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Evaluation of the effects of ghrelin and metformin on sperm parameters, testosterone hormones, and immunohistochemical and immunofluorescent markers in an experimental diabetes model

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Abstract: Testicular dysfunction, a severe secondary complication of diabetes, negatively affects reproductive health. Ghrelin is a peptide that plays a role in many metabolic events, such as stimulating growth hormones and regulating appetite, blood pressure, and reproduction. Metformin has antiinflammatory and antioxidant properties and is widely used to treat type 2 diabetes. Therefore, in this study, to investigate the effects of ghrelin and metformin on testicular function in experimental diabetic rats given streptozotocin, placed on a high-fat diet, and that had their sperm parameters evaluated, testosterone hormone, caspase 3 and iNOS expression, and histopathological examinations were performed. In total, 38 Wistar albino male rats were randomized into 4 groups (a control group, diabetes group, diabetes + ghrelin group, and diabetes + metformin group) with different numbers. It was determined that spermatozoa concentration, motility, and testosterone levels were lower ($p < 0.001$), caspase 3 and iNOS expression levels ($p < 0.05$) were higher, and abnormal sperm rates were higher ($p < 0.001$) in the diabetic group compared to the other groups. Metformin and ghrelin administered to the diabetes group increased spermatozoa concentration, motility, and testosterone levels ($p < 0.001$) and decreased caspase 3 and iNOS expression levels, as well as abnormal sperm rates ($p < 0.05$). Based on these findings, we propose that ghrelin and metformin can be used to protect reproductive health in testicular dysfunction caused by diabetes.

Key words: Caspase 3, iNOS, ghrelin, metformin, sperm

1. Introduction

Diabetes is a metabolic disease that occurs when insulin is not secreted in sufficient amounts or exhibits functionality issues if it is. The incidence of diabetes, which is characterized by hyperglycemia as a result of increased blood sugar levels, is constantly increasing [1,2]. Hyperglycemia's long duration causes serious problems in various systems [2]. It was reported that diabetes disrupts reproductive function in men as a result of changes in spermatogenesis, steroidogenesis, sperm quality, and the normal histological structure of the testicles. In addition, various studies have indicated that body and testicular weight and antioxidant enzyme levels decreased in diabetic rats [3–5]. Testicular oxidative stress, inflammation, and germ cell apoptosis lead to decreasing fertility rates [6].

Ghrelin is a peptide hormone produced in the stomach and released in the circulation [7]. Ghrelin's synthesis by the reproductive organs signifies its autocrine and/or paracrine actions on the gonads [8]. Its functional receptor, Growth Hormone Secretagogue Receptor 1a, is also expressed at different hypothalamic–pituitary gonadal axis levels. Ghrelin sometimes regulates different aspects of the female and male reproductive functions, from germ cell production to embryo development. Through its various biological functions, such as energy metabolism by promoting fat deposition and food intake, ghrelin can be a key indicator of energy status and fertility (nutrient–gene expression) [7]. It plays a significant role in regulating key testicular functions, such as testosterone secretion, Leydig cell proliferation, and the gene expressions of prime functional proteins in the seminiferous tubule [8]. It is thought that ghrelin localized in Leydig and Sertoli cells also plays a role in controlling spermatogenesis [9,10].

Taking low doses of streptozotocin (STZ) and having a high-fat diet are ideal ways to evaluate antidiabetic agents in type 2 diabetes [11,12]. Metformin, with its antiinflammatory and antioxidant properties, is widely used in treating type 2 diabetes [13–15]. It was reported

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that metformin is preferred in treating type 2 diabetes because it inhibits gluconeogenesis in the liver [16]. It also inhibits insulin resistance by increasing insulin-mediated glucose uptake in the skeletal muscles [17,18]. Metformin can directly modify testicular and ovarian function through activated protein kinase complex-dependent and independent mechanisms. Its effects include improved sperm function and fertilization rates, enhanced oocyte quality and embryo development, and reduced miscarriage rates [19]. Metformin treatment improves testicular steroidogenesis and spermatogenesis and reduces testicular oxidative damage in experimental diabetic conditions [20,21].

Testicular dysfunction, a severe secondary complication of diabetes, negatively affects reproductive health. Many studies exist on the harmful effects of diabetes on reproductive health. However, unfortunately, there is still no complete prescription for its treatment. This study aimed to investigate the impact of ghrelin and metformin on sperm parameters, testosterone hormones, and caspase 3 and iNOS expression in experimental diabetic rats given a high-fat diet and STZ.

2. Materials and methods

2.1. Chemicals

Ghrelin (RP10781-1) was obtained from Genscript, STZ (SO130) from Sigma-Aldrich, and metformin from a local pharmacy.

2.2. Experimental groups and induction of diabetes

The study was performed on 38 male Wistar albino rats. The rats were randomly numbered and placed into 4 groups. The rats were obtained from Van Yüzüncü Yıl University's Experimental Research Center and housed in appropriate laboratory conditions. The animals were given clean drinking water and fed pellet feed. After obtaining the necessary permission from the relevant institution (decision dated 27.04.2023 and numbered 2023/06-05), the experimental phase started.

The rats in the control group were fed standard rat food for the first 21 days. All rats except those in the control group had a high-fat diet (60 kcal, % fat) throughout the experiment. Rats made diabetic by STZ administration were put on a high-fat diet (60 kcal, % fat) for the first 3 weeks before the STZ injection on day 22 of the experiment [22]. On the 22nd day, the blood sugars of all rats were measured, and it was determined that they did not have diabetes. The rats in the control group were then injected with citrate buffer (pH = 4.5, 0.1 mol/L). The remaining rats were injected intraperitoneally (IP) with STZ (40 mg/kg) dissolved in citrate buffer (ph = 4.5, 0.1 mol/L). Diabetes was confirmed in the rats in the 2nd, 3rd, and 4th groups by measuring their blood glucose levels 10 days after STZ injection. For the control group (n = 8), only citrate buffer (1 mL/kg) was administered to the animals. Citrate buffer administration was done on the 22nd day [22]. For the diabetes group (n = 10), STZ dissolved in citrate buffer (40 mg/kg) was given IP [22]. STZ administration was initiated on the 22nd day.

The diabetes + ghrelin group (n = 10) was given STZ (40 mg/kg) and ghrelin (100 µg/kg) IP [23]. STZ administration was performed on the 22nd day. From the 32nd day of the study, ghrelin (100 µg/kg) was administered once a day for 21 days, dissolved in physiological saline, and administered IP. The diabetes + metformin group (n = 10) was given STZ (40 mg/kg) and metformin (100 mg/kg). STZ administration was performed on the 22nd day. From the 32nd day of the study, metformin (100 µg/kg) was administered once a day for 21 days, dissolved in physiological saline, given by gavage [24].

2.3. Sample collection and spermatological examination

At the end of the study, intracardiac blood samples were taken while the rats were under general anesthesia (15 mg/kg xylazine and 50 mg/kg ketamine). The testicles were removed immediately afterward. Euthanasia was achieved after blood samples and testicular tissues were collected. These blood samples were centrifuged at 3500 g × for 10 min. Later, a small incision was made in the scrotum, and the testicles were removed. One testes from each rat was left in fixation solution to be evaluated histopathologically, immunohistochemically, and with immunofluorescence. Care was taken not to cool the other testicle, which was taken for spermatological evaluation. First, motility was determined (at 37 °C) without allowing the cauda part of that testicle to cool. The same cauda part was divided into tiny pieces in physiological saline with the help of a sharp scalpel and a suspended mixture. This mixture was used for concentration and morphological evaluation after incubation for 10 min [25].

Sperm concentration was determined using an Eppendorf tube instead of a routine hemocytometer—a slight modification of the method used by Aksu et al. [26]. In short, 10 µL of the mixture obtained by slicing the cauda epididymis in physiological saline was taken with an automatic pipette and placed in an Eppendorf tube containing 990 µL of Hayem solution. After the tube was vortexed for 15 s, approximately 20 µL of diluted sperm suspension was transferred to the counting chambers of the Thoma slide (HHH, Germany), and 5 min was allowed for all sperm to settle. Counting was done under a light microscope at a magnification of 400.

The mixture obtained by slicing the cauda epididymis in physiological saline was used to determine the ratio of abnormal spermatozoa. Exactly 5 µL of this mixture was taken with an automatic pipette and placed on a clean slide. Equal amounts of eosin-nigrosine dye (1.67% eosin, 10% nigrosine, and 0.1 M sodium citrate) were placed on the same slide and mixed. Immediately after mixing, a smear was made on the slide with the help of a coverslip and dried immediately. The slides were then evaluated under the light microscope at a magnification of 400. Precisely 200 sperm cells were evaluated in each slide and expressed as a percentage [27].

2.4. Testosterone measurement

The chemiluminescence microparticle immunological method was used for serum testosterone measurement. This measurement was made with an Abbott Architect i4000 SR device. The measurement was performed using

the appropriate kit (G6-1080/R01 B2P1WT, Archytect System, Germany; calibration range: 0.00–30.00 ngmol/L; quantitation range: 0.15–64.57 nmol/L; sensitivity: 99%) for the device.

2.5. Histopathological examination

A buffered formaldehyde solution (10%) was used for the fixation of the testicular tissues obtained in the study. The fixed tissues were subjected to routine procedures (alcohol and xylene series, followed by placement in paraffin blocks). Sections obtained from the paraffin blocks were stained with hematoxylin and eosin and evaluated according to the absence or severity of lesions using a light microscope.

2.6. Immunohistochemical examination

Tissue sections placed on adhesive (poly-L-Lysin) slides for immunoperoxidase examination were deparaffinized and dehydrated, and caspase 3 (cat no. ab184787; dilution ratio: 1/100, US) was used as the primary antibody and dripped onto the sections. The sections were incubated with a biotinized secondary antibody for 10–30 min at room temperature (ab236466). They were then incubated, taking into account the instructions for use, and 3-3' Diaminobenzidine was used as a chromogen. Light microscopy (Zeiss Axio, Germany) was used to evaluate the stained sections [25].

2.7. Immunofluorescence examination

Tissue sections were placed on adhesive (poly-L-Lysin) slides for immunofluorescence examination. Deparaffinizing and dehydration processes were then performed, and iNOS (cat no: ab283668; dilution ratio: 1/100, UK) was used as the primary antibody and dropped onto the sections. Subsequently, FITC (cat no. ab6785; dilution ratio: 1/1000) was used as a secondary antibody, and the sections were left in the dark for 45 min. In the next step, DAPI (cat no. D1306; dilution ratio: 1/200, UK) was added, and the sections were left in the dark for 5 min. Finally, the readied sections were closed with a coverslip and examined. Examination was performed under a fluorescent microscope (Zeiss Axio, Germany) [28].

2.8. Statistical analysis

The Shapiro–Wilk test was used to determine whether the testosterone and spermatological data were normally distributed. Since the testosterone and spermatological data in the groups were normally distributed, a One-Way ANAVO test was used to determine the significant differences between the groups for the same parameter. The post-hoc (Tukey HSD) test following ANOVA was used to determine which group caused the differences. The Kruskal–Wallis test (nonparametric) was used to determine the differences between groups for immunohistochemical and immunohistopathological evaluations. The Bonferroni-adjusted Mann–Whitney U test was used to determine which group caused the differences. In determining the positive staining intensity, 5 randomly selected areas in each image (images obtained from immunohistochemical and immunofluorescent staining) were evaluated with the Zeiss Zen Imaging Software program. Immunohistochemical and immunohistopathological findings were converted into semiquantitative data. Following Kruskal–Wallis analysis, a Bonferroni-adjusted Mann–Whitney U test was performed to determine which group caused the differences. The SPSS 21.0 program was used to statistically analyze the histopathological and biochemical evaluations. In the evaluations, $p < 0.05$ was considered significant [29].

3. Results

3.1. Sperm parameters

When the sperm parameter findings were examined, spermatozoa concentration and motility were significantly lower in the diabetes group than in the other groups, and the number of abnormal sperm was significantly higher ($p < 0.001$). It was determined that, while the spermatozoa concentration and motility significantly increased in the groups treated with metformin or ghrelin in addition to STZ, the rate of abnormal sperm also decreased ($p < 0.001$). Improvements in sperm parameters were more pronounced in the diabetes + ghrelin group than in the diabetes + metformin group ($p < 0.001$). The statistical data for findings obtained in the evaluation of sperm parameters are summarized in Table 1.

3.2. Testosterone findings

When serum testosterone values were examined, these values in the diabetes group were dramatically lower than in the control group ($p < 0.001$). Furthermore, although serum testosterone values in the groups given metformin and ghrelin with STZ were lower than in the control group, they were significantly restored and increased compared to the testosterone values of the diabetes group ($p < 0.001$). Additionally, testosterone levels in the diabetes + ghrelin group were higher than those in the diabetes + metformin group ($p < 0.001$). The statistical data for the evaluation of the testosterone hormone are summarized in Table 2.

3.3. Histopathological findings

A normal histological structure was detected in the testicular tissues of the control group rats (Figure 1). However, for rats in the diabetes group, severe degenerative changes in spermatocytes on the tubular walls of the testicular tissues, necrotic changes, and thinning of the tubular wall were observed. The hyperemia detected in the interstitial areas was also severe (Figure 1). In diabetic rats treated with ghrelin, degeneration of spermatocytes in the wall of the tubules and thinning of the tubular wall were mild, and hyperemia was moderate (Figure 1). The findings in the diabetes group treated with metformin were similar to those in the diabetes + ghrelin group. Only interstitial hyperemia was mild (Figure 1). A statistically significant difference ($p < 0.05$) was found between the diabetic group treated with ghrelin and the diabetic group treated with metformin when compared with the diabetic group. The findings obtained from the histopathological evaluation are graphically presented in Figures 2a–2d.

Table 1. Sperm parameter findings.

Groups	Spermatozoa concentration ($\times 10^6$) (mean ± SEM)	Motility (%) (mean ± SEM)	Abnormal spermatozoa rate (%) (mean ± SEM)
Control	116.42 ± 1.27[a]	73.00 ± 1.82[a]	12.71 ± 0.95[d]
Diabetes	32.71 ± 0.95[d]	31.71 ± 10.32[d]	53.28 ± 1.11[a]
Diabetes + ghrelin	66.14 ± 1.06[b]	58.71 ± 1.70[b]	28.14 ± 0.69[c]
Diabetes + metformin	46.42 ± 1.39[c]	48.71 ± 1.38[c]	35.85 ± 1.21[b]
p-value	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$

[a,b,c,d] p: Different letters in the same column represent a statistically significant difference ($p < 0.001$); p-values were obtained according to Tukey's HSD test.

Table 2. Serum testosterone results.

Groups	Testosterone (nmol/L) (mean ± SEM)
Control	6.84 ± 0.31[a]
Diabetes	2.57 ± 0.21[d]
Diabetes + ghrelin	3.49 ± 0.21[b]
Diabetes + metformin	3.14 ± 0.25[c]
p-value	$p \leq 0.001$

[a,b,c,d] p: Different letters in the same column represent a statistically significant difference ($p < 0.001$); p-values were obtained according to Tukey's HSD test.

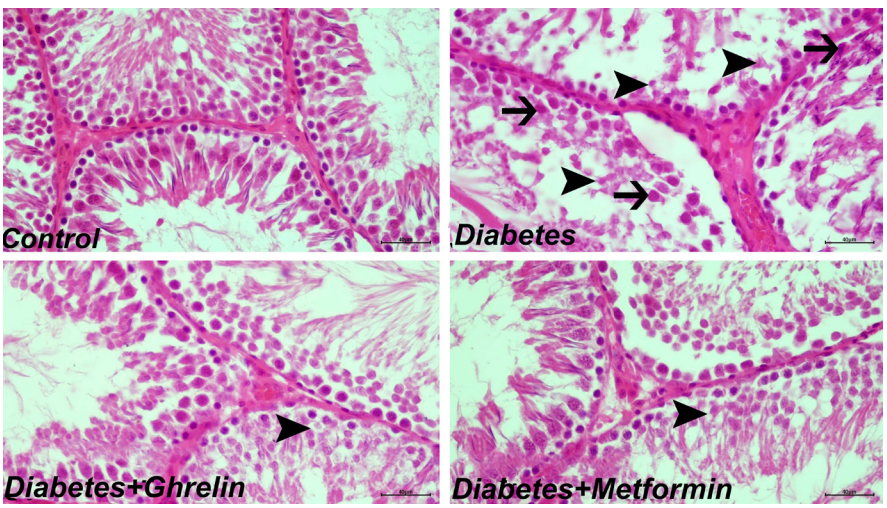
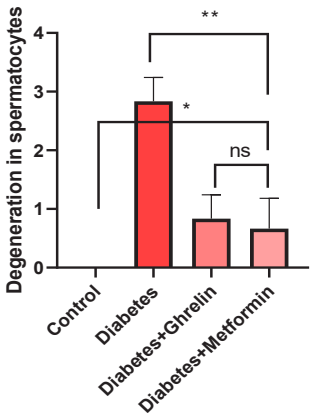


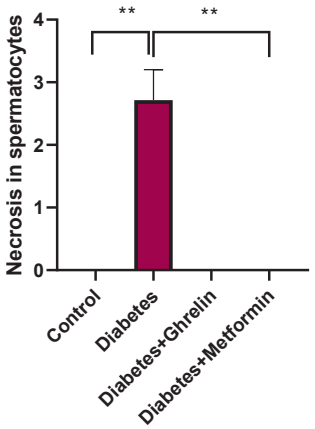
Figure 1. Testicular tissue; degeneration in spermatocytes (arrowheads); necrosis (arrows); thinning of the tubular wall; hyperemia in vessels; H&E; Bar: 40 µm.

3.4. Immunohistochemical findings

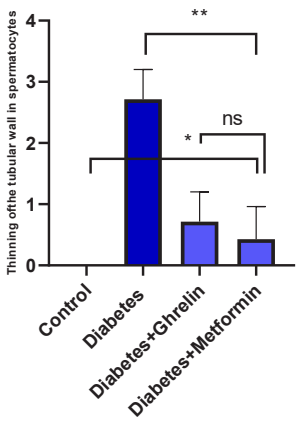
For the immunohistochemical evaluation of the groups, while caspase 3 expression was negative in the control group, it was detected intensely in spermatocytes in the diabetes group (Figure 3). Caspase 3 expression was mildly detected in the ghrelin-administered diabetic group and the metformin-administered diabetic group (Figure 3). When comparing the diabetes + ghrelin and the diabetes + metformin groups with the diabetes group, the difference was significant ($p < 0.05$).



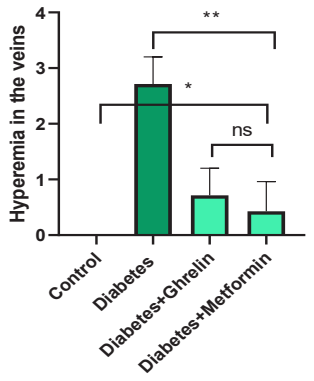
**,* = p < 0.05; ns= no significant difference; (n = 10).
Figure 2a. Histopathologic findings.



** = p < 0.05; (n = 10).
Figure 2b. Histopathologic findings.



**,* = p < 0.05; ns = no significant difference; (n = 10).
Figure 2c. Histopathologic findings.



**,* = p < 0.05; ns = no significant difference; (n = 10).
Figure 2d. Histopathologic findings.

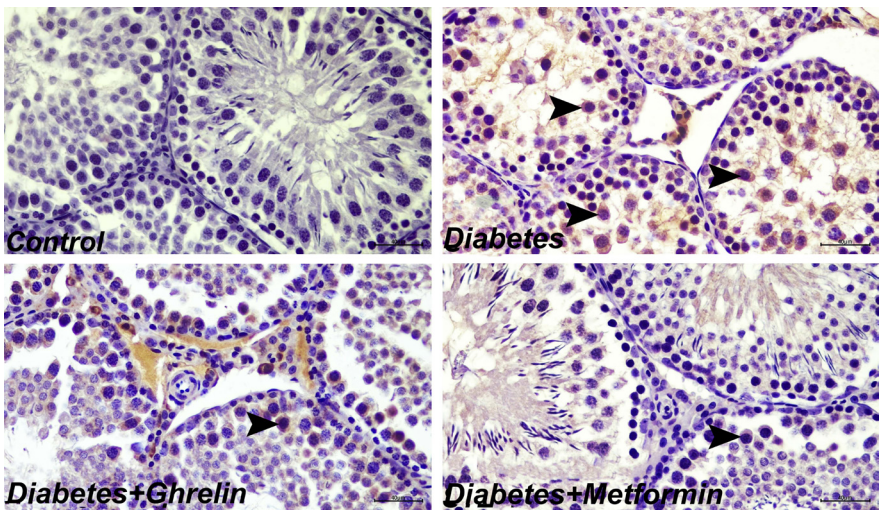


Figure 3. Testicular tissue; expression of cytoplasmic caspase 3 in spermatocytes (arrowheads); IHC-P; Bar: 40 µm.

3.5. Immunofluorescence findings

As a result of the immunofluorescence evaluation in the testicular tissues of the groups, while iNOS expression was negative in the control group, it was intense in the diabetes group (Figure 4). iNOS expression in the intertubular spaces was mild in the diabetes + ghrelin and diabetes + metformin groups (Figure 4), and there was a significant difference ($p < 0.05$) in iNOS expression between these 2 groups and the diabetes group. The statistical data for immunohistochemical and immunofluorescence evaluation of testicular tissues are summarized in Table 3.

4. Discussion

Diabetes has undesirable effects on multiple organ functions and negatively affects reproduction. Therefore, several strategies have been developed to treat diabetes and prevent or delay diabetes-related complications. However, an effective treatment method still does not exist. It is of critical importance to protect and guarantee reproductive health in the treatment protocols applied.

Evaluation of sperm parameters is paramount in the clinical examination of male fertility. In the current study, abnormal deviations occurred in the sperm parameters

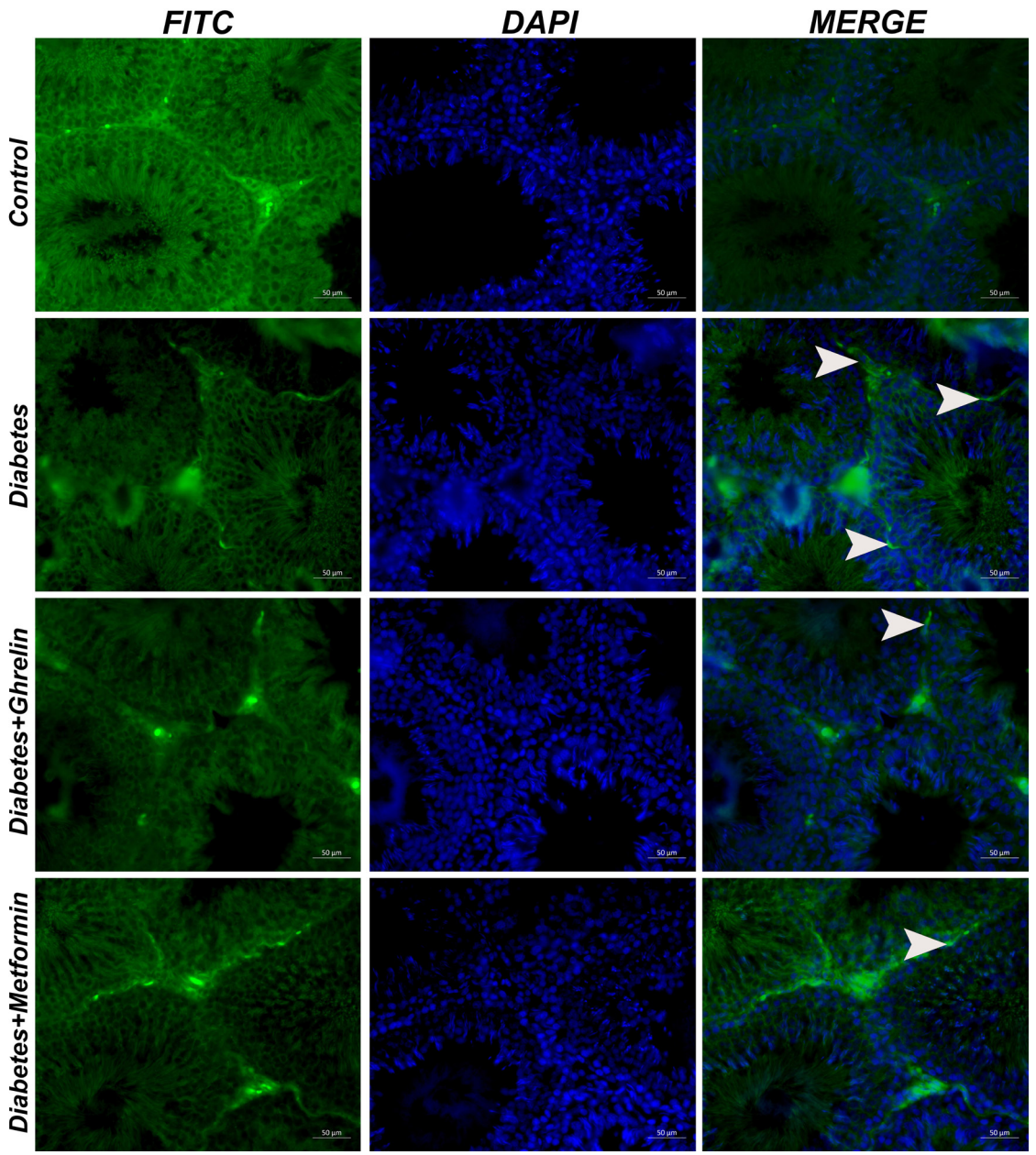


Figure 4. Testicular tissue; iNOS expression in intertubular spaces (arrowheads); IF; Bar: 50 µm.

Table 3. Statistical data of immunohistochemical and immunofluorescent findings in the testicular tissue.

Groups	caspase 3 expression (mean ± SEM)	iNOS expression (mean ± SEM)
Control	19.3 ± 1.52[a]	20.42 ± 2.07[a]
Diabetes	81.47 ± 3.85[b]	79.51 ± 4.08[b]
Diabetes + gherelin	40.57 ± 1.91[c]	40.44 ± 2.87[c]
Diabetes + metformin	41.08 ± 3.16[c]	40.37 ± 2.53[c]
p-value	$p \leq 0.05$	$p \leq 0.005$

[a,b,c] p: Different letters in the same column represent statistically significant differences ($p < 0.05$); p-values were obtained according to the adjusted Mann–Whitney U test.

of the diabetes group, which supports previous findings in the literature. While there were significant decreases in spermatozoa concentration and motility, there were significant increases in abnormal spermatozoa concentration [25,30,31]. The low spermatozoa concentration of the diabetic group may have been caused by decreased testosterone levels. Furthermore, the low testicular glucose utilization of the Sertoli cells may also have been responsible because Sertoli cells play an important role as an energy source in sperm development [32]. Metformin and ghrelin administered to the diabetic group brought the sperm parameters to the levels of the control group. These findings are consistent with previous studies on the effects of metformin and ghrelin on sperm parameters [3,30,33]. The improvement in parameters may have been mediated by the antioxidant properties of ghrelin and its control of insulin and glucose levels [3]. Additionally, the lowering of the glucose level by metformin may have been effective in this improvement [21].

Most testosterone is produced in the testicles. In this study, the testosterone level in the diabetes group was found to be low, similar to previous diabetes studies [3,25]. It was shown that the hypothalamic-pituitary-testicular axis is negatively affected by diabetes, which may be the reason for the decrease in testosterone levels in the diabetes group [3]. However, exogenously administered ghrelin significantly restored testosterone levels. This result is consistent with previous studies [3,34]. However, it differs from another study [35]. This difference may be because ghrelin's effect on testosterone levels depends on nutritional status [36]. Metformin administered in the present study also produced significant increases in testosterone levels. However, serum testosterone levels were higher in the diabetes + ghrelin group than in the diabetes + metformin group. Nna et al. [6] reported that the increase in the number of Leydig cells affected the increase in the testosterone level caused by metformin.

The abnormal changes (degenerative and necrotic changes, interstitial edema) detected in the testicular structure of the diabetes group in the present study are consistent with another study [3]. However, in the current study, ghrelin and metformin administered to diabetic groups reduced the severity of testicular structure deterioration. The effects of ghrelin and metformin on testicular histoarchitecture have been reported in previous studies and are in line with this study's findings [3,37].

It has been reported that diabetes mellitus forces spermatocytes to apoptosis by increasing oxidative stress in testicular tissues. Caspase 3 (also known as executioner caspase), the final apoptosis pathway, was preferred in this study since it is irreversible. It has been reported in the literature that diabetes increases oxidative stress in cells with a hyperglycemic effect, and, accordingly, cells are dragged into apoptosis [38,39]. In this study, caspase 3 expression, which was evaluated this way, was observed to be severe in the diabetes group. The result obtained is consistent with the literature [31,40,41]. A literature review shows that metformin prevents tissue apoptosis by down-regulating the caspase 3 level [6]. The reported findings are consistent with those from our study. The current research shows that ghrelin is as effective as metformin on caspase 3. The results obtained about ghrelin are also compatible with the literature [42] because, besides its metabolic impact, it inhibits apoptosis by producing antiapoptotic effects on various cell types [43].

In this study, iNOS expression was severe in the testicular tissues of the diabetes group. In previously reported studies, iNOS expression was high in the diabetes group [6,31,40]. However, ghrelin administered to the diabetes group decreased the expression level of iNOS in this study, which is consistent with the literature [43]. Additionally, metformin administration had an effect on lowering the iNOS expression level. In this sense, the results obtained here support the results of previously reported studies [6,41].

Our findings determined that ghrelin and metformin had positive effects, including improved sperm quality parameters in diabetes induced by STZ and a high-fat diet and increased testosterone levels. Ghrelin administered to the diabetic group increased testosterone levels more than metformin. Additionally, ghrelin and metformin reduced caspase 3 and iNOS expression levels in the diabetic rats. Based on these results, ghrelin and metformin should be considered part of therapeutic treatments to protect reproductive health in diabetic patients.

References

1. Atta MS, Almadaly EA, El-Far AH, Saleh RM, Assar DH et al. Thymoquinone defeats diabetes-induced testicular damage in rats targeting antioxidant, inflammatory and aromatase expression. International Journal of Molecular Sciences 2017; 18 (5): 919. https://doi.org/10.3390/ijms18050919
2. Shaikh H, Shrivastava VK, Amir M. Diabetes mellitus and impairment of male reproductive function: role of hypothalamus pituitary testicular axis and reactive oxygen species. Iranian journal of Diabetes and Obesity 2017; 8 (1): 41-50.
3. Elseed MY, Gaber M, Shatla IM, Abdrabbou RM. Protective effect of ghrelin on testicular functions in adult male diabetic albino rats. Al-Azhar Medical Journal 2016; 45 (1): 195-208. https://doi.org/10.12816/0026285
4. Ibrahim MN, Asalah AK, Abd-Alaleem DI, Moursi SMM. Effect of ghrelin on testicular functions in streptozotocin induced type 1 diabetic rats. Zagazig University Medical Journal 2014; 20 (2): 312-326. https://doi.org/10.21608/ZUMJ.2014.4358
5. Sönmez MF, Karabulut D, Kiliç E, Akalin H, Sakalar C et al. The effects of streptozotocin-induced diabetes on ghrelin expression in rat testis: biochemical and immunohistochemical study. Folia Histochemica et Cytobiologica 2015; 53 (1): 26-34. https://doi.org/10.5603/FHC.a2015.0006
6. Nna VU, Abu Bakar AB, Ahmad A, Eleazu CO, Mohamed M. Oxidative stress, NF-κB-mediated inflammation and apoptosis in the testes of streptozotocin-induced diabetic rats: Combined protective effects of Malaysian propolis and metformin. Antioxidants 2019a; 8(10): 465. https://doi.org/10.3390/antiox8100465
7. Dupont J, Maillard V, Coyral-Castel S, Ramé C, Froment P. Ghrelin in female and male reproduction. International Journal of Peptides 2010; 158102. https://doi.org/10.1155/2010/158102
8. Dutta S, Biswas A, Sengupta P, Nwagha U. Ghrelin and male reproduction. Asian Pacific Journal of Reproduction 2019; 8(5): 227-232. https://doi.org/10.4103/2305-0500.268144
9. Barreiro ML, Gaytan F, Castellano JM, Suominen JS, Roa J et al. Ghrelin inhibits the proliferative activity of immature Leydig cells *in vivo* and regulates stem cell factor messenger ribonucleic acid expression in rat testis. Endocrinology 2004; 145 (11): 4825-4834. https://doi.org/10.1210/en.2004-0732
10. Tena-Sempere M, Barreiro ML, González LC, Gaytán F, Zhang F-P et al. Novel expression and functional role of ghrelin in rat testis. Endocrinology 2022; 143 (2): 717-725. https://doi.org/10.1210/endo.143.2.8646
11. DE Magalhães DA, Kume WT, Correia FS, Queiroz TS, Allebrandt Neto EW et al. High-fat diet and streptozotocin in the induction of type 2 diabetes mellitus: A new proposal. Anais da Academia Brasileira de Ciências 2019; 91 (1): e20180314. https://doi.org/10.1590/0001-3765201920180314
12. van Losenoord W, Levendal RA, Frost CL. Cannabis and metformin on diabetic male Wistar rat sperm and reproductive organ parameters. Journal of Diabetes and Metabolic Disorders 2022; 21 (2): 1451-1458. https://doi.org/10.1007/s40200-022-01079-z
13. Kim SH, Kim SC, Ku JL. Metformin increases chemosensitivity via gene downregulation encoding DNA replication proteins in 5-Fu resistant colorectal cancer cells. Oncotarget 2017; 8 (34): 56546-56557. https://doi.org/10.18632/oncotarget.17798
14. Park MJ, Lee SY, Moon SJ, Son HJ, Lee SH et al. Metformin attenuates graft-versus-host disease via restricting mammalian target of rapamycin/signal transducer and activator of transcription3 and promoting adenosine monophosphate-activated protein kinase-autophagy for the balance between T helper 17 and Treges. Translational Research 2016; 173: 115-130. https://doi.org/10.1016/j.trsl.2016.03.006
15. Saisho Y. Metformin and inflammation: Its potential beyond glucose-lowering effect. Endocrine, Metabolic & Immune Disorders Drug Targets 2015; 15 (3): 196-205. https://doi.org/10.2174/1871530315666150316124019
16. Zhang S, Xu H, Yu X, Wu Y, Sui D. Metformin ameliorated diabetic nephropathy in a rat model of low-dose streptozotocin-induced diabetes. Experimental and Therapeutic Medicine 2017; 14 (1): 383-390. https://doi.org/10.3892/etm.2017.4475
17. Pernicova I, Korbonits M. Metformin–mode of action and clinical implications for diabetes and cancer. Nature Reviews Endocrinology 2014; 10 (3): 143-56. https://doi.org/10.1038/nrendo.2013.256
18. Tan MH, Alquraini H, Mizokami-Stout K, MacEachern M. Metformin: From research to clinical practice. Endocrinology and Metabolism Clinics of North America 2016; 45 (4): 819-843. https://doi.org/10.1016/j.ecl.2016.06.008
19. Faure M, Bertoldo M J, Khoueiry R, Bongrani A, Brion F et al. Metformin in reproductive biology. Frontiers in Endocrinology 2018; 9: 675. https://doi.org/10.3389/fendo.2018.00675
20. Derkach KV, Bakhtyukov AA, Romanova IV, Zorina II, Bayunova LV et al. The effect of metformin treatment on the basal and gonadotropin-stimulated steroidogenesis in male rats with type 2 diabetes mellitus. Andrologia 2020; 52 (11): e13816. https://doi.org/10.1111/and.13816
21. Naghibi M, Nasrabadi HT, Rad JS, Farashah MSG, Mohammadnejad D. The effects of metformin and forskolin on sperm quality parameters and sexual hormones in type II diabetic male rats. Andrologia 2022; 54 (7): 1605-1617. https://doi.org/10.1111/and.14426
22. Furman BL. Streptozotocin-induced diabetic models in mice and rats. Current Protocols in Pharmacology 2015; 70: 5.47.1-5.47.20. https://doi.org/10.1002/0471141755.ph0547s70
23. Qiu WC, Wang ZG, Lv R, Wang WG, Han XD et al. Ghrelin improves delayed gastrointestinal transit in alloxan-induced diabetic mice. World Journal of Gastroenterology 2008; 14 (16): 2572-2577. https://doi.org/10.3748/wjg.14.2572
24. Alhaider AA, Korashy HM, Sayed-Ahmed MM, Mobark M, Kfoury H et al. Metformin attenuates streptozotocin-induced diabetic nephropathy in rats through modulation of oxidative stress genes expression. Chemico-Biological Interactions 2011; 192 (3): 233-242. https://doi.org/10.1016/j.cbi.2011.03.014

25. Belhan S, Yıldırım S, Karasu A, Kömüroğlu AU, Özdek U. Investigation of the protective role of chrysin within the framework of oxidative and inflammatory markers in experimental testicular ischaemia/reperfusion injury in rats. Andrologia 2020; 52 (9): e13714. https://doi.org/10.1111/and.13714

26. Aksu EH, Akman O, Özkaraca M, Ömür AD, Uçar, Ö. Effect of Maclura pomifera extract against cisplatin-induced damage in reproductive system of male rats. Journal of the Faculty of Veterinary Medicine Kafkas University 2015; 21(3): 397-403

27. Turk G, Atessahin A, Sonmez M, Ceribasi AO, Yuce A. Improvement of cisplatin-induced injuries to sperm quality, the oxidant–antioxidant system, and the histologic structure of the rat testis by ellagic acid. Fertility and Sterility 2008; 89: 1474-1481. https://doi.org/10.1016/j.fertnstert.2007.04.059

28. Köktürk M, Yildirim S, Yiğit A, Ozhan G, Bolat İ et al. What is the eco-toxicological level and effects of graphene oxide-boramidic acid (GO-ED-BA NP)?: In vivo study on zebrafish embryo/larvae. Journal of Environmental Chemical Engineering 2022; 10 (5): 108443. https://doi.org/10.1016/j.jece.2022.108443

29. Sulukan E, Şenol O, Baran A, Kankaynar M, Yıldırım S et al. Nano-sized polystyrene plastic particles affect many cancer-related biological processes even in the next generations; zebrafish modeling. Science of The Total Environment 2022; 838 (Pt 3): 156391. https://doi.org/10.1016/j.scitotenv.2022.156391

30. Nna VU, Abu Bakar AB, Ahmad A, Mohamed M. Down-regulation of steroidogenesis-related genes and its accompanying fertility decline in streptozotocin-induced diabetic male rats: Ameliorative effect of metformin. Andrology 2019b; 7 (1): 110-123. https://doi.org/10.1111/andr.12567

31. Sayed MM, Abd El-Rady NM, Gomaa WMS, Hosny A, Gomaa AMS. Antioxidant, antiapoptotic, and antifibrotic abilities of L-Arginine ameliorate the testicular dysfunction in diabetic rats. Tissue and Cell 2023; 82: 102036. https://doi.org/10.1016/j.tice.2023.102036

32. Hamden K, Jaouadi B, Carreau S, Aouidet A, El-Fazaa S et al. Potential protective effect on key steroidogenesis and metabolic enzymes and sperm abnormalities by fenugreek steroids in testis and epididymis of surviving diabetic rats. Archives of Physiology and Biochemistry 2010; 116 (3): 146-155. https://doi.org/10.3109/13813455.2010.486405

33. Salimnejad R, Soleimani Rad J, Mohammad Nejad D, Roshangar L. Effect of ghrelin on total antioxidant capacity, lipid peroxidation, sperm parameters and fertility in mice against oxidative damage caused by cyclophosphamide. Andrologia 2018; 50 (2): e12883. https://doi.org/10.1111/and.12883

34. Dallak M. Unacylated ghrelin stimulates steroidogenesis in lean rats and reverses reproductive dysfunction in high fat diet-fed rats. Systems Biology in Reproductive Medicine 2019; 65 (2): 129-146. https://doi.org/10.1080/19396368.2018.1523971

35. Ramírez ND, Luque EM, Jones XM, Torres PJ, Espinoza MJM et al. Modulatory effects of ghrelin on sperm quality alterations induced by a fructose-enriched diet. Heliyon 2019; 5 (11): e02886. https://doi.org/10.1016/j.heliyon.2019.e02886

36. Chen VP, Gao Y, Geng L, Parks RJ, Pang YP et al. Plasma butyrylcholinesterase regulates ghrelin to control aggression. Proceedings of the National Academy of Sciences of the United States of America 2015; 112 (7): 2251-2256. https://doi.org/10.1073/pnas.1421536112

37. Khorasani MK, Ahangarpour A, Khorsandi L. Effects of crocin and metformin on methylglyoxal-induced reproductive system dysfunction in diabetic male mice. Clinical and Experimental Reproductive Medicine 48(3), 221-228. https://doi.org/10.5653/cerm.2020.04259

38. Rashid K, Sil PC. Curcumin ameliorates testicular damage in diabetic rats by suppressing cellular stress-mediated mitochondria and endoplasmic reticulum-dependent apoptotic death. Biochimica et Biophysica Acta 2015; 1852(1): 70-82. https://doi.org/10.1016/j.bbadis.2014.11.007

39. Wang Y, Zhang Z, Guo W, Sun W, Miao X et al. Sulforaphane reduction of testicular apoptotic cell death in diabetic mice is associated with the upregulation of Nrf2 expression and function. American Journal of Physiology 2014; 307(1): E14-E23. https://doi.org/10.1152/ajpendo.00702.2013

40. Kushwaha S, Jena GB. Telmisartan ameliorates germ cell toxicity in the STZ-induced diabetic rat: Studies on possible molecular mechanisms. Mutation Research 2013; 755 (1): 11-23. https://doi.org/10.1016/j.mrgentox.2013.04.013

41. Nna VU, Abu Bakar AB, Ahmad A, Mohamed M. Diabetes-induced testicular oxidative stress, inflammation, and caspase dependent apoptosis: the protective role of metformin. Archives of Physiology and Biochemistry 2020; 126 (5): 377-388. https://doi.org/10.1080/13813455.2018.1543329

42. Zhang Y, Ying B, Shi L, Fan H, Yang D et al. Ghrelin inhibit cell apoptosis in pancreatic beta cell line HIT-T15 via mitogen-activated protein kinase/phosphoinositide 3-kinase pathways. Toxicology 2007; 237(1-3): 194-202. https://doi.org/10.1016/j.tox.2007.05.013

43. Granado M, Chowen JA, García-Caceres C, Delgado-Rubín A, Barrios V et al. Ghrelin treatment protects lactotrophs from apoptosis in the pituitary of diabetic rats. Molecular and Cellular Endocrinology 2009; 309(1-2): 67-75. https://doi.org/10.1016/j.mce.2009.06.006