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Gliclazide alone or in combination with atorvastatin ameliorated reproductive damage in streptozotocin-induced type 2 diabetic male rats

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ABSTRACT

Objectives: Type 2 diabetes (T2DM) is one of the most serious challenges of the 21st century with life-threatening complications and excessive health care costs. In diabetic patients, the main goal in T2DM treatment is the regulation of both blood glucose and lipid levels. For that, Gliclazide (GLZ), an oral antidiabetic, and Atorvastatin (ATV), a lipid lowering agent, are widely used drugs as combination. Diabetes has been reported severe impacts on male reproductive system; however, data obtained about ATV and GLZ treatment alone or in combination are conflicted or insufficient. Herein the effects of ATV and GLZ on male reproductive system in type 2 diabetic male rats have been investigated in the present study.

Methods: T2DM was induced by high-fat diet and single injection of streptozotocin (STZ) (35 mg/kg) in young adult male Sprague-Dawley rats. The diabetic rats were given ATV (10 mg/kg), GLZ (10 mg/kg) and ATV/GLZ (1:1, 10 mg/kg) combination by oral gavage for 28 days. The hormone levels were determined in the cardiac blood samples; and the histopathological and ultrastructural analyses were conducted in the testicular tissues and epididymal sperms.

Results: It was observed that diabetes had severe effects on testicular tissue and spermatogenesis. ATV treatment did not affect sperm count and testes structure ($p > 0.05$), however ameliorated sperm morphology ($p < 0.05$). GLZ treatment increased sperm count, and improved sperm morphology, testes structure and spermatogenesis ($p < 0.05$). ATV/GLZ combination treatment enhanced sperm morphology and improved testicular structure ($p < 0.05$) while did not affect sperm count ($p > 0.05$).

Conclusion: GLZ treatment regenerated testicular damage and sperm parameters whether alone or in combination with ATV in diabetic rats without affecting hypothalamic-pituitary-gonadal axis.

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1. Introduction

Diabetes is one of the most serious challenges of the 21st century because of both increased prevalence through industrialization, urbanization, socio-economic impacts, and changed lifestyle habits (Hu, 2011). Diabetes, which has global burden of 415 million, is a leading cause of morbidity and mortality worldwide. Type-2 diabetes (T2DM) prevalence in children and adults is dramatically increasing every passing day in worldwide (Mandal

et al., 2017). According to the Centres for Disease Control and Prevention (CDC) National Diabetes Statistics Reports (CDC, 2017), T2DM accounts for up to 95% of all diabetes cases. T2DM is characterized by hyperglycaemia due to abnormalities in insulin secretion and/or action resulting from altered carbohydrate, lipid and protein metabolism. In long term, untreated T2DM can cause life-threatening micro- and microvascular complications such as neuropathy, nephropathy, sexual dysfunction and myocardial infarction (Nathan, 2015).

The first option in the prevention and treatment of T2DM is a good glycaemic control through proper nutrition combining with increased physical activity (Hossain et al., 2007); however, oral antidiabetics play crucial role when the lifestyle reforms are not sufficient. GLZ, a second generation of sulphonylurea oral antidiabetics, is mostly prescribes when weigh loss is not required and other oral antidiabetics are not suitable due to side effects. Additionally, GLZ is effective in reducing the risk of cardiovascular

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diseases (CVDs) through reduction of platelet adhesion and aggregation, and induction of fibrinolysis (Sarkar et al., 2011).

Highly heterogeneous structure of T2DM and concomitant conditions such as dyslipidaemia, obesity and hypertension lead to insufficiency of monotherapies; thus, combination of oral antidiabetics and lipid lowering drugs with different mechanisms of action provides a better glycaemic control and lipid profile (Ahmed et al., 2012). Statins or 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are widely used lipid lowering drugs which decrease the risk of CVDs approximately 30% together with lifestyle reforms in T2DM (ADA, 2018). ATV, a second generation of statins, reduces cholesterol synthesis by directly inhibition of HMG-CoA reductase and obliquely up-regulation of low-density lipoprotein (LDL) receptors and reduction in very-low-density lipoprotein (VLDL) synthesis which is precursor of LDL (Kapur and Musunuru, 2008). Although, statins have been considered to negatively affect glycaemic control due to various pharmacokinetics and pharmacodynamics interactions; yet, it is inevitable to use for diabetic dyslipidaemia according to the risk-benefit relationship (Bang and Okin, 2014; Barylski et al., 2014; Ruscica et al., 2014).

It is well established that diabetes is strongly associated with subfertility and/or infertility among men (Bener et al., 2009; La Vignera et al., 2009). Several reports indicated that, various molecular mechanism, such as altered reproductive hormone levels, neuropathy and increased oxidative stress, are responsible for structural damage and dysfunction of testicular tissue and sperm in diabetes. Indeed, it is indicated that modulation of blood glucose level and treatment of dyslipidaemia could reverse the damage (Agbaje et al., 2007; Jangir and Jain, 2014).

Even if several studies proved that diabetes has severe impacts on male reproductive system, the effects of ATV and GLZ treatment are conflicted; moreover, there is no data about their combination treatment. Thus, in the present study, we aimed to investigate the effects of ATV and GLZ alone and in combination on male reproductive system in T2DM using high-fat diet/STZ treated rat model.

2. Material and methods

2.1. Chemicals

STZ was obtained from Sigma Aldrich (Munich, Germany) and freshly dissolved in physiological saline solution before use. ATV calcium (Batch No. 0370411001) and GLZ (Batch No. 101106154) were kindly gifted from Deva Holding (Istanbul, Turkey). High-fat chow (58% fat, 20% protein, 22% carbohydrate) (Hou et al., 2012) was supplied by MBD Laboratory Animal Feed Commerce (Istanbul, Turkey). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), phosphate buffered saline (PBS) were purchased from Multicell Wisent (Quebec, Canada) and all other chemicals purchased from Merck Millipore (Munich, Germany) or Sigma Aldrich (Munich, Germany) at analytical grade. Plastic wares were purchased from Eppendorf (Nijmegen, Nederland) or Nest Biotechnology (Munich, Germany), polylysine coated microscope slides and covers were purchased from Thermo Scientific (Munich, Germany), and microtome blades (Feather A35) were purchased from MST Medical (Bursa, Turkey).

2.2. Study design

A total of 40 young adult (12 week old) male Sprague-Dawley rats with a body weight of 250–300 g were obtained from the Institute of Experimental Medicine of Istanbul University. Rats were placed in standard cages with 4–5 animals per each, maintained in a room temperature of 23–25 °C on a 12 h light/12 h dark cycle

and fed with high-fat chow and tap water *ad libitum* during the whole period of experiment. All experimental protocols were carried in accordance with the standards for human care and use of laboratory animals which is approved by the Local Ethical Committee of Istanbul University (2013/124).

After two-week high-fat diet, diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (35 mg/kg in physiological saline solution) except those in control group that received a single i.p. injection of physiological saline solution (Mahmoud et al., 2012; Abo-elmatty et al., 2013). Blood glucose levels were determined from a drop of blood from tail vein by using an automated glucose analyser device (eSensor, Visgeneer Inc., Taiwan) a week after STZ injection. Rats with blood glucose level of 150–280 mg/dL were considered as diabetic and included in the experiment (Okyar et al., 2001). The rats in control and diabetic (STZ) groups received sterile water by oral gavage for 28 days. ATV and GLZ were suspended in sterile water and well mixed before the use. Diabetic rats in ATV (STZ + ATV), GLZ (STZ + GLZ) and combination (STZ + ATV/GLZ) treatment groups were treated with 10 mg/kg ATV (Schmechel et al., 2009; Kubatka et al., 2011), 10 mg/kg GLZ (Hamed and Malek, 2007; Sarkar et al., 2011) and 10 mg/kg ATV:GLZ (1:1) combination, respectively, by oral gavage for 28 days. All treatments were done in the morning (09.30 h–10.00 h) since circadian rhythms is play important role in hormone production, cell regeneration and other biological activities (Bertani et al., 2010; Neumann et al., 2018).

Rats were sacrificed on day 28 under anaesthesia with 2.5 mg/kg Rompun (Bayer, Istanbul, 20 Turkey; i.p.) and 12.5 mg/kg Ketalar (150086; Pfizer, NY; i.p.) and cardiac blood samples were taken for hormone analysis. Testes and epididymis were dissected and sperm were collected from the epididymis. Light microscopy analysis and transmission electron microscopy analysis were conducted.

2.3. Body and testis weights

All animals were weighed in every three days and at the end of the study. After scarification, testes and epididymis were cleaned from peripheral structures carefully and their weights were recorded. Somatic index which was calculated by dividing the testis weight to body weight was evaluated (Nelli et al., 2013).

2.4. Hormone analysis

Approximately 4 mL of blood collected by cardiac puncture, was centrifuged at 2.000g for 20 min at 4 °C, and immediately stored in –20 °C for further analyses. Serum testosterone (T), oestrogen (E2), progesterone (PROG), luteinizing hormone (LH), follicle stimulating hormone (FSH), gonadotropin releasing hormone (GnRH) and inhibin B (INHB) levels were determined by the enzyme-linked immunosorbent assay (ELISA) method using commercial kits (YH Bioscience, China) according to manufacturer's instructions. The optical density (OD) was measured at 450 nm with a reference wavelength of 620–630 nm using Epoch microplate spectrophotometer (BioTek Inc., Germany).

2.5. Sperm evaluations

Left and right caudal epididymides were dissected out separately. Then, sperms were diluted into DMEM/F12 supplemented with 10% FBS. Sperms collected from left epididymis were centrifuged at 500g for 2 min at 4 °C. Sperm count was conducted on Makler counting chamber (Sefi-Medical Ins., Israel) under Olympus BX61 research microscope (Olympus Co., Japan) at 200x magnification. Ten fields were analyzed for each sample and two counts were performed, and then averaged. Sperms collected from right

epididymis were smeared and dried onto polylysine coated micro-scope slides, then stained with Diff-Quick (GBL, Turkey) to evaluate morphological changes. Head and tail abnormalities of randomly selected 200 sperm were evaluated under light microscope at 200× magnification (Akram et al., 2012; Taib et al., 2013).

2.6. Morphometric evaluations

Diameter of seminiferous tubules and thickness of seminiferous epithelium were evaluated in H&E stained sections under Olympus BX61 digital microscope (Olympus Co., Japan) at 200× magnification by using Olympus DP2-BSW computerized image analysis system software (Olympus Co., Japan). Measurements were performed with randomly selected circular 20 seminiferous tubules from different fields of section. Diameter of seminiferous tubules was considered as shortest diameter and thickness of epithelium was recorded as distance between closest sperm to lumen and basal membrane (Khaneshi et al., 2013).

2.7. Histopathological evaluations

Right testis were immediately fixed in Davidson's modified fixative and kept at 4 °C for 24 h, and then second fixation was done in 10% neutral buffered formalin solution for 1 h. The fixed tissues were dehydrated in a graded ethanol series and toluene, and then embedded in paraffin by using an automated tissue processor (Spin Tissue Processor STP-120, Thermo Scientific, Germany). Sections of 5 µm thickness were cut using a microtome (HM 430; Thermo Fisher Scientific, Germany), were stained with H&E. Slides were photographed under Olympus BX61 digital microscope (Olympus Co., Japan) attached with a computerized digital camera (DP72; Olympus, Tokyo, Japan) at various magnifications.

2.8. Immunostaining of Claudin11 and occludin

Paraffin embedded testis sections were immunostained for evaluation of intracellular junctions through determining expression and localization of claudin11 and occludin in seminiferous epithelium. Deparaffined and rehydrated sections were kept in 10 mM citrate buffer (pH = 6.0) for 10 × 1 minute in microwave oven for antigen retrieval. After cool down at room temperature, sections were incubated with 3% H₂O₂ to block endogenous peroxidase activity and with blocking solution to prevent unspecific binding of antibody. Sections were then incubated with claudin11 (Santa Cruz, H-107; sc-25711; diluted 1/100) and occludin (Invitrogen, 40-4700; diluted 1/50) antibodies overnight at 4 °C in a humidified chamber. Finally, sections were incubated with 3-amino-9-ethylcarbazole (AEC) and counterstained with Mayer's hematoxylin. Images of each section were captured and recorded by using Olympus BX61 digital microscope (Olympus Co., Japan) at 400× magnification. The intensity of occludin and claudin expressions in seminiferous tubules were semi-quantitatively evaluated under a light microscope by the following categories: 1+ (weak, but detectable staining), 2+ (moderate or distinct staining), 3+ (intense staining) and 4+ (very intense staining). The various intensities within these areas were determined at different times by two investigators in a blind fashion and the average scores obtained from evaluations were statistically compared among the groups.

2.9. Ultrastructural evaluations by transmission electron microscopy (TEM)

For the ultrastructural analysis left testis were fixed in 2.5% glutaraldehyde rinsed in Millonig's phosphate buffer (pH = 7.4) and then post-fixed in 1% osmium tetroxide. Tissue pieces, then,

embedded in pure araldite and polymerized at 45 °C for 24 h followed by at 60 °C for 48 h. Araldite blocks were sectioned to 0.5 µm with glass knives by using a Leica Reichert UM2 ultratome (Leica Microsystems, Austria) and suitable areas of semi-thin sections were determined by staining with toluidine blue. Ultra-thin sections of 40–50 nm were collected on 200-mesh naked copper grids by using Leica Reichert UM3 ultratome (Leica Microsystems, Austria) and contrasted with uranyl acetate and lead citrate. Ultra-thin sections were evaluated with a JEOL JEM-1011 transmission electron microscope (JEOL Inc., USA) (Chemes, 2013).

2.10. Statistical analysis

Statistical analyses were performed with SPSS v.21.0 software (Chicago, IL, USA). Multiple groups were compared using one-way analysis of variance (ANOVA) followed by the Tukey HSD post hoc comparisons. Data were expressed as mean ± SEM (the standard error of the mean) and considered statistically significant at $p < 0.05$.

3. Results

3.1. Blood glucose Level, body and testis weights

The blood glucose levels, body and testis weights of rats were presented in Table 1. The blood glucose levels in STZ group were significantly higher compared to those in the control group ($p < 0.05$). The blood glucose levels significantly decreased in STZ + GLZ group at the end of study ($p < 0.05$). The testis weights were lower in STZ + ATV group ($p < 0.05$) compared with both control and STZ groups; however, the somatic index was almost same in all groups.

3.2. Hormone levels

The hormone levels were slightly elevated in STZ group; and, decreased insignificantly in STZ + ATV, ATZ + GLZ and STZ + ATV/GLZ groups compared to diabetic rats (Table 2). Interestingly, serum T level was significantly lower in the combination group than that of the STZ group ($p < 0.05$), whereas, INHB and LH levels were slightly higher.

3.3. Sperm count and morphology

The sperm count and morphological characteristics were presented in Table 3. The number of sperm was significantly lower in STZ group ($p < 0.05$), STZ + ATV group ($p < 0.01$) and STZ + ATV/GLZ group ($p < 0.01$) compared to those in the control group. A significant increase of sperm number was observed in STZ + GLZ group compared to STZ group and it was comparable with the control group ($p < 0.05$).

Head and tail abnormalities in diabetic rats were significantly higher compared to those in the control group ($p < 0.01$). ATV and GLZ treatment alone or in combination attenuated the abnormal sperm characteristics ($p < 0.01$).

3.4. Morphometric evaluations

Diameter of seminiferous tubules and thickness of epithelium were correlated in all groups (Fig. 1). Both parameters were decreased in STZ group compared to those in the control group ($p < 0.05$). GLZ treatment alone or in combination improved morphometric characteristics in diabetic rats ($p < 0.05$); however, any significant difference did not observed with ATV treatment.

Table 1

Blood glucose level, body and testis weights in all groups. STZ, diabetic rats; STZ + ATV, diabetic rats in Atorvastatin treatment; STZ + GLZ, diabetic rats in Gliclazide treatment; STZ + ATV/GLZ, diabetic rats in Atorvastatin/Gliclazide combination treatment.

Parameter	Control	STZ	STZ + ATV	STZ + GLZ	STZ + ATV/GLZ
Blood glucose (mg/dL)					
Day 1	111.00 ± 3.42	222.92 ± 6.74 [*]	237.71 ± 11.51 [*]	226.85 ± 13.98 [*]	255.23 ± 15.15 [*]
Day 14	118.40 ± 3.50	200.85 ± 10.04	249.71 ± 10.77	170.35 ± 9.84	224.38 ± 18.34
Day 28	114.00 ± 2.39	195.42 ± 12.84	240.85 ± 15.03	137.30 ± 6.10 [†]	189.46 ± 14.89
Initial body weight (g)	278.00 ± 6.04	267.20 ± 3.55	257.50 ± 2.80	268.30 ± 4.54	265.50 ± 5.62
Final body weight (g)	303.20 ± 7.72	296.90 ± 6.75	275.50 ± 6.43	287.00 ± 9.04	279.20 ± 10.22
Testis weight (g)	1.54 ± 0.03	1.52 ± 0.01	1.41 ± 0.03 [†]	1.51 ± 0.02	1.45 ± 0.03
Somatic index	0.56 ± 0.02	0.57 ± 0.01	0.55 ± 0.01	0.56 ± 0.02	0.55 ± 0.01

Data were expressed as mean ± SEM.

^{*} $p < 0.05$ compared to control group.

[†] $p < 0.05$ compared to STZ group.

Table 2

Hormone levels in all groups. T, testosterone; E2, oestrogen; PROG, progesterone; LH, luteinizing hormone; FSH, follicle stimulating hormone; GnRH, gonadotropin releasing hormone; INHB, inhibin B. STZ, diabetic rats; STZ + ATV, diabetic rats in Atorvastatin treatment; STZ + GLZ, diabetic rats in Gliclazide treatment; STZ + ATV/GLZ, diabetic rats in Atorvastatin/Gliclazide combination treatment.

Hormone	Control	STZ	STZ + ATV	STZ + GLZ	STZ + ATV/GLZ
T (nmol/L)	11.27 ± 0.18	11.95 ± 0.28	10.89 ± 0.24	11.08 ± 0.51	10.39 ± 0.37 [†]
E2 (ng/L)	86.12 ± 1.207	87.62 ± 1.446	87.03 ± 1.672	82.38 ± 2.780	83.23 ± 1.228
PROG (ng/mL)	47.28 ± 1.645	53.66 ± 2.094	50.93 ± 1.502	50.70 ± 0.839	50.82 ± 1.046
LH (mIU/mL)	4.41 ± 0.114	4.50 ± 0.114	4.68 ± 0.171	4.15 ± 0.148	4.55 ± 0.103
FSH (mIU/mL)	5.80 ± 0.280	6.21 ± 0.223	5.95 ± 0.243	5.66 ± 0.217	6.10 ± 0.145
GnRH (pg/mL)	147.16 ± 5.382	160.20 ± 5.293	150.69 ± 6.402	149.81 ± 4.202	152.49 ± 5.143
INHB (ng/L)	197.81 ± 7.485	206.08 ± 10.445	198.70 ± 9.963	182.70 ± 5.973	218.01 ± 25.379

Data were expressed as mean ± SEM.

[†] $p < 0.05$ compared to diabetic group.

Table 3

Sperm count and morphological characteristics in all groups. STZ, diabetic rats; STZ + ATV, diabetic rats in Atorvastatin treatment; STZ + GLZ, diabetic rats in Gliclazide treatment; STZ + ATV/GLZ, diabetic rats in Atorvastatin/Gliclazide combination treatment.

	Control	STZ	STZ + ATV	STZ + GLZ	STZ + ATV/GLZ
Sperm count (millions/mL)	543.60 ± 34.60	400.36 ± 32.01 [*]	350.80 ± 16.87 [*]	463.50 ± 39.69 [†]	377.86 ± 11.84 [†]
Sperm morphology (mean)					
Normal	194.6	172.8	185.9	185.8	179.4
Detached tail	0.8	3.3	1.7	1.6	1.7
Detached head	1.4	8.2	2.8	5.3	4.0
Flattened head	0.2	0.6	0.1	0.2	0.6
Pin head	–	0.1	0.1	0.3	0.4
Bent neck	2.0	3.4	3.0	2.0	3.4
Bent tail	0.8	5.1	4.0	3.1	6.1
Coiled tail	0.2	6.5	2.4	1.7	4.4
Total abnormal sperm count	5.4	27.2	14.1	14.2	20.6
Abnormal sperm (%)	2.7	13.6 [*]	7.05 ^{*†}	7.1 [†]	10.3 ^{*†}

Data were expressed as mean ± SEM.

^{*} $p < 0.05$ compared to control group.

[†] $p < 0.05$ compared to STZ group.

3.5. Histopathological observations

Testicular tissue of control animals exhibited normal structure with round seminiferous tubules and clusters of normal Leydig cells in the interstitial area among the tubule profiles. Epithelium of the seminiferous tubules was structurally intact; all types of cells in the tubules had normal cellular attachment. The Sertoli cells and germ cells exhibited normal nuclear and cytoplasmic characteristics. The Sertoli cells demonstrated typical, irregular nuclei and well-defined cytoplasm, which appeared granular.

The spermatogonia, oval in shape, were closely associated with the basal lamina. The spermatocytes showed various degrees of condensation of their nuclei and were closely associated with Sertoli cell cytoplasm. Spermatids were embedded in the cytoplasm of the Sertoli cells and mature spermatozoa were observed in the

lumen (Fig. 2A). However, in STZ group, some of the seminiferous tubules were irregular in shape and they appeared shrunken and separated from each other. Cells of germinal epithelium had abnormal cellular attachment leading to disorganization and destruction of germinal epithelium and epithelium was separated from the basal membrane in some of the seminiferous tubules. A few of the tubules showed signs of nuclear pyknosis or degeneration in the spermatogenic cells. Immature germ cells and cellular debris were observed in the lumen of some tubules. The interstitial area among seminiferous tubules was expanded (Fig. 2B).

In the STZ + ATV group, irregularly shaped deformed tubules and destructed testicular epithelium were observed in some of the tubules. Spermatogenic arrest was still evident in some tubules compared to the STZ group. Cytoplasmic vacuoles in the seminiferous tubule epithelium were noted in several tubules. In few of the

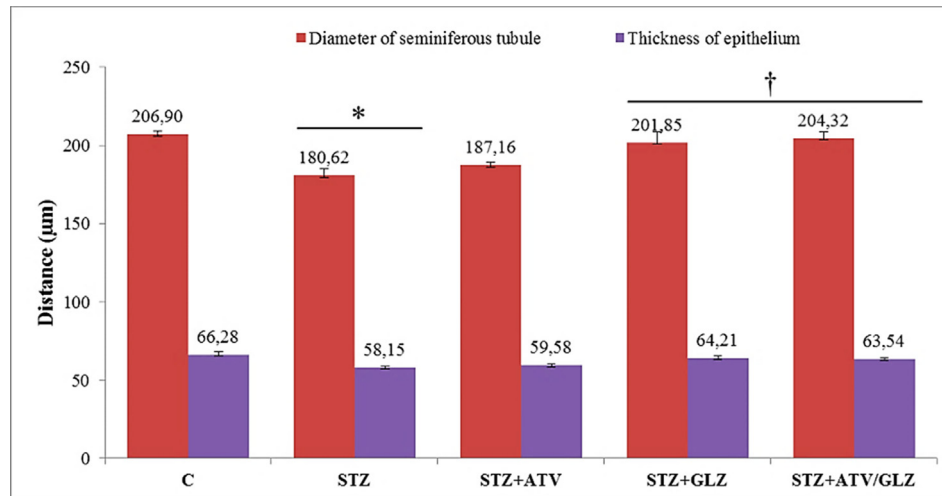


Fig. 1. Diameter of seminiferous tubules and thickness of epithelium in all groups. STZ, diabetic rats; STZ + ATV, diabetic rats in Atorvastatin treatment; STZ + GLZ, diabetic rats in Gliclazide treatment; STZ + ATV/GLZ, diabetic rats in Atorvastatin/Gliclazide combination treatment. * $p < 0.05$ compared to control group. † $p < 0.05$ compared to STZ group.

tubules epithelium was separated from the basal membrane. Immature germ cells and cellular debris were observed in the lumen of some of the seminiferous tubules in this group as in the STZ group. The expansion of the interstitial area was lesser than STZ group (Fig. 2C).

In the STZ + GLZ group, the seminiferous epithelium was comparable with those in the control group. Most of the seminiferous tubules were observed normal in a way that germ cells in various stages of spermatogenesis were normally visible. Thus, conservation of proper spermatogenesis was evident. The Sertoli cells and germ cells showed closer associations with minimal loss of cellular attachments in several tubules. The lumen contained packed mature spermatozoa (Fig. 2D).

In STZ + ATV/GLZ group, abruption in between the epithelial cells of some of the seminiferous tubules was observed. Epithelium was separated from the basal membrane in few of the seminiferous tubules as well. Immature germ cells and cellular debris were noted in the lumen. Size of the seminiferous tubules was pretty much same with those in the control group, and the interstitial area between seminiferous tubules were not enlarged; however it was depleted or appears to contain amorphous material. Histological abnormalities found in this group were less severe than those observed in STZ + ATV group (Fig. 2E).

3.6. Immunostaining of Claudin11 and occludin

In all animals, claudin-11 immunostaining was localized in a linear fashion at the basal compartment corresponding to the blood testes barrier region (Fig. 2F–J); and occludin was localized in both the basal and the adluminal compartments in the seminiferous epithelium (Fig. 2K–O). In STZ group, claudin 11 and occludin expression significantly decreased compared to the control group ($p < 0.05$). The expressions were not different between the STZ and STZ + ATV groups ($p > 0.05$). The level of the expressions in the STZ + GLZ group statistically increased compared to that of the STZ and STZ + ATV groups, respectively ($p < 0.05$). While the expressions in the STZ + ATV/GLZ group statistically increased compared to that of the STZ group, it was statistically decreased compared to STZ + GLZ group.

3.7. Ultrastructural observations

Regular ultrastructure and typical epithelium of the seminiferous tubules with normally ordered Sertoli cells and germ cells

were observed in the control group. In spermatids, mitochondria were placed in the periphery and acrosomal cap formation was normal. Sperms in the lumen had typical axonemal structure (Fig. 3A–C).

However, in STZ group, basal membrane was irregular in shape and Sertoli cells were partly separated from the basal membrane. Numerous lipid droplets, which some of them were quite large, were observed in Sertoli cell cytoplasm. Mitochondria were degenerated, enlarged and lost their crista, thus vacuole-like appearance was noticed. Germinal epithelium was degenerated due to abnormal cellular attachments between Sertoli and germ cells. Some of the germ cell nuclei were noticed to be pyknotic as a sign of apoptosis. Multiple lipid droplets and vacuoles were observed in the cytoplasm of spermatids. Presence of a large number of apoptotic cells close to lumen was observed. Additionally, immature and abnormal cells and cellular debris were observed in the lumen (Fig. 3D–F).

In STZ + ATV group, irregular basal membrane was thickened due to collagen accumulation. Sertoli cells and spermatogonia were partly separated from basal membrane. Vacuoles and fewer but large lipid droplets were determined in both Sertoli cells and spermatids. Late spermatids were found in the basal compartment of epithelium as well as immature spermatids were observed in the lumen. Also, spermatids were separated from each other; and, myelin figures were noticed in the cytoplasm of late spermatids (Fig. 3G–I).

In STZ + GLZ group, the ultrastructure of testicular tissue was ameliorated compared to that in STZ group. Basal membrane was regular and the thickness was normal. Sertoli cells were closely associated with germ cells, and germinal epithelium was intact. Mitochondria of spermatids and acrosomal cap formation appeared normal. Axonemal structure of sperms in the lumen was normal (Fig. 3J–L).

In STZ + ATV/GLZ group, basal membrane maintained its irregularity; however, thickening was not seen as in STZ + ATV group. The number of lipid droplets in Sertoli cells was lower compared to those in STZ group; however, existing lipid droplets were quite big. Mitochondria in Sertoli cells lost their crista and vacuole-like appearance was noticed. Cellular attachments were tighter compared to those in STZ group as well as STZ + ATV group. Also, lipid droplets were noticed in spermatids. In the lumen, sperms with abnormal tail structure were observed as well as normal sperms (Fig. 3M–O).

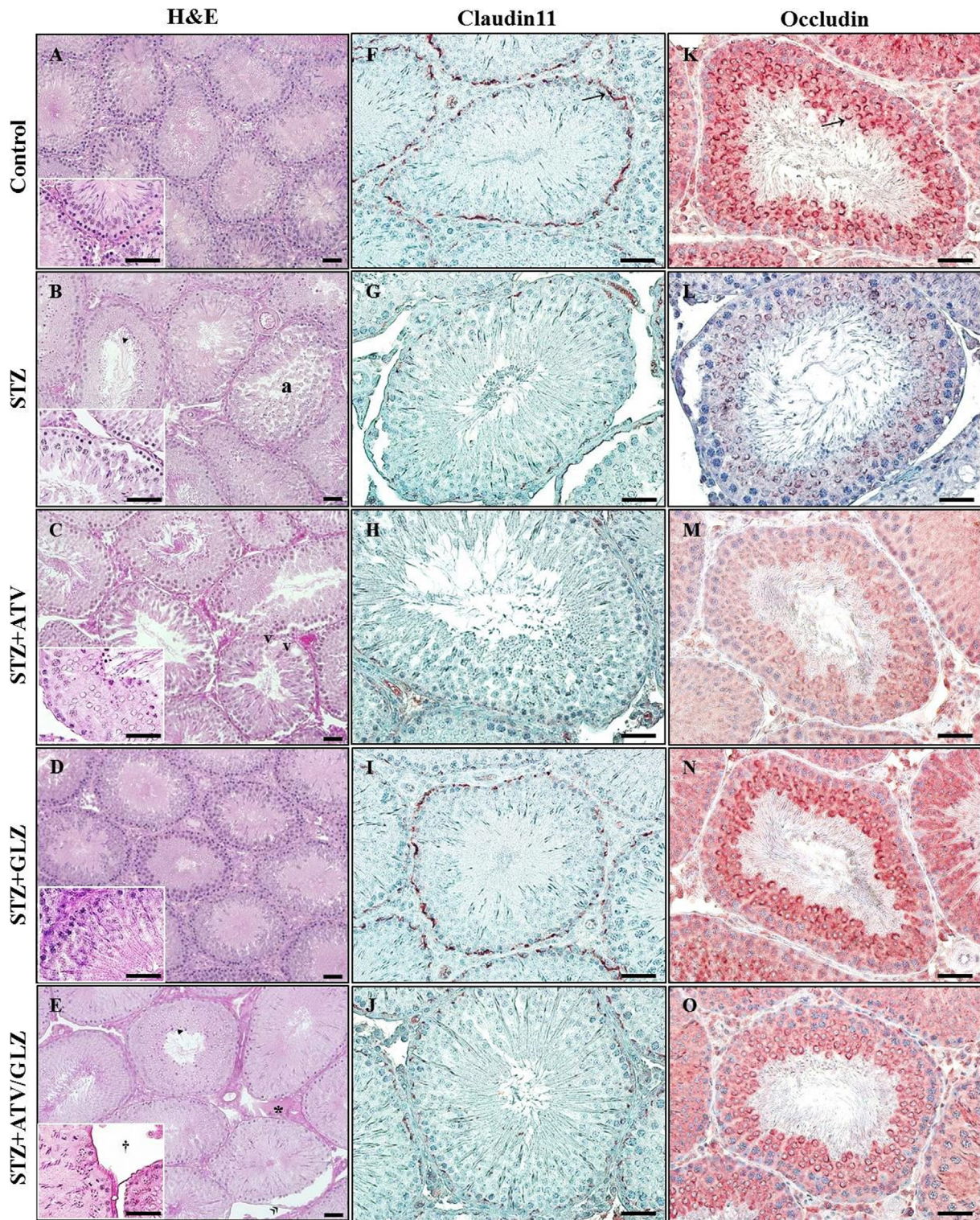


Fig. 2. Normal morphology of seminiferous tubules, interstitial area and cellular attachments (A), claudin11 (→, F) and occludin (→, K) expression. Irregular and atrophied (*, B) seminiferous tubules, immature germ cells in lumen (▶, B), abnormal cellular attachments (insert, B) and loss of cellular tight junctions (G, L) in diabetic rats. Disorganised germinal epithelium, cytoplasmic vacuoles (v, C), immature germ cells in lumen (insert, C), slightly increased claudin11 (H) and occludin (M) expression in ATV treated diabetic rats. Round seminiferous tubules with minimal loss of cellular attachments and normal interstitial area (D), well organized tight junctions (I, N) comparable to control group in GLZ treated diabetic rats. Immature germ cells in lumen (▶, F), amorphous material in interstitial area (*, F), depleted interstitial area (†, insert, F), increased expression of claudin11 (J) and occluding (O) in ATV/GLZ combination group. Staining: H&E (first column); Immunohistochemistry: Claudin11 (second column) and occludin (third column); Scale bars: 50 μ m. STZ, diabetic rats; STZ + ATV, diabetic rats in Atorvastatin treatment; STZ + GLZ, diabetic rats in Gliclazide treatment; STZ + ATV/GLZ, diabetic rats in Atorvastatin/Gliclazide combination treatment.

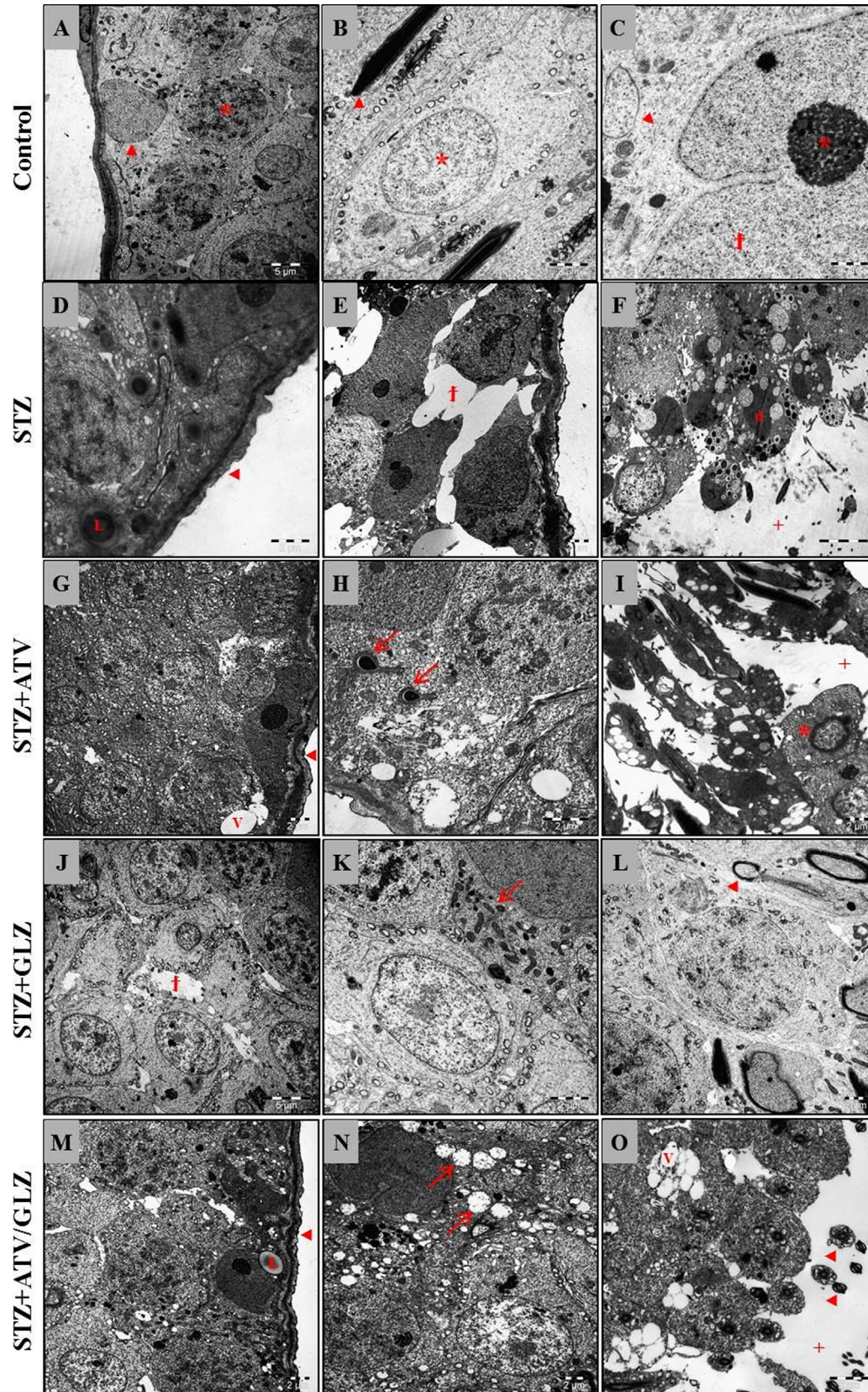


Fig. 3. Regular ultrastructure of Sertoli cell nuclei (▶, A), spermatogenic cell (*, B) and head of sperm (▶, B) of a spermatid, nucleus (†, C) and nucleolus (*, C) of a Sertoli cell, and a smooth endoplasmic reticulum (▶, C) in control. Lipid droplets (L, D), irregular basal membrane (▶, D), abnormal cellular attachments and separations (†, E), abnormal cells and cellular debris (d, F) in the lumen (+, F) in diabetic rats. Vacuoles (v, G), irregular and thickened basal membrane (▶, G), late spermatids (→, H) and immature spermatid (▶, I) in the lumen (+, I) in STZ + ATV group. Minimal loss between spermatogenic cells (†, J), normal mitochondria (→, K) and acrosomal cap of a spermatid (▶, L) in STZ + GLZ group. Irregular basal membrane (▶, M), a lipid droplet (L, M), swollen mitochondria without crista (→, N), vacuoles (v, O), and abnormal and normal sperm tails (▶, O) in the lumen (+, O) in STZ + ATV/GLZ group. STZ, diabetic rats; STZ + ATV, diabetic rats in Atorvastatin treatment; STZ + GLZ, diabetic rats in Gliclazide treatment; STZ + ATV/GLZ, diabetic rats in Atorvastatin/Gliclazide combination treatment.

4. Discussion

STZ causes pancreatic β -cell death through DNA alkylation and production of nitric oxide and reactive oxygen species resulting in impaired insulin-glucose homeostasis and ultimately diabetes (Szkudelski, 2001; Lenzen, 2008). A high-fat diet and low-dose single injection of STZ were adopted to induce T2DM in rats according to previous studies (Skovsø, 2014; Gheibi et al., 2016). In our study, the mean blood glucose level was significantly and constantly higher in diabetic rats during the course of diabetes; and, the mean bodyweight was slightly increased due to nutritional composition and relatively shorter regimen. Thus, a stable model of T2DM rat was successfully developed.

Spermatogenesis and sperm functions are strongly related with hypothalamic-pituitary-gonadal axis. Alterations in GnRH signalling affect both LH and FSH secretion that stimulates steroidogenesis and spermatogenesis, respectively, in Leydig and Sertoli cells (Schoeller et al., 2012). INHB closely correlated with FSH levels as a substantial negative feedback signal (Pierik et al., 1998). In the present study, LH, FSH and INHB levels remained unchanged as well as GnRH levels.

It has been reported in several studies that diabetes is associated with decreased levels of testosterone which is aromatized to E2 in mostly adipose tissue (Zhao et al., 2010; Hussein and Al-Qaisi, 2012); whereas, some other studies reported that testosterone levels remain unchanged (Doustmohammadian et al., 2013; Natah et al., 2013). In the present study, the mean testosterone level was significantly decreased only in STZ + ATV/GLZ group. Navarro-Casado et al. (2010) showed a direct correlation between serum testosterone and gonadotropin levels. Thus, it can be suggested that unaffected GnRH release from the pituitary gland may have prevented the decrease of testosterone in the other exposure groups except STZ + ATV/GLZ group. Explanation of the sex hormones levels is complex; thus, evaluation and harmonisation of various factors are needed (Kim and Halter, 2014). The confliction on the results of the various studies can be related to inter-species differences, study duration and/or method selection.

In the present study, reduced sperm count and increased head and tail abnormalities were observed in diabetic rats similar to previous reports (Scarano et al., 2006; Giribabu et al., 2014). Although, testosterone levels did not change, it is thought that sperm defects in diabetic rats may be caused by oxidative stress which was shown in previous studies (Matkovics et al., 1996; Vignera et al., 2012). In our study, GLZ treatment both increased sperm number and attenuated the abnormal sperm characteristics; however, ATV treatment only improved the sperm morphology. It can be suggested that regenerated blood glucose and antioxidant properties of GLZ (Nelli et al., 2013) ensured improvements in sperm quality and quantity. On the other hand, insignificant reduction in testosterone and somatic index in STZ + ATV group may prevent the increment in sperm quantity. Additionally, percentage of abnormal sperm in STZ + ATV/GLZ group was observed higher than individual treatments; thereby, in combination, ATV may be attributed to suppress the positive effects of GLZ with contribution of decreased testosterone.

Histopathological and morphometric evaluations clearly showed that diabetic condition impaired the spermatogenesis and the data are consistent with earlier reports (Khaneshi et al., 2013; Sisman et al., 2014; Orman et al., 2015). In STZ + ATV group, the histopathological findings were similar to diabetic condition; however, interstitial area was less expanded. The insignificant increase in the decreased seminiferous tubule diameter may be answer for this recovery. GLZ treatment was improved the testicular structure and increased the morphometric characteristics as comparable with control group. Cellular attachments and mature

spermatozoa were evidence of proper spermatogenesis (Ross et al., 2015). Although, epithelial damage was observed in STZ + ATV/GLZ group, thickness of epithelium and size of seminiferous tubules were similar to control group. Interestingly, interstitial area was not enlarged but depleted; thereby, it can be suggested that reduced levels of testosterone may be associated. Herewith, our histopathological findings support that GLZ has an efficient ameliorative effect on testicular tissue in diabetic condition.

Tight junctions between Sertoli cells, which claudin11 and occludin are the key intracellular adhesion proteins, create a unique microenvironment for the apical germ cells by separating them from general circulation (Jiang et al., 2014; McCabe et al., 2016). These junctions are essential for physical support as well as glucose metabolism in maintenance of spermatogenesis (Alves et al., 2013). Reduced expression levels of claudin11 and occludin or complete absence in some seminiferous tubules indicated that diabetes impaired tight junctions in consistent with previous reports (Lui et al., 2003; Ricci et al., 2009). ATV treatment did not cause any changes in the histo-architecture of diabetic testes; on the contrary, GLZ treatment strongly improved the tight junctions. ATV/GLZ combination slightly increased the expression levels of claudin11 and occludin; therefore, it can be suggested that combination treatment is not substantial enough as GLZ alone. Moreover, histopathological observations and morphometric evaluations supported the immunohistochemical data.

Marked ultrastructural changes were occurred in diabetic condition in agreement with previous reports (Kianifard et al., 2012; Wankeu-Nya et al., 2013). Mitochondrial degeneration can be associated with altered lipid metabolism due to insulin resistance and/or absence (Saltiel and Kahn, 2001), and increased uptake of fatty acids (De Fronzo and Abdul-Ghani, 2008). Moreover, it has been reported that maintaining energy requirement for spermatogenesis by other pathways than glucose metabolism may lead to changes in organelle structures in Sertoli cells (Hassan and Abdel Moneium, 2001). Sertoli cells has a primary role in spermatogenesis, thus, presence of apoptotic cells resulted by structural damage of Sertoli cells indicates an incomplete maturation process in diabetic condition. ATV treatment slightly improved the ultrastructure of diabetic testes. Since, Sertoli cells and spermatogonia were partly separated, fewer and big lipid droplets were observed, and late spermatids in the basal compartment and immature spermatids in lumen were found, it can be suggested that altered spermatogenesis is still evident. GLZ treatment almost completely ameliorated the ultrastructure of diabetic testes; however, minimal loss of cellular attachments and some deformed Sertoli cells were observed. Although, irregular basal membrane, lipid droplets and vacuole-like appearance were noticed in combination group, cellular attachments were quite intact. It is thought that ATV may suppress the regenerative effects of GLZ when the combination treatment improves the testicular structure to some extent but is not as effective as GLZ alone.

5. Conclusion

High-fat diet/STZ-induced T2DM impaired reproductive functions of male rats through reduced sperm count, altered sperm morphology, damaged testicular structure and impaired cell-to-cell junctions. ATV treatment did not substantially improve reproductive functions as well as did not cause any further damages. However, GLZ treatment alone or in combination with ATV ameliorated testicular structure and sperm parameters. Our results may be particular interest for providing information about the effects of commonly used multidrug therapy in T2DM on male reproductive system.

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Ethical approval

All experimental protocols were carried in accordance with the standards for human care and use of laboratory animals which is approved by the Local Ethical Committee of Istanbul University (2013/124).

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its files.

Authors' contributions

All authors contributed substantially to the manuscript, and have met the criteria for authorship. EO performed animal treatments and hormone analysis. EO, TEY and ZS performed the animal surgeries, tissue extraction, histopathological examinations and all other bench experiments, analyzed the data and drafted the manuscript. EG supervised the histopathological examinations, data analysis and drafted the manuscript. AO supervised animal treatments, data analysis and drafted the manuscript. EO and GO conceived the study, designed the experiments and finalized the manuscript. All authors read, edited and approved the final manuscript.

Competing interests

The authors declare that there are any non-financial competing interests.

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