Effects of vitamin D on apoptosis and betatrophin in the kidney tissue of experimental diabetic rats

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Summary. The aim of this study is to investigate the effects of vitamin D on betatrophin and apoptosis in rats kidney tissue using an experimental diabetes model created with streptozotocin (STZ). 41 male Wistar-albino breed rats were assigned to 5 groups, which included 3 groups consisting of 7 animals each and 2 groups consisting of 10 animals each. The control group received no treatments. Single-dose 0.1 M sodium buffer was administered ip to the Buffer group. 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. Subjects with a glucose level exceeding 250 mg/dl were accepted to be diabetic. The Diabetes + Vitamin D group was injected ip with 50 mg/kg single-dose STZ by dissolving the material in 0.1 M sodium buffer. Once diabetes was established, 200 IU/day vitamin D was administered orally. Rats in all groups were decapitated in the end of the experiment, their kidney tissues were promptly extracted and TUNEL stained with immunohistochemistry. Additionally, serum samples acquired from all groups were evaluated with regard to total antioxidant status (TAS) and total oxidant status (TOS) levels. The histological and biochemical analyses of the Control, Buffer, and Vitamin D groups revealed similar serum TOS and TAS levels, and TUNEL positivity and betatrophin immunoreactivity. While the Diabetes group showed significantly higher TOS levels and TUNEL positivity compared to the Control group, their TAS levels and betatrophin immunoreactivity were significantly reduced. The Diabetes+Vitamin group demonstrated significantly lower TOS levels and TUNEL positivity compared to the Diabetic group, and their TAS levels and betatrophin immunoreactivity increased significantly. In conclusion; experimental diabetes was found to increase TOS and apoptotic cells and decrease TAS and betatrophin levels in kidney tissue in experimental diabetes, and that administering VitD as treatment caused a decrease in TOS and apoptotic cells and an increase in TAS and betatrophin levels. It was concluded that future studies needed to investigate various experimental diabetes times so that the role of diabetes in the pathophysiology of its effect on kidney tissue could be uncovered.

Key words: streptozotocin, diabetes mellitus, kidney, betatrophin

Introduction

Diabetes mellitus (DM) is a chronic disease characterized by microvascular and macrovascular complications that appear due to an insulin deficiency or issues connected to the effects of insulin (1). Diabetes is accountable for 30–40% of the causes of kidney disease in the United States in the recent term, and is also among the most significant long-term complications leading to mortality and morbidity (2). Recent
studies have found increased free oxygen radicals and lipid peroxidation to play a role in the pathogenesis of numerous diseases (3). Studies performed on rats with experimental diabetes showed significantly higher free oxygen radicals and lipid peroxidation, and determined that oxidative stress was involved in the etiology and progression of diabetes (4). Furthermore, it was found that the chronic complications of diabetes could appear in connection to increased oxidative stress and changes in antioxidant capacity (5, 6).

In the study they conducted on rats in 2008, Arthur D. Melton and colleagues (7) identified a protein that was released from the liver in insulin resistance and lead to replication in beta cells in the pancreas. This protein named betatrophin brought about a significant improvement in the regenerative treatment, which aims to increase beta cell mass and insulin secretion in Diabetes Mellitus (7).

Betatrophin belongs to the family of angiopoietin-like proteins and is encoded by the C19orf80 gene. In rats, it is expressed in the liver, white adipose cells, and brown adipose cells; while in humans, it is only expressed in the liver (8, 9). Betatrophin, which is among the novel biomarkers that are known to be involved in diabetes and lipid metabolism; bears the names ANGPTL8, RIFL, and lipasin due to its effect on fasting/feeding regulation and the regulation of lipoprotein lipase (LPL) activity (10, 11). The first study performed on rats showed that ANGPTL8(betatrophin)-/- rats had low levels of circulating triglyceride (TG) (12). This protein was previously named RIFL by Ren and colleagues (10), who showed that it was expressed in the adipose tissue and liver and was regulated via nutrients and hormonal factors (10).

The discovery of ANGPTL8 as a hormone that could increase beta cell proliferation was considered a scientific breakthrough (13). ANGPTL8 was shown to increase in cases of insulin resistance where beta-cell proliferation and insulin production increase (7). These findings, particularly the ability of ANGPTL8 to increase beta-cell proliferation, were later questioned (14, 15). Similarly, many studies have reported increased ANGPTL8 levels in Type 2 DM subjects. A phenomenon of ANGPTL8 resistance was suggested in individuals with Type 2 DM whose beta cells did not respond to increased ANGPTL8 levels (16, 17).

Vitamin D is a group of sterols regulating calcium and phosphorus metabolisms that is among fat-soluble vitamins, and differently from other vitamins, has hormone and hormone-precursors that can be produced in the body (18-20). Several studies researching the relationship between vitamin D and Diabetes Mellitus have stated that 25(OH)D, which is a vitamin D indicator, is reduced in those with Type 2 diabetes in comparison to those without diabetes, that plasma vitamin D levels influence insulin resistance, that insulin sensitivity and release decrease when these levels are inadequate, and that insulin receptors in promonocyte cells are reduced (21). It was shown by another study that insulin resistance decreased in patients with Type 2 diabetes who were administered supplemental vitamin D (11). Vitamin D was shown to have antioxidant effects (12). Accordingly, one study that investigated the relationship of oxidative stress with vitamin D and calcium balance determined that, under oxidative stress, calcium balance and mitochondrial membrane potentials were altered (13). This change causes damage to the mitochondria and DNA, and leads the cell to undergo programmed termination; apoptosis. Apoptosis may be induced by disturbance of intracellular electron balance, oxidative stress, mitochondrial defects, and inadequacy of the antioxidant system (24).

This study aims to investigate the effects of vitamin D on betatrophin and apoptosis in the kidney tissue of rats with experimental diabetes.

**Materials and methods**

This study was conducted at Firat University Experimental Research Center in collaboration with the Department of Histology at Firat University Faculty of Medicine, and approval was obtained from Firat University Animal Studies Ethics Committee.

**Laboratory Animals**

Male Wistar albino rats used in the experiments were supplied from Firat University Experimental Research Center.
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Induction of Diabetes
In order to create diabetes in 20 rats to be used in this section 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) using a 26 gauge insulin injector. Blood was obtained from the tail vein after 72 hours, and rats with a fasting blood glucose > 250 mg/dl in the glucometer device were accepted to be diabetic. Blood glucose was measured by Glucostix (Myles, Ekhart, IN). For fasting blood glucose levels of the rats, blood samples were obtained between 8-10 am following 8-10 hours of fasting.

Formation of the Experimental Groups
41 8-12-week-old Wistar-albino breed rats weighing between 200-220 gr were distributed to 5 groups:

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

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

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 were measured and recorded routinely at the beginning and in the end of the experiment.

Group IV (Diabetic group) (n=10): Single-dose 50 mg/kg STZ was administered ip by being dissolved in 0.1 M sodium buffer (pH: 4.5). Subjects with blood glucose levels exceeding 250 mg/dl in blood obtained from the tail vein following 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). Subjects with blood glucose levels exceeding 250 mg/dl in blood obtained from the tail vein following 72 hours were considered diabetic. Once experimental diabetes was established, 50 IU/day Vitamin D was administered orally using a dropper every day throughout the experimental period. Glucose levels were measured and recorded at the beginning and in the end of the experiment.

Extraction of Samples
Rats in all groups were weighed at the end of the experiment, administered with ketamine(75 mg/kg)+xylazine(10 mg/kg) ip, and decapitated under anesthesia. Kidney tissues of the rats were promptly extracted after decapitation. Kidney tissues from all groups were fixed in a 10% formaldehyde solution for histological assessment.

Biochemical Work
Measurements of Total Antioxidant (TAS) and Total Oxidant (TOS) Levels
TAS and TOS levels were measured based on the total antioxidant activity and total oxidant activity methods described in the literature (25, 26). Measurement results were presented in units of µmol/l.

TUNEL Method
5-6 µm sections obtained from paraffin blocks were transferred to polylysine slides. Cells that underwent apoptosis were identified with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA) based on the manufacturer’s instructions. Tissues were deparaffinized with xylene, passed through graded alcohol series, and washed with phosphate buffered saline (PBS). The tissues were first incubated with 0.05% proteinase K for 10 minutes, and were then incubated with 3% hydrogen peroxide for 5 minutes to prevent endogenous peroxidase activity. The tissues were washed again with PBS, incubated with Equilibration Buffer for 6 minutes, and then were incubated with working solution (70% µl Reaction Buffer+30% TdT Enzyme) for 60 minutes at 37ºC in a humid environment. The tissues were then kept in Stop/Wash Buffer for 10 minutes and treated with Anti-Digoxigenin-Peroxidase for 30 minutes. Apoptotic cells were visualized with the diaminobenzidine (DAB) substrate. The sections were counterstained
with Harris hematoxylin and covered with the appropriate covering solution. The developed preparations were inspected under a Leica DM500 microscope and images were captured (Leica DFC295). In evaluating TUNEL staining, nuclei stained blue with Harris hematoxylin were considered normal, and the cells where the nuclei demonstrated a brown stain were considered apoptotic. At least 500 normal and apoptotic cells were counted in randomly chosen regions of the sections under 10x magnification. The Apoptotic index (AI) was calculated according to the ratio of apoptotic cells to total (normal+apoptotic) cells and statistical analyses were conducted.

**Immunohistochemistry**

4-6 mm sections were obtained from paraffin blocks and transferred to polylysine slides. Deparaffinized tissues were passed through graded alcohol series and boiled in a microwave oven (750W) for 7+5 minutes in pH:6 Buffer solution for antigen retrieval. In order to ensure cooling after being boiled, the tissues were kept at room temperature for 20 minutes, 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 to prevent endogenous peroxidase activity (Hydrogen Peroxide Block, TA–125-HP, Lab Vision Corporation, USA). In order to avoid non-specific background staining, 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 Corporation, USA) solution for 5 minutes. They were incubated with the primary antibody betatrophin (betatrophin Polyclonal Antibody, PA5- 38043, Invitrogen, USA) diluted 1:200 for 60 minutes at room temperature in a humid environment. Following the primary antibody treatment, the tissues were washed with PBS for 3x5 minutes and then incubated with the secondary antibody (biotinylated Goat Anti-Polyvalent (anti-rats/rabbit IgG), TP–125-BN, 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 they were simultaneously washed with PBS once an image signal was obtained under the light microscope. Tissues counterstained with Mayer’s hematoxylin were then passed through PBS and distilled water, and covered with the suitable covering solution (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA). The developed preparations were examined 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 presented as mean ± standard deviation. The SPSS software (Version 22) was utilized for the statistical analyses. One-way ANOVA and Posthoc Tukey tests were used for cross-group evaluations. p-values<0.05 were considered statistically significant.

**Results**

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

TUNEL staining performed to determine apoptotic cells under light microscopy was evaluated and TUNEL positivity was determined in the tubular cells in kidney tissue (red arrow). Control (A), Buffer (B), and Vitamin D (C) groups manifested similar TUNEL positivity patterns. The Diabetes (D) group
manifested increased TUNEL positivity with statistical significance when compared to the Control group (p<0.05). TUNEL positivity of the Diabetes+Vitamin D group was significantly reduced when compared to the Diabetes group (p<0.05). Apoptotic index (%)

Betatrophin

Evaluations of the immunohistochemical staining, which was done to investigate betatrophin immunoreactivity under light microscopy revealed; Betatrophin immunoreactivity in the parts of the tubules facing the lumen in kidney tissue (red arrow). Betatrophin immunoreactivity in G (Glomerule) kidney tissue was similar for the Control (A), Buffer (B) and
Vitamin D (C) groups. For the Diabetes (D) group, betatrophin immunoreactivity was significantly reduced compared to the control group (p<0.05). Betatrophin 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).

According to the biochemical work done to assess serum TAS levels of all groups, the Control, Buffer, and Vitamin D groups had similar TAS levels. The Diabetes group had lower TAS levels when compared to the Control group with statistical significance (p<0.05). The Diabetes+Vitamin D group had increased TAS levels compared to those of the Diabetes group with statistical significance (p<0.05) (Figure 6).

According to the biochemical work done to assess serum TOS levels of all groups, the Control, Buffer, and Vitamin D groups had similar TOS levels. The Diabetes group had higher TOS levels when compared to the Control group with statistical significance (p<0.05). The Diabetes+Vitamin D group had reduced TOS levels compared to those of the Diabetes group with statistical significance (p<0.05) (Figure 7).

Figure 3. Apoptotic Index
Values are presented as mean±standard deviation
a Compared to the control group
b Compared to the diabetes group (p<0.05)

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

The prevalence of diabetes mellitus is gradually increasing in our country and around the world, and as a consequence, the associated complications are observed more frequently. Among the chronic complications of Diabetes that markedly reduce survival times and impair the life quality of patients are macro and microvascular complications, particularly coronary artery disease and diabetic nephropathy (27-29).

Betatrophin belongs to the family of angiopoietin-like proteins and is encoded by the C19orf80 gene. In rats, it is expressed in the liver, white adipose cells, and brown adipose cells; while in humans, it is only expressed in the liver (8, 9). Betatrophin, which is among the novel biomarkers that are known to be involved in diabetes and lipid metabolism; bears the names ANGPTL8, RIFL, and lipasine due to its effect on fasting/feeding regulation and the regulation of lipoprotein lipase (LPL) activity (10, 11). The first study performed on rats showed that ANGPTL8(betatrophin)-/- rats had low levels of circulating triglyceride (TG) (12). This protein was previously named RIFL by Ren and colleagues, who showed that it was expressed in the adipose tissue and liver and was regulated via nutrients and hormonal factors (10). This protein named betatrophin brought about a remarkable improvement in the regenerative treatment, which aims to increase beta cell mass and insulin secretion in Diabetes Mellitus (30). Several studies have shown increased levels of serum betatrophin in obesity and diabetes (31-33).

Numerous studies conducted to investigate the relationship between vitamin D and Diabetes Mellitus have stated that 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 insulin resistance, that insulin sensitivity and release decrease in cases where these levels...
are lacking, and that insulin receptors in promonocyte cells are reduced (34). Another study showed that insulin resistance decreased with supplemental vitamin D in patients with Type 2 diabetes (35).

Diabetes Mellitus is linked to greater production of reactive oxygen types, inadequacy of antioxidant defense mechanisms, and higher oxidative stress as a consequence (36, 37). Calcium balance and mitochondrial membrane potential undergo changes under oxidative stress. This change results in damage to the mitochondria and DNA, and causes the cell to undergo programmed death; apoptosis (38). Apoptosis may be induced by a disturbance of the intracellular electron balance, oxidative stress, mitochondrial defects, and inadequacy of the antioxidant system (39).

In this study, the kidney tissues of the DM group demonstrated significantly higher apoptosis compared to the Control group, and apoptosis was significantly lower in the DM+Vit D group. TOS levels, which were inspected as the increased apoptosis in the DM group could be linked to oxidative stress, also showed a significant increase. Moreover, reduced apoptosis shown by the DM+Vit D group may be connected to the antioxidant effect of Vit D. Accordingly, there was a significant decrease in the TOS levels of the DM+Vit D group.

Chen et al. (40) stated in their study that serum betatrophin levels were significantly higher in type 2 DM patients with various stages of albuminuria and that betatrophin could be a novel endocrine regulator associated with the development of diabetic nephropathy.

In our study, as a hormone discovered in relation to the energy metabolism in the recent years, betatrophin immunoreactivity was investigated using immunohistochemical staining for the reason that it could be involved in the pathogenesis of diabetic nephropathy. Betatrophin immunoreactivity was significantly reduced in the kidney tissues of the DM group when compared to the control group, and was higher in the DM+Vit D group with statistical significance.

It is also known that the glomerular basal membrane, tubular basal membrane, and Bowman capsule thicken during the development of diabetic nephropathy. Although diabetic nephropathy has generally been considered primarily a glomerular disease, recently, the rate of functional disruption is considered more consistent with tubulointerstitial fibrosis (41).

Arthur D. Melton and colleagues (42) reported that the overexpression of ANGPTL8 “betatrophin” in the rats liver caused a 17-fold increase in β-cell proliferation and a 3-fold increase in β-cell mass. Moreover, they showed that ANGPTL8 was released into circulation and then bound to β-cells via an unidentified receptor that leads to β-cell proliferation and mass (42).

Jiao and colleagues (43) showed that the transplantation of human and rats islets treated with insulin receptor antagonist (S961) under the kidney capsule later resulted in an increase in ANGPTL8 levels and beta-cell proliferation in the transplanted rats islets (43).

Betatrophin levels, which decreased in kidney tissues of the DM group and increased in the DM+Vit D group, suggest that its low levels in the late course of DM may be due to its use and exhaustion in the kidney tissue. The immunohistochemical study we conducted determined the area of betatrophin immunoreactivity as the areas of tubular cells facing the lumen.

Conclusion

In conclusion, experimental diabetes was determined to be connected to increased TOS and apoptotic cells and decreased TAS and betatrophin levels in the kidney tissue, and it was found that VitD administered as treatment resulted in a decrease in TOS and apoptotic cells and an increase in TAS and betatrophin levels. It was concluded that future studies needed to investigate various experimental diabetes times so that the role of diabetes in the pathophysiology of its effect on kidney tissue could be uncovered.

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

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