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ORLISTAT /GEMFIBROZIL AMELIORATE TESTICULAR DYSFUNCTION IN STREPTOZOTOCIN-INDUCED DIABETES IN RATS VIA TARGETING VEGF/ NO / PROGRAMMED CELL DEATH FACTOR 4 SIGNALING PATHWAYS

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One of the complex chronic consequences of diabetes is testicular dysfunction, which is well-known yet still has a murky origin. Objective: With the aim of preventing testicular dysfunction in diabetic rats, we looked into the potential effects of orlistat and gemfibrozil on vascular endothelial growth factor (VEGF) as a marker of angiogenesis, nitric oxide (NO) as an oxidative stress marker, and programmed cell death factor 4 (PDCD4) as an apoptotic marker. Methods: Streptozotocin (STZ, 40 mg/kg/i.p. once) was used to induce diabetes in adult rats, and the treated groups received treatment started one week before induction. Rats were sacrificed three weeks later, and blood samples were obtained for hormonal analysis. Additionally, testicular tissue was examined for testicular oxidative stress, inflammatory markers, and apoptotic markers. Additionally, testicular VEGF and androgen receptors were evaluated, and testicular damage was identified. Results: The results revealed a notable decrease in PDCD4 signaling along with a considerable improvement in inflammatory markers, and oxidative stress status. Conclusion: Our data highlight the significance of VEGF/NO/ PDCD4 signaling pathways in the genesis of testicular dysfunction as a consequence of diabetes mellitus and point to the positive benefits of orlistat and gemfibrozil, either separately or in combination, in reducing testicular damage in diabetic rats.

Keywords : *Gemfibrozil- Orlistat- angiogenesis- Oxidative stress- Apoptosis.*

INTRODUCTION

Diabetes mellitus prevalence is rising quickly in individuals of all ages, and it is a systemic metabolic condition with numerous macro and micro-vascular health issues[1]. Negative effects of diabetic hyperglycemia on both female and male reproductive functions are known[2]. Male reproductive system issues caused by diabetes include erectile dysfunction, aberrant semen modification, and dysfunctional ejaculation[3]. Additionally, testicular dysfunction is a related issue whose specific cause is still not fully understood[4].

Gemfibrozil is a fibrate medication used for decreasing cholesterol[5&6]. In addition to being a PPAR-activator, it also demonstrated anti-inflammatory and anti-oxidative stress properties, although its exact mode of action is unknown[7&8].

By suppressing gastric and pancreatic lipases, the obesity drug orlistat changes the

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nutrients absorption in the gastrointestinal tract and excretes around 30% of the fat consumed[9]. It has shown promise in lowering obesity-related comorbidities like endothelial dysfunction and metabolic syndrome in people in addition to helping them lose weight[10&11].

For wound healing and tissue regeneration, the physiological angiogenesis process, which is the creation of new blood vessels from pre-existing ones, is of utmost importance. Vascular endothelial growth factor (VEGF) controls angiogenesis, which causes innervation of vessels and causes new blood vessels growth. Angiogenesis is critical in this regard for specific organs, including testicular and ocular tissue[12&13].

A unique tumour suppressor gene, called "Programed Cell Death Protein 4" (PDCD4), has recently been identified. Numerous studies have shown that PDCD4 contributes to the development of a number of metabolic illnesses by disrupting gut microbiota, oxidative stress, insulin resistance, the chronic inflammatory response, and glucose and lipid metabolism abnormalities[14].

Determining the effects of Orlistat and Gemfibrozil, each alone or together, on testicular dysfunction in the diabetes model as well as the underlying molecular processes pertaining to apoptotic and oxidative reactions, involving the VEGF/nitric oxide (NO)/PDCD4 or mitochondrial apoptotic cell death factor signaling pathways, was the goal of the current experimental work.

MATERIAL AND METHODS

Drugs and chemicals

Orlistat, gemfibrozil and Streptozotocin were obtained from (Sigma-Aldrich, Egypt).

Experimental animals

The research was performed at the Faculty of Medicine, Tanta University, Egypt. In the animal house of the Pharmacology Department, 50 male Sprague-Dawley (SD) rats (6–8 weeks old) weighing 120–150 g were stored under specified conditions of humidity (50%), temperature (23°C), and lighting (12 hours of light/12 hours of darkness). We fed rats a typical laboratory diet and allowed unlimited access to tap water ad libitum. As per the institutional animal care instructions, all animals got humane attention. Tanta University's Experimental Animal Ethical Committee gave the approval to the work protocol **(approval code: 35926/10/22)**. One week was allowed for acclimatization prior to the start of the experiment.

Induction of a Diabetes model by STZ

All groups from 2 to 5 were deprived of food for 12 hours but with free access to water before the induction of D.M. For this, 20 mmol of freshly prepared citrate buffer (Sigma-Aldrich) dissolved 200 mg of streptozotocin, which was administered intraperitoneally (IP)[15] in a dose of 40 mg/kg[16]. Through a tail puncture, a glucometer (Beurer GmbH, Ulm, Germany) measured the blood glucose levels after 48 hours Diabetic animals were those with blood glucose levels above 200 mg/dL.

Experimental design and treatment protocol

This research involved 50 male Sprague-Dawley (SD) rats. Randomly, the animals were sorted into 5 equal groups of ten rats each:

- **Group I:** acted as a standard control group, receiving saline vehicles (i.p.) once, as well as 0.5% CMC and distilled water through oral gavage.
- **Group II:** Served untreated diabetic group caused by STZ as a single intraperitoneal (i.p.) injection and were received vehicles of 0.5% CMC and distilled water via oral gavage.
- **Group III:** were recieved orlistat at a dose of 10 mg/kg/day[17]. Orlistat was delivered orally via gavage after being suspended in 0.5% CMC and purified water.
- **Group IV:** were given gemfibrozil at a dose of 100 mg/kg/day[18]. gemfibrozil in a 0.5% CMC solution was administered by oral gavage.
- **Group V:** were administered both orlistat and gemfibrozil in the same dosing regimen as described previously.
- The treatment procedure with CMC and distilled water vehicles (for group I and II) and oral drugs (for group III, IV, and V) began one week before diabetes induction and continued until the trial ended on the 21st day.

Sample collection

At the completion of the experiment (the 21st day), all rats were sacrificed under ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively) anesthesia. We centrifuged blood samples at 3000 ×g (centrifugal force) for 15 min and kept at -20 °C till usage. Rats' testis were excised, weighed, and then rinsed with a phosphate-buffered saline (PBS) solution (pH 7.4) to eliminate any red blood cells or clots. The left testis was promptly frozen in liquid nitrogen and then maintained at -80 °C for RNA extraction while the right testis was fixed in Bouin solution for histomorphological study.

Hormone Analysis

Testosterone was evaluated utilizing commercial kits (Mouse/Rat Testosterone ELISA Kit from Abcam, Tokyo, Japan, Cat # ab285350) in accordance with the guidelines of the manufacturer. We assessed the optical density (OD) at 450 nm.

Testicular oxidative stress evaluation

The testis homogenate was aliquoted and kept at -80°C till usage. Spectrophotometric kits were used to evaluate the activity of SOD and glutathione peroxidase (GPx) in each sample aliquot (Biodiagnostic, Giza, Egypt). SOD activity (Catalog No: SD 25 21) was evaluated using the technique illustrated by Sun et al.[19], with absorbance measured at 480 nm. Additionally, the activity of GPx (Catalog No: SD 25 11) was measured using the procedures described by[20], which are dependent on the GSH oxidation by a hydrogen peroxide substrate. The thiobarbituric acid-reactive compounds test was utilized to determine MDA levels, and absorbance was evaluated at 532 nm[21]. (Catalog No: SD 25 29). Testicular inducible nitric oxide synthase (**iNOS**) activity was evaluated by the ELISA kit (MyBioSource, Catalog # MBS723326).

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was obtained from testicular tissues utilizing Trizol (Invitrogen; Thermo Fisher Scientific, Inc.) For assessing the RNA quality, the A260/A280 ratio was analyzed utilizing the NanoDrop® ND–1000 Spectrophotometer (NanoDrop Technologies; Wilmington, DE, USA) The estimated purity used for any given RNA was between 1.8 and 2.0, followed by cDNA synthesis utilizing the (**Applied Biosystems™**, **USA)**, GAPDH was utilized as an internal standard of mRNA expression. The real-time-PCR was carried out in an Mx3005P Real-Time PCR System (Agilent Stratagene, USA) utilizing TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Cat. # P725 or P750) **(Enzynomics, Korea)** following the instructions of the manufacturer. We performed PCR was in a 20 µl reaction mixture having 5 µl cDNA, 1 µl of 10 pM of each primer (forward and reverse), 10 µl SYBR Green master mix, and H_2O PCR grade up to 20 µl. PCR cycling conditions involved an initial denaturation at 95°C for 12 minutes to activate the chemically modified PCR DNA polymerase followed by 40 cycles of denaturation for 10 seconds at 95°C, annealing for 15 seconds at 60°C, and extension for 30 seconds at 72°C. The oligonucleotide-specific primers were formed by Sangon Biotech (Beijing, China). (Table 1) Outcomes are presented as fold-changes in comparison to the control group following the $2^{-\Delta\Delta CT}$ technique[22].

Histological Examination

Each group right testis was divided longitudinally into two halves, fixed in Bouin solution for 4-5 hours until it hardened, and then prepared for paraffin blocks. After processing, it was wax-embedded in paraffin. Then, tissues were groupified into parts (5 mm thick), and stained with Periodic acid Schiff reaction (PAS) for identification of neutral mucopolysaccharides, hematoxylin and eosin (H&E) and Masson trichrome for identification of collagen fibers[23].

Table 1: Primers utilized for real-time PCR analysis

Gene name	Primer sequence		Gene accession number
	Forward:	Reverse:	
Bax	CGAATTGGCGATGAACTGGA	CAAACATGTCAGCTGCCACAC	NM_017059.2
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA	NM_016993.1
Casp-3	GAGACAGACAGTGGAACTGACGATG	GGCGCAAAGTGACTGGATGA	NM_012922.2
p53	CTACTAAGGTCGTGAGACGCTGCC	TCAGCATACAGGTTTCCTTCCACC	NM_030989.3
Gapdh (Rat)	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	NM_017008.4
TNF-α	ACTGAACTTCGGGGTGATCG	GCTTGGTGGTTTGCTACGAC	NM_012675.3
NF-κB	CGCGGGGACTATGACTTGAA	AGTTCCGGTTTACTCGGCAG	NM_199267.2
L-1β	GACTTCACCATGGAACCCGT	GGAGACTGCCCATTCTCGAC	NM_031512.2
Pcdc4	GGATGTCCCACACTCATACTC	GACCTCCATCTCCTTCACTTAC	NM_022265.2

Immunohistochemical examination

Testicular immunoexpression of VEGF and androgen receptor (AR) was assessed by immunohistochemistry staining on 5 µm sections of the testis. Briefly, the testis sections that had been embedded in paraffin were dewaxed and rehydrated. Using a primary polyclonal rabbit anti-rat antibody, serial sections were stained for VEGF (Cat. # PA1-21796 Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.A) and for AR (ab3510, Abcam, Cambridge, Massachusetts, USA). The immunological response was demonstrated using diaminobenzidine (DAB). When the DAB reaction's brownish colouring was seen in the sections, it was quickly rinsed off. It was done to counterstain with Mayer's hematoxylin. Negative controls were prepared by the exclusion of the primary antibodies[24].

Morphometric study

We gathered images utilizing a Leica microscope (DM3000, Leica, Germany) coupled with a Leica camera (DFC-290, Leica, Germany). Ten distinct, selected fields randomly from every slide were investigated for:-

1. Mean diameter of seminiferous tubules was assessed in H&E stained sections.
2. Mean thickness of germinal epithelium in H&E stained sections.
3. Mean area percentage (area %) of reaction for PAS stain
4. Mean area percentage (area %) of collagen fiber content (in Masson trichrome-stained sections).
5. Mean colour intensity of VEGF immunopositive cells (in DAB-stained sections).
6. Mean colour intensity of AR immunopositive cells (in DAB-stained sections).

Statistical analysis

The statistical programme for the social sciences (SPSS) version 21.0 was utilized to statistically evaluate the results as mean standard deviation (IBM Corp., Armonk, NY, USA). A post-hoc test was employed after one-way analysis of variance (ANOVA) for many comparisons to assess the statistical significance across experimental groupes. The Pearson test was used to examine correlations. P value less than 0.05 was regarded as significant.

Sample size

Assuming the mean testicular SOD was 18.9±7.34 vs 25.6±5.71 in control vs orlistat group. At 80% power and 95% CI, the estimated sample will be 55 rats, with 11 rats in each group open EPI.

RESULTS AND DISCUSSION

Results

Effect of Orlistat/ Gemfibrozil on *testicular oxidative stress*

Administration of STZ led to oxidative stress of testicular tissues as suggested by significantly increased levels of prooxidants MDA and iNOS activity levels in the diabetes group II in comparison with control group I, antioxidants like SOD and GPx activity levels were significantly lower. Similarly, the total GSH level elevated markedly in the DM group relative to the control group. Conversely, these alterations were markedly enhanced in the treated groupes administrated either orlistat or gemfibrozil or both when compared with the control. Combination therapy in group V was significantly lower than groupes III and IV in iNOS level and also significantly higher in GPX level than both groupes (III and IV).

Effect of Orlistat/ Gemfibrozil on *mRNA levels of apoptosis-related markers*

The mRNA expressions of p53, Bax, and caspase-3 were markedly higher in DM group (II) relative to the control group (I). In contrast to the control group, Bcl2 expression was downregulated in the diabetic-treated rats. Both models of orlistat/ Gemfibrozil intervention, either alone or combined, significantly enhanced the anti-apoptotic pathway via the downregulation of P53 and upregulation of Bcl2, Bax, and Casp3 in comparison to the diabetic and control groupes. The proportion of Bax to Bcl2 (Bax/Bcl2) was computed as 1.0, 2.3 ± 1.1, 1.47 ± 0.35, 1.48 ± 0.37, 1.2 ± 0.3$ for the control, diabetic, diabetes group treated by orlistat, diabetes group treated by gemfibrozil, diabetes group treated by both orlistat and gemfibrozil, respectively.

In comparison to controls, diabetic rats' testicular expression of PDCD4 mRNA was considerably greater ($p<$ 0.001). Orlistat / Gemfibrozil treatment, either alone or combined, markedly reduced the expression of mRNA of PDCD4 in diabetic rats compared to the control and diabetic group.

A combination of both treatments significantly decreased PDCD4, P53, and caspase 3 expressions in group V in comparison to group III. Meanwhile, comparing group V with IV was significantly lowered in PDCD4, P53, and caspase-3 expressions and higher in BCL2 expression level.

Table 2: Imapct of Orlistat/ Gemfibrozil on testicular oxidative stress parameter in DM.

	GroupI (n=11)	**GroupII (n=11)**	**GroupIII (n=11)**	**GroupIV (n=11)**	**GroupV (n=11)**	**P-value**
SOD activity (unit/mg protein)	2.39±0.06$^{\$}$	1.01±0.1$^{\$*}$	2.3±0.05$^{\$*}$	2.3±0.09$^{\$*}$	2.37±0.07$^{\$}$	0.001
GPx activity (unit/mg protein)	42.9±0.7$^{\$}$	26±1.14$^{\$*}$	36.1±1.57$^{\$*}$	34.9±1.3$^{\$*}$	40.9±1.54$^{\$\#@}$	0.001**
MDA level (nmol Eq /mg protein)	1.25±0.048$^{\$}$	5.0±0.6$^{\$*}$	2.2±0.15$^{\$*}$	2.35±0.15$^{\$*}$	1.8±0.169$^{\$\#}$	<0.001**
iNOS activity (ng/mg protein)	2.2±0.08$^{\$}$	4.18±0.296$^{\$*}$	2.8±0.227$^{\$*}$	2.84±0.13$^{\$*}$	2.32±0.089$^{\$\#@}$	0.001

p<0.001

*p<0.05 compared to the control

$^{\$}$p<0.05 compared to DM

$^{@}$ p<0.05 compared to orlistat treated group III

$^{\#}$ p<0.05 compared to gemfibrozil-treated group IV

Table 3: Impact of Orlistat/ Gemfibrozil on mRNA expression of apoptosis-related genes.

	Group I (n=11)	Group II (n=11)	Group III (n=11)	Group IV (n=11)	Group V (n=11)
P53	$1.0^{\$}$	$1.57\pm0.12^{\$*}$	$1.19\pm0.54^{\$*}$	$1.28\pm0.06^{\$*}$	$1.05\pm0.048^{\$\#@}$
BAX	$1.0^{\$}$	$1.58\pm0.13^{\$*}$	$1.28\pm0.09^{\$*}$	$1.23\pm0.09^{\$*}$	$1.13\pm0.075^{\$}$
BCL2	$1.0^{\$}$	$0.75\pm0.057^{\$*}$	$0.87\pm0.03^{\$*}$	$0.85\pm0.033^{\$*}$	$0.95\pm0.033^{\$\#}$
BAX/BCL2 ratio	$1.0^{\$}$	$2.3\pm0.33^{\$*}$	$1.47\pm0.106^{\$*}$	$1.48\pm0.11^{\$*}$	$1.2\pm0.09^{\$}$
Caspase -3	$1.0^{\$}$	$2.1\pm0.067^{\$*}$	$1.37\pm0.09^{\$*}$	$1.6\pm0.09^{\$*}$	$1.1\pm0.045^{\$\#@}$
PDCD4	$1^{\$}$	$1.75\pm0.12^{*\$}$	$1.32\pm0.09^{*\$}$	$1.32\pm0.1^{*\$}$	$1.1\pm0.039^{\$\#@}$

$p<0.001$
*$p<0.05$ compared to the control.
$^{\$}p<0.05$ compared to DM.
$^{@}$ $p<0.05$ compared to orlistat treated group III.
$^{\#}$ $p<0.05$ compared to gemfibrozil-treated group IV.

Effect of Orlistat/ Gemfibrozil on mRNA levels of inflammation-related markers

The levels of mRNA of the pro-inflammatory markers, IL-1β, TNF-α, and NF-κB, were markedly increased in the DM group relative to the NC group. These negative impacts were diminished in the other three models of Orlistat/ Gemfibrozil or both interventions with TNF-α, NF-β, and IL-1β markedly lower in group V relative to group I. Significant lower expression levels of IL-1β and TNF-α were detected when comparing group V with group III and IV.

Effect of Orlistat/ Gemfibrozil on serum testosterone level in diabetic rats

In comparison to the control, STZ treatment markedly reduced testosterone levels (p 0.05). Compared to the diabetic group, the group treated with Orlistat/ Gemfibrozil or both had an obvious rise (p 0:05) in testosterone hormone levels. When compared to groupes III and IV, group V demonstrated an obvious elevation in testosterone levels.

Table 4: Impact of Orlistat/ Gemfibrozil on mRNA expression of inflammatory markers related genes .

	I (n=11)	II (n=11)	III (n=11)	IV (n=11)	V (n=11)
NF-β	$1.0^{\$}$	$1.6\pm0.1^{\$*}$	$1.18\pm0.06^{\$*}$	$1.2\pm0.07^{\$*}$	$1.05\pm0.03^{\$}$
TNF-α	$1.0^{\$}$	$1.75\pm0.08^{\$*}$	$1.32\pm0.08^{\$*}$	$1.37\pm0.08^{\$*}$	$1.1\pm0.039^{\$\#@}$
IL-1β	$1.0^{\$}$	$1.73\pm0.08^{\$*}$	$1.41\pm0.08^{\$*}$	$1.58\pm0.069^{\$*}$	$1.055\pm0.03^{\$\#@}$

$p<0.001$
*$p<0.05$ compared to the control
$^{\$}p<0.05$ compared to DM
$^{@}$ $p<0.05$ compared to orlistat treated group III
$^{\#}$ $p<0.05$ compared to gemfibrozil-treated group IV

Table 5: Effect of Orlistat/ Gemfibrozil on serum testosterone level in diabetic rats**.**

	I (n=11)	II (n=11)	III (n=11)	IV (n=11)	V (n=11)
$\bar{X}\pm SD$	$5.65\pm0.27^{\$}$	$4.33\pm0.139^{*\$}$	$5.0\pm0.1^{*\$}$	$4.84\pm0.169^{*\$}$	$5.57\pm0.29^{\$\#@}$

$p<0.001$
*$p<0.05$ compared to the control
$^{\$}p<0.05$ compared to DM
$^{@}$ $p<0.05$ compared to orlistat treated group III
$^{\#}$ $p<0.05$ compared to gemfibrozil-treated group IV

Effect of on testis histological features

Hematoxylin and eosin stained sections

The testicular samples of **group 1 (control group)** showed the normal histological architecture of the testis; large seminiferous tubules were closely packed together with minimal interstitial tissue containing interstitial cells of Leydig. The germinal epithelial lining of the seminiferous tubules was thick, with numerous layers of cells ordered from the thin basement inwards: spermatogenic cells, spermatocytes, spermatids, and sperms in the lumens of the tubules **(Fig. 1 A& F).** Sections from **group II** showed significant loss of testicular architecture. The seminiferous tubules were distorted; some were shrunken, and others appeared collapsed and heavily infiltrated by mononuclear cellular infiltration. Some tubule lumens were empty of sperm. Meanwhile, several lumens revealed exfoliated pyknotic nuclei. The germinal epithelium of numerous seminiferous tubules seemed thin, with degeneration and pyknosis of many nuclei. The interstitial space revealed acidophilic exudate **(Fig. 1 B&G). Group III** showed mild noticeable improvement in the histopathological picture compared to group 2; the seminiferous appeared larger but its germinal epithelium revealed pyknosis of its nuclei. In the interstitial area, acidophilic material deposition was found **(Fig. 1C &H**). **Group IV** revealed obviously normal testicular architecture with minimal signs of tubular damage. Germinal cells were obviously similar to normal (**Fig. 1D &I).** Moreover, sections from group V showed similar to normal histological outcomes with large seminiferous tubules tightly packed together. Spermatogenic cells, spermatocytes, spermatids, and sperm made up the seminiferous tubules' thick germinal epithelial lining with little interstitial tissue containing Leydig interstitial cells **(Fig. 1E &J).** Morphometric analysis of the mean diameter of the seminiferous tubules and mean thickness of the germinal epithelium exhibited a highly marked reduction in group II in comparison to group I. (Table 6)**.**

PAS stained sections

PAS-stained sections of the testis demonstrated a strong PAS-positive reaction in the basement membrane of the seminiferous tubules **(Fig. 2 A)**. Group II depicted a faint PAS-positive reaction. The basement membranes of the seminiferous tubules were partially disrupted **(Fig. 2 B)**. As opposed to that, sections from group III revealed weak interrupted PAS-positive basement membranes **(Fig. 2 C)**. Moreover, sections from group IV & group V depicted a near-normal strong PAS-positive reaction **(Figs. 2 D &E, respectively)**. Groupes II & III showed a significantly lower mean area percentage of PAS-stained sections as compared to group I in morphometric measurements. However, a marked elevation was revealed in groupes IV &V. (Table 6).

Masson Trichrome stained sections

The amounts of collagen fibers around seminiferous tubules; tunica albuginea and interstitial spaces were minimal in Masson trichrome-stained sections of the testis in the control group **(Fig. 2 F)**. Group II revealed that the number of collagen fibers had markedly elevated, particularly in the interstitial tissue and tunica albuginea **(Fig.2 G).** The tunica albuginea and the interstitial spaces between the seminiferous tubules in group III exhibited moderate collagen fiber deposition **(Fig.2 H).** As opposed to that, group IV demonstrated a small number of collagen fibers in the tunica albuginea and between the seminiferous tubules **(Fig.2 I).** Meanwhile, few collagen fibers are visible in group V **(Fig.2 J).** The mean area percentage of Masson trichrome stained sections from group II showed a marked elevation as compared to group I. Though, group V displayed a marked reduction area percentage of collagen fibers in comparison with group II (Table 6).

VEGF immunohistochemical stained sections

Immunostained sections from the control group (group I) showed few cells with faint positive VEGF immunohistochemical reaction expressed by a brownish cytoplasmic coloration **(Fig. 3A).** Although, sections from group II revealed plenty of cells with a high positive VEGF immunoexpression **(Fig. 3B).** Group III sections; as opposed to that, revealed several cells with a moderately positive VEGF immunoreactivity **(Fig. 3C).** But group IV sections demonstrated some cells with a positive VEGF reaction **(Fig. 3D)**. Sections from group V displayed a VEGF reaction that

was nearly normal **(Fig. 3E)**. Morphometric analysis of the mean color intensity of positive VEGF reaction depicted a marked elevation in group II and group III compared to the control group. While group V demonstrated a marked reduction in the mean color intensity of VEGF immunoreactivity as compared with groupes 2 &3. As opposed to that, there was a non-significant difference in VEGF immunoexpression between group I and group V (Table 6).

AR immunohistochemical stained sections

The control group (group I) had a robust positive AR immunoreaction in the cytoplasm in the AR immunostained sections **(Fig. 3 F)**. Sections of group II had very faint cytoplasmic AR immunoreactivity **(Fig. 3 G)**. Group III demonstrated a weakly positive AR immunoreaction **(Fig. 3 H)**. In group IV, there was a moderate immunostaining reaction for AR **(Fig. 3 I)**. However, group V sections showed more intense AR immunostaining **(Fig. 3 J)**. Morphometric analysis of the mean color intensity of positive AR immunoreactions in groupes II and III demonstrated a significant reduction when compared to the control group. In comparison to group II, there was a considerable elevation in AR immunoexpression in group V. (Table 6)

Table 6: showing statistical analysis of morphometric measurements in the different studied groups.

Parameters/ Groupes	**Group I**[a] (control group) (n=10)	**Group II**[b] **(**Untreated diabetic group) (n=10)	**Group III**[c] (Orlistat treated diabetic group) (n=10)	**Group IV**[d] (Gemfibrozil treated diabetic group) (n=10)	**Group V**[e] (Orlistat+ gemfibrozil treated diabetic group) (n=10)
Mean diameter of seminiferous tubules (µm)	302.13± 5.98[b,c]	181.93± 7.13[a,c,d,e]	260.44± 10.18[a,b,e]	288.02± 13.01[b]	297.51± 6.88[b,c]
Mean thickness of germinal epithelium (µm)	84.14± 1.75[b,c]	57.09± 1.59[a,c,d,e]	67.93± 2.01[a,b,e]	68.25± 2.93[b]	80.99± 3.01[b,c]
Mean area percentage% of PAS stain	24.83± 1.53[b,c]	12.59± 0.921[a,d,e]	15.58± 0.97[a,d,e]	19.96± 1.08[b,c]	22.77± 1.45[b,c]
Mean area percentage% of Masson trichrome stain	9.95± 0.33[b,c,d]	18.14± 0.74[a,c,d,e]	15.21± 0.47[a,b,d]	12.33± 0.88[a,b,c]	10.34± 0.62[b,c]
Mean color intensity of VEGF immunohistochemical staining	14.93± 1.04 [b,c,d]	47.64± 1.14[a,c,d,e]	37.74± 0.84 [a,b,d,e]	24.47± 0.89 [a,b,c,e]	15.96± 0.79 [b,c,d]
Mean color intensity of AR immunohistochemical staining	60.7± 1.35 [b,c,d]	36.83± 1.48 [a,c,d,e]	43.31± 1.97 [a,b,d,e]	52.38± 1.66 [a,b,c,e]	58.89± 1.82 [b,c,d]

a–e One-way ANOVA test with Tukey's post hoc test was utilized to assess whether there was a significant difference between the groupes at *p <0.05. A indicates significance from group I, b indicates significance from group II, c indicates significance from group III, d indicates significance from group IV, and e indicates significance from group V. Vascular endothelial growth factor, often known as VEGF, and AR: androgen receptor.

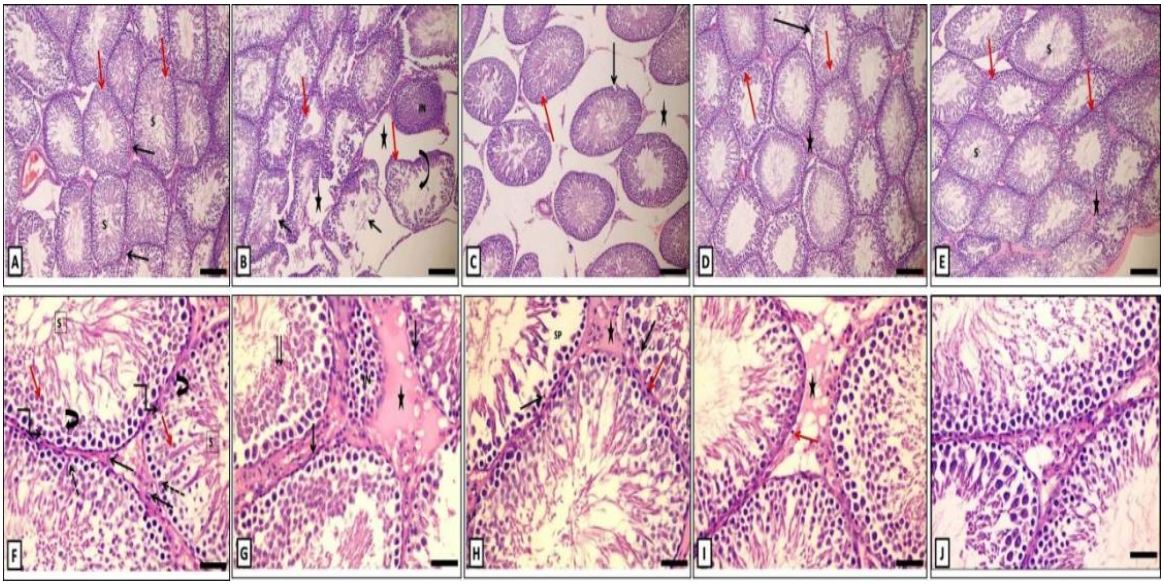

Fig. 1: Photomicrographs of **H&E stained** sections from rat's testis showing **A)** In class I normal histological architecture of testis**;** large closely packed seminiferous tubules lined by thick germinal epithelium (red arrows) with abundant sperms (S) in the lumen of each tubule and minimal interstitial tissue in between (black arrows). **B)** in class II, testicular architecture is disturbed; shrunken, widely separated seminiferous tubules (stars). Some tubules depict thin germinal epithelium (red arrows), and their lumens are devoid of sperms (curved arrow); the others are obliterated by heavy mononuclear cellular infiltration (IN). The basement membrane of many seminiferous tubules is interrupted (black arrows). **C)** In class III, the seminiferous tubules appear larger and lined by thicker germinal epithelium (red arrow). But they are still widely separated (star), and the basement membrane is interrupted (black arrow). **D)** In class IV, the seminiferous tubules are closely packed with minimal interstitial space. Meanwhile, it is filled with homogenous eosinophilic exudate (star). The germinal epithelium appears thicker (red arrow). Interrupted widely separated basement membrane (black arrow) could be seen. **E)** In class V, the testis seems histologically normal. Normal closely packed large seminiferous tubules with thick germinal epithelial lining and abundant central sperms (S). Notice, few interstitial spaces are filled with homogenous eosinophilic exudate (star). **At higher magnification: F)** class I shows normal seminiferous tubules lined with normal germinal epithelium; spermatogonia (zigzag arrows), primary spermatocytes (curved arrows), spermatids (red arrows) and numerous sperms in the lumens. Sertoli cells (dashed arrows) could be seen in between the spermatogenic cells. Leydig interstitial cells can also be detected (black arrows). **G)** Class II showing disturbed seminiferous tubules. Numerous spermatogenic cells have pyknotic nuclei (arrows). The interstitial space displays mononuclear infiltrates (IN) and Eosinophilic exudate (star). Many exfoliated pyknotic nuclei (double arrows) could be noticed. **H)** Class III demonstrates a moderate unorganized epithelium of seminiferous tubules with wide spaces (SP) between cells, and Pyknotic nuclei can be seen in some spermatogenic cells (black arrows) and some interstitial cells of Leydig (red arrows). Acidophilic material deposition (star) is present in the interstitial space. **I)** class IV shows mild wide interstitial spaces with acidophilic material (star), and few interstitial cells of Leydig have pyknotic nuclei (arrow) **J)** class V the architecture of seminiferous tubules is relatively near to the normal with normal germinal epithelium and narrow interstitial spaces. (A-E x100, scale bar = 200 μm), (F-J x400, scale bar = 50 μm)].

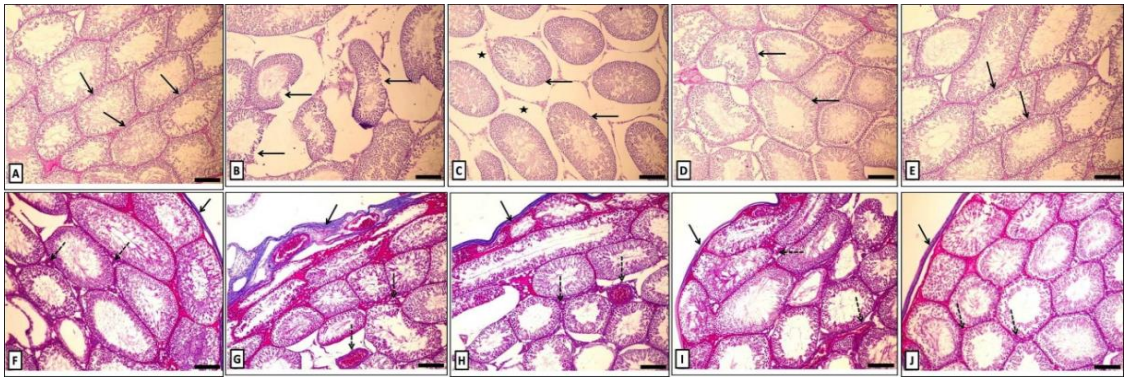

Fig.2: (A-E) Photomicrographs of **PAS-stained** testis sections: **A)** class I revealing germinal epithelium sitting on a uniformly thin, strongly PAS-positive basement membrane (arrows). **B)** Class II showing faint PAS-positive reaction in the partial interrupted basement membrane of the seminiferous tubules (arrows) with wide spaces in between (stars). **C)** Class III displaying apparent weak PAS-positive reaction in the basement membranes (arrows) of the seminiferous tubules**. D)** Class IV demonstrating a great PAS-positive reaction in the basement membrane (arrows) of the seminiferous tubules. **E)** Class V revealing a very great PAS-positive reaction in the basement membranes of most of the seminiferous tubules. (F-J) Photomicrographs of **Masson's trichrome-stained** testis sections: **F)** class I exhibiting thin tunica albuginea (arrow) surrounds the seminiferous tubules, with only a few collagen fibres (dashed arrow) in between seminiferous tubules and around the blood vessels. **G)** Class II displaying markedly thick tunica albuginea with visible layer separation (arrow) and increased collagen fiber deposition (dashed arrows) in between seminiferous tubules and around the blood vessels. **H)** Class III showing moderate collagen fibers deposition in the tunica albugina (arrow) and in between the seminiferous tubules (dashed arrows). **I)** Class IV showing mild collagen fibers deposition in the tunica albuginea (arrow) and in between the seminiferous tubules (dashed arrows). **J)** Class V revealing minimal collagen fibers in the tunica albuginea (arrow) and in between the seminiferous tubules (dashed arrows). (Magnification ×200, scale bar=100 µm).

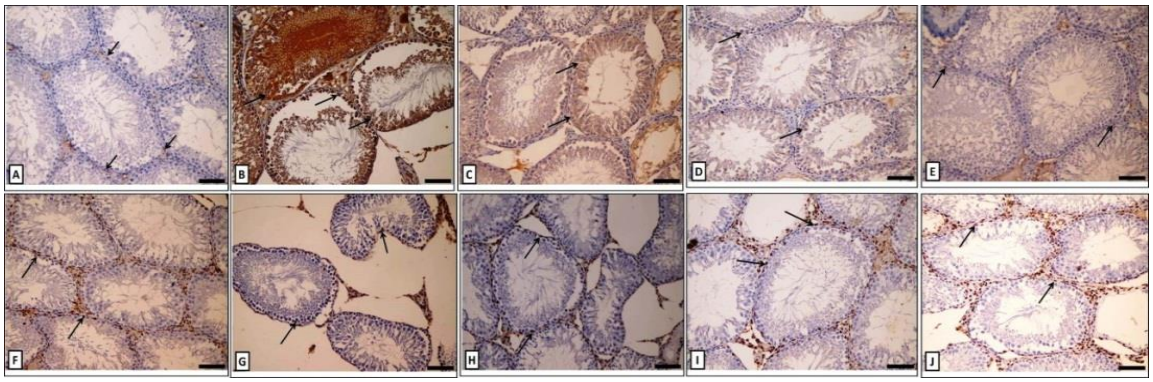

Fig. 3: Photomicrographs of immunohistochemical stained sections from rat's testis:- **(A-E) VEGF immunohistochemical staining** showing **A)** in class I: few cells with faint positive immunoreaction (arrows), **B):** in class II: numerous cells with very great positive immunoreaction (arrows), **C)** in class III: many cells with moderate positive immunoreaction (arrows), **D)** in class IV: some cells with mild positive immunoreaction, **E)** in class V: few cells with weak positive immunoreaction (arrows). **(F-J) AR immunohistochemical staining** showing: **F)** in class I: many cells with strong positive immunoreaction (arrows), **G):** in class II: very weak immunoreaction (arrows), **H)** in class III: few cells with positive immunoreaction (arrows), **I)** in class IV: some cells with strong positive immunoreaction, **J)** in class V: multiple cells with great positive immunoreaction (arrows). **[Magnification X200, scale bar = 100 µm].**

Discussion

The main cause of the changes in endoneurial metabolism, such as those seen in the advanced glycation, polyol pathway, essential fatty acid metabolism, protein kinase C, neurotropic factors, and nerve blood flow, is hyperglycemia (25, 26). Numerous pieces of evidence indicate that a number of crucial mechanisms, including the suppression of testosterone synthesis, oxidative stress induction, and the activation of mitochondria-dependent apoptosis, have been believed to have a vital effect on the incidence of testicular dysfunction as a complication of diabetes and, consequently, could lead to temporal and complete infertility[27].

Since diabetes mellitus has been linked to testicular dysfunction in both humans and rats, an animal model of diabetes was induced using STZ in the current work[28]. According to the current study's testicular oxidative stress status, our results are consistent with earlier research that demonstrated that orlistat or gemfibrozil therapy dramatically reduced the levels of oxidative stress in various rat models[29]. Our results coincides with previous studies as regard to antioxidant effect of each ortilstat and gemfibrozil on testicular tissue in different models respectively[30&31].

We proposed that a decrease in enzyme synthesis, an increase in enzyme breakdown, or the overproduction of ROS results in the inactivation of the enzymes could all contribute to the decline in antioxidant enzyme activity[32] with more significant antioxidant effect as a novel combination.

Apoptosis is a distinct mechanism of programmed cell death which is brought on by specific circumstances and controlled by a number of genes[33]. Additionally, one of the main causes of decreased male fertility has been attributed to the death of testicular spermatogenic cells[34]. The activation of Bax/Bcl-2 and the start of caspase may cause male germ cells to die, and the NF-kB has a vital effect in encouraging the transcription of genes included in this process[33].

A rise in oxidative stress can stimulate Bax, disrupt mitochondrial role, and stimulate the caspase pro-apoptotic system, based on a few researches that significantly revealed more apoptotic cell death and injury in obese mice and rats models. These studies involved Fas-based and mitochondrial-based apoptotic pathways[35&36].

In the current work, we revealed that both apoptotic pathways are stimulated in diabetic rats, as suggested by marked elevation in Bax and Casp-3 mRNA expressions observed in the untreated group. Additionally, the untreated group's Bcl-2 mRNA expression was much lower than it was in the normal group that may be related to the elevated oxidative stress. In comparison to the control group, the orlistat-treated rats displayed a considerable enhancement in the mRNA expression of these pro- and anti-apoptotic indicators. This might be as a result of its protective impact against oxidative stress, which results in fewer instances of apoptotic cell death.

Clinical investigations show that people with diabetes have chronic inflammation in a variety of tissues as a result of the release of certain signaling proteins that cause inflammatory reactions[37]. Orilstat was found to reduce oxidative stress linked inflammatory reaction in myocardial tissue which simulate its effect in testicular tissue in our study[38].

Our findings are consistent with earlier research[39], which showed that the presence of oxidative damage in tissues indicates a likelihood of inflammation since the two are connected and share some common activation stimuli (ROS). Previous researches have observed testicular inflammation in diabetic rats, which is compatible with our current results. Additionally, elevated levels of TNF-, iNOS, and IL-6 as well as NF-B stimulation have been seen in the testes of diabetic rats.

The significant drop in testosterone levels found in diabetic rats in the current work was consistent with numerous other studies[40]; the tissues of the testes may have experienced excessive oxidative stress and apoptosis, which exacerbate testicular failure[41].

It's important to be aware that orlistat, gemfibrozil, or combinations thereof were capable of reducing testicular damage, as shown by a decrease in the sex hormone concentrations, which suggested a protective ability of orlistat, gemfibrozil, or a superior combination thereof. Furthermore, the elevated levels of TNF- and NF-B that are recognized to enhance the apoptotic pathway, suggest the inclusion of the extrinsic apoptotic pathway[42].

Furthermore, earlier research pointed out the role of previously discussed pathogenic pathways that underlie testicular dysfunction in people with uncontrolled diabetes. However, growing experimental and clinical data have revealed that angiogenesis may has a vital role in the pathophysiology of the disease. Angiogenesis is a natural adaptation, but in some cases, such as during pathological situations like diabetic retinopathy and solid organ cancers, it loses its normal control and causes disruptions in organ function[43].

VEGF Vascular endothelial growth factor (VEGF), also identified as a neurotrophic and angiotrophic factor, causes endothelial cells to proliferate and makes the artery wall more permeable[13]. VEGF is produced by Sertoli and Leydig cells, which also have VEGF receptors[44]. VEGF is regarded as a crucial modulator of angiogenesis and plays a role in germ-cell homeostasis[45].

Additionally, poor cellular oxygenation can increase VEGF synthesis, which, if unchecked, leads to irregular angiogenesis (46). As VEGF regulates proliferation of endothelial cell and maintains a sufficient blood supply, keeping VEGF levels within the proper range represents a primary target in organs where microcirculation is especially important, like the testis and retina[47];Additionally, mammalian testicular microcirculation is essential for basic testicular activities such as spermatogenesis and paracrine and hormonal regulation. Leydig and Sertoli cells secrete VEGF, and the testicular tissue contains VEGF receptors[45]; as a result, treatment approaches focusing on the regulation of angiogenesis may be effective in treating the testicular dysfunction brought on by diabetes mellitus. Therefore, the aim of our work was to reveal how gemfibrozil and orlistat, either separately or together, can reduce testicular dysfunction in diabetic rats through a new mechanism that targets and controls angiogenesis and endothelial cell proliferation.

Our findings suggested that gemfibrozil treatment effectively attenuated the testicular dysfunction-in the diabetic rats . These findings are due to its inhibitory effects on inflammation and angiogenesis that occur in testicular tissue as a complication of hyperglycemia through inhibition of VEGF signalling pathways. These results were in concomitant with recent data that has illustrated the role of of PPAR modulation in the angiogenesis process[48]. Briefly, activation of PPARα represses inflammatory responses, via inhibition of the cytokine-induced vascular cell adhesion molecule-1 (VCAM-1), thus limiting the recruitment of inflammatory cells to the activated endothelium. This is, in part, mediated through the inhibition of the nuclear factor κB (NFκB) activation. In addition, PPARα negatively interfere with NF-κB and activator protein (AP)-1 transcriptional activities[49&50].

In Addition, PPARα antiangiogenic functions are partially mediated through decreased vascular endothelial growth factor (VEGF) expression and an increase of antiangiogenic endostatin and thrombospondin (TSP)-1[51].

Our findings also suggested that orlistat possesses a beneficial effect in improving testicular dysfunction in diabetic rats, potentially by attenuating the level of oxidative stress and inflammatory markers, thereby reducing the oxidative stress, inflammation, and apoptotic changes. In addition, orlistat has obvious effect on endothelial function and angiogenesis which could be mediated through its ability to inhibits endothelial cell fatty acid synthase thus, blocks the synthesis of fatty acids, and prevents endothelial cell proliferation . Moreover, orlistat prevents the display of the vascular endothelial growth factor (VEGF) receptor on the endothelial cell surface thus could be useful as an antiangiogenic drug.[52] Hence, orlistat, which acts by inhibiting gastrointestinal lipase, may also be considered as a valuable therapeutic agent in attenuating testicular damage in D.M via its unique antiangiogenic mechanism .

Furthermore, Assessing the precise functions of PDCD4, a tumour-suppressor gene with many functions that are important for many biological processes like protein translation, apoptosis, signal transduction, and induction of inflammatory mediators, has received a lot of interest in recent years[53].

As cells go through apoptosis, PDCD4 expression is elevated, which is consistent with the expectations regarding apoptosis induction. Inducing apoptosis in hepatoma cells through the overexpression of PDCD4 results in the

activation of the pro-apoptotic member of the BAX, Bcl2 protein family, the release of cytochrome C from mitochondria, and the activation of caspases 9, 8, and 3. But the mechanism underlying BAX activation is still not well understood[54]. As previously shown, In HeLa cells, the loss of PDCD4 increased pro-caspase 3 expressions, which increased levels of active caspase 3 and induced apoptosis without pro-apoptotic stimuli. It has also been demonstrated that PDCD4 knockdown causes apoptosis[55].

Moreover, PDCD4 has been linked to the production of inflammation, according to numerous studies. PDCD4 knockdown mice have much lower life spans than their wild-type siblings and spontaneous malignancies, predominantly B-lymphoma, but they are resistant to the production of inflammatory illnesses like autoimmune diabetes and encephalomyelitis[56].

Additionally, PDCD4 has lately been identified as a new inflammation and apoptosis gene[57] and is intimately connected to the pathogenesis of type 2 diabetes. This suggests that it can control both inflammation and carcinogenesis[58]. The exact function of PDCD4 in type 2 DM and the related mechanisms, however, are not fully described.

Since regulation of apoptosis and angiogenesis is closely dependent on PDCD4 regulation, it has been suggested that compounds that target the regulation of PDCD4 expression level and inhibit the generation of ROS should be able to prevent the negative impact of diabetes on testicular dysfunction[59].

Recently, few studies suggested that PDCD4 could control VEGF levels in an NF-kB-dependent manner[60]. Moreover, Western blot analysis demonstrated that inhibiting NF-kB activity restored VEGF expression in cells with depleted levels of PDCD4, demonstrating that PDCD4 knockdown's ability to induce VEGF is based on the NF-kB pathway[61] Thus PDCD4 is an important factor in angiogenesis besides its role in inflammation and cancer prevention.

To our knowledge, this is the first time orlistat or gemfibrozil have been used as PDCD4 inhibitors, and we found that they ameliorate testicular dysfunction in the rat model.

Our findings suggested that orlistat/gemfibrozil treatment effectively attenuated symptoms in the testicular dysfunction-induced groupes. These findings are due to the modulatory and inhibitory effects of angiogenesis and inflammation that occur in testicular tissue as a complication of hyperglycemia through inhibition of VEGF, NO, and PDCD4 signalling pathways.

The goal of this study was to evaluate the effects of gemfibrozil and orlistat, each alone or in combination, on STZ-induced testicular dysfunction and their novel potential mechanisms for targeting angiogenesis and apoptosis. Our in vivo experiment showed that gemfibrozil and orlistat successfully regulate both apoptosis and angiogenesis while improving inflammatory and oxidative responses, thus attenuating testicular dysfunction in diabetic rats. Further studies will be necessary to both validate and expand upon these findings.

Ethical Approval

The research methodology was performed as per the Tanta University Faculty of Medicine's Local Committee of Research and Medical Ethics. **(approval code: 35926/10/22)**.

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أورليستات / جمفبروزيل يحسن اعتلال الخصية الوظيفي في مرض السكري الناجم عن الستربتوزوتوسين في الجرذان عن طريق استهداف مسارات إشارات عامل موت الخلايا المبرمج VEGF / NO / 4

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اعتلال وضعف الخصية هو اختلال مزمن مصاحب لمرض السكري في الرجال مع عدم وجود تفسيرات علمية واضحة لهذا الاعتلال. في هذه الدراسة تم استخدام الأورليستات وهو دواء يستخدم لعلاج السمنة وجيمفبروزيل وهو دواء يستخدم لخفض الدهون الثلاثية في الدم ، وفي هذه الدراسة قد هدفنا إلى استهداف دورهما المحتمل كل على حده أو مجتمعين في الوقاية من اعتلال الخصية المزمن نتيجة السكري النوع الأول في جرذان التجارب. تم تحفيز مرض السكري في فئران التجارب بواسطة ستريبتوزوتوسين وبدأ العلاج قبل أسبوع واحد من التحريض في المجموعات المعالجة. بعد ثلاثة أسابيع تم التضحية بالجرذان وتم جمع عينات الدم. كما تم تقييم الإجهاد التأكسدي للخصية ، وعلامات الالتهاب والاستماتة. علاوة على ذلك ، تم الكشف عن تلف الخصية باستخدام بصبغة الهيماتوكسيلين والأيوزين وتم تقييم عامل النمو البطاني الوعائي للخصية الأندروجين عن طريق التلوين الكيميائي النسيجي المناعي. أظهرت النتائج تحسنًا ملحوظًا في تفاعل البوليميراز المتسلسل ، ومضادات الالتهاب تضرر الخلايا و تحسن مضادات الأكسدة مع انخفاض ملحوظ في عامل موت الخلايا المبرمج ٤ تشير هذه النتائج إلى آثارها المفيدة في التخفيف من تلف الخصية مع تسليط الضوء على مسارات الإشارات الأساسية التي ساهمت في مسبباتها.