

ANTOX REPRO-PROTECTIVE SYNERGISTIC ROLE WITH INSULIN IN A STREPTOZOTOCIN-INDUCED DIABETIC RAT MODEL (BIOCHEMICAL, HISTO-MORPHOMETRIC AND IMMUNOHISTOCHEMICAL STUDY)

BY

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ABSTRACT

Background: Diabetes-related reproductive dysfunction is considered a major health concern. Its pathogenesis is closely linked to oxidative stress. Antox is a combination of antioxidants (selenium and vitamins A, C and E); its protective role was documented previously in animal models. **Aim of the work:** was to assess the repro-protective synergistic role of Antox with insulin use on testicular and epididymal alterations in a streptozotocin-induced diabetic rat model. **Material and Methods:** Thirty-six rats were allocated into six equal groups: (I) not given any medication; (II) (positive control) received intraperitoneal injection of citrate buffer; (III) (Diabetic) received intraperitoneal injection of streptozotocin (50 mg/kg). The three groups were left without treatment for four weeks. (IV) (Diabetic/Insulin), (V) (Diabetic/Antox), and VI (Diabetic/Insulin/Antox) received streptozotocin, followed by four weeks of daily subcutaneous insulin injection (1U/100 g), oral Antox (10 mg/kg/day), or both insulin and Antox, respectively. By study end, blood, semen, testis and epididymis samples were collected for biochemical, histo-pathological and tissue homogenate analyses. **Results:** Diabetic animals presented decreased body, testis weight, serum testosterone, sperm count, motility and impaired testicular redox homeostasis compared with control groups. Furthermore, deteriorated testicular and epididymis cytoarchitecture, with significantly decreased spermatogenesis scoring with decreased α -smooth muscle actin and androgen receptor immune-expression were detected. Insulin and Antox co-treatment to diabetic rats effectively mitigated the aforementioned parameters, compared with either drug administered alone. **Conclusion:** The present study clarified the potential synergistic effects of combined insulin and Antox treatment against diabetes-induced reproductive alterations. Therefore, Antox could be considered as an adjuvant therapy in diabetic patients.

Keywords: Albino rat, testis, diabetes, microanatomy and oxidative stress

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that results in hyperglycemia due to problems with insulin secretion and/or activity (Nethengwe *et al.*, 2022). Notably, oxidative stress results from increased free radical generation or compromised antioxidant defenses, leading to diabetes (Maritim *et al.*, 2003). Additionally, it results in long-term problems such as sexual dysfunction, renal failure, foot ulcers, loss of vision, etc. (Ding *et al.*, 2015). Male infertility is one of the main issues that can result from DM (Alsenosy *et al.*, 2019). Moreover, Nna *et al.* (2017) found a strong relationship between the frequency of male infertility and the percentage of diabetic men.

Currently, the research focuses on using medicinal plants high in antioxidants to lessen oxidative stress in diabetes mellitus, particularly because oxidative stress is associated with problems of DM, such as male infertility. The male gonads (testes and epididymis) are destroyed by oxidative stress, which inhibits spermatozoa production and storage and further contributes to male infertility (Tian *et al.*, 2020).

A crucial strategy of treatment regarding the creation of effective combinations of insulin or oral anti diabetic medications with natural compounds or even combinations of various natural antioxidants for supplementation in both diabetic individuals and experimental models (Yurie *et al.*, 2017). The mechanisms

of action underlying the advantages of these combinations in glycemic control and in the mitigation of the development of numerous diabetes problems had been clarified as a result (Yonamine *et al.*, 2016). A new combination therapy for diabetes mellitus that combines insulin with natural antioxidants aims to achieve effective glycemic control without side effects (Gutierrez *et al.*, 2019).

Antox is a combination of antioxidants (selenium and vitamins A, C and E), its protective role was documented previously in many animal models of Diabetes mellitus. Co-treatment with vitamin E was effective in reversing induced toxicity (Jahan *et al.*, 2014). Concurrent use of vitamin C revealed a protective role and significantly reduced histological alterations and chromosomal abnormalities in induced testicular injury (Amal and Fawzy, 2013). Additionally, it had been claimed that vitamin A therapy reduces the negative reproductive effects (Sujatha *et al.*, 1999). Through an antioxidant action, restoration of the histopathological image, and repair of DNA damage, treatment with selenium lessens the damaging effect induced in the testis (Alharthi *et al.*, 2020).

AIM OF THE WORK

The aim of this work was the assessment of the repro-protective synergistic role of Antox with insulin use on testicular and epididymis alterations in a streptozotocin-induced diabetic rat model.

MATERIAL AND METHODS

I- Material:

Chemicals: Streptozotocin (STZ) was purchased from Sigma. Antox and commercial insulin (Mixtard 30/70; Novo Nordisk) were purchased from a local pharmacy.

II-Experimental Design:

For sample size calculation, assuming that the mean of superoxide dismutase (SOD) in the Antox group is 5.32 ± 0.47 and of the control group 4.8 ± 0.38 the sample size was 36 (6 in each group) by using open EPI, CI is 95%, and test power is 80%. Thirty-six adult male albino rats, weighing 150 and 200 g, were used in the work. The rat trials were authorized by Zagazig University's Institutional Animal Care and Use Committee (approval number: ZU-IACUC/3/f/305/2022), and all trial protocols complied with the guidelines in the Guide for the Care and Use of Laboratory

Animals. Rats were put into six groups were randomly divided.

- Six rats from Group I (the negative control) were used as a standard control. They were not given any medication and were left alone for a period of four weeks.
- Six rats from Group II (the positive control group) received a single intraperitoneal injection of citrate buffer (0.1 M, PH = 4.5) and were then left for four weeks.
- Six rats from Group III (Diabetic) received a single intraperitoneal injection of 50 mg/kg of STZ liquefied in citrate buffer, and they were then left for four weeks (Naziroğlu *et al.*, 2004).
- Six rats were placed in Group IV (Diabetic/Insulin), which received a single intraperitoneal injection of 50 mg/kg of STZ liquefied in citrate buffer. They also received a daily subcutaneous injection of commercial insulin (Mixtard 30/70; Novo Nordisk) at a dose of 1 U/100 gm for four weeks (Liao *et al.*, 2010).
- Six rats in Group V (Diabetic/Antox) received an intraperitoneal injection of STZ at a dose of 50 mg/kg, followed by an oral administration of Antox at a rate of 10 mg/kg/day for four weeks (Amal and Mona, 2009)
- Six rats in Group VI (Diabetic/Insulin/Antox) were given intraperitoneal injections of STZ at a dose of 50 mg/kg, insulin at a dose of 1 U/100 g/day (S.C.), and Antox orally at a dose of 10 mg/kg/day for four weeks.
- Using a single intraperitoneal injection of streptozotocin (Sigma, 45 mg/kg in 0.1 M citrate buffer, pH 4.5), diabetes was established. Blood sugar values over 250 mg/dl 48 hours after STZ injection verified the establishment of diabetes (Sisman *et al.*, 2014). Throughout the trial period, blood glucose was checked once a week. After administering 50 mg/kg pentobarbital intraperitoneally four weeks after the STZ injection, blood samples were taken for study of hormonal and serum glucose levels, and testicular and epididymal tissues were removed for biochemical and histological review. While the left testes and epididyma were

used for the histopathological study, the right testes were maintained for the tissue homogenate analysis.

III-Measurement of sperm parameters

Sperms were collected using the diffusion method (*Seed et al., 1996*), which involved removing the end of the vas deferens from each group by the study end and submerging it in 3 mL of Hank's balanced salt solution. After 10 minutes, a hemocytometer was used to count the sperm (*Flora et al., 2011*).

IV-Sexual hormone measurement

The serum testosterone level was measured quantitatively by solid phase radioimmunoassay (RIA). Enzyme-linked immune sorbet assay (ELISA) kits were used to calculate the amount of the serum sexual hormone (testosterone) (DRG ELISA Kit, Germany) (*Richards et al., 1999*).

V-Tissue specimens

The right and left both testes and epididyma were slowly dissected from each group after receiving one injection (75 mg/kg BW) (*Silverman et al., 2014*), placed on an ice-cold plate, and dried. Half of each group's specimens were submerged in 10% neutral buffered formalin solution for two hours to harden the samples for light microscopic examination. The remaining specimens from each group were kept at -80 °C for additional homogenization. They were homogenized in 50 mM Tris-HCl pH 7.4 and 300 mM sucrose using a tissue homogenizer, producing a 10% (w/v) homogenate (Heidolph Instruments, Donau, Germany). The supernatant from the centrifuged homogenate was used to measure malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPX) levels for 15 minutes at 4 °C.

VI-Biochemical Analysis

Measurement of MDA, SOD and GPX in tissue homogenate: (*Shivarajashankara et al., 2001*)

VII-Histopathological examination

Each testicle's formalin's fixed; paraffin-embedded blocks were divided into 5 m sections and stained with 'Masson's Trichrome and H&E stains. Light microscopy (Leica, Germany) was used for the histopathological inspection of the slides stained with the H&E and MT stains to look for structural and degenerative changes in the

testis' seminiferous tubules and interstitial space, furthermore, in the epididymis (*Silverman et al., 2014*).

VIII- Immunohistochemistry (IHC)

This was executed by using method according to *Taylor et al., (2013)*. All stained slides were evaluated under light microscopy by the Image Analysis Unit of the Human Anatomy and Embryology Department at Zagazig University (LEICA ICC50 W).

IX- Morphometric examination

For each group, six rats, morphometric analysis was done. Along with the presence of well-distinguished cross testicular and epididymal sections from the six animals/group, representative fields across the images taken by the light microscope at 400x magnification were chosen after using special staining (Masson's Trichrome) and immunostaining with the anti-AR and anti-SMA antibodies. At the Anatomy and Embryology Department of Zagazig University, we used Image J analysis software (Fiji image j; 1.51 n, NIH, USA) to count the number of AR-in 6 animals/group and the mean values were reported. Also calculated in the representative fields of the Masson's trichrome and -SMA-stained sections were the percentages of Masson's trichrome- and -SMA-positive areas, respectively (*Elewa et al., 2019*).

X- Spermatogenesis scoring

The following categories for spermatogenesis were created using the Johnsen scoring system: 10 represents complete spermatogenesis and perfect tubules; 9 represents numerous spermatozoa but disorganized spermatogenesis; 8 represents only a few spermatozoa; 7 represents no spermatozoa but numerous spermatids; 6 represents only a few spermatids; 5 represents no spermatozoa or spermatids but numerous spermatocytes; 4 represents only few spermatocytes; 3 represents only spermatogonia, 2 represents no germ cells; and 1 represents neither germ cells nor Sertoli cells (Johnsen, 1970).

STATISTICAL ANALYSIS:

Continuous variables were shown as mean±SD if the data were regularly distributed, while heterogeneous data were characterized using median and inter quartile

range (IQR). When variances were equal, one-way analysis of variance (ANOVA) and post hoc (LSD) were employed to examine group differences; when variances were unequal, Kruskal-Wallis test and Dunn's multiple comparison test were applied. The Kolmogorov-Smirnov test was used to check for normality, and $P < 0.05$ was decided upon as the threshold for statistical significance. The Graph Pad Prism software, version 5.0, was utilized for all statistical calculations.

RESULTS

A-Body and testis weights

The body weight and testis weights were significantly lower in the diabetic rats (group III) compared to the control rats (groups I and II). However, body weight and testis weights were significantly increased in animals treated with insulin alone (group IV), Antox alone (group V) and both (group VI), but still significantly different from those in control rats, as presented in (Table 1).

B-Sperm analysis

The mean of sperm count and motility's percentage for the group III were significantly reduced as compared to that of the groups I and II. In animals treated with insulin alone (group IV), Antox alone (group V) and both (group VI), sperm count and motility were significantly improved (Table 2).

C- Biochemical Results

Serum Glucose level analysis

There was a significant increase in serum glucose level in group III as compared to groups (I and II). The data revealed that insulin use in group (IV) significantly decreased the level of serum glucose ($P < 0.05$).

There was also a significant decreased level in Antox administered group (V), with statistically significant decrease in the insulin + Antox administered group (VI) (Table 3).

Serum testosterone level analysis

There was a significant decrease in testosterone level in group III as compared to groups (I and II). The data revealed that insulin use significantly elevated the level of serum sex hormone (testosterone) ($P < 0.05$). There was also a significant elevated level in Antox administered group (V), with statistically significant increase ($P < 0.05$) in the insulin+ Antox administered group (VI) to approach the control 'groups' values (Table 3).

Effect on oxidative stress markers

MDA, SOD and GPX activities were assessed. SOD values were significantly lower in group III than in groups I and II. However, these values in the group IV were significantly higher than those in group III. Meanwhile, GPX values in group (III) were significantly lower than those in groups I and II. However, these values were significantly higher in group (IV) than in group (III). In contrast, MDA values were significantly higher in group (III) than in group (I and II), but these values decreased significantly in group (IV). Furthermore, there was also a significant elevated levels of SOD and GPX in Antox administered group (V) in comparison with group (III) with statistically significant decrease in comparison with group (IV), with statistically significant increase in the insulin + Antox administered group (VI) in contrary with MDA levels (Table 4).

Table (1): Shows animals' body and testicular weights in all experimental groups.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Body weight (g)	186± 10.20	188.5± 8.09	121.3± 4.92 ^a	138.8± 7.62 ^{a,b}	137.3± 5.68 ^{a,b}	171.7±7.23 ^{a,b,c,d}
Testis weight (g)	2.198± 0.128	2.197±0.129	0.93±0.12 ^a	1.41±0.07 ^{a,b}	1.22±0.08 ^{a,b,c}	1.74±0.04 ^{a,b,c,d}

(n=6) ^a $p < 0.05$ in comparison with both groups I & II
^c $p < 0.05$ in comparison with group IV

^b $p < 0.05$ in comparison with group III
^d $p < 0.05$ in comparison with group V

Table (2): Shows Sperm count and Sperm motility in all experimental groups.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Sperm Count ($10^6/ml$)	89.5± 1.87	81.5± 2.42	28.5± 3.08 ^a	55.0± 3.57 ^{a,b}	50.17± 1.72 ^{a,b,c}	76.83±2.31 ^{a,b,c,d}
Sperm motility (%)	96.33± 3.38	92.17±3.18	66.33±3.61 ^a	79.5±1.87 ^{a,b}	76.33±2.25 ^{a,b}	88.33±2.16 ^{b,c,d}

(n=6) ^a p <0.05 in comparison with both groups I&II
^c p <0.05 in comparison with group IV

^b p <0.05 in comparison with group III
^d p <0.05 in comparison with group V

Table (3): Shows serum glucose and testosterone levels in all experimental groups.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Blood glucose level (mg/dl)	82.33± 6.5	87.33± 3.01	314.0± 18.8 ^a	234.3±13.8 ^{a,b}	272.0± 7.72 ^{a,b,c}	159.7±7.5 ^{a,b,c,d}
Testosterone level (ng/ml)	2.92±0.09	2.78±0.08	0.24±0.04 ^a	0.57±0.05 ^{a,b}	1.8±0.11 ^{a,b,c}	2.27±0.1 ^{a,b,c,d}

(n=6) ^a p <0.05 in comparison with both groups I&II
^c p <0.05 in comparison with group IV

^b p <0.05 in comparison with group III
^d p <0.05 in comparison with group V

Table (4): Shows effect on oxidative stress markers in all experimental groups.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
MDA (nmol/mg)	32±2.608	39.17 ±2.48	149.7 ±10.7 ^a	82.67±4.54 ^{a,b}	104.8 ±5.9 ^{a,b,c}	70.17 ±4.0 ^{a,b,c,d}
SOD (U/mg)	31.5±2.88	32.17±3.31	9.5±1.871 ^a	20.5±1.871 ^{a,b}	15.5±1.87 ^{a,b,c}	27.33±1.63 ^{a,b,c,d}
GPX (ng/mg)	27.5±1.87	25.5±1.87	11.00±0.89 ^a	17.5±1.049 ^{a,b}	13.92±1.28 ^{a,b,c}	20.5±1.18 ^{a,b,c,d}

(n=6) ^a p <0.05 in comparison with both groups I&II
^c p <0.05 in comparison with group IV

^b p <0.05 in comparison with group III
^d p <0.05 in comparison with group V

D-Histological results

Hematoxylin and eosin stain of the testis

Rat's testis in both group (I and II) showed normal testicular tissue which was formed of closely regular seminiferous tubules lined by stratified germinal epithelium. Limited spaces (interstitium) in between the tubules which contain blood vessels and interstitial cells. Germinal epithelium consisted of spermatogonia, spermatocytes, spermatids with spermatozoa in the lumen of seminiferous tubules (**Fig. 1a and b**). On the other hand in group (III) the testicular tissue was greatly affected with dark stained and shrunken nuclei and greatly affected Sertoli cells. Nevertheless, there were multiple

spacing areas and notable decreased sperm amount in the tubular lumen (**Fig. 1c**).

All these findings were greatly disappeared with insulin use in group IV (**Fig. 1d**).

On Antox use in group V the findings in group III were slightly improved with still present some dark stained and shrunken nuclei of the stratified germinal epithelium (**Fig. 1e**). In group VI and combined use of Antox and insulin there was marked improvement in all histological findings with nearly normal histological picture of the testicular tissue (**Fig. 1f**).

Hematoxylin and eosin stain of the epididymis

Rat's epididymis in both group I and II showed regular outlined tubules with sperms inside the lumen and narrow intertubular spaces. The tubules were lined with pseudostratified epithelial lining with stereocilia, the epithelium appeared low columnar to cuboidal. The Epithelium is constituted of principal cells and basal cells. (Fig. 2a and b).

On the other hand in group III the tubules show stratification of the lining epithelium. The epithelial lining of the tubule shows vacuolations and increased intercellular spaces with cellular debris in the lumen (Fig. 2c). All these findings greatly disappeared with insulin use in group IV except for few areas of detached basement membrane (Fig. 2d). On Antox use in group V the findings in group III were slightly improved with still present some dark stained and shrunken nuclei with increased intercellular spaces (Fig. 2e). In group VI and combined use of Antox and insulin there was marked improvement in all histological findings with nearly normal histological picture of the epididymal tissue (Fig. 2f).

Special stain of the testis and epididymis with 'Masson's trichrome (MT) Masson's trichrome stained sections were examined, group I and II showed minimal collagen fibers in sub-capsular region (Fig. 3a and b). Group III showed marked collagen fibers deposition with its characteristic blue discoloration in sub capsular region (Fig. 3c).

In the groups IV, V and VI, there were with the least collagen deposition in co- administration of insulin and Antox in group VI (Fig. 3d, e and f). On the other hand, 'Masson's trichrome epididymal stained sections were examined, group I and II showed minimal collagen fibers in interstitial region (Fig.4a and b).

Group III showed marked collagen fibers deposition with its characteristic blue discoloration in interstitial regions (Fig. 4c).

In the groups IV, V and VI, there were the least collagen deposition in co- administration of insulin and Antox in group VI (Fig. 4d, e and f).

E-Immunohistochemical sections results

An analysis of the myoid cells in the peritubular region in testis and epididymis

Peritubular myoid cells were assigned for localization and assessment using immunohistochemical expression for α -SMA (PTMCs). In group I and II, seminiferous and epididymis tubules were encircled by peritubular α -SMA that was robustly expressed (Fig. 5a and b, 6a and b).

In contrast, the peritubular region of the seminiferous and epididymis tubules showed weak expressions in group III (Fig. 5c, 6c). In PTMCs, insulin therapy in group IV partially recovered α -SMA expressions (Fig. 5d, 6d). On the other hand, Antox use in group V minimally recovered α -SMA expressions (Fig. 5e, 6e).

Furthermore, the combined use of Insulin and Antox in group VI greatly recovered the expressions (Fig. 5f, 6f).

An analysis of the anti-Androgen receptor sensitive cells in the testis and epididymis

Our immunohistochemical results of anti-androgen receptors (AR) revealed significantly reduced AR expression in Leydig cells and spermatogonia nuclei. AR immune-positive cells were clearly observed in group I and II (Fig. 7a and b, 8a and b), infrequently demonstrated in the group III (Fig. 7c, 8c) and insulin therapy in group IV partially restored the expressions (Fig. 7d, 8d). On the other hand, Antox use in group V minimally restored the expression (Fig. 7e, 8e). Furthermore, the combined use of Insulin and Antox in group VI greatly restored the expressions (Fig. 7f, 8f).

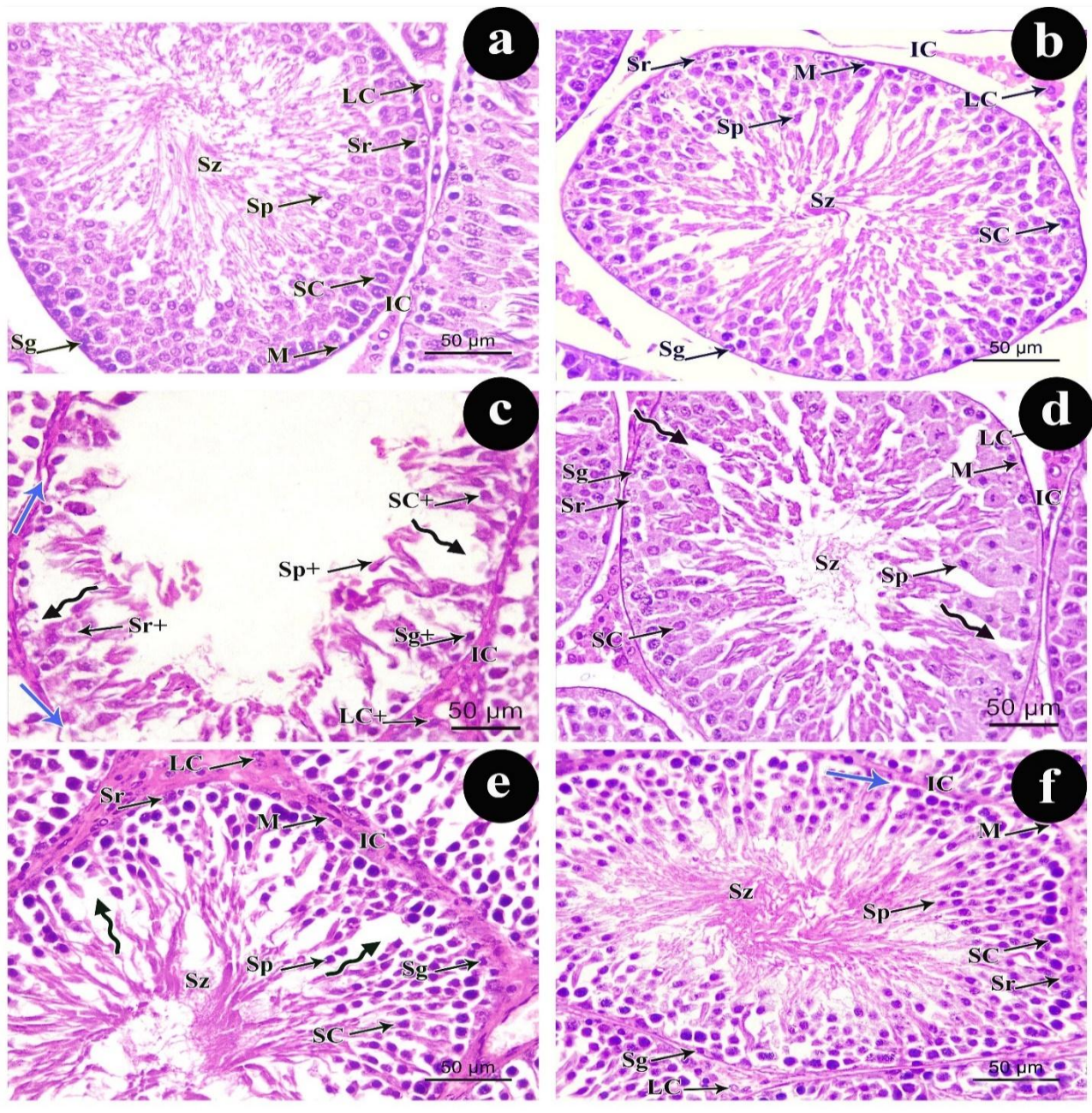


Figure (1): Photomicrographs of histological sections in testis (seminiferous tubules) of adult rat in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f). The sections show crowded seminiferous tubules lined by stratified germinal epithelium with interstitium in between (IC) contains Leydig cells (LC). The basement membrane is lined with myoid cells (M) with Sertoli cells (Sr), spermatogonia (Sg), spermatocytes (SC), spermatids (SP), with spermatozoa in the lumen (SZ). In affected sections there are irregular Sertoli cells (Sr+) irregular spermatogonia (Sg+), dark stained spermatocytes (SC+), irregular spermatids (SP+), dark stained Leydig cell nuclei (LC+), intercellular spaces (zigzag arrows), areas of detached basement membrane (blue arrows).

(H and E x 400, Scale bar 50 µm)

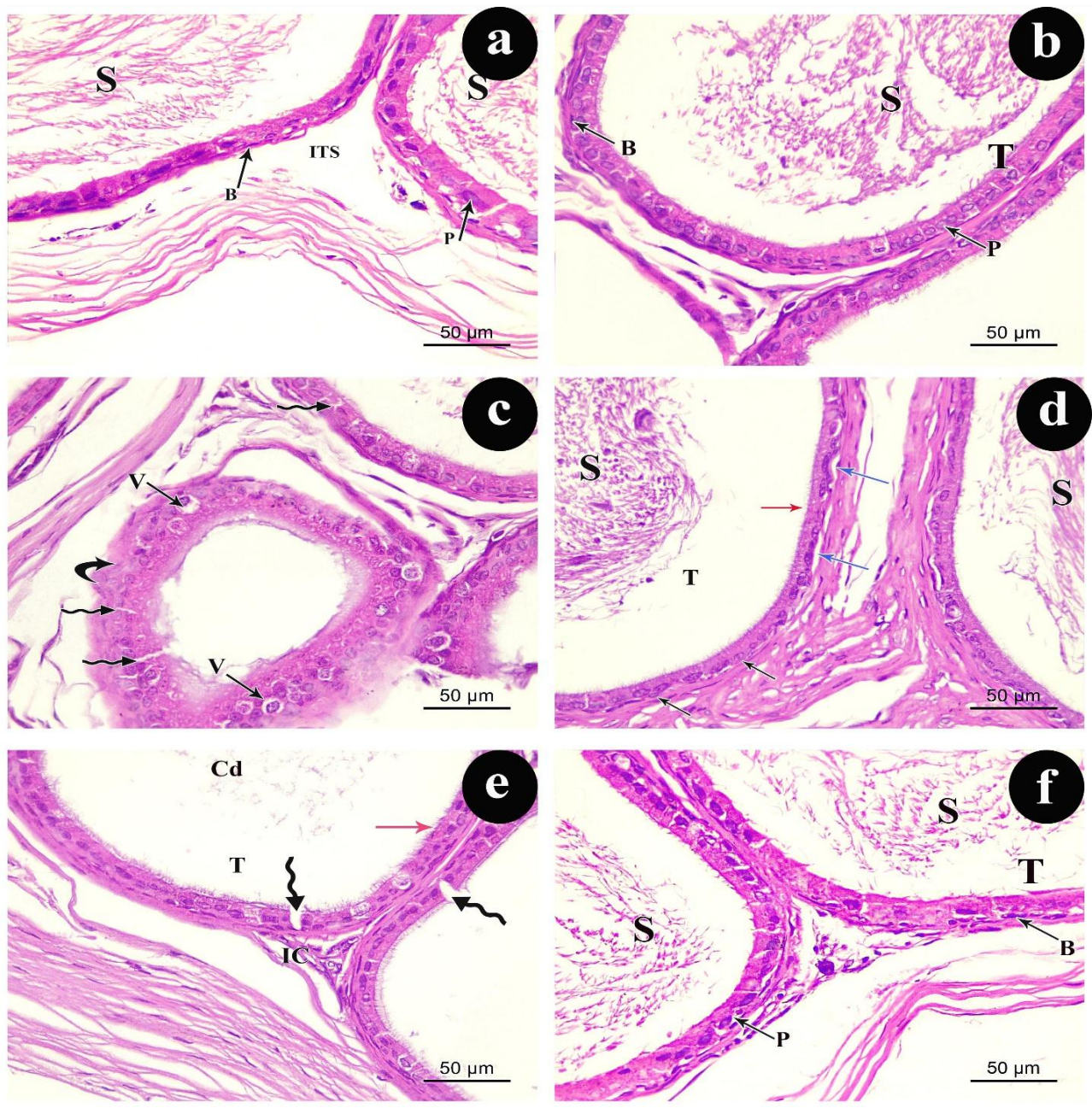


Figure (2): Photomicrographs of histological sections in epididymis of rats in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f). The sections show outlined tubules (T) with sperms (S), inter-tubular spaces (ITS). The Epithelium is constituted of principal cells (P) and basal cells (B), stratification of the lining epithelium (curved arrow) vacuolations: (V), intercellular spaces: (zigzag arrows) stereo-cilia: (red arrow) areas of detached basement membrane: (blue arrows), cellular debris: (Cd) and interstitium (IC).

(H and E x 400, Scale bar 50 µm)

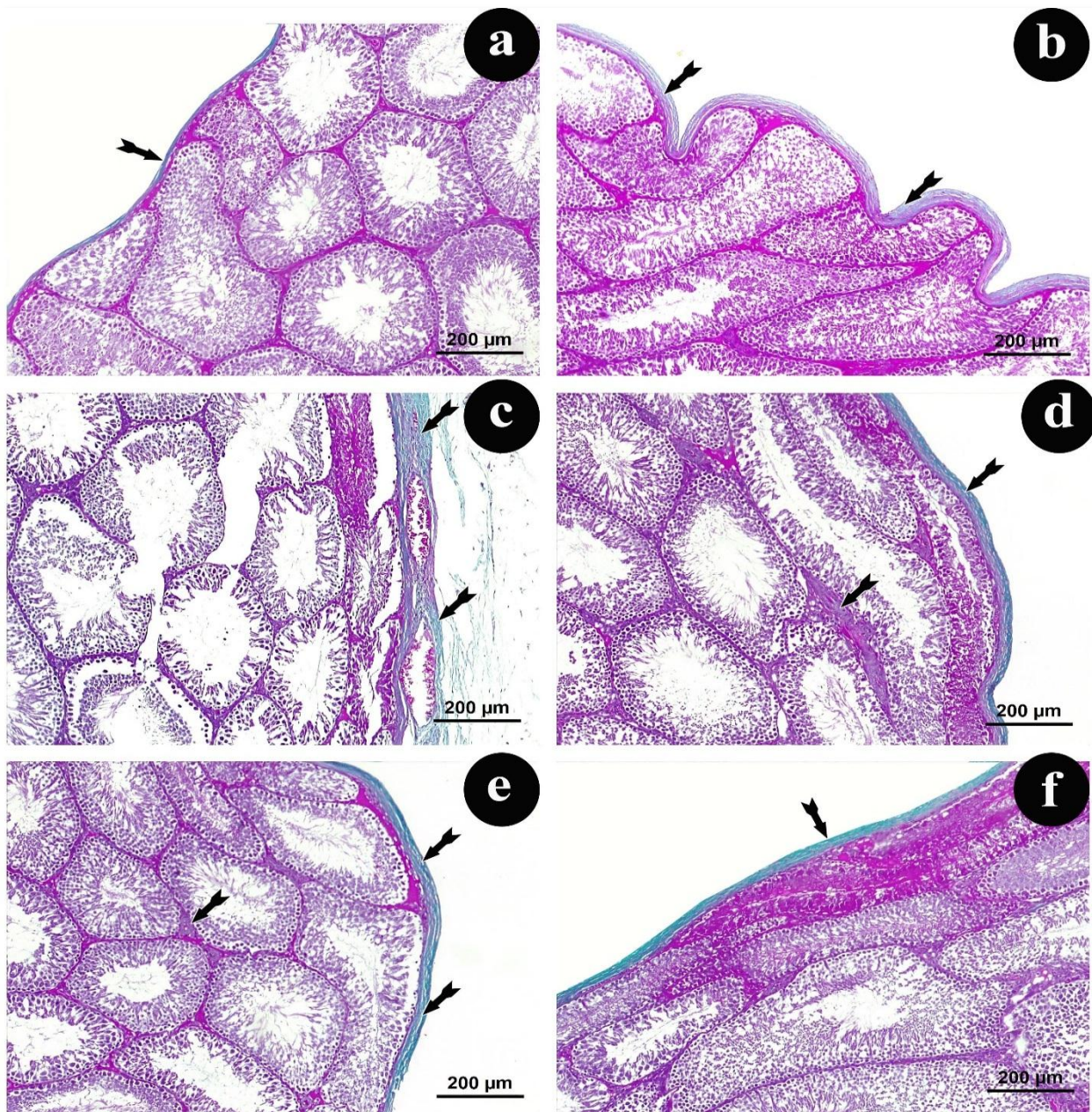


Figure (3): Photomicrographs of Masson trichrome stained sections of testicular sections of rats in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f). In all sections, appeared areas of collagen fibers deposition (tailed arrows).

(Masson Trichrome x 100, Scale bar 200 μm)

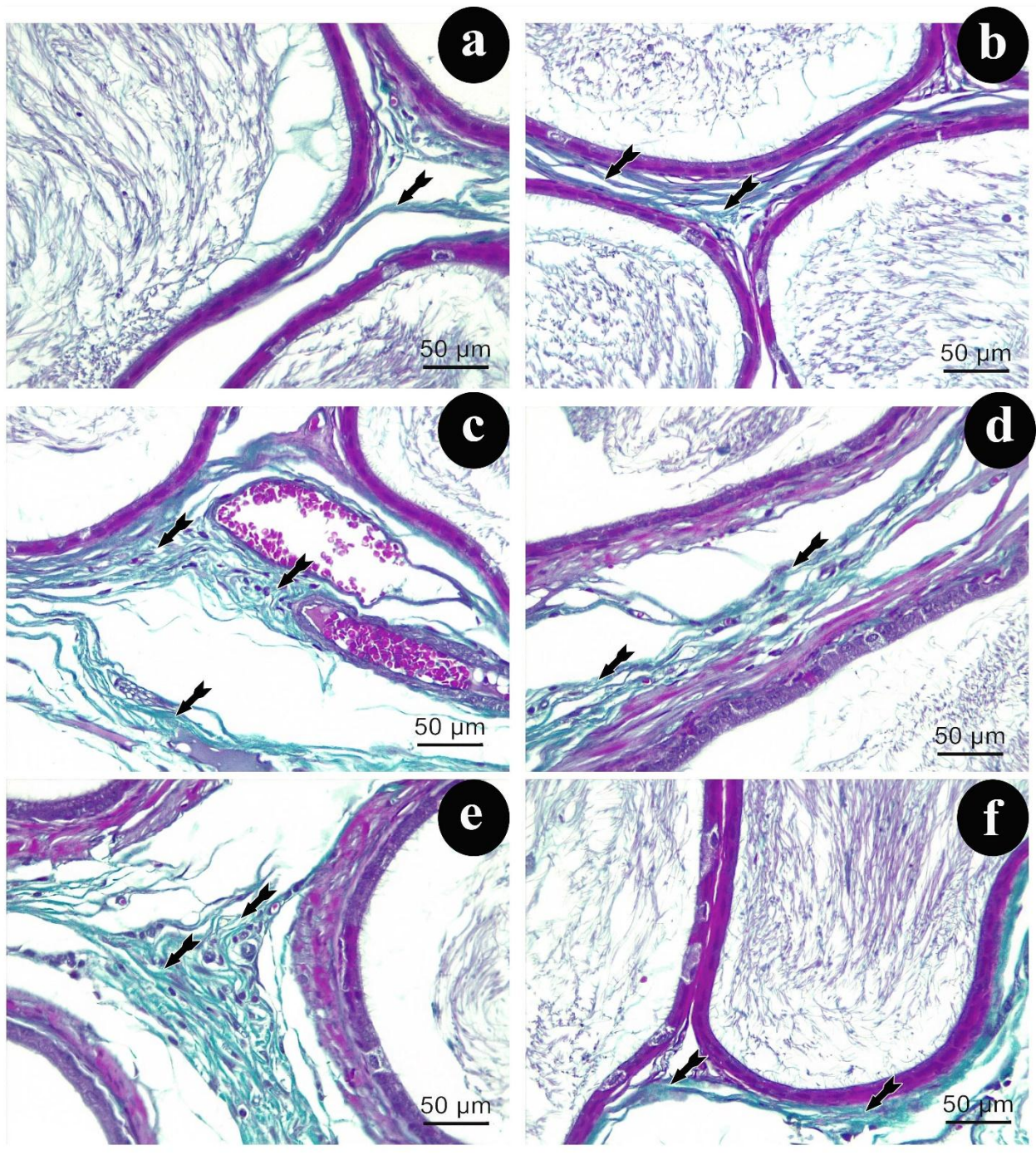


Figure (4): Photomicrographs of Masson trichrome stained sections of epididymis in rats in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f). In all sections, appeared areas of collagen fibers deposition (tailed arrows).

(Masson Trichrome x 400, Scale bar 50 μm)

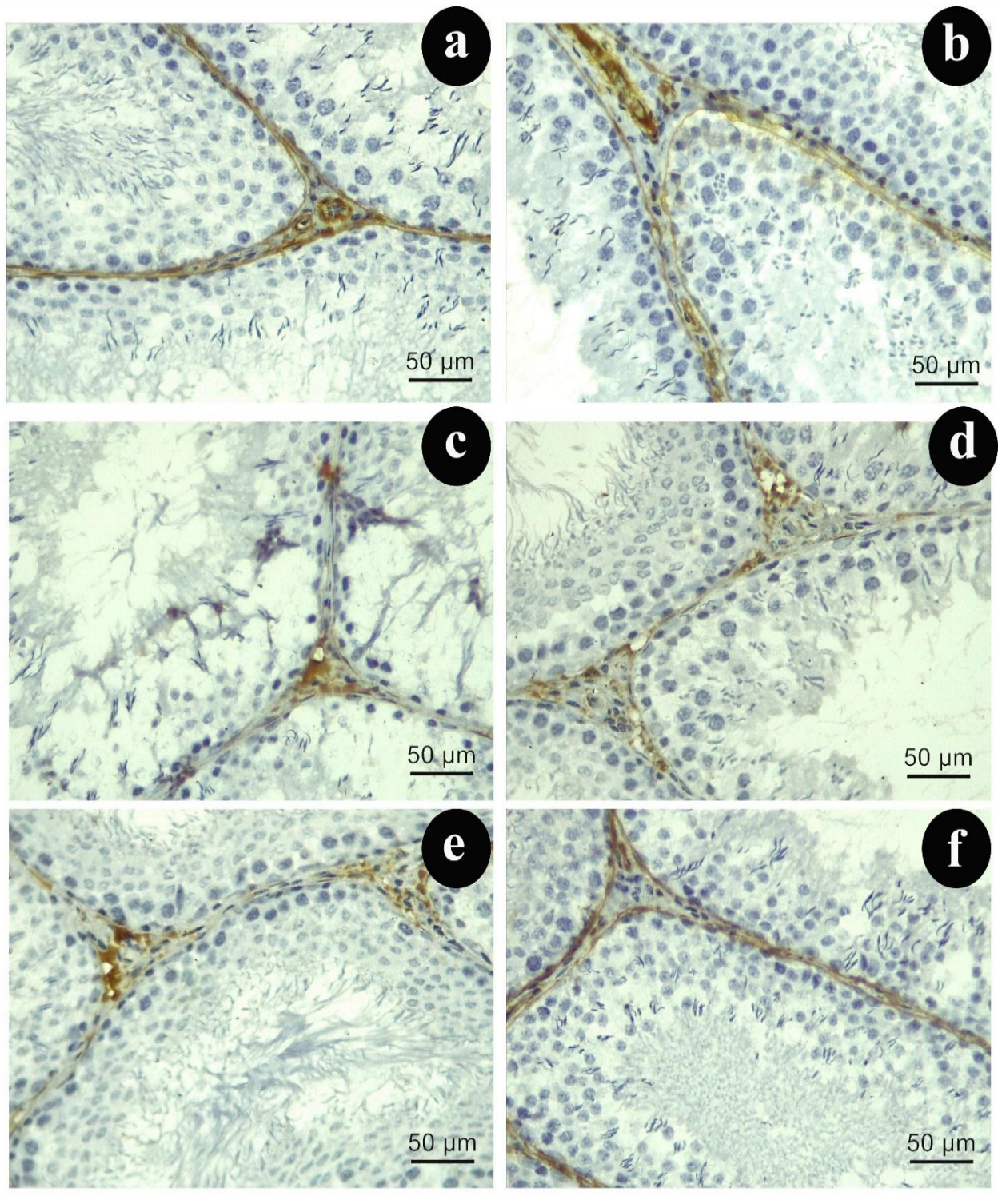


Figure (5): Photomicrographs of α -Smooth muscle actin (α -SMA) immune-expression of testis in rats in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f).

(α -SMAx400, Scale bar 50 μ m)

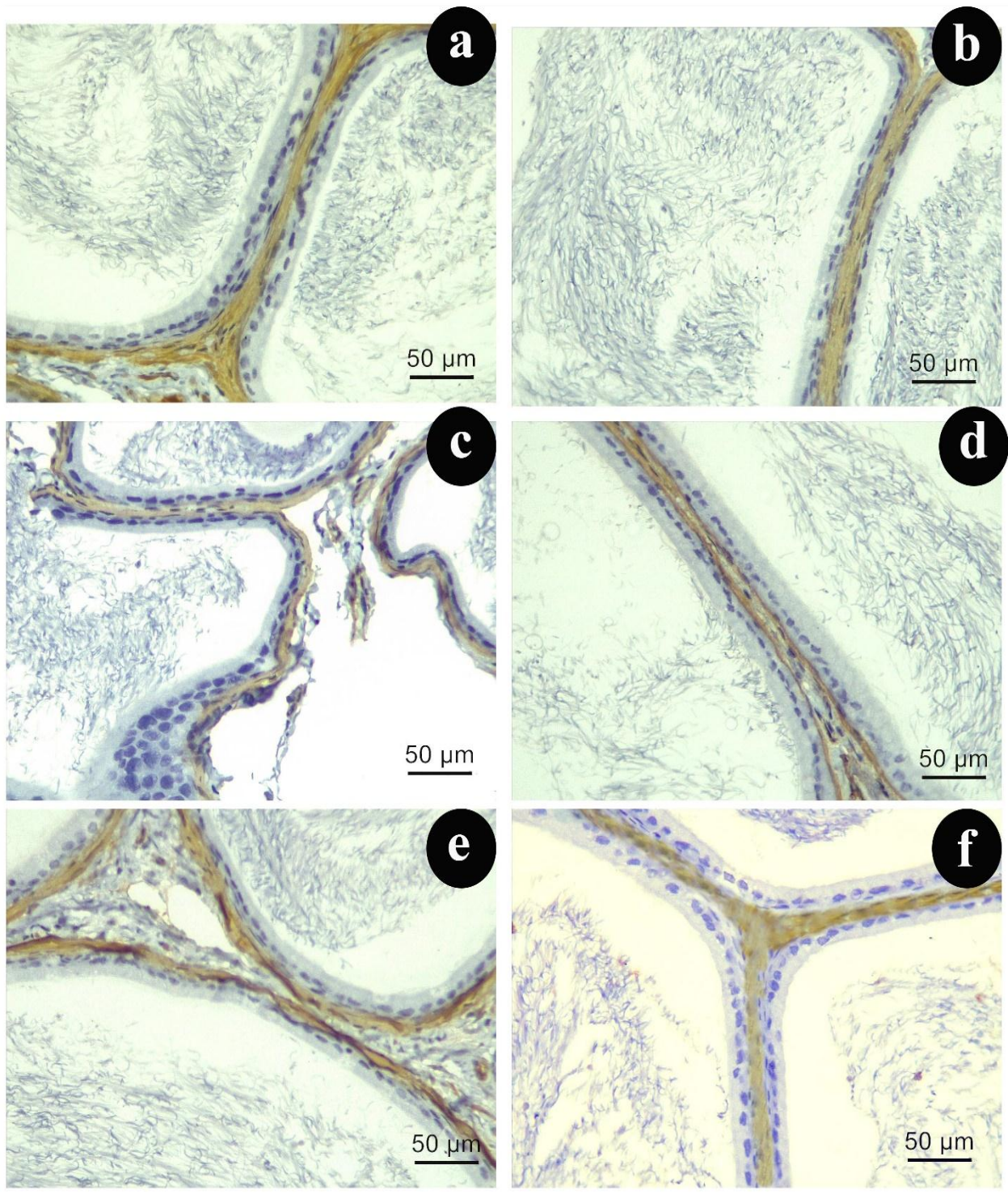


Figure (6): Photomicrographs of α -Smooth muscle actin (α -SMA) immune-expression of epididymis in rats in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f). (α -SMAx400, Scale bar 50 μ m)

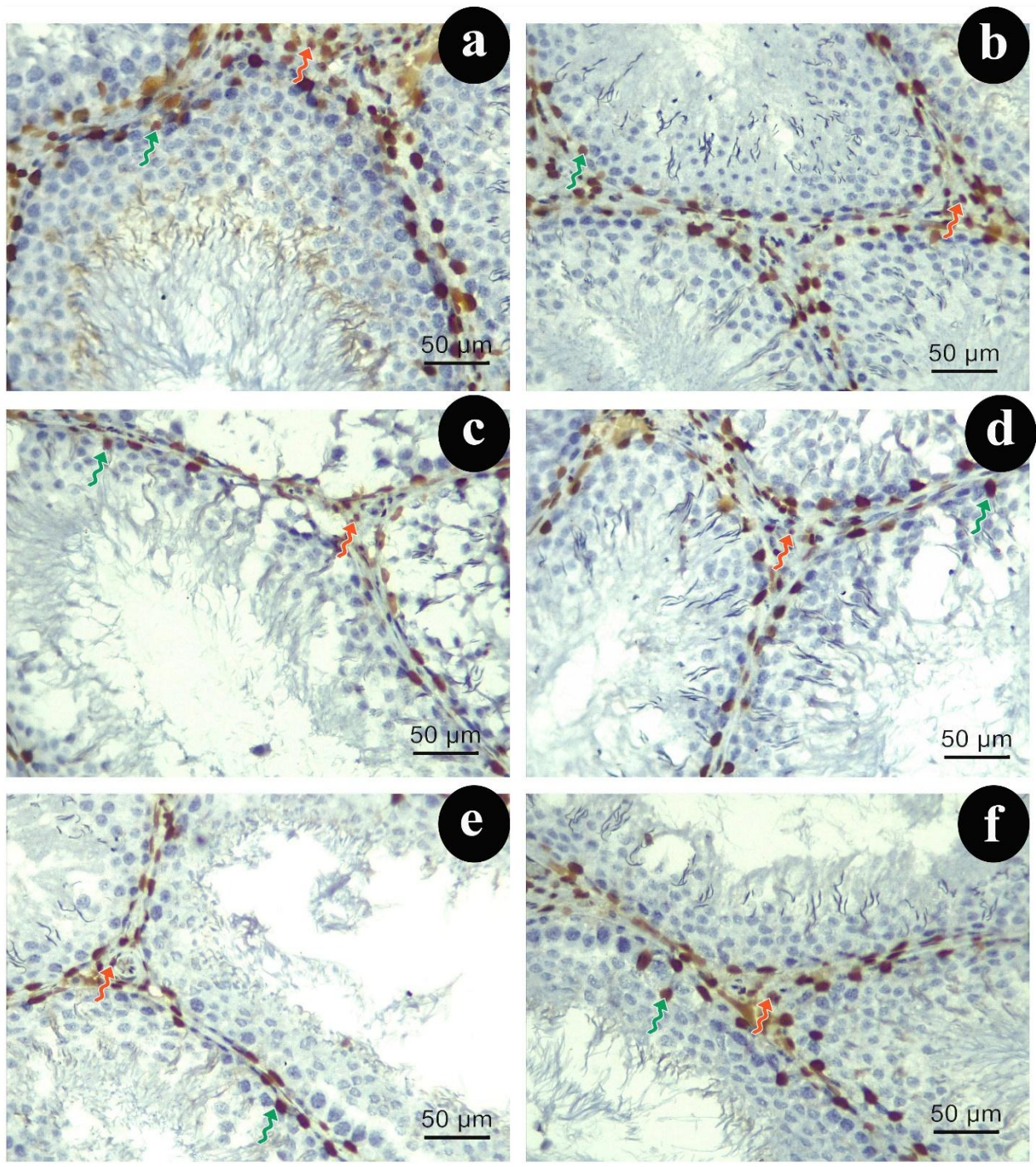


Figure (7): Photomicrographs of Androgen Receptor (AR) immune-expression of testis in rats in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f). In all sections, appeared immune positive cells in Leydig cells (red zigzag arrows) and in stratified germinal epithelium (green zigzag arrow). (AR x 400, Scale bar 50 µm)

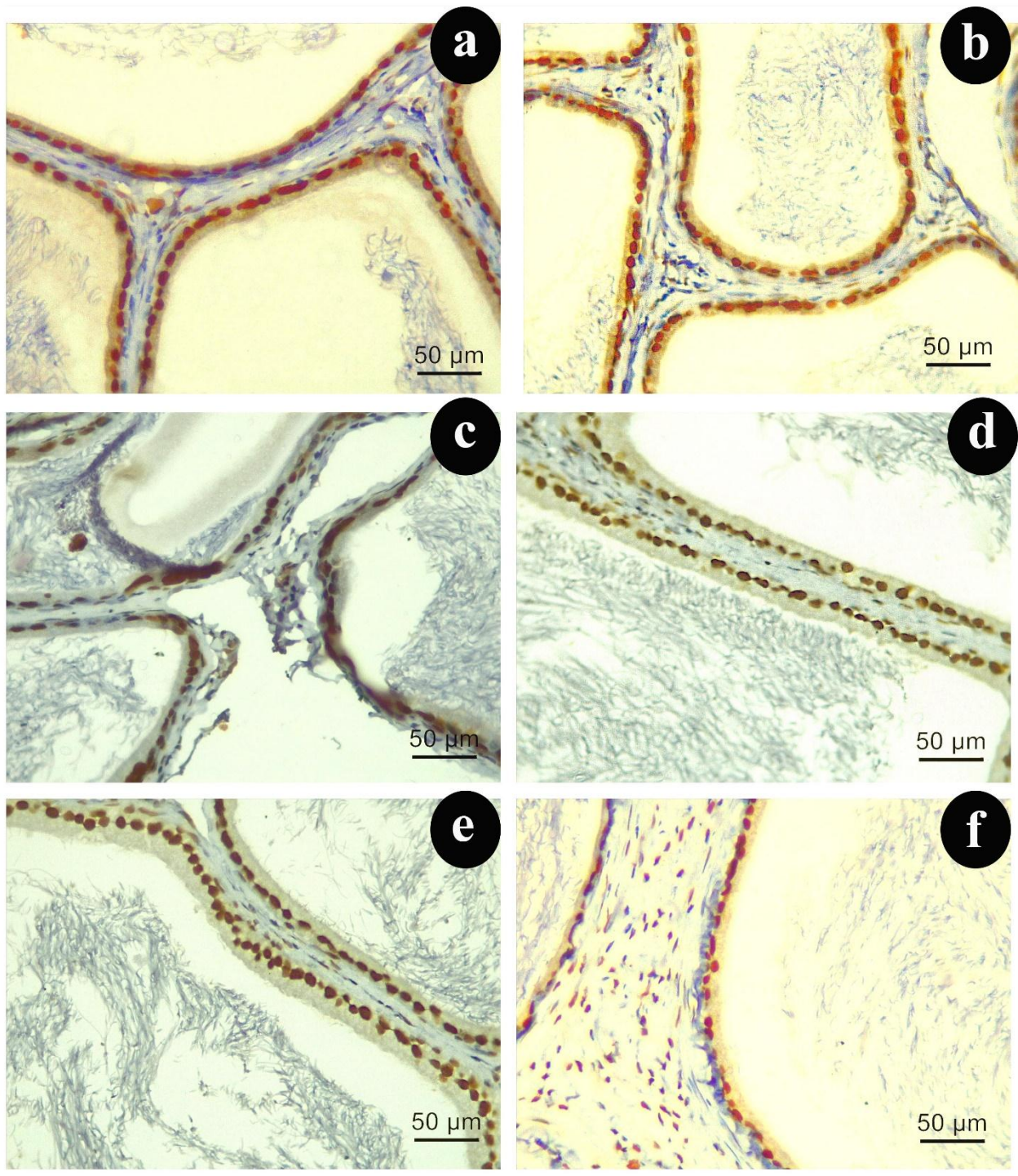


Figure (8): Photomicrographs of Androgen Receptor (AR) immune-expression of epididymis in rats in all experimental groups, group I (a), group II (b), group III (c), group IV (d), group V (e) and group VI (f).

(AR x 400, Scale bar 50 µm)

F-Histo-Morphometric Results

The area percentage of 'Masson's trichrome positive reaction in both testis and epididymis sections was significantly increased in the diabetic rats (group III) in comparison with the control rats (groups I and II). However, it was significantly decreased in animals treated with insulin alone (group IV), Antox alone (group V) -despite the statistically significant increase in comparison with group (IV) - and both (group VI), but still significantly different from those in control rats, as presented in (Table 5).

On the other hand, the area percentage of α -SMA positive reaction in both testis and epididymis sections was significantly decreased in the diabetic rats (group III) in comparison with the control rats (groups I and II). However, it was significantly increased in animals treated with insulin alone (group IV), Antox alone (group V) -despite the statistically significant decrease in comparison with group (IV) - and both (group VI), but still significantly different from those in control rats, as presented in (Table 5).

Nevertheless, the cell count for positive cells of Anti- AR reaction in both testis and

epididymis sections was significantly decreased in the diabetic rats (group III) in comparison with the control rats (groups I and II). However, it was significantly increased in animals treated with insulin alone (group IV), Antox alone (group V) -despite the statistically significant decrease in comparison with group (IV) - and both (group VI), but still significantly different from those in control rats, as presented in (Table 5).

G-Histopathological Scoring Results

Johnson's score was used to assess the tubular injury in all experimental groups. There were no histopathological changes in the control groups (I and II). Diabetes in group (III) caused intense injury to the tubules and spermatogenesis process, with statistically significant decrease in the scoring in comparison with groups (I and II) ($P < 0.005$). These damages were improved in group (IV) ($P < 0.005$) compared with group (III) with statistically non-significant increase in the score. On the other hand, there was a statistically significant increase in scoring as regards groups (V and VI) with remarkable improved scoring in group (VI) (Table 6).

Table (5): shows different morphometric parameters in all experimental groups.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Masson's Trichrome (%) in testis	1.88±0.07	2.07±0.17	19.49±1.69 ^a	7.58±0.63 ^{a,b}	9.33±0.7 ^{a,b,c}	3.55±0.52 ^{a,b,c,d}
Masson's Trichrome (%) in epididymis	6.33±0.98	6.81±0.79	19.12±1.21 ^a	10.66±0.95 ^{a,b}	13.50±0.91 ^{a,b,c}	8.51±0.55 ^{a,b,c,d}
α - SMA (%) in testis	6.26±0.45	6.3±0.37	1.57±0.26 ^a	3.4±0.38 ^{a,b}	4.39±0.36 ^{a,b,c}	5.29±0.39 ^{a,b,c,d}
α - SMA (%) in epididymis	8.31±0.37	7.83±0.52	2.7±0.26 ^a	4.73±0.22 ^{a,b}	3.61±0.31 ^{a,b,c}	5.55±0.22 ^{a,b,c,d}
Anti-AR Cell count in testis	73±3.4	70±2.28	21.50±1.87 ^a	42.83±2.48 ^{a,b}	32.17±1.47 ^{a,b,c}	53±2.09 ^{a,b,c,d}
Anti-AR Cell count in epididymis	76.67±2.16	79.50±1.87	30.50±1.87 ^a	46.33±2.16 ^{a,b}	40.83±1.47 ^{a,b,c}	56.5±1.87 ^{a,b,c,d}

(n=6) ^a $p < 0.05$ in comparison with both groups I&II
^c $p < 0.05$ in comparison with group IV

^b $p < 0.05$ in comparison with group III
^d $p < 0.05$ in comparison with group V

Table (6): Johnson's scoring in all experimental groups.

Johnson's Scoring	Group I	Group II	Group III	Group IV	Group V	Group VI
Median (IQR)	10.0 (1.0)	10.0 (1.0)	1.0 (1.0) ^a	5.0 (1.0)	4.0 (1.0) ^a	8.0 (1.0) ^{a,b}

(n=6) ^a $p < 0.05$ in comparison with both groups I&II
^c $p < 0.05$ in comparison with group IV

^b $p < 0.05$ in comparison with group III
^d $p < 0.05$ in comparison with group V

DISCUSSION

The increasing prevalence of diabetes mellitus, particularly at a younger age, represents a major health challenge, as it may negatively impact male fertility throughout the active reproductive age (*Bhandari and Sapra, 2020*). Since oxidative stress had been implicated as a key player in the pathogenesis of diabetes-induced testicular dysfunction (*Tian et al., 2020*), the current research was designed to assess the repro-protective synergistic role of multi-antioxidants (Antox) with insulin on testicular and epididymal alterations in a diabetic rat model.

Based on *Furman, (2021)* previous work, diabetes induction was performed in the current study by using a single high STZ dose to destroy pancreatic islet β cells, eliminating any possibility of endogenous insulin-synthesizing activity that might confound the results; effective establishment of diabetes was confirmed by elevated serum glucose levels (over 250 mg/dl) (*Sisman et al., 2014*). The present study illustrated significant body weight loss in diabetic rats, compared with the controls. This finding could be ascribed to insulin deficiency-induced protein depletion and lipolysis (*Guyton and Hall, 2006*). The diabetic group also exhibited a significant testis weight decrement, suggesting testicular structural alterations. These findings were consistent with other reports on diabetic models (*Kotian et al., 2019, Samir et al., 2021*). Meanwhile, insulin therapy to diabetic rats induced significant amelioration of diabetes-induced body and testis weight loss. Previous researches attributed the beneficial effect of insulin on body weight to its well-known anabolic effect. Interestingly, insulin and Antox co-treatment elicited more obvious improvement in body and testis weights, compared with either drug administered alone (*Kolb et al., 2018*).

In the current work, diabetic rats revealed significantly reduced serum testosterone levels as well as sperm count, and motility compared to the controls. In accordance, recent evidence revealed strong association of diabetes with low testosterone levels and altered sperm parameters (*Zhong et al., 2021*). Indeed, lack of spermatozoa cytoplasmic antioxidant enzymes and the rich

content of polyunsaturated fatty acids in their plasma membranes rendered them particularly exposed to oxidative stress (*Aitken and Curry, 2011*). The fatty acids in the sperm membranes are attacked by reactive oxygen species (ROS), resulting in lipid peroxidation with subsequent loss of membrane integrity and increased permeability, eventually leading to reduced sperm motility and vitality (*Alahmar, 2019*). Unfortunately, ROS-induced sperm damage accounts for 30% to 80% of male infertility cases (*Agarwal et al., 2004*). Oxidative stress had also been reported to negatively impact steroidogenesis process, leading to reduced testosterone biosynthesis (*Leisegang et al., 2022*). On the other hand, insulin therapy in diabetic rats promoted significant amelioration of diabetes-induced testosterone deficiency and sperm parameter alterations. Consistent with our findings, (*Kang et al., 2021*) had recorded elevated testosterone levels in type 1 diabetic patients receiving insulin therapy. In this regard, insulin treatment had been shown to modulate the hypothalamic-pituitary-testicular axis function and, thus, regulate testosterone levels and eventually restore fertility (*Schoeller et al., 2012*). In addition, an in vitro study on mouse Leydig cells proved that insulin upregulates steroidogenesis via direct modulation of Leydig cell functions (*Leisegang and Henkel, 2018*). It was noteworthy that, insulin and Antox co-treatment to diabetic rats, in the present study, effectively increased testosterone levels and the tested sperm parameters, relative to insulin and Antox monotherapy, suggesting their potential complementary roles. In the same context, serum sex hormones and sperm parameters were reportedly improved in infertile men treated with oral antioxidants for 6 months (*Saylam and Çayan, 2020*). Furthermore, in vitro Leydig cell models treated with different antioxidant agents demonstrated enhanced testosterone synthesis (*Leisegang et al., 2022*).

In the present research, impaired cellular redox homeostasis was observed in non treated diabetic rats, as evidenced by overproduction of the lipid peroxidation marker, MDA, and reduced levels of the antioxidant enzymes; SOD and GPX.

Similarly, altered oxidant/antioxidant status was previously reported in diabetic patients and animal models (*Almulathanon et al., 2021; Salah et al., 2022*). Excessive advanced glycation end products and free hydroxyl radicals induced by chronic hyperglycemia are undoubtedly involved in diabetes-induced oxidative stress and testicular dysfunction (*Tian et al., 2020*). Insulin treatment to our diabetic rat models considerably mitigated diabetes-induced oxidative stress. Consistent with our results, insulin was considered a potent antioxidant (*Okon and Umoren, 2017*). It restored redox balance indirectly via normalization of blood glucose levels, and directly by potentiation of antioxidative defense system (*Okon and Umoren, 2017; Yaribeygi et al., 2019*). Importantly, the combined insulin and Antox treatment to diabetic rats elicited more effective improvement of testicular redox balance than the drug administered alone. In fact, antioxidants are well-documented to enhance male fertility by minimizing the toxic testicular impacts of oxidative stress (*De Luca et al., 2021*). They reduce oxidative damage directly by reacting with free radicals or indirectly by inhibiting free radical activity (*Balbi et al., 2018*). Although high dosages of single antioxidant compounds have been shown to disturb the cellular redox status, multi-antioxidants have been established to promote synergistic protective influences, providing effective treatment for male infertility (*Arafa et al., 2020*). Vitamins C and E supplementation to diabetic patients for 3 months considerably increased SOD and GPX activities and improved insulin action (*Rafiqhi et al., 2013*). Non-enzymatic antioxidants including selenium and vitamins A, C and E interrupted lipid peroxidation chain reactions; their combination seems to be **promising** (*Balbi et al., 2018; Leisegang et al., 2022*)

The smooth muscle-like PTM cells were necessary for normal spermatogenesis. Their contractile nature aided in the transport of immotile spermatozoa to the epididymis (*Uchida et al., 2020*). α -SMA was a cytoskeletal protein highly expressed in PTM cells, and it was considered a main contractility marker of PTM cells (*Vickers,*

2017). The present investigation revealed a significant reduction of α -SMA expression in PTM cells, as well as, increased testicular collagen fiber deposition in diabetic rats, reflecting loss of a smooth muscle phenotype and impaired contractility (*Welsh et al., 2009*). In this regard, the phenotypic switch of PTM cells in subfertility or infertility cases was witnessed by lack of PTM cell contractility markers, and testicular fibrosis (*Welter et al., 2013*). The observed decline of α -SMA expression in PTM cells could be referred to excessive levels of testicular ROS and prostaglandin metabolites (*Mayer et al., 2018*). In addition, hyperglycemia-induced metabolic disturbances and oxidative stress were implicated in the pathogenesis of diabetes-mediated collagen changes (*Bondarenko, 2019*). On the other side, insulin and/or Antox treated diabetic rats displayed significantly increased α -SMA expression in PTM cells as well as decreased testicular collagen fiber deposition, compared to diabetic rats. These changes were more evident on combined insulin and Antox administration, indicating further improvement of PTM cell contractility and sperm transport.

Although germ cells did not express functional ARs (*De Gendt et al., 2004*), androgens are well established to regulate spermatogenesis via binding to ARs in somatic testicular cells (*Roberts and Chauvin, 2019*). Sertoli cells were considered the main target for androgen action (*Verhoeven et al., 2018*). In fact, AR signaling pathway in Sertoli cells was crucially important for regulating the biological processes in Sertoli cells, maintaining the integrity of the blood testis barrier, proliferation and differentiation of spermatogonial stem cells, Sertoli cell-spermatid adhesion and sperm-released (*Zhang et al., 2022*). Therefore, the present lack of AR expression in Sertoli cells contributed partially to distorted Sertoli cells, wide intercellular spaces and defective spermatogenesis detected in the testicular tissue of diabetic rats. Additionally, diabetes-induced oxidative stress was considered a major contributor to altered spermatogenesis and germ cell damage (*Agarwal et al., 2014*).

Leydig cell AR signaling is indispensable for the development of fully functional Leydig cells (*O'Hara et al., 2015*). Accordingly, the noticed decline of Leydig cell AR expression in the non-treated diabetic group of the present study could be partly accountable for altered steroidogenesis, and lower testosterone levels recorded in this group. In harmony with previous reports (*Salah et al., 2022*), the current investigation further clarified that insulin treatment mitigated diabetes-induced testicular tissue alterations and significantly increased testicular AR expression. Concomitant administration of insulin and Antox promoted more improvement of the aforementioned parameters, compared with either drug administered alone. In this context, Antox beneficial role against heavy metal toxicity was illustrated by preservation of testicular micro-architecture and serum testosterone levels in arsenic, lead and cadmium intoxicated rats (*Hassanin et al., 2010b; Hassanin et al., 2010a; Labib and Galal, 2021*). Improved testicular antioxidant status, testosterone levels and testicular expression of α -SMA and ARs could provide a plausible explanation for ameliorated testicular architecture in the treated diabetic groups.

The present histological data of the diabetic group displayed stratification and vacuolations of the epididymis epithelial lining with increased intercellular spaces and luminal cellular debris. Additionally, immunohistochemical analyses revealed the reduction of epididymis expression of α -SMA and ARs in diabetic rats. In agreement with the current findings, (*Singh et al., 2009*) and (*Korejo et al., 2016*) illustrated degenerative epididymis alterations and secretory dysfunction in diabetic rats. Indeed, the smooth muscle cells of the epididymis were fundamental for spermatozoa transport (*Elfgan et al., 2018*), and their obvious affection in our diabetic rat models, proved by decreased epididymis α -SMA expression, reflected impaired contractile activity and sperm transport. During spermatozoa epididymis transit, they matured and acquired motility and fertilization ability (*Robaire and Hinton, 2015*). Since epididymis functions were androgen-dependent (*Elfgan et al.,*

2018), the observed decrement of epididymis AR expression in diabetic rats, in the present study, also indicated profound functional alterations of the epididymis that could impair sperm quality and even aggravate male infertility (*Li et al., 2020*). In the current study, the beneficial effects of Antox and/or insulin treatment on diabetes-induced epididymis damage were witnessed by amelioration of the epididymis cytoarchitecture and up regulation of α -SMA and ARs immune-expression; these effects were more evident on Antox and insulin co-treatment emphasizing their complementary protective roles against diabetes-induced epididymal dysfunction. In alignment with the current results, (*Soudamani et al., 2005*) demonstrated partial preservation of epididymis epithelium on insulin replacement to pre-pubertal diabetic rats. The protective roles of various antioxidants against diabetes-triggered epididymis injury had also been proven in different experimental studies (*Corrêa et al., 2019; Jaul-Faddladdeen, 2022; Sahu et al., 2020*). Furthermore, the antioxidant Malaysian propolis had been shown to enhance the efficacy of metformin against testicular and epididymis toxicities in diabetic rat models (*Nna et al., 2019*).

CONCLUSION

Antox administration alleviated diabetes-induced testicular and epididymal structural and functional alterations by restoring cellular redox homeostasis and up-regulation of α -SMA and AR immune-expression. Antox's beneficial impact was potentiated by its co-administration with insulin, suggesting their possible synergistic effects. Therefore, Antox supplementation could be considered an adjuvant therapy with insulin to protect against reproductive dysfunction and enhance male fertility in diabetic patients.

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الدور التآزري الواقي تناسليا لل انتوكس مع الأنسولين في نموذج السكري المستحث بالستربتوزوتوسين بالجرذان (دراسة الكيمياء الحيوية والنسيج المورفومتري والمناعة الكيميائية النسيجية)

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الملخص العربي

المقدمة: يعتبر الخلل التناسلي المرتبط بالسكري مهدد كبير على الصحة. ترتبط امراضه ارتباطاً وثيقاً بالإجهاد التأكسدي. انتوكس هو مزيج من مضادات الأكسدة (السيلينيوم والفيتامينات A ، C ، E) وتم توثيق دوره الوقائي سابقاً في النماذج الحيوانية.

الهدف: كان الهدف من البحث تقييم الدور التآزري الوقائي ل انتوكس مع استخدام الأنسولين في تغييرات الخصية والبربخ في نموذج الفئران المصابة بداء السكري الناجم عن الستربتوزوتوسين.

المواد والطرق المستخدمة: تم تقسيم ستة وثلاثين فأراً إلى ست مجموعات متساوية (I) :لم يتم إعطاؤها أي دواء (II) . (الضابطة الإيجابية) تلقى الحقن داخل الصفاق من محلول السترات؛ (III) (السكري) تلقى الحقن داخل الصفاق من الستربتوزوتوسين (٥٠ مجم / كجم). تركت المجموعات الثلاث دون علاج لمدة أربعة أسابيع (IV) .السكري / الأنسولين (V) السكري / انتوكس (و) VI السكري / الأنسولين / انتوكس (تلقى الستربتوزوتوسين ، تليها أربعة أسابيع من الحقن اليومي للأنسولين تحت الجلد (١ وحدة / ١٠٠ جم) ، انتوكس عن طريق الفم (١٠ مجم / كجم / يوم) ، أو كلاهما الأنسولين والانتوكس على التوالي. بنهاية الدراسة جمعت عينات الدم والسائل المنوي والخصية والبربخ لتحليل الكيمياء الحيوية والنسيج المرضي وتحليل تجانس الأنسجة.

النتائج: أظهرت الحيوانات المصابة بمرض السكر انخفاضاً في وزن الجسم ، ووزن الخصية ، وهرمون التستوستيرون في الدم ، وعدد الحيوانات المنوية ، والحركة ، وضعف توازن الأكسدة والاختزال في الخصية مقارنة بالمجموعات الضابطة. علاوة على ذلك ، تم الكشف عن تدهور البنية الخلوية للخصية والبربخ ، مع انخفاض معدل تكوين الحيوانات المنوية بشكل ملحوظ مع انخفاض الأكتين العضلي الأملس ومستقبل الأندروجين. خفف العلاج المشترك للأنسولين والانتوكس للفئران المصابة بداء السكري من العوامل المذكورة أعلاه بشكل فعال ، مقارنة بأي دواء يتم إعطاؤه بمفرده.

الخلاصة: أوضحت الدراسة الحالية الآثار التآزرية المحتملة للعلاج المشترك بالأنسولين والانتوكس ضد التغيرات الإنجابية التي يسببها مرض السكري. لذلك ، يمكن الأخذ بعين الاعتبار استخدام انتوكس كعلاج مساعد لمرضى السكري.