Spexin Ameliorates both Functional and Histological Testicular Changes in Type 2 Diabetic Rat Model

Original Article

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ABSTRACT

Background: In both human and rat tissues, the peptide hormone spexin is expressed. Some studies examined its relation to obesity and diabetes. Aim: To investigate the impact of spexin on testicular alterations in type 2 diabetic rats, both functionally and histologically, and potential underlying processes.

Materials and Methods: Four equal groups of twenty-four adult rats were used: control, type 2 diabetes group, type 2 diabetic group treated with metformin, and type 2 diabetic group treated with spexin. Rats were given a high-fat diet for eight weeks and injected single dose of streptozotocin (40 mg/kg) in the 4th week. These rats had developed type 2 diabetes. Normal diet and vehicle were provided to control rats. Treatment groups (III & IV) received either oral metformin (300 mg/kg/day) or intraperitoneal spexin (35 µg/kg/day) for four weeks, starting from the fifth week and ending at the end of the eighth week. Metabolic profile, some inflammatory, oxidative stress markers and serum spexin levels were assessed at the end of experiment. Histological and immunohistochemical assessment of the testis were investigated.

Results: Diabetic groups showed a significant deterioration in metabolic profile, histological structure of the testis, epididymal sperm count and motility, some inflammatory oxidative stress markers while testicular superoxide dismutase and serum spexin were significantly reduced. These alterations were significantly reversed in groups treated with spexin and metformin.

Conclusions: Both Spexin and metformin have almost the same protective effect by attenuating the testicular histological and metabolic alterations caused by type 2 diabetes.

Key Words: D.M, Metformin, Spexin, Testis.

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INTRODUCTION

Insulin resistance and hyperglycemia are two metabolic diseases that define type 2 diabetes mellitus. It comes with numerous consequences since it affects the function of numerous organs, including the heart, testis, kidneys and retina. Insulin is the primary hormone that controls how much glucose is produced and used by human tissues and organs. Obesity, a poor diet and a sedentary lifestyle are major causes of the rise in diabetes worldwide[1].

Normal spermatogenesis and testicular function depend on glucose homeostasis. In diabetes, persistent hyperglycemia increases the oxidative stress state, which can harm the testis and lead to infertility[2].

Several studies revealed a connection between diabetes and a decrease in sperm count and testicular weight with the presence of significant numbers of defective sperm. Additionally, a high prevalence of diabetes-related infertility problems exists, even in younger individuals, Elevated oxidative stress state may result in epithelial damage and germ cell death with a decrease in the number of seminiferous tubules[3].

Also, persistent hyperglycemia is linked to the induction of cellular inflammation and lowers the activity of the cellular antioxidant system like catalase and superoxide dismutase enzymes. The risk of oxidative stress is raised and results in testicular failure. Furthermore, inflammatory cytokines as TNF-α and IL-6 are linked to the genesis of co-morbidities like diabetes, obesity and other conditions as well as the regulation of metabolism[4].

Metformin is the most commonly prescribed glucose-lowering drug for type 2 diabetes due to its safety and efficacy. Also, it has a positive effects on metabolism and cardiovascular health. It is advantageous for the endothelium, skeletal muscles and adipose tissue that are impacted by

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hyperinsulinemia and insulin resistance (IR). As insulin secretagogues or insulin sensitizers, metformin works well whether taken alone or in combination with other medications[5].

Spexin (SPX) is a 14 amino acid peptide hormone which is abundantly present in both central and peripheral tissues. It performs a variety of biological processes, including lowering blood glucose levels, body weight and caloric consumption[6]. SPX can affect food intake, regulate insulin secretion stimulate lipolysis and inhibit lipogenesis in vitro Furthermore, SPX downregulates fatty acid uptake into the hepatocytes[7].

Type 2 diabetes (T2D) and obesity were noted to have reduced circulating Spexin levels. Furthermore, systemic Spexin therapy reduced TNF- and IL-6 levels in both liver, serum of obese diabetic mice, as well as improved metabolic indices like insulin resistance, lipid metabolism and liver function[7].

Spexin therapy's anti-inflammatory, antioxidant and hypoglycemic effects on heart tissue help to lessen diabetes-related cardio metabolic abnormalities in obese type 2 diabetic rats. Also, Spexin injection has been shown to protect renal tissue by decreasing inflammatory infiltration, necrosis, apoptosis and fibrosis in obese rats[8].

Although some researchers reported that type 2diabetes, obesity and associated co-morbidities may be improved by Spexin, the impact of spexin on testicular dysfunction associated with type 2 diabetes and its underlying processes has not been studied. As a result, our goal was to show how the spexin administration could affect the testicular dysfunction in a type 2 diabetes model and explain its possible underlying mechanisms.

MATERIALS AND METHODS

Animals:

We utilized twenty-four adult male albino rats, ranging in weight from 170 - 200 g. They came from the Animal House at Zagazig University's Faculty of Veterinary Medicine.

Ethical approval and care of experimental animals:

The rats were kept at room temperature with a 12-hour light/dark cycle and they were given food and drink. The National Institutes of Health's global criteria for the use and care of laboratory animals were followed throughout the experiment's approach. With approval number ZU-IACUC/3/F/33/2023, it was authorized by Zagazig University's Institutional Animal Care and Use Committee.

Chemicals and preparation:

STZ: sourced from Alfa Aesar in Kandel, Germany, as a white powder (weight: 40,000 Da; CAS: 9011–18 - 1).

Metformin: acquired from CID, Egypt in the form of 500 mg tabs of Cidophage.

Spexin: purchased from Belmont, California, USA, from Phoenix Pharmaceuticals.

Experimental protocol:

The rats were given a week to acclimate before being split equally into 4 groups. **Group I (Control group):** Rats were fed a standard laboratory chow diet from Zagazig University's Faculty of Agriculture, which included 11.4 % fat, 62.8 % carbs, and 25.8 % protein. Throughout the eight-week experiment, they were additionally administered 1 milliliter of regular saline orally via oral gavage. Intraperitoneally (i.p.) 0.01M citrate buffer, pH 4.5 was given at the start of the fourth week to imitate the injections of STZ[7].

Group II (diabetic group): for eight weeks, Rats were given a high-fat diet (HFD) that included cottonseed oil formed of (16.45 % protein, 25.6 % carbs, and 58.0 % fat) mixed to laboratory chow[9] and rats that had fasted overnight were given a single intraperitoneal injection of STZ (40 mg/kg body weight, diluted in 0.01 M citrate buffer, pH 4.5) to induce diabetes starting from the fourth week[10]. A 5 % glucose solution was given to rats to drink overnight in order to alleviate the hypoglycemia brought on by the medication. After receiving a single weekly injection of STZ, blood glucose levels of rats were assessed; those over 200 mg/dl were considered diabetic[11].

Group III (diabetic/ metformin treated group): rats were given the same treatment as the group with diabetes. Moreover, oral gavage doses of metformin (300 mg/kg/day) were administered to rats for four weeks, starting from the fifth week and ending at the eighth week[12].

Group IV (diabetic /Spexin treated group): rats received the same treatment as the group with diabetes. In addition, rats were given spexin dissolved in isotonic saline via intraperitoneal injection (35 μg /kg/day) for 4 weeks, from the beginning of the fifth week till the end of the eighth week'[13].

Estimation of body mass index (BMI): The formula used to estimate BMI was body weight (g) / lenght^2 (cm^2) = BMI (gm/cm^2). When the BMI is greater than 0.68 g/cm^2, it might be utilized as an indicator of obesity[14].

Collecting blood sample and performing a biochemical analysis:

At the end of experiment, fasting rats were anaesthetized and blood samples were taken. After centrifuging the blood, the serum supernatant was kept at -20 °C until it was needed to estimate:

- The glucose oxidase method (Spinreact, Spain) was utilized to estimate blood glucose. Also, to measure insulin, the enzyme-linked immunosorbent assay (ELISA) was utilized.

- Using the following formula, fasting serum insulin (µIU/ml) × fasting serum glucose (mg/dl)/405, the homeostasis model assessment-insulin resistance (HOMA-IR) index was calculated[15].

- Lipid profile estimation in the following manner: The levels of high-density lipoprotein (HDL), triglycerides (TG), and total serum cholesterol (TC) were measured, the following formula for calculating blood low density lipoprotein (LDL) levels was used by Friedewald *et al.*, 1972[16], TC - HDL - TG/5 = LDL (Biosource Europe S.A. in Belgium was the source of the kits).

- Measuring serum levels of spexin with commercial kits (Creative Diagnostics, USA; catalog number: DEIA10757).

- The level of free serum testosterone was determined with the aid of ELISA kits (BioCheck, CA 94405).

- The method of[17], for estimating serum LH level, was employed in the experiment.

- The serum FSH level was estimated using the procedure described in reference[18].

- Evaluation of tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) levels in the serum: kits for TNF-alpha (Catalo number: MBS355371 (Sigma-Aldrich) and IL-6 (Catalo number: MBS355410 (Bio diagnostic-Egypt). Every measurement kit was used in compliance with the instructions supplied by the manufacturers.

Gonadal extraction:

A sodium thiopental injection administered intraperitoneally at a dose of 25 mg/kg body weight was used to sacrifice the rats[19]. The midline of the abdominal wall was incised; the epididymis and testis were carefully removed and dissected. A digital balance was used to measure the testicular weights. At 37°C, the left epididymis of each rat was removed, minced, and then put back together in 2 milliliters of Hank's buffer salt solution (HBSS) [20]. The usual haemocytometric method used to determine the caudal epididymis sperm after 5 minutes at 37°C. Motility and total sperm counts were assessed in the samples that were placed on the glass slides[21].

Sperm count is a quantity of spermatozoa per milliliter of fluid. It is counted under a 400X light microscope. The following formula is used to compute the number of spermatozoa per milliliter of fluid, or sperm concentration. Spermatozoa count = number of counts multiplied by volume, dilution factor, and number of areas counts[22].

At least 200 static sperm were counted for evaluating motility. By dividing the number of viable sperm cells by the total number of sperm cells, the percentage of motility was determined[22]. Additionally, 200 sperm per smear of sperm from each group were checked under a light microscope for malformations.

Evaluation of testicular oxidative stress markers:

Testicular tissue from each group was homogenized in 50 mm potassium phosphate (pH 7.4), centrifuged at 4000 rpm for 15 minutes at 4 ◦C, and the supernatants were stored at −80 ◦C until performing the following estimations:

- Malondialdehyde (MDA) level: kits for MDA (Sunred Bio Shanghai 2010637-11-, CHINA).

- Super oxide dismutase (SOD) Level: kits for SOD (Thermofisher, USA, coiorimetric assay, Ca EI ASODC) were used.

Histological study:

Light microscopic examination:

Following the processing of the testicular tissue samples to create paraffin blocks, serial sections with 5 µm thickness were stained with:

1- Hematoxylin and Eosin (H and E)[23].

2- Mallory trichrome stain[24].

3- Immunohistochemical staining for Ki-67 antibody (Cat. No. MA5- 14520, Thermo Fisher Scientific, Rockford, USA), diluted 1:200 in PBS. Ki-67 is a proliferative marker that is localized in the nucleus of germ cells and may be detected utilizing the Avidin-Biotin-Peroxidase complex technique (25).

Electron microscopy examination:

Ultrathin sections from each rat's testis were produced, and a JEOL transmission electron microscope JEM-2100 was used for its examination in the Electron Microscope Research Laboratory (EMRL) at Mansoura University's Faculty of Agriculture (Egypt)[23].

Histomorphometric study:

The data was gathered using a "Leica Qwin 500" image analyzer computer system (Leica imaging system LtD, Cambridge, England). The height of the germinal epithelium, the area % of collagen fibers, and the area % of Ki 67 immumoreactivity at a magnification of ×400 were measured in ten non-overlapping fields.

Statistical analysis:

A version 20 of "Statistics for Windows SPSS" was used to analyze the biochemical and morphometric measures. Results were shown with their mean standard deviation (SD). For this, the LSD test and one-way analysis of variance (ANOVA) were employed. The differences were considered statistically significant when the probability value (*p*) was less than 0.05, highly significant when p was less than 0.001, and non-significant when p was larger than 0.05[26].

RESULTS

Biochemical results:

Metabolic changes in all group Table 1:

According to diabetic group, there was significant increase in BMI, HOMA-IR, serum (glucose, insulin, TC, TGs, and LDL), and a significant decrease in serum HDL. When compared to the diabetic group, there was a substantial rise in serum HDL and a significant decrease in BMI, HOMA-IR, and serum (glucose, insulin, TC, TGs, and LDL) in the metformin and spexin-treated groups.

Table 1: Metabolic parameters in all studied groups (Number of rats in each group = 6):

Groups / Parameter	Control	Type 2 diabetic	Type 2 diabetic metformin treated	Type 2 diabetic spexin treated
BMI gm / cm^2	0.62 ± 0.05	0.74 ± 0.07[a]	0.64 ± 0.055[b]	0.63 ± 0.052[b]
Serum glucose(mg/dl)	74 ± 12.4	142 ± 9.35[a]	82 ± 9.3[b]	81 ± 9.7[b]
Serum insulin(µIU\ml)	16.8 ± 2.1	25.16 ± 2.9[a]	18.5 ± 1.87[b]	18 ± 1.89[b]
HOMA-IR	3.1 ± 0.9	8.9 ± 1.6[a]	3.88 ± 0.8[b]	3.66 ± 0.9[b]
Serum TC (mg/dl)	90.0 ± 8.34	185.7 ± 13.31[a]	96.43 ± 9.07[b]	99 ± 8.4[b]
Serum TG (mg/dl)	72 ± 8.6	129.66 ± 7.1[a]	78.3 ± 5.5[b]	76.5 ± 7.6[b]
Serum LDL (mg/dl)	36 ± 1.7	137 ± 1.4[a]	46 ± 1.1[b]	47 ± 1.5[b]
Serum HDL (mg/dl)	39.75 ± 6.4	22.3 ± 5.39[a]	34 ± 4.5[a,b]	38 ± 4.66[b]

Inflammatory and Oxidative markers changes in all group Table 2:

According to diabetic group, Serum inflammatory markers (IL6 and TNF-α) and testicular tissue level of oxidative markers (MDA) were significantly elevated, while testicular SOD was significantly lower than in the control group. When compared to the diabetic group, the spexin and metformin-treated groups showed a large increase in testicular SOD and a significant drop in serum inflammatory indicators (IL6 and TNF-α) as well as the testicular tissue level of oxidative markers MDA.

Table 2: Inflammatory and Oxidative parameters in all studied groups (Number of rats in each group= 6):

Groups / Parameter	Control	Type 2 diabetic	Type 2 diabetic metformin treated	Type 2 diabetic spexin treated
Serum IL-6 (pg/ml)	45.3 ± 3.3	64.57 ± 3.1[a]	54.3 ± 3[a,b]	45 ± 3.7[b,c]
Serum TNF(pg/ml)	56 ± 3.7	75 ± 3.7[a]	67 ± 1.87[a,b]	55 ± 3.48[b,c]
Testicular tissue MDA(µg/g tissue)	38.66 ± 4	64.2 ± 3.65[a]	44.8 ± 3.4[a,b]	36.5 ± 3.5[b,c]
Testicular tissue SOD(µg/g tissue)	77.66 ± 8.06	53.33 ± 8.1[a]	74.67 ± 9[b]	76.2 ± 7.8[b]

Changes in levels of spexin, hormones and quality of testis and sperms in all groups Table 3:

According to diabetic group, In contrast to the control group, this study showed a significant and marked drop in testicular weight as well as sperm count, motility, and blood levels of (spexin, testosterone, LH and FSH). When compared to the diabetic group, the spexin and metformin-treated groups showed a significant rise in testicular weight and a significant increase in serum levels of (spexin, testosterone, LH and FSH), sperm count and motility.

Table 3: Spexin, hormones, and quality of testis and sperms in all groups:

Groups / Parameter	Control	Type 2 diabetic	Type 2 diabetic metformin treated	Type 2 diabetic spexin treated
Serum spexin (ng/ml)	1.78 ± 0.06	0.73 ± 0.05[a]	1.24 ± 0.04[a,b]	1.79 ± 0.03[b,c]
Serum LH (mg/dl)	3.06 ± 0.65	2.1 ± 0.47[a]	3.15 ± 0.7[b]	3.1 ± 0.59[b]
Serum FSH (mg/dl)	4.1 ± 0.5	3.35 ± 0.47[a]	4.5 ± 0.54[b]	4.9 ± 0.62[a,b]
Serum testosterone mg/dl	5.7 ± 0.94	3.9 ± 0.63[a]	6.2 ± 0.95[b]	6.4 ± 0.89[b]
Sperm motility %	78.33 ± 4.3	58.66 ± 3.14[a]	77 ± 5[b]	78 ± 4[b]
Sperm count %	71.67 ± 6.8	39.5 ± 1.88[a]	70 ± 6.9[b]	70 ± 3.2[b]
Right Testicular weight (g)	2.04 ± 0.32	1.13 ± 0.12[a]	1.67 ± 0.22[b]	1.89 ± 0.3[b]
Left Testicular weight (g)	2.16 ± 0.52	1.18 ± 0.14[a]	1.87 ± 0.23[b]	2 ± 0.32[b]

Data were expressed as mean ± SD. a means $P < 0.05$ when compared with control group. b means $P < 0.05$ when compared with type 2 diabetic group. c means $P < 0.05$ when compared with type 2 diabetic metformin treated group. BMI, body mass index: HOMA-IR, homeostasis model assessment insulin resistance; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; TNF, tumor necrosis factor alpha; IL6, interleukin-6; SOD, superoxide dismutase; MDA, malondialdehyde; LH, luteinizing hormone; FSH, follicle stimulated hormone.

Correlation between serum spexin level and some studied parameters within the diabetic group (Table 4): Spexin serum level was negatively correlated with each of BMI, serum glucose, serum insulin, HOMA-IR, serum TC, serum TGs, serum LDH, serum IL6, serum TNF-α and serum MDA, but it was positively associated with both serum HDL and serum SOD.

Table 4: Pearson's correlation coefficient (r) between serum spexin level and some studied parameters within the type 2 diabetic group (number of rats= 6):

Correlation / Parameter	R	*P*
BMI	- 0.95	< 0,01
Glucose	- 0.849	< 0,05
Insulin	- 0.850	< 0,05
HOMA-IR	- 0.98	< 0,01
TC	- 0.95	< 0,01
TGs	- 0.89	< 0,05
LDL	- 0.88	< 0,05
HDL	+ 0.814	> 0,05
IL6	- 0.825	< 0,05
TNF-α	- 0.89	< 0,05
MDA	- 0.849	< 0,05
SOD	+ 0.919	> 0,01

BMI, body mass index; HOMA-IR, homeostasis model assessment insulin resistance; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; IL6, interleukin-6 TNF□, tumor necrosis factor alpha; MDA malondialdehyde, SOD, superoxide dismutase.

Histological results :

A) Light microscope results:

1) H and E-stained results:

The control group exhibited seminiferous tubules that were separated by the interstitium. A thin layer of basal lamina encircled seminiferous tubules, which were lined with sertoli cells and stratified germinal epithelium including spermatogonia, primary spermatocytes, and spermatids. Sertoli cells with Large, pale nuclei were seen. Leydig interstitial cells had vesicular nuclei and were round with appearances between seminiferous tubules Figure 1 a. The group with diabetes displayed thicker connective tissue capsules, obvious distortion of tubules and cell loss, which was supported by the wide distance separating the germinal cells and congested blood vessels. The nuclei of most cells were dark with multinucleated giant cell formation. The interstitisum had vacuolation and

homogenous acidophilic materials Figure 1 b. Seminiferous tubules in the metformin-treated group still displayed reduced cellularity and separation between the germinal dark-nucleated cells Figure 1 c. Seminiferous tubules of Spexin group had an almost normal appearance of germinal cells with thin, regular basal lamina. Pale nuclei were visible in the sertoli cells. Leydig's interstitial cells featured rounded vesicular nuclei Figure 1 d.

2) Mallory trichrome results:

Collagen fibers were seen in the control group's typical locations around the blood vessels and seminiferous tubules Figure 2 a. The diabetic group had much higher collagen fiber distribution in the capsule, surrounding the seminiferous tubules, and around the blood vessel Figure 2 b. A reduction in the distribution of collagen fibers surrounding the seminiferous tubules, and around the blood vessel was observed in the group treated with metformin Figure 2 c. The collagen fibers in the group treated with spexin were sparsely and regularly distributed around the blood vessel and seminiferous tubules Figure 2 d.

3) Immunohistochemical results:

Spermatogonia and primary spermatocyte nuclei in the control group displayed an intense positive immunoreaction in sections stained with the anti-Ki-67 antibody Figure 3 a. Few cells in the diabetic group had a positive immunoreaction Figure 3 b. Positive immunoreaction was found in the nuclei of some spermatogonia in the metformin-treated group Figure 3 c. A positive immunoreaction was observed in spermatogonia and certain primary spermatocytes nuclei of the Spexin-treated group Figure 3 d.

B) Electron microscope results:

Basal compartment of control group have basal lamina enclosed with myoid cell that has flat nuclei. On the basal lamina, there was spermatogonic cell with euchromatic nucleus. The cytoplasm of sertoli cells contained mitochondria with tight junction between some of the cells and electron-dense granules. Leydig cell displayed several mitochondria, lipid droplets, and euchromatic nuclei with a peripheral rim of heterochromatin Figure 4 a. The ad luminal compartment revealed early spermatid having large euchromatic nuclei with prominent nucleolus and peripherally placed mitochondria. Parts of late spermatids were observed. Some nuclei of late spermatids appeared with acrosomal caps Figure 4 b. Cross sections of the sperm tails revealed that middle pieces were made up of 2 central singlets and nine doublets microtubules. Cell membranes, mitochondrial sheaths, and nine coarse, dense fibers encircled axoneme Figure 4 c.

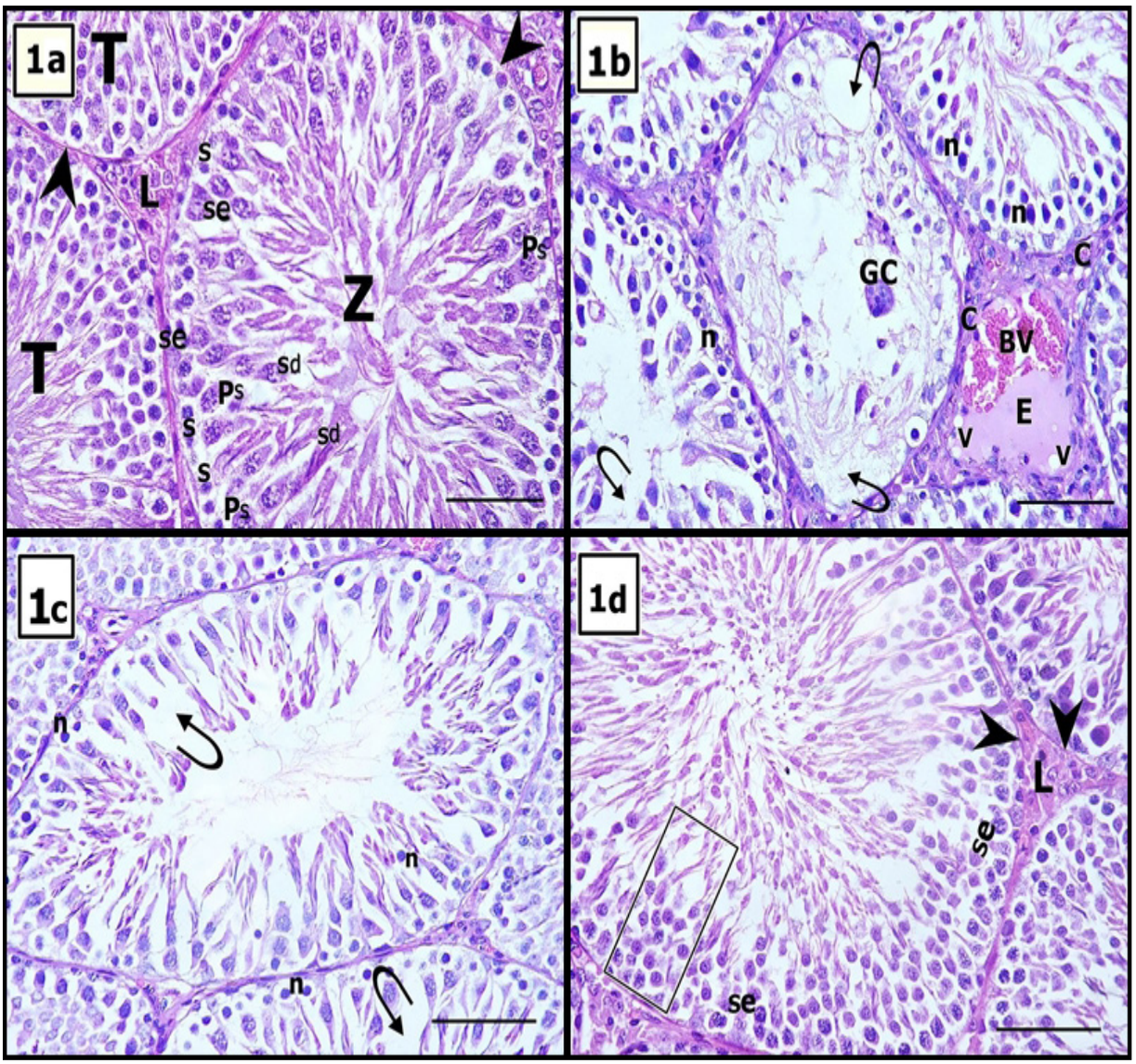

Figure 1: Photomicrographs of control testis stained with H&E (a) seminiferous tubules (T) with interstitium containing Leydig cells (L) and a normal, thin basal lamina (arrow heads). Spermatogonia (S), primary spermatocytes (Ps), and spermatids (Sd) make the germinal epithelium. The nuclei of sertoli cells (Se) are large and pale. Spermatozoa are seen in the tubule lumen (Z). (b) The thick connective tissue capsule (C), marked distorted tubules with wide distance separating germinal cells (curved arrows) that have dark nuclei (n), multinucleated giant cell (GC) and congested blood vessels (BV) are present in the diabetic group. Vacuolation (v) and exudate (E) are visible in the interstitium. (c) Metformin treated group; there are areas of cell loss in seminiferous tubules, (curved arrows). The nuclei of germinal cells are darkly stained (n). (d) Seminiferous tubules with thin, regular basal lamina (arrow heads) and intact germinal epithelium (rectangles) are seen in the spexin-treated group. The nuclei of Sertoli cells (Se) are pale. Leydig's interstitial cells featured rounded vesicular nuclei (L) (H&E X400, Scale Bar X40μm).

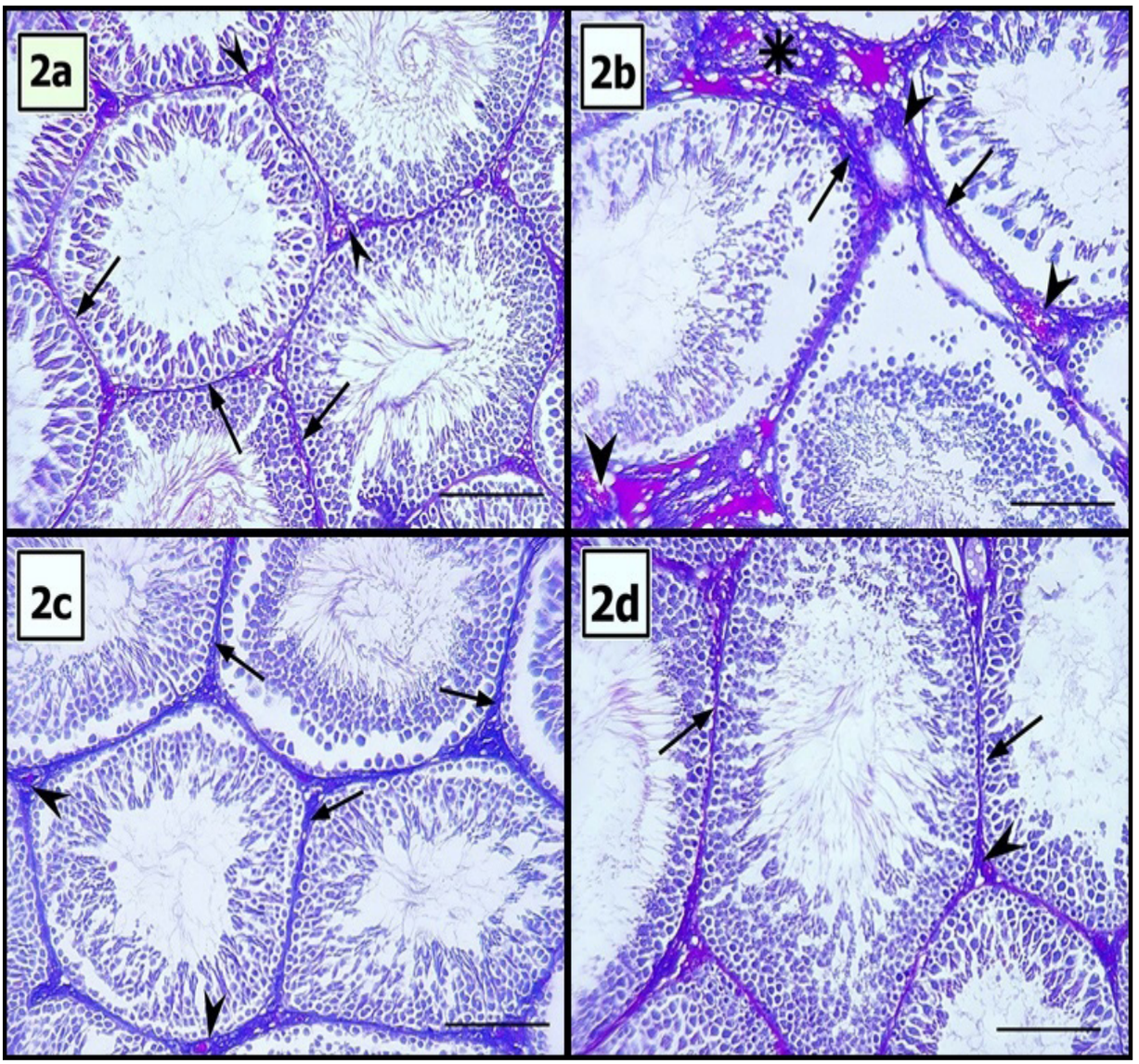

Figure 2: Sections of Mallory trichrome:(a) in the control group's normal distribution of collagen fibers are present around the blood vessels (arrowheads) and seminiferous tubules (arrows) (b) Collagen fiber distribution in the capsule (Asterix), surrounding the seminiferous tubules (arrows) and around the blood vessel (arrowheads) is much marked in the diabetic group (c) A reduction in the distribution of collagen fibers surrounding the blood vessel (arrowheads) and seminiferous tubules (arrows) is observed in the group treated with metformin.(d) The collagen fibers in the group treated with spexin were sparsely and regularly distributed around the blood vessel (arrowheads) and seminiferous tubules(arrows) (Mallory trichrome stainX400 ,Scale Bar X40µm).

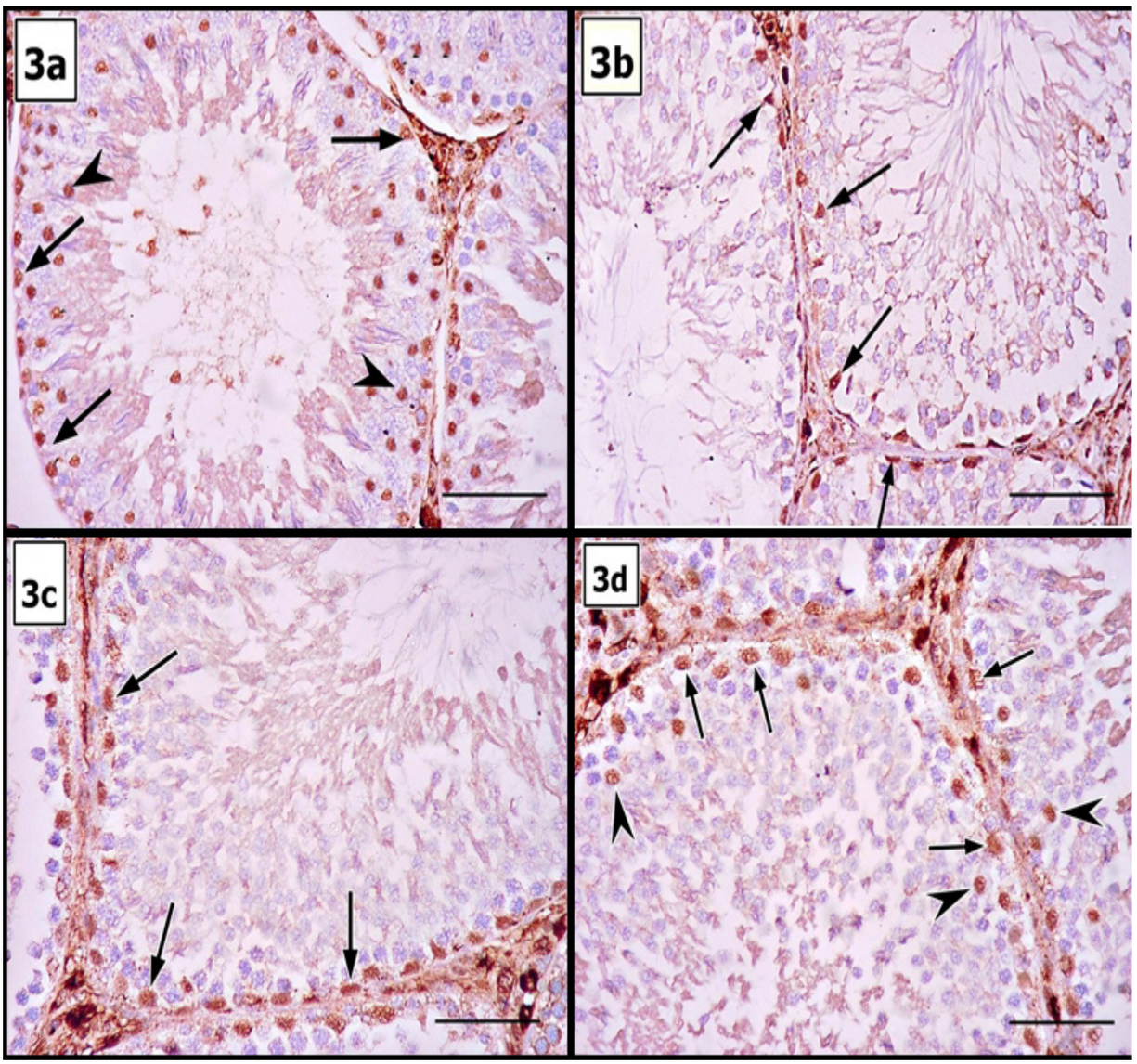

Figure 3: Sections stained with anti-Ki-67 antibody in immunohistochemistry: (a) Spermatogonia (arrows) and primary spermatocyte nuclei (arrowheads) in the control group display an intense positive immunoreaction. (b) Few cells in the diabetic group have positive immunoreaction (arrows). (c) Positive immunoreaction is found in the nuclei of some spermatogonia in the metformin-treated group (arrows). (d) A positive immunoreaction is observed in spermatogonia (arrows) and certain primary spermatocytes (arrowheads) nuclei of the Spexin-treated group (Anti-Ki-67X400, Scale Bar X40µm).

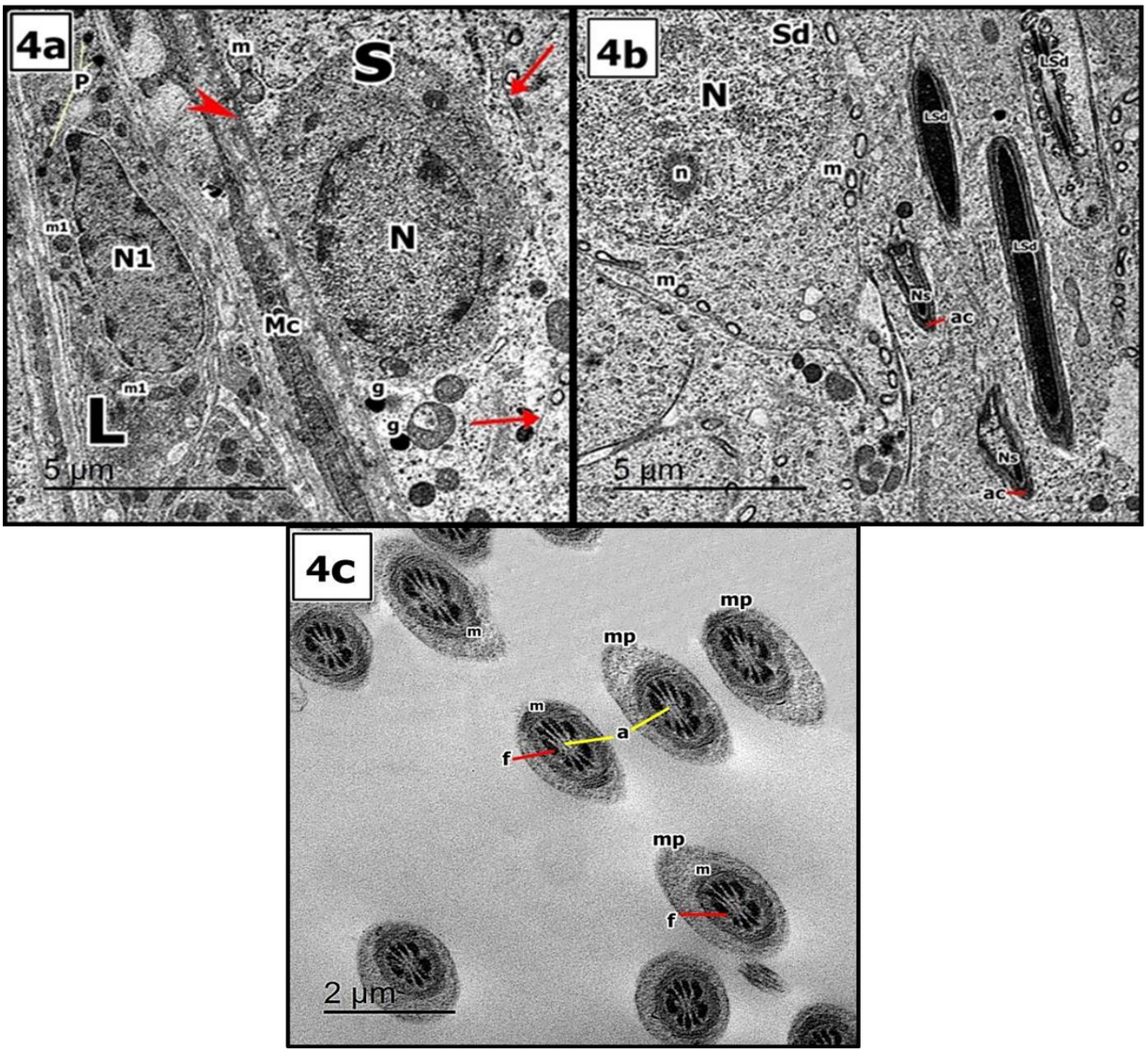

Figure 4: An electron micrograph of the (a) Control testis reveals a: basal compartment shows normal basal lamina (arrowhead) enclosed by myoid cell (Mc) with flat nuclei. Spermatogonic cell (S) have euchromatic nucleus (N). The cytoplasm of the adjacent sertoli cells has mitochondria (m), electron-dense granules (g). Between some cells, tight junctions are present (arrows). Leydig cell (L) displays euchromatic nucleus (N1), numerous mitochondria (m1) and lipid droplets (p) (Direct Mag X 1500). (b) Early spermatid (sd) has large euchromatic nucleus (N) with prominent nucleolus (n) and peripheral mitochondria (m). Parts of late spermatids (LSd) are observed. Some nuclei of late spermatids (Ns) appear with acrosomal caps (ac) (Direct Mag X 1200). (c) Middle pieces (mp) are made up of axoneme (a) that is of two central singlets and nine doublets. Nine thick dense fibers (f), mitochondrial sheaths (m), and cell membranes encircle axoneme (Direct Mag X 2000).

Basal compartment of Group II (Type 2 diabetic group) showed different cells (spermatogonic cell, sertoli cell and primary spermatocyte). The basal lamina appeared irregular and thickened. Spermatogonic cell had nucleus with clumps of heterochromatin and discontinued nuclear envelop. Additionally, areas of cell separation and discontinuous tight junctions were identified Figure 5 a. Another section of the basal compartment showed sertoli cell with irregular shaped nucleus and different shaped mitochondria rested on irregular basal lamina. Primary spermatocyte was seen with nucleus and abnormal mitochondria. Cell separation and discontinuous tight junctions were present Figure 5 b. Early spermatids had shrunken nuclei and peripheral mitochondria. Parts of late

spermatids were seen Figure 5 c. Sections of the sperm tails revealed principle pieces and distorted middle piece Figure 5 d. Leydig cells featured an indented nucleus with mitochondria in the cytoplasm and heterochromatin clusters Figure 5 e.

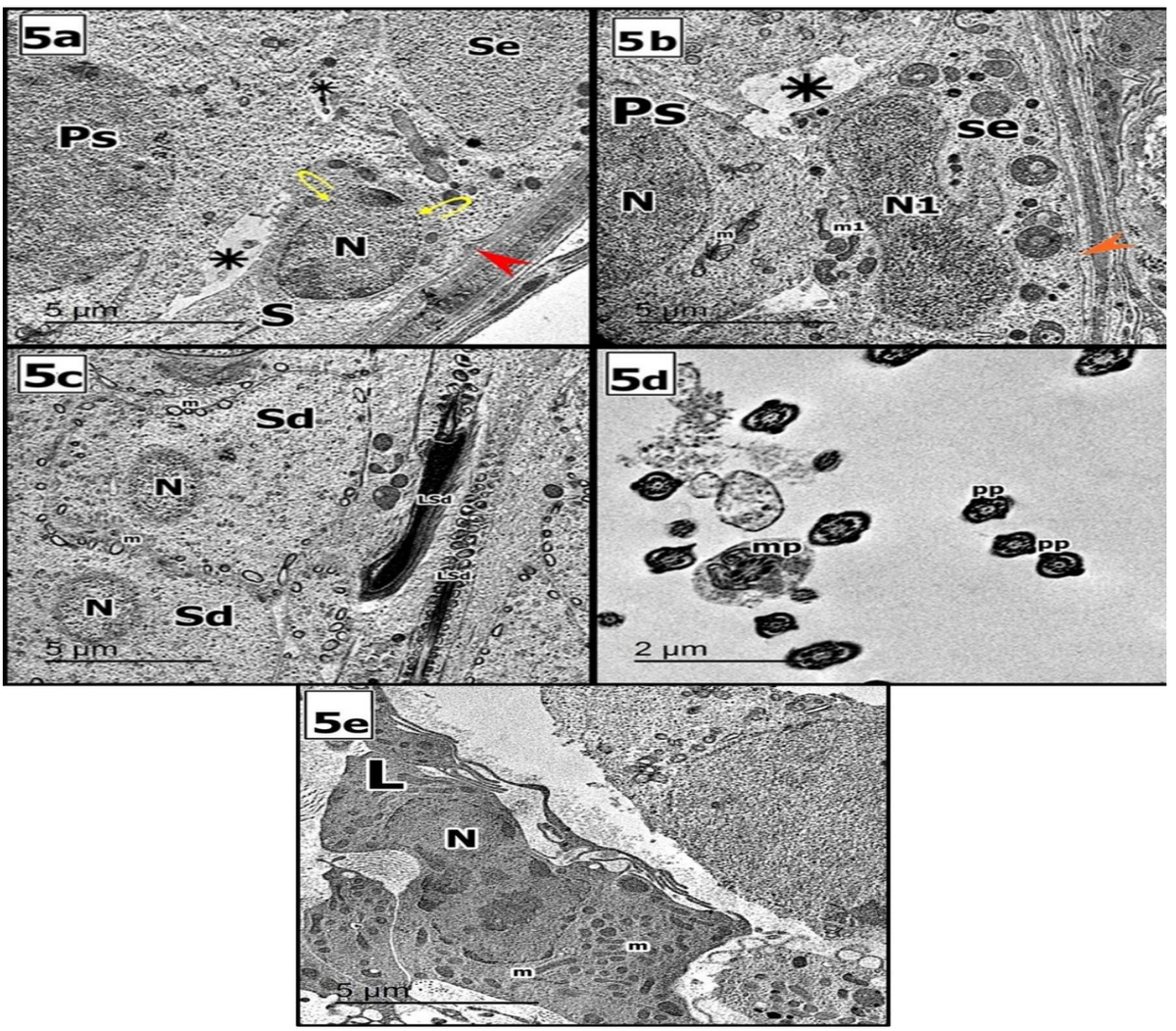

Figure 5: An electron micrograph of the Group II (Type 2 diabetic group) basal compartment shows: (a) different cells (spermatogonic cell (s), sertoli cell (se) and primary spermatocyte (Ps) are present. The basal lamina (arrowhead) appears irregular and thickened. The spermatogonic cell (s) had nucleus (N) with clumps of heterochromatin and discontinued nuclear envelop (curved arrows). Additionally, areas of cell separation and discontinuous tight junctions were identified (asterisks) (Direct Mag X 1200). (b)Another section of the basal compartment shows sertoli cell (se) with irregular shaped nucleus (N1) and different shaped mitochondria (m1) resting on irregular basal lamina (arrowhead). Nucleus (N) and abnormal mitochondria (m) appear in primary spermatocyte (Ps). Cell separation and discontinuous tight junctions are present (asterisks) (Direct Mag X 1200). (c) Early spermatids (sd) have shrunken nuclei (N) and peripheral mitochondria (m). Parts of late spermatids are seen (LSd) (Direct Mag X 1000). (d) Sections of the sperm tails revealed principle pieces and distorted middle piece (Direct Mag X 2500). (e) Leydig cell (L) featured an indented nucleus (N) with mitochondria (m) in the cytoplasm and heterochromatin clusters (Direct Mag X 1200).

Basal compartment of Group III (Type 2 diabetic metformin treated group) revealed some cells (spermatogonic cell, primary spermatocyte and sertoli cell). The basal lamina appeared regular and enclosed by myoid cell. The spermatogonic cell had euchromatic nucleus. The adjacent sertoli cell had nucleus with prominent nucleolus, electron-dense granules and mitochondria. Area of cell separation was still identified Figure 6 a. Early spermatids were visible in the ad luminal compartment. One appeared with large euchromatic nucleus covered by acrosomal cap on its anterior half and peripherally placed mitochondria. Another one revealed small shrunken nucleus Figure 6 b. Leydig cell had euchromatic nucleus with prominent nucleolus and clumps of heterochromatin. Numerous mitochondria were visible in the cytoplasm Figure 6 c.

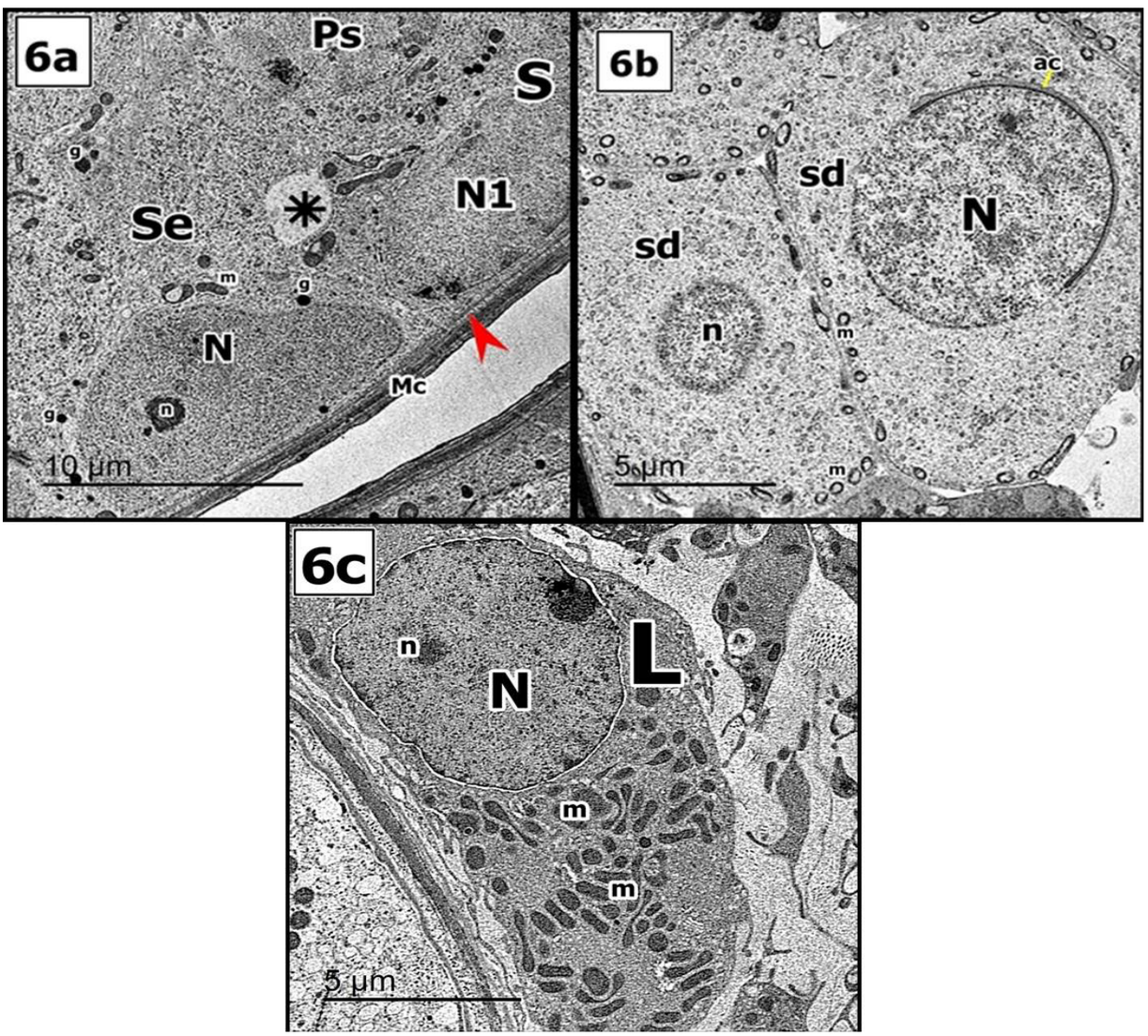

Figure 6: An electron micrograph of Group III (Type 2 diabetic metformin treated group) shows: (a) basal compartment with some cells (spermatogonic cell (s), primary spermatocyte (Ps) and sertoli cell (se). The basal lamina (arrowhead) appears regular and enclosed by myoid cell (Mc). The spermatogonic cell (s) had euchromatic nucleus (N1). The adjacent sertoli cell (se) had nucleus (N) with prominent nucleolus (n), electron-dense granules (g) and mitochondria (m). Area of cell separation was still identified (asterisk) (Direct Mag X 800). (b) Early spermatids are visible in the ad luminal compartment (sd). One appears with large euchromatic nucleus (N) covered by acrosomal cap (ac) on its anterior half and peripherally placed mitochondria (m). Another one reveals a small shrunken nucleus (n) (Direct Mag X 1200). (c) Leydig cell (L) has euchromatic nucleus, clumps of heterochromatin (N) with prominent nucleolus (n). Numerous mitochondria (m) are in the cytoplasm (Direct Mag X 1200).

Group IV (Type 2 diabetic spexin treated group) basal compartment revealed thin, normal basal lamina. On the basal lamina, there was spermatogonic cell with euchromatic nucleus. The cytoplasm of the adjacent sertoli cells had electron-dense granules. The cells next to each other have tight junctions. One primary spermatocyte appeared with euchromatic nucleus Figure 7 a. Another section revealed early spermatid with acrosomal cap covering a large euchromatic nucleus with a prominent nucleolus. Peripherally placed mitochondria were seen Figure 7 b. Leydig cell displayed several mitochondria and euchromatic nuclei with a peripheral heterochromatin rim and SER in its cytoplasm Figure 7 c.

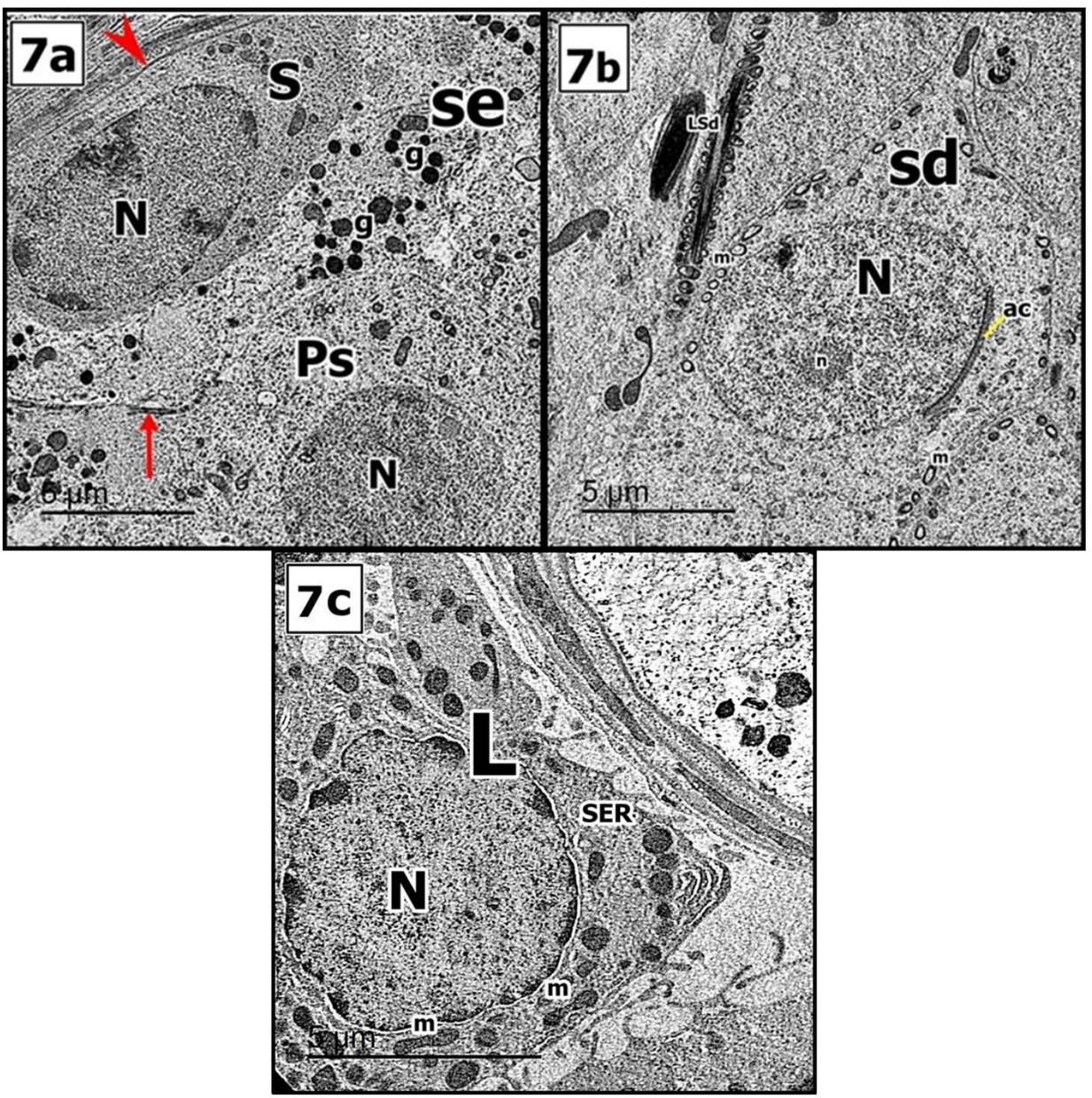

Figure 7: An electron micrograph of Group IV (Type 2 diabetic spexin treated group) revealed (a) basal compartment shows thin, normal basal lamina (arrowhead). Spermatogonic cell (S) having euchromatic nucleus (N). The cytoplasm of the adjacent sertoli cell (se) has electron-dense granules (g). Tight junctions are observed (arrow). One primary spermatocyte (Ps) appears with euchromatic nucleus (N) (Direct Mag X 1000). (b) Early spermatid (sd) has acrosomal cap (ac) covering a large euchromatic nucleus (N) with a prominent nucleolus (n) close to Golgi complex (Gc). Peripherally placed mitochondria (m) are visible, and parts of late spermatids (LSd) are observed (Direct Mag X 1000). (c) Leydig cell (L) displayed several mitochondria (m) and euchromatic nuclei (N) with a peripheral heterochromatin rim and (SER) in its cytoplasm (Direct Mag X 1500).

Morphometric and statistical results:

1- Height of germinal epithelium:

Testicular germinal epithelium height revealed a substantial variation between the groups: group II (diabetic group) and group III (T2D + Metformin group) had the lowest mean values of height, while group I (control group) and group IV (T2D + Spexin group) had the highest mean values. When comparing group I's germinal epithelial height to groups II and III, there was a statistically significant rise. Additionally, When compared to group II, groups III and IV displayed a statistically significant increase in germinal epithelial height. Nonetheless, group IV did not differ significantly from either group I or group III Table 5, Figure 8.

2- Collagen fibers area percent:

There was a notable variation found in the area % of collagen fibers between the groups; group II (the group with diabetes) had the highest mean value of area percent of collagen fiber, whereas group I (the control group) had the lowest mean value. Compared to groups I, III, and IV, group II had an increase in the area % of collagen fiber that was statistically significant. Nonetheless, group I did not differ statistically significantly from groups III and IV. Furthermore, there was no appreciable distinction between groups III and IV Table 5, Figure 8.

3- Area percentage of Ki-67 Immune reaction:

It was found that that the area % of Ki-67 immune response in each group varied significantly. Group II (the diabetic group) had the lowest mean value of area % of Ki-67 immune reaction, while groups I (the control group) and IV (the T2D + Spexin group) had the highest mean values. In comparison to groups II and III, there was a threefold rise in the area % of Ki-67 immune reactivity in group I that was statistically significant. Comparing group III and IV to group II, there was a statistically significant increase in the area % of Ki-immune reaction. In contrast, group IV did not differ significantly from group I or group III Table 5, Figure 8.

Table 5: Comparing the germinal epithelium height, area percent of collagen fiber, and area percent of Ki-67 Immune reaction in the studied groups:

Groups	Group I (Control group)	Group II (Diabetic group)	Group III (T2D + Metformin ttt group)	Group IV (T2D + Spexin ttt group)	f	*P* value	LSD
	Mean ± SD Range	Mean ± SD range	Mean ± SD range	Mean ± SD Range			
Epithelial height	74.13 ± 2.2 (70.5 - 77)	39.23 ± 3.41 (33.9 - 43.4)	69.72 ± 4.23 (63.4 - 74.3)	71.25 ± 1.56 (69 - 73)	174.114	< 0.001**	*P*1 < 0.001** *P*2 = 0.002* *P*3 = 0.115 *P*4 < 0.001** *P*5 < 0.001** *P*6 = 0.392
Area percent collagen	3.02 ± 0.44 (2.5 - 3.6)	14.35 ± 0.71 (13.3 - 15.3)	3.3 ± 0.15 (3.1 - 3.5)	3.18 ± 0.31 (2.7 - 3.6)	931.037	< 0.001**	*P*1 < 0.001** *P*2 = 0.287 *P*3 = 0.528 *P*4 < 0.001** *P*5 < 0.001** *P*6 = 0.658
KI-67 expression	55.32 ± 1.33 (53.4 - 57.5)	17.17 ± 1.17 (15.2 - 18.3)	52.93 ± 0.68 (52.1 - 54)	54.05 ± 1.42 (52.4 - 56.2)	1465.04	< 0.001*	*P*1 < 0.001** *P*2 = 0.002* *P*3 = 0.079 *P*4 < 0.001** *P*5 < 0.001** *P*6 = 0.118

One way ANOVA test
LSD (least significant difference)
**highly significant.
*P*1 group I vs Group II.
*P*2 group I vs Group III.
*P*3 = group I vs Group IV.
*P*4 = group II vs Group III.
*P*5 = group II vs Group IV.
*P*6 = group III vs Group IV.

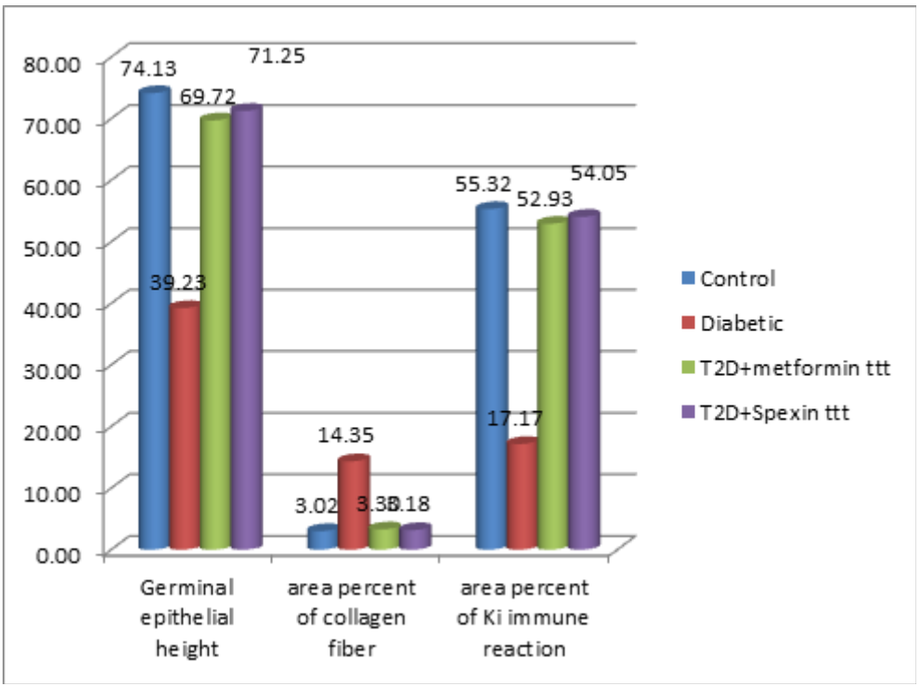

Figure 8: Bar chart comparing the germinal epithelium height, area % of collagen fiber, area % of Ki-67 Immune reaction in the studied groups.

DISCUSSION

Type 2 diabetes (T2D) is the most common metabolic condition and represents more than 90 % of diabetic cases. The hallmark of type 2 diabetes is insulin resistance (IR). It may cause long-term issues that harm the testicles, eyes, and nerves[27]. In the present study, all findings validated the presence of testicular dysfunction in rats with T2D which was alleviated by both metformin and spexin therapy.

The current results verified a significant rise in BMI and metabolic abnormalities in the T2D group compared to the control group. These abnormalities included reduced serum levels of HDL and increased serum levels of LDL, TC and TG with increased serum levels of insulin, insulin resistance and hyperglycemia that indicate disturbed glucose homeostasis. These results were supported by many studies[28].

Also, data of this work revealed significant increase in inflammatory and oxidative stress markers in diabetic group as increasing levels of (serum IL6, TNF, Testicular MDA with decreased testicular SOD). These changes could be resulted from hyperglycemia. In hyperglycemic conditions, there is excessive production of superoxide anion radical (O2-) which induces oxidative stress, inhibits antioxidant systems leading to damage of DNA and biomolecules with increased inflammatory markers[29].

In agreement with other studies[3], some hormonal changes in diabetic group were confirmed in our study by significant reduction in serum level of (testosterone, FSH, LH) and decreased weight of testes, sperm count and motility), According to Zhong *et al.*[30], obesity and diabetes are related to decreased sperm volume, concentration and motility as well as lower testosterone levels, all of which are signs of testicular dysfunction and spermatogenic disruption.

Spexin has been implicated in the development of type 2 diabetes and obesity-induced insulin resistance. Low spexin levels were related negatively with BMI, insulin, and HOMA-IR, and they were shown to be lower in obese and diabetic patients[31]. Behrooz *et al.*[32] reported correlation between spexin, insulin and HOMA-IR In obese children. Furthermore, a negative correlation was discovered between the levels of body weight, total cholesterol, and spexin.

In this study, further evidence for notable disruptions in testicular function may come from the significant drop in spexin serum levels in the

diabetic group and the negative correlation of spexin with several metabolic (BMI, HOMA-IR, glucose, insulin, TC, TG, and LDL), inflammatory, and oxidative markers (IL6, TNF and MDA). This finding could support a potential function for spexin in the pathophysiology of diabetic testicular alterations. There is controversy on the regulation of spexin in diabetes; some studies supported our findings, while others disagreed[7, 33].

H and E sections from the diabetic group showed thicker connective tissue capsule and congested blood vessels. This result is in line with Shaban *et al.*[34] who mentioned that oxidative stress is a secondary cause of inducing fibroblasts to synthesis excessive collagen fibers. In accordance with de Oliveira *et al.*[35], the same group showed distorted seminiferous tubules with massive epithelial desquamations, reduction in the mean epithelial height, vacuolization, darkly stained nuclei, multinucleated giant cell formation and wide separation between germinal cells.

The interstitial tissue had vacuolation and homogenous acidophilic material; this was in line with Celik *et al.*[36] who stated that the cause could be due to inadequate venous drainage, increased vascular permeability, and excessive lymphatic exudates. Uyar *et al.*[37] indicated that a substantial formation of oxygen free radicals induces apoptosis and autophagy of germ cells.

Also, Diabetic group revealed a marked increase in the collagen fibers in the capsule surrounding the seminiferous tubules and the blood vessel. This finding was supported by collagen fiber area % that statistically analyzed. Kurus *et al.*[38] proposed that oxidative stress could promote the genes responsible for collagen synthesis, leading to an increase in collagen deposition. These results were in line with their findings.

In same group, fewer germinal cells had positive Ki-67 immunoreaction compared to control group which suggests reduced proliferation of germinal cells. Ronconi *et al.*[39] attributed the findings due to generation of free radicals and damage of proteins and nucleic acids. Also, there is a direct toxic effect on spermatogenic cells, Leydig cell and Sertoli cell resulting in reduced epithelial height in diabetic group.

Ultrastructurally, this group revealed discontinuous nuclear envelop and heterochromatin clumps in the nuclei of spermatogenic cells, areas of broad cell separation and discontinuous tight junctions. These results are in consistent with Shaban *et al.*[34] who defined the expanded intercellular gaps due to the destruction of the Sertoli cells' processes that fill spaces between the spermatogenic cells resulting in its sloughing and exfoliating into the lumen. According to Mohamed *et al.*[40] ROS cause disruption of the blood-testis barrier's tight connections, allowing more water and harmful substances to enter between the spermatogenic cells.

Also, diabetic group showed an irregular thick basal lamina that either due to a slower rate of proteolysis or an accelerated rate of collagen deposition[41]. Abnormal shaped early spermatids with small shrunken nucleus and peripheral mitochondria appeared with abnormal distorted middle piece. Clinical studies have also revealed that oxidative stress may result in decreased sperm count, motility, an increase in aberrant forms and function problems[42].

Spexin is one of the regulating factors in metabolic syndrome, moreover, it was found that it improves cardio-metabolic disturbances in obese T2D rats and protects the renal tissue[8].The impact of Spexin on testicular dysfunction connected to type-2 diabetes has not been extensively studied. Therefore, the purpose of this research was to examine how Spexin affects testicular alterations in people with type 2 diabetes. The effectiveness of spexin in treating T2D was assessed by contrasting it with the standard drug, metformin.

In comparison to the diabetic group, the T2D spexin treated group showed improved IR and BMI and lower serum levels of glucose, insulin, TC, TG, and LDL and increased serum levels of HDL, according to our study. Walewski *et al.*[43] who found that spexin inhibited the absorption of long-chain fatty acids into adipocytes, decreased insulin resistance, improved dyslipidemia, decreased food intake, and enhanced body weight loss in obese rats, corroborated these findings.

Additionally, spexin-treated T2D mice had decreased glucose and cholesterol levels. Additionally, in T2D mice, spexin decreased body weight, insulin resistance, and the glycolated haemoglobin test (HbA1c). Furthermore, in diet-induced obese mice, long-term spexin infusion reduced hepatic steatosis and decreased insulin, free fatty acids, triglycerides, and HOMA-IR[44].

According to Said *et al.*[45] the anti-inflammatory and anti-oxidative action of Spexin was demonstrated by the considerable decrease in testicular MDA and

serum levels of (IL6 and TNF-α), as well as the significant rise in testicular SOD in the T2D Spexin treated group.

According to Kolodziejski *et al.*[7] reported that spexin indirectly affect inflammatory indicators by altering the level of leptin in T2D obese mice and increase adiponectin. Adiponectin is a protective adipokine that lowers inflammation and insulin resistance and its concentration is down regulated in obese T2DM.

Also, one of the genes most frequently down regulated in obesity is spexin. When, premature adipocytes exposed to spexin resulting in reduced adipose tissue inflammation, activation of adipocyte lipolysis and a reduction in the differentiation of cells into mature adipocytes[42]. The current results showed an increase in serum level of (testosterone, FSH, LH) and increased weight of testes, sperm count and motility in T2D spexin treated group compared to diabetic group but there wasn't any significant correlation between spexin and (testosterone, FSH, LH).

On contrary, in fish model, there were studies showing that spexin can inhibit LH and FSH secretion[46]. These data show that the effect of spexin on the reproductive axis may be type-specific, and further investigations are needed to find out whether it is effective in various types of reproduction. One of the present study restriction is that the results may differ from those obtained on fish due to the study's methodology on rats. Furthermore, a limited number of rats were employed in this investigation.

Metformin-treated diabetic group showed an evident improvement in metabolic, inflammatory, and oxidative alterations, which in turn led to a reduction in testicular dysfunction. Additionally, the data demonstrated an increase in serum spexin levels in the metformin-treated T2D group in contrast to the diabetic group. Metformin partially worked by controlling the production of spexin, which made T2D rats more sensitive to insulin and reduced their body weight. These results were supported by Polianskyte-Prause *et al.*[47].

Seminiferous tubules in metformin-treated group's revealed some improvement although, separation between the germinal cells with dark nuclei are still present which was in accordance with Nna *et al.*[48].Metformin can raise testosterone levels, preventing germ cell death, enhancing antioxidant activity, and improve sperm quality in obese and insulin-resistant patients. Also, metformin has been proven to ameliorate the alterations in the germinal cells caused by diabetes[49].

In addition, Metformin reduces respiration and increases lactate output by Sertoli cells by inhibiting the activity of the respiratory electron transport chain in mitochondria. As lactate is the main source for male germ cells energy, its availability controls the metabolic activity and survival of spermatocytes[50].

Spexin treated group showed almost normal appearance of seminiferous tubules with thin, regular basal lamina and intact germinal epithelium. Sertoli cells and Leydig's cells had vesicular nuclei. Türkel *et al.*[51] reported that Spexin alters cytokine levels such as TNF-α and IL-6 in many different disease models so it has an anti-inflammatory action.

The same group shows minimal amount and regular distribution of the collagen fibers surrounding the seminiferous tubules and the blood vessel. Spexin acted in diabetic nephropathy through its anti-fibrotic action and significantly inhibited TGFβ1, type IV collage and PI3 K/AKT/ERK activation. Also, spexin reduced tissue inhibitor of metalloproteinase 1 and reduced metalloproteinase-2 levels that regulates the fibrosis[52].

Spexin treated group revealed positive immunoreactivity in the spermatogonia and several primary spermatocyte nuclei which was in agreement with Abulfadle *et al.*[53]. It was found that Spexin is a significant regulator of cell proliferation, differentiation, and inhibitor of apoptosis of different cell types by activation of kinases pathways, including the upregulation of mitogen-activated kinase[54].

CONCLUSION

According to the study's results, Spexin as well as the traditional drug, metformin, could significantly ameliorate metabolic and testicular damage induced by type 2 diabetes by decreasing insulin resistance, dyslipidemia, oxidative stress, inflammation, apoptosis and fibrosis.

CONFLICT OF INTEREST

There is no potential conflict of interest among the authors.

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الملخص العربي

الاسبكسين يحسن كلاً من تغيرات الخصية الوظيفية والنسيجية في نموذج الجرذ السكري من النوع الثاني

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الخلفية: يوجد هرمون الببتيد سبيكسين في كل من أنسجة الإنسان والجرذان وأثبتت بعض الدرسات علاقته بالسمنة ومرض السكري. مرض السكري له آثار سلبية على الجهاز التناسلي الذكري حيث تتضرر الخصية وينخفض تكوين الحيوانات المنوية.

الهدف من البحث: يهدف هذا البحث إلى دراسة تأثير السبيكسين على التغيرات الوظيفية والنسيجية في خصية الجرذان الذكرية المصابة بمرض السكري من النوع الثاني، والآليات الأساسية المحتملة.

المواد وطرق البحث: تم استخدام أربعة وعشرين فأرًا بالغًا وتقسيمهم إلى أربع مجموعات متساوية مكونة من: المجموعة الضابطة، مجموعة مرضى السكري من النوع 2، مجموعة مرضى السكري من النوع 2 المعالجة بالميتفورمين، ومجموعة مرضى السكري من النوع 2 المعالجة بالسبيكسين. تم إعطاء الفئران نظامًا غذائيًا غنيًا بالدهون لمدة ثمانية أسابيع وتم حقنها داخل الصفاق بالستربتوزوتوسين (40 مجم / كجم) في الأسبوع الرابع. وقد أدى ذلك إلى إصابة الفئران بمرض السكري من النوع الثاني. تم توفير نظام غذائي عادي ومركبة للسيطرة على الفئران. حيث أعطيت مجموعات العلاج إما الميتفورمين عن طريق الفم (300 ملغم / كغم / يوم) أو سبيكسين داخل الصفاق (35 ميكروغرام / كغم / يوم) لمدة أربعة أسابيع، بدءاً من الأسبوع الخامس وتنتهي في نهاية الأسبوع الثامن. تم تقييم المظهر الأيضي وبعض دلالات الألتهاب والأكسدة ومستويات سبكسين في نهاية التجربة. ثم تم التحقيق في التقييم النسيجي والمناعي للخصية.

النتائج: أظهرت مجموعة مرضى السكري تدهوراً ملحوظاً في التمثيل الغذائي، والبنية النسيجية للخصية، وعدد الحيوانات المنوية وحركتها في البربخ، وبعض علامات الإجهاد التأكسدي الالتهابي، في حين انخفض بشكل ملحوظ سوبر اوكسيد ديسموتاز في نسيج الخصية والسبيكسين في الدم. عند مقارنة المجموعات المعالجة بالسبيكسين والميتفورمين مع مجموعة مرضى السكري، تم عكس هذه التغيرات بشكل ملحوظ.

الاستنتاج: يتمتع الاسبكسين و الميتفورمين بالقدرة على تحسين التغيرات النسيجية والتمثيل الغذائي في الخصية بشكل كبير والتي يسببها مرض السكري من النوع 2.