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Histological investigation of the protective effect of metformin on testis and sperm parameters in obese rats with type 2 diabetes mellitus

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Ethics Committee Approval
The study was performed in Sakarya University Animal Laboratory in accordance with international guidelines, after approval from the Animal Care and Use Ethics Committee was taken(04.09.2019/30).
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Diabetes mellitus (DM) is thought to have adverse effects on the male reproductive system. Metformin (MTF) is a widely used drug in the treatment of DM. This study hypothesized that MTF can reduce the harmful effects of DM on the male reproductive system in addition to diabetes treatment.
Methods: Twenty-one adult (15-18 g, 11-12 months) C57BL6 male mice were randomly assigned to three groups, as follows: The Control group (C), Diabetes group (D), and Diabetes + Metformin (D+MTF) treatment group. Groups D and D+MTF were fed a diet composed of 60% fat for four weeks. DM was created by administering a single dose of 30 mg/kg streptozotocin intraperitoneally (i.p.). MTF was given through gavage at a dose of 300 mg/kg/day. At the end of the experiment, sperm parameters were evaluated in the testicular tissue. Histomorphology features and immune expressions of VEGF, Caspase-3, and Ki-67 were evaluated in testicular tissue sections stained with Hematoxylin and Eosin (HE).
Results: Sperm concentration, motility, and morphological characteristics of diabetic mice were significantly reduced compared to control, and MTF-treated mice. Seminiferous tubule diameters and Johnsen scores were significantly higher in the control and D+MTF groups when compared to the diabetic group. VEGF and Ki-67 immune expression were significantly higher in the control and D+MTF groups compared to the D group (P=0.001, and P=0.002, respectively). Because of D, Caspase-3 positive cell density was higher in the D group compared to the control and D+MTF groups (P=0.002).
Conclusion: Diabetes mellitus had adverse effects on sperm parameters and the testicular tissue. High glucose levels with decreased VEGF immune expression damaged spermatogenic cells and decreased proliferation and differentiation due to impaired intratesticular vascularity. MTF treatment reduced the damage in spermatogenic cells and contributed to the differentiation and division of cells through the restoration of vascularization.

Keywords: Metformin, Type 2 Diabetes, Obese

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Introduction

Diabetes mellitus (DM) is one of the most common persistent diseases affecting more than one hundred million people worldwide. It is a metabolic disease characterized by hyperglycemia that causes long-term damage to many organs, including the heart, eye, kidney, nervous system, and the vascular system. Diabetes develops a result of a deficiency in insulin secretion (T1DM) or a decrease in the sensitivity of tissue to insulin (T2DM) [1, 2].

DM may cause chronic hyperglycemia-related reproductive complications. It has been reported that high glucose causes increased oxidative stress and apoptosis in testicular cells, thus causing infertility [3-5]. A study revealed that increased oxidative stress in diabetic rats caused DNA damage in the testis, loss of sperm cells, and delay in spermatogenesis [5]. Similarly, another study proved that DM caused apoptotic cell death in germ cells in testicular tissue in rats, and the condition was the leading cause of infertility [3].

High blood glucose levels can harm blood vessels and cause endothelial disorders. Therefore, DM is considered an essential risk factor for cardiovascular diseases [6]. Vascular endothelial growth factor (VEGF), also known as vascular permeability, is a component of vessel wall endothelial cells and an angiogenic factor that causes proliferation and increased permeability [7, 8]. VEGF was effective in proliferation and differentiation during the spermatogenesis process [9]. It is known that DM causes testicular cell death by inducing the apoptosis pathway. Experimental studies have shown that cellular apoptosis results from testicular damage because of oxidative stress induced by hyperglycemia [10].

Despite increasing knowledge on T2DM risk factors, the incidence and prevalence of the disease continue to increase globally. Early diagnosis and safe and effective treatments can reduce morbidity and mortality by preventing or delaying complications [11]. MTF, a dimethyl biguanide, is a hypoglycemic drug usually prescribed for the control of T2DM. MTF has a direct eliminating effect against ROS. It improves the level of oxidative stress in DM by supporting the impaired antioxidant defense system [12].

In T2DM obesity cases, MTF treatment improves sperm abnormalities and increases the decreased sperm count. In humans, serum testosterone and LH pulsatility may be increased in individuals treated with MTF for few months [13]. This indicates that MTF can modulate testicular steroidogenesis and increases pituitary LH pulsatility, regulating Leydig cell functions [14].

This study aims to histopathological and immunohistochemically investigate the effects of MTF on obese mice with T2DM in terms of sperm parameters and testicular tissue.

Materials and methods

Test animals and ethical statement

In this study, twenty-one 11-12 week*old C57BL6 male mice, weighing an average of 15-18 grams, were used. The study was performed in the Sakarya University Animal Laboratory per the international guidelines after approval was obtained from the Animal Care and Use Ethics Committee (04.09.2019/30). All mice were adapted to the laboratory environment for one week (kept in wire cages, 12/12 h light/dark-light cycle, at 22°C, temperature, in 50-60% humidity) before the study began. The 21 mice in our study were divided into three groups, regardless of any trait. There were seven mice in each group.

Test design

Control Group (C): Mice in this group were fed average mouse food (consisting of 14.3% protein, 4% fat, 65.4% starch, and 16.3% sugar, fibers, vitamins, and minerals) and tap water for eight weeks. No further action was taken.

Three days after DM was induced, 14 animals were again randomly divided into two groups composed of 7 mice, as the diabetes and metformin treatment groups.

Diabetes Group (D): Mice in this group were underwent the diabetes induction protocol. Hyperglycemia was induced by feeding with HFD for four weeks. T2DM was created by administering 30mg/kg STZ intraperitoneally. Animals continued to be fed with HFD for another four weeks after they developed DM.

Diabetes and Metformin Group (D+M): After DM was created with the administration of 30mg/kg STZ IP, MTF (300mg/kg/day) [15] was dissolved in 0.09% isotonic solution and given through gavage for four weeks. After DM occurred, the mice were fed an average standard diet. At the end of the eight-week study, the animals were sacrificed using cervical dislocation with overdose anesthesia (pentobarbital 100 mg/kg IP) twenty-four hours after the last treatment. Tissue samples obtained for histopathological examinations were fixed in 10% buffered formalin.

Diabetes protocol

For inducing hyperglycemia in the D groups, mice were fed HFD with a total energy of 25.07 kJ/g, mostly from fats, 20% from protein, and 20% from carbohydrates (52.2% starch, 14%, 3 proteins, 17.2% fat, and 16.3% fibers, sugar, essential vitamins and minerals) [16]. The rats fasted for 16 hours at the end of 4 weeks. After 16 hours of fasting, streptozotocin (STZ), (Cayman Chemical Temno, 13104, USA) was dissolved in 0.05M citrate buffer (pH 4.5) and a single dose of 30 mg/kg was administered intraperitoneally [17]. Fasting blood glucose stages were measured with the glucometer (Roche Diagnostics, Basel, Switzerland) from blood samples taken from the tail vein 72 hours after STZ administration. Mice with fasting blood glucose levels of 150-250 mg/dL were considered hyperglycemic and used in the test.

Sperm collection

Mature sperm cells were obtained from the epididymis with the stripping method. Epididymal sperms were counted using the method described by Yokoi et al. [18]. At the end of the experiment, 10 µl of the epididymal sperm suspension was obtained from the three groups in phosphate buffer saline (PBS), and sperm count was performed in the Makler counting chamber. Their concentrations were recorded by multiplying by the dilution ratio. The morphology of 200 spermatozoa were evaluated after staining of the samples of each mouse in the three groups, which were examined at 40X magnification under the light microscope, and their percentages were calculated. Sperm morphology was evaluated according to the Kruger criteria [19].

Testicular histopathology

For evaluation under the light microscope, testicular tissue samples of mice were fixed with Bouin's fixative and embedded in paraffin. Before sectioning, samples were cooled at -20°C. Sections of 3-5 µm thickness sliced with Thermo Scientific Microm HM40E (Otto-Hahn-Strasse1a 69190 Walldorf, Germany) microtome were placed on slides after they were opened in a gelatine (Gelatine, Foodland, Ewald-GelatineGmbH, Meddersheimer Str 50, 55566 BadSobernheim, Germany) in hot water bath. Sections were stained with H-E. (Merck KGa A 6427 Darmstadt, Germany) to examine the histological structure.

The germinal layer thickness of seminiferous tubules

Testicular tissue samples were embedded in paraffin after fixation and tissue determination using 10% neutral buffered formalin. To evaluate seminiferous tubule diameters morphometrically, we took 5-micron thick sections from tissue blocks. Then the areas were stained with HE. More than 20 sections were taken from each block. The germinal layer of the seminiferous tubules was measured using the NIS-Element (USA) camera and software. Mean diameters of the germinal layer (µm) of the seminiferous tubules were measured for each testis [21].

VEGF, Caspase-3, and KI-67 IHC staining

The tissue samples, cut in 4 microns from the paraffin-embedded blocks, were deparaffinized and washed with decreasing concentrations of alcohol solutions. The preparations in citrate buffers were subjected to heat treatment for 20 minutes in the microwave. After that, all preparates were placed in blocks of 3% H2O2 with endogen peroxidase activity. The primary antibodies were Caspase-3(Genetex), VEGF(Genetex), and Kİ-67(Genetex), in 1/300 ratio, after which the secondary antibodies (UltraVisionLarge Volume DetectionSystem Anti-rabbit by LabVision, HRP) were evaluated. The producing company's procedures were implemented in each step. Diamino benzidine (DAB) was used to make the paint visible. Mayer's hematoxylin was used for contrast coloring. The preparations were covered by the mounting medium (Aqueous Mounting Medium by ScyTek). The area and intensity of brown staining were assessed using ImageJ software (ImageJ, NIH, Bethesda, MD).

Statistical analysis

Statistical analyses were performed using the SPSS 22.0 package program (SPSS Inc. and LeadTech. Inc. Chicago. USA). Numerical data were presented as mean (standard deviation) (SD). Shapiro-Wilk test was used to check the normality of distribution. The One-way ANOVA and the Kruskal Wallis tests were used to compare more than two variables. The Tukey HSD was used for variables with homogeneous in-group significance and variances, and Tamhane's T2 test was utilized for non-homogeneous variables. *P*-values of <0.05 were considered significant.

Results

Body weight and blood sugar levels

The body weight and blood glucose values of the mice in the experimental groups are shown in Table 1. At the beginning of the study, the mean body weight and blood glucose levels were similar between the three groups ($P>0.05$). A statistically significant increase was observed in the D and D+M groups' body weights compared to the C group at week 4 ($P=0.006$, $P=0.006$, respectively). Mean body weight at the end of week 8 was significantly higher in the D+M when compared to the C and D groups ($P=0.001$ for both).

At the end of week 4, a significant increase was observed in mean blood glucose levels in groups D, and D+M fed with HFD compared to group C fed with standard mice food ($P<0.001$ for both). There was no significant difference in blood glucose levels between the D and D+M groups ($P>0.05$). After MTF treatment for 4 weeks, blood glucose levels in the D+M group were significantly lower compared to the untreated group D ($P<0.001$), while they were significantly higher than in group C ($P<0.001$).

Light microscopic results of sperm parameters

The results of light microscopic evaluation of sperm parameters are presented in Table 2. DM caused a significant decrease in all sperm parameters. Sperm density, percent motility, percent motility/immobility in situ, and percent of cells with normal morphology decreased significantly ($P<0.05$). These parameters were significantly decreased in the D group, and the mean values of the D+M group were close to those of the C group ($P<0.001$ for comparisons of all parameters).

Table 1: Body weights and blood glucose values of study groups at baseline, 4th and 8th weeks

Groups (n= 7)	Body weight (g) Mean(SD)			Blood sugar (mg/dL) Mean(SD)		
	Baseline	4th week	8th week	Baseline	4th week	8th week
C	16.57(0.34)	19.17(0.57)	22.35(0.40)	89.50(1.22)#	91.02(1.83)#	91.57(1.03)
D	16.44(0.28)	20.18(0.55)*	29.50(1.03)	230.28(17.21)	138.22(8.18)	271.31(9.08)
D+MTF	16.47(0.24)	22.52(0.47)**	17.20(0.36)**	181.37(68.02)	120.80(23.61)	140.72(2.95)**

C: Control, D: Diabetes, D+MTF: Diabetes and Metformin treatment group.SD:Standard deviation, *$P<0.05$ compared with C and D+MTF, **$P<0.001$ compared with C and D, # $P<0.001$ compared with DM and DM+MTF.

Table 2: Microscopic sperm parameters evaluation results in all groups

Parameters	C Mean(SD)	D Mean(SD)	D+MTF Mean(SD)	*P*-value
Sperm Count (millions)	29.14(3.89)	16.43(1.27)	26.57(1.51)	<0.001
Motility (%)	32.86(4.80)	17.57(2.51)	27.57(2.07)	<0.000
				0.024
On-site mobility (%)	17.142(4.80)	11.43(2.44)	13.57(2.44)	0.016
Immotile (%)	50(7.07)	71(4.51)	58.86(2.34)	<0.001
				0.011
				0.001
Morphology (%)	25(2.94)	14.43(3.10)	22.86(2.34)	<0.001
Johnsen Score	8.86(0.69)	6(0.82)	8.43(0.53)	<0.001

C: Control group, D: Diabetes group, D+MTF: Diabetes and Metformin treatment group, SD: Standard deviation

Histopathological findings and Johnsen score

Under the light microscope (Figure 1), the testicular tissue belonging to group C showed a normal seminiferous tubule and spermatogenic structure (Figure 1-A), and tight connective tissue. The tunica albuginea, and seminiferous tubules within the testicular lobules separated by septa extending interiorly from the capsule seemed normal. In the testes of group D (Figure 1-B), there was severe atrophy in most seminiferous tubules, decrease in the number and layer of spermatogenic cells, and separation of spermatogenic arrests, and intercellular junctions. Tissue sections of the testicles (Figure 1-C) taken from the D+M treated group had areas close to the healthy histological seminiferous tubule organizational structure similar to group C. Furthermore, spermatozoa accumulated in the spermatogenetic layers and seminiferous tubule lumen. Separations were less observed in the intercellular connections in the seminiferous tubules of these groups compared to group D (Figure 1). There was no significant difference in terms of the Johnsen scores between the C and D+M groups ($P>0.05$). The Johsen scores in

both groups were significantly higher than that of the D group. The p values were $P<0.001$ and $P=0.031$, respectively (Table 2).

Seminiferous tubules diameter

The diameters of seminiferous tubules in group D were significantly decreased compared to groups C and D+MTF ($P=0.004$, $P=0.006$, respectively). There was no significant difference between the D+MTF group and the C group (Figure 3).

Immunohistochemical results

VEGF and Ki-67 immune expression increased in C and D+M groups, and Caspase-3 expression decreased (Figure 2). In group D, on the contrary, Caspase-3 immune expression increased, while VEGF and Ki-67 decreased. VEGF and Ki-67 expression significantly increased in groups C and D+M, when compared with group D ($P=0.001$ and $P=0.002$, respectively). Caspase-3 expression was increased in group D compared to groups C and D+M ($P=0.002$). There was no difference between Caspase-3 expression in C and D+M groups ($P>0.05$). Although VEGF and Ki-67 expression increased after metformin treatment in the D+M group, statistical differences were observed compared to the C group. P values were $P=0.002$ and $P=0.001$, respectively.

Figure1: Seminiferous tubule preparations of the study groups, stained with H.E., 100X, 100 scale bar. Control group (C), diabetes group (D), and diabetes metformin treatment group (D+MTF). The typical testicular structure is seen in 1A. In 1B, seminiferous tubule structures are observed with reduced diameters. Distorted seminiferous tubule layers were remarkable in this group. In 1C, seminiferous tubules with increased diameters due to metformin effect are observed. In this group, the seminiferous tubule layers were prominent enough to distinguish.

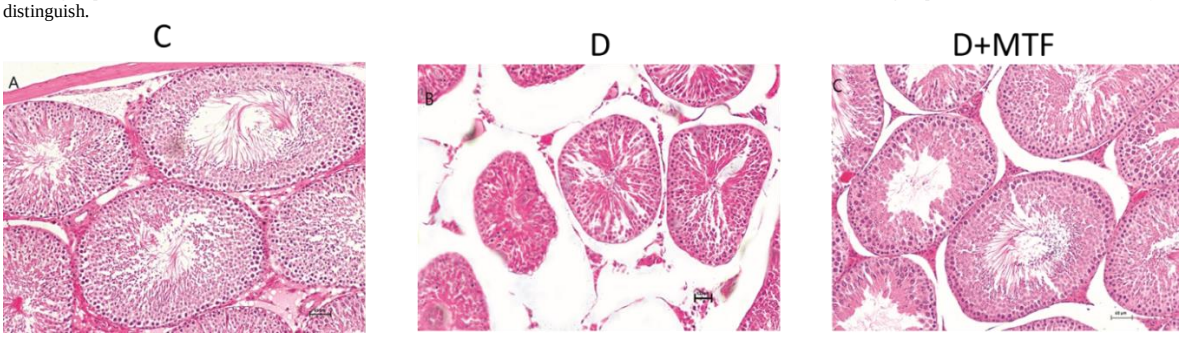


Figure 2: Seminiferous tubule tissue samples. 100X, 50 scale bar. VEGF, Ki-67 Caspase-3 immunoexpression in seminiferous tubule tissue samples belonging to C, D and D+MTF groups. Dark brown marked cells are considered positive. These cells indicate increased expression of the target protein Caspase-3. The black arrow heads in Figure 2-H show the Caspase-3 positive cell.

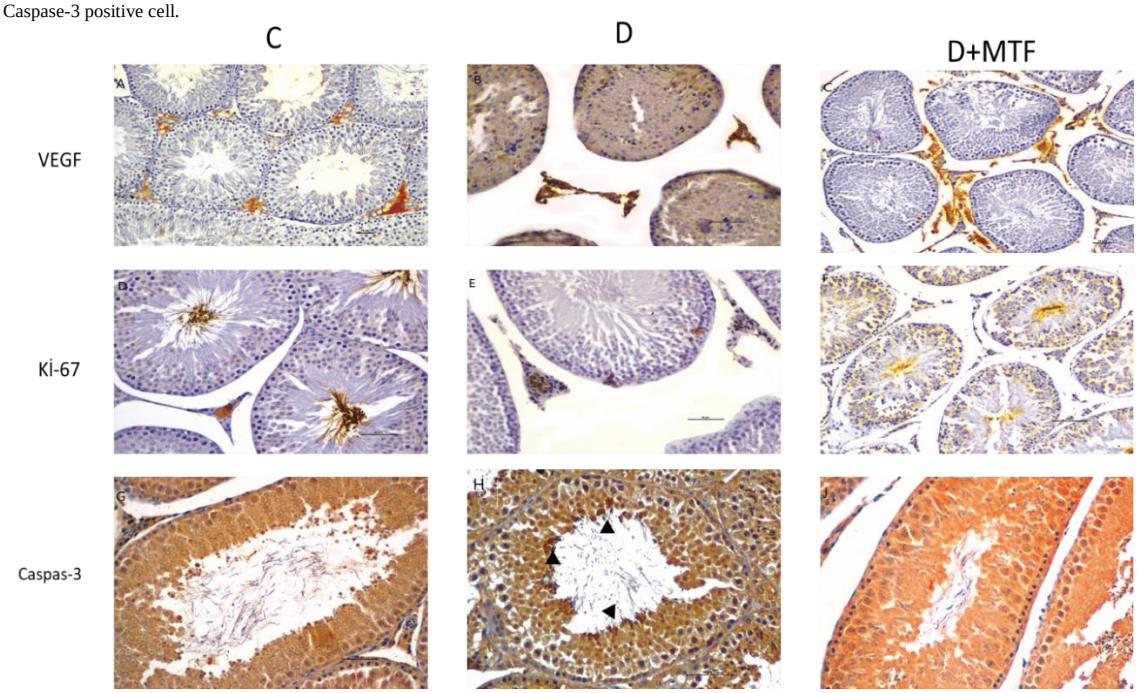
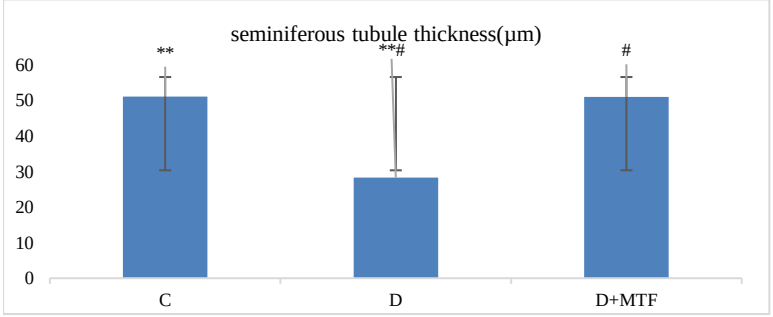


Figure 2: Comparison of seminiferous tubule (st) diameters of the experimental groups. Control group (C), diabetes group (D), and diabetes metformin treatment group (D+MTF). Kruskal Wallis, the continuation of the Mann-Whitney test. ** $P<0.05$ The st diameters of the C group were higher than those of the D group. # $P<0.05$ The st diameters of the D+MTF group were more significant than the D group. There was no statistical difference in ST diameters between the C and D+MTF groups.



Discussion

This study evaluated the protective effect of Metformin in HFD/STZ-induced diabetic testicular tissue and the histological changes using Caspase-3, Ki-67, VEGF immune expression staining and microscopic sperm parameters.

DM is a common health problem that impairs reproductive functions in men and women. In diabetic mice, high glucose levels can impair testicular function and cause infertility [22, 23]. A study stated that in diabetic mice, it also caused a decrease in the diameter of the seminiferous tubules, and damaged spermatogenic cell morphologies and epithelial layers [24].

Clinical studies point out that metformin provides satisfactory results in the treatment of patients with oligoasthenoteratospermia (OAT). It is thought that metformin has significant effects on decreasing insulin and globulin-dependent sex hormone levels and improving serum androgen levels and semen quality in OAT patients [25]. However, the mechanism by which metformin repairs spermatogenic function and the damage caused by obesity is still unknown. The functioning of a normal testis is determined by the cycle of spermatogenic cells formed by normal functioning Sertoli and Leydig cells. When we examined the preparations stained with HE, we observed that HFD-S caused not only obesity but also a decrease in the diameters of the seminiferous tubules. The results proved that the diameters of the seminiferous tubules and the Johnsen score were remarkably higher in the D+M group compared to the untreated D group. These values were significantly lower in group D, which was not treated in the same way, compared with group C. The same situation resulted in decreased sperm parameters and poor-quality sperm. Sperm parameters and in C and D+M groups were significantly higher in comparison with Group D. Studies are indicating that the sperm count, motility, and morphology of the DM groups are of lower quality when compared to the control groups, similar to the results of this study [26]. Niknamand and Mahmoudi [27], reported that sperm count and morphology were significantly disrupted and seminiferous tubule diameters decreased in diabetic rats.

Expression of the Ki67 protein is related to cell division. The interphase Ki67 protein is mainly located in the nucleus on the surface of most chromosomes [28]. In this study, Ki67-positive cell counts increased remarkably in the spermatid of groups C and D+M rats compared to group D. These results indicate the positive effects of Metformin treatment on the diabetic testis during oxidative stress. Previous studies indicated that the proliferative activity of germ cells decreased in the testicular tissues of diabetic mice due to STZ, whereas increases in cell apoptosis were observed [4, 24, 29]. Diabetes is associated with increased oxidative stress, which damages the nuclear DNA of sperm and oocytes. Antioxidant agents are known to help alleviate the oxidative damage associated with DM. In this study, the number of caspase-3-positive cells in the germinal epithelium increased remarkably in group D. On the other hand, the number of Caspase-3 positive cells markedly reduced after Metformin treatment in the D+M group. Similar to our study, in which the lowest caspase-3 levels were seen in group C, studies reported that hyperglycemia-induced apoptosis is regulated by activating the caspase-3 pathway [30] and that Caspase-3 expression is higher in the diabetes groups compared to the control groups [31].

The angiogenic factors, VEGF and VEGF receptors are produced in both the Sertoli and Leydig cells [32]. VEGF induces spermatogonia proliferation and is essential for the homeostasis of germ cells [33]. VEGF also stimulates microvascular permeability and affects the passage of spermatogonia through tight junctions in the Sertoli cells [34]. A study stated that the amount of testicular VEGF decreased in diabetic mice, and the decrease in VEGF level was associated with increased apoptosis and testicular damage [35]. In our study, we observed that VEGF expression was almost undetectable in the testicular tissues of the DM group compared to the control group, in line with the literature. These results suggest that the decrease in VEGF expression resulting from DM may cause decreased endothelial permeability and insufficient angiogenesis, resulting in vascular disorders.

Limitations

The diversity of antibodies used in the evaluation could be increased.

Conclusion

This study showed that type 2 DM induced by HFD and streptozotocin harms the spermatogenesis stage of mice, and causes quantitative, motility-related and morphological changes in semen quality. Deterioration of seminiferous tubule histology increased the apoptosis rate of testicular cells and the deterioration of the intratesticular vascular structure. We think that metformin administration probably regulates glucose metabolism, contributes to the restoration and repair of the damaged intratesticular vascular structure with its effect on weight loss and antioxidant support. We have the opinion that supporting the vascular structure improves semen parameters and reduces spermatogenic cell apoptosis.

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