Vitamin D supplementation alleviates diabetic complications by increasing the amount of irisin in testicular tissues and blood of rats with experimental diabetes

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ber of apoptotic cells and increased the amount of irisin. Vitamin D had an effective role in maintaining the physiological integrity of rat testicular tissues, so vitamin D may be a potent agent to be used in the treatment of diabetes in the future.

Key Words: Experimental diabetes, Testis, Irisin, Vitamin D, Apoptosis.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease in which the organism cannot adequately benefit from carbohydrates, fats, and proteins in the organism due to the deficiency of the insulin hormone or the deficiencies in the effect of the insulin hormone, and it is among the most important public health problems of our era. Long-term diabetes disrupts the structure of all vessels by affecting vascular cells and basement membranes and causes many complications such as microangiopathy, retinopathy, nephropathy, and neuropathy. In addition, in terms of the male reproductive system, DM negatively affects the testicles by disrupting the structures of sperm cells, or by changing the hormone levels that affect the development of sperm cells, or both. Apoptosis is increased in testes with diabetes, and this causes dysfunction in the testicles. Also, oxidative stress increases due to diabetes (due to hyperglycemia), and in this case, it is known that this triggers excessive production of reactive oxygen species (ROS) and free radicals. The increase in oxidative stress in rats with experimental diabetes, and diabetic patients occurs as a result of an excessive increase in ROS and a decrease in the antioxidant defense mechanism. As stated above, the damages caused by diabetes to biological systems in various ways are innumerable.

In recent years, there have been several studies showing that vitamin D plays an important role in the regulation of blood insulin levels and plays a role in glucose metabolism. Vitamin D and its metabolites control apoptosis and inhibit the growth and proliferation of malignant cells. Also, vitamin D has an antioxidant effect by increasing the expression of G6PDH (glucose 6 phosphate dehydrogenase), which has a protective effect against oxidative stress, in addition to its strong anti-proliferative, pro-differentiative, and immunomodulatory effects. Although vitamin D alleviates the harmful complications of diabetes, it has not been fully revealed yet which molecule or molecules mediate other than the above-mentioned mechanisms.

A molecule called irisin, which is responsible for energy metabolism consisting of 112 amino acids and weighing 12.587 Daltons, has been discovered in recent years. It was found that irisin is expressed in almost all biological tissues such as intracranial arteries, kidneys, myelin sheath, neural cells, optic nerve, retina, thyroid, ovaries, Purkinje cells, rectum, salivary glands, eccrine sweat gland, stomach, lungs, tongue, liver, small intestine, dermis and hypodermis of skin, seminiferous tubules, spermaticogenic cells, and Leydig cells in fetal human testicles, Leydig cells in adult human testis and epididymis, and it is first synthesized from skeletal muscle. The main physiological role of irisin is to turn white adipose tissue into brown adipose tissue, causing heat release in the organism and thus mediating weight loss.

Since there is a direct relationship between insulin resistance and obesity and diabetes, it is inevitable that there may be a relationship between irisin mediating weight loss and diabetes. Because irisin decreased in fasted animals and increased in fattened animals, and it was suggested that this might be related to insulin resistance.

Considering the above basic information altogether, the study aimed to reveal (1) the histopathology of testicular tissue, (2) irisin immunoreactivity and apoptosis status, blood TAS and TOS values, (3) testicular tissue supernatant and blood irisin amounts and the effects of vitamin D on these values by experimentally inducing diabetes with STZ in rats.

Materials and Methods

The study was approved by the Animal Experiments Ethics Committee of Firat University with the decision No. 210 in the session numbered 22, and dated 16.12.2015. In this study, 41 Wistar albino male rats, 8-10 weeks old, weighing between 200-220 g, were randomly divided into 5 groups as follows: Control (no treatment, n=7), Sham (a single dose of 0.1 M sodium citrate buffer were administered ip to the rats in this group, n=7), vitamin D group (during the experiment, 50 IU/day of vitamin D was administered to the rats orally every day, via a dropper, n=7). Diabetes group [Streptozotocin was dissolved in 0.1 M sodium citrate buffer (pH: 4.5) and administered to rats as a single dose of 50 mg/kg ip, n=10] and Diabetes+vitamin-D group [Streptozotocin was dissolved in 0.1 M sodium citrate buffer (pH: 4.5) and administered to rats as a single dose of 50...
mg/kg ip, n=10]. Rats were fed ad libitum and kept in standard cages at 22-25°C ambient temperature, 12 hours of light (07:00-19:00) and 12 hours of darkness (19:00-07:00) in the FUDAM animal laboratory.

In our study, the formation of experimental diabetes with Streptozocin (STZ) was performed as follows, based on the study that previously created experimental diabetes with STZ. In order to induce diabetes, Streptozocin (STZ, Zanosar, Sicor Pharmaceuticals, Inc., Tel Aviv, Israel) was dissolved in 0.4 mL (0.1 M) sodium citrate buffer (pH: 4.5) with a 26 gauge insulin injector, and a single dose of 50 mg/kg was given ip to 20 rats in the diabetes and diabetes+vitamin D groups. Blood samples were drawn from the tail vein of the rats 72 hours after this administration. Rats with fasting blood glucose of >250 mg/dL as a result of measuring the blood taken in a glucometer device were considered as diabetic. Measurement of blood glucose was performed with the Glucostix (Miles, Elkhart, IN, USA) device. In order to determine the fasting blood glucose levels of rats, blood samples were taken between 8-10 am after 8-10 hours of fasting. After the experimental diabetes was established, vitamin D 50 IU/day was administered to the animals orally, via a dropper, every day during the experimental period of 8 weeks.

**Collection, Storage, and Analysis of Biological Samples**

For biochemical analysis, half of the blood from the tail vein was taken into normal biochemistry tubes and the other half into EDTA tubes containing aprotinin (for irisin analysis). These blood samples were immediately centrifuged at 4,000 rpm (1,792 g) and stored at -80°C until studied.

Rats were decapitated under anesthesia by administering ketamine (75 mg/kg) + xylazine (10 mg/kg) ip immediately after blood collection from the tail vein. Testicular tissues of the rats were quickly removed immediately after decapsulation. For the histological study, the right testis was placed in Bouin’s solution and detected. Left testis (200 mg fresh tissue) was taken for biochemical study and stored at -80°C.

**Preparation and Storage of Tissue Homogenates**

200 mg testicular tissue from each rat was washed three times with phosphate buffer solution to remove the blood and other debris. Then, each tissue was placed in Eppendorf tubes containing 500 KIU/mL, 200 mL of zirconium oxide beads with 0.5 mm diameter were placed in each Eppendorf tube. Phosphate buffer was added to the samples taken so that the final volumes of all samples were 1 mL. The caps of microcentrifuge tubes were closed and placed in the homogenizer (Bullet Blender, BBY24M-CE, NY, USA). Tissues were homogenized in a homogenizer for 5 minutes. The supernatant was transferred to another Eppendorf tube. After centrifugation at 4,000 rpm for another 10 minutes, the supernatant was separated to be used in ELISA studies and the resulting tissue supernatants were stored at -80°C until studied.

**Biochemical Analyses**

**Glucose measurement**

With the Glucostix (Miles, Elkhart, IN, USA) glucometer device, glucose was measured from biological samples.

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**Table I.** Demographic, obstetrics, and fetal characteristics of vitamin D replacement and control group.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=20)</th>
<th>Group 2 (n=20)</th>
<th>Group 3 (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>400IU/day Vitamin D</td>
<td>600 IU/day Vitamin D</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.6±5.03</td>
<td>28.3±3.50</td>
<td>27.1±4.11</td>
</tr>
<tr>
<td>VD level (ng/mL)*</td>
<td>15.9±4.07</td>
<td>14.5±2.09</td>
<td>23.2±4.02</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.80±1.02</td>
<td>2.87 ±1.03</td>
<td>2.43±0.20</td>
</tr>
<tr>
<td>Parity</td>
<td>1.78 ±0.33</td>
<td>1.67±0.55</td>
<td>1.43±0.21</td>
</tr>
<tr>
<td>Gestational age at birth (weeks)</td>
<td>36.5±6.44</td>
<td>37.3±3.04</td>
<td>36.8±5.01</td>
</tr>
<tr>
<td>Fetal birth weight (gr)</td>
<td>2840.8±302.6</td>
<td>2887.7±230.5</td>
<td>2790.5±320.4</td>
</tr>
<tr>
<td>GDM (n/%)</td>
<td>0</td>
<td>1 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>PIH (n/%)</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Mode of delivery (n/%)</td>
<td>13 C/S (65%),</td>
<td>15 C/S (75%),</td>
<td>14 C/S (70%),</td>
</tr>
<tr>
<td></td>
<td>7 vaginal birth (35%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: Indicates the existence of statistical significance between groups (One-way ANOVA). Parameters without an asterisk indicate that there is no statistical significance between groups.
the blood taken from the tail vein at the beginning of the experiment, at the end of the experiment, and at regular intervals following the STZ application.

**TAS and TOS Measurements**

TAS\(^{21}\) and TOS\(^{22}\) were determined by the Erel technique in an autoanalyzer (Olympus, Hamburg, Germany), using Rel Assay Kits (Mega Tip Ind. Trade. Co. Ltd, Gaziantep, Turkey) and the unit of TAS was given as Trolox Equiv./L\(^{21}\), while the unit of TOS was µmol H\(_2\)O\(_2\) Equiv./L\(^{22}\).

**Measurement of Irisin Levels**

Irisin levels in the blood and testicular supernatants of rats were determined according to the manufacturer’s instructions specified in the catalogs of the Irisin Enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). While the intra assay coefficient of variance (CV) value of the ELISA kit used was 8%, the inter assay CV value was 12%. The minimum measurement limit of the kit was 0.05 ng/mL. In order to demonstrate the accuracy of irisin measurements in tissue supernatants, biochemical assay validity tests (linearity, recovery, specificity, sensitivity, intra and inter assay tests) were performed as previously described\(^{19}\). Irisin level was determined to be measured with the same sensitivity in tissue supernatants. Automatic washer Bio-Tek ELX50 (BioTek Instruments, Winooski, VT, USA) device was used for plate washing, Bio-Tek ELX800 device was used for absorbance readings and Panasonic printer was used for printing results. Test results were expressed as ng/mL.

**Tissue Follow-Up, Sectioning and Staining of Tissues by Hematoxylin-Eosin Method**

Right testis tissues from all rats were fixed in Bouin’s solution (Biognost, Zagreb, Croatia) and then passed through routine histological follow-up series\(^{23}\). Then, testicular tissues were embedded into the paraffin blocks, and 5-6 μm thick sections were obtained from these paraffin blocks with a microtome (Leica, Wetzlar, Germany). Sections were stained with the hematoxylin-eosin method\(^ {24}\). The sections stained were examined and photographed under a research microscope\(^ {25}\).

**Staining of Tissues with the Immunohistochemical Method**

The immunoperoxidase method was used for the immunohistochemical analyses in the study\(^ {26,27}\). For immunohistochemical staining, 5-6 μm thick sections from paraffin blocks were taken on polylysine slides. Sections taken were deparaffinized and passed through graded alcohol series. After this process, it was boiled for 15 minutes in a microwave oven (750 W) in citrate buffer solution (pH=6) for antigen retrieval. After boiling, the tissues were kept at room temperature for approximately 20 minutes to cool. Tissues cooled at room temperature were washed with PBS (phosphate buffered saline, Sigma-Aldrich, Saint Louis, MO, USA) 3 times for 5 minutes, for a total of 15 minutes. Then, they were incubated with hydrogen peroxide block solution for 5 minutes to prevent endogenous peroxidase activity in the tissues (Hydrogen peroxide Block, Lab Vision Corporation, Fremont, CA, USA). The tissues were washed with PBS for 3x5 minutes, and Ultra V Block (Lab Vision Corporation, Fremont, CA, USA) solution was applied for 5 minutes to prevent non-specific binding. After these procedures, the tissues were incubated with the primary antibody (irisin Rabbit Polyclonal H-067-17, Phoenix Pharmaceuticals, Burlingame, CA, USA) diluted at 1/200 concentration for 60 minutes at room temperature in a humidified environment. Following the primary antibody application, the tissues were washed with PBS for 3x5 minutes and then incubated with secondary antibody [biotinylated Goat Anti-Polyvalent (anti-mouse/rabbit IgG), TP-125-BN, Lab Vision Corporation, Fremont, CA, USA] for 30 minutes at room temperature in a humidified environment. After the secondary antibody application, the tissues were washed again with PBS for 3x5 minutes and incubated with Streptavidin Peroxidase (Lab Vision Corporation, Fremont, CA, USA) for 30 minutes at room temperature in a humidified environment. Following the incubation, the tissues were washed again with PBS. 3-amino-9-ethylcarbazole (AEC) Substrate+AEC Chromogen (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, Lab Vision Corporation, Fremont, CA, USA) solution was dropped onto the tissues and as soon as the image signal was obtained under the light microscope, the tissues were washed with PBS. Tissues were counterstained with Mayer’s hematoxylin. After counterstaining, the tissues were rinsed with PBS and distilled water. After that, the stained tissues were covered with the appropriate mounting medium (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, Fremont, CA, USA). The prepared sections were examined and imaged under the Leica DM500 microscope (Leica, Wetzlar, Germany). Rat heart tissue was
used as the positive control tissue. Rabbit IgG was used as the negative control. In the immunohistochemical staining, histoscore (prevalence x severity) was performed based on the prevalence (0.1: <25%, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and severity (0: none, +0.5: very little, +1: little, +2: moderate, +3: severe) of immunoreactivity.

TUNEL Staining of Tissues
Terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) method was used in order to reveal apoptosis. It was performed in accordance with the manufacturer's procedure (ApopTag plus peroxidase in situ apoptosis detection kit, Merck, Darmstadt, Germany). In the evaluation of TUNEL staining, nuclei observed in blue color with Harris hematoxylin were considered normal, while the cells with brown nuclear staining were considered apoptotic. At least 500 normal and apoptotic cells were counted in randomly selected areas using 10X magnification in the sections. The apoptotic index was calculated by dividing apoptotic cells to total cells (normal+apoptotic) and statistical analyses were performed accordingly. Rat heart tissue was used as the positive control tissue. Instead of a working solution, a reaction buffer was applied as the negative control.

Statistical Analysis
Statistical analyses were performed using the SPSS 22 program (IBM Corp., Armonk, NY, USA) and the data obtained were recorded as the mean ± standard deviation. One-way ANOVA and post hoc Tukey tests were used for intergroup evaluation. The value p < 0.05 was considered statistically significant.

Results
While the live weight measured at the end of the experiment in rats belonging to the control, sham, and vitamin D groups showed a statistically significant increase compared to the live weight measured at the beginning of the experiment, it was shown that the live weight measured at the end of the experiment in the rats in diabetes and diabetes+vitamin D groups showed a statistically significant decrease compared to the live weight measured at the beginning of the experiment in contrast to the control, sham, and vitamin D groups (p < 0.05) (Table I).

Glucose levels were similar at the beginning and end of the experiment in the control, Sham (Citrate buffer), and vitamin D groups. It was detected that the blood glucose levels measured at the end of the experiment in rats in diabetes and diabetes+vitamin D groups increased statistically significantly compared to the blood glucose levels measured at the beginning (p < 0.05). Table I shows the initial and final blood glucose levels of the experimental animals.

No statistically significant difference was found when blood TAS and TOS values of control, Sham (Citrate buffer), and vitamin D groups were compared among themselves. However, it was found that TAS values showed a statistically significant decrease in the diabetes group, while TOS values increased statistically significantly (p < 0.05). In addition, TAS levels significantly increased in the diabetes+vitamin D group compared to the diabetes group, while it was found that TOS levels statistically significantly decreased (p < 0.05). Table I shows serum TAS and TOS levels of all groups.

When blood TAS and TOS values of control,
Sham (Citrate buffer) and vitamin D groups were compared among themselves, no statistically significant difference was found. However, blood and tissue amounts in the diabetes group showed a statistically significant decrease, while the irisin amount showed a statistically significant increase in the Diabetes+vitamin D group when compared to the diabetes group \((p < 0.05)\). Table II shows the tissue and blood irisin levels of all groups.

Spermatogenic series cells in the seminiferous tubules of the testicular tissues and Leydig cells in the interstitial space of rats belonging to control, Sham, and vitamin D groups were normal in appearance according to the results of hematoxylin-eosin staining (Figure 1A, 1B, 1C). There was no separation in the basement membranes of the testicular tissues of these groups. Clear edema in the interstitial spaces between the seminiferous tubules (Figure 1Da, 1Db), separation in Leydig cells (Figure 1Da, 1Db), marked congestion around some seminiferous tubules (Figure 1Dc), basal membrane separation (Figure 1Db), and atrophic seminiferous tubules in some areas were observed in testicular tissues belonging to the diabetes group (Figure 1D). Atrophic tubule structure and congestion around seminiferous tubules were not observed when the testis tissues belonging to the diabetes+vitamin D group were examined. However, although there was a decrease in edema in the interstitial areas (Figure 1Ea), basement membrane separation was present, but it was milder than the basement membrane separation in the diabetes group (Figure 1Eb).

Rat heart tissue was used as the positive control tissue (Figure 2B). Breast tissue was used as the negative control tissue and no TUNEL positivity was observed (Figure 2A). When TUNEL staining results in testicular tissues belonging to control (Figure 2C), sham (Figure 2D) and vitamin D (Figure 2E) groups were examined, apoptotic cells were observed in the spermatogenic series cells in the seminiferous tubules in a small number, and no apoptotic cells were detected in Leydig cells in the interstitial area (Figure 2C, 2D, 2E). When apoptotic cells in the testicular tissues of the diabetes group (Figure 2F) were compared to the control, sham, and vitamin D groups, they were found to be statistically significantly increased in the spermatogenic series cells in the seminiferous tubules \((p < 0.05)\).

Apoptotic cells in the testicular tissues of the diabetes+vitamin D (Figure 2G) group were found to be statistically significantly increased in the spermatogenic series cells in the seminiferous tubules compared to the control group and decreased compared to the diabetes group \((p < 0.05)\). No apoptotic cells were observed in Leydig cells in the interstitial area. The apoptotic index is given in Table III.

No irisin immunoreactivity was observed in the negative control tissue (PBS was applied instead of irisin antibody) (Figure 3A). Irisin immunoreactivity was detected in rat heart tissue used as the positive control tissue (Figure 3B). Immunoreactivity of irisin in testicular tissues of all rats was observed in Leydig cells in the interstitial area. Irisin immunoreactivity was observed in Leydig cells in the interstitial area in testicular tissues of control (Figure 3C), sham (only citrate buffer was given, Figure 3D), and vitamin D group (Figure 3E), and the intensity of irisin immunoreactivity was similar in these three groups. There was a decrease in irisin immunoreactivity \((p < 0.05)\) (Figure 3F) when the diabetes group was compared to the control (Figure 3C) and sham (only citrate buffer was given) groups (Figure 3D), and an increase in irisin immunoreactivity was observed with the administration of vitamin D. The irisin immunoreactivity in the testicular tissues of the diabetes+vitamin D group was found to be statistically significantly decreased when compared to the control group, but there was a statistically significant increase when compared to the diabetes group \((p < 0.05)\) (Figure 3G). The irisin immunoreactivity scores of all groups are given in Table III.

### Table II. Comparison of blood and tissue irisin amounts of experimental and control groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C n = 7</th>
<th>S n = 7</th>
<th>Vit D n = 7</th>
<th>DM n = 10</th>
<th>DM + Vit D n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>6.65 ± 0.77</td>
<td>7.37 ± 0.67</td>
<td>7.44 ± 0.71</td>
<td>2.80 ± 0.43</td>
<td>7.42 ± 0.66</td>
</tr>
<tr>
<td>Tissue</td>
<td>6.02 ± 0.22</td>
<td>6.80 ± 0.42</td>
<td>6.52 ± 0.92</td>
<td>2.25 ± 0.36</td>
<td>6.40 ± 0.47</td>
</tr>
</tbody>
</table>

C, Control; DM, Diabetes Mellitus; S, Sham; Vit D, Vitamin D. Values are given as mean ± standard deviation. a, vs. Control group \((p < 0.05)\); b, vs. DM group \((p < 0.05)\).

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**Discussion**

It has been reported\[^{30}\] that diabetes causes various dysfunctions in biological systems and organ...
Irisin in rats with diabetes and Vit-D

failures, especially eye, kidney, heart, and vascular damage. It has also been reported\textsuperscript{31-34} that diabetes adversely affects gonadal functions in both humans and experimental animals, which leads to low testosterone levels, testicular dysfunction, and inadequate spermatogenesis. Therefore, in this study, the effects of vitamin D on body weights, irisin in testicular tissue, apoptosis, blood irisin levels, TAS and TOS values of experimentally diabetic rats were investigated for the first time using histological and biochemical methods.

The body weight at the end of the experiment in rats of the control, sham, and vitamin D groups was found to be increased when compared to the

![Figure 1](image-url)

Figure 1. Hematoxylin and Eosin staining in testicular tissues. A, Testicular tissue of the control group. Sertoli cell (black arrow), spermatogonium (red arrow), primary spermatocyte (yellow arrow), secondary spermatocyte (brown arrow), spermatozoa (blue arrow), Leydig cell (green arrow); (B), Testicular tissue belonging to the citrate buffer group. Sertoli cell (black arrow), spermatogonium (red arrow), primary spermatocyte (yellow arrow), secondary spermatocyte (brown arrow), spermatozoa (blue arrow), Leydig cell (green arrow); (C), Testicular tissue belonging to Vitamin D group. Sertoli cell (black arrow), spermatogonium (red arrow), primary spermatocyte (yellow arrow), secondary spermatocyte (brown arrow), spermatozoa (blue arrow), Leydig cell (green arrow); (D), Testicular tissue belonging to the diabetes group. Edema in interstitial areas (red stars) (a-b), separation in Leydig cells (red arrows) (a-b), congestion around seminiferous tubules (black stars) (e), basal membrane separation (black arrows) (b), atrophic seminiferous tubule (brown star) (d); (E), Testicular tissue belonging to the diabetes+vitamin D group. Edema in interstitial areas (red star) (a), Basal membrane separation (black arrows) (b). Magnification x 400.

### Table III. Histological evaluations of testicular tissues of experimental and control groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C n = 7</th>
<th>S n = 7</th>
<th>Vit-D n = 7</th>
<th>DM n = 10</th>
<th>DM + Vit D n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNEL Apoptotic Index (%)</td>
<td>4.16 ± 2.13</td>
<td>5.50 ± 2.42</td>
<td>3.66 ± 1.63</td>
<td>10.33 ± 2.65&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>4.33 ± 2.58&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>IHK Irisin Histoscore</td>
<td>2.00 ± 0.58</td>
<td>2.00 ± 0.81</td>
<td>1.85 ± 0.74</td>
<td>0.55 ± 0.21&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.52 ± 0.76&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

C, Control; DM, Diabetes Mellitus; S, Sham; Vit D, Vitamin D. Values are given as mean ± standard deviation. <sup>a</sup>, vs. Control group (p < 0.05); <sup>b</sup>, vs. Sham group (p < 0.05); <sup>c</sup>, vs. Vitamin D group (p < 0.05); <sup>d</sup>, vs. Diabetes Mellitus group (p < 0.05).

Body weight at the beginning of the experiment, while there was a significant decrease in body weight at the end of the experiment compared to the body weight at the beginning of the experiment in diabetes and diabetes+vitamin D groups. Bodyweight loss during the experiment was reported in experimental diabetic mouse and rat studies\textsuperscript{35-38}. Bingöl and Kocamış\textsuperscript{39} in their study on diabetic mice reported that, although not relative to the previous sampling day of each day, mice lost body weight every day compared to that of few days before. In addition, Göçmen et al\textsuperscript{40} reported that there was live weight loss in mice on the 20\textsuperscript{th} and 30\textsuperscript{th} days of the experiment in their study. Also, Wada et al\textsuperscript{36} reported that there was a significant decrease in the live weight of diabetic mice when compared to the mice in the control group 24 weeks after the start of the experiment. Loss of body weight in our study may have resulted from excessive protein degradation in tissues caused by diabetes as Andallu and Varadacharyulu\textsuperscript{41} stated. Also, the insulin hormone organizes the movement of circulating glucose into cells and prevents excessive accumulation of glucose in the blood. The organism weakens in the case of diabetes (in the absence or resistance of insulin) since this organization is disrupted. This could be another possible mechanism of diabetes-induced weight loss observed in animals. The mechanism underlying the weight gain due to vitamin D supplementation in animals is probably due to the fact that vitamin D regulates the insulin level in the blood, facilitating the entry of glucose into cells, and thus both managing and controlling the weight gain. In our study, we reported that rats in the diabetes+vitamin D groups had lower blood glucose levels at the end of the experiment compared to the diabetes group, which supports the mechanism suggested above. Also, diabetes increases the amount of glucose in the blood as shown in this study and previous studies\textsuperscript{42}.

In this study, total antioxidant amounts decreased in the diabetes group while total oxidant amounts increased. Diabetes causes oxidative stress\textsuperscript{4}. The possible reason for the increase in total oxidant levels and decrease in total antioxidant levels in the case of diabetes may be due to the use of antioxidant molecules by cells in order to eliminate oxidative stress caused by diabetes. Moreover, total antioxidant capacity increased with vitamin D supplementation while total oxidant capacity was decreased in our study. This means that vitamin D decreases the damage in biological tissues by increasing the total antioxidant capacity. This is because previous studies\textsuperscript{43,44} have reported that vitamin D decreases oxidative stress and increases antioxidant capacity.
In this study, irisin level in the blood and testicular tissue supernatants was also found to be decreased in the diabetes group when compared to the control group. The possible reason for the decreased irisin level in blood and testicular tissue supernatants may be due to weight loss in case of diabetes. As mentioned above, there was a weight loss due to diabetes in rats. In addition, Huh et al. found decreased levels of circulating irisin after weight loss and suggested that this decrease may be due to a reduction in muscle mass, and our present data support this observation. They also reported that people and rats with diabetes and gestational diabetes had lower serum irisin levels compared to the control group. However, it should be noted that many studies have been performed for serum irisin levels in diabetes, but the results are inconsistent with each other. Again in this study, the irisin level was measured in rat testicular tissue supernatants for the first time and it showed parallelism with blood irisin levels. In other words, the irisin level in the testicular tissues decreased in the case of diabetes. Since there is no other study in the literature, we cannot compare our current results for testicular irises levels and this part needs confirmation. Moreover, in our study, vitamin D supplementation increased irisin levels in blood and testicular tissue supernatants. Possible reasons why vitamin D supplementation may increase irisin may be due to 1) increasing antioxidant capacity, 2) preventing weight loss through regulating insulin sensitivity, and 3) helping the capacity of irisin-secreting tissues by preventing apoptosis in cells.

In this study, there was clear edema in the testicular tissues of diabetic rats, which also caused the separation of Leydig cells in the interstitial areas between the seminiferous tubules, significant congestion around some seminiferous tubules, basement membrane separation, and atrophic seminiferous tubules in some areas. Experimental diabetes study with STZ showed that pathological changes occur in the testicles, tunica albuginea, seminiferous tubules, interstitial connective tissue, and Leydig cells. Also, there was a decrease in spermatogenic series cells in the seminiferous tubules, atrophied tubules, wall thickening in the vessels in the interstitial connective tissue, and inflammatory cell infiltration in testicular tissues of rats of the diabetes.

Vitamin D supplementation eliminates these complications. It probably makes this by regulating the antioxidant capacity, increasing

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**Figure 3.** Irisin immunohistochemical staining in testicular tissues. (A), Negative control tissue; (B), Rat heart tissue that was used as the positive control tissue. Irisin immunoreactivity in cardiac muscle cells (black arrows); (C), Testicular tissue of the control group. Irisin immunoreactivity in Leydig cells (black arrows); (D), Testicular tissue belonging to the citrate buffer group. Irisin immunoreactivity in Leydig cells (black arrows); (E), Testicular tissue belonging to the Vitamin D group. Irisin immunoreactivity in Leydig cells (black arrows); (F), Testicular tissue belonging to the diabetes group. Irisin immunoreactivity in Leydig cells (black arrows); (G), Testicular tissue belonging to the diabetes+vitamin D group. Irisin immunoreactivity in Leydig cells (black arrows). Magnifications: (A), x 40; (B-G), x 400.
lin sensitivity and preventing apoptosis, which is what we have suggested above. This explains why the atrophic tubule structure and the congestion around the seminiferous tubules observed in the testicular tissues of the rats in the diabetes group were not observed in the testicular tissues of the rats in the diabetes+vitamin D group.

Moreover, many studies have also reported that diabetes causes apoptosis. Therefore, in this study, how the apoptosis index changed in rats with experimental diabetes was also investigated. Apoptotic cells increased significantly in spermatogenic cells in the seminiferous tubules in testicular tissues because of diabetes while a significant decrease was detected in apoptotic cells with vitamin D administration. Some studies indicated increased apoptosis in spermatogenic series cells in testicular tissues. The decrease in apoptosis in testicular tissues with vitamin D supplementation shows that the cells are destroyed at a later time point, in other words, the endurance of the cells increases. That is one of the main indicators that vitamin D will take place in some clinical applications in the future.

Conclusions

Clear edema in the Leydig cells located in the interstitial areas between the seminiferous tubules, significant congestion around some seminiferous tubules, basement membrane separation, and formation of atrophic seminiferous tubules in some areas occur in testicular tissues of experimentally diabetic rats. Also, apoptosis occurs because of diabetes, and irisin level decreases both in the circulation and in the testicular tissue supernatants. These negative effects observed in experimental diabetes are alleviated or completely eliminated by vitamin D administration. According to the data of this first study, the clinical scientific contribution of this article is related to the importance of the clinical effectiveness of vitamin D administration in the treatment of male infertility.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

This study was approved by the Animal Experiments Ethics Committee of Firat University.

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