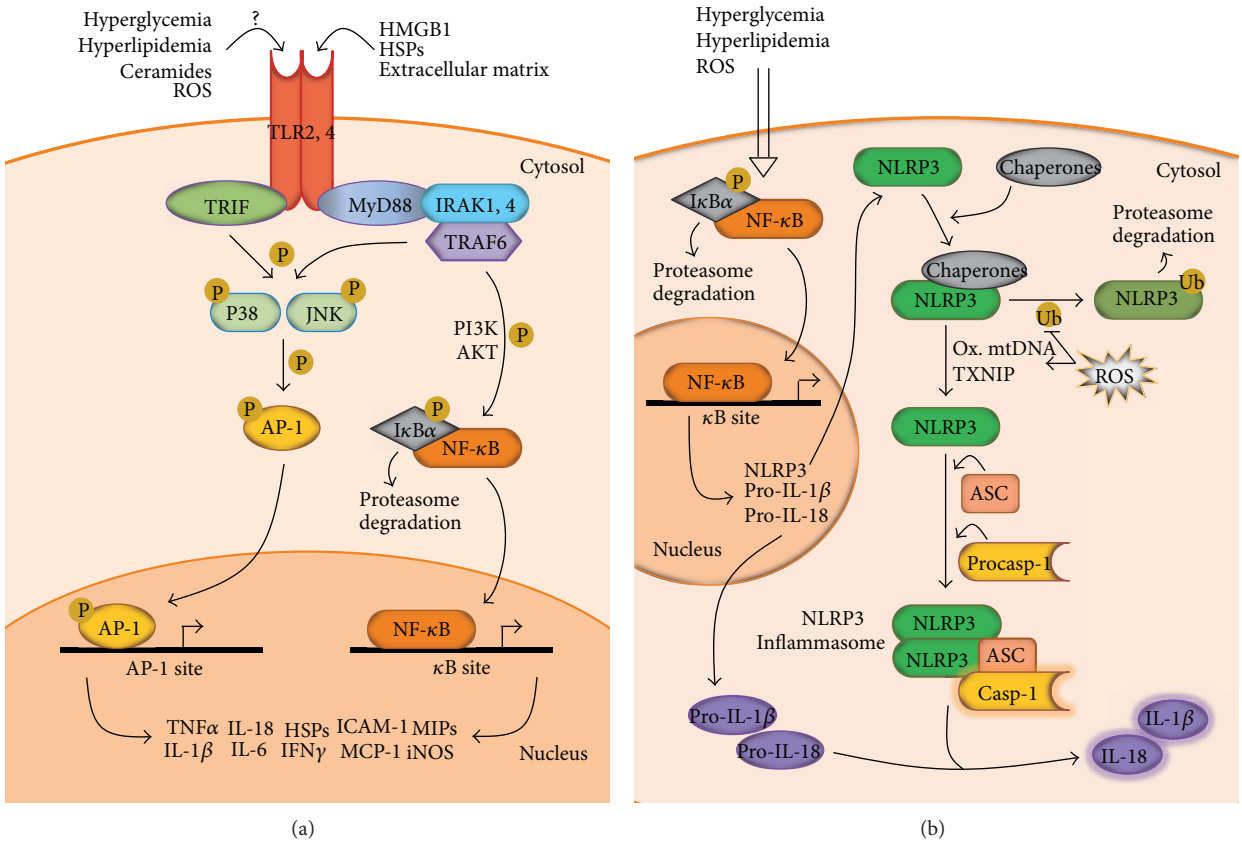

FIGURE 1: TLRs and NLRP3 inflammasome activation in the proinflammatory myocardium. (a) Activation of TLR2 and TLR4, (b) and NLRP3 inflammasome complexes in cardiomyocytes.

[107, 111]. Novel anti-inflammasome properties have been described for classical antidiabetics such as metformin and sulfonylurea. Metformin may affect NLRP3 signaling by enhancing autophagy through AMPK [66, 112] or increasing Sirt1 action [113, 114]. In this line, AICAR, an AMPK agonist, could also restore the formation of autophagosomes and thereby inhibit both caspase-1 and ROS generation in palmitate-treated macrophages [66]. Sulfonylurea glyburide also suppressed the NLRP3-dependent caspase-1 activation and IL-1β release [115]. Finally, Jourdan et al. showed that the blockade of cannabinoid receptor type 1 (CB1R) lowered the levels of NLRP3, ASC, IL-1β, IL-18, NF-κB, and caspase-1 in macrophages from ZDF rats [116].

Beyond the regulation of TLRs and inflammasomes, therapeutic benefit of PPARs and Sirt1 stimulation on T2DM and its cardiac complications has been reported in recent years [117–120]. Interestingly, a PPARα agonist, fenofibrate, decreased TLR4 and MyD88 expression in a model of multiple sclerosis [121]. PPARγ agonist pioglitazone substantially inhibited the expression of TLR2, TLR4, MyD88, and NF-κB in macrophages from obese T2DM mice [75]. Moreover, PPARβ/δ agonist GW0742 impaired NLRP3 inflammasome activity in high-fructose diet-induced diabetic mice [74]. Since TLR4 downregulation was identified as an anti-inflammatory mechanism of the insulin-sensitizer incretin glucagon-like peptide-1 (GLP-1) [122], and considering that a PPARβ/δ agonist markedly upregulated GLP-1 in obese T2DM mice [123], it is possible that PPARβ/δ stimulation may be a valid therapeutic tool for DCM. In the same way, a bulk of emerging evidence has identified Sirts as a future therapeutic target for diabetic complications. In this sense, small molecule activators of Sirt1 are currently being developed. ZDF rats undergoing this treatment effectively improved whole-body glucose homeostasis and insulin resistance [124]. However, no attempt to measure the impact of Sirt1 enhancement in DCM has been made.

6. Conclusions

TLRs and the inflammasome signaling platforms could be two main breakthroughs on cardiac inflammation. Emerging evidence supports a model in which hyperglycemia and FFA stimulate TLRs as upstream inducers of proinflammatory mechanisms in DCM. TLR-dependent NF-κB and ROS appear to regulate both the priming and posttranslational steps required for the assembly and activation of the inflammasome. However, metabolic dysregulated factors such as PPARs and Sirt1 can downmodulate DCM inflammation by interfering with TLRs and inflammasome signaling. Thus, a new set of potential therapeutic approaches for DCM may

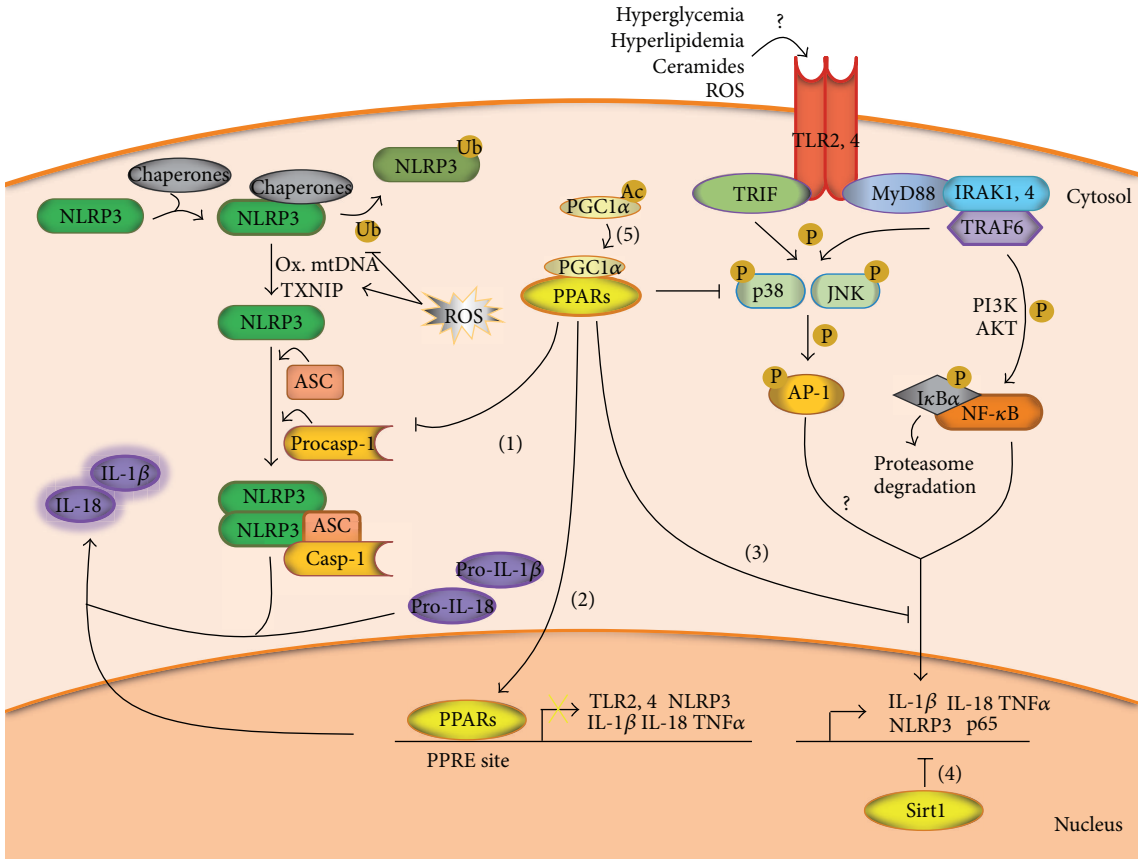

Figure 2: Crosstalk between TLRs, NLRP3 inflammasomes and dysregulated metabolic factors in DCM. PPARs and Sirt1 may control NLRP3 inflammasome and TLR pathways by interfering with the inflammasome assembly (1), proinflammatory gene overexpression (2), and NF-κB signaling (3-4). In addition, Sirt1 could mediate PPARs activation by PGC1α deacetylation (5).

include the stimulation of PPARs and Sirt1 and the inhibition of TLR2, TLR4, and NLRP3. Further, targeting proximal TLR mediators Myd88 and IRAK and the activation steps of the inflammasome may yield some clinical benefit in DCM.

Conflict of Interests

The authors declare that there is no conflict of interests.

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References

[1] S. Boudina and E. D. Abel, "Diabetic cardiomyopathy revisited," *Circulation*, vol. 115, no. 25, pp. 3213–3223, 2007.

[2] S. Rubler, J. Dlugash, Y. Z. Yuceoglu, T. Kumral, A. W. Branwood, and A. Grishman, "New type of cardiomyopathy associated with diabetic glomerulosclerosis," *The American Journal of Cardiology*, vol. 30, no. 6, pp. 595–602, 1972.

[3] Y. F. Zhi, J. B. Prins, and T. H. Marwick, "Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications," *Endocrine Reviews*, vol. 25, no. 4, pp. 543–567, 2004.

[4] X. Palomer, L. Salvado, E. Barroso et al., "An overview of the crosstalk between inflammatory processes and metabolic dysregulation during diabetic cardiomyopathy," *International Journal of Cardiology*, vol. 168, no. 4, pp. 3160–3172, 2013.

[5] H. Taegtmeyer, P. McNulty, and M. E. Young, "Adaptation and maladaptation of the heart in diabetes—part I: general concepts," *Circulation*, vol. 105, no. 14, pp. 1727–1733, 2002.

[6] I. Shiojima and I. Komuro, "Molecular mechanisms of congestive heart failure," *Nippon Rinsho*, vol. 64, no. 5, pp. 826–831, 2006.

[7] W. B. Kannel, M. Hjortland, and W. P. Castelli, "Role of diabetes in congestive heart failure: The Framingham Study," *American Journal of Cardiology*, vol. 34, no. 1, pp. 29–34, 1974.

[8] D. Westermann, S. Rutschow, S. Jäger et al., "Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of angiotensin type 1 receptor antagonism," *Diabetes*, vol. 56, no. 3, pp. 641–646, 2007.

[9] E. Ramírez, M. Klett-Mingo, S. Ares-Carrasco et al., "Eplerenone attenuated cardiac steatosis, apoptosis and diastolic dys-

function in experimental type-II diabetes," *Cardiovascular Diabetology*, vol. 12, no. 1, article 172, 2013.

[10] M. Hamblin, D. B. Friedman, S. Hill, R. M. Caprioli, H. M. Smith, and M. F. Hill, "Alterations in the diabetic myocardial proteome coupled with increased myocardial oxidative stress underlies diabetic cardiomyopathy," *Journal of Molecular and Cellular Cardiology*, vol. 42, no. 4, pp. 884–895, 2007.

[11] F. J. Pashkow, "Oxidative stress and inflammation in heart disease: do antioxidants have a role in treatment and/or prevention?" *International Journal of Inflammation*, vol. 2011, Article ID 514623, 2011.

[12] D. L. Mann, "Inflammatory mediators and the failing heart: past, present, and the foreseeable future," *Circulation Research*, vol. 91, no. 11, pp. 988–998, 2002.

[13] W. Huang and C. K. Glass, "Nuclear receptors and inflammation control: molecular mechanisms and pathophysiological relevance," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 8, pp. 1542–1549, 2010.

[14] X.-Y. Yu, H.-M. Chen, J.-L. Liang et al., "Hyperglycemic myocardial damage is mediated by proinflammatory cytokine: macrophage migration inhibitory factor," *PLoS ONE*, vol. 6, no. 1, Article ID e16239, 2011.

[15] S. Ares-Carrasco, B. Picatoste, A. Benito-Martín et al., "Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 297, no. 6, pp. H2109–H2119, 2009.

[16] E. Haugen, J. Chen, J. Wikström, J. Grönros, L. M. Gan, and L. X. M. Fu, "Parallel gene expressions of IL-6 and BNP during cardiac hypertrophy complicated with diastolic dysfunction in spontaneously hypertensive rats," *International Journal of Cardiology*, vol. 115, no. 1, pp. 24–28, 2007.

[17] T. Hamid, S. Z. Guo, J. R. Kingery, X. Xiang, B. Dawn, and S. D. Prabhu, "Cardiomyocyte NF-κB p65 promotes adverse remodelling, apoptosis, and endoplasmic reticulum stress in heart failure," *Cardiovascular Research*, vol. 89, no. 1, pp. 129–138, 2011.

[18] O. Lorenzo, B. Picatoste, S. Ares-Carrasco, E. Ramírez, J. Egido, and J. Tuñón, "Potential role of nuclear factor κb in diabetic cardiomyopathy," *Mediators of Inflammation*, vol. 2011, Article ID 652097, 9 pages, 2011.

[19] O. Lorenzo, E. Ramírez, B. Picatoste, J. Egido, and J. Tuñón, "Alteration of energy substrates and ROS production in diabetic cardiomyopathy," *Mediators of Inflammation*, vol. 2013, Article ID 461967, 11 pages, 2013.

[20] B. Dong, D. Qi, L. Yang et al., "TLR4 regulates cardiac lipid accumulation and diabetic heart disease in the nonobese diabetic mouse model of type 1 diabetes," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 303, no. 6, pp. H732–H742, 2012.

[21] M. K. Mohammad, M. Morran, B. Slotterbeck et al., "Dysregulated Toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse," *International Immunology*, vol. 18, no. 7, pp. 1101–1113, 2006.

[22] R. C. Coll and L. A. J. O'Neill, "New insights into the regulation of signalling by toll-like receptors and nod-like receptors," *Journal of Innate Immunity*, vol. 2, no. 5, pp. 406–421, 2010.

[23] M.-F. Tsan and B. Gao, "Endogenous ligands of Toll-like receptors," *Journal of Leukocyte Biology*, vol. 76, no. 3, pp. 514–519, 2004.

[24] S. Frantz, R. A. Kelly, and T. Bourcier, "Role of TLR-2 in the activation of nuclear factor κB by oxidative stress in cardiac myocytes," *Journal of Biological Chemistry*, vol. 276, no. 7, pp. 5197–5203, 2001.

[25] T. Kawai and S. Akira, "TLR signaling," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 816–825, 2006.

[26] H. Methe, J.-O. Kim, S. Kofler, M. Weis, M. Nabauer, and J. Koglin, "Expansion of circulating toll-like receptor 4-positive monocytes in patients with acute coronary syndrome," *Circulation*, vol. 111, no. 20, pp. 2654–2661, 2005.

[27] S. Frantz, G. Ertl, and J. Bauersachs, "Toll-like receptor signaling in the ischemic heart," *Frontiers in Bioscience*, vol. 13, no. 15, pp. 5772–5779, 2008.

[28] M. R. Dasu, S. Devaraj, S. Park, and I. Jialal, "Increased Toll-Like Receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects," *Diabetes Care*, vol. 33, no. 4, pp. 861–868, 2010.

[29] S. M. Reyna, S. Ghosh, P. Tantiwong et al., "Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects," *Diabetes*, vol. 57, no. 10, pp. 2595–2602, 2008.

[30] Y. Ma, X. Zhang, H. Bao et al., "Toll-like receptor (TLR) 2 and TLR4 differentially regulate doxorubicin induced cardiomyopathy in mice," *PLoS One*, vol. 7, no. 7, article e40763, 2012.

[31] J. H. Boyd, S. Mathur, Y. Wang, R. M. Bateman, and K. R. Walley, "Toll-like receptor stimulation in cardiomyoctes decreases contractility and initiates an NF-κB dependent inflammatory response," *Cardiovascular Research*, vol. 72, no. 3, pp. 384–393, 2006.

[32] T. Takahashi, "Toll-like receptors and myocardial contractile dysfunction," *Cardiovascular Research*, vol. 78, no. 1, pp. 3–4, 2008.

[33] H. Shi, M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier, "TLR4 links innate immunity and fatty acid-induced insulin resistance," *Journal of Clinical Investigation*, vol. 116, no. 11, pp. 3015–3025, 2006.

[34] A. Schaeffler, P. Gross, R. Buettner et al., "Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-κB pathway in adipocytes links nutritional signalling with innate immunity," *Immunology*, vol. 126, no. 2, pp. 233–245, 2009.

[35] M. B. Fessler, L. L. Rudel, and J. M. Brown, "Toll-like receptor signaling links dietary fatty acids to the metabolic syndrome," *Current Opinion in Lipidology*, vol. 20, no. 5, pp. 379–385, 2009.

[36] Y. Qiao, P. Wang, J. Qi, L. Zhang, and C. Gao, "TLR-induced NF-κB activation regulates NLRP3 expression in murine macrophages," *FEBS Letters*, vol. 586, no. 7, pp. 1022–1026, 2012.

[37] H. J. Ko, Z. Zhang, D. Y. Jung et al., "Nutrient stress activates inflammation and reduces glucose metabolism by suppressing AMP-activated protein kinase in the heart," *Diabetes*, vol. 58, no. 11, pp. 2536–2546, 2009.

[38] M. R. Dasu, S. Ramirez, and R. R. Isseroff, "Toll-like receptors and diabetes: a therapeutic perspective," *Clinical Science*, vol. 122, no. 5, pp. 203–214, 2012.

[39] Y. Zhang, T. Peng, H. Zhu et al., "Prevention of hyperglycemia-induced myocardial apoptosis by gene silencing of Toll-like receptor-4," *Journal of Translational Medicine*, vol. 8, article 133, 2010.

[40] M. J. Kolek, J. F. Carlquist, J. B. Muhlestein et al., "Toll-like receptor 4 gene Asp299Gly polymorphism is associated with reductions in vascular inflammation, angiographic coronary artery disease, and clinical diabetes," *American Heart Journal*, vol. 148, no. 6, pp. 1034–1040, 2004.

[41] A. Riad, H. Meyer zu Schwabedissen, K. Weitmann et al., "Variants of Toll-like receptor 4 predict cardiac recovery in patients

with dilated cardiomyopathy," *The Journal of Biological Chemistry*, vol. 287, no. 32, pp. 27236–27243, 2012.

[42] M. R. Dasu, S. Devaraj, L. Zhao, D. H. Hwang, and I. Jialal, "High glucose induces toll-like receptor expression in human monocytes Mechanism of activation," *Diabetes*, vol. 57, no. 11, pp. 3090–3098, 2008.

[43] M. A. G. Ururahy, M. B. Loureiro, F. P. Freire-Neto et al., "Increased TLR2 expression in patients with type 1 diabetes: evidenced risk of microalbuminuria," *Pediatric Diabetes*, vol. 13, no. 2, pp. 147–154, 2012.

[44] K. Schroder, R. Zhou, and J. Tschopp, "The NLRP3 inflammasome: a sensor for metabolic danger?" *Science*, vol. 327, no. 5963, pp. 296–300, 2010.

[45] B. Vandanmagsar, Y.-H. Youm, A. Ravussin et al., "The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance," *Nature Medicine*, vol. 17, no. 2, pp. 179–189, 2011.

[46] K. Hirao, H. Yumoto, K. Takahashi, K. Mukai, T. Nakanishi, and T. Matsuo, "Roles of TLR2, TLR4, NOD2, and NOD1 in pulp fibroblasts," *Journal of Dental Research*, vol. 88, no. 8, pp. 762–767, 2009.

[47] J. A. Kummer, R. Broekhuizen, H. Everett et al., "Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response," *Journal of Histochemistry and Cytochemistry*, vol. 55, no. 5, pp. 443–452, 2007.

[48] N. A. Bracey, P. L. Beck, D. A. Muruve et al., "The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1beta," in *Experimental Physiology*, pp. 462–472, 2013.

[49] M. Coggins and A. Rosenzweig, "The fire within: cardiac inflammatory signaling in health and disease," *Circulation Research*, vol. 110, no. 1, pp. 116–125, 2012.

[50] C. Jin and R. A. Flavell, "Innate sensors of pathogen and stress: linking inflammation to obesity," *Journal of Allergy and Clinical Immunology*, vol. 132, no. 2, pp. 287–294, 2013.

[51] H. Wen, J. P.-Y. Ting, and L. A. J. O'Neill, "A role for the NLRP3 inflammasome in metabolic diseases—did Warburg miss inflammation?" *Nature Immunology*, vol. 13, no. 4, pp. 352–357, 2012.

[52] A. M. K. Choi and K. Nakahira, "Dampening insulin signaling by an NLRP3 "meta-flammasome"," *Nature Immunology*, vol. 12, no. 5, pp. 379–380, 2011.

[53] E. Latz, T. S. Xiao, and A. Stutz, "Activation and regulation of the inflammasomes," *Nature Reviews Immunology*, vol. 13, no. 6, pp. 397–411, 2013.

[54] F. G. Bauernfeind, G. Horvath, A. Stutz et al., "Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression," *Journal of Immunology*, vol. 183, no. 2, pp. 787–791, 2009.

[55] C. Juliana, T. Fernandes-Alnemri, S. Kang et al., "Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation," *The Journal of Biological Chemistry*, vol. 287, no. 43, pp. 36617–36622, 2012.

[56] K. Shimada, T. R. Crother, J. Karlin et al., "Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis," *Immunity*, vol. 36, no. 3, pp. 401–414, 2012.

[57] R. Zhou, A. Tardivel, B. Thorens, I. Choi, and J. Tschopp, "Thioredoxin-interacting protein links oxidative stress to inflammasome activation," *Nature Immunology*, vol. 11, no. 2, pp. 136–140, 2010.

[58] F. Bauernfeind, A. Rieger, F. A. Schildberg et al., "NLRP3 inflammasome activity is negatively controlled by miR-223," *The Journal of Immunology*, vol. 189, no. 8, pp. 4175–4181, 2012.

[59] C. J. Zuurbier, W. M. Jong, O. Eerbeek et al., "Deletion of the innate immune NLRP3 receptor abolishes cardiac ischemic preconditioning and is associated with decreased Il-6/STAT3 signaling," *PLoS One*, vol. 7, no. 7, article e40643, 2012.

[60] C. Hermansson, A. Lundqvist, C. Wasslavik et al., "Reduced expression of NLRP3 and MEFV in human ischemic heart tissue," *Biochemical and Biophysical Research Communications*, vol. 430, no. 1, pp. 425–428, 2013.

[61] S. Merkle, S. Frantz, M. P. Schön et al., "A role for caspase-1 in heart failure," *Circulation Research*, vol. 100, no. 5, pp. 645–653, 2007.

[62] D. Verma, M. Lerm, R. B. Julinder, P. Eriksson, P. Söderkvist, and E. Särndahl, "Gene polymorphisms in the NALP3 inflammasome are associated with interleukin-1 production and severe inflammation relation to common inflammatory diseases?" *Arthritis and Rheumatism*, vol. 58, no. 3, pp. 888–894, 2008.

[63] D. Verma, E. Särndahl, H. Andersson et al., "The Q705K polymorphism in NLRP3 is a gain-of-function alteration leading to excessive interleukin-1β and IL-18 production," *PLoS ONE*, vol. 7, no. 4, artile e34977, 2012.

[64] A. Dehghan, J. Dupuis, M. Barbalic et al., "Meta-analysis of genome-wide association studies in > 80 000 subjects identifies multiple loci for C-reactive protein levels," *Circulation*, vol. 123, no. 7, pp. 731–738, 2011.

[65] M. Kawaguchi, M. Takahashi, T. Hata et al., "Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury," *Circulation*, vol. 123, no. 6, pp. 594–604, 2011.

[66] H. Wen, D. Gris, Y. Lei et al., "Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling," *Nature Immunology*, vol. 12, no. 5, pp. 408–415, 2011.

[67] E. K. Grishman, P. C. White, and R. C. Savani, "Toll-like receptors, the NLRP3 inflammasome, and interleukin-1beta in the development and progression of type 1 diabetes," *Pediatric Research*, vol. 71, no. 6, pp. 626–632, 2012.

[68] W. Wahli and L. Michalik, "PPARs at the crossroads of lipid signaling and inflammation," *Trends in Endocrinology & Metabolism*, vol. 23, no. 7, pp. 351–363, 2012.

[69] E. M. Burkart, N. Sambandam, X. Han et al., "Nuclear receptors PPARβ/δ and PPARα direct distinct metabolic regulatory programs in the mouse heart," *Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3930–3939, 2007.

[70] J. J. Wright, J. Kim, J. Buchanan et al., "Mechanisms for increased myocardial fatty acid utilization following short-term high-fat feeding," *Cardiovascular Research*, vol. 82, no. 2, pp. 351–360, 2009.

[71] X. Palomer, D. Álvarez-Guardia, R. Rodríguez-Calvo et al., "TNF-α reduces PGC-1α expression through NF-κB and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model," *Cardiovascular Research*, vol. 81, no. 4, pp. 703–712, 2009.

[72] I. Karakikes, M. Kim, L. Hadri et al., "Gene remodeling in type 2 diabetic cardiomyopathy and its phenotypic rescue with SERCA2a," *PloS one*, vol. 4, no. 7, article e6474, 2009.

[73] A. M. Quintela, R. Jimenez, M. Gomez-Guzman et al., "Activation of peroxisome proliferator-activated receptor-b/-d (PPARb/d) prevents endothelial dysfunction in type 1 diabetic rats," *Free Radical Biology & Medicine*, vol. 53, pp. 730–741, 2012.

[74] M. Collino, E. Benetti, M. Rogazzo et al., "Reversal of the deleterious effects of chronic dietary HFCS-55 intake by PPAR-delta agonism correlates with impaired NLRP3 inflammasome activation," *Biochemical Pharmacology*, vol. 85, no. 2, pp. 257–264, 2013.

[75] M. R. Dasu, S. Park, S. Devaraj, and I. Jialal, "Pioglitazone inhibits toll-like receptor expression and activity in human monocytes and db/db mice," *Endocrinology*, vol. 150, no. 8, pp. 3457–3464, 2009.

[76] C. B. Li, X. X. Li, Y. G. Chen et al., "Effects and mechanisms of PPARalpha activator fenofibrate on myocardial remodelling in hypertension," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 11-12, pp. 4444–4452, 2009.

[77] D. Álvarez-Guardia, X. Palomer, T. Coll et al., "PPARβ/δ activation blocks lipid-induced inflammatory pathways in mouse heart and human cardiac cells," *Biochimica et Biophysica Acta*, vol. 1811, no. 2, pp. 59–67, 2011.

[78] R. Genolet, W. Wahli, and L. Michalik, "PPARs as drug targets to modulate inflammatory responses?" *Current Drug Targets*, vol. 3, no. 4, pp. 361–375, 2004.

[79] N. E. Buroker, J. Barboza, and J.-Y. Huang, "The IκBα gene is a peroxisome proliferator-activated receptor cardiac target gene," *FEBS Journal*, vol. 276, no. 12, pp. 3247–3255, 2009.

[80] A. Shimamoto, A. J. Chong, M. Yada et al., "Inhibition of toll-like receptor 4 with eritoran attenuates myocardial ischemia-reperfusion injury," *Circulation*, vol. 114, no. 1, pp. I270–I274, 2006.

[81] R. Rodríguez-Calvo, L. Serrano, T. Coll et al., "Activation of peroxisome proliferator-activated receptor β/δ inhibits lipopolysaccharide-induced cytokine production in adipocytes by lowering nuclear factor-κB activity via extracellular signal-related kinase 1/2," *Diabetes*, vol. 57, no. 8, pp. 2149–2157, 2008.

[82] M. J. Holness, S. Samsuddin, and M. C. Sugden, "The role of PPARs in modulating cardiac metabolism in diabetes," *Pharmacological Research*, vol. 60, no. 3, pp. 185–194, 2009.

[83] F. Zandbergen and J. Plutzky, "PPARα in atherosclerosis and inflammation," *Biochimica et Biophysica Acta*, vol. 1771, no. 8, pp. 972–982, 2007.

[84] S. L. Kempster, G. Belteki, A. J. Forhead et al., "Developmental control of the Nlrp6 inflammasome and a substrate, IL-18, in mammalian intestine," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 300, no. 2, pp. G253–G263, 2011.

[85] G. S. Lee, N. Subramanian, A. I. Kim et al., "The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca2+ and cAMP," *Nature*, vol. 492, no. 7427, pp. 123–127, 2012.

[86] M. Rossol, M. Pierer, N. Raulien et al., "Extracellular Ca2+ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors," *Nature Communications*, vol. 3, p. 1329, 2012.

[87] Y. Qu, S. Misaghi, A. Izrael-Tomasevic et al., "Phosphorylation of NLRC4 is critical for inflammasome activation," *Nature*, vol. 490, no. 7421, pp. 539–542, 2012.

[88] M. E. Kotas, M. C. Gorecki, and M. P. Gillum, "Sirtuin-1 is a nutrient-dependent modulator of inflammation," *Adipocyte*, vol. 2, no. 2, pp. 113–118, 2013.

[89] M. Tanno, A. Kuno, Y. Horio et al., "Emerging beneficial roles of sirtuins in heart failure," *Basic Research in Cardiology*, vol. 107, no. 4, p. 273, 2012.

[90] A. Kauppinen, T. Suuronen, J. Ojala et al., "Antagonistic crosstalk between NF-kappaB and SIRT1 in the regulation of inflammation and metabolic disorders," *Cell Signal*, vol. 25, no. 10, pp. 1939–1948, 2013.

[91] T. Yoshizaki, S. Schenk, T. Imamura et al., "SIRT1 inhibits inflammatory pathways in macrophages and modulates insulin sensitivity," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 298, no. 3, pp. E419–E428, 2010.

[92] P. T. Pfluger, D. Herranz, S. Velasco-Miguel, M. Serrano, and M. H. Tschöp, "Sirt1 protects against high-fat diet-induced metabolic damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 28, pp. 9793–9798, 2008.

[93] R. R. Alcendor, S. Gao, P. Zhai et al., "Sirt1 regulates aging and resistance to oxidative stress in the heart," *Circulation Research*, vol. 100, no. 10, pp. 1512–1521, 2007.

[94] A. Planavila, R. Iglesias, M. Giralt, and F. Villarroya, "Sirt1 acts in association with PPARα to protect the heart from hypertrophy, metabolic dysregulation, and inflammation," *Cardiovascular Research*, vol. 90, no. 2, pp. 276–284, 2011.

[95] F. Yeung, J. E. Hoberg, C. S. Ramsey et al., "Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase," *EMBO Journal*, vol. 23, no. 12, pp. 2369–2380, 2004.

[96] T. F. Liu, B. K. Yoza, M. El Gazzar, V. T. Vachharajani, and C. E. McCall, "NAD+-dependent SIRT1 deacetylase participates in epigenetic reprogramming during endotoxin tolerance," *Journal of Biological Chemistry*, vol. 286, no. 11, pp. 9856–9864, 2011.

[97] H. Zhu, Y. Yang, Y. Wang, J. Li, P. W. Schiller, and T. Peng, "MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1," *Cardiovascular Research*, vol. 92, no. 1, pp. 75–84, 2011.

[98] F. Arslan, M. B. Smeets, L. A. J. O'Neill et al., "Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody," *Circulation*, vol. 121, no. 1, pp. 80–90, 2010.

[99] H. Ehrentraut, C. Weber, S. Ehrentraut et al., "The toll-like receptor 4-antagonist eritoran reduces murine cardiac hypertrophy," *European Journal of Heart Failure*, vol. 13, no. 6, pp. 602–610, 2011.

[100] R. C. Barber, D. L. Maass, D. J. White, L.-Y. E. Chang, and J. W. Horton, "Molecular or pharmacologic inhibition of the CD14 signaling pathway protects against burn-related myocardial inflammation and dysfunction," *Shock*, vol. 30, no. 6, pp. 705–713, 2008.

[101] W. Piao, S. N. Vogel, and V. Y. Toshchakov, "Inhibition of TLR4 signaling by TRAM-derived decoy peptides in vitro and in vivo," *The Journal of Immunology*, vol. 190, no. 5, pp. 2263–2272, 2013.

[102] N. Matsunaga, N. Tsuchimori, T. Matsumoto, and M. Ii, "TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules," *Molecular Pharmacology*, vol. 79, no. 1, pp. 34–41, 2011.

[103] P. Chansrichavala, U. Chantharaksri, P. Sritara, and S. C. Chaiyaroj, "Atorvastatin attenuates TLR4-mediated NF-κB activation in a MyD88-dependent pathway," *Asian Pacific Journal of Allergy and Immunology*, vol. 27, no. 1, pp. 49–57, 2009.

[104] G. Földes, S. von Haehling, D. O. Okonko, E. A. Jankowska, P. A. Poole-Wilson, and S. D. Anker, "Fluvastatin reduces increased

blood monocyte Toll-like receptor 4 expression in whole blood from patients with chronic heart failure," *International Journal of Cardiology*, vol. 124, no. 1, pp. 80–85, 2008.

[105] J. Yang, H. Jiang, J. Yang et al., "Valsartan preconditioning protects against myocardial ischemia-reperfusion injury through TLR4/NF-κB signaling pathway," *Molecular and Cellular Biochemistry*, vol. 330, no. 1-2, pp. 39–46, 2009.

[106] H. L. Wen, Z. S. Liang, R. Zhang et al., "Anti-inflammatory effects of triptolide improve left ventricular function in a rat model of diabetic cardiomyopathy," *Cardiovascular Diabetology*, vol. 12, p. 50, 2013.

[107] D. Westermann, S. Van Linthout, S. Dhayat et al., "Cardioprotective and anti-inflammatory effects of interleukin converting enzyme inhibition in experimental diabetic cardiomyopathy," *Diabetes*, vol. 56, no. 7, pp. 1834–1841, 2007.

[108] D. Y. Fann, S. Y. Lee, S. Manzanero et al., "Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated neuronal death in ischemic stroke," *Cell Death & Disease*, vol. 4, article e790, 2013.

[109] C. M. Larsen, M. Faulenbach, A. Vaag et al., "Interleukin-1-receptor antagonist in type 2 diabetes mellitus," *The New England Journal of Medicine*, vol. 356, no. 15, pp. 1517–1526, 2007.

[110] J. Geiler and M. F. McDermott, "Gevokizumab, an anti-IL-1β mAb for the potential treatment of type 1 and 2 diabetes, rheumatoid arthritis and cardiovascular disease," *Current Opinion in Molecular Therapeutics*, vol. 12, no. 6, pp. 755–769, 2010.

[111] R. Stienstra, L. A. B. Joosten, T. Koenen et al., "The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity," *Cell Metabolism*, vol. 12, no. 6, pp. 593–605, 2010.

[112] R. J. Shaw, K. A. Lamia, D. Vasquez et al., "Medicine: The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin," *Science*, vol. 310, no. 5754, pp. 1642–1646, 2005.

[113] P. W. Caton, N. K. Nayuni, J. Kieswich, N. Q. Khan, M. M. Yaqoob, and R. Corder, "Metformin suppresses hepatic gluconeogenesis through induction of SIRT1 and GCN5," *Journal of Endocrinology*, vol. 205, no. 1, pp. 97–106, 2010.

[114] Z. Zheng, H. Chen, J. Li et al., "Sirtuin 1-mediated cellular metabolic memory of high glucose via the LKB1/AMPK/ROS pathway and therapeutic effects of metformin," *Diabetes*, vol. 61, no. 1, pp. 217–228, 2012.

[115] M. Lamkanfi, J. L. Mueller, A. C. Vitari et al., "Glyburide inhibits the Cryopyrin/Nalp3 inflammasome," *Journal of Cell Biology*, vol. 187, no. 1, pp. 61–70, 2009.

[116] T. Jourdan, G. Godlewski, R. Cinar et al., "Activation of the Nlrp3 inflammasome in infiltrating macrophages by endocannabinoids mediates beta cell loss in type 2 diabetes," *Nature Medicine*, vol. 19, no. 9, pp. 1132–1140, 2013.

[117] S. Ares-Carrasco, B. Picatoste, E. Camafeita et al., "Proteome changes in the myocardium of experimental chronic diabetes and hypertension: role of PPARα in the associated hypertrophy," *Journal of Proteomics*, vol. 75, no. 6, pp. 1816–1829, 2012.

[118] R. S. Rosenson, R. S. Wright, M. Farkouh et al., "Modulating peroxisome proliferator-activated receptors for therapeutic benefit? Biology, clinical experience, and future prospects," *American Heart Journal*, vol. 164, no. 5, pp. 672–680, 2012.

[119] X. C. Dong, "Sirtuin biology and relevance to diabetes treatment," *Diabetes Management*, vol. 2, no. 3, pp. 243–257, 2012.

[120] T. I. Lee, Y. H. Kao, Y. C. Chen et al., "Peroxisome proliferator-activated receptors modulate cardiac dysfunction in diabetic cardiomyopathy," *Diabetes Research and Clinical Practice*, vol. 100, no. 3, pp. 330–339, 2013.

[121] J. Xu, M. K. Racke, and P. D. Drew, "Peroxisome proliferator-activated receptor-α agonist fenofibrate regulates IL-12 family cytokine expression in the CNS: relevance to multiple sclerosis," *Journal of Neurochemistry*, vol. 103, no. 5, pp. 1801–1810, 2007.

[122] N. N. Ta, C. A. Schuyler, Y. Li, M. F. Lopes-Virella, and Y. Huang, "DPP-4 (CD26) inhibitor alogliptin inhibits atherosclerosis in diabetic apolipoprotein E-deficient mice," *Journal of Cardiovascular Pharmacology*, vol. 58, no. 2, pp. 157–166, 2011.

[123] M. Daoudi, N. Hennuyer, M. G. Borland et al., "PPARβ/δ activation induces enteroendocrine L cell GLP-1 production," *Gastroenterology*, vol. 140, no. 5, pp. 1564–1574, 2011.

[124] J. C. Milne, P. D. Lambert, S. Schenk et al., "Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes," *Nature*, vol. 450, no. 7170, pp. 712–716, 2007.

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Research Article

Taurine Alleviates the Progression of Diabetic Nephropathy in Type 2 Diabetic Rat Model

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The overexpression of vascular endothelial growth factor (VEGF) is known to be involved in the pathogenesis of diabetic nephropathy. In this study, the protective effects of taurine on diabetic nephropathy along with its underlying mechanism were investigated. Experimental animals were divided into three groups: LETO rats as normal group ($n = 10$), OLETF rats as diabetic control group ($n = 10$), and OLETF rats treated with taurine group ($n = 10$). We treated taurine (200 mg/kg/day) for 20 weeks and treated high glucose (HG, 30 mM) with or without taurine (30 mM) in mouse cultured podocyte. After taurine treatment, blood glucose level was decreased and insulin secretion was increased. Taurine significantly reduced albuminuria and ACR. Also it decreased glomerular volume, GBM thickness and increased open slit pore density through decreased VEGF and increased nephrin mRNA expressions in renal cortex. The antioxidant effects of taurine were confirmed by the reduction of urine MDA in taurine treated diabetic group. Also reactive oxygen species (ROS) levels were decreased in HG condition with taurine treated podocytes compared to without taurine. These results indicate that taurine lowers glucose level via increased insulin secretion and ameliorates the progression of diabetic nephropathy through antifibrotic and antioxidant effects in type 2 diabetes rat model.

1. Introduction

Diabetes mellitus is a condition in which lower state of antioxidant has been observed [1]. The most common microvascular complication of diabetes mellitus is diabetic nephropathy [2]. The mechanisms involved in the pathogenesis of diabetic nephropathy are variable and many growth factors initiate diabetic renal complications [3]. Among growth factors, vascular endothelial growth factor (VEGF) plays a part in this pathogenesis [4]. The expression of VEGF increases in extremely vascularized and quickly growing tumors [5]. VEGF is known to increase vascular permeability to macromolecules [6] and its expression is localized to the epithelial glomerular cells, podocytes, distal tubules, and renal collecting duct in the normal kidney [7]. VEGF is especially increased during the early stages of diabetic nephropathy [8]; therefore, reducing VEGF overexpression may ameliorate diabetic renal disease [9].

Proteinuria is one of the diagnostic criteria for diabetic nephropathy. Recent studies have focused on changes to the glomerular basement membrane (GBM) structure [10, 11]. Nephrin is a podocyte-specific protein [12] and its expression is reduced in the early stages of proteinuria in diabetic patients [13].

Taurine (2-aminoethane sulphonic acid) is present in most mammalian tissue and have various effects such as osmoregulation, bile acid conjugation, cell proliferation, and the viability and prevention of oxidant induced tissues injury. In biological system, taurine has antioxidant effect to stabilize biomembranes, reduce the malondialdehyde, and scavenge

reactive oxygen species [14]. It is another candidate for the treatment of diabetic nephropathy.

Both plasma and platelet taurine concentrations are decreased in type 1 diabetic patients and these levels increase to normal after oral taurine administration [15]. Since Trachtman et al. reported marked reductions in proteinuria in streptozotocin- (STZ)-induced diabetic rats with decreased renal lipid peroxidation after oral supplementation of taurine [16], several reports about the effects of taurine on diabetic nephropathy showed the results of improvements in oxidative stress [17–20] and reductions in TGF-β expression [20]. Recently, taurine administration prevented the occurrence and development of diabetic nephropathy by decreasing blood glucose, improving lipid metabolism and glomerular basement membrane metabolism [21]. These effects of taurine on diabetic nephropathy have not been fully demonstrated and are currently under investigation.

In this study we investigated whether taurine could improve diabetic nephropathy or not and then would like to know the mechanism how it can ameliorate diabetic kidney disease.

2. Materials and Methods

2.1. Reagents. Taurine was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Treatment of Experimental Animals. All experiments were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of Yonsei University at Wonju Campus. Ten male Long-Evans-Tokushima-Otsuka (LETO, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) rats, were used as nondiabetic controls, and 30 male Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats were used for a type 2 diabetes mellitus model and were purchased from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). At 25 weeks of age the rats were classified into three groups as follows: normal control group, diabetic control group, and taurine-treated diabetic group (200 mg/kg/day). Animals were housed at a constant temperature (20 ± 2°C) and humidity level (50–60%) with a 12 hours light and dark cycle. The animals had free access to water and 20% sucrose containing rat chow (Junsei Chemical Company Ltd., Tokyo, Japan) until 45 weeks of age. The chemical treated groups received their treatments by an oral gavage tube from 26 to 45 weeks of age. At 15, 25, 35, and 45 weeks of age, body weights were measured. At 25, 35, and 45 weeks of age, 24 hours urine was collected with metabolic cages in order to measure the urinary albumin (Exocell Nephrat II; Exocell Inc., Philadelphia, PA, USA) and creatinine (Exocell The Creatinine Companion; Exocell Inc.). We then calculated a urinary albumin creatinine ratio (ACR). At 45 weeks of age, all rats were anesthetized with Zoletil50 (Virbac Laboratories, Carros, France) by intraperitoneal injection. Blood samples were taken by cardiac puncture and collected in test tubes containing heparin solution and centrifuged at 1,500 g for 10 minutes in order to obtain plasma. The plasma was stored at −80°C until use. After perfusion with 0.9% saline, both kidneys were removed. One kidney was stored at −80°C for analysis of mRNA and protein expression and the other kidney was embedded with 4% paraformaldehyde for histological examination.

2.3. Measurements of Fasting Blood Glucose, Plasma Insulin, and Adiponectin. Twelve-hour fasting blood glucose levels were analyzed with the LifeScan SureStep (Lifescan, Burnby, Canada) using tail vein blood. Plasma glucose disposal rate (short insulin tolerance test, Kitt; %/min), which indicates the time necessary to reduce the basal glucose level by 50%, was calculated as $0.693/t_{1/2}$, where $t_{1/2}$ was determined from the slope of the glycemic concentrations from 3 to 15 minutes after intravenous injection of regular insulin (1 U/kg). Plasma levels of insulin (Shibayagi Co., Shibukawa, Japan) and adiponectin (AdipoGen Inc., Seoul, Korea) were measured using an ELISA kit. Beta-cell function was determined by homeostasis model assessment of beta-cell function (HOMA-β) which was calculated as follows [22]: fasting insulin (μU/mL) × 20/[fasting glucose (mM/L) − 3.5].

2.4. Determination of Urinary Malondialdehyde (MDA) Levels. A rapid and sensitive fluorometric HPLC method was used to measure urine MDA, which was analyzed by the NeoDin Medical Institute (Seoul, Korea). Urine samples were treated with 0.1125N PCA and 40 mM 2-TBA and subjected to heat at 97°C for 1 hour. To stop the reaction, the samples were put on ice for 20 minutes and then methanol and 20% TCA buffer were added. Samples were mixed and centrifuged at 13,000 g for 6 minutes; then the supernatant was transferred to the insert vial. The fluorescence detector was set to an excitation wavelength of 525 nm and an emission wavelength of 560 nm. The run time was 2 minutes and the flow rate was 1 mL/min.

2.5. Histological Examination of Kidney. Paraffin-embedded kidney tissue was cut into sections 4 μm thick and stained with periodic acid-Schiff (PAS). We examined these sections with an optical microscope that was equipped with a charge coupled device camera (Pulnix, Sunnyvale, CA, USA) in order to obtain the pictures of glomeruli from the outer and middle thirds of the renal cortex. We measured glomerular areas using an image analysis system (GmbH, SIS, Minster, Germany) and calculated glomerular volume using the Weibel and Gomez formula as follows [23]: Glomerular volume (Gv) = $\text{Area}^{1.5} \times 1.38/1.01$ (1.38: shape coefficient, 1.01: size distribution coefficient). About 30 glomeruli were observed in the kidney sections of each rat.

For the evaluation of the ultrastructure of the glomeruli, kidney tissue was thin sectioned and examined under a JEOL transmission electron microscope (JEM-1200EX II, JEOL Ltd., Tokyo, Japan). Electron micrographs were taken at ×30 K for each sample. The numbers of slit pores were counted and divided by the GBM length (10 μm) to arrive at the linear density using an image analysis system. About 10 glomeruli were observed in the kidney sections of each rat.

2.6. Immunohistochemical Staining for VEGF. Four percent paraformaldehyde-fixed kidney tissues were embedded in

Table 1: Biochemical characteristics in experimental groups.

	LETO	OLETF	OLETF + TA
FBG (mg/dL)	90.1 ± 1.36	171.1 ± 14.32*	119.3 ± 10.94†
Insulin (ng/mL)	2.5 ± 0.65	1.0 ± 0.45*	1.6 ± 0.59*,†
Kitt (%/min)	5.9 ± 0.36	1.6 ± 0.49*	2.3 ± 0.54*
HOMA-IR	30.38 ± 2.64	26.12 ± 3.30	27.9 ± 5.47
HOMA-β	1908 ± 249.4	258.7 ± 65.23*	726.8 ± 133.03*,†
ADP (μg/mL)	7.3 ± 1.12	5.9 ± 1.39*	6.6 ± 1.16

Data are expressed as mean ± SEM.; ADP: adiponectin; FBG: fasting blood glucose; HOMA-β: homeostasis model assessment for beta-cell function; HOMA-IR: homeostasis model assessment for insulin resistance; Kitt: short insulin tolerance test; LETO: normal control group; OLETF: diabetic control group; OLETF + TA: taurine-treated diabetic group. * $P < 0.05$ compared with LETO; † $P < 0.05$ compared with OLETF.

paraffin. The kidney tissues were prepared into 4 μm sections and placed on a microscope slide. Paraffin was then removed at 60°C for 1 hour, followed by dehydration in xylene. The sections were subjected to graded alcohols, immersed in distilled water (PBS-T) for 5 minutes, and then incubated in 20 μg/mL proteinase K solution (DAKO, Glostrup, Denmark) for 15 minutes. The slides were washed with PBS-T and incubated in Ultra V block (DAKO) for 5 minutes in room temperature.

Polyclonal anti-VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added in a 1 : 100 dilution and the sections were kept overnight at 4°C. The slides were washed with PBS-T and biotinylated secondary antibody (Santa Cruz Biotechnology Inc.) was added. The antibody binding was visualized using the avidin and biotinylated horseradish peroxidase reaction. To evaluate the VEGF staining, slides were observed using a light-microscope adhered to a charge-coupled camera device (Pulnix, Sunnyvale, CA, USA). We measured the optical density of stained VEGF using an image analysis system (GmbH, SIS, Minster, Germany). About 20 glomeruli were observed in the kidney sections of each rat.

2.7. Expression of Nephrin and VEGF mRNA in the Kidney. Total RNA was isolated and purified from the kidney cortex and concentrations were determined using a microspectrophotometer. For cDNA synthesis, reverse transcription was performed with 1 μg of RNA. The cDNA was prepared using these samples as templates according to protocols provided with a commercially available QuantiTect Reverse Transcription kit (QuantiTect reverse transcription kit; Qiagen, Hilden, Germany). For quantitative real-time PCR, SYBR Green PCR master mix (Applied Biosystem, Foster City, CA, USA) was used in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Quantitative real-time PCR was activated and cDNA was denatured by a preincubation for 15 minutes at 95°C; the template was then amplified for 35 cycles with denaturation for 15 seconds at 94°C, annealing of primers at 58°C for 30 seconds, and extension at 72°C for 30 seconds.

2.8. Immunoblot Analysis of the Kidney. To analyze the protein expression of VEGF in the kidney tissues, a western blot analysis was performed. The kidney cortex samples were homogenized by the TissueLyser II (QIAGEN GmbHm Haan, Germany) in a RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA). The protein samples were heated for 5 minutes at 95°C and then electrophoresed on SDS-PAGE (10%) gels and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Bedford, MA, USA) for 2 hours at 250 mA. The membrane was blocked by 5% skim milk for 1 hour at room temperature and then incubated with rabbit anti-β-actin (1 : 1000) anti-VEGF (1 : 500) and antinephrin antibodies (1 : 1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. The membrane was washed 5 times in Tris-buffer saline/0.1% Tween 20 prior to 1 hour of probing with horseradish peroxidase-conjugated secondary antibody. The blots were detected using an enhanced chemiluminescent substrate (Thermo Fisher Scientific Inc., Fremont, CA, USA).

2.9. Cell Cultures. Conditionally immortalized mouse podocytes (provided kindly by Dr. Mundel of the University of Harvard, Cambridge, MA, USA) were cultured at 37.5°C without γ-interferon in DMEM containing 5.5 mM glucose. Differentiated podocytes were synchronized into quiescence by growing cells in serum-free medium for 24 hours. The cultured podocytes were treated with high glucose media (30 mM), high glucose media with taurine (30 mM). After 24 hours, we measured nephrin and VEGF mRNA expression.

2.10. Reactive Oxygen Species (ROS) Production in Podocytes. For the measurement of ROS production, cultured podocytes were added to dichlorofluorescein diacetate (DCF-DA) at a concentration of 10 μM at 37.5°C for 30 minutes and then analyzed for fluorescence using a fluorometer (GloMax-Multi Jr Single Tube Multimode Reader, Promega Corporation, Madison, WI, USA).

3. Data Analysis

Data are expressed as means ± SEM. Statistical analyses were conducted with SPSS version 18.0 for windows using a one-way ANOVA and Tukey's test. t-tests were used for experiments with only two groups. Differences were considered significant at $P < 0.05$.

4. Results

4.1. Clinical and Biochemical Characteristics of Experimental Rats. Fasting blood glucose levels significantly increased in

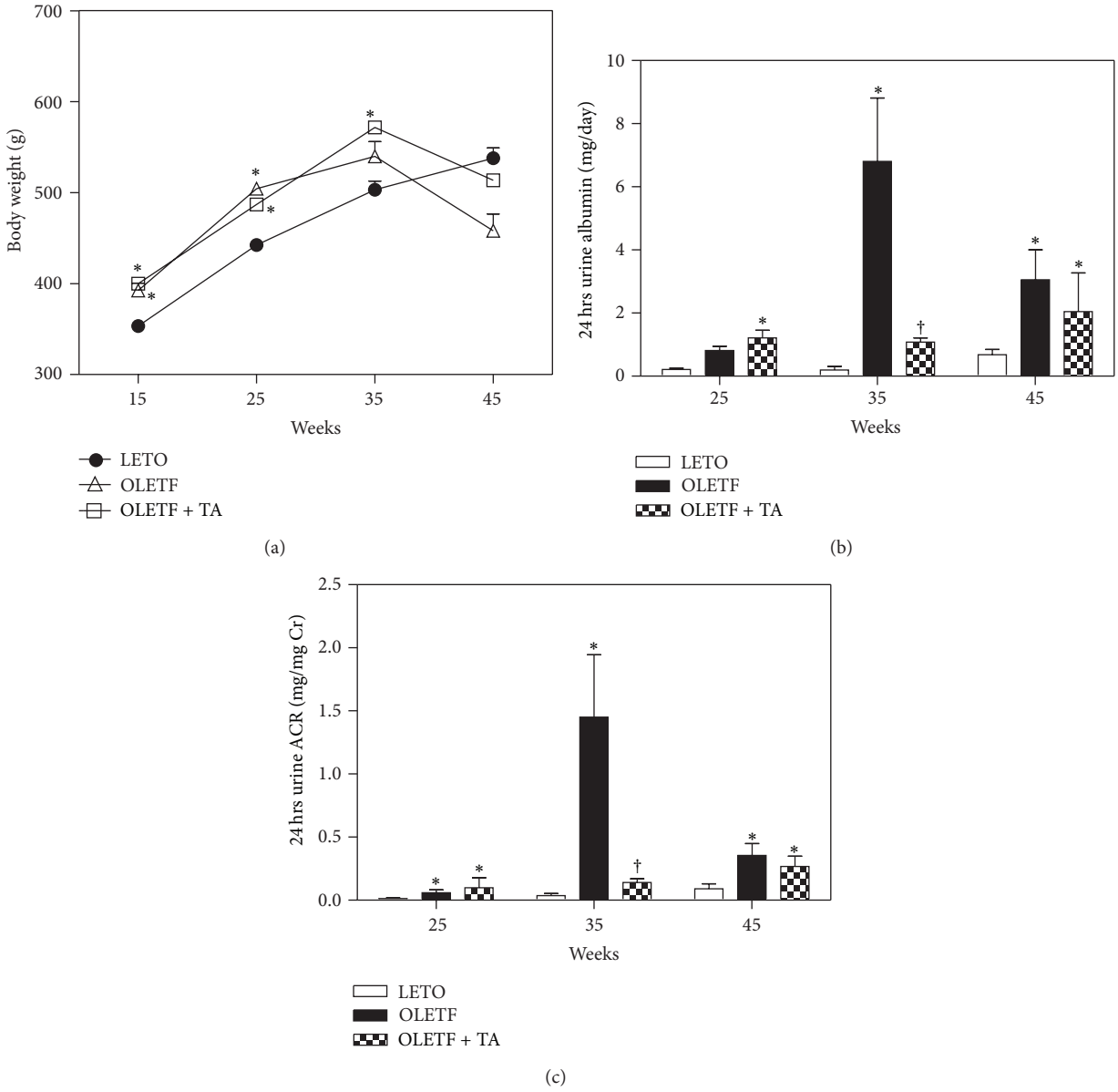

Figure 1: Changes of body weights, 24 hours urine albumin, and ACR in experimental rats on the basis of the duration of diabetes mellitus. (a) The body weights of OLETF and taurine-treated groups were decreased compared to LETO after 35 weeks of age. (b) Urine albumin levels were significantly lower at 35 weeks of age in the taurine-treated diabetic group compared to the diabetic control group. (c) 24 hours urine ACR significantly decreased in the taurine-treated diabetic group compared to the diabetic control group. ACR, albumin creatinine ratio; LETO, normal control group; OLETF, diabetic control group; OLETF + TA, taurine-treated diabetic group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with LETO; $^{\dagger}P < 0.05$ compared with OLETF.

the diabetic control group compared to the normal control group and significantly decreased in the taurine-treated diabetic control group. Insulin levels significantly decreased in the diabetic OLETF and diabetic with taurine groups compared to the normal control group. Among the diabetic groups, insulin levels were significantly higher in the taurine-treated diabetic group (OLETF + TA) compared to the diabetic control group (OLETF). HOMA-β was increased in the taurine-treated diabetic group compared to the diabetic control group. Adiponectin significantly decreased in the diabetic control group compared to the normal control group and increased in the taurine-treated OLETF group, although this result was not statistically significant (Table 1). While the body weights of the animals in the normal control group (LETO) increased continuously until 45 weeks of age, those of the animals in the diabetic OLETF and diabetic with taurine groups decreased slightly after 35 weeks of age (Figure 1(a)). 24 hours urine albumin and ACR significantly decreased in the taurine-treated diabetic group compared to the diabetic control group at 35 weeks; however these differences were not statistically different at 45 weeks (Figures 1(b) and 1(c)).

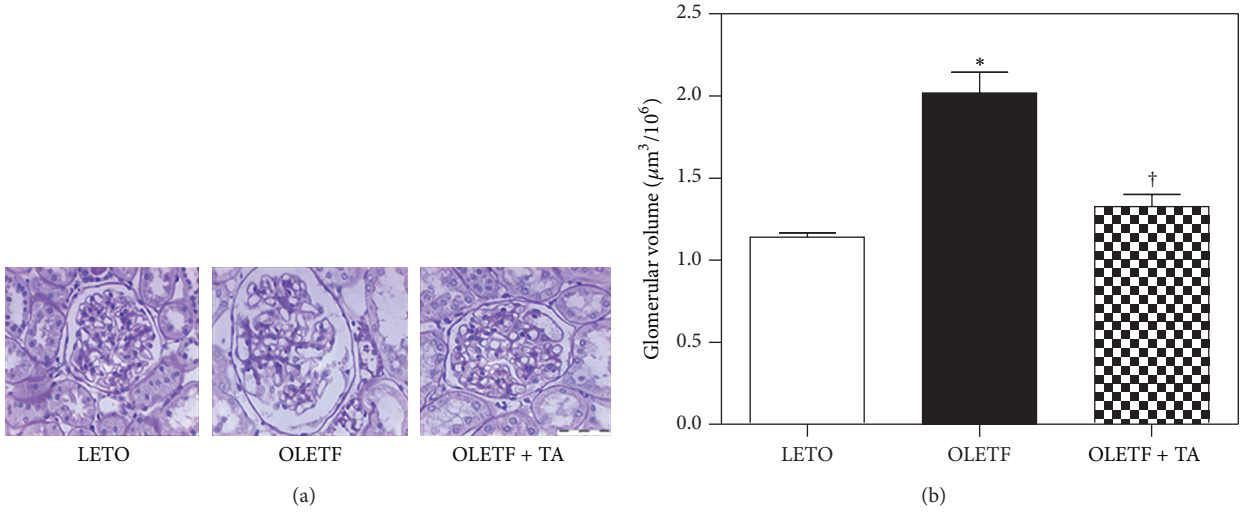

Figure 2: Morphological characteristics of renal glomeruli in the three groups. (a) Cross-sectioned glomeruli were stained with periodic acid-Schiff (×400). (b) The volume of renal glomeruli in the taurine-treated diabetic group decreased compared the diabetic control group. LETO, normal control group; OLETF, diabetic control group; OLETF + TA, taurine-treated diabetic group. Data are expressed as mean ± SEM. $^*P < 0.05$ compared with LETO; $^\dagger P < 0.05$ compared with OLETF.

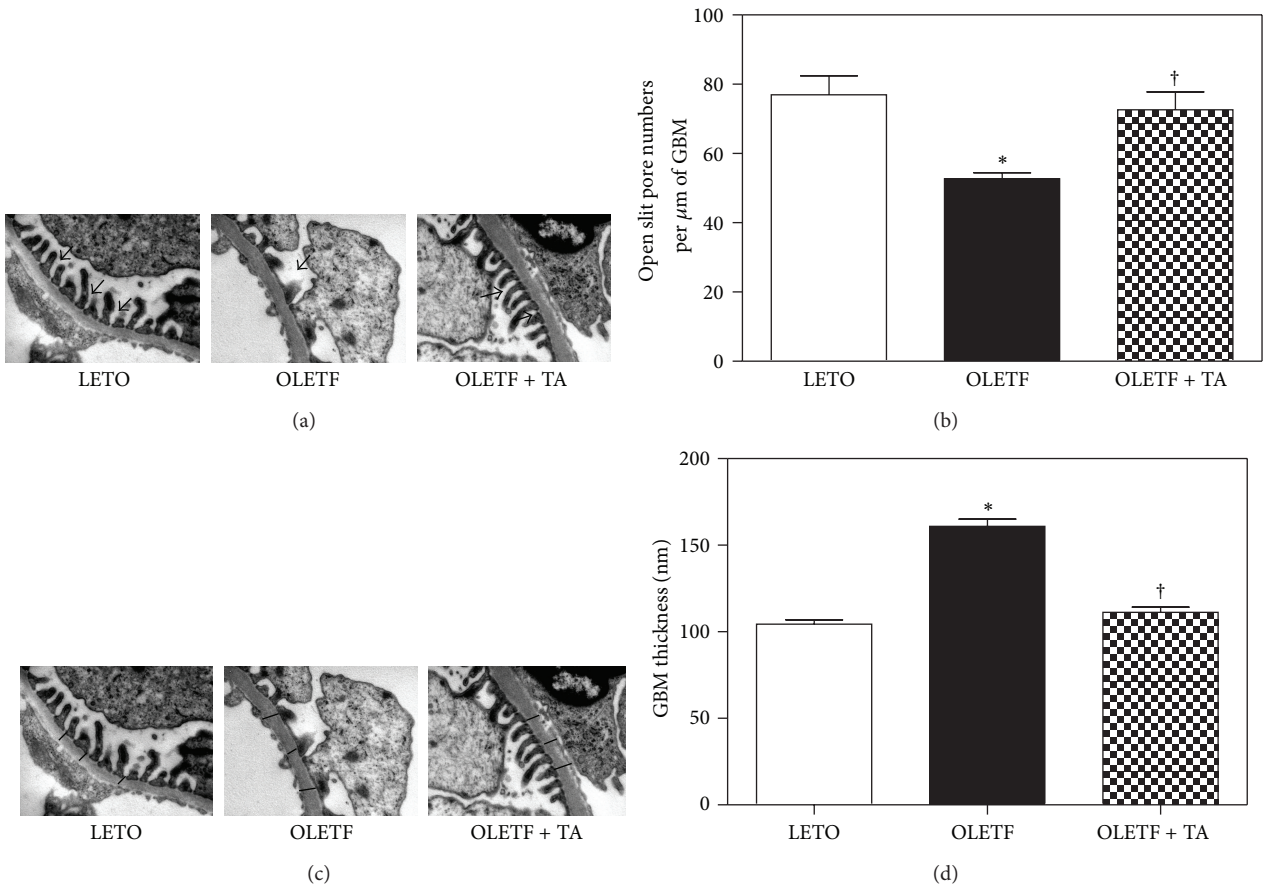

Figure 3: Changes in the numbers of open slit pores and GBM thickness among the three groups. (a) Electron microscopy demonstrated open slit pores (arrows) between the foot processes of the podocytes (×30 K). (b) The numbers of open slit pores significantly increased following taurine treatment when compared to the diabetic control group. (c) Electron microscopy revealed the GBM thickness. Arrows indicate the thickness of the GBM (×30 K). (d) The taurine-treated diabetic group had significantly decreased GBM thickness compared to the diabetic control group. GBM, glomerular basement membrane; LETO, normal control group; OLETF, diabetic control group; OLETF + TA, taurine-treated diabetic group. Data are expressed as mean ± SEM. $^*P < 0.05$ compared with LETO; $^\dagger P < 0.05$ compared with OLETF.

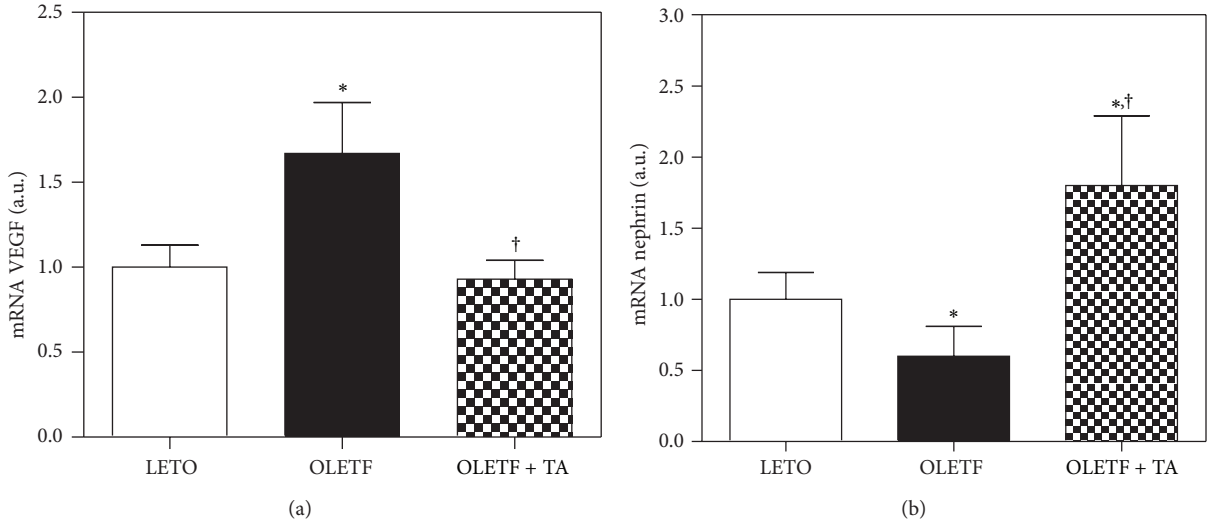

Figure 4: Differences in VEGF and nephrin mRNA expression in the kidney. (a) Renal VEGF mRNA expression decreased in the taurine-treated diabetic group compared to the diabetic control group. (b) Renal nephrin mRNA expression increased in response to taurine treatment. LETO, normal control group; OLETF, diabetic control group; OLETF + TA, taurine-treated diabetic group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with LETO; $^{\dagger}P < 0.05$ compared with OLETF.

4.2. Histological Changes of Renal Glomeruli. The weight of the right kidney increased in the diabetic groups and significantly decreased in the taurine-treated diabetic group (0.42 ± 0.02 g) than in the diabetic control group (0.51 ± 0.02 g). However, there was no statistical significance in the difference in left kidney weight between the diabetic control (0.52 ± 0.02 g) and taurine-treated diabetic groups (0.43 ± 0.03 g). The calculated glomerular volume of the diabetic control group significantly increased compared to the normal control group. In the taurine-treated diabetic group the volume of the renal glomerulus was significantly diminished compared to the diabetic control group (Figure 2).

4.3. Electron Microscopic Morphometry of the Open Slit Pores Number and Glomerular Basement Membrane Thickness. The numbers of open slit pores decreased in the diabetic control group compared to the normal control group and significantly increased in the taurine-treated diabetic group compared to the diabetic control group (Figures 3(a) and 3(b)). The thickness of the GBM was increased in the diabetic control group compared to the normal control group and significantly decreased in the taurine-treated diabetic group, approaching that of the normal control group (Figures 3(c) and 3(d)).

4.4. The mRNA Expression of VEGF, Nephrin, and Type IV Collagen in the Renal Cortex. The renal VEGF mRNA expression, according to quantitative RT-PCR, decreased significantly in the taurine-treated diabetic group compared to the diabetic control group (Figure 4(a)). On the other hand, renal nephrin expression significantly decreased in the diabetic control group compared to the normal control group. In the taurine-treated diabetic group, nephrin levels increased significantly compared to both the diabetic and normal control groups (Figure 4(b)). The type IV collagen mRNA increased in diabetic control group (1.6 ± 0.8) compared to normal control (1 ± 0.5) and the increased levels were reduced in taurine-treated group (1.3 ± 0.4). The levels were not significant in each group (data not shown).

4.5. The Renal VEGF and Nephrin Expression in the Kidney. By the immunohistochemistry, the renal VEGF expression decreased in the taurine-treated diabetic group compared to diabetic control group (Figures 5(a) and 5(b)) and by western blot, the VEGF expression decreased in the taurine-treated diabetic groups, although this difference was not statistically significant (Figures 5(c) and 5(d)). The nephrin levels increased in tuarine-treated diabetic group compared to diabetic control group. But the nephrin levels of diabetic control group also increased compared to normal control group. The levels were not significant different between each group (Figures 5(e) and 5(f)).

4.6. Antioxidant Effect of Taurine. To determine the oxidative stress, we examined MDA levels in urine samples collected over 24 hours at 45 weeks of age. MDA in the diabetic control group increased compared to the normal control group. MDA was lower in the taurine-treated diabetic group than in the diabetic group, although this difference was not statistically significant (Figure 6).

4.7. The Effects of Taurine on VEGF, Nephrin, and ROS in Mouse Cultured Podocytes. ROS formation significantly decreased after taurine treatment (Figure 7). VEGF mRNA expression increased in podocytes treated with high glucose (HG) compared to those treated with normal glucose (NG) and nephrin mRNA expression reduced in mouse cultured podocytes treated with high glucose (HG) compared to those

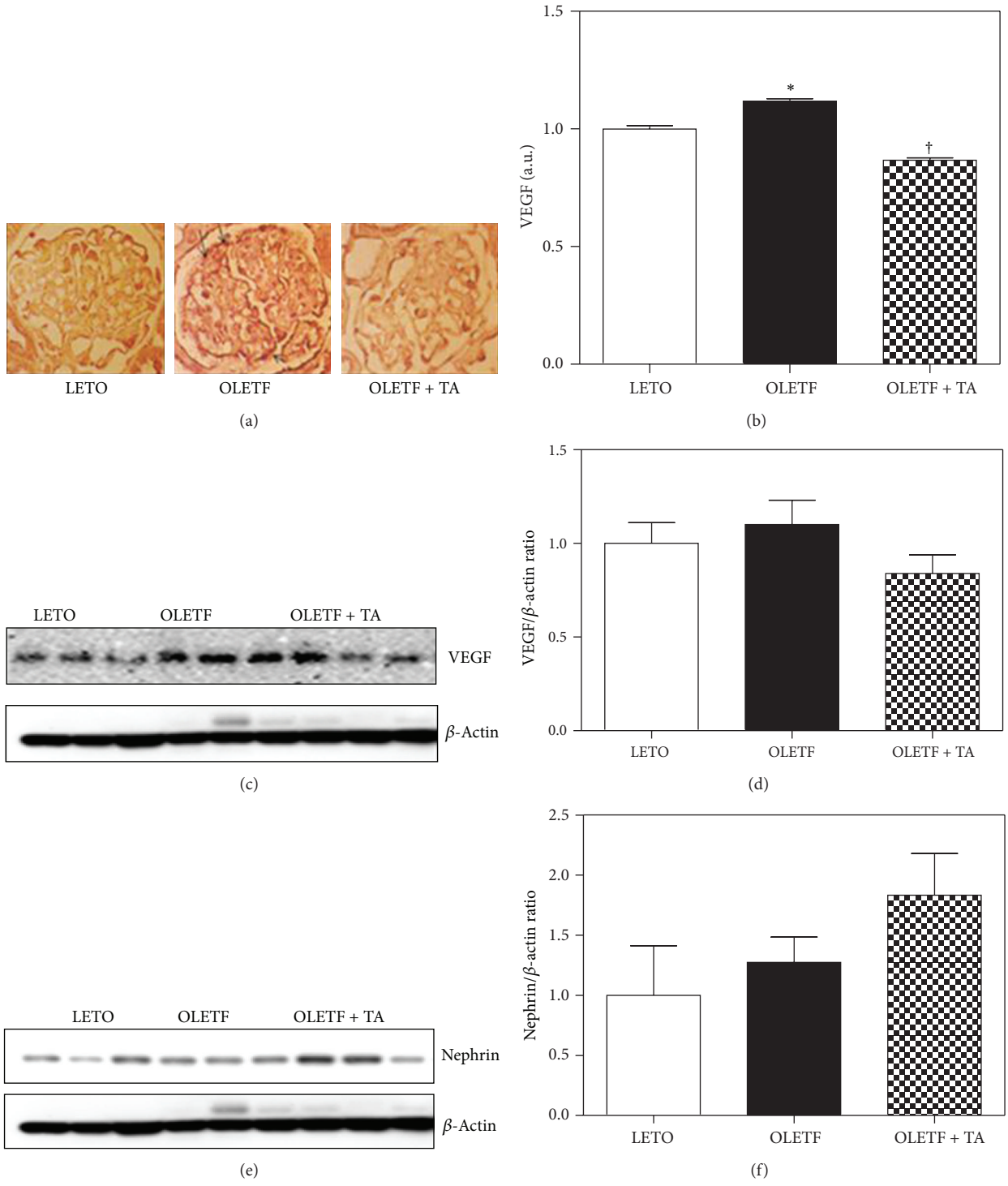

Figure 5: Effects of taurine on the expression of VEGF and nephrin in renal cortex. (a) Glomerular VEGF immunohistochemistry (arrows) in each group (×400). (b) Optical densities of VEGF in the glomeruli decreased significantly in the taurine-treated diabetic group compared to the diabetic control group. ((c), (d)) In the taurine-treated diabetic group, renal VEGF expression by western blot immunostaining decreased compared to that of the diabetic control group. ((e), (f)) The nephrin levels increased in taurine-treated group compared to diabetic control group. However the nephrin levels also increased in diabetic control group compared to normal control. But the VEGF and nephrin levels are not statistically significant. LETO, normal control group; OLETF, diabetic control group; OLETF + TA, taurine-treated diabetic group; VEGF, vascular endothelial growth factor. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with LETO; $^{\dagger}P < 0.05$ compared with OLETF.

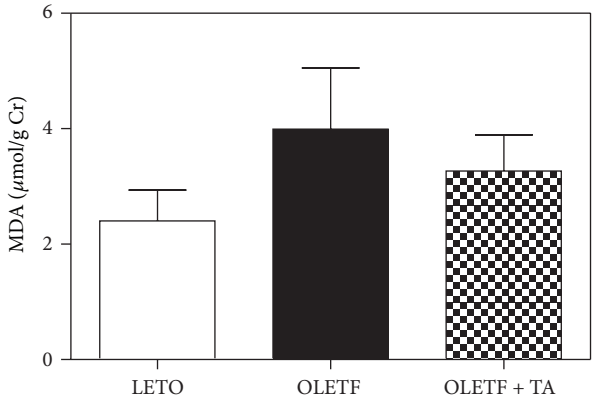

Figure 6: Changes in 24 hours urinary MDA levels at 45 weeks of age. In the diabetic control group, MDA increased compared to the normal control group. MDA decreased in the taurine-treatment group compared to the diabetic control group. However, there was no statistical significance in this difference. MDA, malondialdehyde; LETO, normal control group; OLETF, diabetic control group; OLETF + TA, taurine-treated diabetic group.

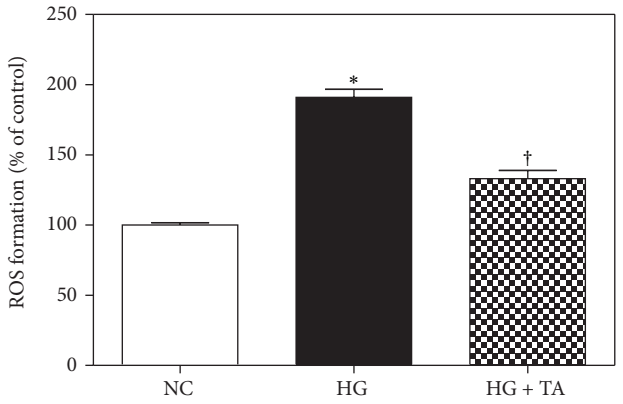

Figure 7: Changes in ROS formation in podocytes among the three groups. The taurine-treated high glucose group demonstrated a significantly decreased ROS production. NC, normal glucose; HG, high glucose; HG + TA, taurine-treated high glucose. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with NG; $^{\dagger}P < 0.05$ compared with HG.

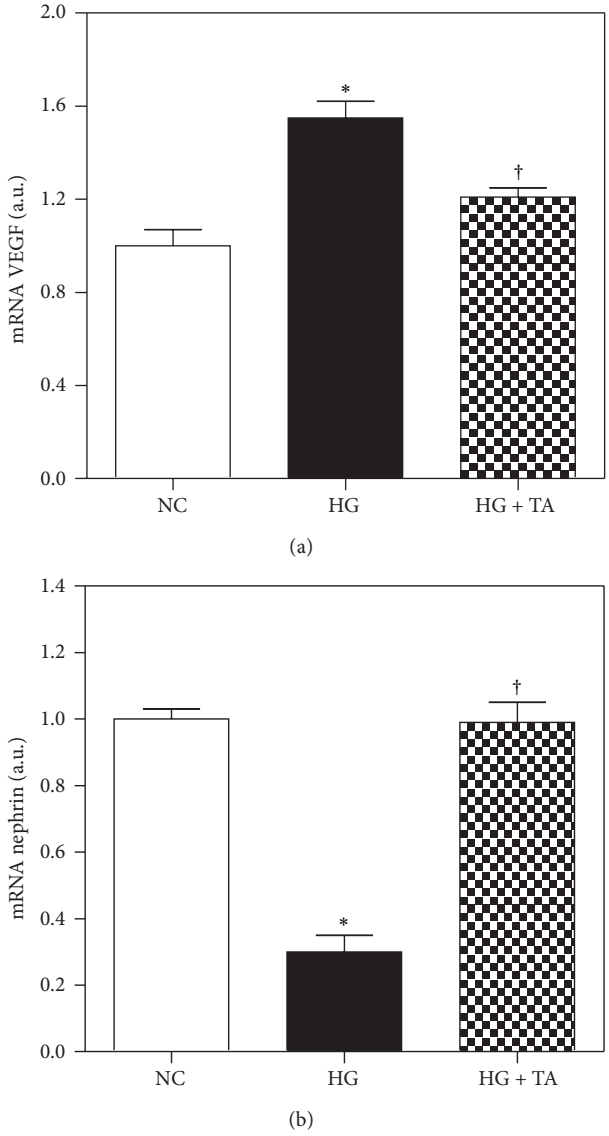

Figure 8: VEGF and nephrin mRNA expression by quantitative real-time PCR in mouse cultured podocytes. (a) VEGF mRNA expression significantly decreased in the taurine-treated HG group compared to that of the HG group. (b) In the taurine-treated high glucose group, nephrin expression increased compared to the high glucose group. NC, normal glucose; HG, high glucose; HG + TA, taurine-treated high glucose. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with NG; $^{\dagger}P < 0.05$ compared with HG.

treated with normal glucose (NG). But the changes significantly recovered to the control level after taurine treatment (Figures 8(a) and 8(b)).

5. Discussion

Since Huxtable compiled the physiological actions of taurine in 1992, many studies of taurine have been reported [24]. Taurine is one of the most abundant amino acids in the mammalian organs, and has a potent antioxidant property [16, 26].

In diabetic nephropathy, taurine treatment reduces proteinuria and albuminuria and prevents glomerular hypertrophy [16, 17, 19], mesangial extracellular matrix expansion [20], and hypertrophy in renal tubular epithelial cells [27].

Our focus of this study was to understand molecular targets of taurine in diabetic nephropathy. Our data demonstrate that treating the diabetic group with taurine led to decreased ACR and also ameliorated the glomerular volume, GBM thickness, and the numbers of open slit pores compared to the diabetic control group. Increased oxidative stress is one of the reasons for the pathogenesis of diabetic nephropathy [28–30]. Several studies have also reported the prevention of diabetic renal disease after taurine treatment and its association with decreased ROS formation [16–20]. Our study showed that urinary MDA level was lower in the taurine-treated diabetic

group than diabetic control group. However, there was no significant difference between the groups.

The diabetes control and complications trial (DCCT) and some clinical studies have demonstrated that lowering high blood glucose levels prevents the development and progression of diabetic renal disease [31, 32]. In our study, fasting blood glucose levels decreased significantly in the taurine-treated diabetic group compared to the diabetic control group, along with the increment of HOMA-β in the taurine treatment. Taurine may act as a regulator of insulin secretion [33], and hence the protective effect of taurine on diabetic nephropathy may be accomplished by blood glucose lowering through improved insulin secretion. Moreover, studies suggested that taurine treatment diminishes the rate of renal gluconeogenesis and also promoting the transformation of glucose to glycogen [18, 21, 34].

Nephrin is a podocyte-specific protein [12] and its reduction is related to increased glomerular hyperpermeability in diabetic nephropathy [35]. Diminished nephrin expression and altered nephrin localization were shown in patients with nephropathy in both type 1 and type 2 diabetes [13]. Nephrin gene expression varies according to glomerular size [36]. Changes in nephrin expression are associated with the extent of proteinuria in diabetic nephropathy [37]. Our data indicate that nephrin mRNA expression was increased significantly in the taurine-treated high glucose group compared to the high glucose group. This result implies that taurine prevent glomerular hyperpermeability through increased expression of nephrin. The other antioxidant agent, resveratrol (RSV) which is potent free radical scavenger, attenuates renal dysfunction and oxidative stress in STZ induced diabetic model rats and increases the nephrin levels in diabetic kidneys [38, 39].

VEGF is a major controller of angiogenesis and vascular permeability [40]. Renal VEGF is especially increased during the early stages of diabetic renal disease and reducing VEGF overexpression may ameliorate diabetic renal disease [8, 9]. Although the mechanism of VEGF-induced proteinuria in diabetic nephropathy is unclear, Unemori et al. observed the high vascular permeability by renal VEGF stimulates collagenase production and proteolytic disruption of the endothelial basement membrane [41]. Due to increased ROS in diabetic nephropathy, the VEGF and nephrin levels had been shown to be reduced or increased, respectively, to development or progression of proteinuria in diabetic nephropathy [42]. Sun et al. have shown that renal hypoxia and VEGF mRNA level consequently improve renal tubulointerstitial hypoxia of the diabetic rat kidney [43]. Nephrin expression is closely related to VEGF expression because VEGF signaling is essential for the formation and maintenance of a functional glomerular filtration barrier [44, 45].

In this study, not only ROS formation decreased significantly in the high glucose with taurine-treated group compared to the diabetic control group in podocytes but also decreased renal VEGF mRNA in both the kidney and podocytes.

We could not measure blood pressure in animal. In deed lowering arterial pressure is related to improvements in proteinuria. Harada et al. suggested taurine supplementation reduces hypertension in rat. Lowering arterial pressure is related to improvements in proteinuria [46].

6. Conclusion

Taurine may prevent the progression of diabetic nephropathy, possibly by its antioxidant property and also through the recovery in nephrin and reduction in renal VEGF expression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jang Hyun Koh, Eun Soo Lee contributed equally to this work.

Acknowledgment

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References

[1] D. Giugliano, A. Ceriello, and G. Paolisso, "Oxidative stress and diabetic vascular complications," *Diabetes Care*, vol. 19, no. 3, pp. 257–267, 1996.

[2] A. R. Andersen, J. Sandahl Christiansen, and J. K. Andersen, "Diabetic nephropathy in type 1 (insulin-dependent) diabetes: an epidemiological study," *Diabetologia*, vol. 25, no. 6, pp. 496–501, 1983.

[3] A. Flyvbjerg, "Putative pathophysiological role of growth factors and cytokines in experimental diabetic kidney disease," *Diabetologia*, vol. 43, no. 10, pp. 1205–1223, 2000.

[4] D. R. Cha, N. H. Kim, J. W. Yoon et al., "Role of vascular endothelial growth factor in diabetic nephropathy," *Kidney International*, vol. 58, supplement 77, pp. S104–S112, 2000.

[5] K. J. Kim, B. Li, J. Winer et al., "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo," *Nature*, vol. 362, no. 6423, pp. 841–844, 1993.

[6] R. E. Gilbert, D. J. Kelly, A. J. Cox et al., "Angiotensin converting enzyme inhibition reduces retinal overexpression of vascular endothelial growth factor and hyperpermeability in experimental diabetes," *Diabetologia*, vol. 43, no. 11, pp. 1360–1367, 2000.

[7] M. E. Cooper, D. Vranes, S. Youssef et al., "Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes," *Diabetes*, vol. 48, no. 11, pp. 2229–2239, 1999.

[8] D. R. Cha, Y. S. Kang, S. Y. Han et al., "Vascular endothelial growth factor is increased during early stage of diabetic nephropathy in type II diabetic rats," *Journal of Endocrinology*, vol. 183, no. 1, pp. 183–194, 2004.

[9] E. Y. Lee, M. S. Shim, M. J. Kim, S. Y. Hong, Y. G. Shin, and C. H. Chung, "Angiotensin II receptor blocker attenuates overexpression of vascular endothelial growth factor in diabetic podocytes," *Experimental and Molecular Medicine*, vol. 36, no. 1, pp. 65–70, 2004.

[10] K. V. Lemley, K. Blouch, I. Abdullah et al., "Glomerular permselectivity at the onset of nephropathy in type 2 diabetes mellitus," *Journal of the American Society of Nephrology*, vol. 11, no. 11, pp. 2095–2105, 2000.

[11] K. Tryggvason and J. Wartiovaara, "Molecular basis of glomerular permselectivity," *Current Opinion in Nephrology and Hypertension*, vol. 10, no. 4, pp. 543–549, 2001.

[12] V. Ruotsalainen, P. Ljungberg, J. Wartiovaara et al., "Nephrin is specifically located at the slit diaphragm of glomerular podocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 14, pp. 7962–7967, 1999.

[13] S. Doublier, G. Salvidio, E. Lupia et al., "Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin II," *Diabetes*, vol. 52, no. 4, pp. 1023–1030, 2003.

[14] A. Erdem, N. Ü. Gündoğan, A. Usubütün et al., "The protective effect of taurine against gentamicin-induced acute tubular necrosis in rats," *Nephrology Dialysis Transplantation*, vol. 15, no. 8, pp. 1175–1182, 2000.

[15] F. Franconi, F. Bennardini, A. Mattana et al., "Plasma and platelet taurine are reduced in subjects with insulin-dependent diabetes mellitus: effects of taurine supplementation," *American Journal of Clinical Nutrition*, vol. 61, no. 5, pp. 1115–1119, 1995.

[16] H. Trachtman, S. Futterweit, J. Maesaka et al., "Taurine ameliorates chronic streptozocin-induced diabetic nephropathy in rats," *American Journal of Physiology*, vol. 269, no. 3, pp. F429–F438, 1995.

[17] L. Wang, L. Zhang, Y. Yu, Y. Wang, and N. Niu, "The protective effects of taurine against early renal injury in STZ-induced diabetic rats, correlated with inhibition of renal LOX-1-mediated ICAM-1 expression," *Renal Failure*, vol. 30, no. 8, pp. 763–771, 2008.

[18] K. Winiarska, K. Szymanski, P. Gorniak, M. Dudziak, and J. Bryla, "Hypoglycaemic, antioxidative and nephroprotective effects of taurine in alloxan diabetic rabbits," *Biochimie*, vol. 91, no. 2, pp. 261–270, 2009.

[19] J. Das and P. C. Sil, "Taurine ameliorates alloxan-induced diabetic renal injury, oxidative stress-related signaling pathways and apoptosis in rats," *Amino Acids*, vol. 43, no. 4, pp. 1509–1523, 2012.

[20] S. Higo, S. Miyata, Y. J. Qing, R. Kitazawa, S. Kitazawa, and M. Kasuga, "Taurine administration after appearance of proteinuria retards progression of diabetic nephropathy in rats," *Kobe Journal of Medical Sciences*, vol. 54, no. 1, pp. 35–45, 2008.

[21] S. Lin, J. Yang, G. Wu et al., "Preventive effect of taurine on experimental type II diabetic nephropathy," *Journal of Biomedical Science*, vol. 17, supplement 1, p. S46, 2010.

[22] D. R. Matthews, J. P. Hosker, and A. S. Rudenski, "Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man," *Diabetologia*, vol. 28, no. 7, pp. 412–419, 1985.

[23] P. H. Lane, M. W. Steffes, and S. M. Mauer, "Estimation of glomerular volume: a comparison of four methods," *Kidney International*, vol. 41, no. 4, pp. 1085–1089, 1992.

[24] R. J. Huxtable, "Physiological actions of taurine," *Physiological Reviews*, vol. 72, no. 1, pp. 101–164, 1992.

[25] C. E. Wright, H. H. Tallan, Y. Y. Lin, and G. E. Gaull, "Taurine: biological update," *Annual Review of Biochemistry*, vol. 55, pp. 427–453, 1986.

[26] H. Trachtman, R. Del Pizzo, S. Futterweit et al., "Taurine attenuates renal disease in chronic puromycin aminonucleoside nephropathy," *American Journal of Physiology*, vol. 262, no. 1, pp. F117–F123, 1992.

[27] J. S. Huang, L. Y. Chuang, J. Y. Guh, Y. Yang, and M. Hsu, "Effect of taurine on advanced glycation end products-induced hypertrophy in renal tubular epithelial cells," *Toxicology and Applied Pharmacology*, vol. 233, no. 2, pp. 220–226, 2008.

[28] D. Suzuki, T. Miyata, N. Saotome et al., "Immunohistochemical evidence for an increased oxidative stress and carbonyl modification of proteins in diabetic glomerular lesions," *Journal of the American Society of Nephrology*, vol. 10, no. 4, pp. 822–832, 1999.

[29] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[30] H. Ha and K. H. Kim, "Pathogenesis of diabetic nephropathy: the role of oxidative stress and protein kinase C," *Diabetes Research and Clinical Practice*, vol. 45, no. 2-3, pp. 147–151, 1999.

[31] "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group," *The New England Journal of Medicine*, vol. 329, no. 14, pp. 977–986, 1993.

[32] R. Turner, "Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)," *The Lancet*, vol. 352, no. 9131, pp. 837–853, 1998.

[33] W. J. Lamoreaux, C. Cuttitta, A. Santora, J. F. Blaize, J. Tachjadi, and A. El Idrissi, "Taurine regulates insulin release from pancreatic beta cell lines," *Journal of Biomedical Science*, vol. 17, supplement 1, p. S11, 2010.

[34] H. O. Goodman and Z. K. Shihabi, "Supplemental taurine in diabetic rats: effects on plasma glucose and triglycerides," *Biochemical Medicine and Metabolic Biology*, vol. 43, no. 1, pp. 1–9, 1990.

[35] D. J. Kelly, P. Aaltonen, A. J. Cox et al., "Expression of the slit-diaphragm protein, nephrin, in experimental diabetic nephropathy: differing effects of anti-proteinuric therapies," *Nephrology Dialysis Transplantation*, vol. 17, no. 7, pp. 1327–1332, 2002.

[36] J. J. Kim, J. J. lin, D. S. Jung et al., "Differential expression of nephrin according to glomerular size in early diabetic kidney disease," *Journal of the American Society of Nephrology*, vol. 18, no. 8, pp. 2303–2310, 2007.

[37] R. G. Langham, D. J. Kelly, A. J. Cox et al., "Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: effects of angiotensin converting enzyme inhibition," *Diabetologia*, vol. 45, no. 11, pp. 1572–1576, 2002.

[38] K. H. Chen, C. C. Hung, H. H. Hsu, Y. Jing, C. Yang, and J. Chen, "Resveratrol ameliorates early diabetic nephropathy associated with suppression of augmented TGF-β/smad and ERK1/2 signaling in streptozotocin-induced diabetic rats," *Chemico-Biological Interactions*, vol. 190, no. 1, pp. 45–53, 2011.

[39] C. C. Chang, C. Y. Chang, Y. T. Wu, J. Huang, T. Yen, and L. Hung, "Resveratrol retards progression of diabetic nephropathy through modulations of oxidative stress, proinflammatory cytokines, and AMP-activated protein kinase," *Journal of Biomedical Science*, vol. 18, no. 1, p. 47, 2011.

[40] P. Carmeliet, V. Ferreira, G. Breier et al., "Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele," *Nature*, vol. 380, no. 6573, pp. 435–439, 1996.

[41] E. N. Unemori, N. Ferrara, E. A. Bauer, and E. P. Amento, "Vascular endothelial growth factor induced interstitial collagenase expression in human endothelial cells," *Journal of Cellular Physiology*, vol. 153, no. 3, pp. 557–562, 1992.

[42] G. Wolf, S. Chen, and F. N. Ziyadeh, "From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy," *Diabetes*, vol. 54, no. 6, pp. 1626–1634, 2005.

[43] H. K. Sun, Y. M. Lee, K. H. Han et al., "Phosphodiesterase inhibitor improves renal tubulointerstitial hypoxia of the diabetic rat kidney," *The Korean Journal of Internal Medicine*, vol. 27, no. 2, pp. 163–170, 2012.

[44] V. Eremina, H. J. Baelde, and S. E. Quaggin, "Role of the VEGF-A signaling pathway in the glomerulus: evidence for crosstalk between components of the glomerular filtration barrier," *Nephron-Physiology*, vol. 106, no. 2, pp. p32–p37, 2007.

[45] C. A. Bertuccio, "Relevance of VEGF and nephrin expression in glomerular diseases," *Journal of Signal Transduction*, vol. 2011, Article ID 718609, 9 pages, 2011.

[46] H. Harada, T. Tsujino, Y. Watari, H. Nonaka, N. Emoto, and M. Yokoyama, "Oral taurine supplementation prevents fructose-induced hypertension in rats," *Heart and Vessels*, vol. 19, no. 3, pp. 132–136, 2004.

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Research Article

Body Mass Index and Retinopathy in Type 1 Diabetic Patients

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Aim. To investigate whether body mass index (BMI) independently or in correlation with other risk factors is associated with diabetic retinopathy (DR) progression. *Methods*. The study included 176 patients with type 1 diabetes divided into three groups according to DR status: group 1 (no retinopathy; $n = 86$), group 2 (mild/moderate nonproliferative DR; $n = 33$), and group 3 (severe/very severe NPDR or proliferative DR; $n = 57$). *Results*. A significant deterioration of HbA_1c, an increase in total cholesterol, systolic, diastolic blood pressure, and diabetic nephropathy with the progression of retinopathy were found. DR progression was correlated with diabetes duration, HbA_1c, hypertension, total cholesterol, and the presence of nephropathy. In patients without nephropathy, statistical analyses showed that progression of retinopathy increased significantly with higher BMI (gr. 1: 24.03 ± 3.52, gr. 2: 25.36 ± 3.44, gr. 3: 26.93 ± 3.24; $P < 0.01$). A positive correlation between BMI and a significant deterioration of HbA_1c, an increase in cholesterol, triglycerides, and hypertension was observed. *Conclusion*. BMI in correlation with HbA_1c, cholesterol, and hypertension appears to be associated with the progression of DR in type 1 diabetic patients without nephropathy. However, additional studies are required to investigate the pathogenic role of obesity and weight loss in retinal diabetic complications particularly relating to nephropathy.

1. Introduction

Diabetes mellitus is the most frequent endocrine disease in developed countries estimated to have affected 366 million people worldwide and is expected to nearly double by 2030. This growing trend is observed for both type 2 and type 1 diabetes where type 1 accounts for 5–10% of the total cases and its increased incidence is seen across the world in various population studies with the range between 2% and 5% [1–4]. Type 1 diabetes is an autoimmune disease mediated by a combination of genetic and environmental triggers, however, its increasing incidence worldwide cannot be explained by genetic factor alone. Recent studies suggest an alternative possible cause of this epidemic, namely, the role of infections, early childhood diet, environmental pollutants, insulin resistance, and obesity [4].

The global increase of diabetes has a significant impact on the prevalence of diabetic complications among which diabetic retinopathy (DR) takes an important place [5, 6]. DR is a leading cause of acquired blindness in working-age adults and has been estimated to represent 12% of blindness in developed countries [7, 8]. The prevalence of retinopathy increases with the duration of diabetes and is related to hyperglycemia, hypertension, hyperlipidemia, pregnancy, nephropathy, and anemia with genetic factors also having a specific role [9–17]. DR is characterized by the loss of pericytes, hypertrophy of the basement membrane, microaneurysm formation, increased vascular permeability,

capillary occlusions, neovascularization, and fibrovascular proliferation. The predominant cause of visual loss in diabetic patients results primarily from intraocular angiogenesis (proliferative diabetic retinopathy, PDR) and leakage of the retinal vessels (diabetic macular edema, DME) [7, 13].

Since DR has become a main cause of vision loss and blindness worldwide intense focus on the early prevention of DR and the benefit of controlling modifiable risk factors has become increasingly important. Although numerous studies have confirmed the role and importance of established risk factors for the development and progression of DR [9, 10], the evidence from new recent trials have nonetheless shown that risk reduction for DR with better glucose and blood pressure management has its limits [18, 19]. Thus, a better comprehension of the role of other modifiable risk factors including obesity in the development of DR becomes even more valuable. The evidence supporting a relationship between high body mass index (BMI) and increased risk of DR is inconclusive [20–33]. Some studies have demonstrated a relationship between obesity or higher BMI and an increased risk of DR [20, 22–29] whilst others have yielded conflicting results [30–33].

Considering that obesity is becoming increasingly prevalent in today's society and since it can be managed by lifestyle intervention particularly exercise, nutrition, and education, studying its effect on diabetic complications has certain logic and benefits. Thus the aim of the present study was to investigate whether obesity independently or in association with other established risk factors influences DR development in type 1 diabetic patients.

2. Patients and Methods

This cross-sectional study was performed in collaboration with the Ophthalmology Departments of three Croatian Hospitals in accordance with the Declaration of Helsinki and approved by the Ethics Committee of each Hospital. The patients included in the study received both written and oral information concerning the study and signed a written informed consent.

2.1. Patients. A total of 176 patients with type 1 diabetes were included in the study. All of them were on insulin therapy. Type 1 diabetes was defined according to the American Diabetes Association classification [2]. Patients with immunologic, infectious or inflammatory diseases, and malignancies as well as those taking cytostatics or corticosteroids, pregnant women, and patients with other eye diseases (mature cataract, uveitis, age-related macular degeneration) were not included in the study.

2.2. Methods. This study consisted of patients with diabetes who underwent their regular medical and ophthalmological checkups during a one-year period. All patients who met the inclusion criteria were invited to partake in the study and signed the consent form. Blood samples for laboratory analyses were collected between 08:00 and 10:00 am after a 12-hour overnight fast and complete clinical and ophthalmic examinations were done.

2.2.1. Blood Samples. Glycated hemoglobin value (HbA_1c), total cholesterol, triglycerides, and HDL cholesterol were measured. HbA_1c was determined by an automated immunoturbidimetric assay (reference values 3.5–5.7%) [34]. Total cholesterol, triglycerides, and HDL cholesterol were measured by the enzymatic colorimetric tests (reference values: total cholesterol <5.00 mmol/L; triglycerides <1.70 mmol/L; HDL cholesterol >1.0 mmol/L) [35, 36].

2.2.2. Anthropometric Parameters. BMI was calculated by dividing weight and height squared (kg/m^2). A balance-beam scale was used to measure weight, and height was measured using a wall-mounted stadiometer with patients in their underwear and without shoes. Recommended value of BMI among men was considered <23 and among women <22 kg/m^2 with a normal range being between 18.5 and 24.9 kg/m^2 [37].

2.2.3. Clinical Parameters. Blood pressure was measured with an ambulatory sphygmomanometric device after a 5 min rest and the mean of three measurements was used. All three measurements were conducted during the same visit. Hypertension was defined as blood pressure >130/80 mmHg or the use of antihypertensive treatment.

2.2.4. Ophthalmologic Examination. Complete eye examination included best corrected visual acuity (BCVA), Goldmann applanation tonometry, slit lamp biomicroscopy of the anterior eye segment, binocular indirect slit lamp fundoscopy, and fundus photography after mydriasis with topically administrated 1% tropicamide and 5% phenylephrine eye drops. Color fundus photographs of two fields: macular field and disc/nasal field of both eyes were taken using a 45° fundus camera (VISUCAM, Zeiss) according to the Europe and Diabetes Study (EURODIAB) retinal photography methodology [38]. Macular field: where the exact centre of the optic disc is laid at the nasal end of the horizontal meridian of the field view. Disc/nasal field: where the optic disc is positioned one disc-diameter in from the temporal edge of the field on the horizontal meridian. The EURODIAB classification scheme was applied since it uses two-field 45° fundus photography and standard photographs to grade retinal lesions [38]. Patients were divided into three groups according to the DR status: group 1 (no retinopathy; $n = 86$), group 2 (mild/moderate nonproliferative DR (NPDR); $n = 33$), and group 3 (severe/very severe NPDR or proliferative diabetic retinopathy (PDR); $n = 57$). The status of the seriously affected eye was the basis on which severity of retinopathy was determined.

2.2.5. Diabetic Nephropathy. Diabetic nephropathy has been categorized and classified into stages based on the values of urinary albumin excretion (UAE): microalbuminuria and macroalbuminuria [39].

Table 1: Basic characteristics of type 1 diabetic patients ($n = 176$) divided into three groups according to diabetic retinopathy status.

	Group 1 ($n = 86$)	Group 2 ($n = 33$)	Group 3 ($n = 57$)	P
Sex (m/f)**	46.51/53.49	54.55/45.45	53.14/43.86	ns
Age (years)*	37.19 ± 16.62	45.06 ± 12.56	41.40 ± 11.23	<0.05
Diabetes duration (years)*	12.94 ± 6.77	23.03 ± 8.94	24.67 ± 7.20	<0.01

*mean ± SD **%; percentage.
Group 1: no retinopathy, group 2: mild/moderate nonproliferative diabetic retinopathy; group 3: severe/very severe nonproliferative diabetic retinopathy or proliferative diabetic retinopathy.

2.3. Statistical Analyses. Descriptive statistics (n, mean ± standard deviation) for all analyzed variables were conducted. Differences in distributions of continuous data were determined by One-Way ANOVA [40]. Scheffe's post hoc test was used. Differences in distributions of categorical data were evaluated by Chi-square test (χ^2). The relationship between DR, obesity (defined by BMI), and risk factors was analyzed using Pearson's correlation test. Data were analyzed with IBM SPSS software version 12.0 [41]. In all the analyses, P value of less than 0.01 and 0.05 were considered statistically significant.

3. Results

This study included 176 patients with type 1 diabetes (90 (51.1%) males, and 86 (48.9%) females) with a mean age of 40.03 ± 14.66 years. The mean age at onset was 21.70 ± 13.05 years and the mean duration of diabetes was 18.63 ± 9.27 years. According to the DR status, they were divided into three groups: group 1 (no retinopathy; $n = 86$), group 2 (mild/moderate NPDR; $n = 33$), group 3 (severe/very severe NPDR or PDR; $n = 57$). Incidence of any form of retinopathy in the examined patients ($n = 176$) was 51% of which 10.8% had severe to very severe NPDR and 21.6% had PDR.

Table 1 presents descriptive statistics of basic characteristics of type 1 diabetic patients divided into three groups according to DR status. There was no significant difference in gender between investigated groups. One-Way ANOVA was applied to compare means of age between the three observed groups (45.06 ± 12.56 years versus 37.19 ± 16.62 and 41.40 ± 11.23 years; $P < 0.05$). Scheffe's post hoc test demonstrated only the significant difference between groups 1 and 2 with a test significance of 5%.

Duration of diabetes was significantly different between the three groups (23.03 ± 8.94 and 24.67 ± 7.20 years versus 12.94 ± 6.77 years; $P < 0.01$). Scheffe's post hoc test demonstrated the significant difference between groups 1 and 2, as well as between groups 2 and 3 with a test significance of 1%.

Table 2 presents descriptive statistics of metabolic and clinical parameters of type 1 diabetic patients divided into three groups according to their DR status. We observed a significant deterioration of HbA_1c ($P < 0.01$) and a significant increase in total cholesterol ($P < 0.01$) as well as systolic ($P < 0.01$) and diastolic blood pressure ($P < 0.05$) with the progression of retinopathy. Scheffe's post hoc test demonstrated the significant difference in mean HbA_1c value between the groups 1 and 3, as well as between the groups 2 and 3 and in mean total cholesterol value between the groups 1 and 3 with test significance of 1%. The same test demonstrated the significant difference in mean systolic and diastolic blood pressure between the groups 1 and 3 with test significance of 1% for systolic and 5% for diastolic blood pressure.

Patients with NPDR and PDR (groups 2 and 3, resp.) were more often treated with hypolipidemics ($P < 0.01$) and ACE-inhibitors ($P < 0.01$) than patients with no retinopathy (group 1); this was observed by Scheffe's post hoc test with test significance of 1%. We also observed a significant increase in diabetic nephropathy ($P < 0.01$) with the progression of DR.

There was no significant difference in BMI between investigated groups (Table 3). In order to investigate the specific relationship between BMI and DR, patients were additionally divided according to their diabetic nephropathy status into two groups (with/without nephropathy). The group with nephropathy included the patients on dialysis due to diabetic nephropathy whilst the group without nephropathy consisted of patients with normoalbuminuria or microalbuminuria. In type 1 diabetic patients without nephropathy mean BMI was significantly different ($P < 0.01$) (Table 4). Scheffe's post hoc testdemonstrated the significant difference in BMI between the groups 1 and 3 with test significance of 5% (Table 5).

In type 1 diabetic patients with nephropathy, there was no significant difference in BMI between the groups divided according to the DR status (data not shown).

Body mass index was significantly positively correlated with HbA_1c ($r = 0.156$), total cholesterol ($r = 0.256$), triglycerides ($r = 0.288$), and systolic ($r = 0.349$) and diastolic blood pressure ($r = 0.282$), whereas significant negative correlations between BMI and HDL-cholesterol ($r = -0.155$) as well as diabetic nephropathy ($r = -0.132$) were observed. No correlation between BMI and diabetes duration ($r = 0.136$) was detected. These data are shown in Table 6.

DR was significantly positively correlated ($P = 0.01$) with diabetes duration ($r = 0.524$), HbA_1c ($r = 0.375$), total cholesterol ($r = 0.252$), and systolic ($r = 0.175$) and diastolic blood pressure ($r = 0.194$) as well as diabetic nephropathy ($r = 0.761$). No correlations between diabetic retinopathy and BMI ($r = -0.053$), triglycerides ($r = 0.108$), and HDL-cholesterol ($r = -0.029$) were observed. These data are shown in Table 7.

In type 1 diabetic patients without nephropathy, DR was significantly positively correlated ($P = 0.01$) with BMI ($r = 0.247$), HbA_1c ($r = 0.298$), total cholesterol ($r = 0.378$), and systolic ($r = 0.386$) and diastolic blood pressure ($r = 0.302$). In these patients, no correlations between diabetic retinopathy, triglycerides ($r = 0.160$), and HDL-cholesterol ($r = 0.081$), were seen. These data are shown in Table 8.

In type 1 diabetic patients on hemodialysis due to diabetic nephropathy, DR was significantly positively correlated only with HbA_1c ($r = 0.499$), while significant negative correlation between DR and BMI($r = -0.400$) was detected. In these patients, no correlations between diabetic retinopathy

Table 2: Metabolic and clinical parameters of type 1 diabetic patients ($n = 176$) divided into three groups according to diabetic retinopathy status.

	Group 1 ($n = 86$)	Group 2 ($n = 33$)	Group 3 ($n = 57$)	P
HbA_1c (%)*	7.25 ± 1.56	7.47 ± 1.44	8.56 ± 1.32	<0.01
Total cholesterol (mmol/L)*	4.74 ± 1.08	5.18 ± 1.01	5.48 ± 1.17	<0.01
Triglycerides (mmol/L)*	1.42 ± 0.77	1.41 ± 0.81	1.66 ± 0.80	ns
HDL-cholesterol (mmol/L)*	1.28 ± 0.41	1.35 ± 0.36	1.28 ± 0.38	ns
Insulin dosage (IU/kg)*	0.71 ± 0.21	0.77 ± 0.16	0.72 ± 0.17	ns
Systolic blood pressure (mmHg)*	127.21 ± 13.89	135.76 ± 20.75	136.40 ± 20.62	<0.01
Diastolic blood pressure (mmHg)*	79.77 ± 10.06	81.82 ± 9.03	84.65 ± 11.66	<0.05
Antihypertensive treatment**	32.56	60.61	57.89	<0.01
Hypolipidemic treatment**	25.58	39.39	54.39	<0.01
Nephropathy**	5.81	24.24	78.95	<0.01

*mean ± SD **%; percentage.
Group 1: no retinopathy, group 2: mild/moderate nonproliferative diabetic retinopathy; group 3: severe/very severe nonproliferative diabetic retinopathy or proliferative diabetic retinopathy; HbA_1c: glycated hemoglobin value; nephropathy: patients with type 1 diabetes on hemodialysis due to diabetic nephropathy.

Table 3: Body mass index (BMI) of type 1 diabetic patients ($n = 176$) divided into three groups according to diabetic retinopathy status.

	Group 1 ($n = 86$)	Group 2 ($n = 33$)	Group 3 ($n = 57$)	P
BMI (kg/m^2)*	24.06 ± 3.48	25.17 ± 3.22	24.43 ± 4.28	ns

*mean ± SD.
Group 1: no retinopathy, group 2: mild/moderate nonproliferative diabetic retinopathy; group 3: severe/very severe nonproliferative diabetic retinopathy or proliferative diabetic retinopathy; BMI: body mass index.

Table 4: Body mass index (BMI) of type 1 diabetic patients ($n = 124$) without nephropathy divided into three groups according to diabetic retinopathy status.

	Group 1 ($n = 81$)	Group 2 ($n = 25$)	Group 3 ($n = 18$)	P
BMI (kg/m^2)*	24.03 ± 3.52	25.36 ± 3.44	26.93 ± 3.24	<0.01

*mean ± SD.
Group 1: no retinopathy, group 2: mild/moderate nonproliferative diabetic retinopathy; group 3: severe/very severe nonproliferative diabetic retinopathy or proliferative diabetic retinopathy; BMI: body mass index.

and total cholesterol ($P = -0.160$), triglycerides ($r = -0.125$), HDL-cholesterol ($r = -0.046$), and systolic ($r = -0.049$) and diastolic blood pressure ($r = -0.054$) were observed. These data are shown in Table 9.

4. Discussion

Although type 1 accounts for only 5–10% of total cases of diabetes, it still represents a significant public health problem due to its rising incidence and prevalence as well as high risk of complications including retinopathy. The incidence of any stage of DR in patients with type 1 diabetes is higher than in type 2 and the advanced forms of DR with visual impairment and blindness usually develop throughout the patients most productive age [1–6]. According to the study, conducted to estimate the global prevalence of DR, age standardized prevalence of any form of DR in type 1 diabetic subjects aged 20–79 years after 10 to 20 years of diabetes duration was 55.55% and 86.22% after more than 20 years. In the same study, the prevalence of PDR after 10 to 20 years was 19.46% and 40.36% after more than 20 years [42]. In our investigation, the mean duration of diabetes was 18.63 ± 9.27 years with the prevalence of any form of retinopathy being 51% while 21.6% of patients had PDR. Furthermore, our results also confirm previous findings that the main risk factors for DR are diabetes duration, prolonged poor glycaemic control, hypertension, and high triglyceride levels [7–10, 15–17]. We found significant differences depending on the duration of diabetes ($P < 0.01$) and the level of glycaemic control ($P < 0.01$) between investigated groups. Advanced stages of DR were present in patients with a longer duration of diabetes and higher values of HbA_1c. Additionally, a significant difference in the level of systolic ($P < 0.01$) and diastolic blood pressure ($P < 0.05$) as well as total triglyceride levels ($P < 0.01$) between the groups according to their DR status was found.

There are continuous efforts to correlate and explain the dynamic relationship between diabetic retinopathy and nephropathy, the two major microvascular complications of diabetes. Current data suggests that the presence of a preexisting retinopathy or nephropathy may contribute to the development of another, independent of established risk factors for microvascular complications especially in type 1 diabetic patients [43]. Our findings confirm the relationship between DR and nephropathy with obtained results indicating the existence of significant differences for the presence of nephropathy (patients on hemodialysis due to diabetic nephropathy) between the studied groups ($P < 0.01$). The strongest positive correlation of DR with investigated metabolic and clinical parameters was found for the presence

Table 5: *Scheffe's post-hoc test*: dependent variable body mass index (BMI) of type 1 diabetic patients ($n = 124$) without nephropathy was divided into three groups according to diabetic retinopathy status.

(I) diab. ret.	(J) diab. ret.	Mean difference (I–J)	Std. error	Sig.	95% Confidence interval	
					Lower bound	Upper bound
Group 1	Group 2	−1.32886	0.75492	0.216	−3.1985	0.5408
	Group 3	−2.89267*	0.89892	0.007	−5.1190	−0.6663
Group 2	Group 1	1.32886	0.75492	0.216	−0.5408	3.1985
	Group 3	−1.56381	1.04668	0.331	−4.1561	1.0285
Group 3	Group 1	2.89267*	0.89892	0.007	0.6663	5.1190
	Group 2	1.56381	1.04668	0.331	−1.0285	4.1561

*The mean difference is significant at the 0.05 level.

Table 6: Correlation between body mass index and metabolic and clinical parameters in type 1 diabetic patients ($n = 176$).

	Body mass index (BMI) Correlation test*
Diabetes duration (years)	0.136
HbA_1c (%)	0.156*
Total cholesterol (mmol/L)	0.256**
Triglycerides (mmol/L)	0.288**
HDL-cholesterol (mmol/L)	−0.155**
Systolic blood pressure (mmHg)	0.349**
Diastolic blood pressure (mmHg)	0.282**
Nephropathy	−0.132

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).
HbA_1c: glycated hemoglobin value; nephropathy: patients with type 1 diabetes on hemodialysis due to diabetic nephropathy.

Table 7: Correlation between diabetic retinopathy and diabetes duration, body mass index, and metabolic and clinical parameters in type 1 diabetic patients ($n = 176$).

	Diabetic retinopathy Correlation test*
Diabetes duration (years)	0.524**
BMI (kg/m^2)	−0.053
HbA_1c (%)	0.375**
Total cholesterol (mmol/L)	0.252**
Triglycerides (mmol/L)	0.108
HDL-cholesterol (mmol/L)	−0.029
Systolic blood pressure (mmHg)	0.175*
Diastolic blood pressure (mmHg)	0.194**
Nephropathy	0.761**

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).
BMI: body mass index; HbA_1c: glycated hemoglobin value; nephropathy: patients with type 1 diabetes on hemodialysis due to diabetic nephropathy.

of nephropathy ($r = 0.761$). DR was also significantly positively correlated with diabetes duration ($r = 0.524$), total cholesterol ($r = 0.252$), HbA_1c ($r = 0.375$), systolic ($r = 0.175$), and diastolic blood pressure ($r = 0.194$).

Table 8: Correlation between diabetic retinopathy and diabetes duration, body mass index, and metabolic and clinical parameters in type 1 diabetic patients without nephropathy ($n = 124$).

	Diabetic retinopathy Correlation test*
BMI (kg/m^2)	0.247**
HbA_1c (%)	0.298**
Total cholesterol (mmol/L)	0.378**
Triglycerides (mmol/L)	0.160
HDL-cholesterol (mmol/L)	0.081
Systolic blood pressure (mmHg)	0.386**
Diastolic blood pressure (mmHg)	0.302**

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).
BMI: body mass index; HbA_1c: glycated hemoglobin value.

Table 9: Correlation between diabetic retinopathy and diabetes duration, body mass index, and metabolic and clinical parameters in type 1 diabetic patients with nephropathy ($n = 52$).

	Diabetic retinopathy Correlation test*
BMI (kg/m^2)	−0.400**
HbA_1c (%)	0.499**
Total cholesterol (mmol/L)	−0.160
Triglycerides (mmol/L)	−0.125
HDL-cholesterol (mmol/L)	−0.046
Systolic blood pressure (mmHg)	−0.049
Diastolic blood pressure (mmHg)	−0.054

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).
BMI: body mass index; HbA_1c: glycated hemoglobin value.

Since current treatment options [7–10, 15–17, 44, 45] fail to entirely eliminate the risk of microvascular diabetic complications, there is a continuing need for the development of new management strategies. Increasing interest is addressed to modifiable risk factors particularly obesity due to its rising incidence and established association with diabetes. To date, the relationship between BMI and DR has been examined in a number of epidemiologic studies giving conflicting results [20–33]. This inconsistency may be partly explained

by methodological differences, diversity in study participants, lack of comprehensive anthropometric measurements, inadequate clinical sample size, and particularly racial or ethnic differences [31, 32].

Although several biological theories are proposed, the exact pathophysiological mechanisms supporting the relationship between higher BMI and DR are not entirely clear. Metabolic syndrome, increased oxidative stress, and inflammation due to their association with both obesity and DR have been implied as possible connecting pathogenic mechanisms [8, 14, 20, 46]. Likewise, growing attention has been directed towards the role of vasoproliferative factors in the pathogenesis of DR, whereby their concentrations have been found to be higher in the vitreous of eyes with PDR [47, 48]. Similarly in the serum of obese individuals elevated angiogenic factors have been detected providing additional evidence of the possible bond between obesity and PDR [46, 49]. Obesity and type 1 diabetes are widespread dysmetabolic disorders whose long term complications include severe impairment of the vascular system [50–52]. Several studies have suggested that weight gain may also be involved in the onset as well as long-term progression of type 1 diabetes and may contribute to its rising incidence as confirmed in type 2 [51, 53]. Type 1 diabetes is an inflammatory disease in which its very onset is caused by an inflammatory reaction via lymphocyte-mediated destruction of pancreatic beta cells. This is further followed by a chronic state of low-intense body inflammation episodically aggravated by hyperglycemic fluctuations. Obesity is also a disorder associated with elevated inflammation, oxidative stress, and insulin resistance with growing evidence suggesting that an interrelationship with diabetes could include these mechanisms [52–54]. Epidemiological data have identified hyperlipidemia and hypertension to be connected with obesity as risk factors for DR [28, 50]. In fact, metabolic syndrome encompassing these conditions has also been shown to be associated with retinopathy [55] where many overweight type 1 diabetic patients are difficult to treat and require a relatively high dose of insulin to achieve adequate glycemic control [55, 56]. It is possible that metabolic syndrome and its associated insulin resistance although usually associated with type 2 diabetes may also be a clinical feature for some type 1 diabetic patients [56]. Endothelial dysfunction (ED) as an early indicator of DR is also present in obesity and is characterised by increased levels of adhesion molecules. ED caused by oxidative stress and inflammation processes both connected to diabetes and obesity plays a significant role in the pathogenesis of diabetic arterial wall damage [57–59].

It is generally established that in obese and diabetic individuals, inflammatory and oxidative processes are found to be consistently elevated. The level of inflammatory activation seems to be proportional to severity of obesity in overweight individuals and the quality of glycemic control in type 1 diabetic patients, respectively. Obesity increases the prevalence of several risk factors known to be involved in DR onset and development including inflammatory markers. According to newer concepts, adipose tissue is an active endocrine proinflammatory organ that secretes a large number of bioactive molecules, named adipokines, such as interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) leptin, and adiponectin. They regulate body weight homeostasis, influence coagulation, lipid levels, inflammation, oxidative stress, and insulin resistance as well as atherosclerosis and diabetes occurrence. Plasma leptin levels which are elevated in obese individuals and which correlate positively with BMI and insulin resistance [60, 61] have also been related to hypertensive and diabetic retinopathy [61]. Recent findings show that leptin promotes vascular endothelial cell proliferation and angiogenesis in vitro and neovascularisation in vivo [60]. Conversely, adiponectin levels correlate negatively with visceral and subcutaneous fat areas [62, 63] with low adiponectin levels associated with obesity, type 2 diabetes, and insulin resistance [59]. An increased level of adiponectin was found in diabetic and nondiabetic subjects with impaired kidney function as well as in type 1 diabetic patients without complications and particularly in those with diabetic nephropathy [62–65]. In our study, there were no statistically significant differences in BMI between, the investigated groups according to DR status. However, after excluding patients with nephropathy, BMI was significantly higher in those with advanced stages of DR ($P < 0.01$). Moreover, in patients without nephropathy, a statistically positive correlation between DR and BMI was found ($r = 0.247$; $P < 0.01$) whilst in patients with nephropathy, this correlation was negative ($r = -0.400$; $P < 0.01$).

These obtained findings may be connected to adipokines particularly adiponectin and may be the result of the contradictory effect of obesity and impaired renal function on adiponectin levels [53]. Adiponectin is conversely correlated with BMI, and since kidney dysfunction increases its levels in obese diabetic patients with nephropathy, this mutual influence may have a nullifying effect. In patients without nephropathy, a potential action of adiponectin can be linked to obesity and its impact on oxidative stress and inflammatory markers and therefore DR development may be more pronounced.

Our study has shown that BMI in correlation with poor glycaemic control, hypertension, and dyslipidemia appears to be associated with the progression of DR in type 1 diabetic patients without nephropathy. Several findings indicate the existence of mutual pathogenic mechanisms including inflammation, oxidative stress, and endothelial dysfunction between retinopathy and nephropathy. However, it is not entirely clear in what phase of those complications specific mechanisms dominate or at what stage and to what extent they overlap. Moreover, it is questionable whether these pathogenetic mechanisms impact differently on distinct stages of retinopathy or nephropathy development, whereby a slight modification in action of just one bioactive molecule may cause an entirely different consequential outcome. This is of particular relevance since in chronic kidney disease due to impaired renal biodegradation and elimination, various active protein levels may be increased. Further, it is well established that ED correlates with the progression of renal disorder where it is inactive at the very beginning of the disease yet has an active role in the progression of the glomerulosclerosis causing nephropathy deterioration [66]. Likewise in patients with DR without nephropathy, it is

possible that endothelial lesions and dysfunction have less expressed or no influence on retinopathy development.

Obtained results illustrate the value of obesity assessment as a possible modifiable risk factor which may consequently have potential clinical implications on the management of DR. A possible limitation of our study may stem from the cross-sectional design itself in which accurate determination of observed correlation findings may be limited. It would therefore be advantageous to present the course of change in BMI as a very dynamic parameter over time by conducting a prospective study addressing this specific question. Findings showing that BMI is associated with the presence and severity of DR in patients without nephropathy open up implications for further research and intervention in order to elucidate the role of oxidative stress, inflammation, body weight change, and their interaction in the pathogenesis of DR particularly relating to nephropathy. In patients with DR and without nephropathy, the obtained results may also have clinical implications since in these patients, a tight regulation of body weight managed by lifestyle intervention is highly advisable. In this case, alongside glycaemic, hypertension, and lipid control, we may have an additional changeable risk factor which could influence DR development and onset itself. A better and more detailed understanding of all the facets linking obesity and diabetes with the components of the underlying inflammatory and oxidative dysregulation represents the source of future ability for the successful prevention and management of DR.

Conflict of Interests

There is no conflict of interests regarding the publication of this paper.

References

[1] L. Y. Melendez-Ramirez, R. J. Richards, and W. T. Cefalu, "Complications of type 1 diabetes," *Endocrinology and Metabolism Clinics of North America*, vol. 39, no. 3, pp. 625–640, 2010.

[2] American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 32, supplement 1, pp. S62–S67, 2009.

[3] D. M. Maahs, N. A. West, J. M. Lawrence, and E. J. Mayer-Davis, "Epidemiology of type 1 diabetes," *Endocrinology and Metabolism Clinics of North America*, vol. 39, no. 3, pp. 481–497, 2010.

[4] G. P. Forlenza and M. Rewers, "The epidemic of type 1 diabetes: what is it telling us?" *Current Opinion in Endocrinology, Diabetes and Obesity*, vol. 18, no. 4, pp. 248–251, 2011.

[5] J. E. Shaw, R. A. Sicree, and P. Z. Zimmet, "Global estimates of the prevalence of diabetes for 2010 and 2030," *Diabetes Research and Clinical Practice*, vol. 87, no. 1, pp. 4–14, 2010.

[6] G. Roglic, N. Unwin, P. H. Bennett et al., "The burden of mortality attributable to diabetes: realistic estimates for the year 2000," *Diabetes Care*, vol. 28, no. 9, pp. 2130–2135, 2005.

[7] S. E. Moss, R. Klein, and B. E. K. Klein, "The 14-year incidence of visual loss in a diabetic population," *Ophthalmology*, vol. 105, no. 6, pp. 998–1003, 1998.

[8] L. M. Aiello, "Perspectives on diabetic retinopathy," *American Journal of Ophthalmology*, vol. 136, no. 1, pp. 122–135, 2003.

[9] Diabetes Control and Diabetes Control and Complication Trial / Epidemiology of Diabetes Interventions and Complications Research Group, "Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy," *The New England Journal of Medicine*, vol. 342, no. 6, pp. 381–389, 2000.

[10] I. M. Stratton, E. M. Kohner, S. J. Aldington et al., "UKPDS 50: risk factors for incidence and progression of retinopathy in type II diabetes over 6 years from diagnosis," *Diabetologia*, vol. 44, no. 2, pp. 156–163, 2001.

[11] S. Kaštelan, M. Tomić, J. Pavan, and S. Orešković, "Maternal immune system adaptation to pregnancy—a potential influence on the course of diabetic retinopathy," *Reproductive Biology and Endocrinology*, vol. 8, article 124, 2010.

[12] S. Kaštelan, M. Tomić, J. Salopek-Rabatić et al., "The association between the HLA system and retinopathy development in patients with type 1 diabetes mellitus," *Collegium Antropologicum*, vol. 37, supplement 1, pp. 65–70, 2013.

[13] M. D. Davis, "Diabetic retinopathy: a clinical overview," *Diabetes Care*, vol. 15, no. 12, pp. 1844–1874, 1992.

[14] M. Tomić, S. Ljubić, S. Kaštelan, A. Gverović Antunica, A. Jazbec, and T. Poljičanin, "Inflammation, haemostatic disturbance and obesity: possible link to pathogenesis of diabetic retinopathy in type 2 diabetes," *Mediators in Inflammation*, vol. 2013, Article ID 818671, 10 pages, 2013.

[15] The Diabetes Control and Complications Trial Research Group, "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus," *The New England Journal of Medicine*, vol. 329, no. 14, pp. 977–986, 1993.

[16] A. K. Sjolie, J. Stephenson, S. Aldington et al., "Retinopathy and vision loss in insulin-dependent diabetes in Europe: The EURODIAB IDDM Complications Study," *Ophthalmology*, vol. 104, no. 2, pp. 252–260, 1997.

[17] B. Karamanos, M. Porta, M. Songini et al., "Different risk factors of microangiopathy in patients with type I diabetes mellitus of short versus long duration. The EURODIAB IDDM Complications Study," *Diabetologia*, vol. 43, no. 3, pp. 348–355, 2000.

[18] J. W. J. Beulens, A. Patel, J. R. Vingerling et al., "Effects of blood pressure lowering and intensive glucose control on the incidence and progression of retinopathy in patients with type 2 diabetes mellitus: a randomised controlled trial," *Diabetologia*, vol. 52, no. 10, pp. 2027–2036, 2009.

[19] F. Ismail-Beigi, T. Craven, M. A. Banerji et al., "Effect of intensive treatment of hyperglycaemia on microvascular outcomes in type 2 diabetes: an analysis of the ACCORD randomised trial," *The Lancet*, vol. 376, no. 9739, pp. 419–430, 2010.

[20] S. Kaštelan, M. Tomić, A. Gverović Antunica, S. Ljubić, J. Salopek-Rabatić, and M. Karabatić, "Body mass index—a risk factor for retinopathy in type 2 diabetic patients," *Mediators of Inflammation*, vol. 2013, Article ID 436329, 8 pages, 2013.

[21] N. Chaturvedi, J. H. Fuller, R. J. Jarrett et al., "Mortality risk by body weight and weight change in people with NIDDM: The WHO Multinational Study of Vascular Disease in Diabetes," *Diabetes Care*, vol. 18, no. 6, pp. 766–774, 1995.

[22] M. Henricsson, L. Nyström, G. Blohmé et al., "The incidence of retinopathy 10 years after diagnosis in young adult people with diabetes: results from the nationwide population-based Diabetes Incidence Study in Sweden (DISS)," *Diabetes Care*, vol. 26, no. 2, pp. 349–354, 2003.

[23] UK Prospective Diabetes Study (UKPDS) Group, "Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34)," *The Lancet*, vol. 352, no. 9131, pp. 854–865, 1998.

[24] H. A. van Leiden, J. M. Dekker, A. C. Moll et al., "Risk factors for incident retinopathy in a diabetic and nondiabetic population: The Hoorn Study," *Archives of Ophthalmology*, vol. 121, no. 2, pp. 245–251, 2003.

[25] M. Dirani, J. Xie, E. Fenwick et al., "Are obesity and anthropometry risk factors for diabetic retinopathy? The diabetes management project," *Investigative Ophthalmology & Visual Science*, vol. 52, no. 7, pp. 4416–4421, 2011.

[26] L. Zhang, G. Krzentowski, A. Albert, and P. J. Lefebvre, "Risk of developing retinopathy in diabetes control and complications trial type 1 diabetic patients with good or poor metabolic control," *Diabetes Care*, vol. 24, no. 7, pp. 1275–1279, 2001.

[27] X. Li and Z. Wang, "Prevalence and incidence of retinopathy in elderly diabetic patients receiving early diagnosis and treatment," *Experimental and Therapeutic Medicine*, vol. 5, no. 5, pp. 1393–1396, 2013.

[28] C. E. M. De Block, I. H. De Leeuw, and L. F. Van Gaal, "Impact of overweight on chronic microvascular complications in type 1 diabetic patients," *Diabetes Care*, vol. 28, no. 7, pp. 1649–1655, 2005.

[29] R. Klein, B. E. K. Klein, and S. E. Moss, "Is obesity related to microvascular and macrovascular complications in diabetes? The Wisconsin Epidemiologic Study of Diabetic Retinopathy," *Archives of Internal Medicine*, vol. 157, no. 6, pp. 650–656, 1997.

[30] R. Klein, B. E. Klein, S. E. Moss, M. D. Davis, and D. L. DeMets, "The Wisconsin Epidemiologic Study of Diabetic Retinopathy. III. Prevalence and risk of diabetic retinopathy when age at diagnosis is 30 or more years," *Archives of Ophthalmology*, vol. 102, no. 4, pp. 527–532, 1984.

[31] L. S. Lim, E. Shyong Tai, P. Mitchell et al., "C-reactive protein, body mass index, and diabetic retinopathy," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 9, pp. 4458–4463, 2010.

[32] R. Raman, P. K. Rani, P. Gnanamoorthy, R. R. Sudhir, G. Kumaramanikavel, and T. Sharma, "Association of obesity with diabetic retinopathy: sankara nethralaya diabetic retinopathy epidemiology and molecular genetics study (SN-DREAMS Report no. 8)," *Acta Diabetologica*, vol. 47, no. 3, pp. 209–215, 2010.

[33] G. K. Dowse, A. R. G. Humphrey, V. R. Collins et al., "Prevalence and risk factors for diabetic retinopathy in the multiethnic population of Mauritius," *American Journal of Epidemiology*, vol. 147, no. 5, pp. 448–457, 1998.

[34] M. Vučić Lovrenčić and E. Topić, "Hemoglobin A1c: standardizacija 'zlatnog standarda'," *Biochemia Medica*, vol. 16, no. 1, pp. 25–36, 2006.

[35] P. N. M. Demacker, G. J. M. Boerma, and H. Baadenhuijsen, "Evaluation of accuracy of 20 different test kits for the enzymic determination of cholesterol," *Clinical Chemistry*, vol. 29, no. 11, pp. 1916–1922, 1983.

[36] M. W. McGowan, J. D. Artiss, D. R. Strandbergh, and B. Zak, "A peroxidase-coupled method for the colorimetric determination of serum triglycerides," *Clinical Chemistry*, vol. 29, no. 3, pp. 538–542, 1983.

[37] S. K. Kumanyika, E. Obarzanek, N. Stettler et al., "Population-based prevention of obesity: the need for comprehensive promotion of healthful eating, physical activity, and energy balance: a scientific statement from American Heart Association Council on Epidemiology and Prevention, Interdisciplinary Committee for prevention (formerly the expert panel on population and prevention science)," *Circulation*, vol. 118, no. 4, pp. 428–464, 2008.

[38] S. J. Aldington, E. M. Kohner, S. Meuer, R. Klein, and A. K. Sjolie, "Methodology for retinal photography and assessment of diabetic retinography: The EURODIAB IDDM Complications Study," *Diabetologia*, vol. 38, no. 4, pp. 437–444, 1995.

[39] KDOQI, "KDOQI clinical practice guidelines and clinical practice recommendations for diabetes and chronic kidney disease," *American Journal of Kidney Diseases*, vol. 49, supplement 2, no. 2, pp. S12–S154, 2007.

[40] R. R. Sokal and F. J. Rohlf, *Biometry: The Principles and Practice of Statistics in Biological Researchedition*, W. H. Freeman, New York, NY, USA, 3rd edition, 1995.

[41] StatSoft, *Electronic Statistics Textbook*, StatSoft, Tulsa, Okla, USA, 2011, http://www.statsoft.com/Textbook.

[42] J. W. Y. Yau, S. L. Rogers, R. Kawasaki et al., "Global prevalence and major risk factors of diabetic retinopathy," *Diabetes Care*, vol. 35, no. 3, pp. 556–564, 2012.

[43] P. Romero-Aroca, M. Baget-Bernaldiz, J. Reyes-Torres et al., "Relationship between diabetic retinopathy, microalbuminuria and overt nephropathy, and twenty-year incidence follow-up of a sample of type 1 diabetic patients," *Journal of Diabetes and Its Complications*, vol. 26, no. 6, pp. 506–512, 2012.

[44] The Diabetes Control and Complications Trial Research Group, "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus," *The New England Journal of Medicine*, vol. 329, no. 14, pp. 977–986, 1993.

[45] P. Reichard, B.-Y. Nilsson, and U. Rosenqvist, "The effect of long-term intensified insulin treatment on the development of microvascular complications of diabetes mellitus," *The New England Journal of Medicine*, vol. 329, no. 5, pp. 304–309, 1993.

[46] J. V. Silha, M. Krsek, P. Sucharda, and L. J. Murphy, "Angiogenic factors are elevated in overweight and obese individuals," *International Journal of Obesity*, vol. 29, no. 11, pp. 1308–1314, 2005.

[47] L. P. Aiello, R. L. Avery, P. G. Arrigg et al., "Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders," *The New England Journal of Medicine*, vol. 331, no. 22, pp. 1480–1487, 1994.

[48] A. Praidou, S. Androudi, P. Brazitikos, G. Karakiulakis, E. Papakonstantinou, and S. Dimitrakos, "Angiogenic growth factors and their inhibitors in diabetic retinopathy," *Current Diabetes Reviews*, vol. 6, no. 5, pp. 304–312, 2010.

[49] S. Miyazawa-Hoshimoto, K. Takahashi, H. Bujo, N. Hashimoto, and Y. Saito, "Elevated serum vascular endothelial growth factor is associated with visceral fat accumulation in human obese subjects," *Diabetologia*, vol. 46, no. 11, pp. 1483–1488, 2003.

[50] D. W. Haslam and W. P. T. James, "Obesity," *The Lancet*, vol. 366, no. 9492, pp. 1197–1209, 2005.

[51] I. M. Libman, M. Pietropaolo, S. A. Arslanian, R. E. LaPorte, and D. J. Becker, "Changing prevalence of overweight children and adolescents at onset of insulin-treated diabetes," *Diabetes Care*, vol. 26, no. 10, pp. 2871–2875, 2003.

[52] B. Tran, S. Oliver, J. Rosa, and P. Galassetti, "Aspects of inflammation and oxidative stress in pediatric obesity and type 1 diabetes: an overview of ten years of studies," *Experimental Diabetes Research*, vol. 2012, Article ID 683680, 7 pages, 2012.

[53] G. L. King, "The role of inflammatory cytokines in diabetes and its complications," *Journal of Periodontology*, vol. 79, no. 8, pp. 1527–1534, 2008.

[54] J. F. Keaney Jr., M. G. Larson, R. S. Vasan et al., "Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 3, pp. 434–439, 2003.

[55] T. Y. Wong, B. B. Duncan, S. H. Golden et al., "Associations between the metabolic syndrome and retinal microvascular signs: the atherosclerosis risk in communities study," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 9, pp. 2949–2954, 2004.

[56] M. McGill, L. Molyneaux, S. M. Twigg, and D. K. Yue, "The metabolic syndrome in type 1 diabetes: does it exist and does it matter?" *Journal of Diabetes and Its Complications*, vol. 22, no. 1, pp. 18–23, 2008.

[57] L. F. Van Gaal, I. L. Mertens, and C. E. De Block, "Mechanisms linking obesity with cardiovascular disease," *Nature*, vol. 444, no. 7121, pp. 875–880, 2006.

[58] G. De Mattia, M. C. Bravi, O. Laurenti et al., "Endothelial dysfunction and oxidative stress in type 1 and type 2 diabetic patients without clinical macrovascular complications," *Diabetes Research and Clinical Practice*, vol. 79, no. 2, pp. 337–342, 2008.

[59] E. Fuentes, F. Fuentes, G. Vilahur, L. Badimon, and I. Palomo, "Mechanisms of chronic state of inflammation as mediators that link obese adipose tissue and metabolic syndrome," *Mediators of Inflammation*, vol. 2013, Article ID 136584, 11 pages, 2013.

[60] M. R. Sierra-Honigmann, A. K. Nath, C. Murakami et al., "Biological action of leptin as an angiogenic factor," *Science*, vol. 281, no. 5383, pp. 1683–1686, 1998.

[61] G. Uckaya, M. Ozata, Z. Bayraktar, V. Erten, N. Bingol, and I. C. Ozdemir, "Is leptin associated with diabetic retinopathy?" *Diabetes Care*, vol. 23, no. 3, pp. 371–376, 2000.

[62] M. Matsuda, F. Kawasaki, K. Yamada et al., "Impact of adiposity and plasma adipocytokines on diabetic angiopathies in Japanese type 2 diabetic subjects," *Diabetic Medicine*, vol. 21, no. 8, pp. 881–888, 2004.

[63] M. Tomić, S. Ljubić, and S. Kaštelan, "The role of inflammation and endothelial dysfunction in the pathogenesis of diabetic retinopathy," *Collegium Antropologicum*, vol. 37, supplement 1, pp. 51–57, 2013.

[64] A. P. Doumatey, J. Zhou, H. Huang et al., "Circulating adiponectin is associated with renal function independent of age and serum lipids in west Africans," *Internal Journal of Nephrology*, vol. 2012, Article ID 730920, 8 pages, 2012.

[65] N. M. Habeeb, O. I. Youssef, A. A. Saab, and E. S. El Hadidi, "Adiponectin as a marker of complications in type I diabetes," *Indian Pediatrics*, vol. 49, no. 4, pp. 277–280, 2012.

[66] A. S. Astrup, L. Tarnow, L. Pietraszek et al., "Markers of endothelial dysfunction and inflammation in type 1 diabetic patients with or without diabetic nephropathy followed for 10 years: association with mortality and decline of glomerular filtration rate," *Diabetes Care*, vol. 31, no. 6, pp. 1170–1176, 2008.

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Research Article

Honokiol Protected against Heatstroke-Induced Oxidative Stress and Inflammation in Diabetic Rats

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We aimed at investigating the effect of honokiol on heatstroke in an experimental rat model. Sprogue-Dawley rats were divided into 3 groups: normothermic diabetic rats treated with vehicle solution (NTDR+V), heatstroke-diabetic rats treated with vehicle (HSDR+V), and heatstroke rats treated with konokiol (0.5–5 mg/ml/kg) (HSDR+H). Sixty minutes before the start of heat stress, honokiol or vehicle solution was administered. (HSDR+H) significantly (a) attenuated hyperthermia, hypotension and hypothalamic ischemia, hypoxia, and neuronal apoptosis; (b) reduced the plasma index of the toxic oxidizing radicals; (c) diminished the indices of hepatic and renal dysfunction; (d) attenuated the plasma systemic inflammatory response molecules; (e) promoted plasma levels of an anti-inflammatory cytokine; (f) reduced the index of infiltration of polymorphonuclear neutrophils in the serum; and (g) promoted the survival time fourfold compared with the (HSDR+V) group. In conclusion, honokiol protected against the outcome of heatstroke by reducing inflammation and oxidative stress-mediated multiple organ dysfunction in diabetic rats.

1. Introduction

Among the patients with heatstroke at the Mecca Pilgrimage, most of them had diabetes with hyperglycemia [1]. Indeed, compared with nondiabetic rats, diabetic rats were more susceptible to heat stroke occurrence [2, 3]. Most of the heatstroke manifestations (e.g., excessive hyperthermia associated with a systemic inflammatory response that led to multiple organ dysfunction in which the brain dysfunctions predominated) could be reproduced by exposing the streptozotocin-induced diabetic rats to hot environments [4]. The heatstroke-diabetic rats (HSDR) displayed excessive hyperthermia, brain inflammation, ischemia and oxidative damage, and multiple organ dysfunction or failure.

Honokiol was one of main constituents of the Chinese herb, Magnolia officinalis, and had a variety of pharmacological actions, including anti-inflammatory effects [5], antimicrobial activity [6, 7], and antioxidative effects [8]. However, it was not known whether hypothalamic ischemia and oxidative stress with systemic inflammation and multiple organ dysfunction in HSDR could be affected by honokiol.

Heatstroke was a form of hyperthermia associated with a systemic inflammatory response that led to multiple organ dysfunction, in which central nervous system (CNS) disorders predominated [2]. Based on triad of factors (hyperthermia, CNS disorders, and a history of heat stress), anesthetized rodents all displayed a uniform response which was similar to responses of humans with heatstroke [3].

The present study was to assess the effects of heat stress on body core temperature (T_{co}), mean arterial pressure (MAP), hypothalamic values of cerebral blood flow (CBF), partial pressure of O_2 (PO_2), cellular ischemia marker (e.g., glutamate), organ damage markers (e.g., glycerol and lactate dehydrogenase), toxic oxidizing radicals (e.g., nitric oxide and dihydroxybenzoic acid), and serum levels of inflammatory markers (e.g., interleukin-1β, interleukin-6, tumor necrosis factor-α, and myeloperoxidase) in HSDR with or without honokiol pretreatment.

2. Methods

2.1. Animals. Adult male Sprague-Dawley rats (weighing 223 to 256 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals which were allowed to become acclimated for at least one week were housed four per cage at an ambient temperature (T_a) of 22 ± 1°C with a 12-h light/dark cycle and were supplied with rat chow and tap water *ad libitum*. The experimental protocol was approved by the Animal Ethic Committee of the Chi Mei Medical Center (Tainan, Taiwan) under guidelines of the National Science Council. Animal care and experiments were according to the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH publication number 85-23 revised 1996). Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approximately 480 minutes in duration) by a single intraperitoneal dose of urethane (1.4 g/kg body weight). At the end of the experiments, control rats and all rats that had survived heat stroke were sacrificed with an overdose of urethane.

2.2. Surgery and Physiological Parameter Monitoring. The right femoral artery and vein of rats under urethane anesthesia were cannulated with polyethylene tubing (PE50), for blood pressure monitoring and drug administration. The T_{co} temperature (T_{co}) was monitored continuously by means of a thermocouple, while MAP was monitored continuously with a pressure transducer.

2.3. Induction of Diabetes. Diabetes was induced by injecting streptozotocin (Sigma, St. Louis, MO, USA) at 30 mg/mL/kg of body weight in the tail veins of unanesthetized rats. The animals were maintained for 4-5 weeks before heat stress was applied. At the day of thermal experiments, the right femoral artery and vein of rats under general anesthesia were cannulated with polyethylene tubing (PE50) for blood pressure monitoring and drug administration or biochemical determination, respectively.

2.4. Introduction of Heatstroke. In the present study, the core temperature (at about 37°C) of the anesthetized animals was maintained with an infrared light lamp except in the heat stress experiments. Heatstroke was induced by putting the animals in a bold heating pad of 43°C controlled by circulating hot water. The instant in which MAP dropped to ~50 mmHg was found to be about 60 minutes after the start of heat stress (Figure 1). At 60 minutes, the heating was removed and the animals were allowed to recover at room temperature (26°C). Survival time values (interval between the start of heat stress and animal death) were determined. It was seen in Figure 1 that the heated rats displayed both hyperthermia (~42.5°C) and hypotension (~50 mmHg) at 60 minutes, suggesting the occurrence of heatstroke [3].

2.5. Experimental Groups. Animals were assigned randomly to one of the following three major groups: normothermic diabetic rats treated with vehicle solution (NTDR + V), heatstroke-diabetic rats treated with vehicle solution (HSDR + V), and heatstroke-diabetic rate treated with honokiol (HSDR + H). Sixty minutes before the start of heat stress, honokiol ($H_8C_2O_2$) (0.5 mg, 1.5 mg, or 5.0 mg per mL per kg of body weight; Sigma-Aldrich, Saint Louis, MO, USA) or vehicle (DMSO) solution (1 mL per kg body weight) was administered via the veins.

2.6. Measurement of Cerebral Blood Flow (CBF) and Partial Pressure of Oxygen (PO_2) in the Hypothalamus. A 100 μm diameter thermocouple and two 230 μm fibers were attached to the oxygen probe. This combined probe measured oxygen, temperature, and microvascular blood flow. The measurement required OxyLite and OxyFlow instruments. OxyLite 2000 (Oxford Optronix Ltd., Oxford, UK) was a 2-channel device (measuring PO_2 and temperature at two sites simultaneously), whereas OxyFlo 2000 was a 2-channel Laser Doppler perfusion monitoring instrument. This combined probe was implanted stereotaxically into the right hypothalamus according to the stereotaxic coordinates of Paxinos and Watson [9] to measure both CBF and PO_2 in the hypothalamus.

2.7. Determination of Serum Levels of the Toxic Oxidizing Radicals. For determination of NO_x^- and DHBA, blood samples were taken 0 and 60 minutes after the start of heat exposure. The NO_x^- concentrations in the dialysates were measured with the Eicomo-20 NO_x^- analysis system (Eicom, Kyoto) [10]. The concentrations of hydroxyl radicals were measured by a modified procedure based on the hydroxylation of sodium salicylates by hydroxyl radicals, leading to the production of 2,3-DHBA and 2,5-DHBA [11].

2.8. Quantification of Organ Function and Injury. For determination of creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were estimated in blood samples collected at 0 and 60 minutes after the start of heat stress. The serum levels of creatinine, BUN, ALT, AST, and ALP were determined by spectrophotometry (HITACHI7600, Tokyo, Japan). In addition, LDH was measured to evaluate the extent of organ injury by Fuji DRI-CHEM 3030 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.9. Measurement of Serum IL-1β, IL-6, TNF-α, IL-10, and ICAM-1 Levels. Blood samples were collected 0 and 60

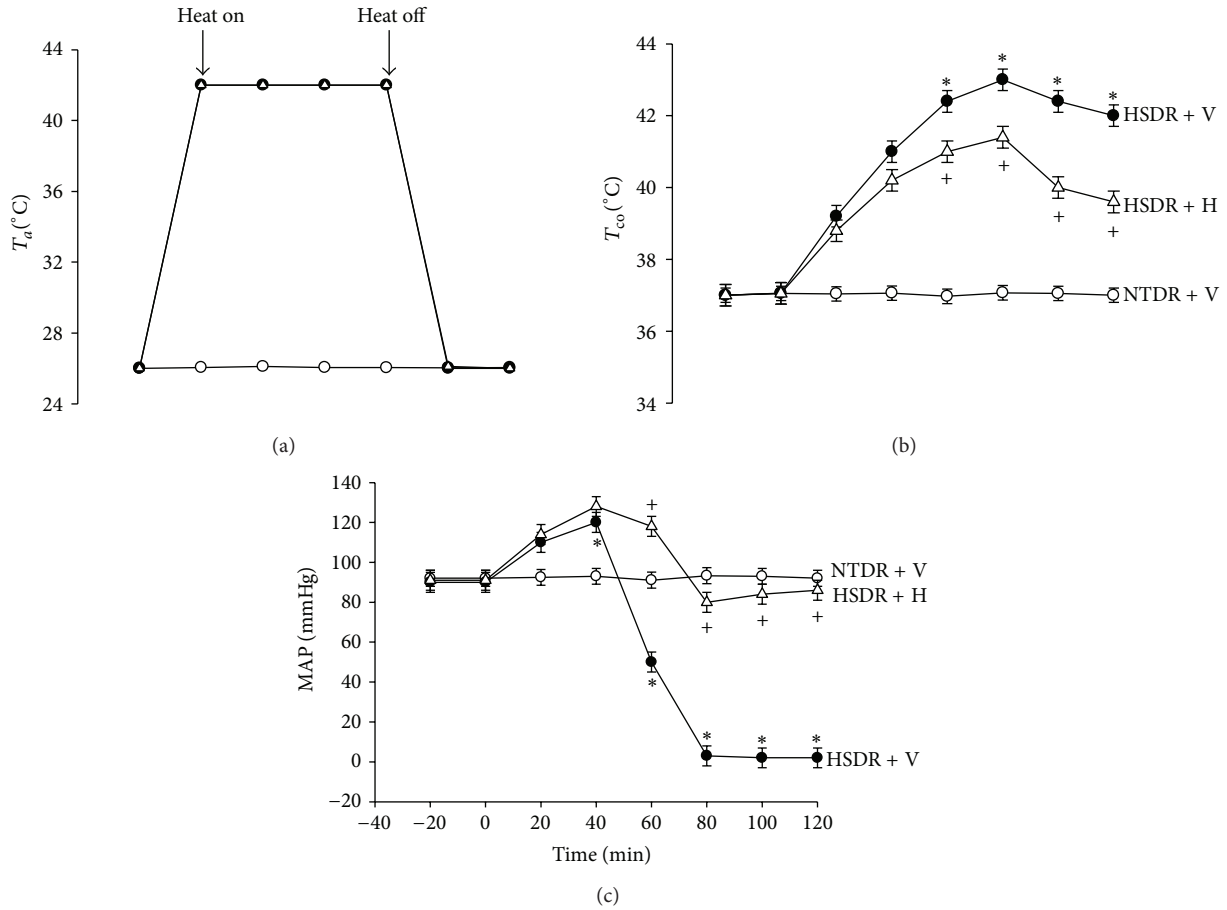

Figure 1: Values of both body core temperature (T_{co}) and mean arterial pressure (MAP) during different ambient temperatures (T_a) for normothermic diabetic rats treated with vehicle solution (NTDR + V; ○), heatstroke-diabetic rats treated with vehicle solution (HSDR + V; ●), and heatstroke-diabetic rats treated with honokiol (HSDR + H; △). Data were means ± standard deviation (SD) of eight animals per group. $^{*}P < 0.05$, in comparison with the (○) group; $^{+}P < 0.05$, in comparison with the (●) group.

minutes after the start of heat stress and stored at −80°C until they could be assayed. We used commercially available ELISA kits for the determination of serum IL-1β, IL-6, TNF-α, IL-10, and ICAM-1 levels (Quantikine, R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instruction.

2.10. Measurement of E-Selectin. Peripheral polymorphonuclear (PMN) cells were isolated from the whole blood of rats and treated with heparin (100 units/mL). Erythrocytes were allowed to sediment for 30 minutes after the addition of 3 mL of 6% dextran (weight/volume in PBS) to 10 mL blood. After sedimentation, the plasma containing leukocytes was centrifuged twice at 300 g for 5 min each. The precipitates were mixed with 70% osmolality-adjusted Percoll and centrifuged at 30,000 g for 30 min at 26°C. The PMN-riched layer was fractionated. Each fraction was washed twice with Hanks' balanced salt solution, and the cell number was counted. The purity of the PMNS was determined to exceed 95% by Giemsa Staining Cells (1×10^6 cells/tube) which were incubated with a rabbit polyclonal antibody to (CD62E (ab18981; Abcam PIC332, Cambridge, UK) or control. After washing, the cells were stained with a secondary antibody (goat polyclonal to rabbit IgG-H & L [FITC] • [ab6717]; Abcam PIC). Cells were incubated for 1 hour at 4°C and washed. They were mixed with oligasaccharides and incubated for 20 minutes, and then coincubated with KM93 for 60 minutes. The fluorescence intensity of cells was analyzed with a FACStar (Becton Dickinson).

2.11. Determination of Myeloperoxidase (MPO) Activity. A spectrophotometric method [12] was used to determine MPO activity in the serum. A 100 μL aliquot of serum was mixed with 900 μL of 50 mmol/L phosphate buffer (pH-6.0) containing 0.167 mg/mL of o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. One unit of peroxidase activity equaled the amount of enzyme decomposing 1 μmol of hydrogen peroxide which was calculated from the oxidation of o-dianisidine using an absorption coefficient of 11.3/mM/cm at 460 nm.

2.12. Assessment of Plasma Corticosterone and ACTH. Plasma corticosterone and ACTH were assayed using Corticosterone Double Antibody RIA kit (MP Biomedicals, Solon, Oh, USA)

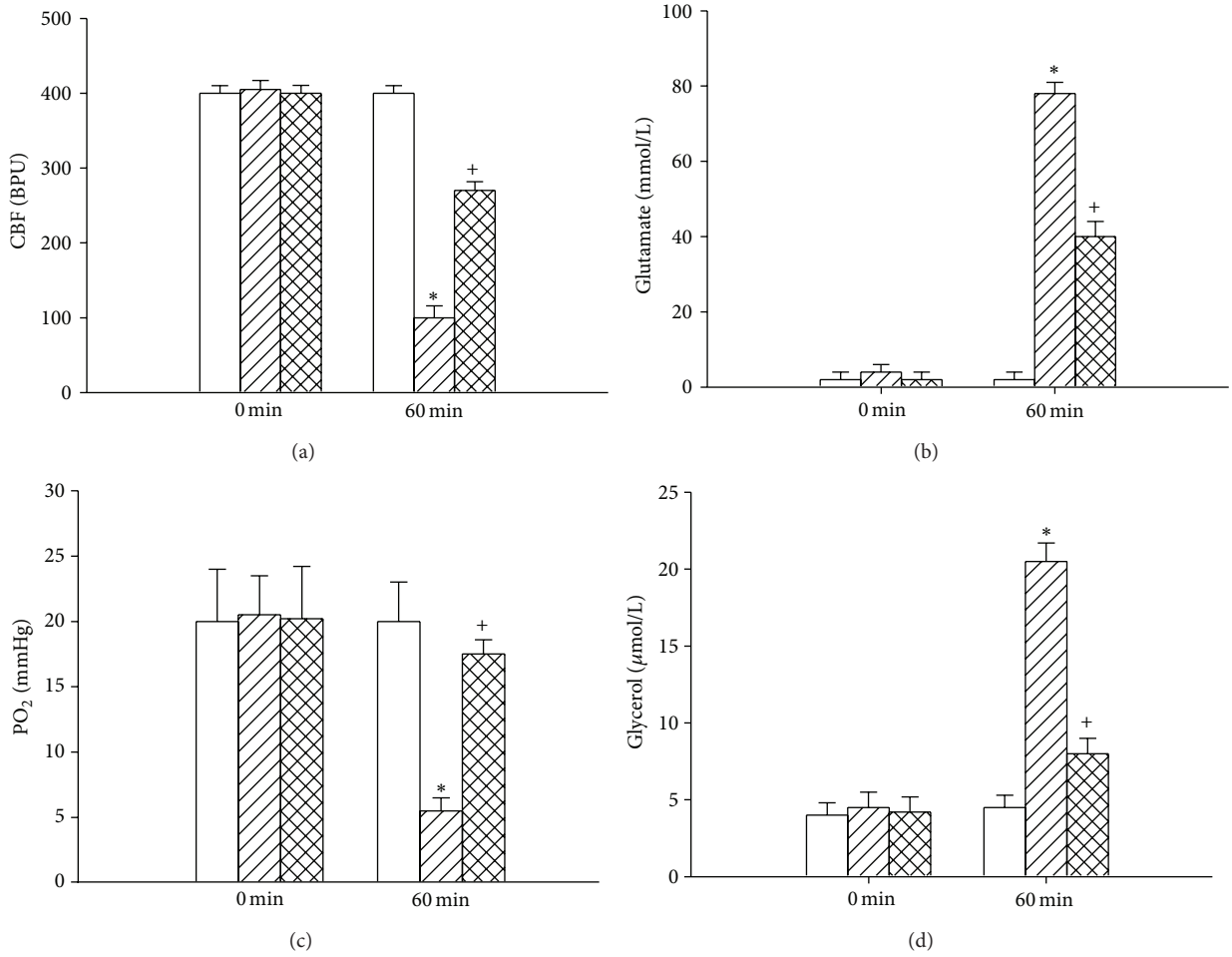

Figure 2: Values of hypothalamic cerebral blood flow (CBF), brain partial pressure of O_2 (PO_2), and cellular levels of glutamate and glycerol for the (NTDR + V) group (white bar), (HSDR + V) group (dashed bar), and (HSDR + H) group (crossed bar). The values were obtained at 0 or 60 min after the initiation of heat exposure in heatstroke rats or the equivalent times in the (NTDR + V) group. $^{*}P < 0.01$ in comparison with the (white bar) group; $^{+\,+}P < 0.05$ in comparison with the (dashed bar) group. All heatstroke groups had heat exposure (43°C) withdrawn exactly at 60 min and were then allowed to recover at room temperature (26°C). Bars were the mean ± SD of eight rats for each group. Please see the legends of Figure 1 for explanation of the abbreviations.

Table 1: Body weight, plasma glucose, and plasma insulin concentration in normal and streptozotocin-induced diabetic rats.

Groups of animals	Body weight (g)	Plasma glucose (mg/dL)	Plasma insulin (μU/mL)
Normal rats	301 ± 7 (8)	213 ± 8 (8)	52 ± 6 (8)
Diabetes	256 ± 9 (8)	465 ± 17* (8)	10 ± 4* (8)

$^{*}P < 0.01$ compared to normal rats.

and ACTH (Rat, Mouse)-RIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA), respectively. All analyses were performed according to manufacturer's instruction.

2.13. Statistical Analysis. All data were expressed as means ± standard deviation. One-way analysis of variance with Tukey's multiple comparisons test was used for serum markers and physiological parameters. Significant differences were established at $P < 0.05$. For all statistical analyses, SPSS soft ware version 10.0 (SPSS Inc., Chicago, IL, USA) was used.

3. Results

Diabetic rats had smaller body weight, higher plasma glucose levels, and lower plasma insulin when compared to healthy rats (Table 1).

The survival time values for (HSDR + V) rats were decreased from the (NTDR + V) control values of 480 ± 3 min to new values of 79 ± 3 min after the start of heat stress (Table 2). Heatstroke-diabetic rats (HSDR) treated with H (0.5, 1.5, and 5.0 mg/mL/kg of honokiol) 60 minutes before heat stress had significantly and dose-dependently higher

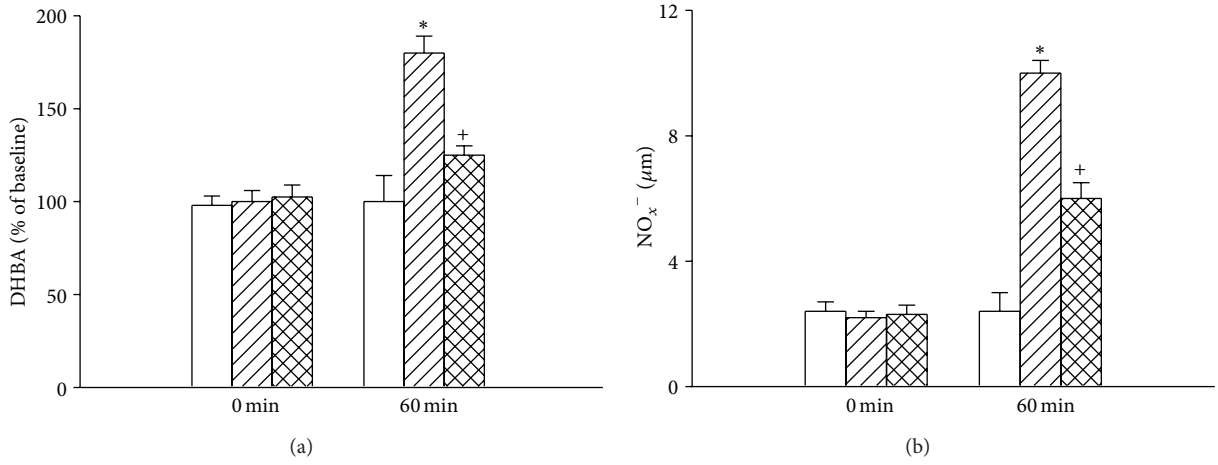

Figure 3: Values of hypothalamic levels of dihydroxybenzoic acid (DHBA) and nitric oxide metabolites (NO_x^-) for the (NTDR + V) group (white bar), (HSDR + V) group (dashed bar), and (HSDR + H) group (crossed bar). The values were obtained at 0 or 60 min after the initiation of heat exposure in heatstroke rats or the equivalent times in the (NTDR + V) group. $^*P < 0.01$ in comparison with the (white bar) group; $^+P < 0.05$ in comparison with the (dashed bar) group. Bars were the mean ± SD of eight rats for each group. See the legends of Figure 1 for explanation of the abbreviations.

Table 2: The survival time values for normothermic diabetic rats (NTDR) treated with vehicle (NTDR + V), heatstroke-diabetic rats treated with vehicle (HSDR + V), and heatstroke-diabetic rats treated with honokiol (HSDR + H).

Treatment groups	Survival time (min)
(1) NTDR + V	480 ± 3 (8)
(2) HSDR + V	79 ± 3 (8)*
(3) HSDR + H (0.5 mg/mL/kg)	114 ± 5 (8)+
(4) HSDR + H (1.5 mg/mL/kg)	186 ± 9 (8)+
(5) HSDR + H (5.0 mg/mL/kg)	273 ± 15 (8)+

All heatstroke rats which had heat exposure (43°C) were withdrawn exactly at 60 minutes and then allowed to recover at room temperature (26°C). Data are mean ± SD, followed by number of animals in parentheses. Normothermic controls (NTDR + V) were killed about 480 minutes after experiment (or at the experimental end) with the urethane overdose.

*$P < 0.01$, in comparison with Group 1.

+$P < 0.05$, in comparison with Group 2 (Dunn's test followed by Kruskal-Wallis test). Vehicle solution or melatonin was adopted 60 minutes before the start of heat stress.

values of survival time (114–273 min) than those treated with vehicle. However, in the following experiments, only the effects of one dose of 1.5 mg/mL/kg of honokiol were on determining other parameters during heatstroke.

Figure 1 showed the effects of heat exposure (43°C for 60 minutes) on both T_{co} and MAP in (NTDR + V) rats, (HSDR + V) rats, and (HSDR + H) rats. As shown in this figure, sixty minutes after the start of heat exposure, the values of MAP in the (HSDR + V) group were significantly lower than those in the (NTDR + V) group (~51 mmHg versus ~92 mmHg; $P < 0.05$). On the other hand, the values of T_{co} in the (HSDR + V) group were significantly higher than those in the (NTDR + V) group (~42.2°C versus ~37.3°C; $P < 0.01$). Heat-induced hypotension and hyperthermia were all significantly reduced by honokiol pretreatment as shown in (HSDR + H) group.

Figure 2 showed that the hypothalamic levels of PO_2 and cerebral blood flow in the (HSDR + V) rats were significantly lower at 60 minutes after the start of heat exposure than in the (NTDR + V) group (~100 BPU versus ~400 BPU for CBF; ~5 mmHg versus ~20 mmHg for PO_2; $P < 0.05$). In contrast, the hypothalamic levels of glutamate and glycerol in the (HSDR + V) rats were significantly higher at 60 minutes after the start of heat exposure (~82 mmol/L versus ~3 mmHg for glutamate; ~22 μmol versus ~5 μmol for glycerol; $P < 0.05$). Heat-induced hypothalamic hypoxia, ischemia, and damage at 60 minutes were all significantly reduced by pretreatment with honokiol.

Figure 3 showed that the hypothalamic levels of 2,3-DHBA and NO_x^- in the (HSDR + V) rats were significantly higher at 60 minutes after the start of heat exposure than in the (NTDR + V) rats (~180% versus ~100 mmHg for DHBA; ~9.6 μM versus ~2.6 μM for NO_x^-; $P < 0.05$). As shown in this figure, heat-induced increased hypothalamic levels of all these parameters were significantly reduced by honokiol pretreatment in the (HSDR + H) rats.

Figure 4 showed that the hypothalamic values of TUNEL-positive cells in the (HSDR + V) were significantly higher at 60 minutes after the start of heat stress than that in the (NTDR + V) rats (~148 cells versus ~5 cells; $P < 0.01$). As shown in this figure, heat-induced increased hypothalamic values of TUNEL-positive cells were significantly reduced by honokiol pretreatment in the (HSDR + H) rats.

Figure 5 showed that the serum values of IL-1β, IL-6, TNF-α, E-selectin, ICAM-1, lactate dehydrogenase, and MPO for (HSDR + V) were significantly higher at 60 minutes after the start of heat stress than that in the (NTDR + V) rats. Heat-induced increased values of these parameters were all significantly reduced by honokiol pretreatment in the (HSDR + H) rats. In addition, serum levels of IL-10 in the (HSDR + H) rats were significantly higher than that in the (HSDR + V) rats at 60 minutes after the start of heat stress.

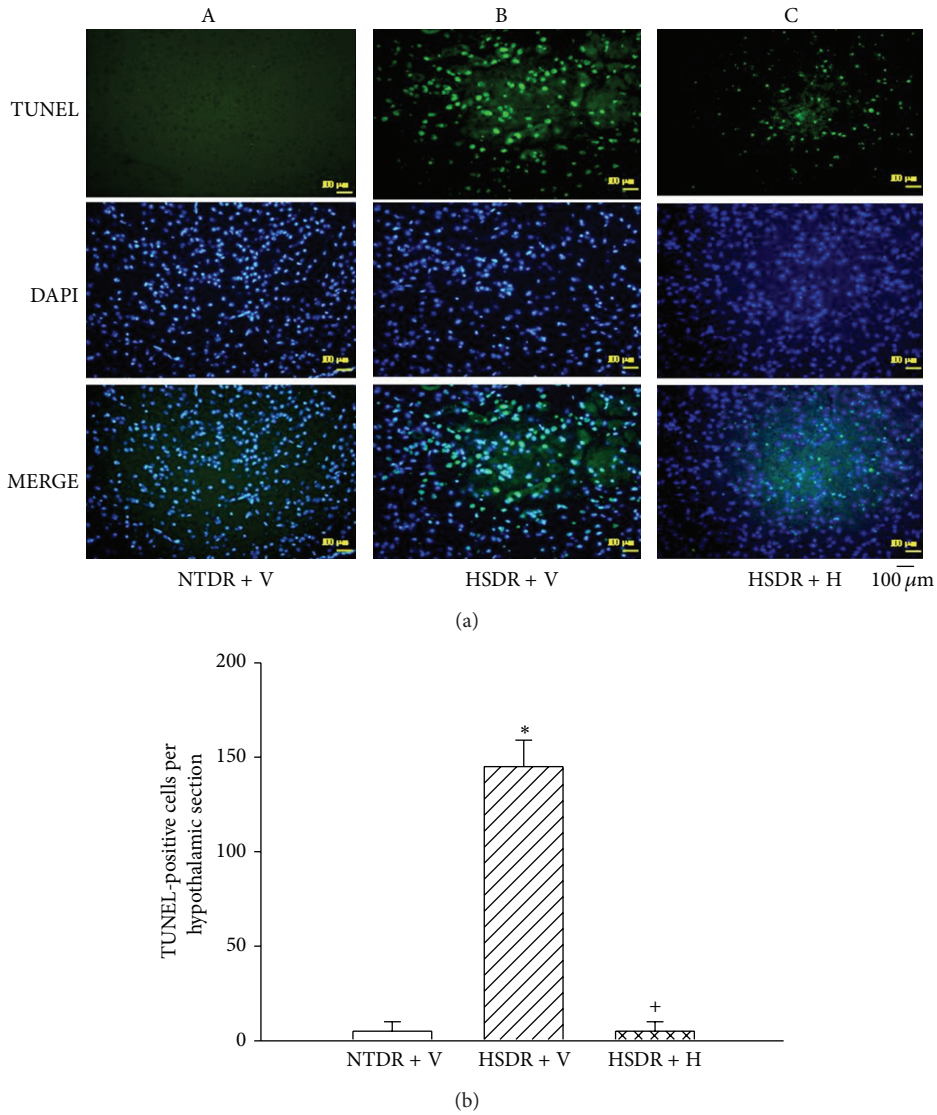

Figure 4: Values of hypothalamic TUNEL-positive cells for the (NTDR + V) group (white bar), (HSDR + V) group (dashed bar), and (HSDR + H) group (crossed bar). The values were obtained at 60 min after the initiation of heat exposure in heatstroke rats or the equivalent times in the (NTDR + V) group. $^{*}P < 0.01$ in comparison with the (white bar) group; $^{+}P < 0.01$ in comparison with (dashed bar) group. Bars were each the mean ± SD of eight rats of reach group. Top panels depict the representative photographs for TUNEL staining in a (NTDR + V) rat, a (HSDR + V) rat, and a (HSDR + H) rat. See the legends of Figure 1 for the abbreviation.

Table 3 showed that the plasma levels of both ACTH and corticosterone in the (HSDR + V) rats were significantly higher at 60 minutes after the start of heat stress than that in the (NTDR + V) rats. Additionally, (HSDR + H) rats had significantly ($P < 0.05$) higher plasma levels of both ACTH and corticosterone than (HSDR + V) rats.

Table 4 showed that the serum level of BUN, creatinine ALT, AST, and AP in the (HSDR + V) rats were significantly higher at 60 min after the start of heat stress than that in the (NTDR + V) rats. Heat-increased serum levels of BUN, creatinine, ALT, AST, and AP in the (HSDR + V) rats were significantly reduced by honokiol pretreatment in the (HSDR + H) rats.

4. Discussion

Severe heat stress induced excessive hyperthermia, splanchnic vasoconstriction, and multiple organ dysfunction or failure. During heatstroke, excessive hyperthermia facilitates the leakage of endotoxin from the intestine to the circulating blood and resulted in leukocytes infiltration and excessive activation of endothelial cells [2, 13]. Tissue ischemia, hypoxia, and damage secreted proinflammatory cytokines which led to excessive production or release of the toxic oxidizing radicals like NO_x^- and DHBA and resulted in multiple organ damage [14]. Amplification of the resultant activated inflammatory state induced accumulation of free radicals

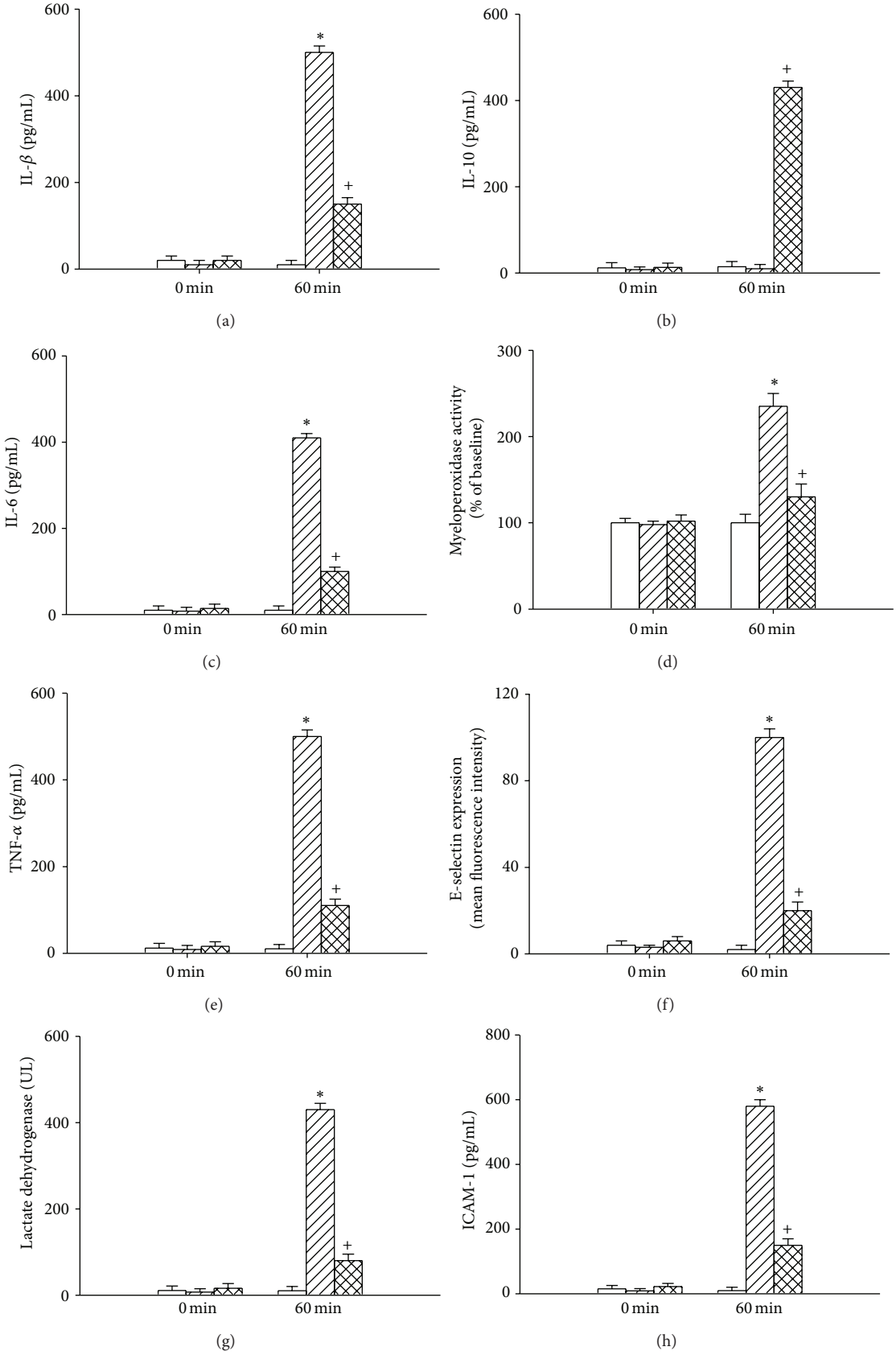

Figure 5: Values of serum interleukin-1β (IL-β), IL-6, tumor necrosis factor-α (TNF-α), E-selectin, ICAM-1, lactate dehydrogenase, and myeloperoxidase (MPO) for the (NTDR + V) group (white bar), (HSDR + V) group (dashed bar), and (HSDR + H) group (crossed bar). The values obtained at 0 or 60 min after the initiation of heat exposure in heatstroke rats or the equivalent times in the (NTDR + V) group. $^{*}P < 0.01$ in comparison with the (white bar) group; $^{+}P < 0.05$ in comparison with the (dashed bar) group. Bars were the mean ± SD of eight rats for each group.

Table 3: The plasma levels of both adrenocorticotrophic-hormone (ACTH) and corticosterone for (NTDR + V) rats, (HSDR + V) rats, and (HSDR + H) rats.

Treatment groups/time	ACTH ($pg \cdot mL^{-1}$)	Corticosterone ($ng \cdot mL^{-1}$)
(1) NTDR + V:		
0 min	412 ± 108 (8)	139 ± 25 (8)
60 min	429 ± 113 (8)	145 ± 23 (8)
(2) HSDR + V:		
0 min	407 ± 99 (8)	128 ± 21 (8)
60 min	1063 ± 164* (8)	428 ± 23* (8)
(3) HSDR + H:		
0 min	421 ± 120 (8)	133 ± 24 (8)
60 min	1965 ± 185+ (8)	747 ± 33+ (8)

All heatstroke rats which had heat exposure (43°C) were withdrawn exactly at 60 minutes and then allowed to recover at room temperature (26°C). Data are man ± SD, followed by number of animals in parentheses.

*$P < 0.05$, in comparison with Group 1.

+$P < 0.05$, in comparison with Group 2 (Dunn's test followed by Kruskal-Wallis test); vehicle solution (V) or honokiol (H) was adopted 60 min before the start of heat exposure.

Table 4: The serum levels of blood urea nitrogen (BUN), creatinine (C), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphate (AP) for (NTDR + V) group, (HSDR + V) group, and (HSDR + H) group.

Treatment groups/time	BUN (mmol/L)	C (mmol/L)	ALT (U/L)	AST (U/L)	AP (U/L)
(1) NTDR + V:					
0 min	8.4 ± 0.7 (8)	28 ± 2 (8)	36 ± 3 (8)	112 ± 9 (8)	308 ± 22 (8)
60 min	8.6 ± 0.6 (8)	26 ± 3 (8)	34 ± 4 (8)	115 ± 7 (8)	314 ± 24 (8)
(2) HSDR + V:					
0 min	8.6 ± 0.8 (8)	26 ± 3 (8)	35 ± 4 (8)	114 ± 8 (8)	301 ± 21 (8)
60 min	22 ± 0.9* (8)	72 ± 4* (8)	135 ± 6* (8)	556 ± 7* (8)	583 ± 27* (8)
(3) HSDR + H:					
0 min	8.5 ± 0.7 (8)	25 ± 4 (8)	32 ± 4 (8)	109 ± 8 (8)	299 ± 24 (8)
60 min	11 ± 1.1+ (8)	48 ± 5+ (8)	79 ± 5+ (8)	196 ± 6+ (8)	402 ± 19+ (8)

All heatstroke rats which had heat exposure (43°C) were withdrawn exactly at 60 minutes and then allowed to recover at room temperature (26°C). Data are mean ± SD, followed by number of animals in parentheses.

*$P < 0.01$, in comparison with Group 1.

+$P < 0.05$, in comparison with Group 2 (Dunn's test followed by Kruskal-Wallis test). Vehicle solution (V) or honokiol (H) was adopted 60 min before the start of heat stress.

and resulted in augmentation of local tissue injury [15]. This systemic inflammation response led to polymorphonuclear cells sequestration in both the lung and liver and resulted in multiple organ dysfunction or failure [16]. In particular, heat-induced multiple organ dysfunction might be associated with hypothalamo-pituitary-adrenocortical (HPA) axis impairment [17]. Their data showed that intolerance to heat exposure was associated with an HPA axis impairment, possibly related to changes occurring in the inflammatory mediators levels.

As mentioned in Section 1, the patients with diabetes were susceptible to heat stroke at Mecca Pilgrimage [1]. Our present study further showed that heat-induced multiple organ dysfunction or failure in streptozotocin-induced diabetic rats was related to tissue inflammation and oxidative stress. Especially, pretreatment with honokiol significantly (a) attenuated hyperthermia, hypotension and hypothalamic ischemia, hypoxia, and neuronal apoptosis, (b) reduced the plasma index of the toxic oxidizing radicals including NO_x^- and 2,3-DHBA, (c) diminished the indices of hepatic and renal dysfunction including creatinine, blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactate hydrogenase, (d) attenuated the plasma systemic inflammatory response molecules like soluble intercellular adhesion molecule-1, E-selectin, tumor necrosis factor-alpha, interleukin-1β, and interleukin-6, (e) promoted plasma levels of an anti-inflammatory cytokine, interleukin-10, (f) reduced an index of infiltration of polymorphonuclear neutrophils in the serum as measured by myeloperoxidase activity, (g) enhanced the plasma levels of corticosterone and adrenocorticotrophic hormone, and (h) promoted the survival time fourfold in HSDR. Hence, we suggested that isolating honokiol from Magnolia officinalis might be a potential adjunct in the prevention of heatstroke. In fact, the biological activities of honokiol include potent inhibition of lipid peroxidation [8, 18], scavengers of hydroxyl radicals [19], inhibition of leukotriene synthesis in leukaemia cells [20], and inhibition of UV-induced mutations [21].

Serum molecules like IL-1β, IL-6, TNF-α, ICAM-1, and E-selectin were involved in the pathophysiology of systemic

inflammatory response syndromes [22–25]. Activation of both neutrophils and endothelial cells is associated with overproduction of these systemic inflammatory response syndrome molecules in patients [2] or rats [26] with heatstroke. In the present study, both hyperthermia and overproduction of these systemic inflammatory response syndrome molecules in rats could be significantly attenuated by honokiol. In addition, honokiol pretreatment facilitated the production of serum IL-10, an anti-inflammatory cytokine, [27] that occurred during heatstroke. Thus, honokiol might downregulate the extent of activated inflammation via attenuating the heatstroke-induced excessive hyperthermia. The severity of heat illness was believed to depend on the degree of hyperthermia and its duration [28].

It was promoted that decreased heat tolerance was associated with HPA axis impairment [17]. As shown in the present study, hypothalamic ischemia, hypoxia, and neuronal apoptosis were associated with HPA impairment (evidenced by insufficient release of both ACTH and corticosterone) in HSDR which could be significantly reversed by honokiol. It was likely that honokiol might increase heat tolerance by reducing HPA axis impairment (as reflected by producing more ACTH and corticosterone in plasma in response to heat stress) in rats. The hypothesis is supported by our previous finding showing that glucocorticoids reduced interleukin-1 concentration and resulted in neuroprotective effects in rat heatstroke [29].

It should be mentioned that the normal rats should release more ACTH and corticosterone in response to heat stress than the normothermic controls (nonetheless, the data are not reported here). However, the plasma levels of both ACTH and corticosterone in heated diabetic rats should be lower than that of heated nondiabetic rats. The reduced release of both ACTH and corticosterone displayed by heated diabetic rats could be reversed by honokiol supplement.

5. Conclusion

In summary, we demonstrated that honokiol protected against hyperthermia, hypotension, and multiorgan injury in HSDR. The beneficial effects of honokiol might be on the reduction of (a) systemic inflammatory response syndrome molecules like TNF-α, IL-1βIL-6, ICAM-1, MPO, and E-selectin in the plasma, (b) the toxic oxidizing radicals like NO_x^- and 2,3-DHBA, in the plasma, (c) the organ injury indicators like LDH, creatinine, blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase in the plasma, and (d) an indicator for leukocytes accumulation like MPO in the serum, and thus leading to maintenance of HPA axis function and increased survival in rats with heatstroke-associated multiple organ dysfunction. Our data suggested that honokiol was useful in reducing both inflammation and oxidative stress; lowering heat-induced multiple organ dysfunction.

Conflict of Interests

The authors report no conflict of interests related to this study or the findings specified in this paper.

Disclosure

The results presented in this paper have not been published previously in whole or part, presented in abstract form. All the authors have contributed substantially to the research, preparation and production of the paper and approved its submission to the Journal.

References

[1] S. S. Al-Harthi, O. Karrar, S. A. Al-Mashhadani, and A. A. Saddique, "Metabolite and hormonal profiles in heat stroke patients at Mecca Pilgrimage," *Journal of Internal Medicine*, vol. 228, no. 4, pp. 343–346, 1990.

[2] A. Bouchama and J. P. Knochel, "Heat stroke," *The New England Journal of Medicine*, vol. 346, no. 25, pp. 1978–1988, 2002.

[3] S. H. Chen, M. T. Lin, and C. P. Chang, "Ischemic and oxidative damage to the hypothalamus may be responsible for heat stroke," *Current Neuropharmacology*, vol. 11, no. 2, pp. 129–140, 2013.

[4] K. L. Lee, K. C. Niu, M. T. Lin, and C. S. Niu, "Attenuating brain inflammation, ischemia, and oxidative damage by hyperbaric oxygen in diabetic rats after heat stroke," *Journal of the Formosan Medical Association*, vol. 112, no. 8, pp. 454–462, 2013.

[5] J.-P. Wang, T.-F. Ho, L.-C. Chang, and C.-C. Chen, "Anti-inflammatory effect of magnolol, isolated from Magnolia officinalis, on A23187-induced pleurisy in mice," *Journal of Pharmacy and Pharmacology*, vol. 47, no. 10, pp. 857–860, 1995.

[6] A. M. Clark, F. S. El-Feraly, and W. S. Li, "Antimicrobial activity of phenolic constituents of Magnolia grandiflora L," *Journal of Pharmaceutical Sciences*, vol. 70, no. 8, pp. 951–952, 1981.

[7] K. Y. Ho, C. C. Tsai, C. P. Chen, J. S. Huang, and C. C. Lin, "Antimicrobial activity of honokiol and magnolol isolated from Magnolia officinalis," *Phytotherapy Research*, vol. 15, no. 2, pp. 139–141, 2001.

[8] Y. Lo, C. Teng, C. Chen, C. Chen, and C. Hong, "Magnolol and honokiol isolated from Magnolia officinalis protect rat heart mitochondria against lipid peroxidation," *Biochemical Pharmacology*, vol. 47, no. 3, pp. 549–553, 1994.

[9] G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Acdemic Press, New Yourk, NY, USA, 1982.

[10] H. Togashi, K. Mori, K. Ueno et al., "Consecutive evaluation of nitric oxide production after transient cerebral ischemia in the rat hippocampus using in vivo brain microdialysis," *Neuroscience Letters*, vol. 240, no. 1, pp. 53–57, 1998.

[11] C. Yang and M. Lin, "Oxidative stress in rats with heatstroke-induced cerebral ischemia," *Stroke*, vol. 33, no. 3, pp. 790–794, 2002.

[12] K. M. Mullane, R. Kraemer, and B. Smith, "Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium," *Journal of Pharmacological Methods*, vol. 14, no. 3, pp. 157–167, 1985.

[13] D. M. Hall, G. R. Buettner, L. W. Oberley, L. Xu, R. D. Matthes, and C. V. Gisolfi, "Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 280, no. 2, pp. H509–H521, 2001.

[14] F. Feihl, B. Waeber, and L. Liaudet, "Is nitric oxide overproduction the target of choice for the management of septic shock?" *Pharmacology and Therapeutics*, vol. 91, no. 3, pp. 179–213, 2001.

[15] P. K. Kim and C. S. Deutschman, "Inflammatory responses and mediators," *Surgical Clinics of North America*, vol. 80, no. 3, pp. 885–894, 2000.

[16] R. R. Neviere, G. Cepinskas, W. S. Madorin et al., "LPS pretreatment ameliorates peritonitis-induced myocardial inflammation and dysfunction: role of myocytes," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 277, no. 3, pp. H885–H892, 1999.

[17] V. Michel, A. Peinnequin, A. Alonso, A. Buguet, R. Cespuglio, and F. Canini, "Decreased heat tolerance is associated with hypothalamo-pituitary-adrenocortical axis impairment," *Neuroscience*, vol. 147, no. 2, pp. 522–531, 2007.

[18] H. Haraguchi, H. Ishikawa, N. Shirataki, and A. Fukuda, "Antiperoxidative activity of neolignans from Magnolia obovata," *Journal of Pharmacy and Pharmacology*, vol. 49, no. 2, pp. 209–212, 1997.

[19] J. Taira, T. Ikemoto, K. Mimura, A. Hagi, A. Murakami, and K. Makino, "Effective inhibition of hydroxyl radicals by hydroxylated biphenyl compounds," *Free Radical Research Communications*, vol. 19, supplement 1, pp. S71–S77, 1993.

[20] Y. Hamasaki, E. Muro, S. Miyanji et al., "Inhibition of leukotriene synthesis by honokiol in rat basophilic leukemia cells," *International Archives of Allergy and Immunology*, vol. 110, no. 3, pp. 278–281, 1996.

[21] S. Fujita and J. Taira, "Biphenyl compounds are hydroxyl radical scavengers: their effective inhibition for UV-induced mutation in Salmonella typhimurium TA102," *Free Radical Biology and Medicine*, vol. 17, no. 3, pp. 273–277, 1994.

[22] J. A. Morecroft and L. Spitz, "The role of inflammatory mediators in necrotizing enterocolitis," *Seminars in Neonatology*, vol. 2, no. 4, pp. 273–280, 1997.

[23] J. Boldt, M. Wollbruck, D. Kuhn, L. C. Linke, and G. Hempelmann, "Do plasma levels of circulating soluble adhesion molecules differ between surviving and nonsurviving critically ill patients?" *Chest*, vol. 107, no. 3, pp. 787–792, 1995.

[24] G. S. Kansas, "Selectins and their ligands: current concepts and controversies," *Blood*, vol. 88, no. 9, pp. 3259–3287, 1996.

[25] K. Goda, T. Tanaka, M. Monden, and M. Miyasaka, "Characterization of an apparently conserved epitope in E- and P-selectin identified by dual-specific monoclonal antibodies," *European Journal of Immunology*, vol. 29, no. 5, pp. 1551–1560, 1999.

[26] W. Liu, C. Chen, N. Foo et al., "Human umbilical cord blood cells protect against hypothalamic apoptosis and systemic inflammation response during heatstroke in rats," *Pediatrics and Neonatology*, vol. 50, no. 5, pp. 208–216, 2009.

[27] T. J. Standiford, R. M. Strieter, N. W. Lukacs, and S. L. Kunkel, "Neutralization of IL-10 increases lethality in endotoxemia: cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor," *Journal of Immunology*, vol. 155, no. 4, pp. 2222–2229, 1995.

[28] Y. Shapiro, T. Rosenthal, and E. Sohar, "Experimental heatstroke: a model in dogs," *Archives of Internal Medicine*, vol. 131, no. 5, pp. 688–692, 1973.

[29] C. Liu, C. Chien, and M. Lin, "Glucocorticoids reduce interleukin-1β concentration and result in neuroprotective effects in rat heatstroke," *Journal of Physiology*, vol. 527, part 2, pp. 333–343, 2000.

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Research Article

Bixin and Norbixin Have Opposite Effects on Glycemia, Lipidemia, and Oxidative Stress in Streptozotocin-Induced Diabetic Rats

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The present study investigated the effects of oral administration of annatto carotenoids (bixin (BIX) and norbixin (NBIX)) on glucose levels, lipid profiles, and oxidative stress parameters in streptozotocin (STZ)-induced diabetic rats. Animals were treated for 30 days in the following groups: nondiabetic control, diabetic vehicle, diabetic 10 mg/kg BIX, diabetic 100 mg/kg BIX, diabetic 10 mg/kg NBIX, diabetic 100 mg/kg NBIX, diabetic metformin, and diabetic insulin. Blood glucose, LDL cholesterol, and triglyceride levels were reduced in the diabetic rats treated with BIX. BIX treatment prevented protein oxidation and nitric oxide production and restored superoxide dismutase activity. NBIX treatment did not change most parameters assessed, and at the highest dose, it increased LDL cholesterol and triglycerides levels and showed prooxidant action (increased protein oxidation and nitric oxide levels). These findings suggested that BIX could have an antihyperglycemic effect, improve lipid profiles, and protect against damage induced by oxidative stress in the diabetic state. Because NBIX is a water-soluble analog of BIX, we propose that lipophilicity is crucial for the protective effect of annatto carotenoids against streptozotocin-induced diabetes.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that continues to be a major health problem worldwide. It affects approximately 6% of the population worldwide or 371 million individuals [1]. DM is characterized by hyperglycemia and alterations in carbohydrate, fat, and protein metabolism [2]; impairments in antioxidant enzymes [3]; high oxidative stress-induced damage to pancreatic β cells [4]. This disorder is caused either by a deficiency in the production of insulin or by a hyperinsulinemic condition that is accompanied by tissue resistance to insulin [2].

In addition, plasma triglycerides, low-density lipoprotein (LDL), and total cholesterol levels are usually elevated in diabetes, whereas the levels of high-density lipoproteins (HDL) are reduced [5]. The dyslipidemia associated with the hyperglycemia and changes in the sensitivity or reactivity of the vascular smooth muscle to neurotransmitters and circulating hormones plays a role in the accelerated development of atherosclerotic vascular disease, which is a major long-term complication of diabetes in humans [6].

Hyperglycemia has been hypothesized to contribute to oxidative stress either through the direct generation of ROS or by altering the redox balance [7]. This is thought to occur via

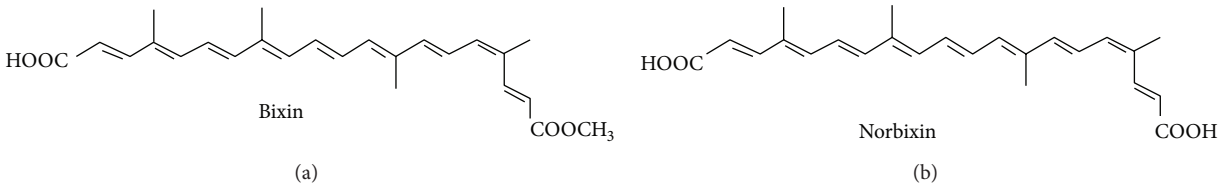

Figure 1: Chemical structure of annatto seed carotenoids.

several well-studied mechanisms, including increased polyol pathway flux, increased intracellular formation of advanced glycation end products (AGEs), activation of protein kinase C, or overproduction of superoxide by the mitochondrial electron transport chain [7, 8]. The polyol pathway leads to a NADPH-dependent reduction of glucose to sorbitol via aldose reductase. Under hyperglycemic conditions, the glucose flux through the polyol pathway is increased, which decreases the levels of NADPH and contributes greatly to an overall redox imbalance in the cell that leads to oxidative stress [8]. In addition, hyperglycemia increases the production of AGEs, which are formed through the covalent binding of aldehyde or ketone groups from reducing sugars to free amino groups of proteins, creating a Schiff base. Glucose alone can also undergo autoxidation to form reactive carbonyl intermediates, of which glyoxal and methylglyoxal are the two main intermediates. These reactive carbonyl intermediates then complete a complex series of chemical rearrangements to yield irreversible AGE structures [9]. Another important source of ROS is the overproduction of superoxide by the mitochondrial electron transport chain. This overproduction occurs under hyperglycemic conditions because the number of substrates entering the Krebs cycle is greatly increased, and consequently, the number of reducing equivalents donating electrons to the mitochondrial electron transport chain is also increased [8].

Therefore, antioxidant compounds such as tea catechins, resveratrol, and garlic acid have been demonstrated to have benefits in the prevention and treatment of diabetic complications caused by oxidative stress [10–12]. The seeds of *Bixa orellana* L., a native shrub from tropical America, are a rich source of antioxidant carotenoid pigments that are largely used by the food coloring industry. This pigment, which is known as annatto (E160b), contains approximately 80% bixin (BIX; Figure 1(a)), an unusual lipid-soluble carotenoid that has a free carboxyl and an esterified carboxyl end group [13]. The hydrolytic removal of the methyl ester group from BIX by saponification generates norbixin (NBIX; Figure 1(b)), a water-soluble carotenoid also found in annatto preparations but at lower amounts than BIX [14]. These annatto carotenoids have been shown to have important antioxidant effects [15].

In addition, annatto extract can cause hypoglycemic episodes in normoglycemic dogs that are mediated by increased insulin plasma levels and possibly by increased affinity of insulin for its receptors [16]. However, this effect is dependent on the solvent used to obtain the annatto extract because oily extracts have a hypoglycemic effect, whereas ethanolic extracts have a hyperglycemic effect in normoglycemic dogs [16, 17]. In addition, NBIX has been shown to have opposing effects in normoglycemic rats and mice: it has a hyperglycemic effect in rats but a hypoglycemic effect in mice [18]. In fact, a study using adipocyte cell culture showed that BIX regulated the mRNA expression of PPAR-γ target genes that are involved in adipogenesis and improved insulin sensitivity [19]. Furthermore, it has been shown that both BIX and NBIX are agonists of the PPAR-α receptor, which regulates the expression of genes involved in fatty acid oxidation and carbohydrate metabolism [20].

These previous studies in cell culture and normoglycemic healthy animals have provided important evidence that annatto carotenoids may have positive effects on carbohydrate metabolism. Thus, studies in animal models of diabetes could help to determine the pharmacological potential of these carotenoids to control risk factors for diabetes complications. Given that oxidative stress has been associated with the pathogenesis of various diabetic complications [3, 5] and given the antioxidant effects of annatto in other biological models, we hypothesized that the annatto carotenoids could also directly counteract the oxidative stress associated with diabetes in addition to acting on the glucose metabolism. Thus, the present study investigated whether BIX and NBIX could prevent the hyperglycemia, dyslipidemia, and oxidative stress associated with streptozotocin (STZ)-induced diabetic rats, which is a well-characterized animal model of diabetes [21]. The effects of these annatto carotenoids were compared to the effects of standard hypoglycemic drugs used in the treatment of diabetes.

2. Materials and Methods

2.1. Chemicals and Solutions. STZ, DL-dithiothreitol (DTT), glutathione, 5,5′-dithiobis(2)-nitrobenzoic acid (DTNB), sodium nitrate, vanadium (III) chloride, sulphanilamide, N-(1-naphthyl)ethylenediamine, glutathione reductase, epinephrine hydrochloride, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate, and L-glutathione oxidized disodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). The orthophosphoric acid, hydrochloric acid, potassium iodide, acetic acid, and sodium hydroxide were purchased from Vetec (Brazil). Metformin (99%) was from Valdequimica (São Paulo, SP, Brazil) and the NPH insulin (100 IU/mL) used was commercially purchased (Lilly, Indianapolis, IN, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

BIX oily solution (10%) and NBIX aqueous solution (10%) were donated by Christian Hansen (Brazil). BIX and NBIX

solutions at 5 and 50 mg/mL were prepared by dilution in distilled water containing 10% Tween 80 and 0.5% ethanol. The vehicle aqueous solution contained 10% Tween 80 and 0.5% ethanol. Metformin was dissolved in water at 50 mg/mL. The solutions were prepared three times a week and stored in amber bottle at 2 to 8°C until use.

2.2. Animals and Streptozotocin-Induced Diabetes. Adult male Wistar rats (70–90 days; 200–350 g) from the Central Animal House of the Federal University of Santa Maria were housed under controlled temperature (23 ± 1°C) and humidity (56%) on a 12-hour light-dark cycle with free access to food (Supralab, São Leopoldo, RS, Brazil) and water. Before the beginning of the experiments, animals were adapted in cages for 20 days. All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals (Council of European Communities, 1986) and were approved by the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria (protocol no.: 089/2011). Diabetes was induced by a single i.p. injection of STZ (1 mL/kg b.w., 60 mg/kg b.w.) dissolved in sodium-citrate buffer (0.05 M, pH 4.5) [21]. The control group received vehicle (sodium-citrate buffer, 1 mL/kg b.w.). In order to reduce death due to hypoglycemic shock, STZ-treated rats received glucose (20%, 2 mL/kg b.w.) by gavage 6 hours after diabetes induction. In addition, during the first 24 h after STZ administration a 5% glucose solution was offered to the animals instead of water. Then, the animals were kept for 15 days with free access to food and water before glycemia evaluation [21]. Blood glucose levels were measured with an automatic analyzer donated by Roche of Brazil (Advantage, Boehringer Mannheim, Indianapolis, IN, USA). Only animals with fasting glycemia over 200 mg/dL were considered diabetic.

2.3. Experimental Design. The animals were randomly divided into eight groups (6 rats per group): nondiabetic control + vehicle, diabetic + vehicle, diabetic + 10 mg/kg BIX, diabetic + 100 mg/kg BIX, diabetic + 10 mg/kg NBIX, diabetic + 100 mg/kg NBIX, diabetic + 100 mg/kg metformin, and diabetic + insulin (4 IU). Vehicle, BIX, NBIX, and metformin were given by gavage (up to 2 mL/kg b.w.) as a daily administration for 30 days. The insulin dose was fractionated (2 IU doses) and administered intradermically twice a day for 30 days. BIX and NBIX doses were chosen based on a previous study [18]. During all the experiments the animals had free access to food and water. The animals were weighed before the diabetes induction and once a week along the experimental period.

After the 30 days of treatment, animals were fasted overnight and anesthetized with xylazine (10 mg/kg body weight) and ketamine (75 mg/kg body weight) and blood samples were collected by cardiac puncture into heparinized tubes to measure the activity of antioxidant enzymes. Another blood sample was collected without anticoagulant to obtain serum for the biochemical analyses and to assess the products of advanced protein oxidation (AOPP) and nitric oxide (NOx) levels.

2.4. Biochemical Analyses. The serum concentrations of glucose, total cholesterol, triglycerides, and HDL were determined using colorimetric kits (Doles, Goiania, GO, Brazil). The LDL cholesterol level was calculated according to Friedewald et al. [22]. Fructosamine was measured using a kinetic kit assay (Gold Analisa, Belo Horizonte, MG, Brazil). Insulin was measured by radioimmunoassay (Immunotech, Beckman Coulter Company, Marseille, France).

2.5. Biomarkers of Oxidative Stress. Serum NOx levels were assessed as nitrite/nitrate content measured on Cobas MIRA as previously described [23]. The oxidation of proteins was evaluated by determining the products of advanced protein oxidation (AOPP) [24]. Glutathione peroxidase (GPx) activity was determined by spectrophotometry using glutathione reductase and NADPH [25]. Catalase (CAT) activity was assessed by spectrophotometry, using hydrogen peroxide as previously described [26]. Superoxide dismutase (SOD) activity was determined by spectrophotometry using epinephrine [27]. To measure thioredoxin reductase (TrxR) activity, total blood was hemolyzed with four volumes of cold Milli-Q water and centrifuged at 9,000 ×g for 15 min at 4°C. The supernatant was then diluted and used for an enzyme assay based on the reduction of DTNB to 5′-thionitrobenzoic acid (TNB) at 412 nm [28]. Glutathione reductase (GR) activity was determined using oxidized glutathione and NADPH. The method is based on the oxidation of NADPH, which is indicated by a decrease in absorbance at 340 nm [29].

2.6. Statistical Analysis. The results were expressed as the mean ± SEM. Data were analyzed for statistical significance by one-way ANOVA followed by Duncan's test using the Statistica 7.0 and differences were considered significant at $P < 0.05$.

3. Results

3.1. Body Weight, Blood Glucose, Fructosamine, and Insulin Levels. STZ-induced diabetes increased blood glucose levels 4-fold compared to the nondiabetic control rats ($P < 0.05$; onset levels in Figure 2(a)). Diabetic rats treated for 30 days with BIX (10 or 100 mg/kg) had a reduction in blood glucose levels compared to the diabetic vehicle group ($P < 0.05$; end levels in Figure 2(a)). This reduction was similar to that observed after treatment with metformin, but it was lower than that observed after treatment with insulin, which reduced blood glucose levels to a value similar to the nondiabetic control group ($P < 0.05$; end levels in Figure 2(a)). In contrast, the diabetic rats treated with NBIX had no change in blood glucose levels compared to the diabetic vehicle group (end levels in Figure 2(a)).

Fructosamine levels increased 2-fold in the diabetic animals compared to the nondiabetic control rats ($P < 0.05$; Figure 2(b)). The treatment with BIX (10 or 100 mg/kg) reduced fructosamine levels of diabetic rats and this effect was similar to that of metformin ($P < 0.05$; Figure 2(b)). Insulin was the most effective treatment to reduce fructosamine levels of diabetic rats ($P < 0.05$), whereas NBIX had no effect

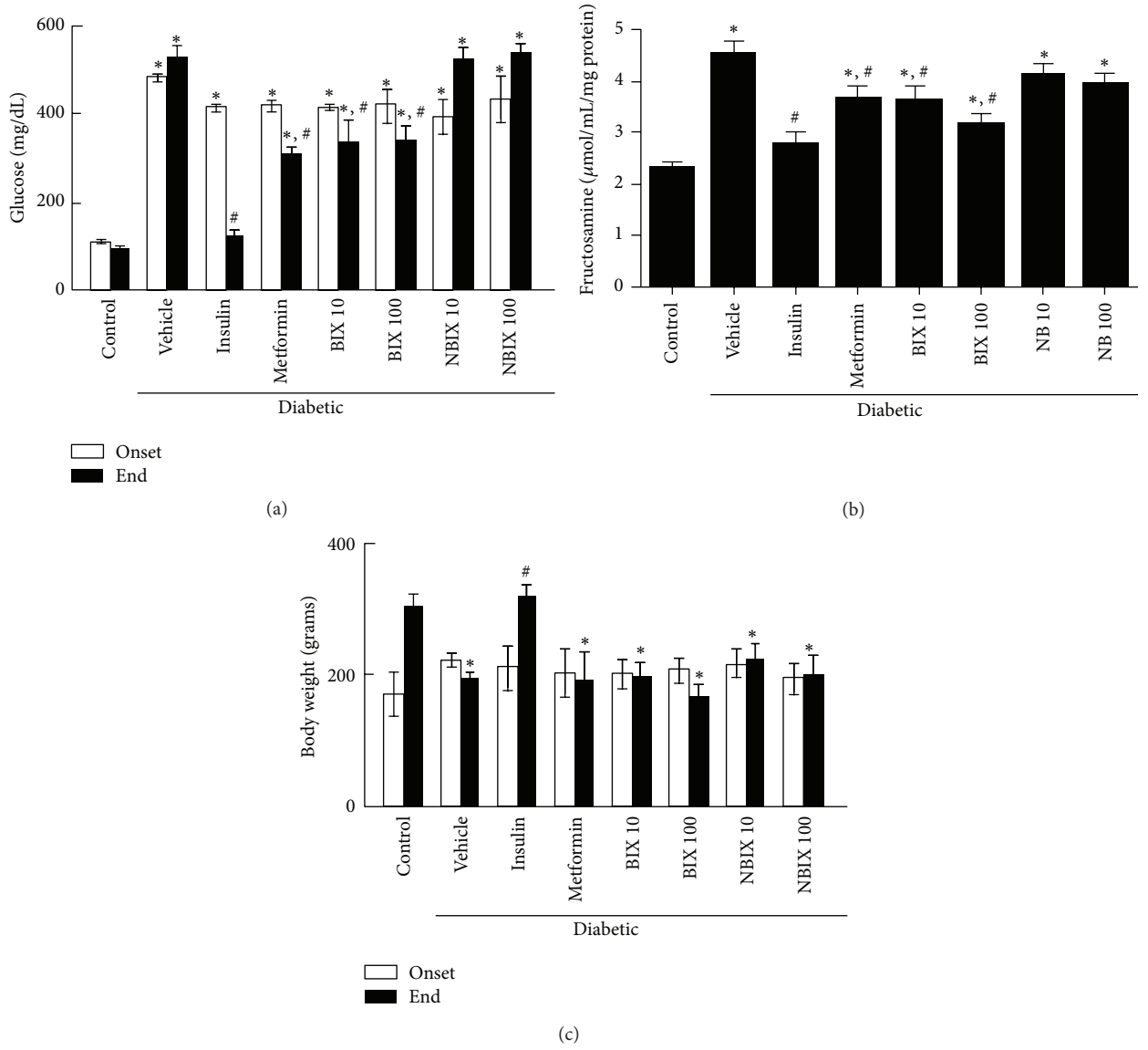

Figure 2: Blood glucose (a) and fructosamine levels (b) and body weight (c) of streptozotocin-induced diabetic rats. Blood glucose levels and body weight were assessed at the onset and end of treatment with vehicle, bixin, norbixin, insulin, or metformin. Fructosamine levels were assessed at the end of the treatment. Data are expressed as means ± SEM ($n = 6$ per group). ANOVA followed by Duncan's test: $^{*}P < 0.05$ versus the respective nondiabetic control; $^{\#}P < 0.05$ versus the respective diabetic vehicle. BIX 10: 10 mg/kg bixin; BIX 100: 100 mg/kg bixin; NBIX 10: 10 mg/kg norbixin; NBIX 100: 100 mg/kg norbixin.

(Figure 2(b)). Fasting blood insulin levels were not different among groups at the end of the treatment (data not show).

No significant difference was observed in the body weight among groups at the onset of the treatment (Figure 2(c)). However, after 30 days diabetic rats treated with vehicle, BIX, NBIX, or metformin had lower body weight than diabetic rats treated with insulin and nondiabetic control animals (Figure 2(c); $P < 0.05$). This occurred because only these two latter groups gained weight during the experimental period (53 and 79%, resp.), whereas the other groups had no body weight change.

3.2. Lipid Profile. Total and HDL cholesterol levels were similar between nondiabetic and diabetic rats (Figures 3(a) and 3(b)). However, treatment with metformin decreased the total cholesterol levels of diabetic rats, whereas 100 mg/kg NBIX increased these levels compared to the diabetic vehicle group ($P < 0.05$; Figure 3(a)). Treatment with insulin or 10 mg/kg NBIX decreased HDL cholesterol levels compared to the nondiabetic and diabetic vehicle rats, whereas treatment with 100 mg/kg BIX increased HDL levels compared to the diabetic vehicle rats (Figure 3(b); $P < 0.05$).

On the other hand, the LDL cholesterol and triglycerides levels were significantly increased in the diabetic vehicle group compared to nondiabetic control rats ($P < 0.05$; Figures 3(c) and 3(d)). Treatment with BIX (10 or 100 mg/kg) or metformin reduced LDL cholesterol levels of diabetic rats to values similar to those of the nondiabetic control rats

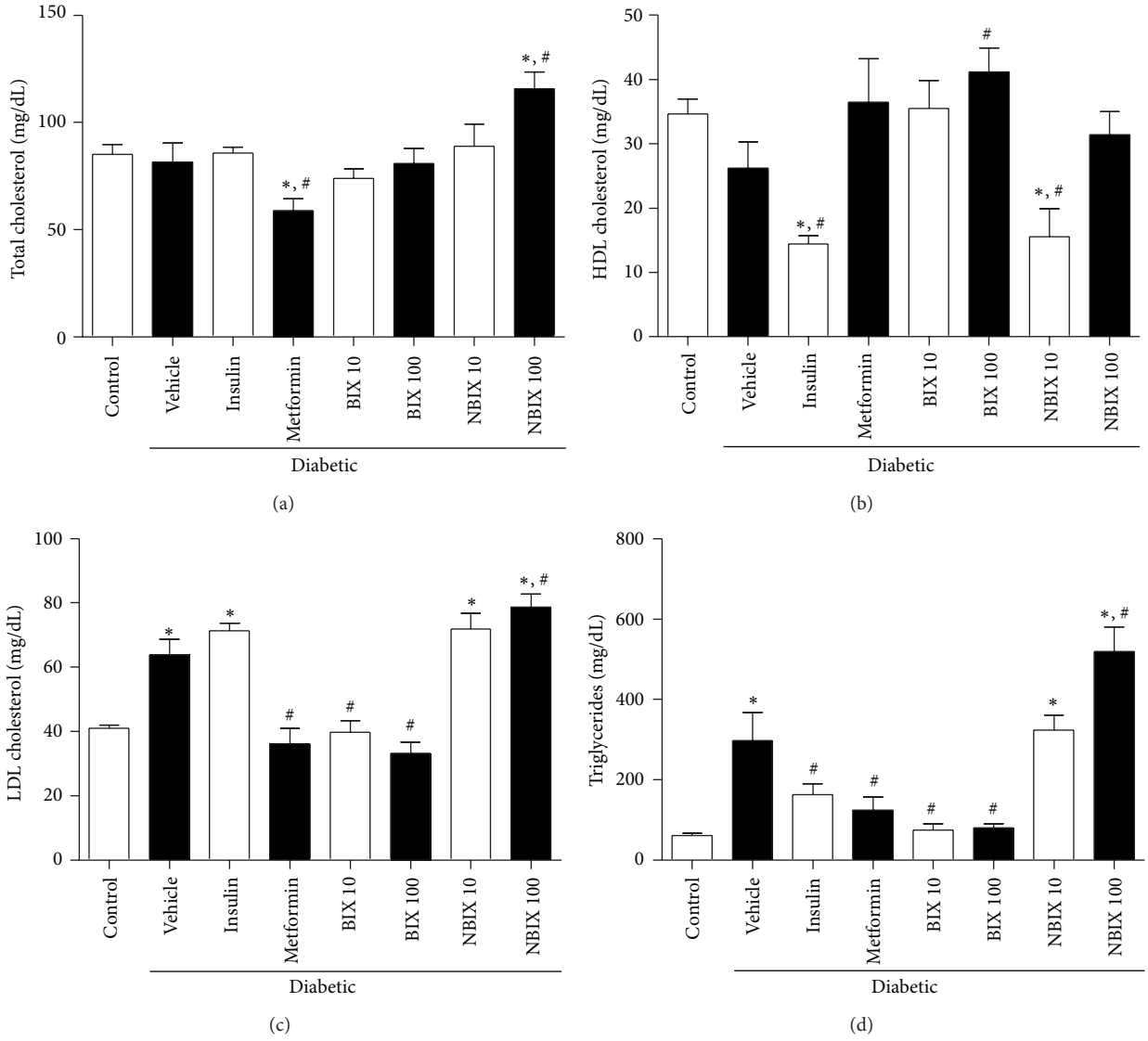

Figure 3: Lipid profile of streptozotocin-induced diabetic rats: total cholesterol (a), HDL cholesterol (b), LDL cholesterol (c), and triglycerides (d). Data are expressed as means ± SEM ($n = 6$ per group). ANOVA followed by Duncan's test: $^{*}P < 0.05$ versus nondiabetic control; $^{\#}P < 0.05$ versus diabetic vehicle. BIX 10: 10 mg/kg bixin; BIX 100: 100 mg/kg bixin; NBIX 10: 10 mg/kg norbixin; NBIX 100: 100 mg/kg norbixin.

(Figure 3(c); $P < 0.05$). However, insulin or 10 mg/kg NBIX did not change LDL levels compared to the diabetic vehicle group, whereas 100 mg/kg NBIX increased these levels ($P < 0.05$; Figure 3(c)). Treatment with BIX (10 or 100 mg/kg) reduced triglycerides levels ($P < 0.05$) to values similar to those of the nondiabetic control group and this effect was similar to that observed after insulin or metformin treatment (Figure 3(d)). NBIX at 10 mg/kg did not change triglycerides levels but at 100 mg/kg it increased these levels when compared to the diabetic vehicle rats (Figure 3(d); $P < 0.05$).

3.3. Biomarkers of Oxidative Stress. The oxidation of proteins, as assessed by AOPP, was increased in diabetic vehicle rats compared to the nondiabetic control group (Figure 4(a); $P < 0.05$). Treatment with BIX up to 100 mg/kg decreased the AOPP levels of diabetic rats, similar to that observed with metformin (Figure 4(a); $P < 0.05$). The diabetic rats treated with insulin also had no increase in AOPP levels compared to the nondiabetic control group, albeit its AOPP level was not different from the diabetic vehicle group ($P < 0.05$; Figure 4(a)). On the other hand, NBIX treatment (100 mg/kg) increased protein oxidation compared to the diabetic vehicle rats (Figure 4(a); $P < 0.05$).

The NOx serum levels were increased in the diabetic vehicle rats compared to the nondiabetic control ($P < 0.05$; Figure 4(b)). This increase was not observed after treatment with insulin, metformin, 100 mg/kg BIX, or 10 mg/kg NBIX (Figure 4(b)). In fact, treatments with 100 mg/kg BIX or 10 mg/kg NBIX were more effective than insulin or metformin to prevent this increase in NOx levels, because they caused a significant decrease of these levels compared to the diabetic vehicle group ($P < 0.05$; Figure 4(b)). However,

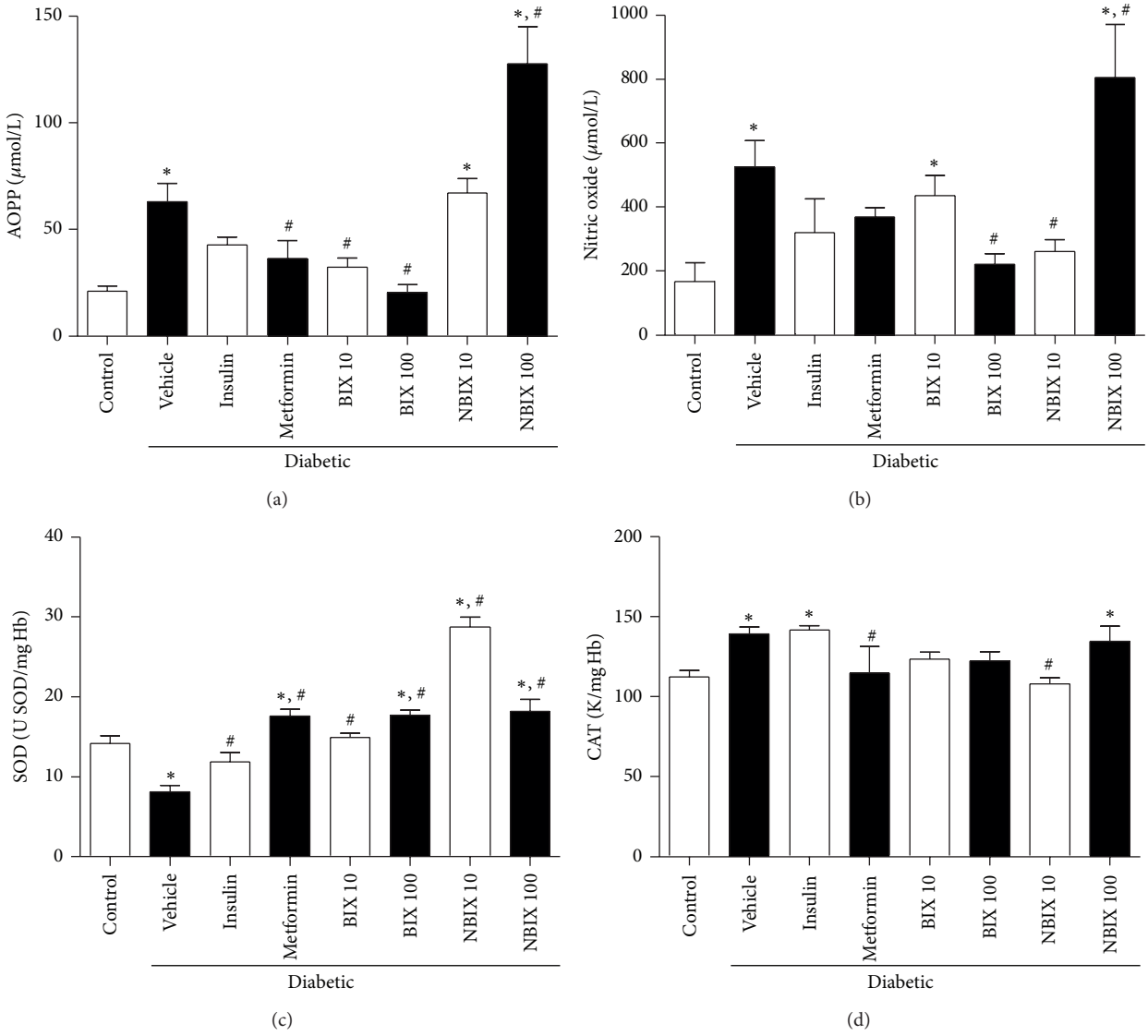

Figure 4: Oxidative stress parameters of streptozotocin-induced diabetic rats: AOPP levels (a), nitric oxide levels (b), superoxide dismutase (SOD) (c), and catalase activity (CAT) activity (d). Data are expressed as mean ± SEM ($n = 6$ per group). ANOVA followed by Duncan's test: $^{*}P < 0.05$ versus nondiabetic control; $^{\#}P < 0.05$ versus diabetic vehicle. BIX 10: 10 mg/kg bixin; BIX 100: 100 mg/kg bixin; NBIX 10: 10 mg/kg norbixin; NBIX 100: 100 mg/kg norbixin.

treatment with 100 mg/kg NBIX increased NOx levels of diabetic rats compared to the diabetic vehicle group ($P < 0.05$; Figure 4(b)).

Superoxide dismutase activity was decreased in the diabetic vehicle rats compared to the nondiabetic control group (Figure 4(c); $P < 0.05$), but this decrease was recovered in all diabetic treated rats ($P < 0.05$; Figure 4(c)). In fact, the treatment with metformin, 100 mg/kg BIX, or NBIX (10 or 100 mg/kg) not only recovered the decrease of superoxide dismutase activity caused by diabetes, but also increased superoxide dismutase activity compared to the nondiabetic control (up to 2-fold for 10 mg/kg NBIX) (Figure 4(c)). Catalase activity was increased in the diabetic vehicle rats compared to the nondiabetic control group (Figure 4(d); $P < 0.05$), but this change was not observed in the diabetic rats treated with BIX (10 or 100 mg/kg), NBIX (10 mg/kg), or metformin (Figure 4(d)), indicating a protective effect of these treatments.

The activity of glutathione peroxidase was not altered by diabetes (Table 1). However, the treatment with insulin or 100 mg/kg NBIX increased glutathione peroxidase activity compared to the nondiabetic control and to the diabetic vehicle group ($P < 0.05$; Table 1). The activities of glutathione reductase and thioredoxin reductase were not altered by diabetes, but diabetic rats treated with 100 mg/kg BIX had higher enzyme activities than the diabetic vehicle group or the nondiabetic control group ($P < 0.05$; Table 1). In contrast, diabetic rats treated with 10 mg/kg NBIX had a decrease

Table 1: Effect of bixin and norbixin treatment on oxidative stress parameters in the blood of streptozotocin-induced diabetic rats.

Groups	GPx (nmol NADPH/min/g Hb)	GR (nmol NADPH/min/mg Hb)	TrxR (nmol DTNB reduced/min/mg Hb)
Nondiabetic control	26.3 ± 5.9	20.6 ± 1.2	155.5 ± 8.2
Diabetic vehicle	25.0 ± 1.6	32.0 ± 0.7	138.0 ± 16.5
Diabetic + insulin	49.6 ± 9.2$^{*,\#}$	18.2 ± 2.9	172.7 ± 21.3
Diabetic + metformin	29.8 ± 8.3	23.9 ± 11.1	142.1 ± 11.4
Diabetic + bixin (10 mg/kg)	11.4 ± 3.7	19.2 ± 3.9	149.8 ± 6.5
Diabetic + bixin (100 mg/kg)	19.7 ± 6.5	61.8 ± 10.9$^{*,\#}$	264.3 ± 27.0$^{*,\#}$
Diabetic + norbixin (10 mg/kg)	26.0 ± 9.5	18.0 ± 1.2	96.3 ± 11.2$^{*,\#}$
Diabetic + norbixin (100 mg/kg)	62.2 ± 3.2$^{*,\#}$	17.2 ± 2.3	150.9 ± 12.7

Data are expressed as means ± SEM ($n = 6$ per group, except for the metformin and insulin groups that had 5 animals). ANOVA followed by Duncan's test: $^{*}P < 0.05$ versus nondiabetic control; $^{\#}P < 0.05$ versus diabetic vehicle.

in thioredoxin reductase activity compared to the diabetic vehicle group and to the nondiabetic control group ($P < 0.05$; Table 1).

4. Discussion

STZ-induced diabetes models are the most used animal models of diabetes because they reproduce in full the pathogenesis of this disease. STZ is accumulated via GLUT2 transporter in the pancreatic β cells, which are then massively destroyed [30]. Moreover, models of insulin-deficient diabetes result in increased GLUT4 phosphorylation, which may render GLUT4 less sensitive to acute regulation by insulin [31]. In our study this model was effectively reproduced and all diabetic groups showed 4-fold increase in blood glucose levels.

In this study, we found that treatment with BIX (10 or 100 mg/kg) for 30 days reduced blood glucose as well as fructosamine levels of diabetic rats. The antihyperglycemic effect of BIX seems to have occurred soon after the start of the treatment, because the decreased level of fructosamine indicates that protein glycation was reduced in the last three weeks [32]. This antihyperglycemic effect could be explained by the activation of PPAR-γ receptors by BIX, a mechanism that was demonstrated *in vitro* in adipocytes cultures [33]. In fact, agonists of this receptor, like the thiazolidinediones, are known to have anti-diabetic effects that are related to the stimulation of adipocyte differentiation leading to increased number of insulin sensitive small adipocytes [33, 34]. The antihyperglycemic effect of BIX was similar to that achieved by the oral anti-diabetic metformin, which is mainly used to treat noninsulin-dependent diabetes in humans. Metformin reduces plasma glucose levels by enhancing tissue sensitivity to insulin and it is not effective in the absence of insulin. Thus, we cannot rule out that the antihyperglycemic effect of BIX could be related to changes in GLUT4 expression or due to GLUT4 dephosphorylation and attenuation of hepatic PEPCK gene expression as previously demonstrated for metformin [35].

In contrast to BIX, the treatment with NBIX did not reduce fasting blood glucose levels. The difference between these structurally similar annatto carotenoids could be related to their different polarity. BIX has a lipophilic character, whereas NBIX has a hydrophilic character. Our results suggest that NBIX had no effect in the PPAR-α or γ receptors in this diabetes model. In fact, the cell culture studies on the activation of PPAR receptors by annatto carotenoids showed a lower potency for NBIX compared to BIX [17, 20].

In addition to the antihyperglycemic effect, BIX (10 and 100 mg/kg) completely prevented the increase in serum triglycerides and LDL cholesterol levels that occurred in diabetic rats. Moreover, the highest dose of BIX also increased the HDL levels of diabetic rats. This effect is similar to that recently demonstrated for an aqueous extract of annatto in a model of hypercholesterolemia in rats [36]. Interestingly, our results demonstrated that BIX was more effective than currently used anti-diabetic drugs to improve the lipid profile in diabetes. Metformin had no beneficial effect on HDL levels, whereas insulin did not prevent changes in LDL levels and it indeed decreased the HDL levels of diabetic rats. In contrast to BIX, the treatment with NBIX did not protect against changes in the lipid levels and at the highest dose it even increased total cholesterol, LDL, and triglyceride levels compared to the diabetic vehicle rats. The beneficial effect of BIX on the lipid profile may be related to the activation of PPAR-α receptor, which increases the expression of genes involved in fatty acid oxidation, fatty acid uptake, and energy consumption in the liver as recently demonstrated in obese KK-Ay mice [20]. This receptor is expressed primarily in tissues with high rates of fatty acid oxidation and peroxisomal metabolism [37] and is activated by several compounds of the isoprenoid family, such as BIX [38, 39]. The effect of NBIX on the PPAR-α receptor was only preliminarily investigated in cell culture assays, where NBIX had a lower potency than BIX to activate the PPAR-α in a GAL4/PPAR-α chimera system [20]. Thus, our results suggest that NBIX is unlikely to activate the PPAR-α receptor in the STZ model of diabetes in rats, which could be related to its lower hydrophobicity compared to BIX [40].

The exposure to high glucose levels may increase the generation of ROS through the nonenzymatic glycation of proteins and glucose auto-oxidation, which leads to oxidative stress and structural and functional tissue damage [41, 42].

The immunological effector molecules in this process are cytokines, namely, interleukin-1 [43], which activates the expression of inducible nitric oxide synthase. High local NOx concentrations induce oxidative stress followed by the destruction of pancreatic β cells and subsequent development of insulin-dependent diabetes mellitus. Moreover, several studies have shown that a single dose of STZ increases the levels of NOx, being responsible for the damage to β cells that leads to diabetes in experimental models [44]. In our study, the diabetic animals had increased NOx levels compared to the nondiabetic control group and this increase was prevented by treatment with the highest dose of BIX and the lowest dose of NBIX. In contrast, treatment with the highest dose of NBIX increased NOx levels to values even higher than the diabetic vehicle group.

AOPP is a suitable biochemical marker for measuring short-term changes in oxidative stress because it assesses the products of plasma protein oxidation, especially albumin oxidation. This marker is increased in inflammatory conditions such as diabetes, atherosclerosis, and renal failure [45]. In the present study, BIX (10 and 100 mg/kg) prevented the increase in AOPP levels caused by the diabetes and this protective effect was similar to that of the antihyperglycemic drugs metformin and insulin. In contrast, NBIX had no protective effect and at the highest dose it further increased AOPP levels of diabetic rats, indicating a prooxidant effect of this carotenoid.

Antioxidant enzymes are the first line of defense against reactive species and oxidative stress [46]. In the present study, diabetic rats had slightly increased catalase activity, which could be interpreted as a defense response against oxidative stress. Despite this response, diabetic rats had increased protein oxidation (AOPP levels). Thus, protein oxidation in diabetic rats could be related to the overproduction of NOx associated with the lower superoxide dismutase activity. Excessive NOx concentrations may react with superoxide anion radical ($O_2^{\bullet-}$) to form peroxynitrite anion ($ONOO^-$), which is a highly reactive oxidizing agent [47]. Treatment with BIX increased superoxide dismutase activity, which probably reduced superoxide anion levels. In addition, it also reduced NOx levels and protein oxidation, which is in agreement with the hypothesis that protein oxidation in diabetic rats was mainly mediated by peroxynitrite. Moreover, the increase in glutathione reductase and thioredoxin reductase activities caused by the treatment with 100 mg/kg BIX could also contribute to its protective effect against oxidative stress in this model of diabetes. NBIX 10 mg/kg decreased NOx levels and increased superoxide dismutase activity of diabetic rats, but it failed to protect against protein oxidation. In fact, NBIX 10 mg/kg increased SOD activity to levels much higher than control. This increased activity of superoxide dismutase along with the reduction of catalase activity leads to the accumulation of H_2O_2, because it is actively produced by superoxide dismutase but not efficiently removed by catalase. This imbalance in the removal of oxidative species was previously reported in various oxidative conditions including alloxan-induced diabetes [48] and is likely contributing to the oxidation of proteins in our study. The treatment with 100 mg/kg NBIX had a marked prooxidant effect by increasing 2-fold the protein oxidation of diabetic rats, which was probably related to the greater increase in NOx levels caused by this treatment.

Thus, BIX also showed antioxidant activity in the STZ-induced model of diabetes, in addition to the antihyperglycemic and antilipidemic effects. This finding is in agreement with the effectiveness of BIX and BIX-enriched annatto extracts to scavenge for both nitrogen and oxygen reactive species *in vitro*, which have been attributed to the electron transfer due to the presence of many conjugated double bonds [49]. Although they had better scavenging capacity for nonradical species (H_2O_2, HOCl, $^1O_2$, and $ONOO^-$) than for radical species ($O_2^{\bullet-}$ and $^{\bullet}NO$), we showed that BIX did decrease NOx levels in STZ-induced diabetes. In addition to the direct scavenging of reactive species, BIX may also enhance the antioxidant defenses, as demonstrated in the present study for superoxide dismutase, glutathione reductase, and thioredoxin reductase activities. The increased activity of these enzymes may be a result of an increased gene expression, since an annatto extract enriched in BIX was recently shown to prevent the decrease in the activity and expression of superoxide dismutase in neutrophils from alloxan-induced diabetic rats [50].

5. Conclusion

BIX, at doses that show no toxic effects [51], was as effective as metformin to decrease blood glucose levels and more effective than metformin and insulin to improve the dyslipidemia in STZ-induced diabetes. Moreover, BIX also prevented the oxidative stress in diabetic rats. Thus, BIX seems to be a promising drug for the diabetes therapy, which may be important considering the known side effects of drugs currently used in human therapy of diabetes [52]. In contrast, the treatment with NBIX, the hydrosoluble analog of BIX, was ineffective to protect against the hyperglycemia and dyslipidemia in STZ-induced diabetes. Furthermore, the highest dose of NBIX increased dyslipidemia and oxidative stress in the STZ-induced diabetes model. Thus, our results suggest that lipophilicity is crucial for the protective effect of annatto carotenoids against STZ-induced diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] International Diabetes Federation, 2012, http://www.idf.org/.

[2] S.-C. Shen, F.-C. Cheng, and N.-J. Wu, "Effect of guava (psidium guajava linn.) leaf soluble solids on glucose metabolism in type 2 diabetic rats," *Phytotherapy Research*, vol. 22, no. 11, pp. 1458–1464, 2008.

[3] M. M. Kesavulu, R. Girl, B. Kameswara Rao, and C. H. Apparao, "Lipid peroxidation and antioxidant enzyme levels in type 2 diabetics with microvascular complications," *Diabetes and Metabolism*, vol. 26, no. 5, pp. 387–392, 2000.

[4] N. Ahmed, "Alloxan diabetes-induced oxidative stress and impairment of oxidative defense system in rat brain: neuroprotective effects of cichorium intybus," *International Journal of Diabetes and Metabolism*, vol. 17, no. 3, pp. 105–109, 2009.

[5] A. Ceriello, "Postprandial hyperglycemia and diabetes complications: Is it time to treat?" *Diabetes*, vol. 54, no. 1, pp. 1–7, 2005.

[6] A. J. Jenkins, T. J. Lyons, D. Zheng et al., "Serum lipoproteins in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Intervention and Complications cohort: associations with gender and glycemia," *Diabetes Care*, vol. 26, no. 3, pp. 810–818, 2003.

[7] F. K. Ahmad, Z. He, and G. L. King, "Molecular targets of diabetic cardiovascular complications," *Current Drug Targets*, vol. 6, no. 4, pp. 487–494, 2005.

[8] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[9] G. Basta, A. M. Schmidt, and R. De Caterina, "Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes," *Cardiovascular Research*, vol. 63, no. 4, pp. 582–592, 2004.

[10] K. B. Pandey and S. I. Rizvi, "Plant polyphenols as dietary antioxidants in human health and disease," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 5, pp. 270–278, 2009.

[11] R. Schmatz, L. B. Perreira, N. Stefanello et al., "Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats," *Biochimie*, vol. 94, no. 2, pp. 374–383, 2012.

[12] R. Ashraf, R. A. Khan, and I. Ashraf, "Garlic (Allium sativum) supplementation with standard antidiabetic agent provides better diabetic control in type 2 diabetes patients," *Pakistan Journal of Pharmaceutical Sciences*, vol. 24, no. 4, pp. 565–570, 2011.

[13] H. D. Preston and M. D. Rickard, "Extraction and chemistry of annatto," *Food Chemistry*, vol. 5, no. 1, pp. 47–56, 1980.

[14] A. Z. Mercadante, A. Steck, and H. Pfander, "Three minor carotenoids from annatto (Bixa orellana) seeds," *Phytochemistry*, vol. 52, no. 1, pp. 135–139, 1999.

[15] R. C. Chisté, A. Z. Mercadante, A. Gomes, E. Fernandes, J. L. F. D. C. Lima, and N. Bragagnolo, "In vitro scavenging capacity of annatto seed extracts against reactive oxygen and nitrogen species," *Food Chemistry*, vol. 127, no. 2, pp. 419–426, 2011.

[16] K. R. M. Russell, E. Y. S. A. Morrison, and D. Ragoobirsingh, "The effect of annatto on insulin binding properties in the dog," *Phytotherapy Research*, vol. 19, no. 5, pp. 433–436, 2005.

[17] K. R. M. Russel, R. M. Karen, E. Y. Morrison, and M. E. West, "The hypoglycaemic effect of annatto (Bixa orellana)," *West Indian Medical Journal*, vol. 40, no. 4, pp. 24–27, 1991.

[18] A. C. S. Fernandes, C. A. Almeida, F. Albano et al., "Norbixin ingestion did not induce any detectable DNA breakage in liver and kidney but caused a considerable impairment in plasma glucose levels of rats and mice," *Journal of Nutritional Biochemistry*, vol. 13, no. 7, pp. 411–420, 2002.

[19] T. Goto, N. Takahashi, S. Hirai, and T. Kawada, "Bixin activates PPARα and improves obesity-induced abnormalities of carbohydrate and lipid metabolism in mice," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 48, pp. 11952–11958, 2012.

[20] N. Takahashi, T. Goto, A. Taimatsu et al., "Bixin regulates mRNA expression involved in adipogenesis and enhances insulin sensitivity in 3T3-L1 adipocytes through PPARγ activation," *Biochemical and Biophysical Research Communications*, vol. 390, no. 4, pp. 1372–1376, 2009.

[21] T. Szkudelski, K. Kandulska, and M. Okulicz, "Alloxan in vivo does not only exert deleterious effects on pancreatic B cells," *Physiological Research*, vol. 47, no. 5, pp. 343–346, 1998.

[22] W. T. Friedewald, R. I. Levy, and D. S. Fredrickson, "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clinical Chemistry*, vol. 18, no. 6, pp. 499–502, 1972.

[23] E. Tatsch, G. V. Bochi, R. D. S. Pereira et al., "A simple and inexpensive automated technique for measurement of serum nitrite/nitrate," *Clinical Biochemistry*, vol. 44, no. 4, pp. 348–350, 2011.

[24] V. Witko-Sarsat, M. Friedlander, C. Capeillère-Blandin et al., "Advanced oxidation protein products as a novel marker of oxidative stress in uremia," *Kidney International*, vol. 49, no. 5, pp. 1304–1313, 1996.

[25] D. E. Paglia and W. N. Valentine, "Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase," *The Journal of Laboratory and Clinical Medicine*, vol. 70, no. 1, pp. 158–169, 1967.

[26] H. Aebi, "Catalase in vitro," *Methods in Enzymology*, vol. 105, pp. 121–126, 1984.

[27] H. P. Misra and I. Fridovich, "The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase," *The Journal of Biological Chemistry*, vol. 247, no. 10, pp. 3170–3175, 1972.

[28] A. Holmgren and M. Bjornstedt, "Thioredoxin and thioredoxin reductase," *Methods in Enzymology*, vol. 252, pp. 199–208, 1995.

[29] I. Carlberg and B. Mannervik, "Inhibition of glutathione reductase by interaction of 2,4,6-trinitrobenzenesulfonate with the active-site dithiol," *FEBS Letters*, vol. 98, no. 2, pp. 263–266, 1979.

[30] S. Lenzen, "The mechanisms of alloxan- and streptozotocin-induced diabetes," *Diabetologia*, vol. 51, no. 2, pp. 216–226, 2008.

[31] N. Begum and B. Draznin, "Effect of streptozotocin-induced diabetes on GLUT-4 phosphorylation in rat adipocytes," *The Journal of Clinical Investigation*, vol. 90, no. 4, pp. 1254–1262, 1992.

[32] E. Ohkawara, Y. Nohara, Y. Kanno et al., "Fructosamine assay using albumin extracted from serum," *Biological and Pharmaceutical Bulletin*, vol. 25, no. 9, pp. 1121–1124, 2002.

[33] N. Takahashi, T. Goto, A. Taimatsu et al., "Bixin regulates mRNA expression involved in adipogenesis and enhances insulin sensitivity in 3T3-L1 adipocytes through PPARγ activation," *Biochemical and Biophysical Research Communications*, vol. 390, no. 4, pp. 1372–1376, 2009.

[34] S. Rocchi, F. Picard, J. Vamecq et al., "A unique PPARγ ligand with potent insulin-sensitizing yet weak adipogenic activity," *Molecular Cell*, vol. 8, no. 4, pp. 737–747, 2001.

[35] J.-T. Cheng, C.-C. Huang, I.-M. Liu, T.-F. Tzeng, and J. C. Chih, "Novel mechanism for plasma glucose-lowering action of metformin in streptozotocin-induced diabetic rats," *Diabetes*, vol. 55, no. 3, pp. 819–825, 2006.

[36] H. de Paula, M. L. Pedrosa, J. V. R. Júnior, F. K. Haraguchi, R. C. dos Santos, and M. E. Silva, "Effect of an aqueous extract of annatto (Bixa orellana) seeds on lipid profile and biochemical markers of renal and hepatic function in hipercholesterolemic rats," *Brazilian Archives of Biology and Technology*, vol. 52, no. 6, pp. 1373–1378, 2009.

[37] P. Lefebvre, G. Chinetti, J.-C. Fruchart, and B. Staels, "Sorting out the roles of PPARα in energy metabolism and vascular homeostasis," *The Journal of Clinical Investigation*, vol. 116, no. 3, pp. 571–580, 2006.

[38] N. Takahashi, M.-S. Kang, K. Kuroyanagi et al., "Auraptene, a citrus fruit compound, regulates gene expression as a PPARα agonist in HepG2 hepatocytes," *BioFactors*, vol. 33, no. 1, pp. 25–32, 2008.

[39] T. Kawada, T. Goto, N. Takahashi, and S. Hirai, "Various Terpenoids derived from herbal and dietary plants function as PPAR modulators and regulate carbohydrate and lipid metabolism," *PPAR Research*, vol. 48, pp. 39–58, 2010.

[40] R. M. Evans, G. D. Barish, and Y.-X. Wang, "PPARs and the complex journey to obesity," *Nature Medicine*, vol. 10, no. 4, pp. 355–361, 2004.

[41] A. C. Maritim, R. A. Sanders, and J. B. Watkins III, "Diabetes, oxidative stress, and antioxidants: a review," *Journal of Biochemical and Molecular Toxicology*, vol. 17, no. 1, pp. 24–38, 2003.

[42] F. Giacco and M. Brownlee, "Oxidative stress and diabetic complications," *Circulation Research*, vol. 107, no. 9, pp. 1058–1070, 2010.

[43] T. Mandrup-Poulsen, K. Bendtzen, H. Nielsen, G. Bendixen, and J. Nerup, "Cytokines cause functional and structural damage to isolated islets of Langerhans," *Allergy*, vol. 40, no. 6, pp. 424–429, 1985.

[44] J. A. Corbett and M. L. McDaniel, "Does nitric oxide mediate autoimmune destruction of β-cells? Possible therapeutic interventions in IDDM," *Diabetes*, vol. 41, no. 8, pp. 897–903, 1992.

[45] R. Gelisgen, H. Genc, R. Kayali et al., "Protein oxidation markers in women with and without gestational diabetes mellitus: a possible relation with paraoxonase activity," *Diabetes Research and Clinical Practice*, vol. 94, no. 3, pp. 404–409, 2011.

[46] B. Halliwell and J. M. C. Gutterdige, *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, UK, 1999.

[47] A. Ceriello, "New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy," *Diabetes Care*, vol. 26, no. 5, pp. 1589–1596, 2003.

[48] R. A. Pinho, M. E. Andrades, M. R. Oliveira et al., "Imbalance in SOD/CAT activities in rat skeletal muscles submitted to treadmill training exercise," *Cell Biology International*, vol. 30, no. 10, pp. 848–853, 2006.

[49] J. Ren and S. H. Chung, "Anti-inflammatory effect of α-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-κB and mitogen-activated protein kinase pathways," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 13, pp. 5073–5080, 2007.

[50] J. V. R. Jnior, G. R. Arajo, B. D. C. Pádua et al., "Annatto extract and β-carotene enhances antioxidant status and regulate gene expression in neutrophils of diabetic rats," *Free Radical Research*, vol. 46, no. 3, pp. 329–338, 2012.

[51] A. R. P. L. Bautista, E. L. T. Moreira, M. S. Batista, M. S. Miranda, and I. C. S. Gomes, "Subacute toxicity assessment of annatto in rat," *Food and Chemical Toxicology*, vol. 42, no. 4, pp. 625–629, 2004.

[52] C. J. Bailey, M. R. C. Path, and R. C. Turner, "Metformin," *The New England Journal of Medicine*, vol. 334, no. 9, pp. 574–579, 1996.

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Research Article

Effect of Gelam Honey on the Oxidative Stress-Induced Signaling Pathways in Pancreatic Hamster Cells

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Background. Oxidative stress induced by reactive oxygen and nitrogen species is critically involved in the impairment of β-cell function during the development of diabetes. *Methods*. HIT-T15 cells were cultured in 5% CO_2 and then preincubated with Gelam honey extracts (20, 40, 60, and 80 μg/mL) as well as quercetin (20, 40, 60, and 80 μM), prior to stimulation by 20 and 50 mM of glucose. Cell lysate was collected to determine the effect of honey extracts and quercetin on the stress activated NF-κB, MAPK pathways, and the Akt (ser473) activated insulin signaling pathway. *Results*. HIT-T15 cells cultured under hyperglycemic conditions demonstrated insulin resistance with a significant increase in the levels of MAPK, NF-κB, and IRS-1 serine phosphorylation (ser307); however, Akt expression and insulin contents are significantly decreased. Pretreatment with quercetin and Gelam honey extract improved insulin resistance and insulin content by reducing the expression of MAPK, NF-κB, and IRS-1 serine phosphorylation (ser307) and increasing the expression of Akt significantly. *Conclusion*. Gelam honey-induced differential expression of MAPK, NF-κB, IRS-1 (ser307), and Akt in HIT-T15 cells shows that Gelam honey exerts protective effects against diabetes- and hyperglycemia-induced oxidative stress by improving insulin content and insulin resistance.

1. Introduction

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces [1]. Loss of β-cell function caused by reduced insulin synthesis and secretion is one of the key events in the pathogenesis of type 2 diabetes. Normal β cells can compensate for insulin resistance by increasing insulin secretion, but insufficient compensation leads to the onset of glucose intolerance [2–4].

Chronic hyperglycemia is a cause of impaired insulin biosynthesis and secretion [5], the progression of which causes insulin resistance and is often accompanied by β-cell degranulation and apoptosis [6]. This process is called "glucose toxicity" and has been demonstrated in various studies in vivo [7] and in vitro [8–10]. Oxidative stress induced by reactive oxygen species (ROS) and nitrogen species produced by several biochemical pathways associated with hyperglycemia (glucose autooxidation, polyol pathway, prostanoid synthesis, and protein glycation) is critically involved in the impairment of β-cell function during the development of type 2 diabetes [11]. It has been reported that extracellular hyperglycemia causes intracellular hyperglycemia in β-cells leading to the induction of ROS [5] which becomes exacerbated, through lack of antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide mutase in the pancreatic islet of diabetic animals [12].

ROS can function as signaling molecules to activate a number of stress sensitive pathways that are linked to insulin resistance, decreased insulin secretion, and content [13], ultimately leading to late complications of diabetes [14]. This transient exposure of β-cells to oxidative stress interrupts the normal coupling of glucose metabolism to insulin secretion by activating stress signaling pathways [15]. The most

extensively studied is the intracellular pathway leading to the activation of the nuclear factor-κB (NF-κB) [16], which plays a critical role in mediating immune and inflammatory responses. Following activation, NF-κB translocates to the nucleus, resulting in the subsequent transcription of genes involved in the production of inflammatory cytokines that promotes the development of insulin resistance [16]. A recent study on bovine endothelial cells found that exposure to hyperglycemia initially increased the production of intracellular ROS, followed by activation of NF-κB [17].

In diabetes, oxidative stress also activates the p38 mitogen activated protein kinase (MAPK) pathway which leads to activation of the serine kinases which promote the degradation of insulin receptor substrate (IRS), thus reducing the insulin signaling activity which is responsible for the development of insulin resistance [18]. A streptozotocin-induced diabetic rat model showed an increase in MAPK activity as compared to controls, which was mediated by the production of ROS [18]. Insulin receptors (IR) are cell surface receptors with α and β subunits, possessing intrinsic tyrosine kinase activity. Insulin binds to the IR and induces tyrosine autophosphorylation of the IR β subunit. The activated IR subsequently phosphorylate their substrates including insulin receptor substrate (IRS-1). Tyrosine phosphorylated IRS-1 recruits a number of SH2 containing signal transducers, which activate several signaling pathways. Serine phosphorylated IRS-1 (ser307) inhibits insulin signal transduction in a variety of cells by steric hindrance of the interaction between IR and IRS-1. In the presence of insulin activated serine kinases, phosphorylation of IRS-1 occurs at the serine307 site, which decreases the IRS-1 tyrosine phosphorylation, thereby decreasing the activation of insulin signaling pathway which may result in insulin resistance [19].

Recently, protein kinase B (PKB or Akt) has been shown to function in the insulin-signaling cascade by phosphorylating transcription factors which are responsible for the transcription and expression of genes related to insulin synthesis and secretion [20, 21]. Therefore Akt is necessary for normal pancreatic β cell function and insulin secretion. Previous studies reported that inactivation of Akt can lead to insulin resistance, decreased β cell mass, and impaired insulin secretion [22, 23]. Our previous study also demonstrated that the following flavonoids quercetin, chrysin, and luteolin present in the Gelam honey also had antioxidant effect [24]. The protective effect of quercetin was significantly higher than that of the other flavonoids which is consistent with the data reported by Lukačínová et al. [25].

The aim of our present study is to determine the effect of Gelam honey extract and quercetin on the stress activated NF-κB and MAPK pathways and IRS-1 serine phosphorylation causing insulin resistance and the Akt activated insulin signaling pathway, causing increase in insulin content.

2. Research Design and Methods

2.1. Extraction of Phenolic Compounds from Honey by Solid Phase Extraction (SPE). Gelam honey samples (Department of Agriculture, Parit Botak, Johor, Malaysia) were subjected to base hydrolysis and extracted with ethyl acetate as described by Wahdan [26] and Seo and Morr [27]. The recovered fractions were combined and dried under nitrogen gas.

2.2. Determination of the Phenolic Content. Phenolic compounds from the extract were assayed using Folin-Ciocalteau assay [28]. Briefly the extract (1 mL) was added to 10% Folin-Ciocalteu reagent (Sigma F9252) and 0.5% sodium carbonate. The contents were thoroughly mixed and allowed to stand for 2 hours. The absorbance of the blue colour that developed after 2 hours was read at 765 nm. Results were expressed in micrograms of gallic acid per gram of the extract, using a standard curve generated with gallic acid (Sigma G7384).

2.3. Determination of the Flavonoid Content. The total flavonoid (TF) content was determined spectrophotometrically [29]. Briefly 1 mL of honey extract or a standard solution of quercetin (Sigma Q4951) (10, 50, 100, 150, 200, 250 μg/mL) in distilled water was added to a 10 mL volumetric flask containing 4 mL of double distilled water, 300 μL of $NaNo_2$ (5%, v/v), and 300 μL of 10% $AlCl_3$. The solution was allowed to stand at room temperature in the dark for 30 minutes and the absorbance was read at 430 nm. The TF content was determined using the standard curve of quercetin (μg/mL). TF content was expressed as μg of quercetin equivalents in 1 g of extract.

2.4. Cell Culture. HIT-T15 cells were cultured according to the instructions provided by ATCC (CRL-1777). The cells were cultured immediately in T-25 cm flask in the F12 K medium (ATCC 30-2004) supplemented with 10% FBS and 1% penicillin and streptomycin at 37°C (5% CO_2 in air). Cells (3rd passage) were trypsinized and subcultured after 5 days incubation, with 80% confluency.

2.5. Treatment of HIT-T15 Cells with Quercetin and Gelam Honey Extract. HIT-T15 cells (5×10^5) were pretreated with Gelam honey extract (20, 40, 60, 80 μg/mL) and quercetin (20, 40, 60, 80 μM) for 24 hours; medium was then replaced with fresh medium. Glucose (Sigma G8769) (20 or 50 mM) was added and the cells were incubated for another 24 hours. To investigate inhibitory effects on Akt signaling pathway, cells were incubated with 5 μM Akt inhibitor VIII (Santa Cruz Biotechnology sc-203173) for one hour before pretreatment with quercetin and honey extract. Glucose (20 or 50 mM) was added and followed by incubation for another 24 hours.

2.6. Cell Lysate Preparation. After treatment the cells were washed twice in PBS and lysed in mammalian cell lysis buffer (Sigma MCL-1) supplemented with protease and phosphatase inhibitors. Insoluble materials were eliminated by centrifugation (12,000 ×g, 10 min, 4°C), and protein concentration in the supernatant was determined by Bradford assay (Bio-Rad Laboratories).

2.7. Measurement of Insulin Content. Insulin content was determined by pretreating 5×10^5 HIT-T15 cells with Gelam honey extract (20, 40, 60, 80 μg/mL) and quercetin (20, 40, 60, 80 μM) for 24 hours following which the medium

was replaced with fresh medium. Then glucose of either 20 mM or 50 mM was added and the cells were incubated for another 24 hours. At the end of this incubation, the cells were centrifuged and the supernatant was removed. The cell pellet was resuspended in PBS and sonicated. The cell lysate was used to estimate the quantity of insulin by ELISA (Crystal Chem Inc, USA). Insulin content was normalized to the total protein concentration [29].

2.8. Western Blot Analysis. Thirty microgram of protein extracts were loaded on 10% SDS-polyacrylamide gel and transferred to activated nitrocellulose membrane. The membrane was blocked with tris-buffered saline (TBS) containing 5% nonfat milk, and incubated with phosphor-Akt (Ser473), phosphor-IRS-1 (Ser307), phospho-p65 NFKB (Ser536), and phospho-p38 MAPK (Thr180/Tyr182) primary antibodies (obtained from Santa Cruz Laboratories) overnight at 4°C. β-actin was used as a loading control. After extensive washes in TBS, membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies and were visualized using chemiluminescence substrate according to the manufacturer's instructions (Amersham Life Sciences, Little Chalfont, UK). Quantitative analysis of the protein was performed by Gel Documentation System (Biospectrum 410, UVP) [30, 31].

2.9. Statistical Analyses. Data were analyzed with one-way ANOVA using SPSS version 16.0 software. The results were expressed as the mean ± standard deviation. $^{*}P$ value < 0.05 was considered to be statistically significant.

3. Results

3.1. Total Phenolic and Flavonoid Content. 10 g of liquefied fresh Malaysian Gelam honey (*Apis mellifera*) was extracted using ethyl acetate and the extract was found to contain 52 μg of gallic acid per gram of extract of total phenolic content and 6.92 μg of quercetin per gram of extract of total flavonoid content.

3.2. Effect of Pretreatment with Quercetin and Gelam Honey Extract on Phospho-p38 MAPK Expression under Normal and Hyperglycemic Conditions. HIT-T15 cells were pretreated with the quercetin at concentrations of 20, 40, 60, 80 μM and Gelam honey extract at concentrations of 20, 40, 60, 80 μg/mL for 24 hours, following which they were cultured with 20 mM (Figures 1(a), 1(c), 1(e)) or 50 mM (Figures 1(b), 1(d), 1(f)) glucose to determine the phosphorylation of MAPK. The data revealed that exposure of HIT-T15 cells to 20 and 50 mM glucose significantly increased the level of phospho-p38 MAPK expression compared to control. Pretreatment with quercetin and Gelam honey extract significantly ($P < 0.05$) reduced the ROS-induced expression of phospho-p38 under 20 mM glucose (Figures 1(a), 1(c), 1(e)) by 56% and 40% in a dose-dependent manner. While pretreatment with quercetin and Gelam honey extract reduced the expression of phospho-p38 significantly ($P < 0.05$) by 69% and 44%, respectively, compared to the cells that were cultured with 50 mM glucose (Figures 1(b), 1(d), 1(f)) alone. The p38 MAPK protein levels from each sample were normalized to their respective β-actin protein amounts ($^{*}P < 0.05$; $^{\#}P < 0.005$ versus glucose-treated group).

3.3. Effect of Pretreatment with Quercetin and Gelam Honey Extract on Phospho-p65 NF-κB Expression under Normal and Hyperglycemic Conditions. HIT-T15 cells were pretreated with the quercetin at concentrations of 20, 40, 60, 80 μM and Gelam honey extract at concentrations of 20, 40, 60, 80 μg/mL for 24 hours, following which they were cultured with 20 mM (Figures 2(a), 2(c), 2(e)) or 50 mM (Figures 2(b), 2(d), 2(f)) glucose to determine the phosphorylation of NF-κB. The phosphorylation of NF-κB was increased in HIT-T15 cells treated with 20 mM and 50 mM glucose alone as compared with control. Pretreatment with the quercetin and Gelam honey extract showed a 50% and 56% decrease ($P < 0.05$) in the expression of phosphorylated NF-κB in a dose dependent manner in the cells that were cultured in 20 mM (Figures 2(a), 2(c), 2(e)) glucose. Pretreatment with quercetin and Gelam honey extract significantly ($P < 0.05$) decrease the expression of phosphorylated NF-κB under 50 mM glucose (Figures 2(b), 2(d), 2(f)) by 36% and 61% in a dose dependent manner. The phosphorylated NF-κB protein levels from each sample were normalized to their respective β-actin protein amounts ($^{*}P < 0.05$; $^{\#}P < 0.005$ versus glucose-treated group).

3.4. Effect of Pretreatment with Quercetin and Gelam Honey Extract on pIRS-1 (ser307) Expression under Normal and Hyperglycemic Conditions. HIT-T15 cells were pretreated with the quercetin at concentrations of 20, 40, 60, 80 μM and Gelam honey extract at concentrations of 20, 40, 60, 80 μg/mL for 24 hours, following which they were then cultured with 20 mM (Figures 3(a), 3(c), 3(e)) or 50 mM (Figures 3(b), 3(d), 3(f)) glucose to determine the phosphorylation of IRS-1 (ser307). The phosphorylation of IRS-1 (ser307) was increased in HIT-T15 cells treated with 20 mM and 50 mM glucose alone as compared with control. Pretreatment with the quercetin and Gelam honey extract showed a 46% and 52% decrease ($P < 0.05$) in the expression of pIRS-1 (ser307) in a dose-dependent manner in the cells that were cultured in 20 mM glucose (Figures 3(a), 3(c), 3(e)). Pretreatment with quercetin and Gelam honey extract significantly ($P < 0.05$) decreased the expression of pIRS-1 (ser307) under 50 mM glucose (Figure 3(b), 3(d), 3(f)) by 40% and 50% in a dose dependent manner. The pIRS-1 protein levels from each sample were normalized to their respective β-actin protein amounts ($^{*}P < 0.05$; $^{\#}P < 0.005$ versus glucose-treated group).

3.5. Effect of Pretreatment with Quercetin and Gelam Honey Extract on pAkt (ser473) Expression under Normal and Hyperglycemic Conditions. HIT-T15 cells were pretreated with the quercetin at concentrations of 20, 40, 60, 80 μM and Gelam honey extract at concentrations of 20, 40, 60, 80 μg/mL for 24 hours, following which they were then cultured with 20 or 50 mM glucose to determine the phosphorylation of Akt

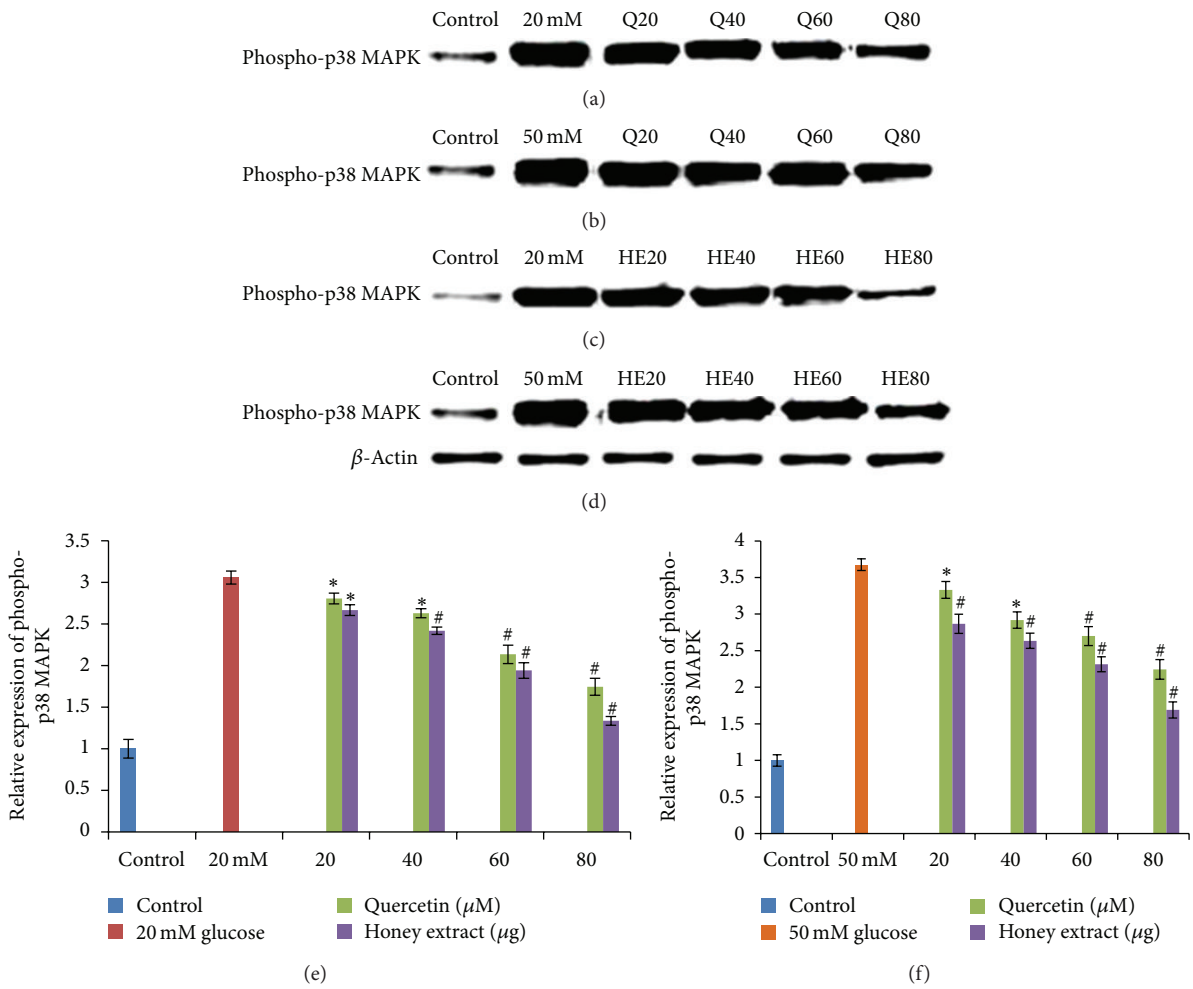

Figure 1: Effect of quercetin and Gelam honey extract on phosphor-p38 MAPK expression. Quantitative analysis and representative western blot analysis of phospho p38 MAPK in HIT-T15 cells pretreated with quercetin and honey extract in cells cultured in 20 mM ((a), (c), (e)) and 50 mM ((b), (d), (f)) glucose. The results were normalized with β-actin antibody. Data were presented as the mean ± standard deviation. (e) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 20 mM glucose alone. (f) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 50 mM glucose alone.

(ser473). Akt (ser473) phosphorylation in HIT-T15 cells was markedly reduced following 20 mM (Figures 4(a), 4(c), 4(e)) and 50 mM (Figures 4(b), 4(d), 4(f)) glucose treatment, but the trend was reversed after pretreatment with quercetin and Gelam honey extract. Pretreatment of the cells with different concentration of quercetin and honey extract for 24 hours significantly increase the expression of pAkt (ser473) up to 32% and 70% respectively, compared to the cells that were cultured alone with 20 mM glucose (Figure 4(a), 4(c), 4(e)). On the other hand, pretreatment with quercetin and honey extract increases the expression of pAkt (ser473) significantly ($P < 0.05$) up to 19% and 54%, respectively, compared to the cells that were cultured alone with 50 mM glucose (Figures 4(b), 4(d), 4(f)). The cells that were exposed to Akt inhibitor VIII prevented the quercetin and honey extract-induced Akt ser473 phosphorylation. The pAkt protein levels from each sample were normalized to their respective β-actin protein amounts ($^{*}P < 0.05$; $^{\#}P < 0.005$ versus glucose-treated group).

3.6. The Effect of Quercetin and Gelam Honey Extract and Akt Inhibitor on Insulin Content of the Cells. To determine the effect of ROS on insulin content, the cells were pretreated with Gelam honey extract (20, 40, 60, 80 μg/mL) and the quercetin (20, 40, 60, 80 μM) before treating the HIT-T15 cells with 20 mM and 50 mM glucose and the insulin content was measured. As shown in Figure 5(a), pretreatment with quercetin and Gelam honey extract increased the insulin content significantly ($P < 0.05$) up to 64% and 78%, respectively, compared to the cells that were cultured alone with 20 mM glucose. Figure 5(b) shows that pretreatment with quercetin and Gelam honey extract increased the insulin content significantly ($P < 0.05$) up to 34% and 48% respectively, compared to the cells that were cultured alone with 50 mM glucose. Exposure of cells to Akt inhibitor VIII for 1 hour before pretreatment with quercetin (20, 80 μM) and Gelam honey extract (20, 80 μg/mL) decreases the insulin content significantly ($P < 0.05$) up to 12% and 6% respectively, compared to the cells that were pretreated with quercetin

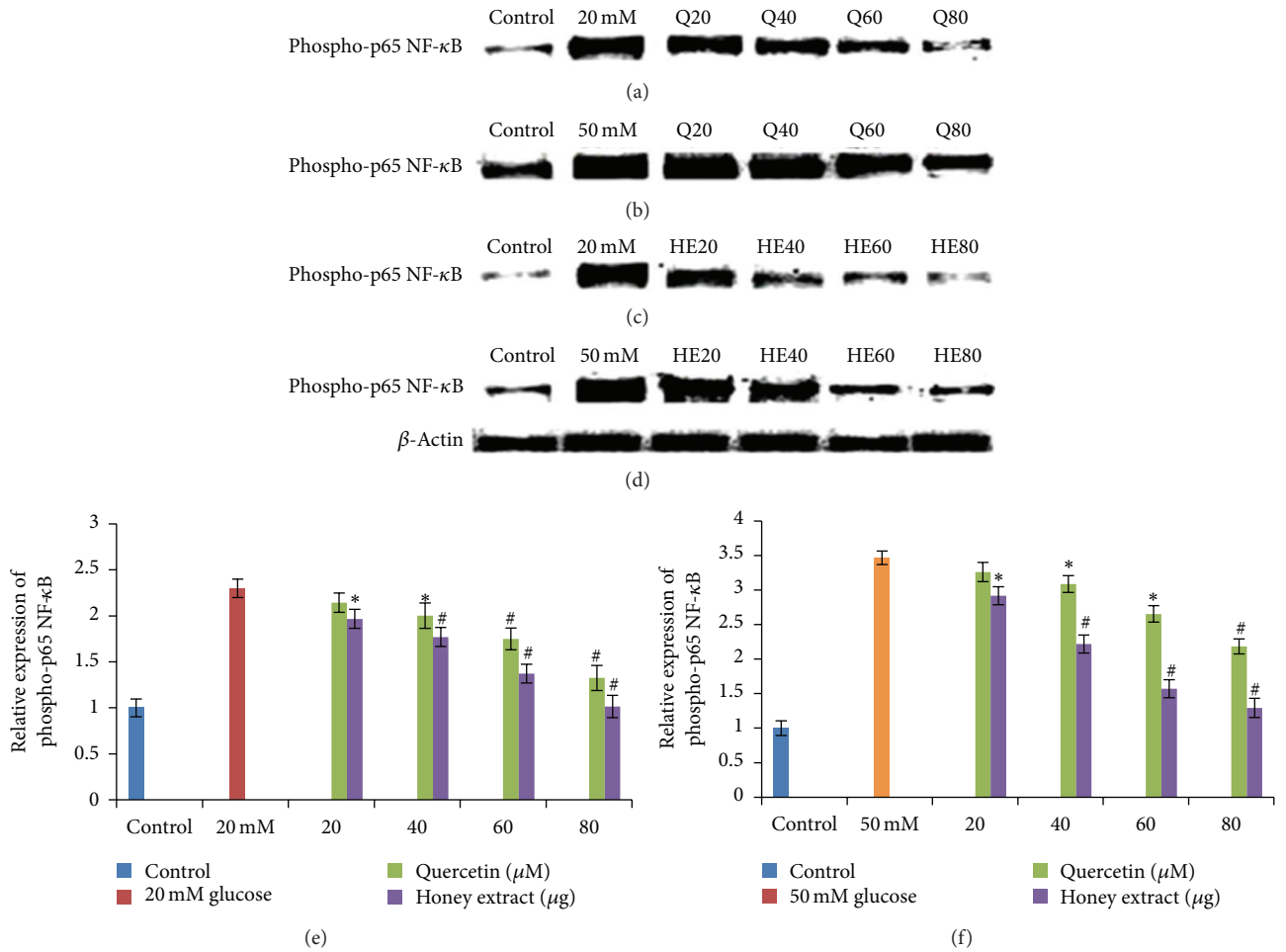

Figure 2: Effect of quercetin and Gelam honey extract on phosphor-p65 NF-κB expression. Quantitative analysis and representative western blot analysis of phospho p65 NF-κB in HIT-T15 cells pretreated with quercetin and honey extract in cells cultured in 20 mM ((a), (c), (e)) and 50 mM ((b), (d), (f)) glucose. The results were normalized with β-actin antibody. Data were presented as the mean ± standard deviation. (e) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 20 mM glucose alone. (f) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 50 mM glucose alone.

and honey extract cultured in 20 mM glucose (Figure 5(a)) without Akt inhibitor. The cells that were exposed to Akt inhibitor VIII for 1 hour and pretreated with quercetin (20, 80μM) and Gelam honey extract (20, 80 μg/mL) decrease the insulin content significantly ($P < 0.05$) up to 10% and 8% respectively, compared to the cells that were pretreated with quercetin and honey extract before culturing in 50 mM glucose (Figure 5(b)) without Akt inhibitor.

4. Discussions

In our previous study, we investigated the antioxidant effect of the Malaysian Gelam honey and some of its flavonoid components (chrysin, luteolin, and quercetin) individually on pancreatic hamster cells (HIT-T15) cultured under hyperglycemic conditions. Our data demonstrated that the cultured cells, pretreated with the extract of the Gelam honey and the different flavonoid components (quercetin, luteolin, and chrysin) at varying concentrations for 24 hours, protected the β cell from oxidative damage caused by ROS induced by hyperglycemia [24]. Therefore in our present study, we determined the effect of Gelam honey extract and quercetin on the stress-activated NF-κB, MAPK pathways and IRS-1 serine phosphorylation causing insulin resistance and the Akt-activated insulin signaling pathway, causing increase in insulin content.

Several studies have demonstrated that flavonoids may reduce hyperglycemia and exert protective effects against nonenzymatic glycation of proteins in animals [32, 33]. Two important studies in streptozotocin-(STZ-) induced diabetes mellitus [33] and alloxan-induced diabetes mellitus in rats [25] have demonstrated that quercetin may even reverse the hyperglycemia close to the normal levels. More recently hyperglycemia has been implicated in the stress-activated signaling pathways such as p-65 NF-κB and p-38 MAPK [17]. Activation of these pathways is linked not only to the development of the late complications of diabetes, but also to insulin resistance and β-cell dysfunction [16].

Another study on bovine endothelial cells has revealed that exposure to hyperglycemia initially increased the

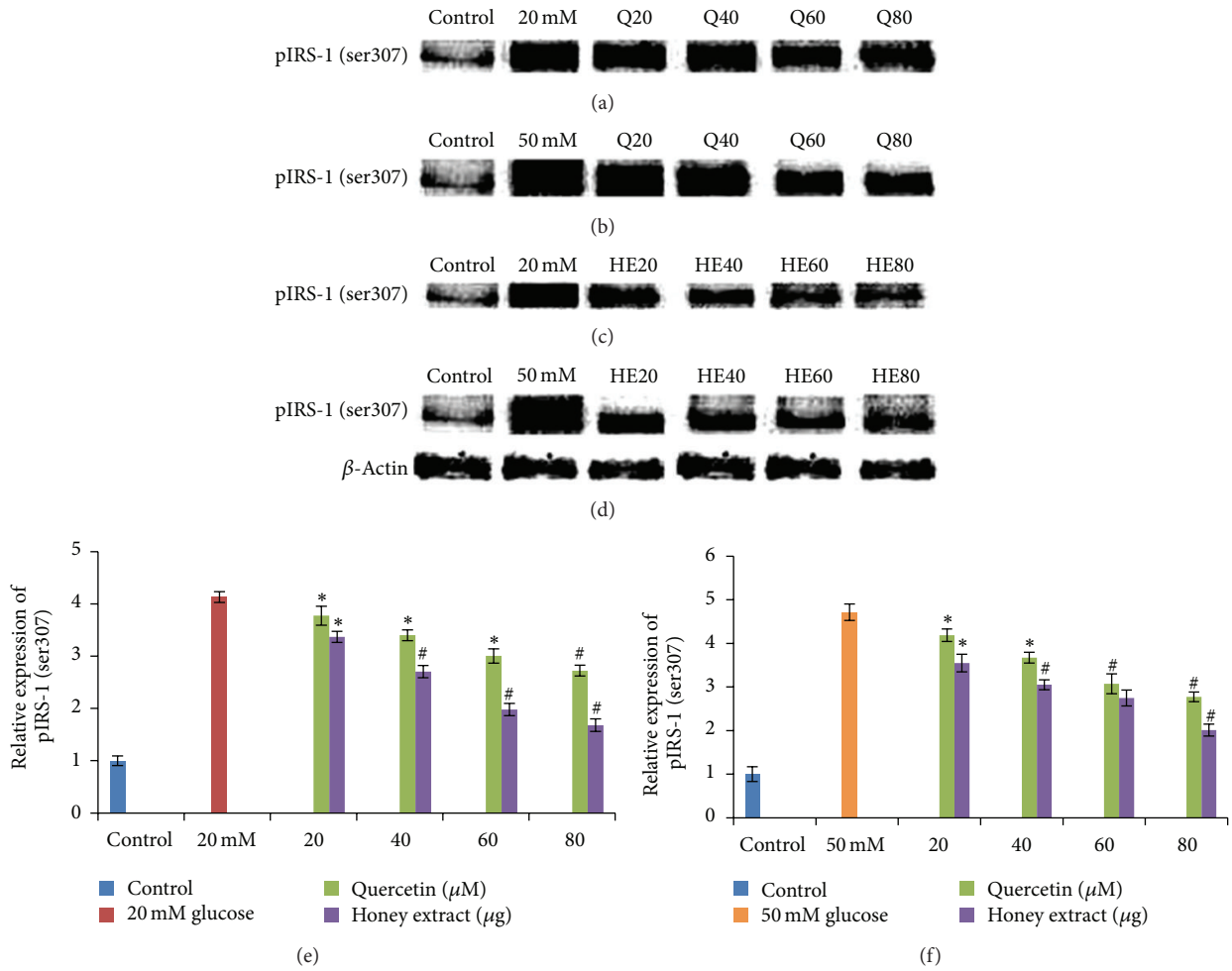

FIGURE 3: Effect of quercetin on pIRS-1 (ser307) expression. Quantitative analysis and representative western blot analysis of pIRS-1 (ser307) in HIT-T15 cells pretreated with quercetin and Gelam honey extract in cells cultured in 20 mM ((a), (c), (e)) and 50 mM ((b), (d), (f)) glucose. The results were normalized with β actin antibody. Data were presented as the mean ± standard deviation. (e) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 20 mM glucose alone. (f) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 50 mM glucose alone.

production of intracellular ROS, followed by activation of p-65 NF-κB, subsequently increasing the PKC activity, the advanced glycation end products (AGE), and sorbitol levels. It has been shown that when hyperglycemia-induced ROS production was reduced, the hyperglycemia-induced effects on NF-κB, PKC, AGE, and sorbitol were also suppressed [34, 35]. In addition, α-phenyl-tert-butylnitrone, a spin-trapping agent that reacts with ROS, significantly reduces the severity of hyperglycemia in both alloxan- and streptozotocin-induced diabetes and inhibits the activation of p-65 NF-κB [36]. It has been shown that both the activation of p-65 NF-κB and the increase in oxidative stress are reduced in rats fed on a diet supplemented with multiple antioxidants [37]. These data indicate that activation of p-65 NF-κB is an initial signaling event caused by ROS that leads to cellular dysfunction and damage [36]. Our findings suggest that in pancreatic hamster cell, quercetin and Gelam honey extracts are able to reduce p-38 MAPK and p-65 NF-κB activation, by its antioxidant effect on ROS. This gives further support that oxidative stress is the initial change induced by high glucose. Our data shows that exposure of HIT-T15 cells to 20 and 50 mM glucose caused a significant increase level of phospho-p38 MAPK (Figure 1) expression and phospho-p65 NF-κB (Figure 2) expression compared to control. Pretreatment with quercetin and Gelam honey extract significantly ($P < 0.05$) reduced the ROS-induced expression of phospho-p38 MAPK (Figure 1) and phospho-p65 NF-κB (Figure 2) under 20 and 50 mM glucose.

Activation of p38 MAPK has been suggested as one of the potential candidates for mediating IRS-1 serine phosphorylation (ser307) by cellular stresses [38–40] causing steric hindrance of the interaction between IR and IRS-1. Our data shows that phosphorylation of IRS-1 (ser307) was increased in HIT-T15 cells treated with 20 and 50 mM glucose alone as compared with control. Pretreatment with the quercetin and Gelam honey extract significantly decreased ($P < 0.05$) the expression of pIRS-1 (ser307) (Figure 3) in a dose-dependent manner in the cells that were cultured in 20 and 50 mM

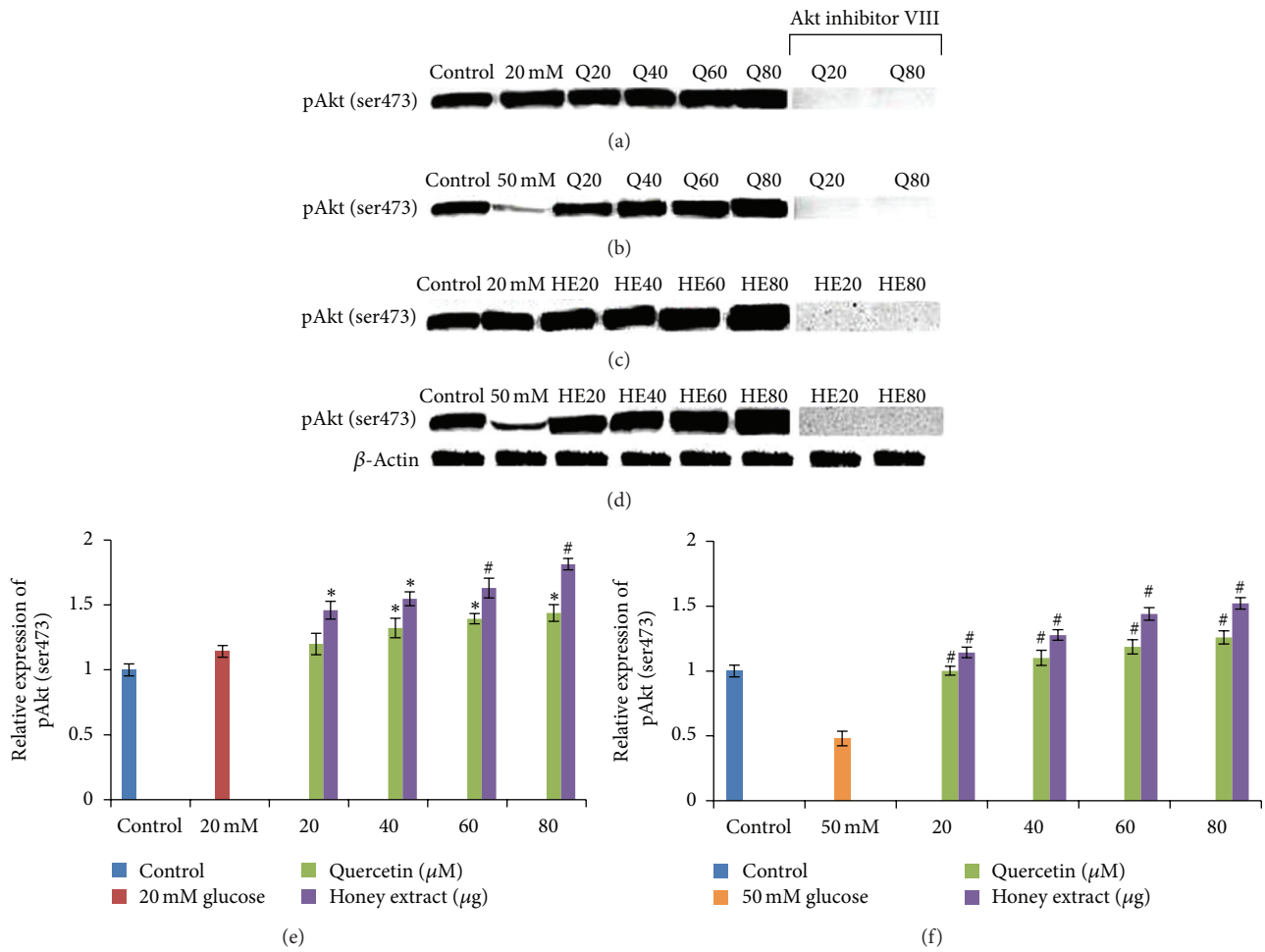

FIGURE 4: Effect of quercetin and Gelam honey extract on pAkt (Ser473) expression.Quantitative analysis and representative western blot analysis of pAkt (Ser473) in HIT-T15 cells pretreated with quercetin and honey extract in cells cultured in 20 mM ((a), (c), (e)) and 50 mM ((b), (d), (f)) glucose. A sustained increase in the level of pAkt (ser473) was observed after pretreatment with quercetin and honey extract. Akt inhibitor VIII prevented the expression Akt ser473 phosphorylation induced by quercetin and honey extract. The results were normalized with β actin antibody. Data were presented as the mean ± standard deviation. (e) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 20 mM glucose alone. (f) $^{*}P < 0.05$; $^{\#}P < 0.005$ quercetin and honey extract treated compared to the 50 mM glucose alone.

glucose (Figure 3). There is possibility that ROS generation, in response to stress stimuli, may promote IRS-1 (ser307) phosphorylation.

Cho et al. in 2001 reported that mice lacking the p-Akt (ser473) protein are insulin resistant, with impaired insulin secretion [41]. Ernesto et al. reported that Akt (ser473) is necessary for normal pancreatic β-cell function and described a novel regulatory role for Akt signaling in insulin secretion [42]. Several studies reported that a decrease in insulin secretion and insulin resistance induced by hyperglycemia has been associated with decreased Akt activity [19, 20, 41, 43, 44]. Our data validates the above statement by showing that Akt (ser 473) (Figure 4) phosphorylation in HIT-T15 cells was markedly reduced following 20 and 50 mM glucose treatment but was reversed after pretreatment with quercetin and Gelam honey extract. Our data suggest that Akt (ser473) (Figure 4) phosphorylation and insulin content (Figure 5) were increased after pre-treatment with quercetin and honey showing the protective effects against β cell dysfunction. The present data shows that treatment with an Akt inhibitor decreased the insulin content significantly (Figure 5) supporting the previous data reported by Cordero et al. [45] that Cocoa flavonoids improve insulin signaling and modulate glucose production via Akt in HepG2 cells.

Growing evidence indicates that ROS are involved in maintaining normal β-cell glucose responsiveness. ROS may have different actions depending on whether the cellular concentrations are either below or above a specific threshold, that is, signaling versus toxic effects. H_2O_2 derived from glucose metabolism is one of the metabolic signals for insulin secretion. Also it has been shown that mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion. ROS produced under short-term exposure, or under nonhyperglycemic conditions may play a role in physiological regulation of glucose induced insulin secretion, while long-term exposure to high glucose induces oxidative stress in β cells [24]. Our data validate the above statement by showing that cells grown under 20 mM glucose (Figure 5(a))

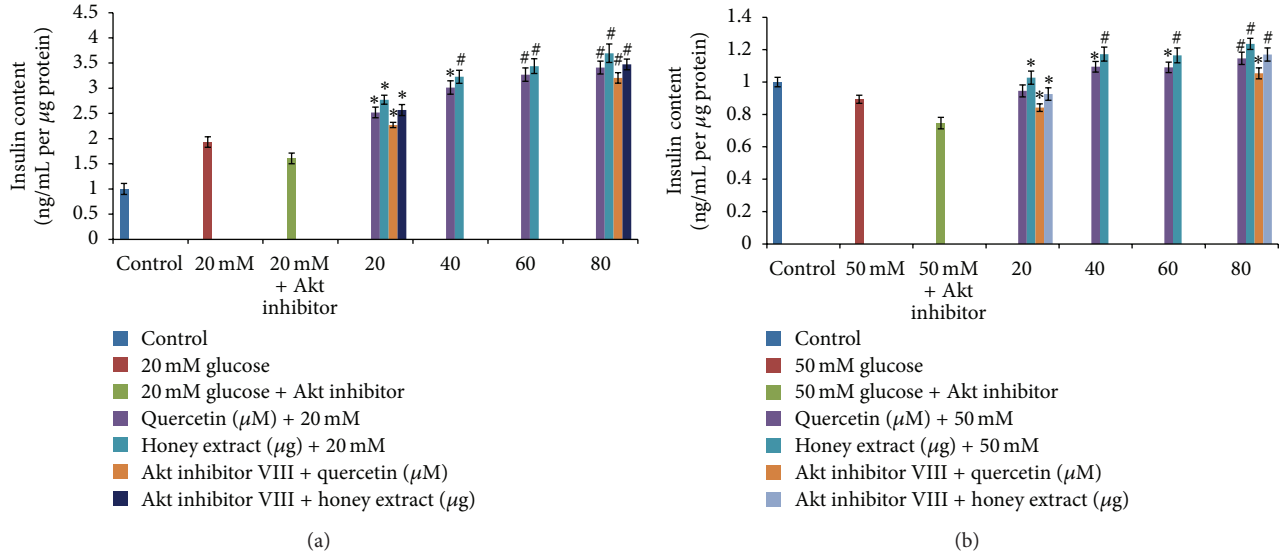

Figure 5: The effect of flavonoids and Gelam honey extract on insulin content. (a) Effect of pretreatment with quercetin and Gelam honey extract and the addition of Akt inhibitor VIII on the insulin content in cells cultured in 20 mM glucose. There was a significant increase in insulin content ($^{*}P < 0.05$) when the cells were pretreated with quercetin and honey. There was a significant decrease in insulin content ($^{*}P < 0.05$) when the cells were treated with Akt inhibitor VIII, before pretreating with quercetin and Gelam honey extract. (b) Effect of pretreatment with quercetin and Gelam honey and the addition of Akt inhibitor VIII on the insulin content in cells cultured in 50 mM glucose. There was a significant increase in insulin content ($^{*}P < 0.05$, $^{\#}P < 0.005$) when the cells were pretreated with quercetin and honey. There was a significant decrease in insulin content ($^{*}P < 0.05$, $^{\#}P < 0.005$) when the cells were treated with Akt inhibitor VIII, before pretreating with quercetin and Gelam honey extract.

under nonhyperglycemic conditions showed an increase in insulin content compared to the controls, while in cells grown under 50 mM glucose (Figure 5(b)) concentration, the insulin content was reduced significantly as compared to controls.

In conclusion, our data suggest the potential use of the extract from Gelam honey in treating diabetes, by modulating the oxidative stress-induced insulin signalling pathways. The data provide further support for the implication of oxidative stress, in β-cell dysfunction. Further studies are required to accurately define the mechanisms involved in diabetic complications at the molecular and biochemical levels.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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References

[1] D. Porte Jr., "β-cells in type II diabetes mellitus," *Diabetes*, vol. 40, no. 2, pp. 166–180, 1991.

[2] R. A. DeFronzo, R. C. Bonadonna, and E. Ferrannini, "Pathogenesis of NIDDM: a balanced overview," *Diabetes Care*, vol. 15, no. 3, pp. 318–368, 1992.

[3] H. Yki-Jarvinen, "Glucose toxicity," *Endocrine Reviews*, vol. 13, no. 3, pp. 415–431, 1992.

[4] A. Vinik, G. Pittenger, R. Rafaeloff, L. Rosenberg, and W. Duguid, "Determinants of pancreatic islet cell mass: a balance between neogenesis and senescence/apoptosis," *Diabetes Reviews*, vol. 4, no. 2, pp. 235–263, 1996.

[5] K. Hideaki, N. Yoshihisa, K. Dan, and M. Takeshi, "Involvement of oxidative stress and the JNK pathway in glucose toxicity," *Review of Diabetic Studies*, vol. 1, pp. 165–174, 2004.

[6] M. Cnop, N. Welsh, J.-C. Jonas, A. Jörns, S. Lenzen, and D. L. Eizirik, "Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes: many differences, few similarities," *Diabetes*, vol. 54, no. 2, pp. S97–S107, 2005.

[7] D. H. Zangen, S. Bonner-Weir, C. H. Lee et al., "Reduced insulin, GLUT2, and IDX-1 in β-cells after partial pancreatectomy," *Diabetes*, vol. 46, no. 2, pp. 258–264, 1997.

[8] R. P. Robertson, H.-J. Zhang, K. L. Pyzdrowski, and T. F. Walseth, "Preservation of insulin mRNA levels and insulin secretion in HIT cells by avoidance of chronic exposure to high glucose concentrations," *Journal of Clinical Investigation*, vol. 90, no. 2, pp. 320–325, 1992.

[9] L. K. Olson, J. B. Redmon, H. C. Towle, and R. P. Robertson, "Chronic exposure of HIT cells to high glucose concentrations paradoxically decreases insulin gene transcription and alters binding of insulin gene regulatory protein," *Journal of Clinical Investigation*, vol. 92, no. 1, pp. 514–519, 1993.

[10] A. Moran, H.-J. Zhang, L. K. Olson, J. S. Harmon, V. Poitout, and R. P. Robertson, "Differentiation of glucose toxicity from beta cell exhaustion during the evolution of defective insulin gene

expression in the pancreatic islet cell line, HIT-T15," *Journal of Clinical Investigation*, vol. 99, no. 3, pp. 534–539, 1997.

[11] D. Giugliano, A. Ceriello, and G. Paolisso, "Oxidative stress and diabetic vascular complications," *Diabetes Care*, vol. 19, no. 3, pp. 257–267, 1996.

[12] M. Tiedge, S. Lortz, J. Drinkgern, and S. Lenzen, "Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells," *Diabetes*, vol. 46, no. 11, pp. 1733–1742, 1997.

[13] J. L. Evans, I. D. Goldfine, B. A. Maddux, and G. M. Grodsky, "Are oxidative stress—activated signaling pathways mediators of insulin resistance and β-cell dysfunction?" *Diabetes*, vol. 52, no. 1, pp. 1–8, 2003.

[14] P. Rösen, P. P. Nawroth, G. King, W. Möller, H.-J. Tritschler, and L. Packer, "The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a congress series sponsored by UNESCO-MCBN, the American diabetes association and the German diabetes society," *Diabetes/Metabolism Research and Reviews*, vol. 17, no. 3, pp. 189–212, 2001.

[15] N. Li, T. Brun, M. Cnop, D. A. Cunha, D. L. Eizirik, and P. Maechler, "Transient oxidative stress damages mitochondrial machinery inducing persistent β-cell dysfunction," *Journal of Biological Chemistry*, vol. 284, no. 35, pp. 23602–23612, 2009.

[16] P. J. Barnes and M. Karin, "Nuclear factor-κB—a pivotal transcription factor in chronic inflammatory diseases," *New England Journal of Medicine*, vol. 336, no. 15, pp. 1066–1071, 1997.

[17] J. M. Kyriakis and J. Avruch, "Sounding the alarm: protein kinase cascades activated by stress and inflammation," *Journal of Biological Chemistry*, vol. 271, no. 40, pp. 24313–24316, 1996.

[18] C. J. Carlson, S. Koterski, R. J. Sciotti, G. B. Poccard, and C. M. Rondinone, "Enhanced basal activation of mitogen-activated protein kinases in adipocytes from type 2 diabetes: potential role of p38 in the downregulation of GLUT4 expression," *Diabetes*, vol. 52, no. 3, pp. 634–641, 2003.

[19] P. Gual, Y. Le Marchand-Brustel, and J.-F. Tanti, "Positive and negative regulation of insulin signaling through IRS-1 phosphorylation," *Biochimie*, vol. 87, no. 1, pp. 99–109, 2005.

[20] D. A. Glauser and W. Schlegel, "The emerging role of FOXO transcription factors in pancreatic β cells," *Journal of Endocrinology*, vol. 193, no. 2, pp. 195–207, 2007.

[21] W. C. Ya, F. H. Chun, S. T. Keh et al., "The role of phosphoinositide 3-kinase/Akt signaling in low-dose mercury-induced mouse pancreatic β-cell dysfunction in vitro and in vivo," *Diabetes*, vol. 55, no. 6, pp. 1614–1624, 2006.

[22] D. J. Withers, J. S. Gutierrez, H. Towery et al., "Disruption of IRS-2 causes type 2 diabetes in mice," *Nature*, vol. 391, no. 6670, pp. 900–904, 1998.

[23] E. Bernal-Mizrachi, S. Fatrai, J. D. Johnson et al., "Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells," *Journal of Clinical Investigation*, vol. 114, pp. 928–936, 2004.

[24] K. Batumalaie, R. Qvist, K. M. Yusof, I. S. Ismail, and S. D. Sekaran, "The antioxidant effect of the Malaysian Gelam honey on pancreatic hamster cells cultured under hyperglycemic conditions," *Clinical and Experimental Medicine*, 2013.

[25] A. Lukačínová, J. Mojžiš, R. Beňačka, O. Rácz, and F. Ništiar, "Structure-activity relationships of preventive effects of flavonoids in alloxan-induced diabetes mellitus in rats," *Journal of Animal and Feed Sciences*, vol. 17, no. 3, pp. 411–421, 2008.

[26] H. A. L. Wahdan, "Causes of the antimicrobial activity of honey," *Infection*, vol. 26, no. 1, pp. 26–31, 1998.

[27] A. Seo and C. V. Morr, "Improved high-performance liquid chromatographic analysis of phenolic acids and isoflavonoids from soybean protein products," *Journal of Agricultural and Food Chemistry*, vol. 32, no. 3, pp. 530–533, 1984.

[28] S. Z. Hussein, K. M. Yusoff, S. Makpol, and Y. A. M. Yusof, "Antioxidant capacities and total phenolic contents increase with gamma irradiation in two types of Malaysian honey," *Molecules*, vol. 16, no. 8, pp. 6378–6395, 2011.

[29] Y. Tanaka, C. E. Gleason, P. O. T. Tran, J. S. Harmon, and R. P. Robertson, "Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 19, pp. 10857–10862, 1999.

[30] M. Federici, M. Hribal, L. Perego et al., "High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program," *Diabetes*, vol. 50, no. 6, pp. 1290–1301, 2001.

[31] M. Lafontaine-Lacasse, G. Doré, and F. Picard, "Hexosamines stimulate apoptosis by altering SIRT1 action and levels in rodent pancreatic β-cells," *Journal of Endocrinology*, vol. 208, no. 1, pp. 41–49, 2011.

[32] O. Coskun, M. Kanter, A. Korkmaz, and S. Oter, "Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and β-cell damage in rat pancreas," *Pharmacological Research*, vol. 51, no. 2, pp. 117–123, 2005.

[33] N. Nuraliev Yu. and G. A. Avezov, "The efficacy of quercetin in alloxan diabetes," *Experimental and Clinical Pharmacology*, vol. 55, no. 1, pp. 42–44, 1992.

[34] S. Marshall, W. T. Garvey, and R. R. Traxinger, "New insights into the metabolic regulation of insulin action and insulin resistance: role of glucose and amino acids," *FASEB Journal*, vol. 5, no. 15, pp. 3031–3036, 1991.

[35] T. Nishikawa, D. Edelstein, X. L. Du et al., "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage," *Nature*, vol. 404, no. 6779, pp. 787–790, 2000.

[36] E. Ho, G. Chen, and T. M. Bray, "Alpha-phenyl-tert-butylnitrone (PBN) inhibits NFκB activation offering protection against chemically induced diabetes," *Free Radical Biology and Medicine*, vol. 28, no. 4, pp. 604–614, 2000.

[37] R. A. Kowluru, P. Koppolu, S. Chakrabarti, and S. Chen, "Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants," *Free Radical Research*, vol. 37, no. 11, pp. 1169–1180, 2003.

[38] M. Fujishiro, Y. Gotoh, H. Katagiri et al., "Three mitogen-activated protein kinases inhibit insulin signaling by different mechanisms in 3T3-L1 adipocytes," *Molecular Endocrinology*, vol. 17, no. 3, pp. 487–497, 2003.

[39] C. De Alvaro, T. Teruel, R. Hernandez, and M. Lorenzo, "Tumor necrosis factor α produces insulin resistance in skeletal muscle by activation of inhibitor kappa B kinase in a p38 MAPK-dependent manner," *Journal of Biological Chemistry*, vol. 279, no. 17, pp. 17070–17078, 2004.

[40] G. Li, E. J. Barrett, M. O. Barrett, W. Cao, and Z. Liu, "Tumor necrosis factor-α induces insulin resistance in endothelial cells via a p38 mitogen-activated protein kinase-dependent pathway," *Endocrinology*, vol. 148, no. 7, pp. 3356–3363, 2007.

[41] H. Cho, J. Mu, J. K. Kim et al., "Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKBβ)," *Science*, vol. 292, no. 5522, pp. 1728–1731, 2001.

[42] B. M. Ernesto, F. Szabolcs, D. J. James et al., "Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet β cells," *Journal of Clinical Investigation*, vol. 7, pp. 928–936, 2004.

[43] A. D. Kohn, S. A. Summers, M. J. Birnbaum, and R. A. Roth, "Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation," *Journal of Biological Chemistry*, vol. 271, no. 49, pp. 31372–31378, 1996.

[44] K. Y. C. Kenneth, S. L. L. Karen, and W. Donghai, "β cells by enhancing protein kinase Akt-dependent expression of SNARE proteins in mice," *Proceedings of the National Academy of Sciences*, vol. 109, pp. 8919–8924, 2012.

[45] H. I. Cordero, M. A. Martín, L. Bravo, L. Goya, and S. Ramos, "Cocoa flavonoids improve insulin signalling and modulate glucose production via AKT and AMPK in HepG2 cells," *Molecular Nutrition & Food Research*, 2013.

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Review Article

Potential Biomarkers of Insulin Resistance and Atherosclerosis in Type 2 Diabetes Mellitus Patients with Coronary Artery Disease

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Type 2 diabetes mellitus patients with coronary artery disease have become a major public health concern. The occurrence of insulin resistance accompanied with endothelial dysfunction worsens the state of atherosclerosis in type 2 diabetes mellitus patients. The combination of insulin resistance and endothelial dysfunction leads to coronary artery disease and ischemic heart disease complications. A recognized biological marker, high-sensitivity C-reactive protein, has been used widely to assess the progression of atherosclerosis and inflammation. Along with coronary arterial damage and inflammatory processes, high-sensitivity C-reactive protein is considered as an essential atherosclerosis marker in patients with cardiovascular disease, but not as an insulin resistance marker in type 2 diabetes mellitus patients. A new biological marker that can act as a reliable indicator of both the exact state of insulin resistance and atherosclerosis is required to facilitate optimal health management of diabetic patients. Malfunctioning of insulin mechanism and endothelial dysfunction leads to innate immune activation and released several biological markers into circulation. This review examines potential biological markers, YKL-40, alpha-hydroxybutyrate, soluble CD36, leptin, resistin, interleukin-18, retinol binding protein-4, and chemerin, as they may play significant roles in insulin resistance and atherosclerosis in type 2 diabetes mellitus patients with coronary artery disease.

1. Review

In 2010, it was estimated that 285 million people had been diagnosed with diabetes mellitus worldwide, a prevalence of 6.4%. This is predicted to increase to 439 million, a prevalence of 7.7%, by 2030 [1]. The number of deaths indirectly linked to diabetes mellitus is estimated to be 3.96 million per year for all age groups, a prevalence of 6.8% [2]. Type 2 diabetes mellitus (T2DM) (previously known as non insulin-dependent diabetes mellitus) accounts for about 90% of diabetic patients worldwide [1]. T2DM is described as a "silent disease" and is characterized by the combination of inadequate insulin secretion due to islet β-cell deterioration and insulin resistance [3]. T2DM is an independent risk factor for the development and mortality of various complications, implicate microvascular problems including retinopathy, neuropathy, and nephropathy; and macrovascular problems including coronary artery disease (CAD) [4, 5].

CAD is one of the most common causes of death among diabetic patients, with a 2- to 3-fold higher prevalence compared with nondiabetic people [2]. CAD is defined conservatively as past myocardial infarction (MI), coronary artery bypass graft (CABG), or percutaneous transluminal

coronary angioplasty through confirmation by review of medical records or a major Q wave on electrocardiogram examination (ECG) in Insulin Resistance Atherosclerosis Study (IRAS) [6]. Concomitant risk factors, including persistent hyperglycemia, dyslipidemia, hypertension, smoking, a family history of the disease, and the presence of micro- and macroalbuminuria, directly increase the mortality risk of CAD among T2DM patients by approximately 2- to 4-fold compared with nondiabetic people [4, 5]. CAD is defined as an accumulation of cholesterol substance build-up in coronary arteries. Anatomically, coronary arteries are blood vessels supplying oxygen to cardiac muscle. Coronary arteries branch off from the aorta into four major arteries. These are the right coronary artery, the left main coronary artery, the left anterior descending artery, and the left circumflex artery. Blockage to any of these arteries due to plaque instability, known as atherosclerosis, leads to angina and ischemic conditions, resulting in CAD and also increasing the potential development of ischemic heart disease and other major cardiovascular diseases (CVDs) [7].

Owing to recent advances in understanding circulating molecular actions between endothelial function and the immune system, several potential biomarkers have been identified that appear to be linked to T2DM and CAD in terms of insulin resistance and atherosclerosis. This review examines these potential biomarkers as a new alternative to determining the status of insulin resistance in T2DM patients with CAD.

2. Mechanism of Insulin Resistance

According to the American Diabetes Association 2013 guidelines, insulin resistance and persistent hyperinsulinemia are found in a variety of medical conditions, including dyslipidemia and hypertension [4, 5]. Insulin resistance has been established as a precursor and acts as a strong factor linking T2DM with CVD. Studies have reported an increased potential risk of diabetic patients acquiring CAD within the past two decades [2, 6]. This is mainly because of insulin resistance development through genetics and environmental factors [8]. Alterations of β-cell function and insulin properties underlay the metabolic syndrome that includes dyslipidemia, hyperglycemia, hypertension, impaired fibrinolysis, and atherosclerosis, thus contributing to lower insulin sensitivity [6].

At a basic level, insulin resistance occurs through the activation of various types of macrophages. Malfunctioning adipocytes and adipose tissue release greater amounts of various proinflammatory cytokines. Subclinical inflammation due to insulin resistance might also correlate with the pathogenesis of all phases of atherosclerosis. This involves low-grade elevation of acute phase reactants, proinflammatory cytokine secretion, and cell adhesion molecules and leads to myocardial infarction, stroke, and other major peripheral vascular diseases. These factors, therefore, increase cardiovascular mortality. Activation of the innate immune system greatly contributes to the occurrence of T2DM and CAD (Figure 1) [9].

Endothelial dysfunction triggered by persistent inflammation due to increased levels of triglycerides (TRYL), free fatty acids (FFA) and low-density lipoprotein (LDL), and decreased levels of high-density lipoprotein (HDL) that eventually leads to alteration of insulin signaling and glucose uptake in muscles and adipocytes.

Inefficient glucose uptake by muscle and adipose tissue leads to insulin resistance or a medical condition known as compensatory hyperinsulinemia. β cells of the pancreatic islets release excessive amounts of insulin in an attempt to compensate for high plasma glucose levels in the blood [9, 10]. Persistent hyperinsulinemia increases serum levels of triglycerides, free fatty acids (FFA), and low-density lipoprotein and decreases serum levels of high-density lipoprotein. Increased levels of circulating FFA in the blood activate the innate immune system to release pr-inflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-6, and IL-1β [11]. The presence of these cytokines leads to an alteration in insulin sensitivity and disruption of glucose homeostasis [11]. The process involves cytokines first mediating insulin signaling mechanisms in adipocytes, muscles, and the liver to increase the occurrence of insulin resistance [11], before disabling liver X receptors (LXRs) causing an increase in the accumulation of cholesterol, thus stimulating hepatic production and secretion of inflammatory markers, including C-reactive protein, plasminogen inhibitor-1, serum amyloid-A, α1-acid glycoprotein, and haptoglobin [12]. Cytokines then stimulate fibrinogen, which acts as an atherosclerotic risk factor and leads to CAD [11]. Finally, cytokines increase production of very low-density lipoprotein and FFA, which leads to the characteristics of diabetic dyslipidemia and the subsequent increase in plaque accumulation [9, 11, 12].

3. Correlation between Insulin Resistance, Endothelial Dysfunction, and Atherosclerosis

The endothelium is located at the interior surface of blood and lymphatic vessels. The endothelium consists of a thin layer of cells defined as being either vascular endothelial cells (cells in direct contact with blood) or lymphatic endothelial cells (cells in direct contact with lymph). The function of the endothelium is to sense mechanical stimuli, such as high pressure and stretching, and hormonal stimuli, such as vasoactive substances. The endothelium plays a role in the regulation of vasomotor functions, stimulates inflammatory processes, and influences hemostasis [13].

Studies suggest that persistent hyperinsulinemia might trigger endothelial dysfunction. In diabetes, the occurrence of insulin resistance is due to an alteration in insulin signaling. Once this alteration happens, phosphorylation of major pathways (e.g., phosphatidylinositol 3-kinase, phosphoinositide-dependent kinase-1, and AKT/protein kinase B pathways), which activate endothelial nitric-oxide synthase (eNOS), is downregulated drastically. Due to this downregulation, the role of eNOS changes from an antiatherogenic effect to a proatherogenic effect, which further contributes to the development of atherosclerosis.

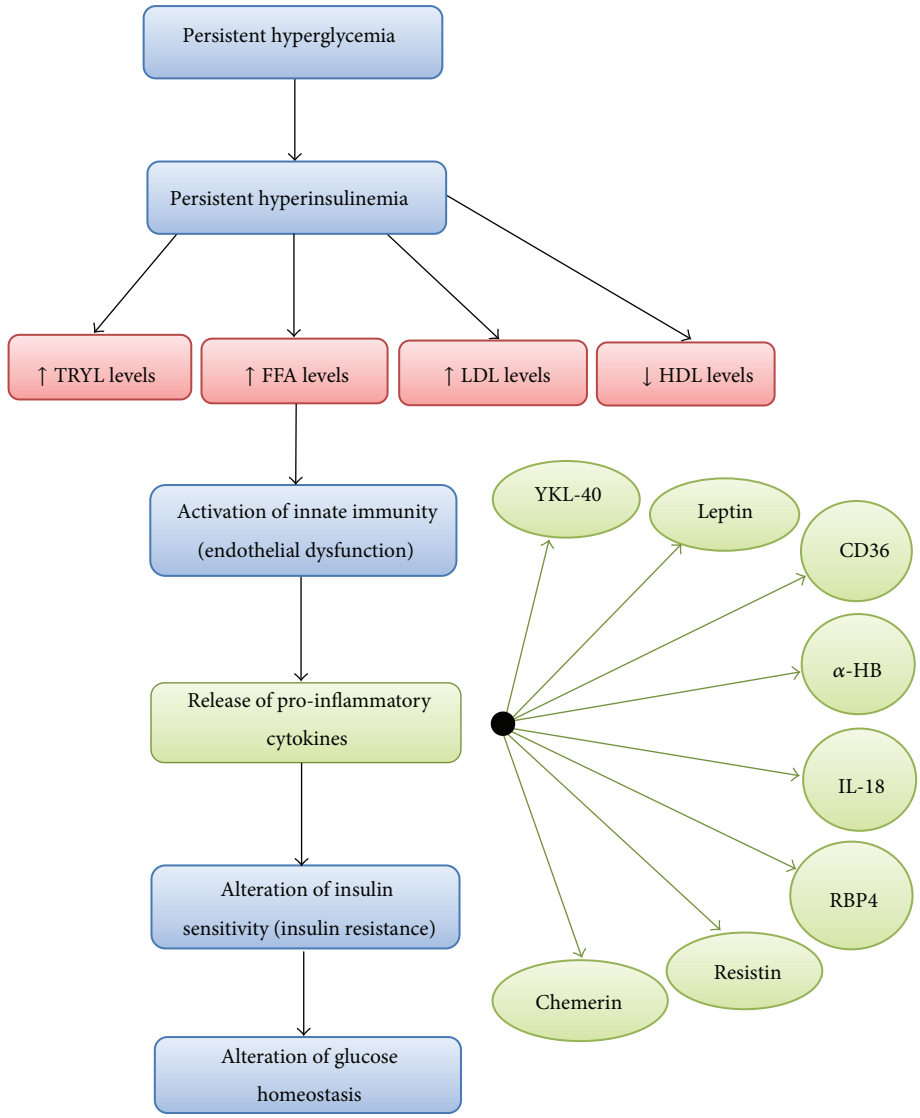

Figure 1: Mechanism of insulin resistance, endothelial dysfunction and proinflammatory secretion.

One of the insulin receptor pathways, mitogen-activated protein kinase, which stimulates mitogenic effects and growth, remains unaffected [13, 14].

A common mechanism of endothelium dysfunction is the depletion of eNOS. According to Willa and Manuel 2003, prolonged decreases of eNOS lead to decreased bioavailability of nitric oxide (NO), which acts as vascular protection by inhibiting inflammation, oxidation, vascular smooth muscle cell proliferation, and migration [14]. Decreased bioavailability of NO, along with low levels of high-density lipoprotein, high levels of small, dense, low-density lipoprotein, high secretion of angiotensin II sensitivity, and high releases of FFA in the blood, worsen is the status of endothelial dysfunction and promotes further atherogenic processes [14]. In addition, the effects of inflammation and reactive oxygen species contribute to decreasing NO bioavailability and stimulate secretion of proinflammatory cytokines, which are termed as possible biomarkers. Identification of these biomarkers might serve as tools for predicting insulin resistance and endothelial dysfunction in T2DM patients with CAD (Figure 2) [14].

The persistent events of hyperinsulinemia lead to insulin resistance and results to T2DM. Frequent hyperinsulinemia due to increased level of triglycerides, FFAs and LDL and decreased HDL, contributes to endothelial dysfunction and interrupts nitric oxide (NO) secretion, increases reactive oxygen species (ROS) and free radicals formation, and interruptions of adhesion molecule expression of chemokine and cytokine release. All the mechanisms contribute to inflammation, atherosclerosis, and CAD. Several cytokines had been discovered and used as biomarkers, strongly supporting the idea that the occurrence of hyperinsulinemia correlates with endothelial dysfunction leading to major diseases, T2DM, and CAD.

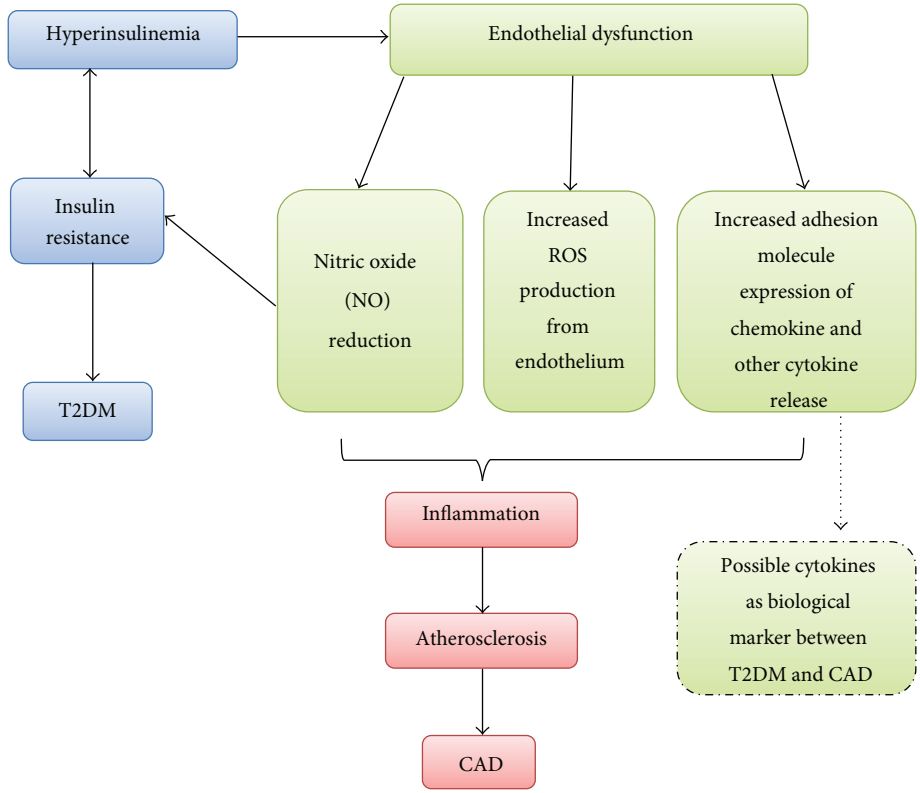

Figure 2: Insulin resistance in T2DM and endothelial dysfunction in CAD development.

4. Potential Biomarkers for Insulin Resistance in T2DM Patients with Coronary Artery Disease

4.1. YKL-40. YKL-40 or alternatively termed as BPR-39 or human cartilage glycoprotein-39, produced by the gene Chitinase 3-like 1 (CH3L1) [15], is a heparin- and chitin-binding lectin without chitinase activity and a member of the mammalian chitinase-like protein cluster [16]. YKL-40 belongs to the glycosyl hydrolase family 18 which consists of enzymes and proteins, includes hydrolytic enzymes named as chitinases from various species including mammalian, bacteria, fungi, nematodes, insects and plants [16]. YKL-40 based on its three NH_2-terminal amino acids, tyrosine (Y), lysine (K), and leucine (L) and its molecular weight of 40 kDa [16], is located at chromosome 1q31-q32 [15], consists of 10 exons and spans about 8 kb of genomic DNA [16], and has a crystal structure [17]. YKL-40 is produced at the site of inflammation [18], secreted by activated macrophages, including activated neutrophils, arthritic chondrocytes, fibroblast-like synovial cells, osteoblasts, and differentiated vascular smooth muscle cells [15].

Even though minor research has been conducted on the exact functions of YKL-40, several studies have reported that YKL-40 is an essential factor in extracellular tissue remodeling involving type 1 collagen fibril formation, a growth factor for fibroblasts and chondrocytes, and also controls mitogenesis by modulating MAP kinase and PI-3 K signaling cascades in fibroblasts [16, 19]. YKL-40's association with migration, reorganization, and adhesion of vascular endothelial cells and vascular smooth muscle cells suggests that it may also play a role in angiogenesis [16, 19].

YKL-40 serum levels increase in patients with acute infections [18] and chronic inflammation [15]. Recent studies have reported that elevated levels of plasma YKL-40 are proportional with the HOMA-IR in T2DM subjects. This indicates that YKL-40 shows some correlation with insulin resistance and dyslipidemia. High levels of YKL-40 also appeared in adult subjects who did not report a medical history of T2DM and CVD comorbidities [20, 21], even at the childhood stage [22]. Other studies have reported elevated levels of plasma YKL-40 and albuminuria detected in both type 1 and type 2 diabetes mellitus patients [21]. The results of these studies suggest that YKL-40 might act as a potential biomarker for endothelial dysfunction, atherosclerosis, insulin resistance, and T2DM [20].

4.2. Alpha-Hydroxybutyrate. Alpha-hydroxybutyrate (α-HB) has been found to be the most significant biomarker associated with insulin sensitivity, diabetes mellitus, and CVD. According to Walter et al. 2010, the underlying biochemical mechanisms of alpha-hydroxybutyrate involve lipid oxidation and oxidative stress [23]. Alpha-hydroxybutyrate acts as an earlier marker of dysglycemia when compared to other biomarkers in the same research, such

as alpha-ketobutyrate (α-KB), creatine, acylcarnitines, and lysoglycerophospholipids [23]. The study showed that the expression of alpha-ketobutyrate served as early indicator in insulin resistance by differentiating the group of normal glucose tolerance-insulin sensitivity (NGT-IS) from normal glucose tolerance-insulin resistance (NGT-IR) among nondiabetic population [23].

Alpha-hydroxybutyrate is an organic acid that is formed as a by-product during production of alpha-ketobutyrate through a reaction catalyzed by lactate dehydrogenase (LDH) or by an LDH isoform in the heart known as alpha-hydroxybutyrate dehydrogenase (α-HBDH) [23, 24]. Elevated levels of alpha-hydroxybutyrate occur due to an increased rate of alpha-ketobutyrate catabolism or inhibition of the products of dehydrogenase that catalyze the conversion of alpha-ketobutyrate to propionyl-CoA [25].

Walter et al. 2010 also reported that elevated levels of alpha-hydroxybutyrate might be associated with insulin resistance by two possible mechanisms [23]. First, the increment of hepatic glutathione stress causes increased production of glutathione, which contributes to the supply of more alpha-ketobutyrate substrate and subsequently results in increased formation of alpha-hydroxybutyrate. Second, increased levels of lipid oxidation lead to increased levels of nicotinamide adenine dinucleotide (NADH or NAD+), are parallel to the concentration of insulin-inhibited free fatty acid (FFA) [25]. Previous study showed positive correlation between steady states of FFA and plasma alpha-hydroxybutyrate in the diabetic cohorts. This supports the idea that increasing amounts of NADH or NAD+ correlate with the reduction of alpha-ketobutyrate to alpha-hydroxybutyrate [23].

4.3. Soluble CD36. CD36 also known as Fatty Acid Translocase (FAT) is a complex multifunctional protein that present as mononuclear phagocytes, serves as a scavenger receptor for oxidized low-density lipoprotein (LDL), cellular transporter of long chain fatty acids in muscles and adipocytes, and apoptotic cells on macrophages [26, 27]. CD36 has been also showed to be involved in several processes, including long-chain fatty acids, advanced glycosylation products, oxidized phosphocholines, collagen, growth-hormone releasing hormone (GNRH), peptides hexarelin and thrombospondin-1 (TSP-1) [28].

In 2008, Handberg et al. studies' has identified the existence and availability of soluble CD36 in cell-free plasma for further research discovery [29]. CD36 has been proposed as a biomarker of macrophage activation and inflammation [30] and atherosclerosis [31]. Several studies report that the expression of this 88 kDa transmembrane glycoprotein CD36 is strongly associated with atherosclerosis, angiogenesis, inflammation, lipid metabolism, platelet activation [31–33], hyperglycemia, and insulin resistance [34]. Oxidized LDL stimulates membrane CD36 expression on the surface of monocytes and macrophages, resulting in an increased atherosclerotic effect, and might be the underlying mechanism causing lipid accumulation in the subendothelial space [27, 30–32].

Previous study discovered that CD36 has the ability to bind and modify LDL that is trapped in arterial wall, contributing to the formation of lipid-engorged macrophage foam cells and initiate atherosclerotic lesions [26, 27, 31]. CD36 interaction with oxidized LDL stimulates a signaling response that act as proinflammatory and proatherogenic where they differentiate into macrophages [26]. The signaling pathway involves activation of Src-family kinases and MAP kinases and Vav family guanine nucleotide exchange factors, thereby contributing to ligand internalization, foam cell formation, and inhibition of migration [26].

Activation mechanism of CD36 pathway started when LDL particles cross the endothelium and become trapped in the intima connective tissue [26]. Under the influence of proinflammatory cytokines, the macrophage produces reactive oxygen and nitrogen species which oxidized the unsaturated phospholipids present in LDL. One oxidized, the LDL particles lose their infinity to its specific LDL receptor but gain affinity for scavenger receptors, including CD36, and internalized by intima macrophages [26]. During internalization, specific oxidized lipids present in oxidized LDL serve as ligands or precursors of ligands for the nuclear hormone receptor peroxisome proliferator-activated receptor γ-dependent (PPAR-γ). This receptor, once engaged and activated, acts as a transcription factor that drives disorderly expression of many metabolic genes, including CD36 [26].

Furthermore, elevated FFA in monocytes and macrophages also stimulate CD36 expression through PPAR-γ-dependent mechanism [26, 31, 33]. Fat accumulation in the human liver results in elevated levels of FFA and lipolysis eventually leads to insulin resistance and diabetic dyslipidemia. Upregulation of CD36 expression in insulin-resistant subjects, which involves an impaired insulin signaling cascade, is another pathological mechanism [30].

Increased levels of CD36 in monocytes among diabetic patients are highly correlated with insulin resistance. Several studies had showed that high levels of CD36 present in prediabetes, overt diabetes, polycystic ovary syndrome (PCOS), and impaired glucose tolerance strongly suggest that CD36 is involved in diabetes [30] and atherosclerosis pathogenesis and acts as inflammation biomarker [31, 32].

4.4. Leptin. Plasma leptin has a strong correlation with obesity, T2DM, CVD, insulin resistance, metabolic syndromes, and inflammatory markers [35]. Leptin, a 16 kDa hormone component of adipokine, is stimulated and secreted specifically by white adipose cells [36] and has been proposed as a biomarker for atherosclerotic CVD [37]. The expression of leptin receptors, mainly in atherosclerotic lesions [38], is involved in a variety of actions, including endothelial activation [39, 40], smooth muscle cell proliferation and calcification [41], and activation of monocytes and adaptive immune responses [42]. Studies have reported that leptin levels are linked to inflammatory and fibrinolysis markers, including C-reactive protein and plasminogen activator inhibitor-1, and are associated with CVD [35, 43].

Leptin is an adipocyte-specific *ob* gene product that has been found to be associated with insulin resistance and diabetes in obesity patients through insulin sensitivity and insulin secretion alterations [44]. Low insulin sensitivity has a pathophysiological effect on metabolic syndromes, including central obesity, dyslipidemia, hyperglycemia, hypertension, impaired fibrinolysis, and atherosclerosis [43]. Leptin secretion by adipocytes might be stimulated by insulin, which directly influences islet β-cell action on insulin levels [45]. Recent research reported a significant association between leptin in coronary heart disease (CHD) and insulin resistance [46].

4.5. Resistin. Resistin and resistin-like molecule protein originates from a family of cysteine-rich secretory proteins produced during adipocyte catabolism in the presence of the thiazolidinediones, a cluster of insulin-sensitizing drugs [36]. Resistin plays a role in the regulation of energy, glucose, and lipid homeostasis [47] and the maintenance of fasting blood glucose levels [48] by modulating hepatic insulin action [37, 49]. Resistin is a macrophage-derived signaling polypeptide hormone with a molecular weight of 12.5 kDa and is 108 amino acids long. It has low circulating levels [50], but in some studies, it has been reported to be upregulated in insulin resistance, T2DM, and CVD [51].

The expression of resistin primarily by monocytes and macrophages is much greater compared with adipocyte catabolism [47]. Resistin may increase the susceptibility of metabolic syndrome (MS) by regulating adiponectin secretion from adipocytes and enhancing hepatic gluconeogenesis by inhibiting the enzymes involved in gluconeogenesis through AMP-activated protein kinase activation [52]. A recent study reported that subjects with premature atherosclerosis have higher levels of plasma resistin compared with subjects with established atherosclerosis [53]. Correlations between insulin sensitivity, adiposity, and T2DM [54] still remain to be fully revealed, even though resistin has been proposed as a potential link between obesity, insulin resistance, and T2DM with CVD [51, 55].

4.6. Interleukin-18. The proinflammatory cytokine, IL-18, is located on chromosome 11q22.2-22.3 [56] and is a member of the IL-1 cluster. Originally, IL-18 was described as an interferon-γ-inducing factor because of its strong ability to stimulate interferon-γ release with the presence of co-stimuli, such as IL-12 or lipopolysaccharide [57]. Recently, studies have suggested that IL-18 is involved in apoptosis and tissue destruction [58], as part of the host defense against infections and neoplasms [57], abnormally expressed in adipose tissue through a mechanism called lipodystrophy [59], and is a predictor of cardiovascular mortality among coronary atherosclerosis subjects [60].

Inflammatory activity by macrophages, monocytes, dendritic cells, osteoblastic stroma cells, and cells of the central nervous system (CNS) stimulates activity of the precursor of IL-18, called pro-IL-18. Pro-IL-18 is cleaved by either caspase-1-dependent conversion [61] or through the FAS ligand, in a caspade-1-independent processing of IL-18 manner [62], to release the active peptide [63]. Once secreted, the active peptide of IL-18 can bind to either the IL-18 receptor or IL-18 binding protein or might be bind to the both. The IL-18 receptor consists of an α-chain and a β-chain. The α-chain is responsible for extracellular binding of IL-18 and the β-chain is responsible for intracellular signal transduction [64]. As for controlling proinflammatory activity, excess amounts of IL-18 secretion will bind to IL-18 binding protein, which result in a free fraction of IL-18 in a negative feedback mechanism. This free fraction of IL-18 is able to activate the β-chain while combination of free fraction of IL-18 and protein-bound IL-18 is able to activate the α-chain [65].

The general mechanisms of IL-18 in the context of insulin resistance involve lineal effects of IL-18 on insulin signaling with or without tumor necrosis factor-α (TNF-α) stimulation in tissues and a secondary response of IL-18 to insulin resistance. Previous research has reported a slight correlation between IL-18 and fasting plasma glucose in T2DM [66] and between IL-18 and fasting plasma insulin in obese women [67]. Persistent circulatory levels of IL-18 have been reported in T2DM subjects in parallel with elevated fasting glucose levels and hyperglycemia [67]. However, IL-18 appears to act as an indicator for insulin resistance but not for β-cell malfunction. It has therefore been suggested that there is a plausible correlation between the functions of IL-18 and type 1 diabetes mellitus (T1DM) [56], T2DM [68], obesity [66], and CVD [60]. Recent investigation showed a strong and clear correlation between IL-18 and insulin resistance in T2DM subjects and also in non-T2DM subjects [68].

4.7. Retinol Binding Protein-4. Retinol binding protein-4 (RBP4) had been identified as the only specific transport protein for retinol (vitamin A) that delivers retinol to tissues from the blood [69]. It is highly expressed in adipose tissue compared with the liver and has a strong association with endothelial function. Research has reported that the region near the RBP4 locus on human chromosome 10q has been linked to an increased risk of T2DM [69]. Fischer et al. reported that decreasing secretion of RBP4 serum levels improved insulin action and showed strong associations between high RBP4 serum levels and insulin resistance [69]. Previously, RBP4 serum levels were found to have an association with insulin sensitivity and to increase in lean and obese, nondiabetic [70], and T2DM subjects [71].

Lack research had been done to discover the exact role of RBP4 in human metabolism as most research has been conducted using glucose transporter-4 knockout mice in an attempt to discover the mechanisms of RBP4 in adipose tissue. However, a recent study reported a strong correlation between RBP4 and insulin resistance in nondiabetic subjects without a medical or family history of diabetes [71].

Investigation showed that high secretion of RBP4 by adipocytes decreased the expression of glucose transporter-4 (GLUT-4) in adipose tissue, which is commonly found in T2DM [71]. A study reported that high circulating levels of serum RBP4 increased the potential for insulin resistance by blocking insulin signaling in muscle, thus increasing hepatic glucose output. However, correlations between RBP4 and

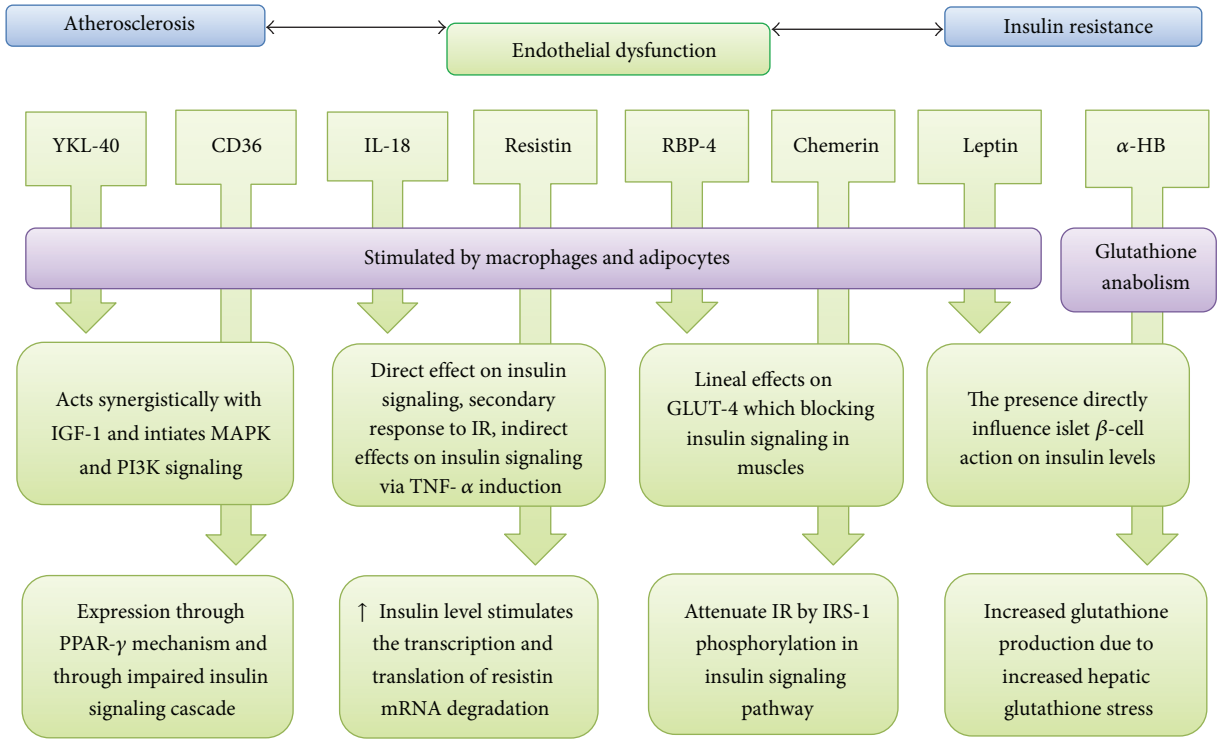

Figure 3: Summary of potential biomarkers mechanism.

vascular endothelium, oxidative stress, low-grade inflammation related to insulin resistance, and diabetic complications are still unclear [71].

4.8. Chemerin. Chemerin (also known as retinoic acid receptor responder 2 and tazarotene-induced gene 2) discovered as 18 kDa adipokine is secreted in the liver, acts as chemotactic agents and is highly stimulated by the innate immune system such as plasmacytoid dendritic cells and macrophages [72]. Chemerin had been discovered as a natural ligand of the chemerin receptor termed as ChemR23, also known as chemokine-like receptor 1 (CMKLR1). Chemerin also involves in intracellular calcium release and phosphorylation of extracellular signal-regulated kinase-1 and -2 (ERK 1/2) [72].

Chemerin is released as an inactive precursor. Through proteolytic cleavage, chemerin is activated by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. Chemerin is produced as a preproprotein, preprochemerin, which requires N-terminal cleavage of a secretion signal peptide before it is secreted as an inactive precursor protein, prochemerin. This proprotein has low biological activity and requires further extracellular C-terminal processing by plasmin, carboxypeptidases or serine proteases of the coagulation, fibrinolytic and inflammatory cascades [73].

These findings showed that chemerin has multiple cleavage sites in the C-terminal domain. In order to reach its maximal anti-inflammatory effects, bioactivity of chemerin is dependently regulated by proteolytic cleavage in the C-terminal region [74]. The presence of chemerin isoforms in hemofiltrate, serum, or ascites has potent chemotactic activity, indicating a proteolytic activation mechanism of chemerin bioactivity. Through mass spectrometry analysis, the isoforms of chemerin have been identified as chem21–154 and chem21–157, respectively; however, the proteases required for the isoforms activation remain unknown [74].

Increased activity of the coagulation cascade and decreased activity of the fibrinolytic cascade have been reported in obesity [75] and T2DM [76, 77], thus indicating the role of chemerin in immune responses [73]. The greater the activity of the serine proteases involved in coagulation, the higher the levels of activated chemerin [78].

A previous study has reported that chemerin is secreted equally in normal and T2DM subjects [77]. In another study, chemerin levels were reported as an independent biomarker of metabolic syndrome [79]. A recent study by Johanna et al. in 2010 reported that chemerin levels correlated with body mass index and waist-to-hip ratio but not with high-density lipoprotein cholesterol, which is highly secreted in obese and T2DM subjects. In terms of a biomarker, elevated levels of chemerin positively correlated with elevated levels of C-reactive protein in overweight and T2DM subjects [78].

5. Summary

The association between the pathophysiology of T2DM and CAD and the presence of biomarkers was summarised in Figure 3. Through endothelial dysfunction along with insulin resistance mechanism, different bioreceptors released biomarkers into the blood circulation to give a signal on

the occurrence of inflammation. The presence of potential biomarkers might reflect an underlying disease pathophysiology which would be essential to predict future events and treatment response indication.

6. Conclusion

Studies have reported strong evidence that suggests YKL-40, α-HB, soluble CD36, leptin, resistin, IL-18, RBP4, and chemerin could be new biomarkers for the pathogenesis of insulin resistance and endothelial dysfunction in T2DM patients. Components of these biological markers have been proposed to act as predictors of cardiovascular events in diabetic patients. However, the exact role of these biomarkers in insulin resistance and associations between biomarkers and disease need to be further elucidated. It is important to have a detailed understanding of the involvement of these biomarkers to clarify the biological action of cytokines and endothelial dysfunction and the occurrence of insulin resistance. In conclusion, these potential biomarkers might provide an alternative diagnostic tool for ensuring optimal management of T2DM patients with CAD.

Abbreviations

CAD:	Coronary artery disease
CMKLR1:	Chemokine-like receptor 1
CVD:	Cardiovascular disease
FAT:	Fatty Acid Translocase
eNOS:	Endothelial nitric-oxide synthase
FFA:	Free fatty acid
GLUT-4:	Glucose Transporter 4
GNRH:	Growth-hormone releasing hormone
HOMA-IR:	Homeostasis model of assessment-insulin resistance
IGF-1:	Insulin growth factor-1
IL:	Interleukin
IR:	Insulin resistance
IRIS:	Insulin Resistance Atherosclerosis Study
IRS-1:	Insulin receptor substrate-1
LDL:	Low-density lipoprotein
MAPK:	Mitogen-activated protein kinase
MS:	Metabolic syndrome
NADH:	Nicotinamide adenine dinucleotide
PI-3K:	Phosphatidylinositol 3-kinase
PPAR-γ:	Peroxisome proliferator-activated receptor γ-dependent
RBP4:	Retinal binding protein-4
ROS:	Reactive oxygen species
TNF-α:	Tumor necrosis factor-α
T1DM:	Type 1 diabetes mellitus
T2DM:	Type 2 diabetes mellitus
TSP-1:	Thrombospondin-1.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contributions

Sharifah Intan Qhadijah Syed Ikmal wrote the paper. Hasniza Zaman Huri, Shireene Ratna Vethakkan, and Wan Azman Wan Ahmad revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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References

[1] J. E. Shaw, R. A. Sicree, and P. Z. Zimmet, "Global estimates of the prevalence of diabetes for 2010 and 2030," *Diabetes Research and Clinical Practice*, vol. 87, no. 1, pp. 4–14, 2010.

[2] G. Roglic and N. Unwin, "Mortality attributable to diabetes: estimates for the year 2010," *Diabetes Research and Clinical Practice*, vol. 87, no. 1, pp. 15–19, 2010.

[3] M. Stumvoll, B. J. Goldstein, and T. W. van Haeften, "Type 2 diabetes: principles of pathogenesis and therapy," *The Lancet*, vol. 365, no. 9467, pp. 1333–1346, 2005.

[4] American Diabetes Association, *Prevention and Management of Diabetes Complications*, American Diabetes Association, Alexandria, Va, USA, 2013.

[5] American Diabetes Association, *Prevention and Management of Diabetes Complications*, American Diabetes Association, Alexandria, Va, USA, 2012.

[6] R. Marian, Z. Daniel, D. Ralph et al., "Insulin sensitivity, insulinemia, and coronary artery disease: the Insulin Resistance Atherosclerosis study," *Diabetes Care*, vol. 27, no. 3, pp. 781–787, 2004.

[7] American Heart Association, http://www.heart.org/HEARTORG/Conditions/More/MyHeartandStrokeNews/Coronary-Artery-Disease-The-ABCs-ofCAD_UCM_436416_Article.jsp.

[8] H. E. Lebovitz, "Type 2 diabetes: anoverview," *Clinical Chemistry*, vol. 45, no. 8, pp. 1339–1345, 1999.

[9] P. Libby, "Inflammation in atherosclerosis," *Nature*, vol. 420, no. 6917, pp. 868–874, 2002.

[10] G. Reaven, "The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals," *Endocrinology and Metabolism Clinics of North America*, vol. 33, supplement 2, pp. 283–303, 2004.

[11] B. Alaa, K. Amira, H. Pierre et al., "Type 2 diabetes mellitus and inflammation: prospects for biomarkers of risk nd nutritional intervention," *Diabetes, Metabolic Syndrome and Obesity*, vol. 3, pp. 173–186, 2010.

[12] A. Sjoholm and T. Nystrom, "Inflammation and the etiology of type 2 diabetes," *Diabetes/Metabolism Research and Reviews*, vol. 22, pp. 4–10, 2006.

[13] D. H. Endemann and E. L. Schiffrin, "Endothelial dysfunction," *Journal of the American Society of Nephrology*, vol. 15, no. 8, pp. 1983–1992, 2004.

[14] A. H. Willa and J. Q. Manuel, "Role of endothelial dysfunction in insulin resistance," *The American Journal of Cardiology*, vol. 92, pp. 10–17, 2003.

[15] M. Rehli, H. Niller, C. Ammon et al., "Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation," *Journal of Biological Chemistry*, vol. 278, no. 45, pp. 44058–44067, 2003.

[16] J. S. Johansen, B. V. Jensen, A. Roslind, D. Nielsen, and P. A. Price, "Serum YKL-40, a new prognostic biomarker in cancer patients?" *Cancer Epidemiology Biomarkers and Prevention*, vol. 15, no. 2, pp. 194–202, 2006.

[17] A. Roslind and J. S. Johansen, "YKL-40: a novel marker shared by chronic inflammation and oncogenic transformation," *Methods in Molecular Biology*, vol. 511, pp. 159–184, 2009.

[18] C. Østergaard, J. S. Johansen, T. Benfield, P. A. Price, and J. D. Lundgren, "YKL-40 is elevated in cerebrospinal fluid from patients with purulent meningitis," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 3, pp. 598–604, 2002.

[19] K. C. Nishikawa and A. J. T. Millis, "gp38k (CHI3L1) is a novel adhesion and migration factor for vascular cells," *Experimental Cell Research*, vol. 287, no. 1, pp. 79–87, 2003.

[20] C. N. Rathcke, J. S. Johansen, and H. Vestergaard, "YKL-40, a biomarker of inflammation, is elevated in patients with type 2 diabetes and is related to insulin resistance," *Inflammation Research*, vol. 55, no. 2, pp. 53–59, 2006.

[21] C. N. Rathcke, F. Persson, L. Tarnow, P. Rossing, and H. Vestergaard, "YKL-40, a marker of inflammation and endothelial dysfunction, is elevated in patients with type 1 diabetes and increases with levels of albuminuria," *Diabetes Care*, vol. 32, no. 2, pp. 323–328, 2009.

[22] I. Kyrgios, A. Galli-Tsinopoulou, C. Stylianou, E. Papakonstantinou, M. Arvanitidou, and A. Haidich, "Elevated circulating levels of the serum acute-phase protein YKL-40 (chitinase 3-like protein 1) are a marker of obesity and insulin resistance in prepubertal children," *Metabolism*, vol. 61, no. 4, pp. 562–568, 2012.

[23] E. G. Walter, B. Kirk, A. L. Kay et al., "α-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population," *PLoS ONE*, vol. 5, supplement 5, Article ID e10883, 11 pages, 2010.

[24] S. B. Rosalki and J. H. Wilkinson, "Reduction of α-ketobutyrate by human serum," *Nature*, vol. 188, no. 4756, pp. 1110–1111, 1960.

[25] S. Landaas, "The formation of 2 hydroxybutyric acid in experimental animals," *Clinica Chimica Acta*, vol. 58, no. 1, pp. 23–32, 1975.

[26] R. L. Silverstein, W. Li, Y. M. Park, and S. O. Rahaman, "Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis," *Transactions of the American Clinical and Climatological Association*, vol. 121, pp. 206–220, 2010.

[27] A. Handberg, K. Levin, K. Højlund, and H. Beck-Nielsen, "Identification of the oxidized low-density lipoprotein scavenger receptor CD36 in plasma: a novel marker of insulin resistance," *Circulation*, vol. 114, no. 11, pp. 1169–1176, 2006.

[28] J. J. D. Maximilliano, X. Nengming, L. C. Adam et al., "Soluble CD36 ectodomain binds negatively charged diacylglycerol ligands and acts as a co-receptor for TLR2," *PLoS ONE*, vol. 4, no. 10, Article ID e7411, 2009.

[29] A. Handberg, M. Skjelland, E. M. Annika et al., "Soluble CD36 in plasma is increased in patients with symptomatic atherosclerotic carotid plaques and is related to plaque instability," *Stroke*, vol. 39, no. 11, pp. 3092–3095, 2008.

[30] C. Liang, S. Han, H. Okamoto et al., "Increased CD36 protein as a response to defective insulin signaling in macrophages," *Journal of Clinical Investigation*, vol. 113, no. 5, pp. 764–773, 2004.

[31] S. Collot-Teixeira, J. Martin, C. McDermott-Roe, R. Poston, and J. L. McGregor, "CD36 and macrophages in atherosclerosis," *Cardiovascular Research*, vol. 75, no. 3, pp. 468–477, 2007.

[32] M. Febbraio and R. L. Silverstein, "CD36: implications in cardiovascular disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 11, pp. 2012–2030, 2007.

[33] E. A. Podrez, T. V. Byzova, M. Febbraio et al., "Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype," *Nature Medicine*, vol. 13, no. 9, pp. 1086–1095, 2007.

[34] A. Handberg, M. Norberg, H. Stenlund, G. Hallmans, J. Attermann, and J. W. Eriksson, "Soluble CD36 (sCD36) clusters with markers of insulin resistance, and high sCD36 is associated with increased type 2 diabetes risk," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 4, pp. 1939–1946, 2010.

[35] F. M. H. van Dielen, C. Van't Veer, A. M. Schols, P. B. Soeters, W. A. Buurman, and J. W. M. Greve, "Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals," *International Journal of Obesity*, vol. 25, no. 12, pp. 1759–1766, 2001.

[36] C. M. Steppan and M. A. Lazar, "Resistin and obesity-associated insulin resistance," *Trends in Endocrinology and Metabolism*, vol. 13, no. 1, pp. 18–23, 2002.

[37] M. W. Rajala and P. E. Scherer, "Minireview: the adipocyte—at the crossroads of energy homeostasis, inflammation, and atherosclerosis," *Endocrinology*, vol. 144, no. 9, pp. 3765–3773, 2003.

[38] S. M. Kang, H. M. Kwon, B. K. Hong et al., "Expression of leptin receptor (Ob-R) in human atherosclerotic lesions: potential role in intimal neovascularization," *Yonsei Medical Journal*, vol. 41, no. 1, pp. 68–75, 2000.

[39] S. Yamagishi, D. Edelstein, X. Du, Y. Kaneda, M. Guzmán, and M. Brownlee, "Leptin induces mitochondrial superoxide production and monocyte chemoattractantprotein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A," *Journal of Biological Chemistry*, vol. 276, no. 27, pp. 25096–25100, 2001.

[40] H. Park, H. M. Kwon, H. J. Lim et al., "Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases in vivo and in vitro," *Experimental and Molecular Medicine*, vol. 33, no. 2, pp. 95–102, 2001.

[41] F. Parhami, Y. Tintut, A. Ballard, A. M. Fogelman, and L. L. Demer, "Leptin enhances the calcification of vascular cells artery wall as a target of leptin," *Circulation Research*, vol. 88, no. 9, pp. 954–960, 2001.

[42] R. Faggioni, K. R. Feingold, and C. Grunfeld, "Leptin regulation of the immune response and the immunodeficiency of malnutrition," *The FASEB Journal*, vol. 15, no. 14, pp. 2565–2571, 2001.

[43] A. M. Wallace, A. D. McMahon, C. J. Packard et al., "Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS)," *Circulation*, vol. 104, no. 25, pp. 3052–3056, 2001.

[44] C. S. Mantzoros, "The role of leptin in human obesity and disease: a review of current evidence," *Annals of Internal Medicine*, vol. 130, no. 8, pp. 671–680, 1999.

[45] S. Margetic, C. Gazzola, G. G. Pegg, and R. A. Hill, "Leptin: a review of its peripheral actions and interactions," *International Journal of Obesity*, vol. 26, no. 11, pp. 1407–1433, 2002.

[46] R. A. Karatela and G. S. Sainani, "Interrelationships of factor VII activity and plasma leptin with insulin resistance in coronary heart disease," *Atherosclerosis*, vol. 209, no. 1, pp. 235–240, 2010.

[47] L. Patel, A. C. Buckels, I. J. Kinghorn et al., "Resistin is expressed in human macrophages and directly regulated by PPARγ activators," *Biochemical and Biophysical Research Communications*, vol. 300, no. 2, pp. 472–476, 2003.

[48] R. R. Banerjee, S. M. Rangwala, J. S. Shapiro et al., "Regulation of fasted blood glucose by resistin," *Science*, vol. 303, no. 5661, pp. 1195–1198, 2004.

[49] E. D. Muse, S. Obici, S. Bhanot et al., "Role of resistin in diet-induced hepatic insulin resistance," *Journal of Clinical Investigation*, vol. 114, no. 2, pp. 232–239, 2004.

[50] S. Galic, J. S. Oakhill, and G. R. Steinberg, "Adipose tissue as an endocrine organ," *Molecular and Cellular Endocrinology*, vol. 316, no. 2, pp. 129–139, 2010.

[51] M. H. Zhang, B. Na, N. B. Schiller, and M. A. Whooley, "Association of resistin with heart failure and mortality in patients with stable coronary heart disease: data from the heart and soul study," *Journal of Cardiac Failure*, vol. 17, no. 1, pp. 24–30, 2011.

[52] Y. Miyamoto, H. Morisaki, Y. Kokubo et al., "Resistin gene variations are associated with the metabolic syndrome in Japanese men," *Obesity Research and Clinical Practice*, vol. 3, no. 2, pp. 65–74, 2009.

[53] M. S. Burnett, J. M. Devaney, R. J. Adenika, R. Lindsay, and B. V. Howard, "Cross-sectional associations of resistin, coronary heart disease, and insulin resistance," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, supplement 1, pp. 64–68, 2006.

[54] B. Youn, K. Yu, H. J. Park et al., "Plasma resistin concentrations measured by enzyme- linked immunosorbent assay using a newly developed monoclonal antibody are elevated in individuals with type 2 diabetes mellitus," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 1, pp. 150–156, 2004.

[55] B. H. Chen, Y. Song, E. L. Ding et al., "Circulating levels of resistin and risk of type 2 diabetes in men and women: results from two prospective cohorts," *Diabetes Care*, vol. 32, no. 2, pp. 329–334, 2009.

[56] K. F. Nolan, D. R. Greaves, and H. Waldmann, "The human interleukin 18 gene IL18 maps to 11q22.2-q22.3, closely linked to the DRD2 gene locus and distinct from mapped IDDM loci," *Genomics*, vol. 51, no. 1, pp. 161–163, 1998.

[57] C. A. Dinarello, D. Novick, A. J. Puren et al., "Overview of interleukin-18: more than an interferon-γ inducing factor," *Journal of Leukocyte Biology*, vol. 63, no. 6, pp. 658–664, 1998.

[58] S. Kashiwamura, H. Ueda, and H. Okamura, "Roles of interleukin-18 in tissue destruction and compensatory reactions," *Journal of Immunotherapy*, vol. 25, supplement 1, pp. S4–S11, 2002.

[59] B. Lindegaard, A. E. Hansen, H. Pilegaard, P. Keller, J. Gerstoft, and B. K. Pedersen, "Adipose tissue expression of IL-18 and HIV-associated lipodystrophy," *AIDS*, vol. 18, no. 14, pp. 1956–1958, 2004.

[60] S. Blankenberg, G. Luc, P. Ducimetière et al., "Interleukin-18 and the risk of coronary heart disease in European men: the Prospective Epidemiological Study of Myocardial Infarction (PRIME)," *Circulation*, vol. 108, no. 20, pp. 2453–2459, 2003.

[61] Y. Gu, K. Kuida, H. Tsutsui et al., "Activation of interferon-γ inducing factor mediated by interleukin-1β converting enzyme," *Science*, vol. 275, no. 5297, pp. 206–209, 1997.

[62] H. Tsutsui, N. Kayagaki, K. Kuida et al., "Caspase-1-independent, Fas/Fas ligand-mediated IL-18 secretion from macrophages causes acute liver injury in mice," *Immunity*, vol. 11, no. 3, pp. 359–367, 1999.

[63] G. Fantuzzi and C. A. Dinarello, "Interleukin-18 and interleukin-1β: two cytokine substrates for ICE (caspase-1)," *Journal of Clinical Immunology*, vol. 19, no. 1, pp. 1–11, 1999.

[64] Z. Kato, J. Jee, H. Shikano et al., "The structure and binding mode of interleukin-18," *Nature Structural Biology*, vol. 10, no. 11, pp. 966–971, 2003.

[65] D. Novick, B. Schwartsburd, R. Pinkus et al., "A novel IL-18BP ELISA shows elevated serum IL-18BP in sepsis and extensive decrease of free IL-18," *Cytokine*, vol. 14, no. 6, pp. 334–342, 2001.

[66] Y. Oikawa, A. Shimada, A. Kasuga et al., "Systemic administration of IL-18 promotes diabetes development in young non-obese diabetic mice," *Journal of Immunology*, vol. 171, no. 11, pp. 5865–5875, 2003.

[67] K. Esposito, A. Pontillo, M. Ciotola et al., "Weight loss reduces interleukin-18 levels in obese women," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 8, pp. 3864–3866, 2002.

[68] C. P. Fischer, L. B. Perstrup, A. Berntsen, P. Eskildsen, and B. K. Pedersen, "Elevated plasma interleukin-18 is a marker of insulin-resistance in type 2 diabetic and non-diabetic humans," *Clinical Immunology*, vol. 117, no. 2, pp. 152–160, 2005.

[69] Q. Yang, T. E. Graham, N. Mody et al., "Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes," *Nature*, vol. 436, no. 7049, pp. 356–362, 2005.

[70] M. C. Young, B. Youn, H. Lee et al., "Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes," *Diabetes Care*, vol. 29, no. 11, pp. 2457–2461, 2006.

[71] S. Gavi, L. M. Stuart, P. Kelly et al., "Retinol-binding protein 4 is associated with insulin resistance and body fat distribution in nonobese subjects without type 2 diabetes," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, supplement 5, pp. 1886–1890, 2007.

[72] S. Roh, S. Song, K. Choi et al., "Chemerin—a new adipokine that modulates adipogenesis via its own receptor," *Biochemical and Biophysical Research Communications*, vol. 362, no. 4, pp. 1013–1018, 2007.

[73] M. C. Ernst and C. J. Sinal, "Chemerin: at the crossroads of inflammation and obesity," *Trends in Endocrinology and Metabolism*, vol. 21, no. 11, pp. 660–667, 2010.

[74] X. Du and L. L. K. Leung, "Proteolytic regulatory mechanism of chemerin bioactivity," *Acta Biochimica et Biophysica Sinica*, vol. 41, no. 12, pp. 973–979, 2009.

[75] B. A. Zabel, S. J. Allen, P. Kulig et al., "Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades," *Journal of Biological Chemistry*, vol. 280, no. 41, pp. 34661–34666, 2005.

[76] Y. Aso, S. Matsumoto, Y. Fujiwara, K. Tayama, T. Inukai, and Y. Takemura, "Impaired fibrinolytic compensation for hypercoagulability in obese patients with type 2 diabetes: association with increased plasminogen activator inhibitor-1," *Metabolism*, vol. 51, no. 4, pp. 471–476, 2002.

[77] K. Bozaoglu, K. Bolton, J. McMillan et al., "Chemerin is a novel adipokine associated with obesity and metabolic syndrome," *Endocrinology*, vol. 148, no. 10, pp. 4687–4694, 2007.

[78] W. Johanna, N. Markus, W. Josef et al., "Systemic chemerin is related to inflammation rather than obesity in type 2 diabetes," *Clinical Endocrinology*, vol. 72, no. 3, pp. 342–348, 2010.

[79] D. Stejskal, M. Karpisek, Z. Hanulova, and M. Svestak, "Chemerin is an independent marker of the metabolic syndrome in a Caucasian population—a pilot study," *Biomedical papers of the Medical Faculty of the University Palacký*, vol. 152, no. 2, pp. 217–221, 2008.