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Protective effect of Stevia on diabetic induced testicular damage: an immunohistochemical and ultrastructural study

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Abstract. – OBJECTIVE: Diabetes mellitus (DM) has been considered a major problem because of its related complications and growing incidence worldwide. Testicular dysfunction has become a predominant diabetic complication characterized by impaired reproductive function and testicular damage. *Stevia rebaudiana Bertoni* has been known for its antioxidant effect on diabetes, inflammation, and obesity. The current study investigates the protective effect of Stevia on diabetic-induced testicular injury.

MATERIALS AND METHODS: Sprague Dawley adult male rats were divided into three groups: the control group, the diabetic group, and the diabetic + Stevia group, type 2 diabetes is induced by a high-fat diet (HFD) and a single dose of 35 mg/kg streptozotocin injection. The effects of Stevia were evaluated regarding biochemical, oxidative stress, histopathological and ultrastructural changes, and immunohistochemical expression of vascular endothelial growth factor (VEGF), vascular cell adhesion molecule-1 (VCAM-1), receptor-interacting serine/threonine-protein kinase 1 (RIPK 1), and caspase 3.

RESULTS: Stevia extract attenuated the diabetic-induced oxidative stress, restored the testicular architecture, and decreased testicular damage, inflammation, necroptosis, and apoptosis by upregulating VEGF and downregulating VCAM 1, RIPK 1, and caspase 3.

CONCLUSIONS: The current study highlights the importance of Stevia as an antioxidant anti-inflammatory that ameliorates diabetic-induced testicular injury by modulating oxidative stress, inflammation, necroptosis, and apoptosis.

Key Words:
Diabetes, Testicular damage, Stevia protective therapy.

Introduction

Diabetes mellitus (DM) has been considered a major concern because of its related complications and growing worldwide incidence[1]. In 2045, 693 million people, or almost 10% of the world's population, are expected to have diabetes[2].

Lately, studies[2] have revealed that testicular dysfunction has become a predominant diabetic complication. Approximately hypotestosteronaemia affects 94.4% of diabetes patients, on average[3], and diabetic individuals have up to ten times the rate of sexual and reproductive problems as nondiabetic patients. decreased testosterone levels and impaired reproductive function characterize diabetes-induced testicular dysfunction[2]. However, the precise mechanism behind diabetic testicular dysfunction remains unknown, and no particular therapies are currently available[4].

Diabetes impacts many biological systems, mostly due to hyperglycaemia-induced reactive oxygen species (ROS) production, resulting in oxidative stress[5]. Diabetes has been linked to lower fertility in male animal models[6] and pregnancy rates in diabetic male couples[7]. These outcomes are owed to oxidative stress, inflammation, and germ cell

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apoptosis[8]. Furthermore, diabetic rats show up-regulated apoptotic pathways in testes, this might inhibit germ cell proliferation and spermatogenesis[9].

Stevia rebaudiana Bertoni has been known for its antioxidant effect on diabetes, inflammation, and obesity[10]. Furthermore, Stevia extract has been shown to control blood glucose, decrease inflammatory markers, and minimize oxidative state in diabetic rats[11,12].

Levels of Malondialdehyde (MDA) and antioxidant enzymes (GSH) have been identified as oxidative stress markers[13]. GSH is a powerful enzyme that protects cells against hydrogen peroxide produced within them[14], whereas MDA is a damaging by-product of fatty acid peroxidation[12].

As part of diabetic-induced testicular failure, DM causes apoptosis, decreased diameter of the seminiferous tubules, and spermatogenetic cells[15]. The two indicators of spermatogenic failure are seminiferous tubule atrophy and spermatogenic cell loss[16]. Testicular apoptosis might be mediated by oxidative stress and decreased angiotrophic substances[17]. Vascular endothelial growth factor (VEGF) is a neurotrophic and angiotrophic factor that promotes endothelial cell proliferation and increases vascular wall permeability. It is also vital for germ cell homeostasis. Both Leydig and Sertoli cells generate VEGF and contain VEGF receptors[16].

Vascular cell adhesion molecule 1 (VCAM-1) is primarily expressed by endothelial cells, and assists the adhesion and migration of immune cells, leading to inflammation. Hyperglycemia-induced oxidative stress causes endothelial dysfunction through inflammation. Additionally, diabetes provokes endothelial dysfunction and inflammation[18].

Necroptosis is a type of controlled cell death that regulates the destiny of individual cells during embryonic development and adulthood[19,20], and it was reported that necroptosis is one of the basic processes of male reproductive system aging[21].

This work aims to study the protective effect of Stevia on diabetic-induced testicular injury through the histopathological, ultrastructural examination, and immunohistochemical study of VEGF, VCAM1, apoptosis, and necroptosis.

Materials and Methods

Animals

In this study, a total of eighteen mature male albino Sprague Dawley rats with a weight range of 180-200 g were utilized. For acclimatization, the rats were housed for two weeks in vented plastic cages under standardized conditions, which included approximately 12 hours of daylight per day and room temperature. The animals were fed with conventional laboratory rat chow and provided with free access to water. It is important to note that all studies were conducted in compliance with the Ethical Committee on Animal Testing's Laws and Regulations, with approval code (R/125), Faculty of Veterinary Medicine, Mansoura University.

Experimental Design

Three groups of six rats each were formed as follows: the control group rats (six nondiabetic rats) were given 2 mL of distilled water orally. The diabetic group rats (6 diabetic rats) were given 2 mL of distilled water orally. The Stevia group rats (6 diabetic rats) receive dissolved Stevia extract in distilled water in a dose of 400 mg/kg via oral gavage for 8 weeks[22]. The Stevia leaves were purchased from Agro-industry Product (SICAP), Cairo, Egypt, and the extract was prepared following the procedure described by El-Mesallamy et al[23]. The experiment lasted eight weeks after diabetes induction.

Induction of TYPE 2 DM

High-fat diet (HFD) was used to feed the rats in the diabetic groups (diabetic and Stevia rats) for 4 weeks, then a single 35 mg/kg of streptozotocin (STZ) intraperitoneal injection[24,25]. ACCU-CHEK glucometer and strips (Roch Diagnostic Co., Germany) were used to check the blood glucose, diabetes was considered with blood glucose equal to or above 200 mg/dl.

Blood Sampling

At the end of the experiment – 8 weeks after induction of diabetes – the rats that had fasted overnight were anesthetized using an intraperitoneal injection of sodium thiopental at a dose of 60 mg/kg. Blood samples were collected by puncturing the left ventricle and placed in EDTA-containing tubes. The samples were then centrifuged to separate the serum. Insulin and testosterone levels were measured using commercially available ELISA kits. The homeostasis model assessment (HOMA) insulin resistance (IR) index was determined using Mathe's method from fasting insulin and fasting blood glucose[26].

Tissue Sampling and Processing

At the time of sacrifices, the testicles were excised, and crossly cut. Part of the testis was fixed in Bouin's solution for H&E staining, the other part was fixed in 10% neutral formalin for

immunohistochemical staining, and other samples were frozen immediately and stored at -80 C for detection of oxidative stress markers.

For evaluation of MDA and GSH, segments of the testis were homogenized (10% w/v) in a pH 7.4 0.1 M Tris-HCl buffer. The homogenates were then centrifuged at 3,000 rpm for 20 minutes at 4°C. The resulting supernatant from the kidney homogenate was employed in assessing oxidative stress indicators, MDA and GSH, using available commercial kits.

Tissue samples were routinely processed in ascending concentrations of ethanol (ethanol 70% for 1 hr, then 95% for 1 hr two times, then 100% for one hour, two changes, xylene, two changes, for one hour), then embedded in melted paraffin. The fixed tissue specimens were trimmed under a fume hood to fit into cassettes and labeled, then, for histological assessment, sections were stained with hematoxylin and eosin[27], and to identify fibrosis, the Masson trichrome stain was used[28].

For the electron microscopy analysis, a small piece of each rat's testis was sliced into pieces and fixed in 4% glutaraldehyde in phosphate buffer (pH 7.2) for 2-4 hours. The samples were then post-fixed in 1% osmium tetroxide. Afterward, they were dehydrated using a series of alcohols and embedded in Spurr resin embedding media. Semithin and ultrathin slices were cut using a Leica Ultracut R ultramicrotome (Austria). The semithin slices, which were about 1 µm thick, were stained with toluidine blue, while uranyl acetate and lead citrate were used to stain the ultrathin sections. Finally, the samples were viewed using a Philips C-100 Bio transmission electron microscopy (Philips/FEI Corporation, Eindhoven, The Netherlands) at 80 kV[29].

Immunohistochemical Staining for Caspase3, VEGF, VCAM1 and RIPK1

Testicular sections (3-5 µm) were used for following the immunoperoxidase method for immunohistochemical staining[25,30], and hydrogen peroxide (3%) was used to stop the endogenous peroxidase activity. The slices were then rinsed three times with phosphate-buffered saline (pH 7.4) and treated in a water bath (95°C, 30 minutes) with sodium citrate buffer (0.01 M, pH 6.0) to recover antigens. After reaching room temperature, the slides were incubated for one hour with BSA (1%). The following primary antibodies were used and incubated overnight at 4°C: anti-caspase-3 (ab2302, Abcam, Cambridge, UK, 1:100)[25], anti-RIPK1 (ab72139, Abcam, Cambridge, UK, 1:200)[31], VCAM1 (ab134047, Abcam, Cambridge, UK, 1:200)[32] and VEGF (ab1316, Abcam, Cambridge, UK, 1:100)[33]. The slices were then treated with horseradish peroxidase-conjugated secondary antibodies (ab7097, Abcam, Cambridge, UK) at 37°C for 0.5 hours, followed by 30 minutes of labeled streptavidin-biotin (DETHP1000, Sigma-Aldrich). The reaction was visualized using Diaminobenzidine/peroxidase substrate DAB (DAKO Company). Finally, the section slides were counterstained with Mayer's hematoxylin for one minute, washed with tap water for eight minutes, dehydrated with ascending grades of ethyl alcohol, cleared with xylol, and the coverslipped.

Morphometric Evaluation

Images were taken with an Olympus® SC100 digital camera (Olympus Soft Imaging Solutions GmbH, Munster, Germany) and a light microscope. Image J software (National Institute of Health, Bethesda, MD, USA) was used to measure various parameters, including the average seminiferous tubule (ST) diameter, spermatogenic epithelium (SE) thickness, ST area (μm^2), ST lumen area (μm^2), SE area (μm^2), SE area ratio, total ST area (μm^2), and total interstitial area (μm^2) according to the methods described by Sziva et al[34]. Additionally, in the Masson trichrome stained sections, the capsular thickness, and the area occupied by collagen fibers were measured, and for the immunohistochemical stained sections, the positive expression of Caspase-3, VEGF, VCAM, and RIPK-1 were measured.

Johnsen Scoring System

Testicular sections were assessed using Johnsen's score on a scale of 1 to 10[35]. To evaluate spermatogenesis, each seminiferous tubule was scored on a scale from 1 to 10 based on the presence or absence of spermatogenic epithelium in the lumen. A score of 10 indicated normal spermatogenesis, with all sperm cell types arranged in an orderly manner, while a score of 1 indicated a complete lack of epithelial maturation.

Statistical Analysis

The data was tabulated, coded, and analyzed using analyzed using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9 (GraphPad San Diego, California, USA). Quantitative data were described using mean, and standard deviation for parametric data after testing normality using the Shapiro-Wilk test. To compare groups, the significance of difference was statistically assessed using analysis of

variance (ANOVA), followed by Tukey's test for a group of comparison, with a $p \leq 0.05$ considered statistically significant, depending on the absence of difference between groups[36].

Results

Biochemical Results

The serum glucose, insulin levels, and HOMA-IR index showed statistically significant increases in the diabetic rats, which Stevia ameliorated. Additionally, there is a non-significant alteration in insulin levels and a significant increase in blood glucose and HOMA-IR between the control and Stevia groups (Figure 1A, B, and C).

When compared to the control rats, the diabetic group's serum testosterone level was considerably lower, but it was increased after Stevia treatment. No significant difference in serum testosterone levels in Stevia-treated rats was found when compared to the control and diabetic rats (Figure 1D).

Stevia considerably lowered MDA levels when compared to the diabetic rats, which dramatically increased MDA levels when compared to the control group. However, as compared to the control group, the MDA level in the Stevia group is still considerably higher. The diabetic group had considerably lower GSH levels. However, in the Stevia group, it was reversed. Stevia restored GSH levels since there was no significant difference between the Stevia and control groups (Figure 1E-F).

Histological Results

Sections from the control rats stained with Hematoxylin and Eosin showed seminiferous tubules formed of regular layers of seminiferous epithelium, germ cells, and Sertoli cells at the base, with Leydig cells between the tubules. The lumen of tubules is filled with spermatids and spermatozoa (Figure 2A-B). In contrast, the diabetic rats showed distorted architecture in the form of vacuolated and necrotic irregular crossly-sectioned seminiferous tubules with a wide lumen and extremely widened interstitial space (Figure 2C-D). Some seminiferous tubules showed complete arrest of spermatogenesis and degeneration of spermatogenic epithelium, resulting in a significantly decreased Johnsen score ($p<0.0001$) (Figure 2C-D, and G). These changes were markedly diminished in the Stevia group as they showed normal regularly sectioned seminiferous tubules, narrow lumen, and relatively widened interstitial space (Figure 2E-F); moreover, the Stevia group showed significantly increased scores when compared with diabetic rats ($p=0.0088$) but still significantly lower than the control group ($p=0.0278$) (Figure 2G).

In Masson trichrome stained sections, the capsule thickness was significantly increased in the diabetic rats, but it was reduced significantly in the Stevia group ($p<0.0001$), although it was still significantly thicker than the control group ($p=0.0072$). The diabetic group showed a significant increase in interstitial fibrosis, but it was significantly decreased in the Stevia group ($p<0.0001$) (Figure 3).

Ultrastructural Results

The electron microscopic examination of the control rats showed normal testicular ultrastructure (Figure 4), In contrast, diabetic rats showed altered structure of seminiferous tubules in the form of distorted Sertoli cells lying on the thickened basal lamina, with the cytoplasm containing lipid droplets and swollen mitochondria (Figure 5A). The spermatogonia show shrunken pyknotic nuclei, primary spermatocytes are distorted with malformed round spermatids (Figure 5B-C). Leydig cells also show shrunken nuclei, lipid droplets in cytoplasm, and swollen mitochondria (Figure 5D). Treatment with Stevia tends to restore the normal architecture of the seminiferous tubules with relatively normal cells (Figure 6).

Histomorphometric Evaluation and Image Analysis

The seminiferous tubule diameters were significantly decreased in the diabetic group compared to the control group ($p=0.002$), and they were significantly increased in the Stevia-treated group ($p=0.03$) when compared with the diabetic group but still significantly decreased when compared with the control group ($p=0.04$) (Figure 7A). Moreover, when compared to the control group, SE thickness decreased significantly in the diabetic group ($p=0.005$) and non-significantly in the Stevia group ($p=0.06$) (Figure 7B).

ST area, area, and SE area were significantly decreased, and the ST lumen significantly increased in the diabetic group ($p=0.003$, 0.0003, and 0.009, respectively). Stevia reverses this effect as it significantly increases SE area and decreases ST lumen area ($p=0.03$ and 0.021 respectively), and ST diameter was not significantly increased ($p=0.26$). Additionally, the SE area ratio is significantly decreased in the diabetic group ($p=0.0001$) when compared to the control and increased in the Stevia group ($p=0.0005$) when compared to the dia-

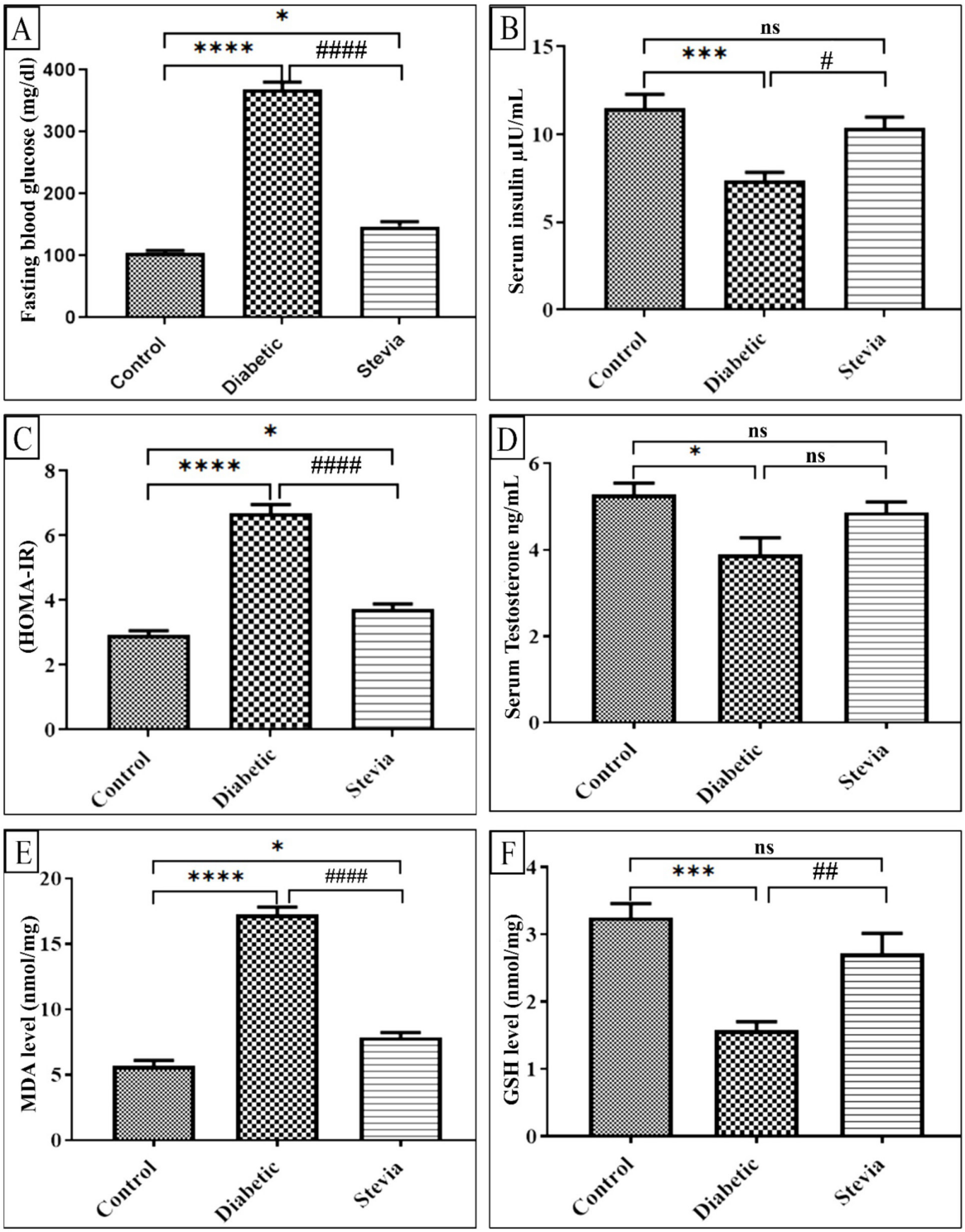

Figure 1. Biochemical results of the different groups. (ns=no significance, *significance *vs.* control group and # significance *vs.* diabetic group, *$p<0.05$, ***$p\leq0.001$, ****$p\leq0.0001$, ##$p\leq0.01$, ###$p\leq0.001$, and ####$p\leq0.0001$). **A**, Fasting blood glucose; (**B,**) Serum insulin; (**C**), HOMA-IR; (**D**), Serum testosterone; (**E**), MDA level; (**F**), GSH level.

betic group (Figure 7C). On the other hand, the spermatogenic epithelium area ratio significantly increased ($p=0.0005$) in the Stevia group when compared with the diabetic group, which showed a significant decrease ($p<0.0001$) when compared to the control group (Figure 7D).

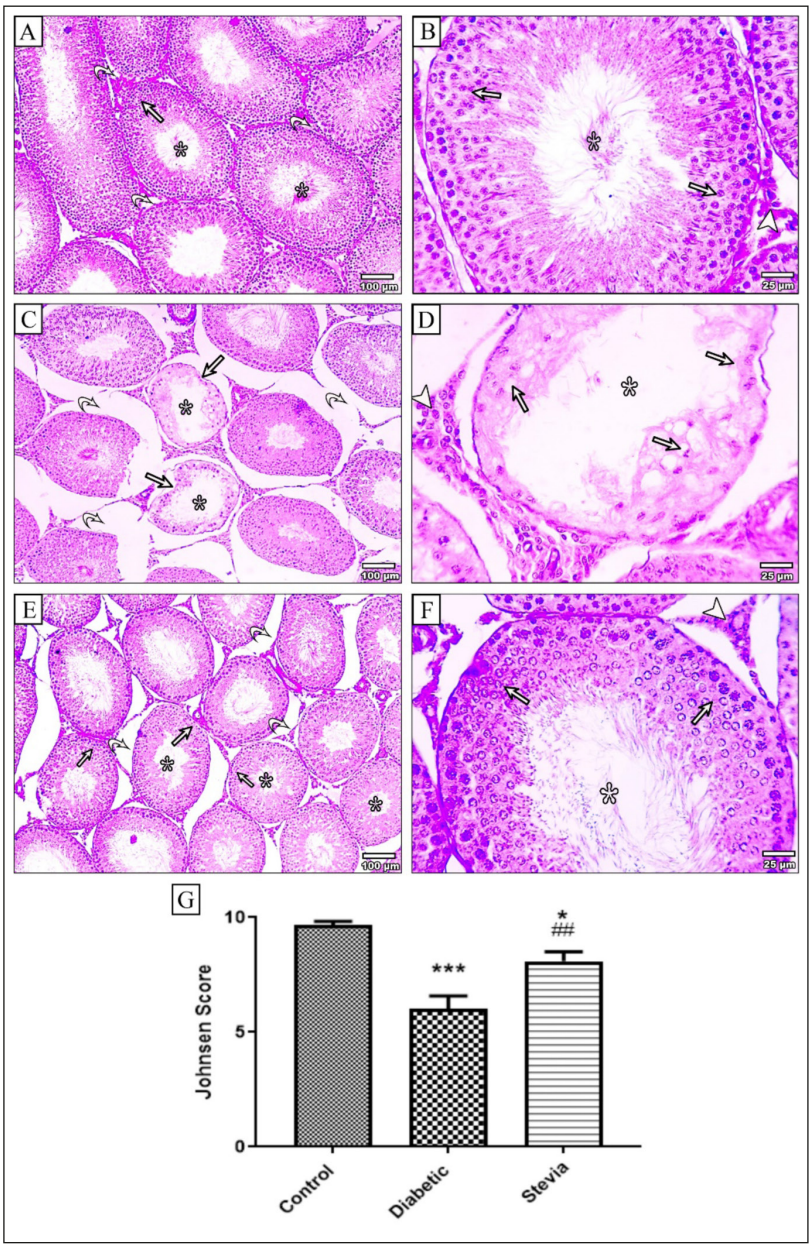

Figure 2. Microscopic images of H&E-stained control group testicular sections (**A-B**) showing typical regular crossly sectioned seminiferous tubules with tiny lumens filled with spermatids and spermatozoa (stars) and bordered with spermatogonia, many layers of spermatocytes (arrows), and Sertoli cells. In the control group, a thin interstitial gap (curved arrows) was found between tubules containing Leydig cells (arrowhead). Diabetic testicular sections (**C-D**) show vacuolated and necrotic irregular cross-sectioned seminiferous tubules (arrows) with a broad lumen (stars) and greatly dilated interstitial space (curved arrows), with no impact on Leydig cells (arrowhead). Normal regular cross-sectioned seminiferous tubules (arrows) with a narrow lumen (stars), normal cells of Leydig (arrowhead) with slightly enlarged interstitial space (curved arrows) in the stevia-treated diabetic group (**E-F**). **A**, (**C**), (**E**), magnification x100 and (**B**), (**D**), and (**F**) magnification x400. G Johnsen score (* significance *vs.* control group and # significance vs. diabetic group, * $p<0.05$, *** $p\leq0.001$, and ## $p\leq0.01$).

As regards the total seminiferous tubule area and the total interstitial area, there was a significant decrease in the total ST area and an increase in the total interstitial area in the diabetic group ($p<0.0001$) when compared to the control group and a significantly increased in Stevia group ($p=0.0002$) when compared to diabetic group (Figure 7E).

The area percentage of immuno-positive area stained against VEGF is significantly decreased in the diabetic rats compared with the control rats ($p<0.0001$); Stevia significantly ameliorates this reduction ($p=0.0135$), but VEGF expression is still significantly lower than the control rats ($p=0.0194$) (Figure 8).

There were weak expressions of VCAM1 RIPK-1 and caspase 3 in the control group, in contrast, the diabetic group showed significantly strong expression ($p<0.0001$), on the other hand, stevia significantly decreased the positive immune expression when compared with the diabetic rats ($p<0.0001$). Moreover, the VCAM1 and RIPK-1 immunoreactive areas were still significantly higher than the control rats ($p=0.0196$ and 0.0175), respectively (Figures 9-11).

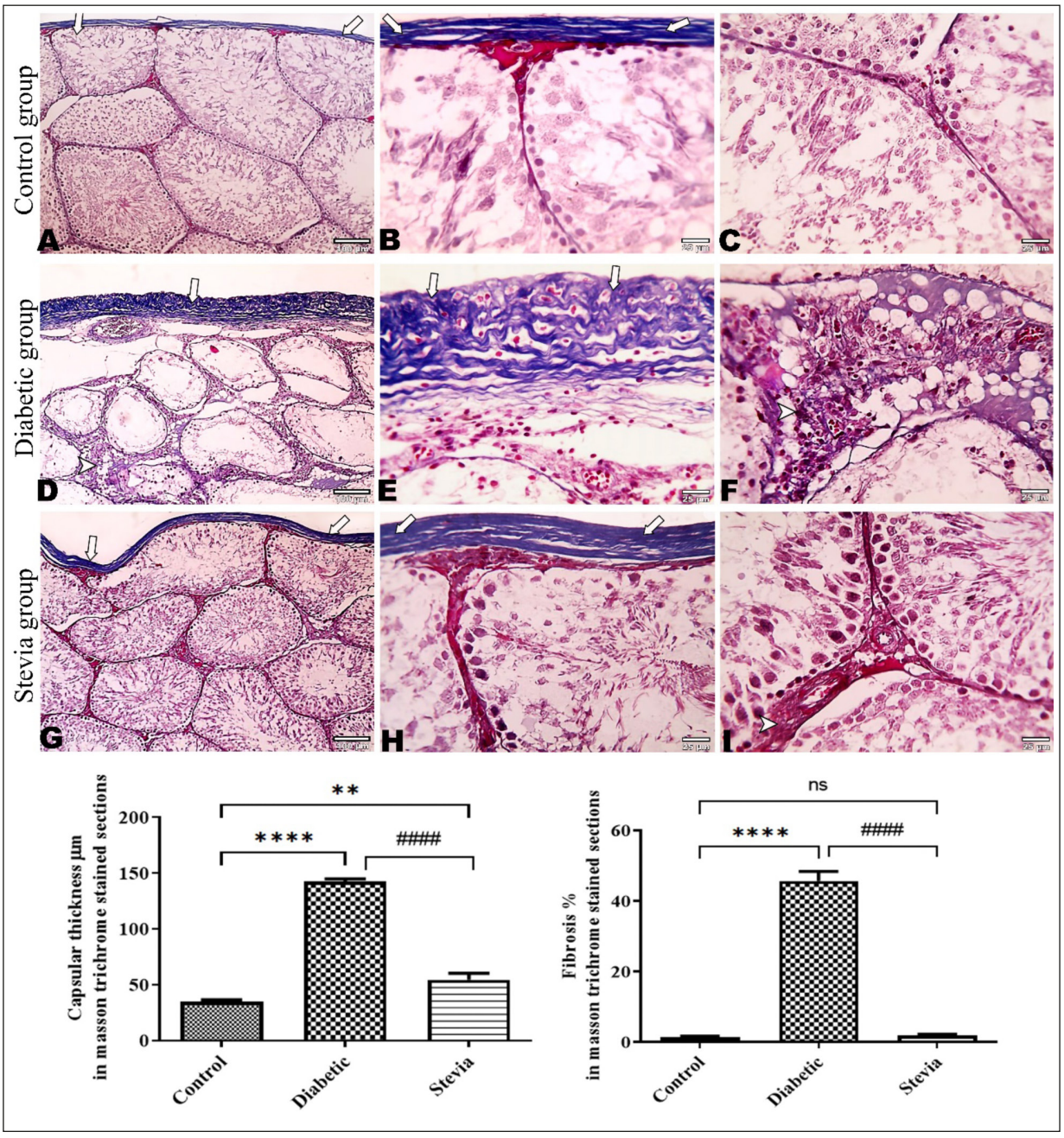

Figure 3. Microscopic pictures of Masson trichrome stained the control group of the testicular section showing normal capsular thickness (arrows) with no interstitial collagen deposition in the control normal group (**A-C**). Crossly sectioned testicular sections from the diabetic group (**D-F**) showed increased capsular thickness (arrows) with excess, bluish-stained interstitial collagen deposition (arrowhead). Crossly sectioned testicular sections from the stevia group (**G-I**) showed markedly decreased capsular thickness (arrows) with no bluish-stained interstitial collagen deposition. [(**A**), (**D**), (**G**), magnification x100, and (**B**), (**C**), (**E**), (**F**), (**H**), (**I**), magnification x400].

Discussion

Diabetes is known to be injurious to many organs, resulting in several complications. As a result, developing efficient methods to eliminate or delay these complications is essential[37].

STZ induces diabetes by causing a fast reduction in β-cells, which results in decreased insulin supply. Hyperglycemia results from insufficient insulin release, which causes oxidative stress via ROS generation[38,39], resulting in an increase in blood glucose, a decrease in serum insulin, a loss of

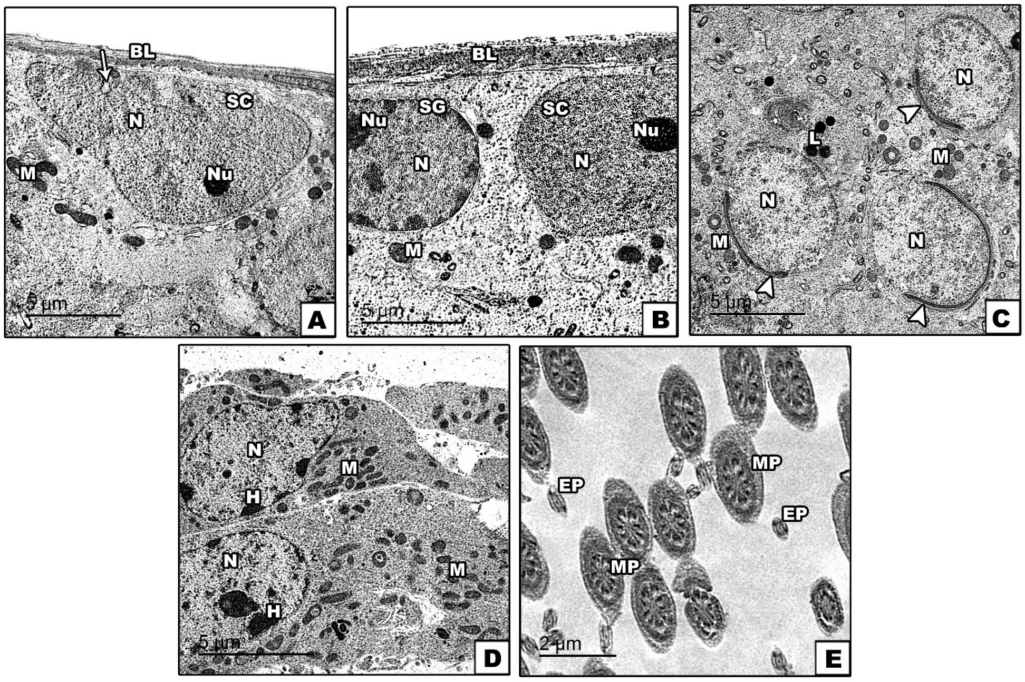

Figure 4. An electron micrograph in the testis of control rats showing (**A-B**) a Sertoli cell (SC), spermatogonium (SG) lying on regular basal lamina (BL). The cell nucleus (N) appears large euchromatic, and oval with a prominent nucleolus (Nu) and well-defined nuclear envelope with a characteristic longitudinal fold (arrow). The cytoplasm shows mitochondria (M). **C**, round spermatid having a central rounded euchromatic nucleus (N). Well-formed acrosomal cap (arrowhead), mitochondria (**M**), and lysosomes (**L**) are found. **D**, Leydig cell nucleus (N) is oval in shape having fine granular euchromatin with a thin rim of peripheral chromatin condensation (H). The cytoplasm shows mitochondria (M). E, normal transverse sections in the middle piece (MP) and end piece (EP) of the sperm tail. [TEM scalebar (**A-D**) 5 micron and (**E**) 2 micron].

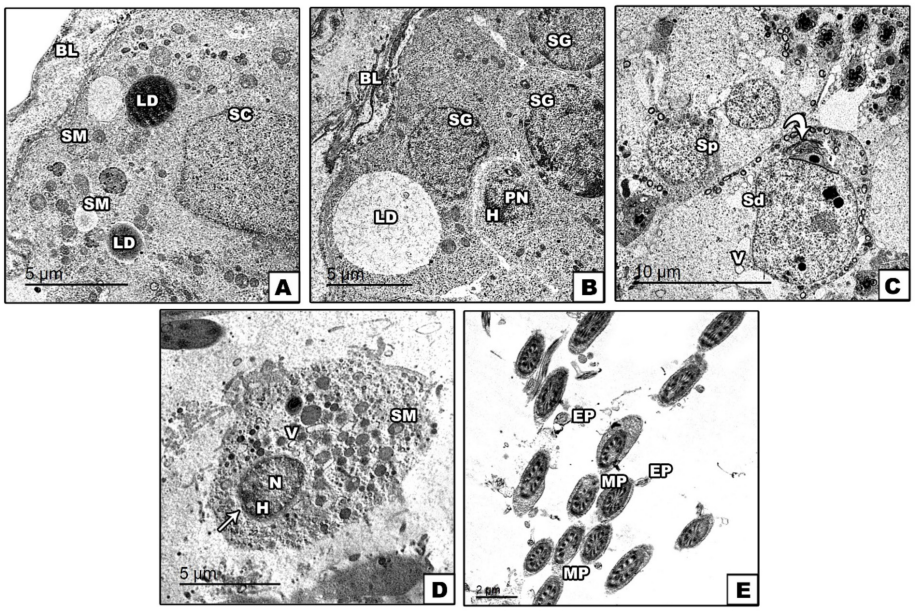

Figure 5. An electron micrograph in the testis of diabetic rats showing (**A**) distorted Sertoli cell (SC) lying on thickened basal lamina (BL), cytoplasm has swollen mitochondria (SM), and lipid droplets (LD). **B**, The spermatogonial nucleus (SG) is irregularly dark shrunken (PN) with peripheral chromatin condensation (H) and a large lipid droplet is found with dilated basal lamina (BL). **C**, distorted primary spermatocyte (Sp) surrounded by an irregularly indented cell membrane, malformed round spermatid (Sd) with deformed Golgi apparatus (curved arrow), vacuoles (V). **D**, a Leydig cell. The cell nucleus (N) is shrunken, with heterochromatin (H) and slightly dilated perinuclear space (arrow). Swollen mitochondria (SM) and vacuoles appear inside the cytoplasm (V). **E**, deformed middle pieces (MP) and slightly distorted end pieces (EP). [TEM scalebar (**A-B, D**) 5 micron, (**C**) 10 micron and (**E**), 2 micron].

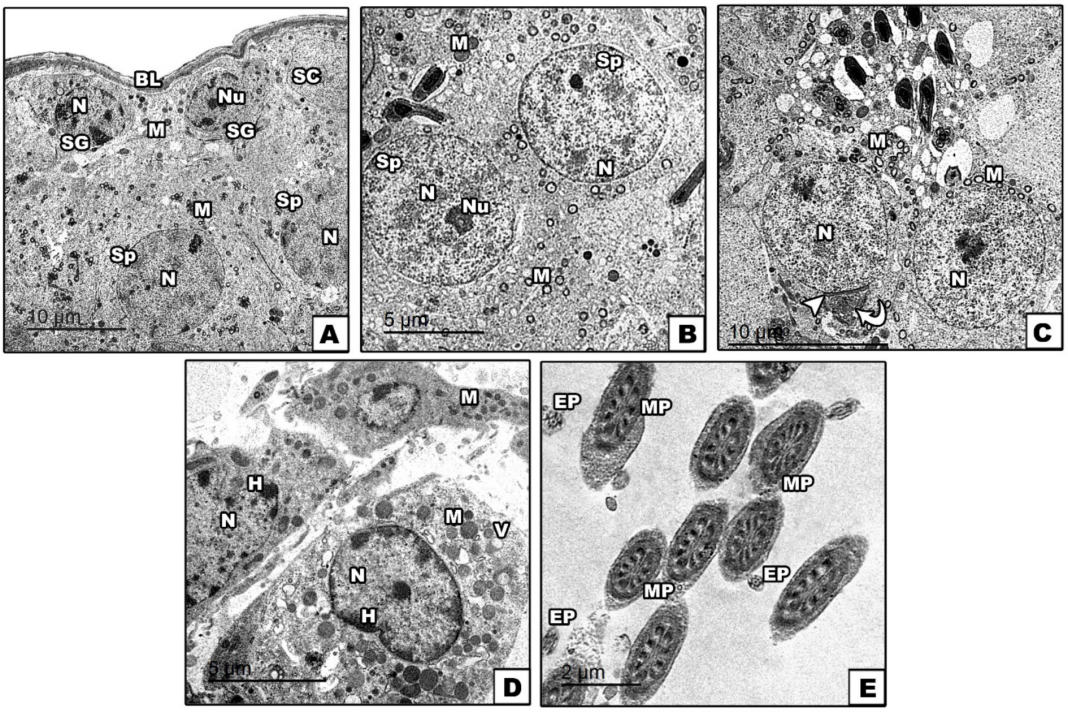

Figure 6. An electron micrograph in the testis of treated rats showing Sertoli cell (SC), spermatogonium (SG), and primary spermatocyte with big rounded euchromatic nucleus (Sp) resting on regular basal lamina (BL) of treated rats' testis (**A-B**). The cell nucleus (N) is euchromatic, and oval, with a prominent nucleolus (Nu) and well-defined nuclear envelope (arrow). The mitochondria (M) are visible in the cytoplasm. The middle rounded euchromatic nucleus (N) of a round spermatid (**C**). The acrosomal cap (arrowhead), mitochondria (M), and cytoplasm are all well-formed, with a prominent Golgi apparatus (curved arrow). **D**, The nucleus of a Leydig cell (N) is oval in shape, with fine granular euchromatin and a thin rim of peripheral chromatin condensation (H). The mitochondria (M) are visible in the cytoplasm. **E**, transverse sections that are almost typical in the middle piece (MP) and end piece (EP) of the sperm tail [TEM scale bar (**A**, **C**) 10 micron, (**B**, **D**) 5 microns, and (**E**)=2 microns].

body weight, and a lower testosterone level, which might be an indicator of testicular injury in diabetic animals as reported before[40]. However, Stevia administration improves all the alterations, indicating its anti-diabetic potential. This study is in line with previous research[41,42] that has proven that Stevia extract decreases blood glucose in diabetic patients. This effect might be mediated by inhibiting glucose absorption in the duodenum of rats[43].

Steviol glycosides, the chemicals that give Stevia extracts their sweet flavor, are assumed to be responsible for Stevia's anti-diabetic properties; however, the non-sweetener part may demonstrate insulinotropic actions[43]. Recent studies[12] have confirmed the role of sativoside in stimulating insulin secretion[42,44]. Besides, Stevia not only increased insulin but also decreased gluconeogenesis, resulting in lower serum glucose levels. Increased HOMA-IR in diabetic rats and a decrease after stevia treatment were recorded in this study, and it is consistent with previous reposts[45,46].

Normal testosterone level is required in spermatogenesis to regulate and maintain the normal seminiferous tubules[47]. We found a significant reduction of testosterone in diabetic rats, which was also recorded in previous research[8,48]. Diabetes decreases plasma testosterone levels, and sperm count, and mediates germ cell apoptosis[49]. The testosterone level was improved after Stevia treatment, which was in line with previous research[50] done on mice. On the other hand, Stevia administration in male rats showed an increased level of testosterone, but it had no statistical significance[51,52].

Insulin has been connected to testosterone secretion due to the presence of insulin-specific receptors on Leydig cells. Also, the reduced insulin in DM has significantly decreased testosterone levels[53]. Oxidative stress is a major contributor to Leydig cell dysfunction in diabetic testes, and antioxidants may be able to reverse these deficits[54]. The antioxidant treatment could be able to improve testosterone levels. Stevia's antioxidant capabilities are ascribed to its high flavonoid, phenol, catalase, and peroxidase concentration[12].

In this study, the diabetic rats showed elevated MDA and reduced GSH levels that indicate oxidative stress, these are in line with previous reports[55-57]. Additionally, MDA was accused of ampli-

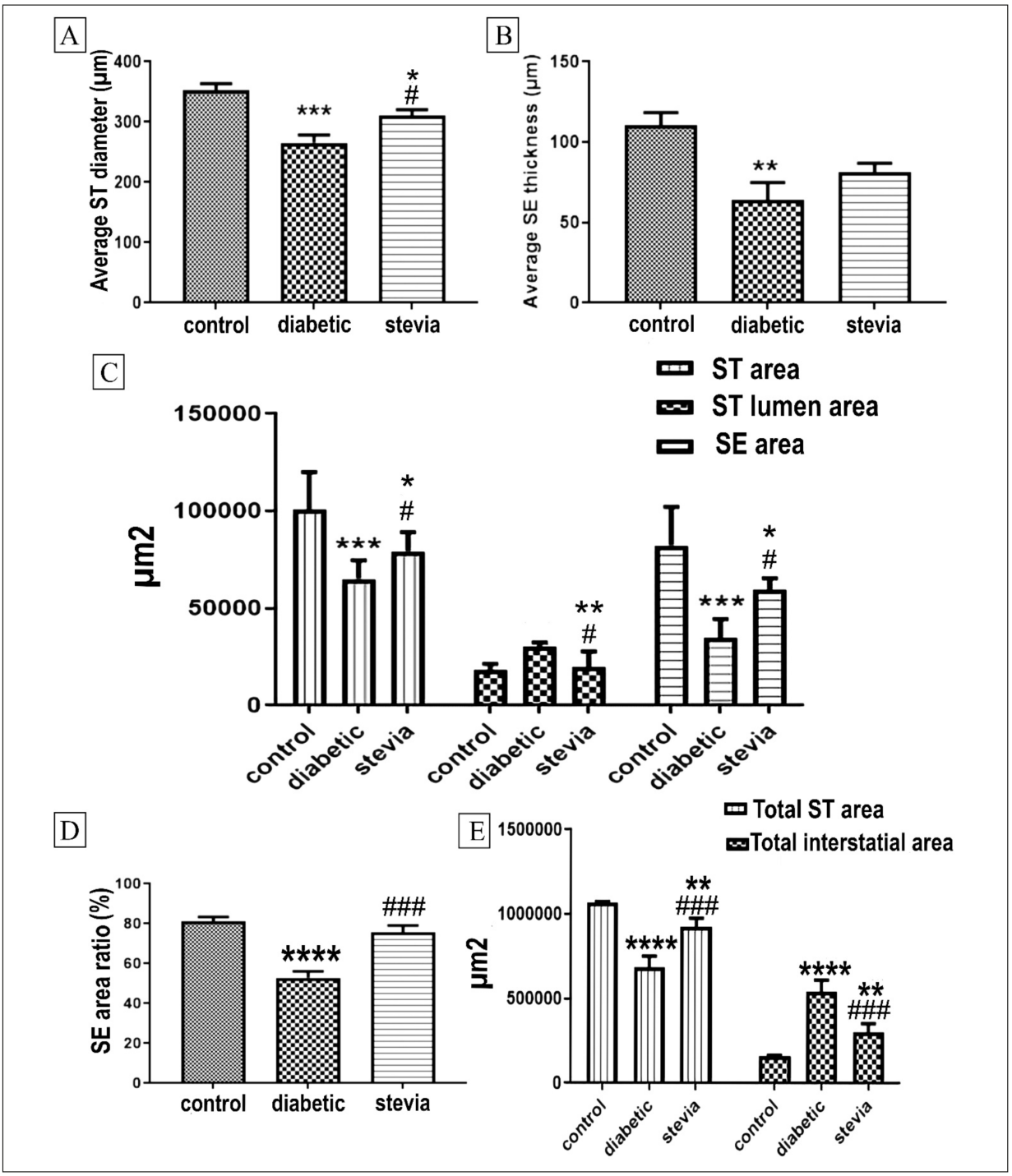

Figure 7. Morphometric evaluation of the seminiferous tubule parameters, (**A**) average seminiferous tubule diameter, (**B**) average spermatogenic epithelium (SE) thickness, (**C**) seminiferous tubule (ST) area, ST lumen area and spermatogenic epithelium area, (**D**) Spermatogenic epithelium area ratio and (**E**) total ST area and interstitial area. The data represented as mean ± SD, ANOVA and followed by Tukey's test, (*significance *vs.* control group and #significance *vs.* diabetic group, * or #means $p<0.05$, ** or ## means $p<0.01$, *** or ### means $p<0.001$ and **** or ####means $p\leq0.0001$).

fying the damages mediated by oxidative stress[58]. A significant reversion of the diabetic effect on MDA and GSH was found in this study following Stevia's administration. Previous studies[59] supported our findings as GSH supplementation was shown to ameliorate the adverse effect of DM on seminiferous tubules. In addition, the antioxidant treatment prevented testicular damage in rodents[60].

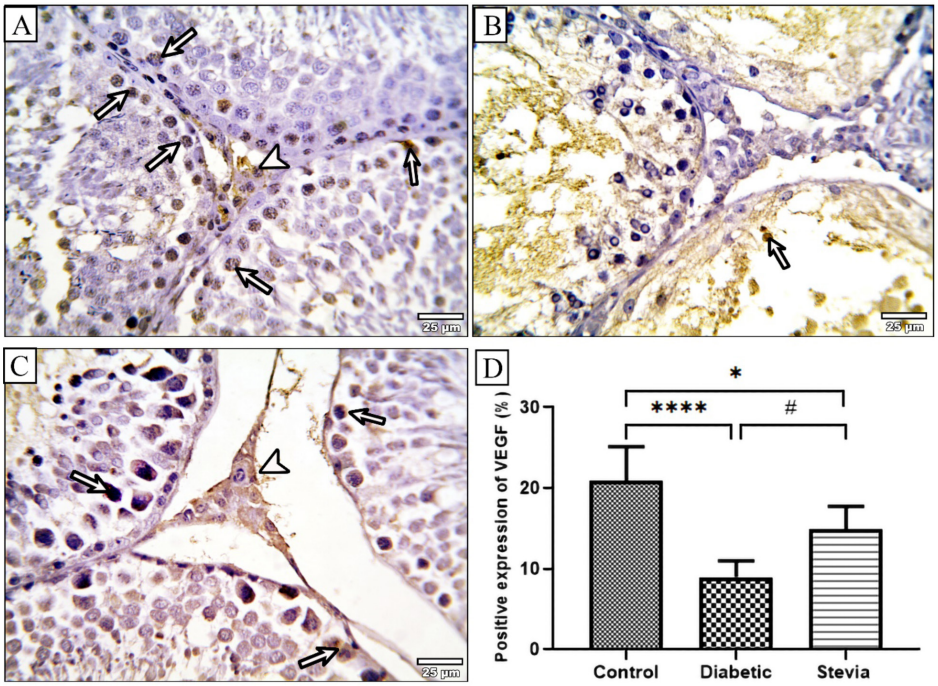

Figure 8. Microscopic images of VEGF immuno-stained cross-sectioned testicular tissue in the control group (**A**) indicate strong positive staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead), the diabetic group (**B**) showed decreased staining and the stevia group (**C**) showing moderate positive staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead) [(**A-B**) magnification x400]. Image analysis (**D**) represents the immunoreactive area (%) in the stained sections (* significance *vs.* control group and # significance *vs.* diabetic group, * or # means $p<0.05$ and **** or #### means $p\leq0.0001$).

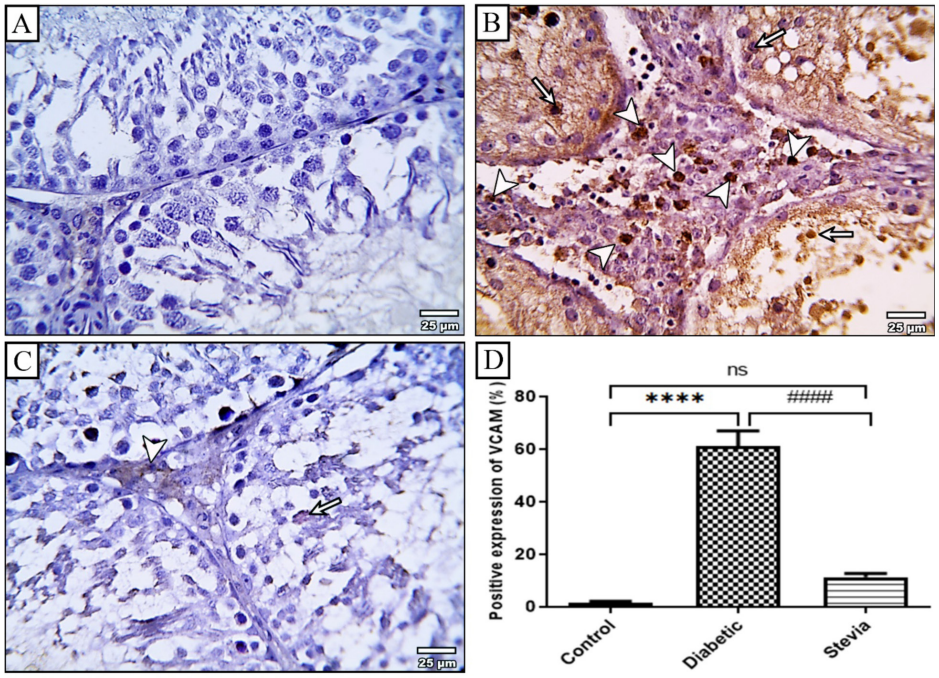

Figure 9. Microscopic images of testicular tissue immuno-stained against VCAM1 and exhibiting negative staining in the control group (**A**). Crossly sectioned testicular tissue from the diabetic group (**B**) showed strong positive brown staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead). Crossly sectioned testicular tissue from the stevia group (**C**) showing weak positive brown staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead) [(**A-B**) and magnification x400]. Image analysis (**D**) represents the immunoreactive area (%) in the stained sections (* significance *vs.* control group and # significance *vs.* diabetic group, * or # means $p<0.05$ and **** or #### means $p\leq0.0001$).

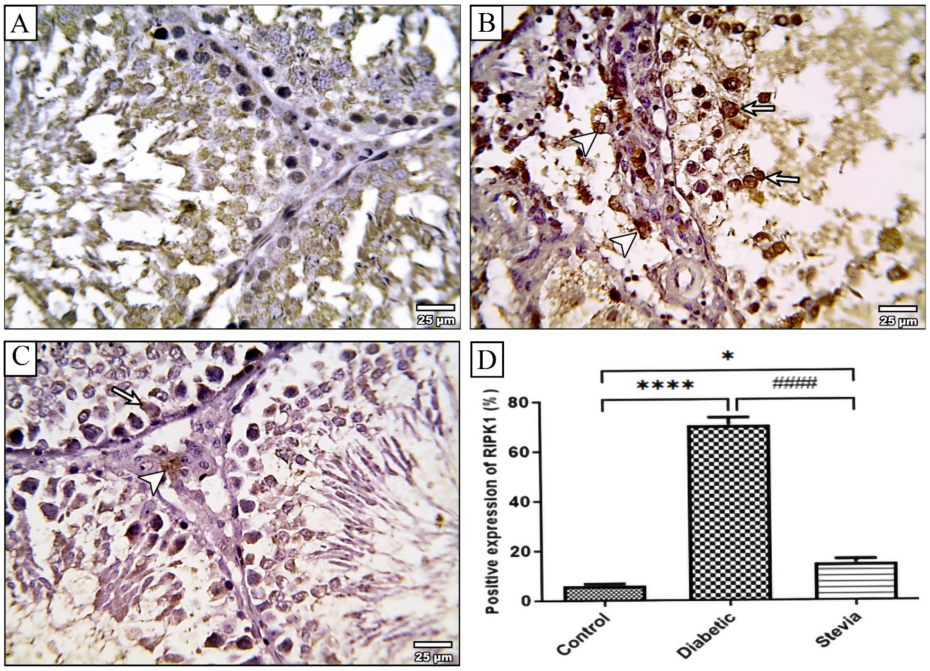

Figure 10. Microscopic images of RIPK1 immuno-stained cross-sectioned testicular tissue in the control group (**A**) demonstrate negative staining. Crossly sectioned testicular tissue from the diabetic group (**B**) showed strong positive brown staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead). Crossly sectioned testicular tissue from the stevia group (**C**) showing weak positive brown staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead) [(**A-B**) and magnification x400]. Image analysis (**D**) represents the immunoreactive area (%) in the stained sections (*significance *vs.* control group and # significance *vs.* diabetic group, * or # means $p<0.05$ and **** or #### means $p\leq0.0001$).

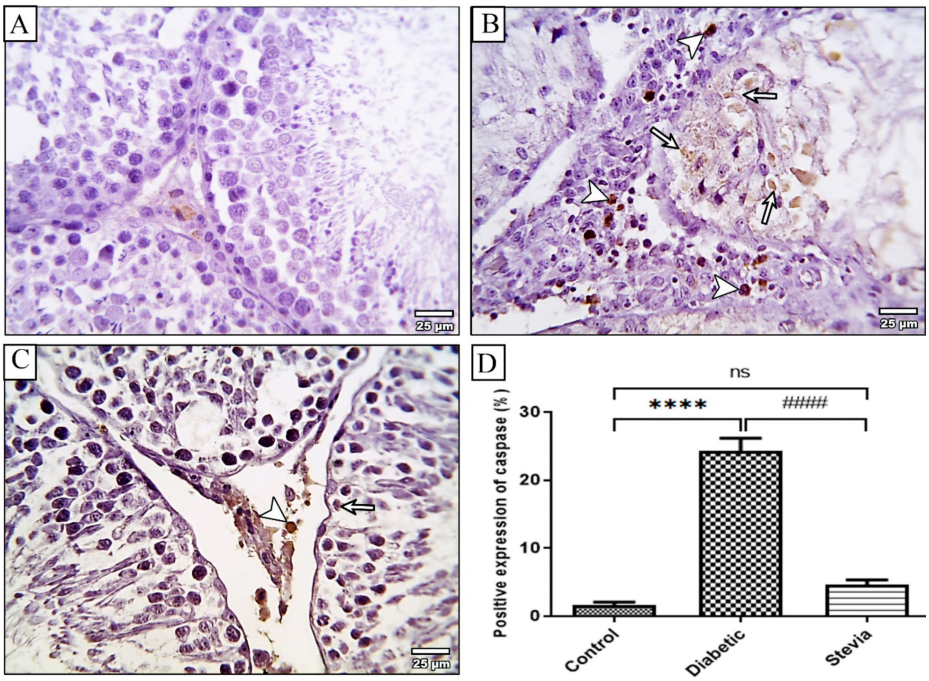

Figure 11. Microscopic images of caspase-3 immuno-stained cross-sectioned testicular tissue in the control group (**A**) indicate negative staining. Crossly sectioned testicular tissue from the diabetic group (**B**) showed moderate positive brown staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead). Crossly sectioned testicular tissue from the stevia group (**C**) showing weak positive brown staining in the tubular epithelium (arrows) and interstitial cells of Leydig (arrowhead) [(**A-B**) and magnification x400)]. Image analysis (**D**) represents the immunoreactive area (%) in the stained sections (* significance *vs.* control group and # significance *vs.* diabetic group, * or # means $p<0.05$ and **** or #### means $p\leq0.0001$).

The major cause of diabetes complications has been identified as oxidative stress[61]. Diabetic-induced testicular oxidative stress is the primary cause of male diabetic reproductive failure[9,62]. Moreover, oxidative stress mediates the apoptotic death of diabetic testis[37,57].

Sustained hyperglycemia has been shown to mediate systemic complications via many mechanisms, predominantly by a rise in ROS generation and oxidative stress, which can directly lead to cellular death, or by depletion of the defensive antioxidant mechanisms, such as glutathione peroxidase synthesis[63].

It has been proven that diabetes has a deleterious effect on testicular structure in the form of seminiferous tubule atrophy and spermatogenetic cell apoptosis[2]. Likewise, Histological investigation revealed that the seminiferous tubule had been disrupted, Sertoli cells had been lost, and Leydig cells had diminished, which has been reported before[64]. As mentioned previously, we observed a decrease in the thickness of the germinal epithelium, which was also noted in a prior study[65]. Interstitial oedema has been reported as well[37]. Furthermore, the seminiferous tubules shrank, and spermatogonia, spermatocytes, and sperm cells became degenerated and vacuolated[66]. We have found that treatment with Stevia could efficiently restore all these changes and attenuate the development of testicular dysfunction which was reported also by Gholizadeh et al[52].

Regarding electron microscopic examination, swollen mitochondria, lipid droplets, and chromatin condensation in Sertoli, Leydig, and spermatogenic cells were seen with subsequent alleviation of these changes in Stevia-treated rats, these changes were reported also by Trindade et al[65]. Spermatids with faulty mitochondria not only produce less ATP but also experience more oxidative stress. The mitochondria are thought to be the major generator of free radicals, and their malfunction is linked to diabetes-related alterations[67].

Few research[68,69] has focused on testicular interstitial fibrosis in DM that impairs testosterone production and spermatogenesis, resulting in infertility and sexual dysfunction. Antioxidants and/or anti-inflammatory medications may be able to prevent diabetic-induced oxidative stress and inflammatory responses[70]. However, testicular interstitial fibrosis induced by long-term hyperglycemia is difficult to be treated[68,69]. Testicular interstitial fibrosis may result in permanent oligozoospermia and persistently low sperm motility[71]. This study confirmed previous literature regarding fibrosis in diabetic rats' testis and recorded its amelioration in the Stevia-treated group and it is the first one to our knowledge that commented on testicular interstitial fibrosis following Stevia administration. DM was found to enhance the expression of TGF-beta1 in testis, and this was parallel with its progression. Moreover, testicular interstitial fibrosis causes Leydig cell apoptosis, decreasing testosterone production and hence the amount and activity of germ cells[71]. Tissue fibrosis is thought to be a reparative process that occurs in reaction to cell loss or a direct hyperglycemic insult. Excessive fibrosis is associated with poor testosterone and decreased sperm production[72,73].

VCAM-1 is a crucial regulator of the adhesion of leukocytes and their movement through endothelial cells by interacting with α4β1 integrin. This integrin found on leukocytes sticks to VCAM-1 present in endothelial cells, leading to the activation of signaling pathways within the activated endothelial cells, which enables the movement of leukocytes[74]. VCAM-1 was initially discovered as a glycoprotein on the surface of endothelial cells[75]. However, under chronic conditions in some diseases, VCAM-1 is also expressed on other cells, including Sertoli cells and cancer cells[76]. Leydig cells have a high expression of CD106 (VCAM-I) and bind to lymphocytes through this protein[77]. Pro-inflammatory cytokines such as TNFα, ROS, oxidized low-density lipoprotein, and high glucose concentration activate VCAM-1 expression[78].

Diabetes causes increased nuclear translocation of the transcription factor NF-κB, resulting in an inflammatory response[79]. In the current work, diabetic rats showed high expression of VCAM-1 supported by previous research[80]. This might be explained as ROS promotes NF-κB-mediated inflammation by increasing the amounts of proinflammatory cytokines, and adhesion molecules[79]. It is reported that stevioside has, anti-inflammatory[81], and anti-apoptosis effects[82] as well as immunomodulatory properties[83], which might explain the reduction of VCAM1 after Stevia treatment.

Under diabetes circumstances, testicular apoptosis, which happens at modest levels during normal spermatogenesis, is greatly amplified[15]. It is predominantly facilitated by stimulation of the mitochondrial-mediated apoptotic pathway[62]. Increased caspase 3 expression in our findings was supported by literature examining STZ-induced diabetes in mice and rats[15,37]. Moreover, oxidative stress is critical to diabetic-induced testicular cell apoptosis. Therefore, enhancing the antioxidant capability of the testis might be a potentially effective method to reduce testicular apoptosis, and hence infertility in

diabetic males, which could explain the reduction of caspase 3 levels in Stevia-treated rats.

The interaction between inflammation and oxidative stress results in a vicious loop that culminates in the activation of the apoptotic signaling pathways. When activated by ROS, inflammatory mediators trigger apoptosis *via* caspase-8 activation, which drives the extrinsic apoptotic pathway[57]. Multiple investigations have shown elevated levels of p53, the Bax/Bcl2 ratio, and caspase-9[9,62], This is an indication that the intrinsic apoptotic signaling pathway is activated. Both paths resulted in caspase 3 activation, as seen in this study.

We have shown that diabetic rats induce RIPK1 expression with a reduction in the Stevia-treated rats. This is the first study to investigate the function of stevia in lowering RIPK1 expression levels in the testis. Inhibiting RIPK1 is a novel treatment strategy for human inflammatory and degenerative diseases[84]. DM has been recorded to increase expression of multiple necroptosis markers as RIPK1, 2, and 3 in β pancreatic cells, with the level of RIPK1 reaching more than 18 folds of the control rats, although they all significantly reduced in Stevia-treated diabetics[85]. Diabetes increases impaired cellular glucose absorption, whereas hyperglycemia promotes glycosylation, which is the primary cause of necroptosis[86]. Moreover, after repressing RIPK1 with a necroptosis-inhibiting drug, the number of seminiferous tubules, and spermatogenesis score were increased, implying that addressing necroptosis has several favorable implications[87].

The development of advanced glycation end products (AGEs) and ROS are both regulators of the necrosomal pathway. The production of AGEs and ROS is dependent on glycolysis[86], which is increased during necroptosis[88]. Interestingly, LaRocca et al[86] reported that elevated glucose suppresses extrinsic apoptosis[86]. This is in line with other reports[89] that revealed glucose uptake hinders apoptosis, but glucose deprivation stimulates it. Although extrinsic apoptosis was inhibited by exposure to hyperglycemia, considerable amounts of caspase-independent death still occurred. Because necroptosis and apoptosis are both induced by the same ligands, this could suggest that hyperglycemia potentiates a shift from apoptosis toward necroptosis[88]. The existence of comorbid diseases, which are frequently present in people with diabetes, or the possible interactions between Stevia and other medications may not have been taken into account in this study. It is possible that the study did not cover the long-term effects of Stevia therapy or any possible adverse effects from prolonged use. To gain a more comprehensive understanding of the potential benefits and limitations of using Stevia to treat testicular injuries induced by diabetes, it is crucial to consider these limitations when interpreting the study's results. Further research is needed to address these concerns.

Conclusions

Generally, this study stated the protective effect of Stevia in experimentally induced diabetic testicular damage, recommending its possible value in clinical applications to prevent testicular damage in diabetic patients.

Authors' Contributions

Conceptualization, methodology, writing-review and editing, supervision, project administration, funding acquisition, M. Elshafey, O. Erfan, E. Risha, A. Badawy, H.A. EBRAHIM, M. EL-Sherbiny, E. Enan, M. Eldesoqui. Analysis, investigation, writing-original preparation, I. El-Shenhaby. Software, Validation, analysis, and Interpretation of Data M. Elshafey, O. Erfan, E. Risha, A. Badawy, H.A. Ebrahim, M. El-Sherbiny, I. El-Shenhaby, E. Enan, M.E. Almadani, M. Eldesoqui. All authors read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Informed Consent

Not applicable.

Ethics Approval

The research design applied in our study meets Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Animal experiments involved in this research were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt (R/125) and conducted following the guiding principle of laboratory research animal species and utilize, issued by the US National Institutes of Health (NIH publication No. 85-23, reviewed 1996).

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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