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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Abstract. – **OBJECTIVE:** Diabetes is an important endocrinological disease that has an increasing incidence in the world and affects all biological tissues including testicles. Therefore, this study aimed to reveal the histological and biochemical effects of vitamin D on irisin, apoptosis, total antioxidant status (TAS), and total oxidant status (TOS) in testicular tissues of rats with experimental diabetes.

MATERIALS AND METHODS: 41 male Wistar rats, 8-10 weeks old, weighing between 200-220 g, were included in the study as the following groups: control group (n=7; no treatment), sham group [only sodium citrate buffer (SCB)] [n=7; single dose 0.1 Molar (M) SCB given intraperitoneally (i.p)], vitamin D group (n=7; 50 IU/day given orally), diabetes group [n=10; single dose 50 mg/kg Streptozotocin (STZ) dissolved in 0.1 M SCB and given i.p (tail vein blood glucose level above 250 mg/dl after 72 hours)] and diabetes+vitamin D group [n=10, single dose 50 mg/kg STZ, dissolved in 0.1 M SCB and given i.p (tail vein blood glucose level above 250 mg/dl after 72 hours) and when diabetes occurs, oral vitamin D administration of 50 IU/day)]. At the end of the 8 weeks experiment, blood was drawn from the tail vein of all rats, they were sacrificed and testicular tissues were taken. While the amount of irisin in the blood and testicular tissue supernatants was analyzed with the Enzyme-Linked Immunosorbent Assay (ELISA) method, TAS and TOS measurements were analyzed with the REL method, testicular tissues were analyzed histopathologically, immunohistochemically, and with the TUNEL method.

RESULTS: When the diabetes group was compared with the control and sham groups, it was reported that the amounts of blood and tissue supernatant irisin and TAS significantly decreased and the TOS was significantly increased; a statistically significant increase in irisin and TAS of blood and tissue supernatants and a significant decrease in TOS were detected when diabetes+vitamin D and diabetes groups were compared among themselves. Similar results were obtained in the immunohistochemical studies. Tissue expressions of irisin decreased in the diabetes group compared to the control and sham groups, while the application of vitamin D increased the tissue expressions of irisin. Additionally, when the numbers of apoptotic cells were compared, it was reported that apoptotic cells in the diabetes group increased significantly compared to the control and sham groups, and vitamin D administration significantly decreased the number of apoptotic cells.

CONCLUSIONS: Taken together, vitamin D administration to diabetic rats decreased the num-

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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[1]. Long-term diabetes disrupts the structure of all vessels by affecting vascular cells and basement membranes[2] and causes many complications such as microangiopathy, retinopathy, nephropathy, and neuropathy[3]. 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[4] that this triggers excessive production of reactive oxygen species (ROS) and free radicals. The increase in oxidative stress in rats with experimental diabetes[5], and diabetic patients occurs as a result of an excessive increase in ROS and a decrease in the antioxidant defense mechanism[6]. As stated above, the damages caused by diabetes to biological systems in various ways are innumerable[5,7].

In recent years, there have been several studies[8-10] 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[11]. 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[12]. 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[13]. 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, spermatogenic cells, and Leydig cells in fetal human testicles, Leydig cells in adult human testis and epididymis, and it is first synthesized from skeletal muscle[13,14]. 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[13,15].

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[16,17].

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[18].

In our study, the formation of experimental diabetes with Streptozotocin (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[18].

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[19].

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 decapitation. 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[20].

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[19].

Biochemical Analyses

Glucose measurement

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

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

	Group 1 (n=20) 400IU/day Vitamin D	Group 2 (n=20) 600 IU/day Vitamin D	Group 3 (n=20)
Age (years)	27.6±5.03	28.3±3.50	27.1±4.11
VD level (ng/mL)*	13.9±4.07	14.5±2.09	23.2±4.02
Gravidity	2.80±1.02	2.87 ±1.03	2.43±0.20
Parity	1.78 ±0.33	1.67±0.55	1.43±0.21
Gestational age at birth (weeks)	36.5±6.44	37.3±3.04	36.8±5.01
Fetal birth weight (gr)	2840.8±302.6	2887.7±230.5	2790.5±320.4
GDM (n/%)	0	1 (5%)	0
PIH (n/%)	2 (10%)	2 (10%)	3 (15%)
Mode of delivery (n/%)	13 C/S (65%), 7 vaginal birth (35%)	15 C/S (75%), 5 vaginal birth (25%)	14 C/S (70%), 6 vaginal birth (30%)

*: 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_2O_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 immunohisto-chemical 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[28].

TUNEL Staining of Tissues

Terminal deoxynucleotidyl transferase dUTP nick and labeling (TUNEL) method was used in order to reveal apoptosis[29]. 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[28].

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,

Table I. Demographic and biochemical comparison of experimental and control groups.

Parameter		C n = 7	S n = 7	Vit D n = 7	DM n = 10	DM + Vit D n = 10
DB	BW (g)	185.00 ± 17.30	194.33 ± 24.85	199.00 ± 18.86	205.33 ± 24.43	180.60 ± 21.33
	GLU (mg/dL)	103.83 ± 9.96	103.00 ± 17.01	109.83 ± 17.61	106.83 ± 10.571	83.50 ± 37.21
DF	BW (g)	272.66 ± 33.03[a]	277.00 ± 31.23[a]	298.40 ± 9.60[a]	151.33 ± 8.77[a]	141.20 ± 16.26[a]
	GLU (mg/dL)	93.50 ± 17.89	108.33 ± 19.46	105.83 ± 14.68	440.16 ± 48.28[a]	478.40 ± 18.21[a]
	TOS	160.00 ± 17.61	151.33 ± 10.26	154.20 ± 18.39	272.25 ± 21.77[b,c,d]	191.80 ± 10.89[e]
	TAS	293.00 ± 17.69	286.50 ± 18.05	286.40 ± 15.82	250.25 ± 11.55[b,c,d]	296.60 ± 17.61[e]

BW, Body Weight; C, Control; DM, Diabetes Mellitus; DF, Diet finish; DB, Diet begin; GLU, Glucose; S, Sham; TAS, Total Antioxidant Status; TOS, Total Oxidant Status; Vit D, Vitamin D. Values are given as mean ± standard deviation. [a], *vs.* Diet Start Group ($p < 0.05$); [b], *vs.* Control Group ($p < 0.05$); [c], *vs.* Sham Group ($p < 0.05$); [d], *vs.* Vitamin D Group ($p < 0.05$); [e], *vs.* Diabetes Mellitus Group ($p < 0.05$).

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

Parameter	C n = 7	S n = 7	Vit D n = 7	DM n = 10	DM + Vit D n = 10
Serum	6.65 ± 0.77	7.37 ± 0.67	7.44 ± 0.71	2.80 ± 0.43[a]	7.42 ± 0.66[b]
Tissue	6.02 ± 0.22	6.80 ± 0.42	6.52 ± 0.92	2.25 ± 0.36[a]	6.40 ± 0.47[b]

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 ($p < 0.05$).

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.

Discussion

It has been reported[30] that diabetes causes various dysfunctions in biological systems and organ

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

Parameters	C n = 7	S n = 7	Vit-D n = 7	DM n = 10	DM + Vit D n = 10
TUNEL Apoptotic Index (%)	4.16 ± 2.13	5.50 ± 2.42	3.66 ± 1.63	10.33 ± 2.65[a,b,c]	4.33 ± 2.58[a,d]
IHK Irisin Histoscore	2.00 ± 0.58	2.00 ± 0.81	1.85 ± 0.74	0.55 ± 0.21[a,b]	1.52 ± 0.76[a,d]

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.* Sham group ($p < 0.05$); [c], *vs.* Vitamin D group ($p < 0.05$); [d], *vs.* Diabetes Mellitus group ($p < 0.05$).

failures, especially eye, kidney, heart, and vascular damage. It has also been reported[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
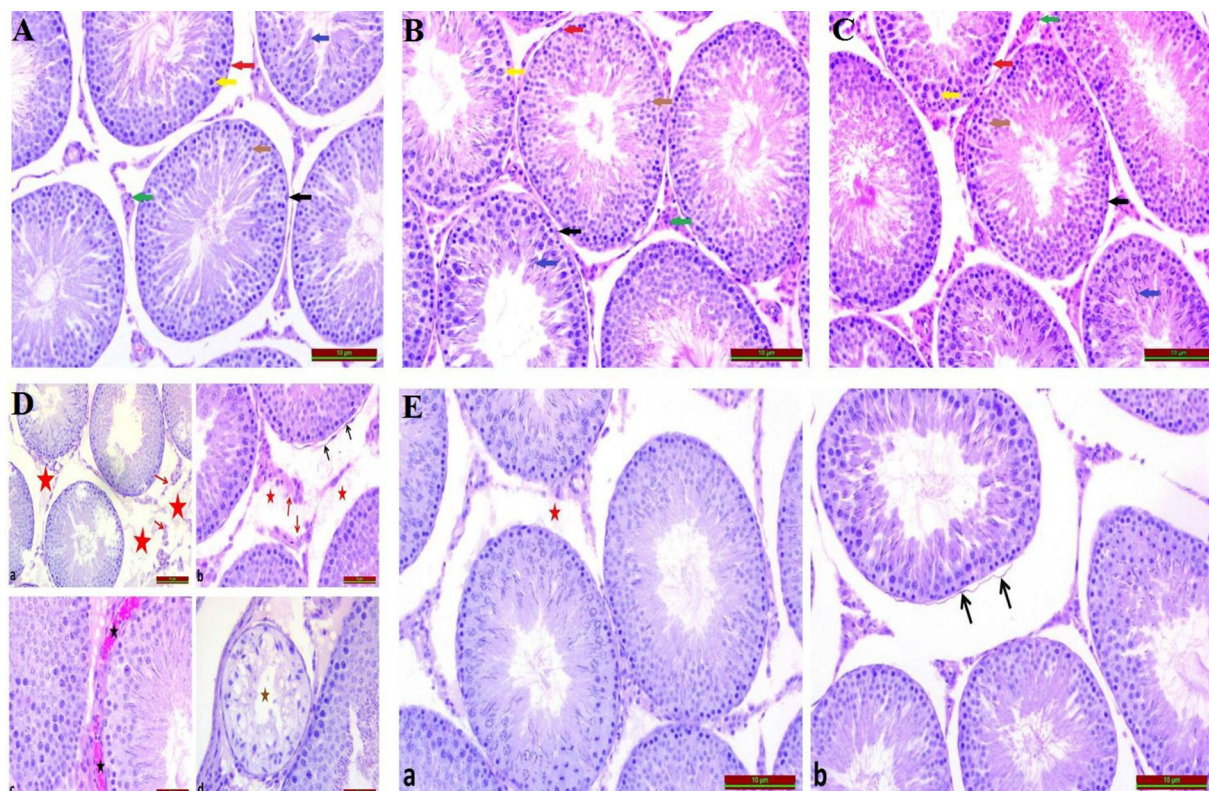

Figure 1. Hematoxylin and Eosin staining in testicular tissues. **A**, Testicular tissue of the control group. Sertoli cell (black arrow), spermatogonium (red arrow), primary spermatocyte (yellow arrow), secondary spermatocyte (brown arrow), spermatozoon (blue arrow), Leydig cell (green arrow); (**B**), Testicular tissue belonging to the citrate buffer group. Sertoli cell (black arrow), spermatogonium (red arrow), primary spermatocyte (yellow arrow), secondary spermatocyte (brown arrow), spermatozoon (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), spermatozoon (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) (**c**), 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.
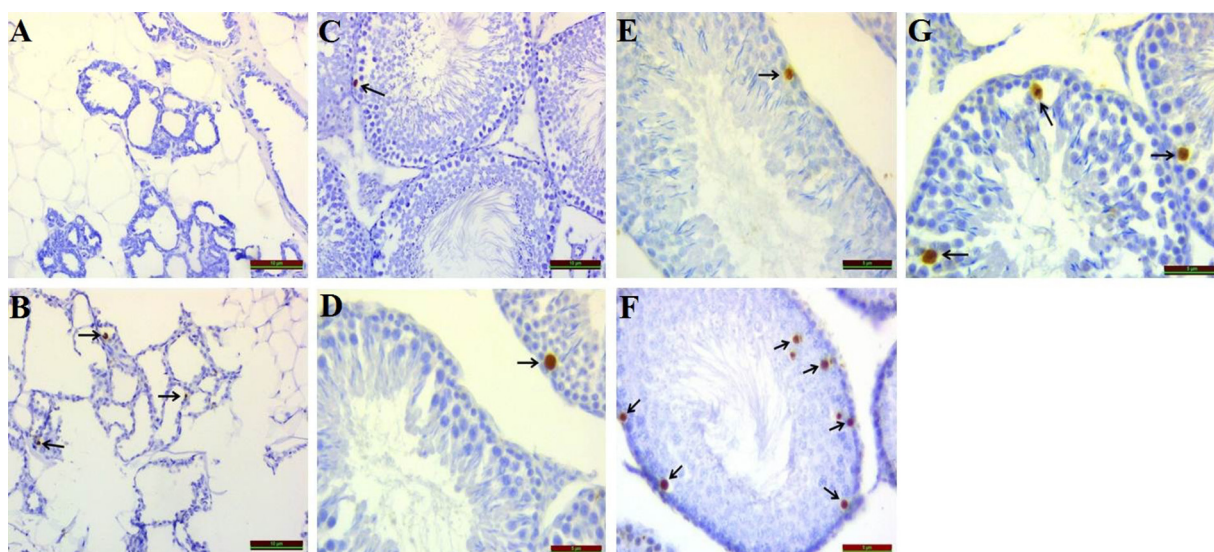
Figure 2. TUNEL staining in testicular tissues. **A**, Negative control tissue; (**B**), Rat breast tissue that was used as the positive control tissue. TUNEL positivity in breast alveolar epithelial cells (black arrows); (**C**), Testicular tissue belonging to the control group. TUNEL positivity in spermatogenic series cells (black arrow); (**D**), Testicular tissue belonging to the citrate buffer group. TUNEL positivity in spermatogenic series cells (black arrow); (**E**), Testicular tissue belonging to Vitamin D group. TUNEL positivity in spermatogenic series cells (black arrow); (**F**), Testicular tissue belonging to the diabetes group. TUNEL positivity in spermatogenic series cells (black arrows); (**G**), Testicular tissue belonging to diabetes+vitamin D group. TUNEL positivity in spermatogenic series cells (black arrows). Magnifications: (**A-B**) x 40; (**C**), x 400; (**D-G**), x 600.

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[35-38]. Bingöl and Kocamış[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[40] reported that there was live weight loss in mice on the 20th and 30th days of the experiment in their study. Also, Wada et al[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[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 mecha-

nism 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[42].

In this study, total antioxidant amounts decreased in the diabetes group while total oxidant amounts increased. Diabetes causes oxidative stress[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[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[45]. As mentioned above, there was a weight loss due to diabetes in rats[46]. In addition, Huh et al[47] 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 observations. They also reported that people[48] and rats with diabetes[49] and gestational diabetes[50] had lower serum irisin levels compared to the control group. However, it should be noted that many studies[48,50-53] have been performed for serum irisin levels in diabetes[48,50,51], but the results are inconsistent with each other[52,53]. 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 iris 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 amounts 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[54-56].

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[32,57-60]. 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[32,57-60].

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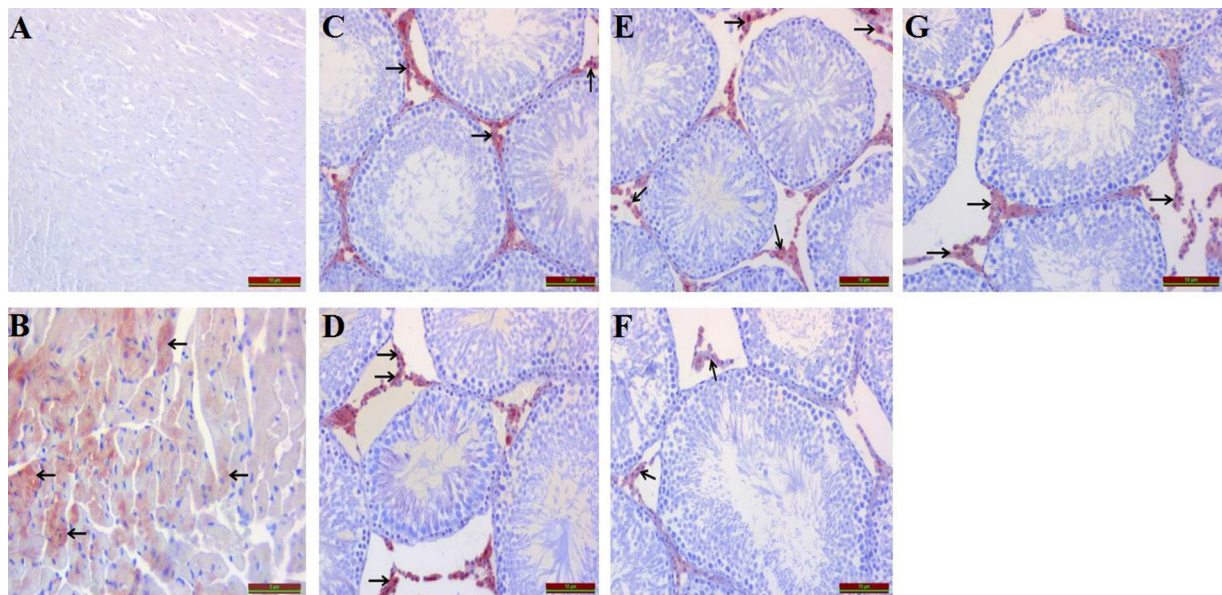

Figure 3. Irisin immunohistochemical staining in testicular tissues. (**A**), Negative control tissue; (**B**), Rat heart tissue that was used as the positive control tissue. Irisin immunoreactivity in cardiac muscle cells (black arrows); (**C**), Testicular tissue of the control group. Irisin immunoreactivity 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[54-56]. 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[61-63] 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[64,65] 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[66,67]. That is one of the main indicators that vitamin D will take place in some clinical applications in the future[56].

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 Fırat University.

Authors' Contributions
MHY, ZKK, KU and SA (Suleyman Aydin) contributed to study conception, design, and data interpretation. MHY, AG, SA (Suna Aydin), BK, ST, BGT, HE, IS and TK contributed to collect data, their analysis and preparing the draft manuscript. All authors contributed to the final approval of the version to be published.

Ethics Approval
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.

Informed Consent
Not applicable.

Availability of Data and Materials
Data are available upon request from the corresponding author.

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References

1) Shah NA, Levy CJ. Emerging technologies for the management of type 2 diabetes mellitus. J Diabetes 2021; 13: 713-724.

2) Daryabor G, Atashzar MR, Kabelitz D, Meri S, Kalantar K. The Effects of Type 2 Diabetes Mellitus on Organ Metabolism and the Immune System. Front Immunol 2020; 11: 1582.

3) Sugimoto K, Murakami H, Deguchi T, Arimura A, Daimon M, Suzuki S, Shimbo T, Yagihashi S. Cutaneous microangiopathy in patients with type 2 diabetes: Impaired vascular endothelial growth factor expression and its correlation with neuropathy, retinopathy and nephropathy. J Diabetes Investig 2019; 10: 1318-1331.

4) Asmat U, Abad K, Ismail K. Diabetes mellitus and oxidative stress-A concise review. Saudi Pharm J 2016; 24: 547-553.

5) Milani E, Nikfar S, Khorasani R, Zamani MJ, Abdollahi M. Reduction of diabetes-induced oxidative stress by phosphodiesterase inhibitors in rats. Comp Biochem Physiol C Toxicol Pharmacol 2005; 140: 251-255.

6) Matough FA, Budin SB, Hamid ZA, Alwahaibi N, Mohamed J. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos Univ Med J 2012; 12: 5-18.

7) Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res 2010; 107: 1058-1070.

8) Takiishi T, Gysemans C, Bouillon R, Mathieu C. Vitamin D and diabetes. Endocrinol Metab Clin North Am 2010; 39: 419-446.

9) Sung CC, Liao MT, Lu KC, Wu CC. Role of vitamin D in insulin resistance. J Biomed Biotechnol 2012; 2012: 634195.

10) Krul-Poel YH, Ter Wee MM, Lips P, Simsek S. Management of Endocrine Disease: The effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes mellitus: a systematic review and meta-analysis. Eur J Endocrinol 2017; 176: R1-R14.

11) Chakraborti CK. Vitamin D as a promising anticancer agent. Indian J Pharmacol 2011; 43: 113-120.

12) Abdul-Razzak KK, Almomany EM, Nusier MK, Obediat AD, Salim AM. Antioxidant vitamins and glucose-6-phosphate dehydrogenase deficiency in full-term neonates. Ger Med Sci 2008; 6: Doc10.

13) Boström P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Boström EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Højlund K, Gygi SP, Spiegelman BM. A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 2012; 481: 463-468.

14) Aydin S, Kuloglu T, Aydin S, Kalayci M, Yilmaz M, Cakmak T, Albayrak S, Gungor S, Colakoglu N, Ozercan IH. A comprehensive immunohistochemical examination of the distribution of the fat-burning protein irisin in biological tissues. Peptides 2014; 61: 130-136.

15) Aydin S. Three new players in energy regulation: preptin, adropin and irisin. Peptides 2014; 56: 94-110.

16) Varela-Rodríguez BM, Pena-Bello L, Juiz-Valiña P, Vidal-Bretal B, Cordido F, Sangiao-Alvarellos S. FNDC5 expression and circulating irisin levels are modified by diet and hormonal conditions in hypothalamus, adipose tissue and muscle. Sci Rep 2016, 6: 29898.

17) Luo Y, Qiao X, Ma Y, Deng H, Xu CC, Xu L. Disordered metabolism in mice lacking irisin. Sci Rep 2020, 10: 17368.

18) Demircan S, Onalan E, Kuloğlu T, Aydın S, Yalçın MH, Gözel N, Dönder E. Effects of vitamin D on apoptosis and betatrophin in the kidney tissue of experimental diabetic rats. Acta Biomed 2020; 91: e2020089.

19) Aydin S. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides 2015; 72: 4-15.

20) Ellenburg JL, Kolettis P, Drwiega JC, Posey AM, Goldberg M, Mehrad M, Giannico G, Gordetsky J. Formalin Versus Bouin Solution for Testis Biopsies: Which Is the Better Fixative? Clin Pathol 2020; 13: 2632010X19897262.

21) Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004; 37: 277-285.

22) Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005; 38: 1103-1111.

23) Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. Methods Mol Biol 2014; 1180: 31-43.

24) Chan JKC. The wonderful colors of the hematoxylin-eosin stain in diagnostic surgical pathology. Int J Surg Pathol 2014; 22: 12-32.

25) Alturkistani HA, Tashkandi FM, Mohammedsaleh ZM. Histological Stains: A Literature Review and Case Study. Glob J Health Sci 2015; 8: 72-79.

26) Hsu SM, Raine L, Fanger H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics. Am J Clin Pathol 1981, 75: 816-821.

27) Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577-580.

28) Aydin S, Kuloglu T, Ozercan MR, Albayrak S, Aydin S, Bakal U, Yilmaz M, Kalayci M, Yardim M, Sarac M, Kazez A, Kocdor H, Kanat B, Ozercan İH, Gonen M, Bilgen M, Balgetir F. Irisin immunohistochemistry in gastrointestinal system cancers. Biotech Histochem 2016; 91: 242-250.

29) Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon withwide-ranging implications in tissue kinetics. Br J Cancer 1972; 26: 239-257.

30) Azgomi RND, Karimi A, Zarshenas MM, Jazani AM. The mechanisms of saffron (Crocus sativus') on the inflammatory pathways of diabetes mellitus: A systematic review. Diabetes Metab Syndr 2022; 16: 102365.

31) Steger RW, Rabe MB. The effect of diabetes mellitus on endocrine and reproductive function. Proc Soc Exp Biol Med 1997; 214: 1-11.

32) Cai L, Chen S, Evans T, Deng DX, Mukherjee K, Chakrabarti S. Apoptotic germ-cell death and testicular damage in experimental diabetes: prevention by endothelin antagonism. Urol Res 2000; 28: 342-347.

33) Alexopoulou O, Jamart J, Maiter D, Hermans MP, De Hertogh R, De Nayer P, Buysschaert M. Erectile dysfunction and lower androgenicity in type 1 diabetic patients. Diabetes Metab 2001; 27: 329-336.

34) Sanguinetti RE, Ogawa K, Kurohmaru M, Hayashi Y. Ultrastructural changes in mouse Leydig cells after streptozotocin administration. Exp Anim 1995; 44: 71-73.

35) Imaeda A, Kaneko T, Aoki T, Kondo Y, Nagase H. DNA damage and the effect of antioxidants in streptozotocin treated mice. Food Chem Toxicol 2002; 40: 979-987.

36) Wada J, Zhang H, Tsuchiyama Y, Hiragushi K, Hida K, Shikata K, Kanwar YS, Makino H. Gene expression profile in streptozotosin induced diabetic mice kidneys undergoing glomerulosclerosis. Kidney Int 2001; 59: 1363-1373.

37) Fujita A, Sasaki H, Ogawa K, Okamoto K, Matsuno S, Matsumoto E, Furuta H, Nishi M, Nakao T, Tsuno T, Taniguchi H, Nanjo K. Increased gene expression of antioxidant enzymes in KKAy diabetic mice but not in STZ diabetic mice. Diabetes Res Clin Pract 2005; 69: 113-119.

38) Hayashi K, Haneda H, Koya D, Maeda S, Isshiki K, Kikkawa R. Enhancement of glomerular heme oxygenase-1 expression in diabetic rats. Diabetes Res Clin Pract 2001; 52: 85-96.

39) Bingöl SA, Kocamış H. Sağlıklı ve diabet oluşturulmuş farelerin böbrek dokusunda katalaz enziminin RT-PCR ile gen ve immunohistokimyasal olarak protein ekspresyonu. Kafkas Univ Vet Fak Derg 2010; 16: 825-834.

40) Gocmen C, Secilmis A, Kumcu EK, Ertuğ PU, Onder S, Dikmen A, Baysal F. Effects of vitamin E and sodium selenate on neurogenic and endothelial relaxion of corpus cavernosum in the diabetic mouse. Eur J Pharmacol 2000; 398: 93-98.

41) Andallu A, Varadacharyulu NC. Antioxidant role of mulberry (Morus indica L. cv. Anantha) leaves in streptozotocin diabetic rats. Clin Chim Acta 2003; 338: 3-10.

42) Gozel N, Genc F, Ozdemir FA, Kuloglu T, Kaya N, Yalcin MH, Kavak-Genc SS, Koca S, Donder E. Effects of Vitamin D on matrix metalloproteinase 9 and apoptosis in experimental diabetic rat kidney tissue. Fresenius Environ Bull 2018; 27: 3766-3775.

43) Sepidarkish M, Farsi F, Akbari-Fakhrabadi M, Namazi N, Almasi-Hashiani A, Maleki Hagiagha A, Heshmati J. The effect of vitamin D supplementation on oxidative stress parameters: A systematic review and meta-analysis of clinical trials. Pharmacol Res 2019; 139: 141-152.

44) Wimalawansa SJ. Vitamin D Deficiency: Effects on Oxidative Stress, Epigenetics, Gene Regulation, and Aging. Biology (Basel) 2019; 8: 30.

45) Feldman AL, Griffin SJ, Ahern AL, Long GH, Weinehall L, Fhärm E, Norberg M, Wennberg P. Impact of weight maintenance and loss on diabetes risk and burden: a population-based study in 33,184 participants. BMC Public Health 2017; 17: 170.

46) Skovsø S. Modeling type 2 diabetes in rats using high fat diet and streptozotocin. J Diabetes Investig 2014; 5: 349-358.

47) Huh JY, Panagiotou G, Mougios V, Brinkoetter M, Vamvini MT, Schneider BE, Mantzoros CS. FNDC5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and II. mRNA expression and circulating concentrations in response to weight loss and exercise. Metabolism 2012; 61: 1725-1738.

48) Akgul Balaban Y, Yilmaz N, Kalayci M, Unal M, Turhan T. Irisin and Chemerin Levels in Patients With Type 2 Diabetes Mellitus. Acta Endocrinol (Buchar) 2019; 15: 442-446.

49) Fouda AA, Gabr NM. Effect of Moderate Swimming Exercise with a Dietary Vitamin D on Serum Irisin Level in Diabetic Rats. AIMJ 2021; 2: 54-61.

50) Kulhan NG, Kulhan M, Turkler C, Ata N, Kiremitli T, Kiremitli S. Could serum levels of irisin be used in gestational diabetes predicting? Taiwan J Obstet Gynecol 2019; 58: 434-437.

51) Xuan X, Lin J, Zhang Y, Zhou L, Xu L, Jia J, Zhao B, Lin Z, Zhu Q, Li L, Wu T, Zhang S, Jiang H, Wang Y. Serum Irisin Levels and Clinical Implication in Elderly Patients With Type 2 Diabetes Mellitus. J Clin Med Res 2020; 12: 612-617.

52) Rana KS, Pararasa C, Afzal I, Nagel DA, Hill EJ, Bailey CJ, Griffiths HR, Kyrou I, Randeva HS, Bellary S, Brown JE. Plasma irisin is elevated in type 2 diabetes and is associated with increased E-selectin levels. Cardiovasc Diabetol 2017; 16: 147.

53) Espes D, Lau J, Carlsson PO. Increased levels of irisin in people with long-standing Type 1 diabetes. Diabet Med 2015; 32: 1172-1176.

54) Tagliaferri S, Porri D, De Giuseppe R, Manuelli M, Alessio F, Cena H. The controversial role of vitamin D as an antioxidant: results from randomised controlled trials. Nutr Res Rev 2019; 32: 99-105.

55) Khosravi ZS, Kafeshani M, Tavasoli P, Zadeh AH, Entezari MH. Effect of Vitamin D Supplementation on Weight Loss, Glycemic Indices, and Lipid Profile in Obese and Overweight Women: A Clinical Trial Study. Int J Prev Med 2018; 9: 63.

56) Tabasi N, Rastin M, Mahmoudi M, Ghoryani M, Mirfeizi Z, Rabe SZ, Reihani H. Influence of vitamin D on cell cycle, apoptosis, and some apoptosis related molecules in systemic lupus erythematosus. Iran J Basic Med Sci 2015; 18: 1107-1111.

57) Cameron DF, Murray FT, Drylie DD. Interstitial compartment pathology and spermatogenic disruption in testes from impotent diabetic men. Anat Rec 1985; 213: 53-62.

58) Anderson JE, Thliveris JA. Morphometry and cytochemistry of Leydig cells in experimental diabetes. Am J Anat 1987; 180: 41-48.

59) Anderson JE, Thliveris JA. Testicular histology in streptozotocininduced diabetes. Anat Rec 1986; 214: 378-382.

60) Öztürk F, Gül M, Ağkadir M, Yağmurca M. Deneysel Diyabetin Sıçan Testislerinde Meydana Getir-

diği Histolojik Değişiklikler. T Klin J Med Sci 2002; 22: 173-178.

61) Tomita T. Apoptosis in pancreatic β-islet cells in Type 2 diabetes. Bosn J Basic Med Sci 2016; 16: 162-179.

62) Costes S, Bertrand G, Ravier MA. Mechanisms of Beta-Cell Apoptosis in Type 2 Diabetes-Prone Situations and Potential Protection by GLP-1-Based Therapies. Int J Mol Sci 2021; 22: 5303.

63) Sudo C, Ogawara H, Saleh AWH, Nishimoto N, Utsugi T, Ooyama Y, Fukumura Y, Murakami M, Handa H, Tomono S, Murakami H. Clinical significance of neutrophil apoptosis in peripheral blood of patientswith type 2 diabetes mellitus. Lab Hematol 2007; 13: 108-112.

64) Murphy CJ, Richburg JH. Implications of Sertoli cell induced germ cell apoptosis to testicular pathology. Spermatogenesis 2015; 4: e979110.

65) Shaha C, Tripathi R, Mishra DP. Male germ cell apoptosis: regulation and biology. Philos Trans R Soc Lond B Biol Sci 2010; 365: 1501-1515.

66) Ding C, Wang Q, Hao Y, Ma X, Wu L, du M, Li W, Wu Y, Guo F, Ma S, Huang F, Qin G. Vitamin D supplement improved testicular function in diabetic rats. Biochem Biophys Res Commun 2016; 473: 161-167.

67) Kazemian E, Akbari ME, Moradi N, Gharibzadeh S, Amouzegar A, Rozek LS, Mondul AM, Khademolmele M, Zarins KR, Ghodoosi N, Shateri Z, Fallah S, Davoodi SH. Assessment the effect of vitamin D supplementation on plasma vitamin D levels, inflammation, and oxidative stress biomarkers based on vitamin D receptor genetic variation in breast cancer survivors: a protocol for clinical trial. J Health Popul Nutr 2021; 40: 46.