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Assessment of Rosemary Oil Role on the Testes of Adult Male Albino Rats with Streptozotocin-Induced Diabetes

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Abstract

Background: **Reactive oxygen species caused by hyperglycemia in diabetes mellitus (DM) are considered a compelling cause of male subfertility and infertility. Insulin or other oral anti-diabetic drugs are not effective in preventing diabetic complications. Rosemary has a hypoglycemic effect with an antioxidant activity and a protective role against testicular toxicity.** ***Aim of work:*** **evaluating the role of rosemary oil in modifying the diabetogenic effect on rat testes.** ***Materials and Methods*****: Six groups of 30 rats, each with five rats, were set up. The groups were +ve Control, rosemary oil, DM, DM + Insulin, DM + Rosemary oil, and DM + Insulin + Rosemary oil. In the diabetic groups, DM was induced with streptozotocin (STZ) (40 mg/kg, I.P.) once. Rosemary oil was administrated (50 mg/kg, I.P) daily in the rosemary oil groups. The insulin groups were treated with subcutaneous insulin (14 IU/kg/day). Treatments in all groups were given for four weeks after confirmation of DM.** ***Results:*** **body weight, blood glucose levels, sperm parameters, hematoxylin & eosin, toluidine blue, and immunostaining sections with morphometric measurements and catalase levels presented improvement in rosemary, insulin, and combined treated groups in an uprising manner.** ***Conclusion:*** **Combined Rosemary oil and insulin ameliorated testicular complications resulting from STZ-induced DM in adult male albino rats.**

Keywords: diabetes, insulin, rosemary, testes, catalase, Bcl2.

Introduction

An overall or relative lack of insulin production or insulin resistance is the underlying cause of diabetes mellitus (DM), an endocrine condition characterized by persistently high blood sugar levels [(1)]. Reports state that the prevalence of DM in persons between the ages of 20 and 79 is expected to increase globally by 10.2% in 2021 to 12.2% in 2045, with Egypt expecting a rise from 20.9% in 2021 to 23.4% in 2045 [(2)]. Serious complications such as retinopathy, neuropathy, nephropathy, and male infertility are linked to DM [(3)]. The reproductive dysfunction induced by DM occurs in approximately 35% to 90% of patients with DM [(4)]. While the primary etiology of diabetic complications is unknown, oxidative stress (OS) has received a lot of attention. in the pathogenesis of different diabetic complications [(5)]. Reactive oxygen species (ROS) caused by hyperglycemia are a worthy cause of male subfertility and infertility [(6)]. B-cell lymphoma protein 2 (Bcl-2) is considered an anti-apoptotic protein that is responsible for regulating cell apoptosis either through inhibiting cytochrome c release from the mitochondria into the cytosol or through binding to the activating factors of apoptosis [(7)]. Excessive ROS production and the subsequent OS stimulate germ cell apoptosis and inhibit the spermatogenesis process [(8)]. This apoptosis was reported by

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a decrease in immunohistochemical expression of Bcl-2 in the testes of diabetic rats [9].

At this time, the existing treatments for DM, including insulin or various oral anti-diabetic medications, are associated with many side effects. In addition to side effects, these drugs are expensive [3]. They do not work to prevent the consequences of diabetes, such as malfunctioning of the testes and genital organs [4]. Current research is focused on finding natural-based treatments with little to no adverse effects to regulate hyperglycemia and prevent diabetic outcomes, particularly male reproductive system dysfunction [3].

The herb rosemary, or Rosmarinus officinalis, is commonly found in the Mediterranean area. This herb is recognized in conventional treatment as an antioxidant and antidiabetic medication. It also has antimicrobial, antidepressant, and anti-inflammatory activities [10]. Rosmarinic acid is a representative of the phenolic acids, and carnosic acid (CA) and carnosol (CS) are highlighted in the phenolic diterpenes; the polyphenols found in this plant are responsible for its bioactivities [11]. The rosemary oil's antioxidant and antibacterial qualities are attributed to its application in the food, medicine, and cosmetics sectors [12].

Rosemary has a hypoglycemic effect and can improve cardiomyopathy and neuropathy in diabetic rats [13,14]. It has a protective role against lithium-induced testicular toxicity [15]. Rosemary also showed improvement in the injured testicular tissue in rats with hyperthyroidism [16]. The purpose of this study was to see how rosemary oil, alone or in conjunction with insulin, affected the testes of adult male albino rats that have developed DM due to streptozotocin (STZ).

Methods

Chemicals: STZ was obtained from Sigma Chemical in St. Louis, MO, USA. Insulin: Mixtard 30 "soluble insulin 30% and isophane insulin 70%" was obtained from Novo Nordisk A/S, Denmark. Egyptian Drug Trading Company. Rosemary oil, density 0.908 g/mL at 25 °C, was obtained from Sigma Chemical in St. Louis, MO, USA.

Animals: Thirty adult male Sprague-Dawley albino rats weighing between 250 and 300 gm at 10 weeks of age were used. Animals were acquired from the animal house of the Faculty of Veterinary Medicine. All rats were kept in special wire mesh cages, housed in a well-ventilated room at average temperature, and provided with food and water ad libitum with a 12-hour dark/light cycle. All rats have been left one week for acclimatization to the new environment. The experiment was performed at the Animal and Experimental House.

Experimental design: Six equal groups of rats (five rats in every group) were selected at random as follows:

Group I: Positive control group (+ve Control): An intraperitoneal (I.P.) injection of ice-cold, pH 4.5, 0.1 M citrate buffer was administered to rats [17].

Group II (rosemary oil group): The rosemary oil (50 mg/kg, I.P.) was given daily [18].

Group III: diabetic group (DM): DM was induced by STZ (40 mg/kg, I.P.) dissolved in ice-cold 0.1M citrate buffer with pH 4.5 [17].

Group IV: diabetic group treated with insulin (DM + Insulin): DM was induced by STZ, then subcutaneous insulin (14 IU/kg/day) was given after confirmation of DM [19].

Group V: diabetic group treated with rosemary oil (DM + Rosemary oil): DM was induced by STZ, then the rosemary oil was given daily after confirmation of DM [18].

Group VI: diabetic group treated with rosemary oil and insulin (DM + Insulin + Rosemary oil): DM was induced by STZ, then the rosemary oil in combination with insulin was given after confirmation of DM.

Induction of DM: To induce DM, a single I.P. injection of freshly generated STZ (40 mg/kg) dissolved in an ice-cold 0.1M citrate buffer with a pH of 4.5 was used. Rats that had fasting blood glucose levels above 250 mg/dl were classified as diabetic rats and employed in the study [17]. The blood was punctured through the caudal vein and analyzed using the Accu-Chek commercial kit (Roche Diagnostics, Mannheim, Germany) weekly until the end of the experiment [20].

Animal scarification: Four weeks after the experiment's start [21], Before being sacrificed by cervical dislocation, rats were given xylazine (10 mg/kg) and ketamine (90 mg/kg) [22].The abdominal wall of each rat was opened, and the epididymis and the testes were extracted [23].

Sperm parameters assessment:

Sperm viability and morphology: In order to extract the epididymal semen, one side's cauda epididymis was removed, and a surgical blade was then used to cut it. One drop of semen was mixed with a drop of eosin and nigrosine. To assess the viability and morphology of sperms, the stained smears were examined using a light microscope at a magnification of ×400. For both, no fewer than 200 spermatozoa from different slide fields in each slide were assessed. Data were expressed as percentages [24].

Sperm count: Using a red blood pipette, semen was extracted out of the cauda epididymis until it reached the 0.5 mark. Normal saline was then added to dilute the semen to the 101 mark. One drop of diluted sperm fluid was used to count sperm using the Neubauer counting chamber under a light microscope at a magnification of ×400. Data were expressed as 10^6 cells/mL [24].

The following formula was used to compute the total sperm count:

Total sperm count /ml = (Dilution Factor x Count in 5 squares x 0.05 $\times 10^6$) [25].

Sperm motility: To extract semen, the cauda epididymis on the opposing side was exposed and sliced with a surgical blade. After that, a 2.9% sodium citrate dehydrate solution was used to dilute it ten times. For every animal, two independent drop preparations were created under a light microscope at a magnification of ×400. Sperm motility was measured and expressed as percentages [24]. For that, 200 spermatozoa were evaluated per animal [26].

Tissue preparation:

The right testis was collected from all groups and prepared for light microscopic evaluation. The remaining testis were also collected from all groups and prepared for assessment of the catalase (CAT) antioxidant marker.

Light microscopic assessment:

Haematoxylin and Eosin (H&E) stained sections assessment: The testes on the right side were immersed overnight at 4°C in Bouin's solution, embedded in paraffin, split into sections 5 µm in thickness, deparaffinized [27], then stained with H&E stain as described by Bancroft and Layton [28].

Right testis specimens measuring about 1 mm were preserved overnight in 2.5% phosphate-buffered glutaraldehyde with pH 7.3, then processed into 1 µm thick, according to Woods and Stirling, semithin sections stained with toluidine blue [29].

Bcl-2 immunostaining:

Testicular sections in paraffin blocks with a thickness of 4 µm were dewaxed, rehydrated, and blocked using 1% goat serum albumin. After incubating the sections with anti-Bcl2 antibodies (DAKO, clone 124, Denmark, 1:100), 3,30-diaminobenzidine tetrahydrochloride

(DAB) was added, and hematoxylin was used as a counterstain [9].

Morphometric study: Fiji Image J (1.51n; National Institute of Health; NIH, Bethesda, MD, USA) was used for morphometric assessment in H&E and Bcl2 stained sections in all groups [30].

- In the H&E-stained sections, two measurements of morphometry were evaluated: the mean diameter of the seminiferous tubule and the height of the germinal epithelium. For both, ×100 magnification photos were used of testicular cross-sections. Five slides representing five different rats were chosen from each group. The two morphometric measurements were then taken, with 15 values for each group, from the three roundest seminiferous tubules on each slide.

1-For the mean of tubular diameter, two diameters were taken in a cross position to calculate the seminiferous tubules' mean diameter.

2- For the mean of the germinal epithelium height, each tubule's four radii were measured, capturing every germinal epithelial cell up to the spermatids. Then, the mean value was calculated [31].

- In Bcl-2 immunostained sections, the Bcl-2 immunostaining mean area percentage was calculated. at ×400 magnification in ten nonoverlapping fields on each slide [32].**Assessment of antioxidant marker:** The remaining testis were collected from all groups for the assessment of CAT. The tissues were homogenized in a pH 7.4 solution containing 50 mM potassium phosphate. Following a 15-minute centrifugation at 4 ℃ and 4000 rpm, the samples were kept at 80 ℃ until analysis [23].CAT (Biodiagnostic, Egypt) was performed according to Aebi. [33] at 510 absorbances. The process depends on the CAT interacting with a certain volume of H_2O_2. A CAT inhibitor was used to stop the reaction after one minute.

Statistical analysis:

SPSS was used to collect and analyze data. (statistical program for social science version 25). Mean and standard deviation (SD) for descriptive data were done. To test differences between study groups, one-way ANOVA was used, followed by a Bonferroni post-hoc test. Probability values (P) less than 0.05 were statistically significant, (P) values less than 0.01 were highly statistically significant and (P) values less than 0.001 were considered to be very highly statistically significant.

Results:

Body weight:

Weight changes were non-statistically significant in both +ve Control and rosemary oil groups. A statistically significant body weight reduction was detected in the DM and DM +rosemary oil groups, while the DM + insulin and DM+ Insulin + Rosemary oil groups presented a statistically significant weight gain as presented in (**Figure 1),** which revealed weight changes in all groups at the beginning of the experiment, 2 weeks later, and just before scarification.

Blood glucose levels:

The groups DM and DM+ rosemary presented a statistically significant increase in the levels of blood glucose, while the DM+ insulin and DM+ insulin + rosemary oil groups presented a statistically significant reduction in it. DM+ insulin+ rosemary oil group presented glucose blood levels approximate to +ve Control and rosemary oil groups as presented in **(Figure 2)**.

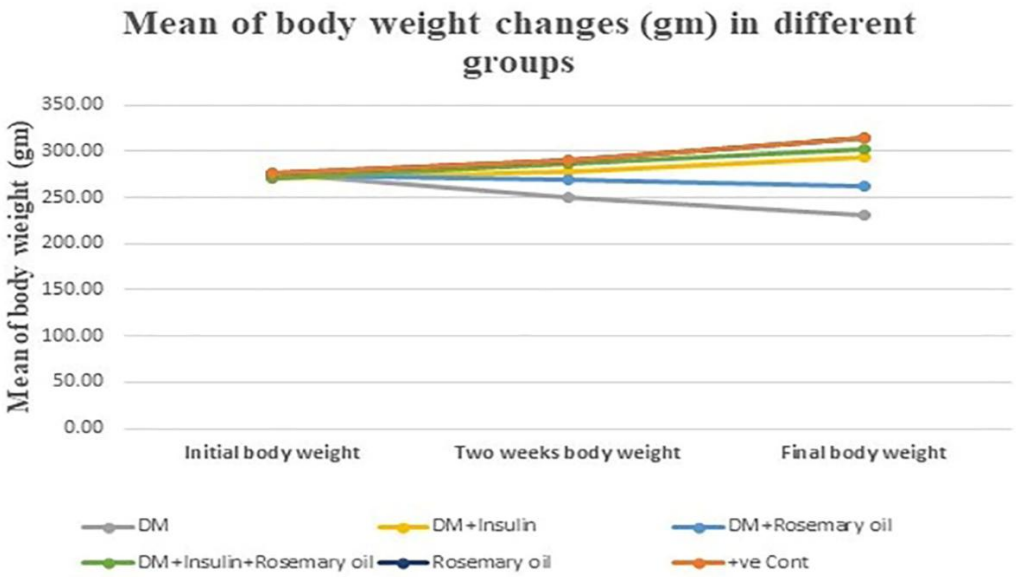

Fig. 1 Mean of body weight changes (gm) throughout the experimental period in diverse groups. Mean ± SD, One-way ANOVA followed by Bonferroni for the post-hoc test.

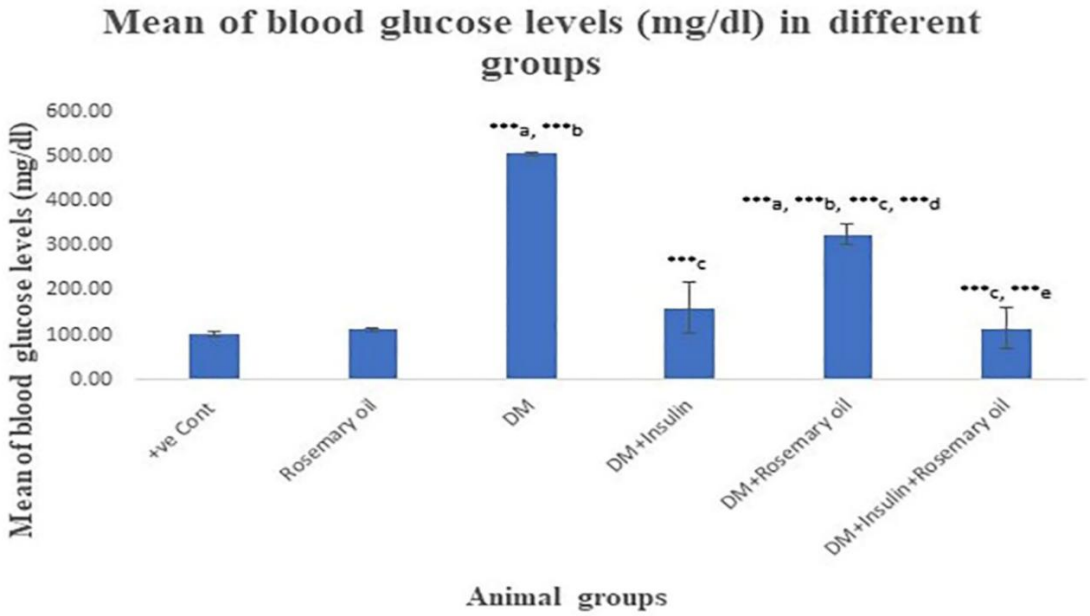

Fig. 2 Mean of blood glucose levels (mg/dl) in diverse groups. Mean ± SD, One-way ANOVA followed by Bonferroni for the post-hoc test. (***), remarkably high, statistically significant at $p < 0.001$. (a): compared to the +ve Control group. (b): compared to the Rosemary oil group. (c): compared to the DM group. (d): compared to DM + Insulin group. (e): compared to DM + Rosemary oil group

Sperm parameters:

A nonsignificant difference was detected in the sperm parameters, including count, viability, motility, and normal morphology, between the +ve Cont, rosemary oil, and DM + Insulin + Rosemary oil groups. While there was a remarkably high decrease of statistical significance in the sperm parameters in the DM, DM + Insulin, and DM + Rosemary oil groups compared to the +ve

Cont, rosemary oil, and DM + Insulin + Rosemary oil groups. However, there was a remarkably high increase statistical significance in the sperm parameters in the DM + Insulin, DM + Rosemary oil, and DM + Insulin + Rosemary oil groups when compared with the DM group, as shown in (**Table 1**). The viable sperm, non-viable sperm, normal sperm, and different forms of sperm abnormalities are presented in **(Figure 3, A-I)**.

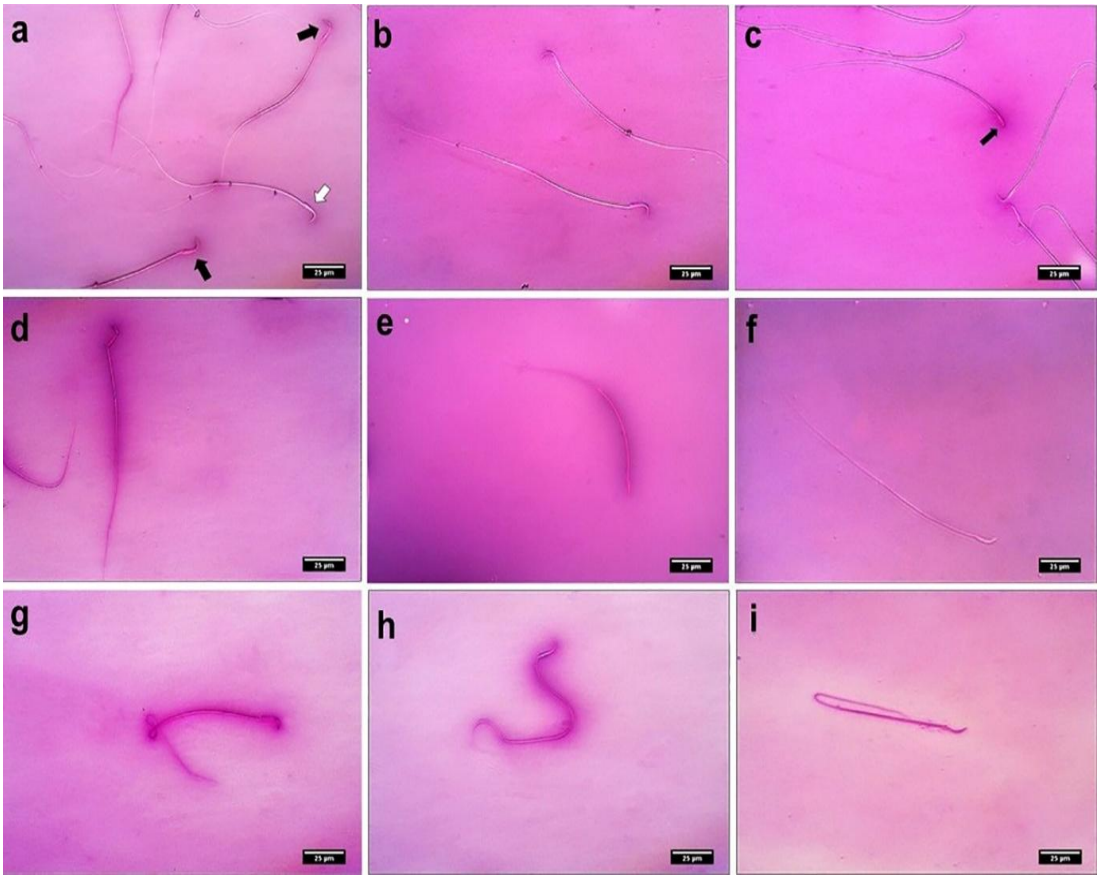

Fig. 3 A photomicrograph showing viable sperm (white arrow) and non-viable sperm (black arrow) **(A)**. morphologically normal sperms **(B)**, amorphous head abnormality (black arrow) **(C)**, bent head abnormality **(D)**, hookless head abnormality **(E)**, banana head abnormality **(F)**, coiled/folded tail abnormality **(G)**, bent head & coiled/ folded tail abnormalities **(H)**, and banana head & coiled/folded tail abnormalities **(I)**. (Eosin & Nigrosine; ×400)

Table.1: The mean of sperm parameters in different groups (N=30):

Animal Groups	Sperm count ($\times 10^6$ / ml)	Sperm motility (%)	Sperm viability (%)	Sperm morphology (%)			
				Normal	Abnormal head	Abnormal tail	Multiple abnormalities
+ve Control	72.6 ± 2.07	71.87 ± 1.32	76.7 ± 1.5	82.84 ± 1.48	2.53 ± 1.09	13.49 ± 0.41	1.14 ± 0.22
Rosemary oil	72.8 ± 3.49	72.85 ± 1.87	77.43 ± 1.73	83.4 ± 1.42	3.01 ± 0.46	12.56 ± 0.95	1.03 ± 0.22
DM	36.2 ± 1.92 ***a, ***b	36.71 ± 1.14 ***a, ***b	41.92 ± 1.31 ***a, ***b	47.46 ± 1.57 ***a, ***b	12.94 ± 1.16 ***a, ***b	29.94 ± 1.09 ***a, ***b	9.67 ± 1.12 ***a, ***b
DM + Insulin	64 ± 1.58 ***a, ***b, ***c	58.21 ± 1.38 ***a, ***b, ***c	67.55 ± 1.29 ***a, ***b, ***c	75.35 ± 1.19 ***a, ***b, ***c	6.53 ± 0.73 ***a, ***b, ***c	16.94 ± 1.17 ***a, ***b, ***c	1.19 ± 0.38 ***c
DM + Rosemary oil	56 ± 1.58 ***a, ***b, ***c, ***d	52.19 ± 0.97 ***a, ***b, ***c, ***d	62.07 ± 1.39 ***a, ***b, ***c, ***d	70.98 ± 0.96 ***a, ***b, ***c, ***d	1.9 ± 0.52 ***c, ***d	22.77 ± 0.75 ***a, ***b, ***c, ***d	4.35 ± 0.98 ***a, ***b, ***c, ***d
DM + Insulin + Rosemary oil	71.4 ± 2.07 ***c, ***d, ***e	70.98 ± 1.65 ***c, ***d, ***e	76.06 ± 0.99 ***c, ***d, ***e	82.19 ± 1.29 ***c, ***d, ***e	2.86 ± 0.38 ***c, ***d	13.82 ± 1.18 ***c, ***d, ***e	1.14 ± 0.53 ***c, ***e

Mean ± SD, One-way ANOVA, Bonferroni test was used for post-hoc test.
(***), very high, statistically significant at $p < 0.001$.
(a): compared to +ve Control group. (b): compared to Rosemary oil group.
(c): compared to DM group. (d): compared to DM + Insulin group.
(e): compared to DM + Rosemary oil group.

Histopathological results:

Hematoxylin and Eosin (H&E) stain:

The +ve Control and rosemary groups' testes revealed densely packed Multiple layers of germinal epithelium line the seminiferous tubules. Spermatozoa were abundant in their lumens. The different types of spermatogenic cells (spermatogonia, primary spermatocytes, early spermatids, and late spermatids) had a normal shape and size. Sertoli cells were discovered between germinal epithelial cells. The interstitial tissue, in between tubules, was formed of clusters of normally shaped Leydig cells with normal blood vessels **(Figure 4, A-B).** The testes in DM group showed irregular-shaped seminiferous tubules, widely separated by haemorrhage and oedema with a marked reduction in their size. They presented an atrophied germinal epithelium with vacuolization. Most tubules were devoid of spermatozoa. There was vacuolization in the interstitial tissue with disorganized Leydig cells and dilated, congested, and thick-walled blood vessels **(Figure 4, C).** The DM+ Insulin group showed an increase in size with more regularity in the shape of most seminiferous tubules. There were diverse types of germinal cells lining the tubules, with variability in spermatozoa numbers. Mild oedema, haemorrhage, and vacuolization were still observed within the germinal epithelium and interstitial tissue. Dilated, congested, thick-walled blood vessels can be detected within interstitial tissue **(Figure 4, D)**. The DM+ rosemary group presented some normal-shaped seminiferous tubules with normal germinal epithelium. Others showed complete atrophy or vacuolization. Some tubules presented improvement in the number of spermatozoa, and some did not. Vacuolization, dilated, congested, thick-walled blood vessels, moderate amounts of haemorrhage, and oedema were still observed within the interstitial tissue **(Figure 4, E)**. The DM+ insulin+ rosemary group showed tightly packed and regular seminiferous tubules with normal diameter, germinal epithelium, and spermatozoa filling their lumens. In between the tubules,

there were clusters of Leydig cells. The blood vessels in the interstitial spaces appeared to be normal. Vacuolization and oedema occasionally appeared within the interstitial tissue **(Figure 4, F).**

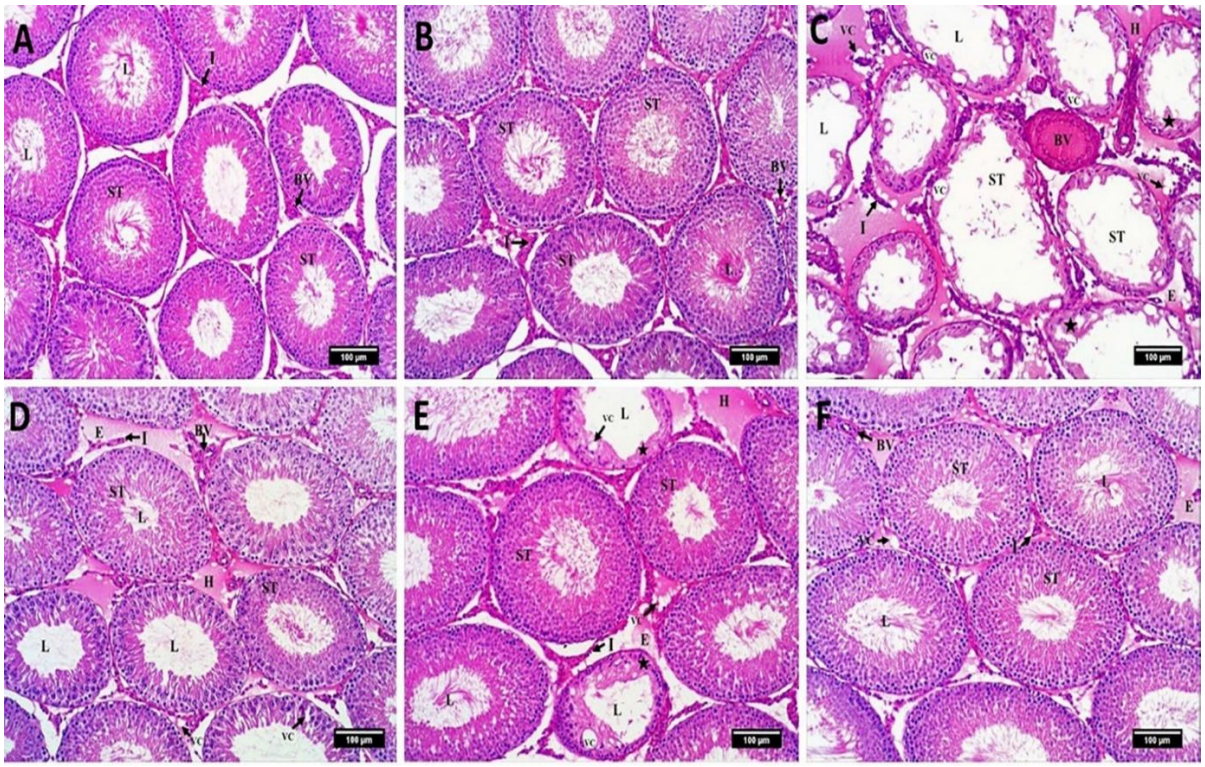

Fig. 4 A photomicrograph of sections in testes of **(A)** +ve Control group **(B)** testis of the rosemary oil group **(A-B)** showing: tightly packed seminiferous tubules (ST) lined by multiple layers of germinal epithelium, lumens (L) full of spermatozoa, and clusters of interstitial tissue (I) with normal blood vessels (BV). **(C)** DM group showing: irregular seminiferous tubules (ST) with atrophic geminal epithelium (⋆), lumens (L) devoid of spermatozoa. hemorrhage (H) and edema (E) widely separating tubules, disorganized interstitial tissue (I) with dilated, congested, thick-walled blood vessels (BV), and vacuolization (VC) within the germinal epithelium and the interstitial tissue. **(D)** DM + Insulin group showing: regularity in most of seminiferous tubules (ST) with normal geminal epithelium., lumens (L) with varying numbers of spermatozoa, vacuolization (VC), and mild hemorrhage (H), edema (E), and dilated, thick-walled blood vessels (BV) in the interstitial tissue (I). **(E)** DM + Rosemary oil group showing: atrophic germinal epithelium (⋆) in some seminiferous tubules (ST), some devoid lumens (L) of spermatozoa. , and disorganized interstitial tissue (I) with moderate hemorrhage (H), edema (E), and vacuolization (VC) **(F)** DM + Insulin + Rosemary oil group showing: restoration of nearly normal testicular tissue, tightly packed seminiferous tubules (ST) lined by multiple layers of germinal epithelium, lumens (L) full of spermatozoa, and clusters of interstitial tissue (I) with normal blood vessels (BV) with a slight edema (E), and vacuolization (VC). (H&E; ×100).

Toluidine blue stain:

Sertoli cells and other spermatogenic cells lined the seminiferous tubules of the +ve Control, and rosemary groups. Sertoli cells were tall, columnar cells with irregular outlines, a broad basal part directed to the basement membrane of the tubules, and a narrow apex. They had ovoid or triangular, prominent nucleoli in euchromatic nuclei and clear cytoplasm. Spermatogonia seemed like small cells having large oval nuclei, eccentric nucleoli, and abundant heterochromatin, which gave them a dark appearance. Primary spermatocytes are the largest of all spermatogenic cells. Their nuclei were large and round, with coarsely

granulated chromosomes. Early spermatids, arranged in several layers near the lumen, were small with round central vesicular nuclei characterized by fine chromatin networks. Late spermatids are elongated cells attached to the apical surfaces of Sertoli cells and scattered between the layers of early spermatids. The clusters of Leydig cells in the interstitial tissue, were cuboidal or polygonal in shape and had prominent nucleoli in large round vesicular nuclei. The basement membrane appeared regular and intact, with flat myoid cells **(Figure 5, A-B)**. The seminiferous tubules in the DM group were lined by a completely atrophic germinal epithelium.

Apoptosis was depicted as a shrinkage of nuclei and condensation of nuclear material into a darkly stained mass (pyknosis). Primary spermatocytes showed nuclei with severely disorganized chromatin. The lumens of tubules were devoid of spermatozoa. Abnormal myoid cells were seen with a thick and irregular basement membrane. Interstitial cells were with abnormal shape, nuclear chromatin margination and vacuolization. In the interstitial tissue, dilated, congested, thick-walled blood vessels appeared. **(Figure 5, C)**. The DM+ Insulin group improved testicular structure more than the DM group, but less than the +ve Cont group. **(Figure 5, D)**. The DM+ rosemary group improved their testicular structure more than the DM group, but less than that of the DM + Insulin group. **(Figure 5-E)**. DM+ insulin+ rosemary group showed more restoration of nearly normal testicular tissue than that of the DM group, DM + Insulin, and DM + Rosemary oil groups **(Figure 5-F)**.

Morphometric assessment:

The height of germinal epithelium and tubular diameter:

When comparing the DM group to the +ve Cont and rosemary oil groups, tubular diameter and the height of the germinal epithelium declined statistically significantly. In comparison to the DM group, the values in the DM + Insulin, DM + Rosemary oil, and DM + Insulin + Rosemary oil groups were significantly higher. The values in DM + Insulin + Rosemary oil group were with non-significant differences compared to the + ve Control group (**Table 2**).

Table.2: The mean of tubular diameter, germinal epithelial height (µm) in different groups (N=30):

Animal groups	Morphometric assessment	
	Tubular diameter (µm)	Germinal epithelial height (µm)
+ve Control	296.69 ± 17.79	115.51 ± 13.4
Rosemary oil	294.31 ± 12.86	110.1 ± 6.21
DM	172.22 ± 10.2 a***, b***	47.87 ± 11.03 a***, b***
DM + Insulin	258.12 ± 24.77 ***a, ***b, ***c	90.94 ± 11.65 ***a, ***b, ***c
DM + Rosemary oil	206.38 ± 21.18 ***a, ***b, ***c, ***d	67.96 ± 6.35 ***a, ***b, ***c, ***d
DM + Insulin + Rosemary oil	297.55 ± 17.92 ***c, ***d, ***e	114.72 ± 12.93 ***c, ***d, ***e

Mean ± SD, One-way ANOVA, Bonferroni test was used for post-hoc test.

(***), very high, statistically significant at $p < 0.001$.

(a): compared to +ve Control group. (b): compared to Rosemary oil group

(d): compared to DM + Insulin group. (c): compared to DM group.

(e): compared to DM + Rosemary oil group.

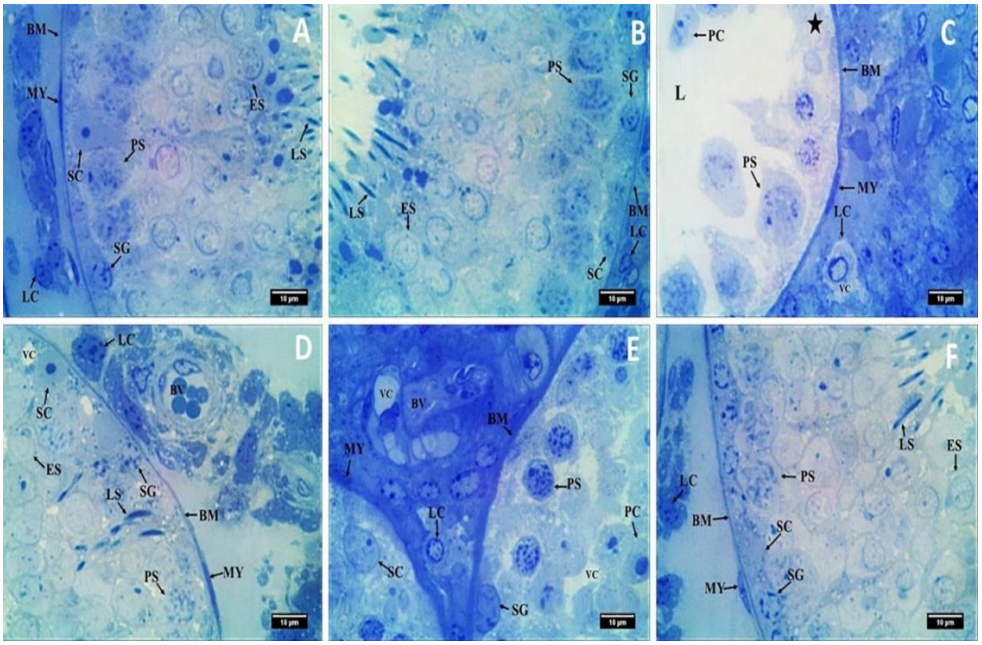

Fig. 5 A photomicrograph of a section in in testes of **(A)** +ve Control group **(B)** testis of the rosemary oil group **(A-B)** showing: seminiferous tubules lined by all types of germinal epithelial cells, spermatogonia (SG), primary spermatocytes (PS), early spermatids (ES), elongated late spermatids (LS), Sertoli cells (SC) resting on normal basement membrane (BM) with flat myoid cells (MY) and cuboidal Leydig cells (LC). **(C)** DM group showing: seminiferous tubule with atrophic germinal epithelium (⋆). disarrangement of their chromatin in primary spermatocytes (PS), pyknotic nuclei (PC) in spermatogenic cells, empty lumen (L) and thick basement membrane (BM) with abnormal myoid cells (MY), disorganized Leydig cells (LC) with nuclear chromatin margination, and vacuolization (VC). **(D)** DM + Insulin group showing: some restoration of germinal epithelial cells with a slightly thick basement membrane (BM) with myoid cells (MY) and disorganized Leydig cells (LC) with a dilated, thick-walled blood vessel (BV) and vacuolization (VC) **(E)** DM + Rosemary oil group showing: a distorted germinal epithelium and cells with pyknotic nuclei (PC), a slightly irregular and thick basement membrane (BM) with myoid cells (MY) and disorganized Leydig cells (LC) with nuclear chromatin margination, vacuolization (VC), and dilated, congested, thick-walled blood vessel (BV). **(F)** DM + Insulin + Rosemary oil group showing: restoration of nearly all germinal epithelial cells resting on normal basement membrane (BM) with flat myoid cells (MY) and normal Leydig cells (LC) (Toluidine blue; ×1000).

Area percentage of Bcl-2 immunostaining: When compared with the +ve Control and rosemary oil groups, a remarkably high decrease of statistical significance in the Bcl-2 immunostaining area percentage was detected in the DM group. When compared to the DM group, the increase in the percentage was statistically significant in the DM + Insulin, DM + Rosemary oil, and DM + Insulin + Rosemary oil groups. The percentage in DM + Insulin + Rosemary oil group is restored to be with a non-significant difference compared to the +ve Control group **(Figures 6-7).**

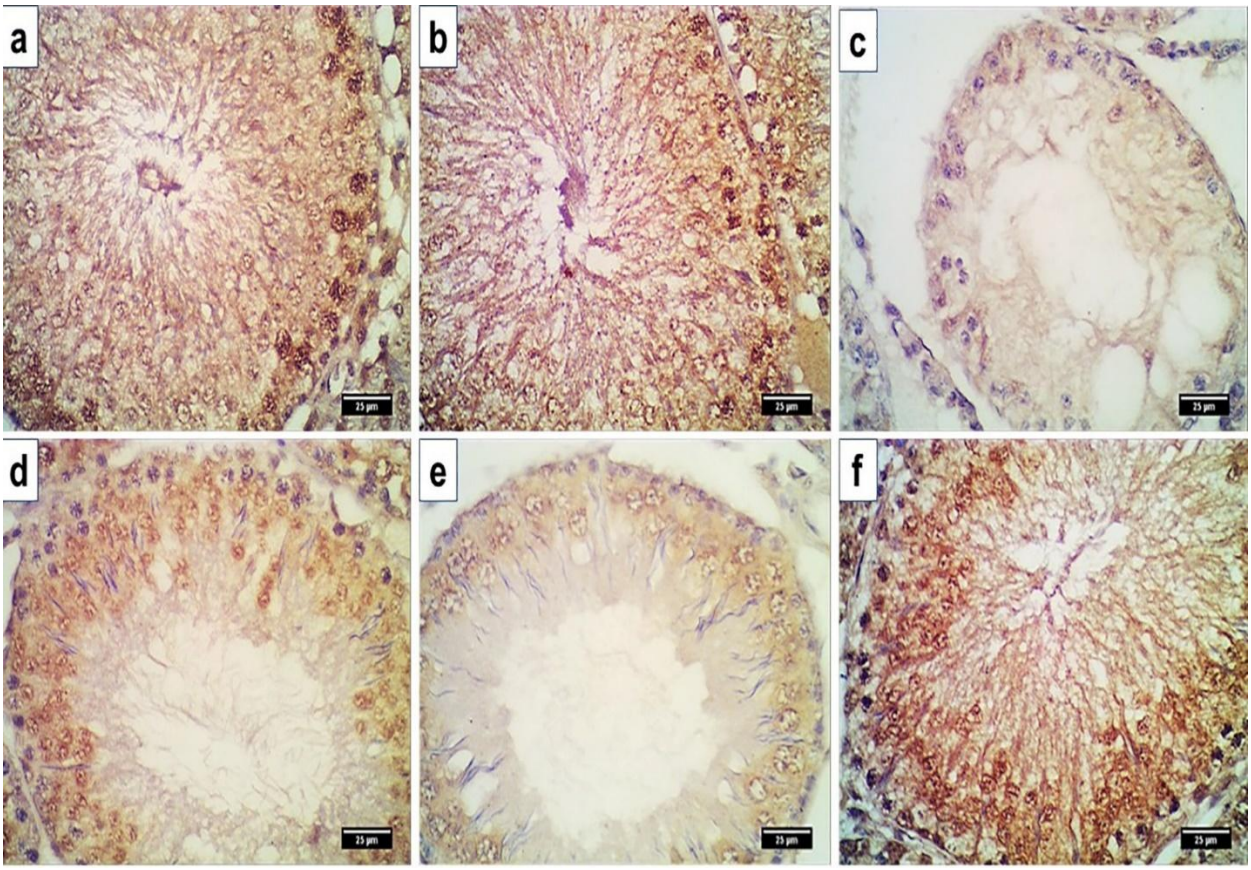

Fig. 6 A photomicrograph of a section in the testis of the +ve Cont group **(A)**, rosemary group **(B)**, DM group **(C)**, DM+ Insulin **(D)**, DM+ rosemary **(E),** and DM+ insulin+ rosemary **(F)**. (Bcl-2 immunostaining; ×400)

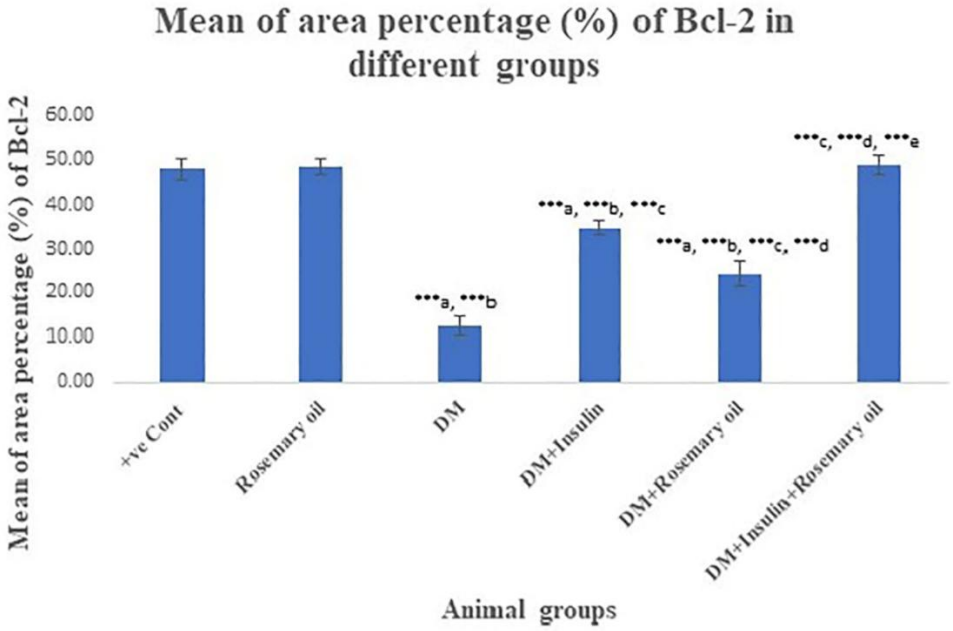

Fig. 7 Mean of area percentage (%) of Bcl-2 immunostaining in distinct groups. Mean ± SD, One-way ANOVA followed by Bonferroni for the post-hoc test (***), remarkably high, statistically significant at $p < 0.001$. (a): compared to +ve Cont. (b): compared to the Rosemary oil group. (c): compared to the DM group. (d): compared to DM + Insulin group. (e): compared to DM + Rosemary oil group.

Antioxidant marker (CAT) in testicular tissue:

There was a nonsignificant difference in the testicular tissue CAT levels between the +ve Control, rosemary oil, and DM + Insulin + Rosemary oil groups. When compared to the +ve Control and rosemary oil groups, a remarkably high, statistically significant drop was noticed in CAT levels in the DM, DM + Insulin, and DM + Rosemary oil groups. However, there was a tremendous, statistically significant rise of CAT values in the DM + Insulin, DM + Rosemary oil, and DM + Insulin + Rosemary oil groups when compared to the DM group. When compared with the DM + Rosemary oil group, the DM + Insulin group had a statistically significant increase in CAT levels. When compared with the DM + Insulin group, the DM + Insulin + Rosemary oil group showed an increase with a statistical significance in CAT values **(Figure 8)**.

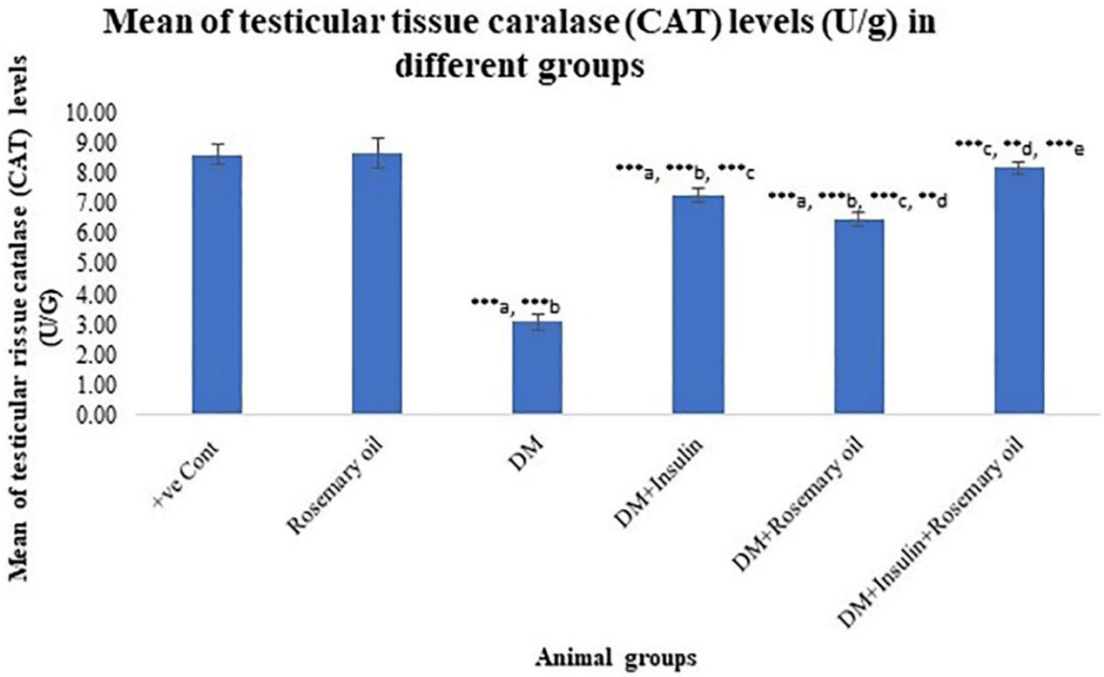

Fig. 8 Mean of testicular tissue CAT levels (U/g) in different groups. Mean ± SD, One-way ANOVA followed by Bonferroni for the post-hoc test. (**) High statistically significant at $p < 0.01$, (***), very high, statistically significant at $p < 0.001$. (a): compared to the +ve Control group. (b): compared to the Rosemary oil group. (c): compared to the DM group. (d): compared to DM + Insulin group. (e): compared to DM + Rosemary oil group.

Discussion:

Persistent hyperglycemia, a hallmark of DM, can lead to progressive tissue damage and organ failure if left untreated [34]. Testicular structural and functional decline are considered serious complications in diabetic male patients, resulting in impairment of fertility in adulthood [35]. Reproductive dysfunction is a result of the relationship between hyperglycemia and increased OS in the testes [36].

While insulin therapy may result in life-threatening hypoglycemia, antidiabetic drugs are associated with side effects like gastrointestinal intolerance, stomach

discomfort, excess weight, water retention, or potential cardiac arrest [37]. Also, they do not work to prevent the consequences of diabetes, such as malfunctioning of the testes and genital organs [4].

In the current study, the DM group lost significantly more weight than the +ve Control group. This decline in the body weight could be the consequence of consuming too much protein, indicating a marked decrease in the carbohydrates accessible to the cells [38]. When compared to the DM group, the DM + Insulin, DM + Rosemary oil, and DM + Insulin + Rosemary oil groups all showed a statistically substantial rise in body weight. That can be explained by the fact that insulin is an anabolic hormone that favors protein synthesis from amino acids throughout the body and energy storage from sources of carbohydrates and fat [39]. The positive impact of rosemary on the body weight can be due to rosemary containing CA, which regulates obese rats' body weight and lipid profile [40]. However, the DM + Rosemary oil groups did not reach the +ve Control level. When compared to the + ve control group, the DM + Insulin + Rosemary oil group demonstrated no significant difference. In controversy, Selmi et al. [41] demonstrated that after receiving rosemary oil treatment, the diabetic rats ended the experiment with their body weight back to normal.

In the current study, the blood glucose level was significantly higher in the DM group than in the +ve Control group. This occurs as a result of STZ's detrimental effects on the pancreatic beta cells [42]. The DM + Insulin, DM + Rosemary oil and DM + Insulin + Rosemary oil groups displayed significantly decreased levels of blood glucose when compared to the DM group. This drop in blood glucose levels with rosemary is attributed to its content of CA, which inhibits tumor necrosis factor-alpha (TNF-α)-mediated insulin resistance by lowering phosphorylation of insulin receptor substrate 1 (IRS1) in adipocytes [43]. A significant drop in the levels of blood glucose was detected in DM+ Insulin + rosemary oil group compared to the DM and DM + Rosemary oil groups. Conversely, Selmi et al. [41] claimed that in diabetic rats, rosemary oil brought blood glucose levels back to normal. However, they used alloxan in the induction of DM and a different dose of rosemary oil, which was (30 mg/kg, I.P.) for 15 successive days. While a nonsignificant difference was detected between the +ve Control, rosemary oil, DM + Insulin and DM + Insulin + Rosemary oil groups.

A statistically significant decline was detected in the viability, count, motility, and normal morphology in the DM group when compared with the +ve Control group. This This DM's negative impact on sperm parameters can be explained by the fact that DM-related OS causes sperm nuclear damage as well as mitochondrial deoxyribonucleic acid (DNA) damage. This is proved by elevated advanced glycation end products (AGEs) as a result of oxidative damage within the reproductive tract, seminal plasma, and sperm of diabetic males [44]. Mitochondria are considered the major sources of ROS [45]. The motility of sperm may be reduced as a result of increased abnormalities with the structural and functional degradation in the tail of sperm. Increased sperm DNA damage and changes in transmembrane mitochondrial potential may both be associated with this effect [46].

The DM + Insulin, DM + Rosemary oil and DM + Insulin + Rosemary oil groups presented a significant rise in the parameters of sperm in comparison with the DM group. Afifi et al. [47] explained insulin's beneficial impact on sperm parameters by its action on pituitary biosynthesis and secretion of follicle-stimulating hormone (FSH) secretion, which play a crucial role in

spermatogenesis. That can be explained by the antidiabetic and antioxidant effects of rosemary [48]. The DM + Insulin and DM + Rosemary oil groups were still lower than those in the +ve Control. In controversy, Sebai et al. [18] demonstrated that diabetic rats restored normal sperm parameters after treatment with rosemary oil. However, Sebai et al. [18] used a different method of DM induction, which was alloxan, and a different weight of rat, which was 220-230 gm. On the other hand, the sperm parameters of the DM + Insulin + Rosemary oil group was with a nonsignificant difference when compared to the + ve Control. This is following Akondi et al., [49] who demonstrated that combination therapy (antioxidant + insulin) was more effective on sperm parameters in rats with DM than using a single treatment (antioxidant or insulin alone). That can be explained by the fact that rosemary increases insulin sensitivity and has an antioxidant action, resulting in an exacerbation of the insulin action [43].

In this study, the histopathological examination of testicular tissue in the DM group presented severely destroyed seminiferous tubules with empty lumens. There was oedema and haemorrhage widely separating the tubules. Vacuolization was observed in the germinal epithelium and interstitial tissue. Dilated, congested, thick-walled blood vessels appeared. There were germinal cells with either pyknotic nuclei or disorganization of chromatin. Also, both the germinal and interstitial tissue showed abnormalities in their shape. The basement membrane was thick, which may be explained by an increase in collagen Controlent. This thickening reduces the testicular cells' blood supply [50]. In addition to that, thick-wall blood vessels in diabetics result in hypoxia-induced cellular injury. Sertoli cells are extremely sensitive to either hyper- or hypo-insulinemia, which occur in DM [51]. The negatively impacted Sertoli cells cause disruption in the metabolism of germinal cells, cytoplasmic vacuolization, and eventual apoptosis [52]. Oedema can be caused by Sertoli cells' phagocytic dysfunction, resulting in hyalinization of the destroyed germ cells [53]. Also, chemical mediators released after tissue degradation increase how permeable the blood vessel wall is, subsequently causing fluid exudates and plasma penetration into the surrounding tissue [54].

Hyperglycemia-induced overproduction of ROS causes intra-testicular OS, inflammation, and germ cell apoptosis, which are linked to DM-induced fertility decrease or decreased reproductive potential in males with DM [55]. Testicular inflammation stimulated by proinflammatory cytokines causes testicular damage, reduction of germinal cells, testicular atrophy, and apoptosis [56]. ROS and OS in the DM can harm Leydig cells and change the hypothalamic-pituitary-gonadal (HPG) axis, which reduces luteinizing hormone (LH) and FSH. These hormones stimulate the Leydig cells, which generate testosterone, and play a vital role in spermatogenesis [57].

The DM + Insulin and DM + Rosemary oil groups showed improvement in the testicular tissue. However, they were not like the normal structure in the +ve Control group. The improvement in the testicular tissue after insulin treatment can be explained by the fact that it regulates the HPG axis by causing the hypothalamus to produce and secrete a gonadotropin-releasing hormone (GnRH) [58]. Tousson et al. [59] explained the positive effect of rosemary by the fact that, it can provide reactive radicals with electrons, converting them into forms that are more stable and inhibiting reactions with incoming biomolecules in biological systems that are susceptible, such as, amino acids, polyunsaturated fatty acids, proteins, DNA,

and carbohydrates. Therefore, it is considered a cytoprotective agent, preventing cell damage from free radicals. The DM + Insulin + Rosemary oil and rosemary oil groups revealed a nearly normal testicular structure. This is because rosemary increases insulin sensitivity and has antioxidant properties, resulting in an exacerbation of insulin action [43,48]. On the other hand, Sayed et al. [60] noticed that both the combined group and the insulin group restored the normal hepatic and renal tissues after induction of DM with no difference between them. However, Sayed et al. [60] used a different dose of STZ, which was 60 mg/kg, and rats of a different age, which was 7 weeks old.

In the existing study, the statistically significant reduction in the tubules' diameter and the height of germinal epithelium in DM group demonstrated the low cellular activity of spermatogenic cells with the failure of differentiation of spermatogonia into primary spermatocytes. Also, atrophied seminiferous tubules with the course of DM suggest that changes in cellular differentiation or otherwise activity resulted in a decrease in spermatozoa production [50].

The DM + Insulin, DM + Rosemary oil and DM + Insulin + Rosemary oil groups presented an improvement with statistical significance in both the germinal epithelium height and tubular diameter compared to the DM group. Studies reported that insulin is important for testicular descent, the proliferation and differentiation of Sertoli cells, spermatogenesis, prostatic growth, and sexual behaviors [58]. Rosemary is considered a cytoprotective and antioxidant agent, as it scavenges free radicals that result in widespread damage to various cell components [61]. On the other hand, the DM + Insulin and DM + Rosemary oil groups' both measurements were still lower than the +ve Control group's. The DM + Insulin + Rosemary oil group displayed a non-significant difference compared to the +ve Control and rosemary oil groups. This agrees with Nna et al., [62] who reported that the combined therapy of an antioxidant and an antidiabetic drug showed an increase with a statistical significance in both measurements in diabetic rats in comparison to either the untreated diabetic or diabetic group treated with antidiabetic alone.

The DM group presented a statistically significant decline in the percentage of area immunostained with Bcl-2 compared to the +ve Control and rosemary oil groups. A previous study observed that, by upsetting the equilibrium between antiapoptotic Bcl-2 proteins and proapoptotic (Bcl-2-associated X protein) Bax, hyperglycemia induced apoptosis. Due to this imbalance, the mitochondrial matrix discharges cytochrome c into the cytosol, raising the amount of caspase 3 that has been cleaved, which facilitates the destruction of DNA by DNases [63]. Apoptosis is linked to Bcl-2 down-expression [64].

The DM + Insulin, DM + Rosemary oil and DM + Insulin + Rosemary oil groups displayed a rise with a statistical significance in the percentage of area immunostained with Bcl-2 compared to the DM group. Insulin has anti-apoptotic action in the testis via down-regulating Bax, up-regulating Bcl-2 proteins, and caspase-3, which can preserve spermatogenesis [65]. Alavi et al. [66] demonstrated that rosemary and its constituents have protective mechanism including the control of mitogen-activated protein kinase (MAPK) signalling pathways and apoptosis. On the other hand, the area percentages of Bcl-2 immunostaining in the DM + Insulin and DM + Rosemary oil groups were still lower than that in the +ve Control group. In comparison to the +ve Control and rosemary oil groups, there was no difference with a significance in the DM +

Insulin + Rosemary oil group. This agrees with Nna et al., [62] who reported that the combined treatment was better than individual antidiabetic drug use. In controversy, Nazmy et al. [67] stated the absence of a significant difference in testicular Bcl-2 between the diabetic group treated using combined therapy and the antidiabetic drug-treated group. However, Nazmy et al. [67] used a different dose of insulin.

In the current study, The DM group presented a decrease with a statistical significance in the activity of CAT in the testicular tissue in comparison to the +ve Control and rosemary oil groups. It is attributed to the OS status induced by DM [36]. ROS disrupts the oxidant/antioxidant system, impairing the antioxidant defense enzymes' capacity to scavenge free radicals [68]. The DM + Insulin, DM + Rosemary oil and DM + Insulin + Rosemary oil groups presented an increase with a statistical significance in the activity of CAT in comparison to the DM group. Aeeni et al. [69] observed that insulin has testicular antioxidant potential, which improves the sperm and germ cells by defending the DNA content. It was observed that rosemary's antioxidant characteristics are due to its high content of isoprenoid quinones as well as CA and CS, which function as free radical chain terminators and chelating agents of ROS [70]. However, the CAT activity in the tissue of testes of the DM + Insulin as well as the DM + Rosemary oil group did not reach the CAT level in the +ve Control group. In controversy, Selmi et al. [41] observed restoring CAT levels to normal levels after treatment with rosemary oil in rats with alloxan-induced DM. However, Selmi et al. [41] measured CAT levels in different organs, which were the liver and kidney. While the DM + Insulin + Rosemary oil group was with a nonsignificant difference when compared with the +ve Control group, which can be clarified by the fact that rosemary promotes the secretion and action of insulin and has antioxidant action [48].

Conclusion:

Treatment with insulin or rosemary oil in STZ-induced DM showed some improvement in the testicular architecture and sperm parameters. However, insulin alone had better results than rosemary oil alone. However, the best results were obtained when both insulin and rosemary oil were combined. According to that, the combination treatment may improve the fertility of diabetic males.

Highlights

- Reactive oxygen species in diabetes mellitus (DM) are considered an important cause of male subfertility and infertility
- Rosemary has a hypoglycemic effect and protective role against testicular toxicity.
- Assessment of body weight, blood glucose levels, sperm parameters, H&E, toluidine blue and immunostaining sections and catalase levels was performed in rosemary, insulin and combined treated groups
- Combined Rosemary oil and insulin ameliorated testicular complications resulted from STZ induced DM.

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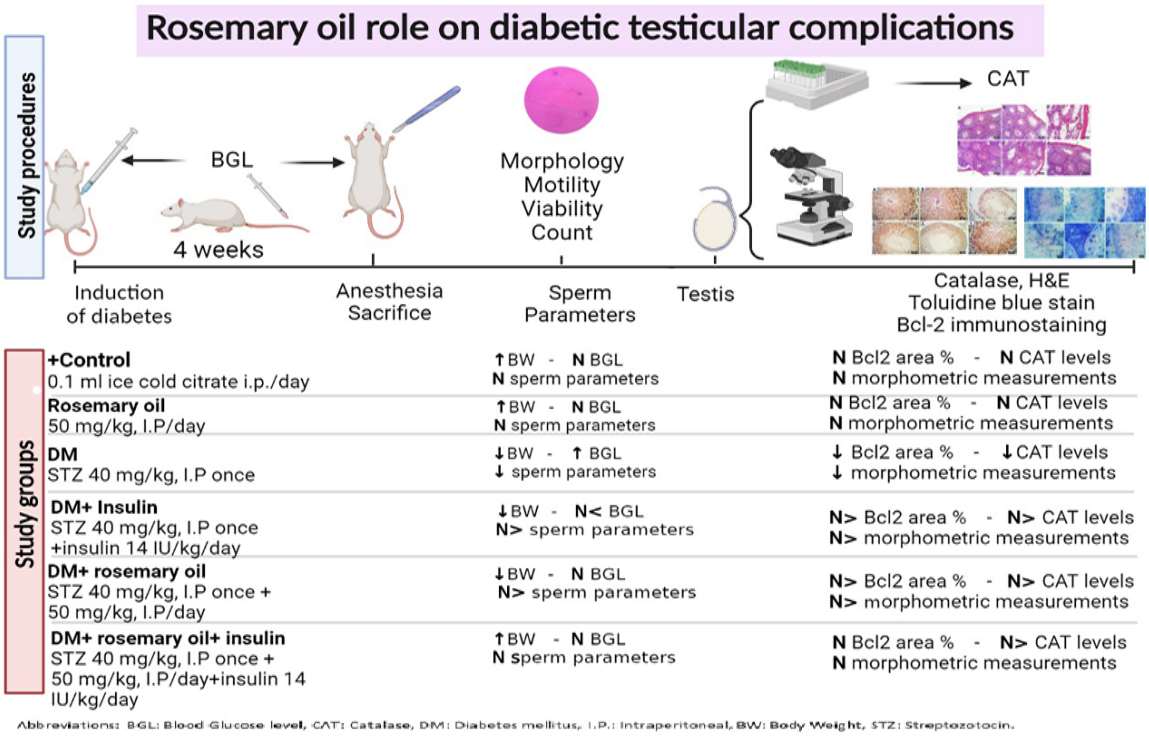
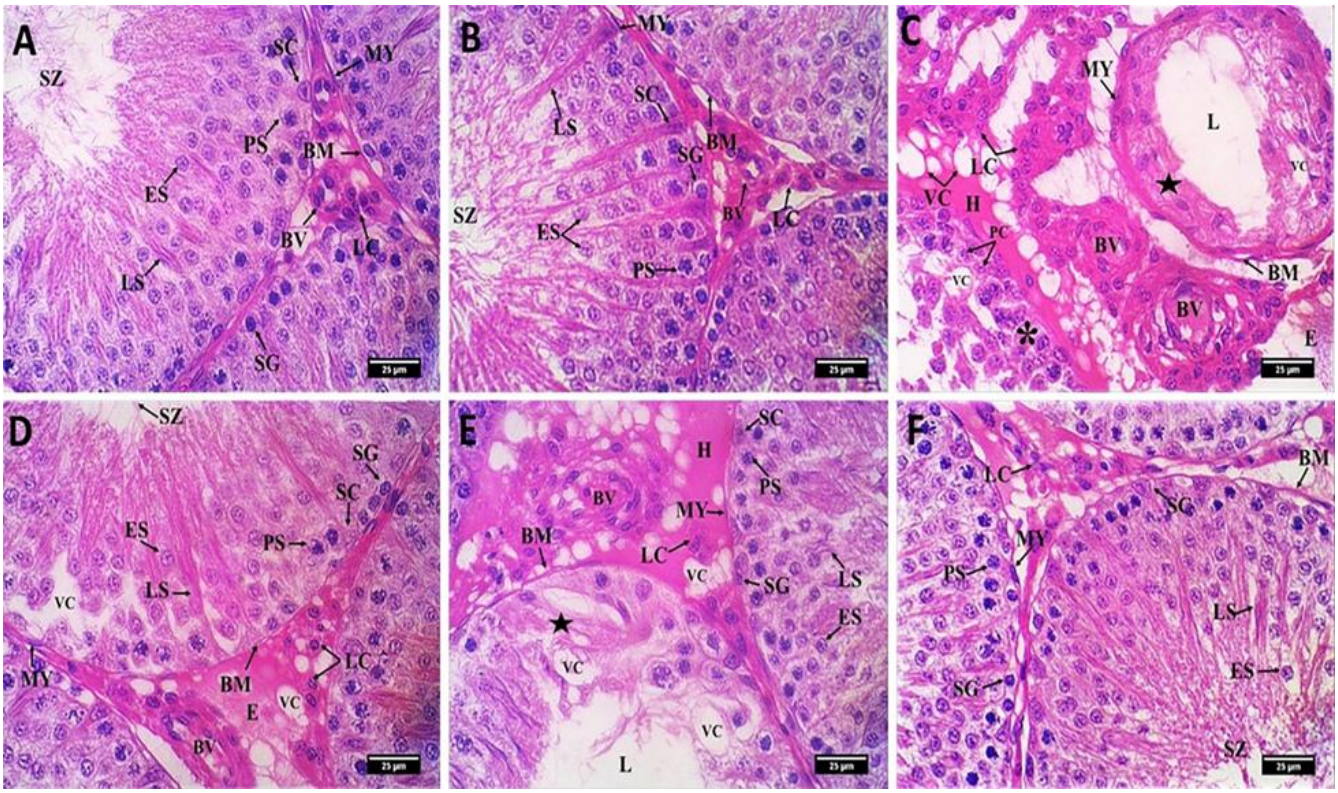
Appendix A



Summary of the methodology and results of the research work



A photomicrograph of a section in the testis of the +ve Cont group **(A),** rosemary oil group **(B),** DM group **(C)** DM + Insulin **(D)**, DM + Rosemary oil **(E)**, and DM + Insulin + Rosemary oil **(F)** showing: seminiferous tubules are lined by different types of germinal epithelial cells; spermatogonia (SG), primary spermatocytes (PS), early spermatids (ES), elongated late spermatids (LS). Sertoli cells (SC), basement membrane (BM), myoid cells (MY), and Leydig cells (LC). DM group **(C)** shows atrophic germinal epithelium (⋆), distorted germinal epithelium (*****), vacuolization (VC), hemorrhage (H), cells with pyknotic nuclei (PC) and (L) lumen is devoid of spermatozoa (H&E; ×400).