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Original Article

Effects of Vitamin D on adropine and apoptosis in kidney tissue

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Abstract

Aim: This study aims to investigate the effects of vitamin D on adropin and apoptosis in rat kidney tissue in the context of the experimental diabetes model created using streptozotocin (STZ).

Material and Methods: 41 male Wistar-albino breed rats of 8-10 weeks were distributed into 5 groups, which consisted of 3 groups with 7 animals each and 2 groups with 10 animals each. No treatments were applied to the control group. The Buffer group was administered with single-dose 0.1 M sodium buffer intraperitoneally (ip). The Vitamin D group was orally administered 200 IU/day vitamin D. The Diabetes group was injected ip with single-dose 50 mg/kg STZ by dissolving the material in 0.1 M sodium buffer.

Results: The biochemical and histological investigations revealed similar serum TOS and TAS levels, and TUNEL positivity and Adropin immunoreactivity for the Control, Buffer, and Vitamin D groups. While TOS levels and TUNEL positivity were significantly higher in the Diabetes group compared to the Control group, TAS levels and Adropin immunoreactivity were significantly lower. The TOS levels and TUNEL positivity were significantly reduced in the Diabetes+Vitamin D group compared to the diabetic group, and TAS levels, adropin immunoreactivity were significantly higher.

Conclusion: In conclusion; it was determined that experimental diabetes increased TOS and apoptotic cells and decreased TAS and adropin levels in the kidney tissue in experimental diabetes, and that Vitamin D administered as treatment decreased TOS and apoptotic cells and increased TAS and Adropin levels. It was concluded that in order to uncover the role of diabetes in the pathophysiology of its effect on kidney tissue, future studies that consider various experimental diabetes times were necessary.

Keywords: Streptozotocin; diabetes mellitus; kidney; adropin

INTRODUCTION

Adropin is a factor that both prevents the development of glucose intolerance and regulates lipid homeostasis by reducing obesity-induced hepatosteatosis and hyperinsulinemia. Adropin has no role in food intake; its fundamental function is to prevent insulin resistance, dyslipidemia, and glucose tolerance. Adropin is made up of 76 amino acids and has a molecular weight of 4499.9 Da. Its half-life has not yet been revealed with certainty. The physiological and biochemical effects of adropin regulate nitric oxide (NO) bioactivity, reduce lipogenic gene expression, decrease dyslipidemia, decrease hepatosteatosis, decrease disrupted glucose tolerance, decrease insulin resistance, and regulate energy balance [1].

Vitamin D is a group of sterols that regulates calcium and phosphorus metabolisms, is among fat-soluble vitamins, and differently from other vitamins, has hormone and hormone-precursors that can be produced in the body [2,3]. It has been stated in numerous studies conducted to investigate the relationship between vitamin D and and diabetes mellitus.25(OH)D, which is a vitamin D indicator, is lower in individuals with Type 2 diabetes compared to those without diabetes, that plasma vitamin D levels affect

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insulin resistance, that insulin sensitivity and release decrease in cases where these levels are lacking, and that insulin receptors in promonocytic cells are reduced [4]. Another study showed that supplemental vitamin D decreased insulin resistance in patients with Type 2 diabetes [5].

Studies have shown that Vitamin D has antioxidant effects [6]. Building on this, a study that evaluated the relationship of oxidative stress with vitamin D and calcium balance showed that calcium balance and mitochondrial membrane potentials were altered under oxidative stress [7]. This change results in damage to the mitochondria and DNA, and causes the cell to undergo programmed death; apoptosis. Disruption of intracellular electron balance, oxidative stress, mitochondrial defects, and inadequate antioxidant stress may induce apoptosis [7].

The aim of this study is to investigate the effects of vitamin D on adropin and apoptosis in the kidney tissue of rats with experimental diabetes.

MATERIAL and METHODS

This study was conducted at Fırat University Experimental Research Center in collaboration with the Department of Histology at Fırat University Faculty of Medicine, and approval was obtained from Fırat University Animal Studies Ethics Committee (Project no: 2017/55).

Induction of Diabetes

In order to create diabetes in 20 rats that would be used in this part of the study, single-dose 50 mg/kg STZ (Streptozocin, Zanosar, Pharmacia, France), which was dissolved in 0.4 ml (0.1 M) sodium-buffer (pH: 4,5), was administered intraperitoneally (ip) with a 26 gauge insulin injector. Blood was drawn from the tail vein after 72 hours, and rats that had fasting blood glucose > 250 mg/dl in the glucometer device were considered diabetic. Glucostix (Myles, Ekhart, IN) did measurement of blood glucose. Blood samples to determine the fasting blood glucose levels of the rats were obtained between 8-10 am after 8-10 hours of fasting.

Laboratory Animals

Male Wistar albino rats used in the experiments were supplied from Fırat University Experimental Research Center.

Formation of the Experimental Groups

41 Wistar-albino breed rats of 8-12 weeks with weights varying between 200-220 gr were assigned to 5 groups:

Group

I (Control Group) (n=7): No procedures were carried out over the 8-week experimental period. Fasting glucose levels at the beginning and in the end of the experiment were measured and recorded.

Group II (Buffer Group) (n=7): Single-dose 0.1 M sodium Buffer was administered ip. Glucose levels at the beginning and in the end of the experiment were measured and recorded.

Group III (Vitamin D group) (n=7): 200 IU/day Vitamin D was administered orally using a dropper every day throughout the 8-week experimental period. Glucose levels at the beginning and in the end of the experiment were measured and recorded routinely.

Group IV (Diabetic group) (n=10): Single-dose 50 mg/kg STZ was administered ip after being dissolved in 0.1 M sodium buffer (pH: 4.5). Those with blood glucose levels above 250 mg/dl in blood drawn from the tail vein after 72 hours were considered diabetic and glucose levels at the beginning and in the end of the experiment were measured and recorded.

Group V (Diabetes+Vitamin D group) (n=10): Single-dose 50 mg/kg STZ was administered ip after being dissolved in 0.1 M sodium buffer (pH: 4.5). Those with blood glucose levels above 250 mg/dl in blood drawn from the tail vein after 72 hours were considered diabetic. After experimental diabetes was created, 50 IU/day Vitamin D was administered orally using a dropper every day during the experimental period. Glucose levels at the beginning and in the end of the experiment were measured and recorded.

Extraction of Samples

After being weighed at the end of the experiment, rats in all groups were administered with ketamine (75mg/kg)+xylazine(10mg/kg) intraperitoneal and decapitated under anesthesia. Following decapitation, kidney tissues of the rats were rapidly extracted. Kidney tissues from all groups were fixed in a 10% formaldehyde solution for histological evaluations.

Biochemical Work

Measurements of Total Antioxidant (TAS) and Total Oxidant (TOS) Levels

Measurements of TAS and TOS levels were performed according to the total antioxidant activity and total oxidant activity methods defined in the literature (8, 9). The unit of the measurement results was µmol / l.

TUNEL Method

5-6 µm sections obtained from paraffin blocks were transferred to polylysine slides. Cells that underwent apoptosis were determined using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA) according to the instructions of the manufacturer.Tissues deparaffinized with xylene were passed through graded alcohol series and washed with phosphate buffered saline (PBS). The tissues incubated with 0.05% proteinase K for 10 minutes were then incubated with 3% hydrogen peroxide for 5 minutes in order to prevent endogenous peroxidase activity. After the tissues were washed again with PBS, they were incubated with Equilibration Buffer for 6 minutes and then were incubated for 60 minutes with working solution (70% µl Reaction Buffer+30% TdT Enzyme) at 37º C in a humid environment. The tissues were then kept in Stop/Wash

Buffer for 10 minutes and treated with Anti-Dioxigenin-Peroxidase for 30 minutes. Apoptotic cells were viewed with the diaminobenzidine (DAB) substrate. The sections were counterstained with Harris hematoxylin and covered with the appropriate covering solution. The developed preparations were evaluated by viewing under a Leica DM500 microscope and their images were captured (Leica DFC295). In the evaluations of TUNEL staining, nuclei stained blue with Harris hematoxylin were considered normal, and the cells where the nuclei manifested a brown stain were considered apoptotic. At least 500 normal and apoptotic cells were counted in randomly selected regions of the sections under 10x magnification. The Apoptotic index (AI) was calculated based on the ratio of apoptotic cells to total (normal+apoptotic) cells and statistical analyses were performed.

Immunohistochemistry

4-6 mm sections obtained from paraffin blocks were transferred to polylysine slides. Deparaffinized tissues were passed through graded alcohol series and boiled in Buffer solution at pH:6 in a microwave oven (750W) for 7+5 minutes for the purpose of antigen retrieval. The tissues which were kept at room temperature for 20 minutes to ensure cooling after being boiled were washed with PBS (Phosphate Buffered Saline, P4417, Sigma-Aldrich, USA) for 3x5 minutes and then were incubated with hydrogen peroxide block solution for 5 minutes in order to prevent endogenous peroxidase activity (Hydrogen Peroxide Block , TA-125-HP, Lab Vision Corporation, USA). The tissues, which had been washed again with PBS for 3x5 minutes, were then treated with the Ultra V Block (TA–125-UB, Lab Vision C.orporation, USA) solution for 5 minutes to prevent non-specific background staining and were incubated for 60 minutes with primary antibodies. **Those were** adropin and betatrophin (anti-adropin antibody, ab122800, Abcam, Cambridge, UK and betatrophin Polyclonal Antibody, PA5-38043, Invitrogen, USA) diluted 1:200, at room temperature in a humid environment. After the tissues underwent the primary antibody treatment, they were washed with PBS for 3x5 minutes and then incubated with the secondary antibody (biotinylated Goat Anti-Polyvalent (anti-mouse / rabbit IgG), TP–125-BN, Lab Vision Corporation, USA) for 30 minutes at room temperature in a humid environment. Following the secondary antibody treatment, the tissues were washed with PBS for 3x5 minutes, incubated with Streptavidin Peroxidase (TS–125-HR, Lab Vision Corporation, USA) for 30 minutes at room temperature in a humid environment, and transferred to PBS. Then the 3-amino-9-ethylcarbazole (AEC) Substrate+AEC Chromogen (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA) solution was dropped onto the tissues and once an image signal was obtained under the light microscope, they were simultaneously washed with PBS. Tissues that were counterstained with Mayer's hematoxylin were then passed through PBS and distilled water, and covered with the appropriate covering solution (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA). The developed preparations were inspected under a Leica DM500 microscope and images were captured (Leica DFC295).

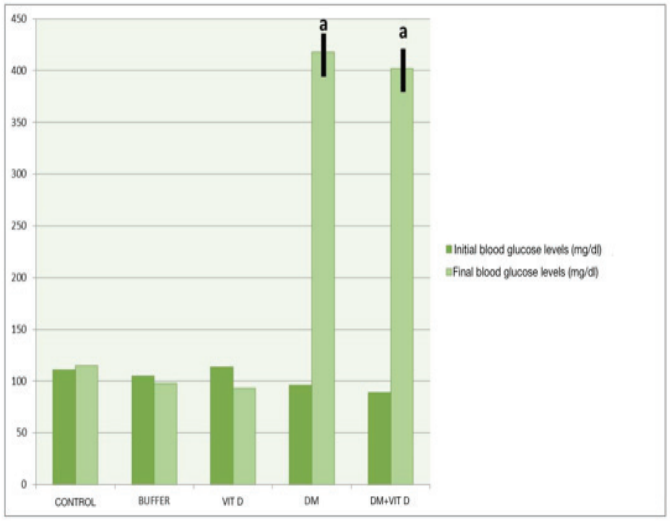
A histoscore was determined based on the diffusiveness (0.1: <25%, 0.4: 26-50%, 0.6: 51-75%, 0.9:76-100%) and intensity (0: none, +0.5: very weak, +1: weak, +2: median, +3: strong) of immunoreactivity in staining. Histoscore= diffusiveness x intensity.

Statistical Analysis

The obtained data were defined as mean±standard deviation. The SPSS Version 22 software was used for the statistical analyses. Cross-group evaluations were done by One-way ANOVA and Posthoc Tukey tests. p-values<0.05 were considered statistically significant.

RESULTS

Evaluations of the initial and final blood-glucose levels of rats in all groups revealed no change in the blood-glucose levels of rats in the Control, Buffer, and Vitamin D groups compared to their initial values. However, blood-glucose levels of rats in the Diabetes and Diabetes+Vitamin D groups were higher than their initial values with statistical significance (p<0.05) (Figure 1).



Values are presented as mean±standard deviation
[a] Compared to the initial blood-glucose levels (p<0.05)

Figure 1. Initial and final blood-glucose levels of the experimental animals (mg/dl)

The results from the evaluations of TUNEL staining performed to determine apoptotic cells under light microscopy revealed TUNEL positivity in the tubular cells in kidney tissue (red arrow). TUNEL positivity was similar for the Control (A), Buffer (B), and Vitamin D (C) groups. TUNEL positivity was found to have increased in the Diabetes (D) group with statistical significance when compared to the Control group (A) (p<0.05). When compared to the Diabetes group, TUNEL positivity of

the Diabetes+Vitamin D group was significantly reduced (p<0.05). Apoptotic index (%) (Figure 2, 3).

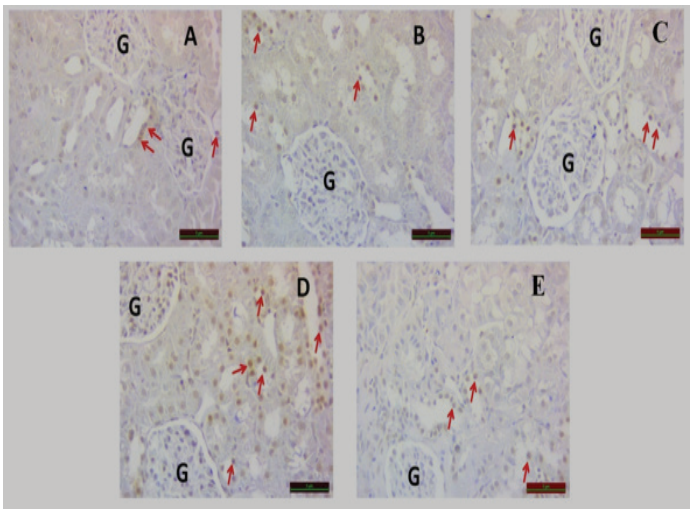
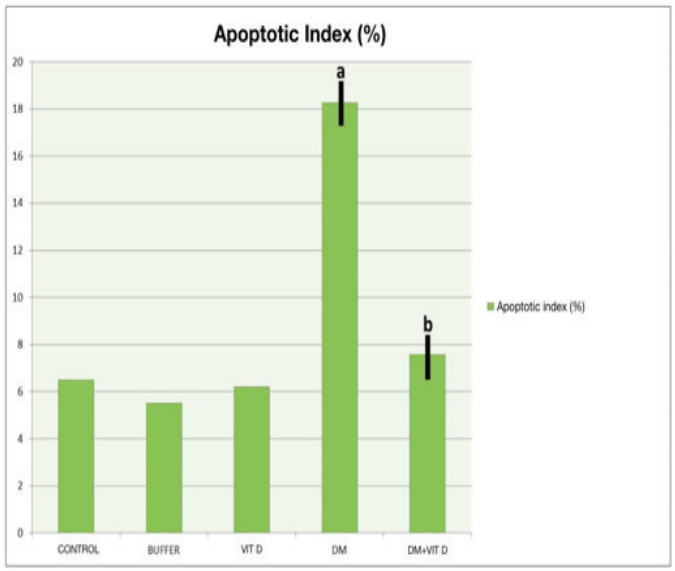


Figure 2. TUNEL positivity (A: control group, B: buffer group, C: vitamin D group, D: diabetes group, E: diabetes + vitamin D group), G: Glomerule



Values are presented as mean±standard deviation
[a] Compared to the control group
[b] Compared to the diabetes group (p<0.05)

Figure 3. Apoptotic Index

Evaluations of the immunohistochemical staining, which was done to investigate adropin immunoreactivty under light microscopy revealed Adropin immunoreactivty in the Glomerules in kidney tissue (red arrow). Adropin immunoreactivity in kidney tissue was similar for the Control (A), Buffer (B) and Vitamin D (C) groups. For the Diabetes (D) group, adropin immunoreactivtity was significantly reduced compared to the control group (p<0.05). Adropin immunoreactivity was found to have increased in the Diabetes+Vitamin D (E) group compared to the Diabetes group with statistical significance (p<0.05). Histoscore (Figure 4, 5).

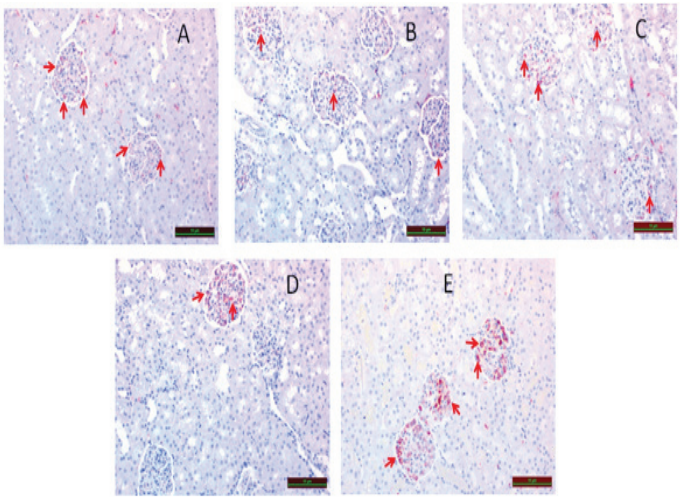
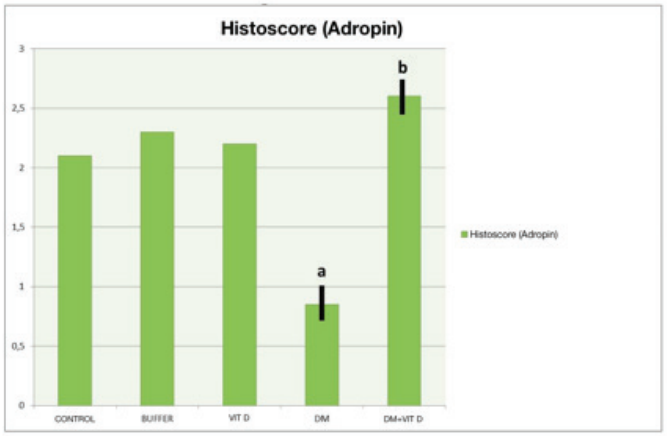
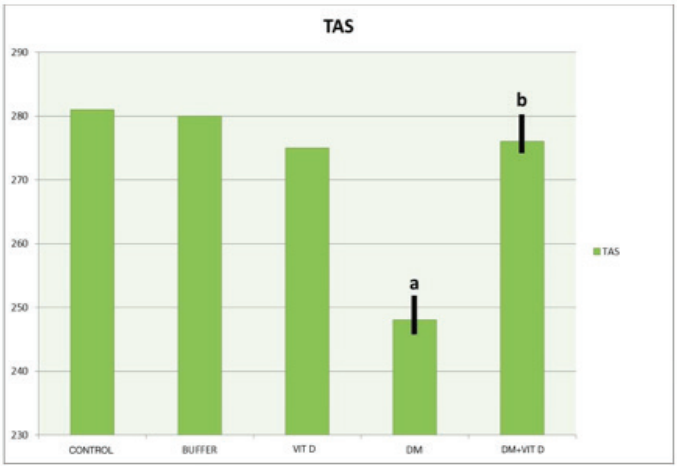


Figure 4. ADROPIN immunoreactivity (A: control group, B: buffer group, C: vitamin D group, D: diabetes group, E: diabetes + vitamin D group), G: Glomerule



Values are presented as mean±standard deviation
[a] Compared to the control group

Figure 5. Adropin Histoscore

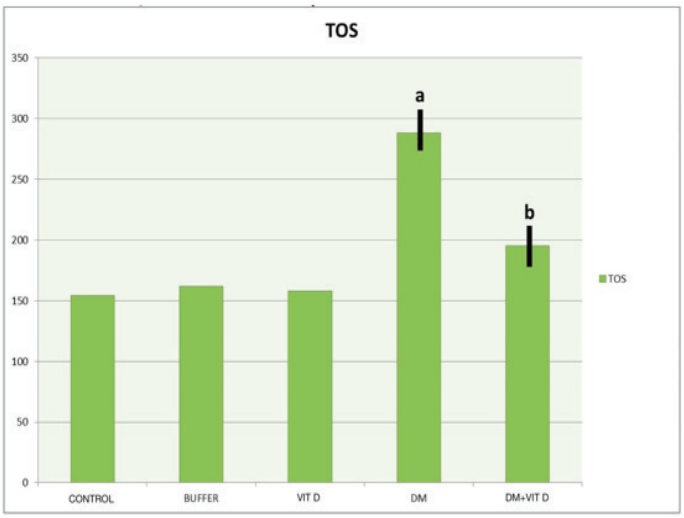


Values are presented as mean±standard deviation
[a] Compared to the control group
[b] Compared to the diabetes group (p<0.05)

Figure 6. Serum TAS levels

Biochemical work done to assess serum TAS levels of all groups revealed that the TAS levels were similar for the Control, Buffer, and Vitamin D groups. TAS levels were reduced in the Diabetes group when compared to the Control group with statistical significance ($p<0.05$). TAS levels of the Diabetes+Vitamin D group were higher than those of the Diabetes group with statistical significance ($p<0.05$) (Figure 6).

Biochemical work done to assess serum TOS levels of all groups revealed that the TOS levels were similar for the Control, Buffer, and Vitamin D groups. TOS levels were higher in the Diabetes group when compared to the Control group with statistical significance ($p<0.05$). TOS levels of the Diabetes+Vitamin D group were lower than those of the Diabetes group with statistical significance ($p<0.05$) (Figure 7).



Values are presented as mean±standard deviation
[a] Compared to the control group
[b] Compared to the diabetes group ($p<0.05$)

Figure 7. Serum TOS levels

DISCUSSION

Diabetes mellitus is a significant public health problem of our time. Its prevalence is continuously increasing in our country and throughout the world, and as a result, related complications are encountered more frequently. Of the chronic complications of Diabetes, macro and microvascular complications, particularly coronary artery disease and diabetic nephropathy, significantly reduce survival times of the patients and impair life quality [10-12].

It has been proven that adropin, which bears the structure of a humoral polypeptide, is produced by multiple biological tissues including the liver, brain, pancreas, kidney, and cerebellum [13,14], and is also related to glucose and lipid metabolisms. Furthermore, it was stated that reduced adropin levels were related to increased adipose tissue and insulin resistance [14]. Adropin immunoreactivity was detected in the glomerules, peritubular interstitial cells and peritubular capillaries in the kidney tissue [15]; in the endocardium, myocardium, and pericardium in heart tissue, and in the serous acini in the pancreas [13]. Physiological and biochemical effects of adropin regulate NO bioactivity, reduce lipogenic gene expression, reduce dyslipidemia, reduce hepatosteatosis, reduce disrupted glucose tolerance, reduce insulin resistance, and regulates energy balance [1].

Numerous studies that have investigated the relationship between vitamin D and Diabetes Mellitus. They have stated that 25(OH)D levels, which is an indicator of vitamin D, are lower in individuals with Type 2 diabetes compared to non-diabetics, that vitamin D levels in plasma affect insulin resistance, and that in the case that these levels are inadequate, insulin sensitivity and release decrease, and insulin receptors in promonocytic cells are reduced [4]. Another study showed that supplemental vitamin D decreased insulin resistance in patients with Type 2 diabetes [5].

Diabetes Mellitus is connected to increased production of reactive oxygen types, inadequacy of antioxidant defense mechanisms, and increased oxidative stress as a result [16,17]. In the case of oxidative stress, calcium balance and mitochondrial membrane potential are altered. This change causes damage to the mitochondria and DNA, and leads the cell to programmed death; apoptosis [18]. Disruption of intracellular electron balance, oxidative stress, mitochondrial defects, and inadequacy of the antioxidant system can induce the emergence of apoptosis [19].

In our study, apoptosis was significantly higher in the kidney tissues of the DM group compared to the Control group and was significantly reduced in the DM+Vit D group with statistical significance. TOS levels, which were checked as the increased apoptosis in the DM group could be related to oxidative stress, also showed a significant increase. Besides, decreased apoptosis in the DM+Vit D group may be explained by the antioxidant effect of Vit D. Accordingly, the TOS levels of the DM+Vit D group presented a significant decrease.

In an animal study by Kumar et al. [20], which was the very first study that was conducted on the topic, a striking significant difference was found between the serum adropin levels of rats fed with nutrients of high carbohydrate-low fat content and those fed with nutrients of high fat-low carbohydrate content. They showed that serum adropin levels in the blood were higher in the case of high fat intake, and that levels of the ENHO gene, which codes adropin, were much higher in the liver tissues of rats fed with nutrients of high fat content in the very same study. The pronounced increase in ENHO gene levels suggested that the adropin hormone could have a role in the lipogenesis metabolism in the liver. Furthermore, it was shown that excess transgenic production of the adropin molecule in rats caused the other genes that play a role in lipogenesis in the liver and adipose tissue at the cellular level to be found at lower levels [20]. Another study showed that the deficiency of the adropin hormone was related to increase in adipose tissue and insulin

resistance and concluded that the adropin molecule could be connected to the glucose metabolism, insulin resistance, dyslipidemia, and metabolic syndrome [21].

In 2010, Lovren et al. [22] revealed the protective potential of adropin in endothelial cells. They determined that adropin activated the vascular endothelial growth factor receptor-2 (VEGFR-2) and its forward signaling pathways phosphoinositide 3-kinase/serin, threonine kinase(PI3K/Akt) and signal-regulated kinases 1/2 (ERK 1/2), hence modulating the expression of adropin eNOS. At the same time, adropin increases the proliferation and migration of endothelial cells as well as the formation of capillary-like structures. It was recently determined that adropin decreased endothelial permeability [22]. Up to now, the mechanisms underlying decreased adropin levels in T2DM patients remain elusive. Studies performed by Gao et al. showed that adropin played a crucial role in modulating glucose utilization in rats [23, 24]. Adropin, which is thought to regulate insulin resistance for the maintenance of glucose homeostasis, is also considered to increase NO secretion and potentially play a role in the repair of endothelial damage with iNOS activation [14]. On the other hand, in our study, immunohistochemical staining was utilized to investigate adropin immunoreactivity, which is a hormone discovered in relation to the energy metabolism in the recent years for the reason that it could be involved in the pathogenesis of diabetic nephropathy. Adropin immunoreactivity was significantly reduced in the kidney tissues of rats in the DM group when compared to the control group, and was higher in the DM+Vit D group with statistical significance. The increased adropin immunoreactivity in the diabetic group in our study could be related to increased oxidative damage. Hyperglycemia was shown to trigger oxidative stress and lead to endothelial dysfunction. Besides, the increased adropin levels in the DM+Vit D group may be explained by a reduction in endothelial dysfunction due to the antioxidant effect of Vit D.

CONCLUSION

In conclusion, it was determined that experimental diabetes increased TOS and apoptotic cells and decreased TAS and adropin levels in the kidney tissue, and that Vitamin D administered as treatment decreased TOS and apoptotic cells and increased TAS and adropin levels. It was concluded that future studies involving various experimental diabetes times were necessary to uncover the role of diabetes in the pathophysiology of its effect on kidney tissue.

Competing interests: The authors declare that they have no competing interest.

Financial Disclosure: There are no financial supports.

Ethical approval: This study was conducted at Fırat University Experimental Research Center in collaboration with the Department of Histology at Fırat University Faculty of Medicine, and approval was obtained from Fırat University Animal Studies Ethics Committee (Project no: 2017/55).

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