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Can troxerutin pretreatment prevent testicular complications in prepubertal diabetic male rats?

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Objective. The vast majority of type 1 diabetes leads to a higher prevalence of reproductive system's impairments. Troxerutin has attracted much attention owing to its favorable properties, including antihyperglycemic, anti-inflammatory, and antiapoptotic effects. This investigation was proposed to evaluate whether pretreatment with troxerutin could prevent apoptosis-induced testicular disorders in prepubertal diabetic rats.

Methods. Fifty prepubertal male Wistar rats were randomly allocated into five groups: control (C), troxerutin (TX), diabetic (D), diabetic+troxerutin (DTX), and diabetic+insulin (DI). Diabetes was induced by 55 mg/kg of streptozotocin applied intraperitoneally. In TX and DTX groups, 150 mg/kg troxerutin was administered by oral gavage. Diabetic rats in DI group received 2–4 U NPH insulin subcutaneously. Troxerutin and insulin treatments were begun immediately on the day of diabetes confirmation. After 30 days, the testicular lipid peroxidation and antioxidant activity, apoptosis process, and stereology as well as serum glucose and insulin levels were assessed.

Results. The results showed that diabetes caused a significant increase in the blood glucose, the number of TUNEL positive cells and tubules, and the malondialdehyde level as well as a significant decrease in serum insulin level compared to controls. The stereological analysis also revealed various alterations in diabetic rats compared to controls. Troxerutin treatment improved these alterations compared to the diabetic group.

Conclusion. Troxerutin-pretreatment may play an essential role in the management of the type-1 diabetes-induced testicular disorders by decreasing blood glucose and modulating apoptosis.

Key words: troxerutin, diabetes, testis, apoptosis, oxidative stress

The growing prevalence of both type 1 and type 2 diabetes is one of the most significant and challenging health problems in the last century (Hsia et al. 2009). The number of patients with type 1 diabetes increases unexpectedly. The vast majority of type 1 diabetes is diagnosed before the age of 30 that leads to a higher prevalence of the impairments in men of reproductive age (La Vignera et al. 2015). It has been reported that about 90% of diabetes are infertile owing to the reduction in sexual function, testicular structural and functional disorders, and spermatogenesis disturbances (Feng et al. 2001). Diabetes induces testicular dysfunction through various mechanisms (Jangir and Jain 2014). One of the crucial mechanisms is hyperglycemia. It causes cell apoptosis in testis through an increase in the reactive oxygen species (ROS) overproduction and oxidative stress (Amaral et al. 2006). It has been well proven that oxidative stress, alterations in antioxidant capacity, and apoptotic cell death can result in the testicular

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cell dysfunction leading to infertility (Agarwal and Saleh 2002; Agarwal and Said 2005). Streptozotocin-induced diabetes is one of the most successful models to study the harmful effects of diabetes on the male reproductive system (King 2012). Therefore, in the present study, the streptozotocin-diabetic rat model was used for the investigation of diabetes effects on testis tissue.

Troxerutin (vitamin P4), as a natural bioflavonoid, can be found in tea, coffee, cereal, vegetables, and a variety of fruits (Panat et al. 2016). This flavonoid has attracted much attention owing to its favorable properties including antihyperglycemic, antioxidant, anti-inflammatory, and antiapoptotic effects (Fan et al. 2009; Zhang et al. 2015; Panat et al. 2016; Yu and Zheng 2017). The antidiabetic effects of troxerutin may be induced by its impacts on the sensitivity of the cells to insulin (Sampath and Karundevi 2014). Sampath and Karundevi (2014) have shown that oral administration of troxerutin (150 mg/kg/day) for 30 days may improve insulin signaling molecules through a significant rise in mRNA and protein of glucose transporter 4 (GLUT4) in type-2 diabetic rats. It can be concluded from the previous studies that troxerutin might affect the activity of antioxidants enzymes and ROS production. Badalzadeh et al. (2015) have reported that troxerutin prevents diabetic vascular abnormalities via improvement of endogenous antioxidative activity.

Moreover, it has been shown that troxerutin can effectively prevent oxidative stress damage and testicular toxicity induced by Nickel in Wistar rats (Elangovan et al. 2016). Troxerutin-pretreatment of the diabetic hearts from rats has reduced the myocardial reperfusion injury via prevention of myocardial apoptosis (Mokhtari et al. 2015). Recently, Kheirollahi et al. (2018) have reported protective effects of troxerutin on testicular torsion-induced damage in the torsion-detorsion models. They have shown that troxerutin may improve Johnson score, sperm count, LH, FSH, and testosterone levels, and had antiapoptotic effects. To the best of our knowledge, studies on the effect of troxerutin in testicular tissue from streptozotocin-induced diabetes are lacking. Therefore, this study was aimed to assess the effect of troxerutin on diabetes-induced testicular oxidative stress and apoptosis process in prepubertal diabetic rats.

Materials and methods

Animals. Fifty prepubertal male Wistar rats (6-week-old, 85–115 g) were obtained from the animal house of Tabriz University of Medical Sciences. Animals were kept under standard conditions (12/12-hour light-dark cycle, 21–25 °C, the relative humidity of 44–56%) with free access to food and water. All protocols conducted according to the Ethics Committee of Animal Research of Tabriz University of Medical Sciences (Ethics approval No: IR.TBZMED.REC.1395.643).

Study design. Animals were randomly allocated into 5 groups (n=10): control (C), troxerutin (TX), diabetic (D), diabetic+troxerutin (DTX), and diabetic+insulin (DI). Diabetes was induced by 55 mg/kg streptozotocin (Sigma-Aldrich, Germany) intraperitoneally dissolved in sodium citrate buffer (pH=4.5) (Oghbaei et al. 2017). Animals in TX and DTX groups received 150 mg/kg troxerutin daily through oral gavage for 30 days (Badalzadeh et al. 2015). Rats in DI group received 2–4 U/day NPH insulin (DarouPakhsh Pharmaceutical Mfg. Co., Iran) subcutaneously for 30 days (Oghbaei et al. 2019). Blood glucose levels were determined by digital glucometer (Norditalia El ettromedicali S.r.I., Italy) 72 hours after the induction, and rats with glucose levels higher than 250 mg/dl were considered diabetic. In order to investigate the preventive effects of troxerutin in the diabetic testicles, all treatments were begun immediately on the day of diabetes confirmation before starting diabetes complications.

Sample collection. At the end of the experimental period, all animals were sacrificed using ketamine-xylazine (80 mg/kg and 10 mg/kg, respectively), and about 5 ml blood samples obtained through the inferior vena cava. Blood samples centrifuged at 3500 rpm for 10 min, and then serum samples were collected and stored at –20 °C until measurements. Furthermore, testes and epididymis were removed and weighed. Then right testis was stored at –80 °C and left testis was fixed in 10% neutral buffered formalin solution until experiments.

Biochemical analyses of serum insulin and blood glucose. The serum insulin concentrations were measured using an insulin ELISA kit (Shanghaicrystal Day Biotech Co., LTD, China) according to the manufacturer's protocols. The blood level of glucose was determined by the digital glucometer (Norditalia El ettromedicali S.r.I., Italy) on the last day of experiments (Oghbaei et al. 2018).

Testicular lipid peroxidation and antioxidant activity. Animal testes were homogenized by cold ice 1.15% KCl to produce 10% homogenate. Oxidative stress parameters were determined following the preparation of supernatant from the testes.

The malondialdehyde (MDA) level was measured spectrophotometrically and using the thiobarbituric

acid reactive substances (TBARS) method. In brief, 0.1 ml of testicular homogenate was mixed with TCA-TBA-HCl (0.2 ml) reagent and centrifuged at 3500×g for 10 min. The absorbance of the supernatant was measured at 535 nm and MDA level was presented as pmol/mg tissue (Pourmemar et al. 2017).

The glutathione peroxidase (GPX) activity was measured by the method of Pourmemar (Pourmemar et al. 2017). Briefly, tissue homogenate (0.2 ml) was mixed with phosphate buffer (0.2 ml, 0.4 M, pH=7), glutathione (0.2 ml), and H_2O_2 (0.1 ml, 0.2 M). TCA (0.4 ml) was added to this mixture following centrifugation at 37 °C for 10 min. After that GPX level was determined following centrifugation for 20 min at 3200×g and presented as mU/mg protein.

The activity of superoxide dismutase (SOD) was measured by RANSOD laboratory kit (Randox Laboratories Ltd, Crumlin, United Kingdom). The SOD activity measurement was based on the xanthine and xanthine oxidase production, which led to the production of superoxide radicals. Red formazan color is produced by the reaction of these radicals with 2-(4-iodophenyl)-3 (4-nitrophenol)-5-phenyl tetrazolium chloride (ITN). The SOD activity was determined using the intensity of inhibition of this reaction at 560 nm through a spectrophotometer and are presented as mU/mg protein (Naderi et al. 2015).

Apoptosis assay. Paraffin sections (5 μm) were deparaffinized, rehydrated, and then for three times washed using nuclease-free phosphate buffer. POD in situ cell death detection kit (Cat No. 116684817910; Roche, Mannheim, Germany) was used for detection of the apoptotic index in seminiferous tubules. The tissue sections were incubated by 0.3% H_2O_2 in methanol at 25 °C for a 30 min to prevent endogenous peroxidase activity. After that, sections were pretreated with 20 μg/ml proteinase K (Roche, Mannheim, Germany) in PBS at 37 °C for 15 min before enzymatic labeling. Tissue sections were incubated by 50 μl of TUNEL reaction mixture in a dark and humidified chamber at 37 °C for 60 min, then hybridized in POD solution for thirty minutes, and stained by 3-3'-diaminobenzidine (DAB) for 15 min. Finally, the tissue sections were counterstained by hematoxylin and apoptotic cells presented as a dark brown nuclear stain under light microscope filtered with blue light. Labeled germ cells were calculated in 20 tubules for each rat and then apoptotic index-1 (AI-1) was explained as the percentage of tubules with at least one TUNEL-positive cells/100 tubules as well as apoptotic index-2 (AI-2) was explained as the percentage of TUNEL-positive cells/100 tubules (Keyhanmanesh et al. 2018).

Stereological studies. After weighing, a small scrape was done on the left testis capsule, and then it was fixed at neutral buffered formalin (10%). The samples were dehydrated using a series of various graded ethanol, cleared with xylene, and embedded in paraffin, respectively. Each paraffin block was cut into nine sections (with 20 μm thickness) then four sections (with 5 μm thickness) using a rotary microtome serially. The first section was obtained by chance, and 20–25 thick sections were selected from each block for stereological studies using a systematic random sampling (SRS) protocol. The selected sections were stained with hematoxylin-eosin (H&E). Stereological analyses were done by the blinded expert, and a total volume of testis was determined by point counting technique and Cavalieri's principle. The volume fraction of structures, the height of germinal epithelium, total length and diameter of seminiferous tubules as well as the numerical density of cells were calculated using version 9 stereo-investigator systems (MBF Bioscience, Micro Bright Field, Inc., Germany) as described previously. All stereological analysis was performed using the SRS protocol (Keyhanmanesh et al. 2019).

Statistical analyses. Data analysis were done using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). All results were presented as mean±SEM. For all variables, One-way ANOVA analysis followed by Tukey's post hoc test was performed. The value of $p<0.05$ was considered as significant.

Results

Effect of troxerutin on serum glucose and insulin levels. Our results revealed that diabetic rats had significantly increased blood glucose compared to the C group ($p<0.001$). Troxerutin and insulin therapy in DTX group significantly decreased blood glucose as compared to the D group ($p<0.001$) (Figure 1).

As shown in Figure 2, serum insulin significantly reduced in the D group as compared to the C group ($p<0.001$). Our results showed that insulin therapy in DI group markedly increased serum insulin compared to the D group ($p<0.001$). However, troxerutin in DTX group did not significantly change the serum insulin as compared to the D group. There was not a significant difference between DTX and DI groups.

Effect of troxerutin on testicular oxidative stress parameters. Figure 3A demonstrates the effect of troxerutin on the level of testicular MDA content. Our results showed that diabetes markedly increased testicular MDA content compared to the C group

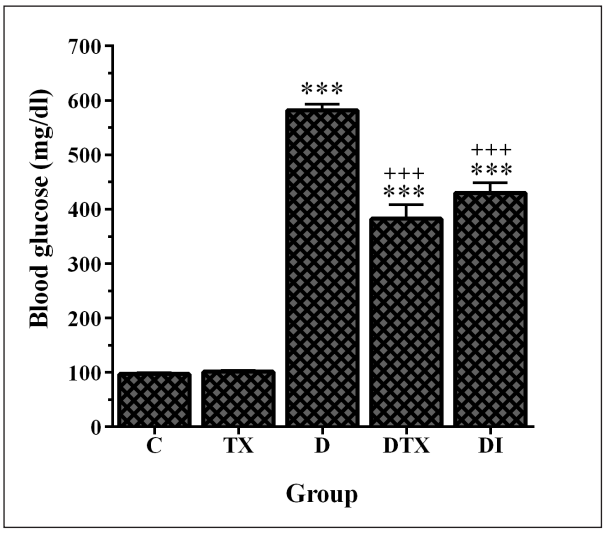

Figure 1. The effects of troxerutin on blood glucose level of different groups. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test (mean±SEM, n=8). Statistical differences between control and experimental groups: ***p<0.001. Statistical differences between diabetic and other experimental groups: +++p<0.001. Abbreviations: C – Control group; TX – Troxerutin group; D – Diabetic group; DTX – Diabetic plus troxerutin group; DI – Diabetic plus insulin group.

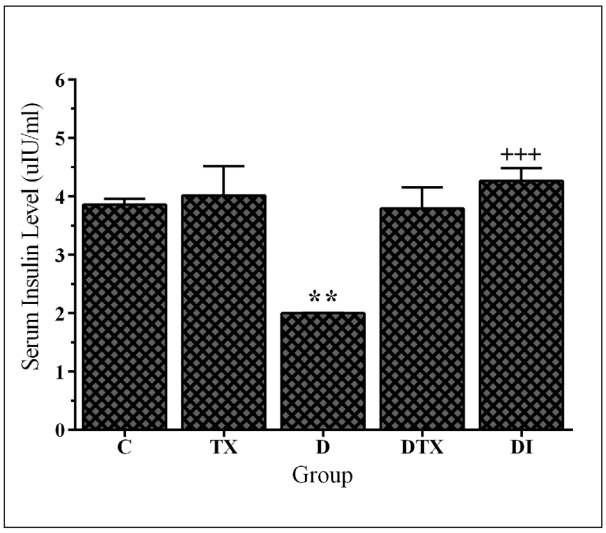

Figure 2. The effects of troxerutin on serum insulin level of different groups. Data were analyzed by using one-way ANOVA followed by Tukey's post hoc test (mean±SEM, n=10). Statistical differences between control and experimental groups: ***p<0.001. Statistical differences between diabetic and other experimental groups: +++p<0.001. Abbreviations: C – Control group; TX – Troxerutin group; D – Diabetic group; DTX – Diabetic plus troxerutin group; DI – Diabetic plus insulin group.

(p<0.01). Insulin therapy noticeably decreased MDA content in the testis tissue as compared to the D group (p<0.05), nevertheless, troxerutin did not significantly change its level as compared to the D group. We did not observe a significant difference between DI and DTX groups. The results also revealed that testicular GPX and SOD activities were non-significantly lower than controls, and treatment with Insulin and troxerutin could not affect them significantly (Figures 3B,C).

Effect of troxerutin on testicular apoptosis. Analysis of the apoptosis data showed that D group had higher apoptotic germ cells (AI-1) and tubular apoptosis index (AI-2) as compared to the C group (p<0.001). Administration of both troxerutin and insulin decreased both apoptotic indices in DTX and DI groups as compared to D group (p<0.001) (Figure 4, Figures 5A,B).

Effect of troxerutin on testicular stereological findings. Testicular morphometric and stereological results showed that the volume and diameter of seminiferous tubule, germinal epithelium volume, and height, and the number of spermatogonia, spermatocytes, round and elongated spermatids, and Sertoli and Leydig cells were significantly reduced in the D group compared to the C group (p<0.001 to p<0.01; Table 1). Moreover, the volume of the lumen, capsule, and interstitial tissue noticeably increased in the D group as compared to controls (p<0.001). There was a marked increase in the volume (p<0.05) and diameter (p<0.01) of seminiferous tubule, germinal epithelium volume (p<0.001), germinal epithelium height (p<0.001), and number of spermatogonia (p<0.01), spermatocytes (p<0.001), round (p<0.01) and elongated (p<0.001) spermatids and Sertoli cell (p<0.01) in the DTX group as compared to the D group. We observed a significant (p<0.001) reduction in the volume of the lumen, capsule, and interstitial tissue in troxerutin treated diabetic rats compared to the D group. On the other hand, there was a significant increase in the diameter (p<0.01) of seminiferous tubule, germinal epithelium volume (p<0.001), germinal epithelium height (p<0.001), and number of spermatogonia (p<0.01), spermatocytes (p<0.001), round (p<0.01) and elongated (p<0.001) spermatids and Sertoli cell (p<0.05) in the DI group as compared to the D group. Furthermore, insulin therapy significantly decreased the volume of lumen, capsule, and interstitial tissue in DI group compared to the D group (p<0.001). However, germinal epithelium height, the volume of interstitial tissue, and the number of spermatogonia, spermatocytes, round and elongated spermatids in DTX and DI groups were significantly different from those of controls (p<0.001 to p<0.01).

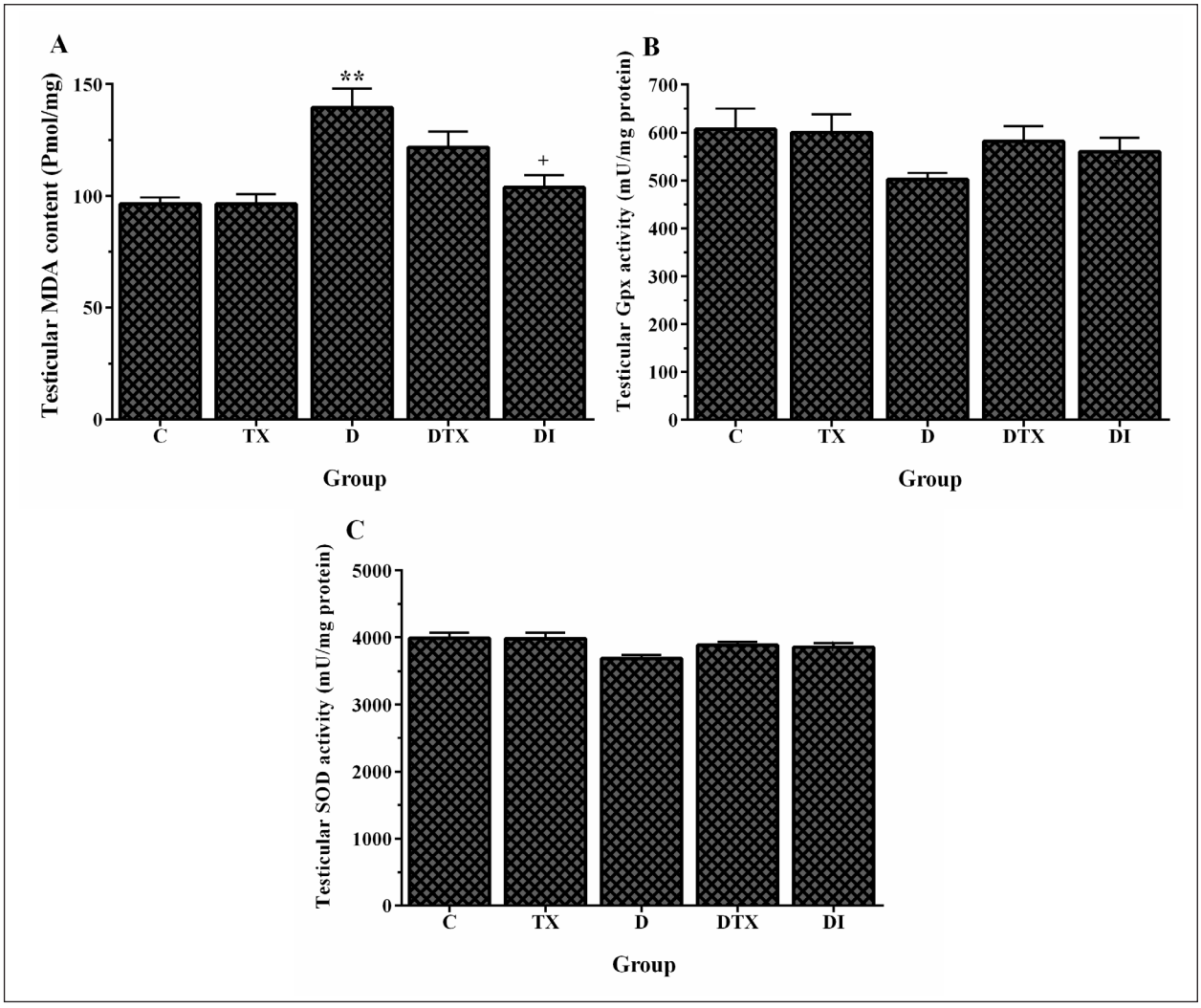

Figure 3. The effects of troxerutin on testicular level of malondialdehyde (MDA) content **(A)**, glutathione peroxidase (GPX) **(B)** and superoxide dismutase (SOD) **(C)** activities of different groups. Data were analyzed by using one-way ANOVA followed by Tukey's post hoc test (mean±SEM, n=10). Statistical differences between control and experimental groups: **p<0.01. Statistical differences between diabetic and other experimental groups: +p<0.05. Abbreviations: C – Control group; TX – Troxerutin group; D – Diabetic group; DTX – Diabetic plus troxerutin group; DI – Diabetic plus insulin group.

Discussion

Type 1 diabetes, as the second most common chronic disease which is diagnosed before the age of thirty, causes a higher prevalence of the pathology in men of reproductive age. It is alarming that infertility associated with diabetes is dramatically increasing in the upcoming years (La Vignera et al. 2015). Therefore, preventive treatments against reproductive complications in diabetes can be of great importance. Accordingly, the study focused on the protective effects of troxerutin on immature testis in prepubertal rats of diabetes. Although categorization of experimental animals based on reproductive age is practically tricky, the age before eight weeks in rats has conservatively classified as prepubertal (Almeida et al. 2000).

Previous experimental and clinical evidence have revealed that troxerutin has favorable effects in various diseases, including cognitive dysfunction and diabetes (Badalzadeh et al. 2015; Mokhtari et al. 2015; Farajdokht et al. 2017). These results suggested that beneficial effects of troxerutin may be mediated by increasing the activity of antioxidant, reducing the level of lipid peroxidation (Badalzadeh et al. 2015), anti-diabetic (Sampath and Karundevi

Table 1
The effect of troxerutin on testicular morphometric and stereological assays in different groups.

Variable	Study group				
	C	TX	D	DTX	DI
Left testicle volume (mm^3)	1358±104.99	1350±36.74	1278±64.06	1356±49.55	1320±35.91
Seminiferous tubule volume (mm^3)	1227.88±96.24	1206.76±31.84	888.84±41.28***	1124.66±35.66^{+}	1095.66±30.57
Germinal epithelium volume (mm^3)	1009.52±78.44	1001.16±23.45	465.70±15.30***	850.90±26.74^{+++}	826.38±23.60^{*+++}
Lumen volume (mm^3)	218.36±22.86	205.60±9.67	423.14±28.88***	273.76±9.23^{+++}	269.28±7.97^{+++}
Capsule volume (mm^3)	21.20±2.94	21.50±3.23	58.34±2.32***	32.92±4.37^{+++}	31.42±2.58^{+++}
Interstitial tissue volume (mm^3)	108.92±9.73	121.74±6.35	330.82±23.28***	198.42±10.27^{**+++}	192.92±7.59^{**+++}
Seminiferous tubule length (m)	16.17±0.87	16.72±0.45	14.54±0.65	16.57±0.82	16.11±0.60
Seminiferous tubule diameter (μm)	438±5.83	434±3.67	400±2.73***	424±1.87^{++}	423±1.22^{*++}
Germinal epithelium height (μm)	133±2.54	134±1.00	103±3.00***	120±1.58^{**+++}	121±1.87^{**+++}
No. of spermatogonia ($\times 10^6$)	60.31±2.54	60.69±2.36	28.06±1.83***	39.29±1.85^{***++}	39.42±1.41^{***++}
No. of spermatocytes ($\times 10^6$)	222.10±6.62	222.48±4.05	119.48±4.22***	187.02±6.74^{**+++}	187.46±7.39^{**+++}
No. of round spermatids ($\times 10^6$)	398.24±19.28	398.38±5.97	241.42±6.46***	310.31±5.03^{***++}	301.06±10.25^{***++}
No. of elongated spermatids ($\times 10^6$)	449.76±14.29	450.80±14.59	259.09±11.40***	354.36±8.54^{***+++}	343.56±12.99^{***+++}
No. of Sertoli cells ($\times 10^6$)	31.93±2.17	32.15±1.40	19.67±0.96***	28.71±0.94^{++}	26.97±1.29^{+}
No. of Leydig cells ($\times 10^6$)	21.42±1.09	21.60±1.11	15.35±1.07**	18.72±0.82	19.02±0.71

Data are presented as mean±SEM (n=6 for each group). Statistical differences between control and different groups: *p<0.05, **p<0.01, ***p<0.001; Statistical differences between diabetic and different groups: $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001.
Abbreviations: C – control group; TX – healthy animals received troxerutin; D – diabetic animals; DTX – diabetic animals received troxerutin; DI – diabetic animals received insulin; No. – number.

2014), anti-inflammatory (Fan et al. 2009), and anti-apoptotic effects (Mokhtari et al. 2015). In our study, the significant decrease in blood glucose level of diabetic rats with troxerutin-pretreatment is provided as an evidence of the anti-hyperglycemic activity of this flavonoid. According to our findings, Yu and Zheng (2017) have reported that troxerutin significantly decreased the level of blood glucose in type 2 diabetic rats. Moreover, the hypoglycemic effect of troxerutin has been shown in mice fed with high fat-fructose diet (Geetha et al. 2014) and in sucrose-induced type 2 diabetic rats (Sampath and Karundevi 2014). However, Zhang et al. (2018) have reported that administration of 60 mg/kg/day troxerutin for 12 weeks could not reduce blood glucose concentrations in type 1 diabetic rats. Also, 150 mg/kg troxerutin in mice fed with a high-calorie diet for 45 days (troxerutin treatment began after 15 days of diet) displayed no decreases in the blood glucose level (Geetha et al. 2014). The contrary possibly is related to the dose and the beginning time of troxerutin administration. Troxerutin reduces blood glucose by increasing glucose uptake and improving the expression of insulin signaling molecules (Sampath and Karundevi 2014).

The oral administration of troxerutin has revealed near average concentrations of blood glucose, GLUT4 proteins level, and serum insulin in diabetic rats (Badalzadeh et al. 2015). In this study, beneficial effects of troxerutin on blood glucose-lowering were not associated with its effect on serum insulin level; hence, these are likely due to an increased glucose uptake, improving the expression of insulin signaling molecules (Sampath and Karundevi 2014; Badalzadeh et al. 2015). In line with our results, troxerutin might not affect insulin secretion in streptozotocin-induced type 1 diabetic models (Badalzadeh et al. 2015). It may be due to that most of the beta cells were destructed by streptozotocin and makes the remained cells less active (Pari et al. 2012). Therefore, in our study, the most important mechanism to regulate glucose may be due to increased glucose uptake and improving the expression of insulin signaling molecules. Nevertheless, in type 2 diabetes pancreatic beta cells are intact, and thus, troxerutin could facilitate insulin secretion (Sampath and Karundevi 2014).

It is well known that diabetes-induced hyperglycemia can affect male infertility through both increasing the production of reactive oxygen species (ROS) and decreasing the efficiency of the antioxi-

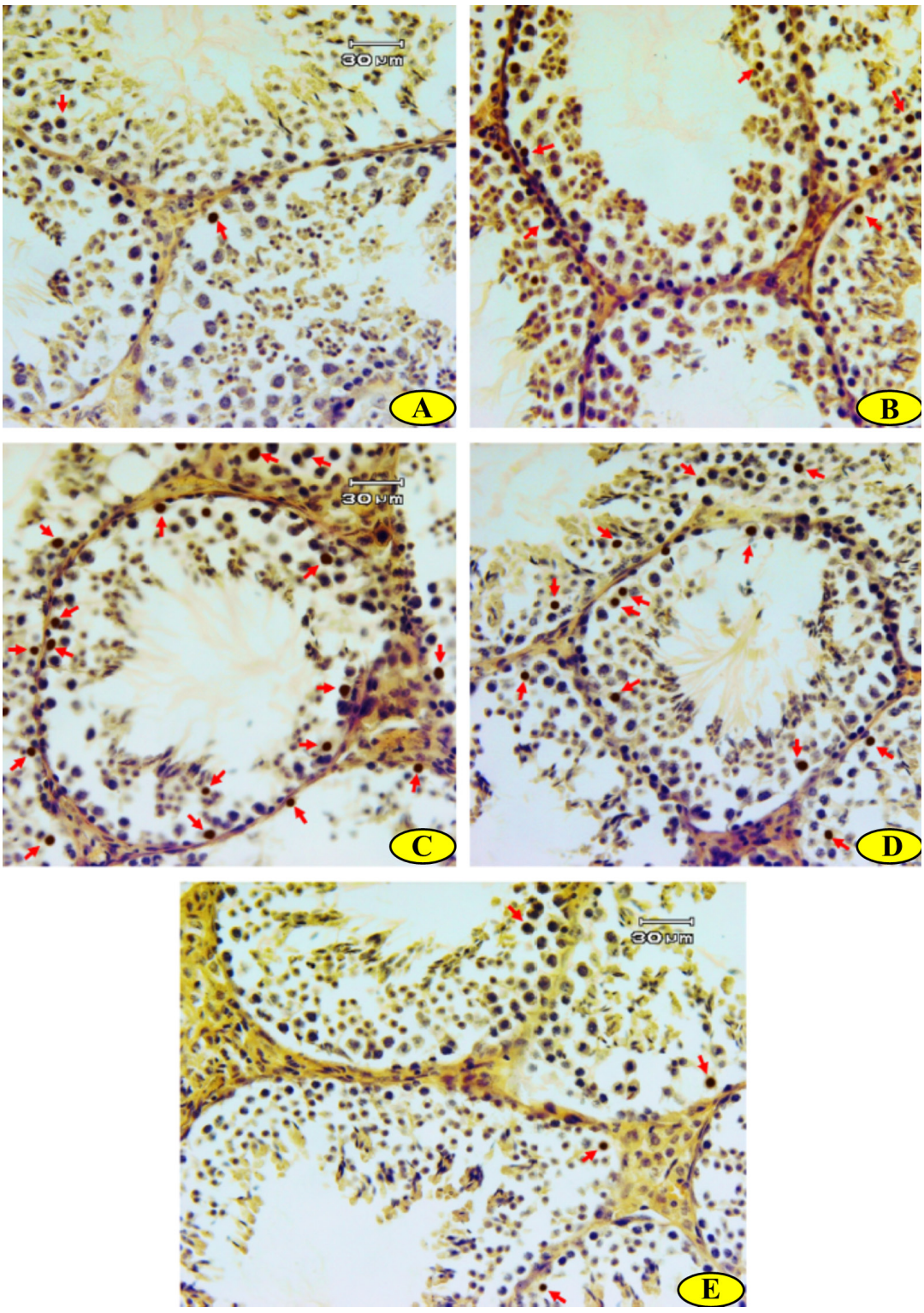

Figure 4. Photomicrographs of TUNEL immunohistochemistry staining for detection of apoptotic cells in the seminiferous tubules of different groups (×400, filtered by blue light). A – control rats; B – troxerutin-treated rats; C – diabetic rats; D – troxerutin-treated diabetic rats; E – NPH insulin-treated diabetic rats. Dark brown nuclei show the TUNEL-positive (apoptotic) cells and blue nuclei display normal cells.

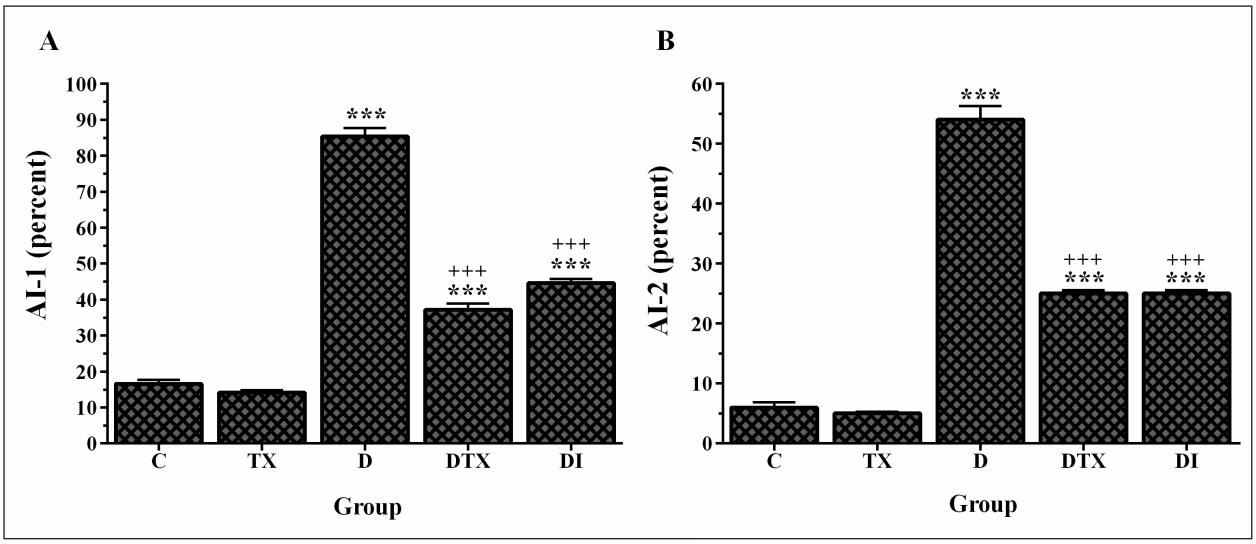

Figure 5. The effect of troxerutin on **(A)** cellular apoptotic germ cell (AI-1) (percent), and **(B)** tubular apoptosis (AI-2) (percent) in the testes of different groups (n=6). Statistical differences between control and experimental groups: ***p<0.001. Statistical differences between diabetic and other experimental groups: +++p<0.001. Abbreviations: C – Control group; TX – Troxerutin group; D – Diabetic group; DTX – Diabetic plus troxerutin group; DI – Diabetic plus insulin group.

dant enzymes in the testicles (Shrilatha and Muralidhara 2007). In this regard, Chandrashekar (2009) has found that prepubertal diabetic rats (4 weeks and six weeks) showed noticeable oxidative damage as evidenced by increased generation of ROS, hydroperoxide and malondialdehyde levels after 15 days. Moreover, Kanter et al. (2012) have reported that streptozotocin-induced diabetic rats (10-week-old) showed significant increases of malondialdehyde (MDA), the main product of lipid peroxidation, and significant reduction of the SOD and GPX antioxidant enzymes activities in the testicle after eight weeks. Elevated activities of catalase (CAT), SOD, and glutathione-s-transferase in the testis of 4-week-old rats also reported, which may suggest adaptive responses by the testes to counteract toxic metabolites (Chandrashekar 2009). Our results showed increased MDA levels as well as unchanged SOD and GPX enzyme activities following four weeks diabetic period, which is according to the report of previous findings in type-1 diabetes mellitus (Badalzadeh et al. 2015). Unlike our finding, attenuated activities of the antioxidant enzymes including SOD, CAT, and GPX in the testicular tissues have been reported following four weeks of streptozotocin-induced diabetes in rats (Badalzadeh et al. 2017). It has been reported that diabetes-induced oxidative stress was forbidden by the antioxidant treatment, which induces up-regulation of testicular antioxidants (Lu et al. 2010). It suggested that flavonoids with potent antioxidant activity could play a protective role in oxidative stress-mediated diabetes. In this regard, the previous study reported that 150 mg/kg troxerutin-pretreatment for four weeks significantly reduced the levels of MDA and increased the antioxidant enzymes CAT, GPX, and SOD activities in the blood of diabetic rats (Badalzadeh et al. 2017). Moreover, troxerutin (60 mg/kg, for 12 weeks) induced a significant increase in SOD activity and decreased MDA content in the hippocampus from streptozotocin-induced diabetic rats (Zhang et al. 2018). However, present findings showed no significant alteration in SOD, CAT, and MDA levels in testicular cells of the diabetic rats received troxerutin-pretreatment for 30 days.

It is well known that apoptotic cell death plays a crucial role in male infertility caused by testicular dysfunction in diabetes (Cai et al. 2000). Zhao et al. (2011) have revealed a marked increase in the ratio of Bax/Bcl-2 and the number of TUNEL-positive germ cells in testis tissue of diabetic mice. An assessment of TUNEL-positive cells is one of the most critical and reliable techniques for studying apoptosis process in the testis (Cai et al. 2000). Our TUNEL-positive cell results showed increased TUNEL-positive germinal cells and tubules in diabetic rats as compared to controls. According to the present study, Aktas et al. (2011) have demonstrated an increased TUNEL reaction of testicular cells in type 1 diabetic rats. Guneli et al. (2008) have also shown TUNEL-positive cells are notably increased in testicular cells of strepto-

zotocin-induced diabetic rats after 14 weeks. Anti-apoptotic effects of troxerutin have been reported in several tissues, including heart (Yu and Zheng 2017), brain (Farajdokht et al. 2017), and testis (Kheirollahi et al. 2018). Farajdokht et al. (2017) have reported that chronic administration of 300 mg/kg troxerutin for 14 days significantly decrease apoptotic cells in the hippocampus.

Moreover, troxerutin administration (150 mg/kg for 4 weeks) attenuated cardiac apoptosis following six weeks of streptozotocin injection (Mokhtari et al. 2015). However, to the best of our knowledge, there is no report on the anti-apoptotic effects of troxerutin on the diabetic testicle. The results of this study showed a significant decrease in the testicular TUNEL-positive germ cells and tubules following troxerutin administration for four weeks in the diabetic group. According to the present study, a recent study showed that troxerutin reduced the number of TUNEL-positive germ cells after testicular torsion (Kheirollahi et al. 2018). Besides, insulin-treated diabetic rats showed decreased both the number of TUNEL-positive germ cells and tubules. According to our results, anti-apoptotic effects of insulin in various cells such as spinal cord (Wu et al. 2007), pancreatic beta-cells (Muller et al. 2006), and myocardial cells (Gao et al. 2002) have reported by previous studies.

Moreover, Gao et al. (2002) have reported that insulin reduced post-ischemic myocardial apoptotic death in in vivo animal experiment through the PI3-kinase-dependent pathway. An increase in nitric oxide (NO) production caused by phosphorylation of endothelial nitric oxide synthase (eNOS) contributes to the anti-apoptotic mechanism of insulin (Gao et al. 2002). It is worth noting that the improvement in the number of tubules with at least one TUNEL-positive cells in animals in the troxerutin treated group was the same as that of the insulin-treated group.

The testicular stereological findings reported atrophic effects of streptozotocin-induced diabetes on the testis tissue (Soudamani et al. 2005). Our stereological results showed decreased seminiferous tubules diameter and volume, germinal epithelium height and volume, the number of spermatogonia, spermatogonia, spermatocytes, spermatids, Sertoli, and Leydig cells in diabetic rats than controls. Moreover, we observed that the volume of the capsule, lumen, and interstitial tissue in diabetic rats was higher than those of the control group. In line with our findings, a reduction in tubular volume and diameter, the number of Sertoli and Leydig cells, spermatogonia, spermatocytes, and spermatids has been shown following streptozotocin-induced diabetes (Soudamani et al. 2005; Kushwaha and Jena 2012; Abbasi et al. 2013). Also, Kianifard et al. (2012) have reported reduced germinal epithelium height, edema in the interstitial tissue, germ cell depletion, decreased cellular population and activity with interruption of spermatogenesis in streptozotocin-induced diabetic rats. Unlike our finding, Yaghoubi et al. (2017) have demonstrated that streptozotocin-induced diabetic mice for 35 days could not considerably influence the testicular volume, germinal epithelium thickness, and the number of Leydig cells. Troxerutin and insulin treatment for 30 days were able to prevent most of these stereological disturbances in diabetic prepubertal rats. Insulin therapy failed to prevent the decreased seminiferous tubule volume and the number of Leydig cells, but troxerutin treatment was not just able to prevent the reduced number of Leydig cells. The beneficial effects of troxerutin and insulin on stereological findings could result from their anti-hyperglycemic and anti-apoptotic effects (Gao et al. 2002; Mokhtari et al. 2015; Jalali et al. 2017). To the best of our knowledge, there is no study on the effects of troxerutin treatment on the testicular morphometric and stereological changes in diabetes.

In conclusion, this study showed that troxerutin could have a protective role in the diabetes-induced reproductive complications prepubertal rats through reducing the blood glucose and modulating the apoptosis process.

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References

Abbasi Z, Tabatabaei SRF, Mazaheri Y, Barati F, Morovvati, H. Effects of sesame oil on the reproductive parameters of diabetes mellitus-induced male rats. World J Mens Health 3, 141–149, 2013.

Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am 29, 817–828, 2002.

Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU Int 95, 503–507, 2005.

Aktas C, Kanter M, Erboga M, Timurkan H. Effects of experimental diabetes on testis proliferations and apoptosis in rats. J Exp Clin Med 28, 94–98, 2011.

Almeida SA, Kempinas WG, Lamano Carvalho TL. Sexual behavior and fertility of male rats submitted to prolonged immobilization-induced stress. Braz J Med Biol Res 33, 1105–1109, 2000.

Amaral S, Moreno AJ, Santos MS, Seica R, Ramalho-Santos J. Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes. Theriogenology 66, 2056–2067, 2006.

Badalzadeh R, Layeghzadeh N, Alihemmati A, Mohammadi M. Beneficial effect of troxerutin on diabetes-induced vascular damages in rat aorta: histopathological alterations and antioxidation mechanism. Int J Endocrinol Metab 13, e25969, 2015.

Badalzadeh R, Chodari L, Ghorbanzadeh V. Troxerutin, a bioflavonoid, improves oxidative stress in blood of streptozotocin-induced type-1 diabetic rats. IJPS 13, 75–86, 2017.

Cai L, Chen S, Evans T, Deng DX, Mukherjee K, Chakrabarti S. Apoptotic germ-cell death and testicular damage in experimental diabetes: prevention by endothelin antagonism. Urol Res 28, 342–347, 2000.

Chandrashekar KN. Evidence of oxidative stress and mitochondrial dysfunctions in the testis of prepubertal diabetic rats. Int J Impot Res 21, 198–206, 2009.

Elangovan P, Jalaludeen AM, Ramakrishnan R, Pari L. Protective effect of troxerutin on nickel-induced testicular toxicity in Wistar rats. J Environ Pathol Toxicol Oncol 35, 133–146, 2016.

Fan SH, Zhang ZF, Zheng YL, Lu J, Wu DM, Shan Q, Hu B, Wang YY. Troxerutin protects the mouse kidney from D-galactose-caused injury through anti-inflammation and anti-oxidation. Int Immunopharmacol 9, 91–96, 2009.

Farajdokht F, Amani M, Bavil FM, Alihemmati A, Mohaddes G, Babri S. Troxerutin protects hippocampal neurons against amyloid beta-induced oxidative stress and apoptosis. EXCLI J 16, 1081–1089, 2017.

Feng SL, Li SH, Wang Y, Chen CC, Gao B. Effect of ligustrum fruit extract on reproduction in experimental diabetic rats. Asian J Androl 23, 71–73, 2001.

Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation 105, 1497–1502, 2002.

Geetha R, Yogalakshmi B, Sreeja S, Bhavani K, Anuradha CV. Troxerutin suppresses lipid abnormalities in the heart of high-fat–high-fructose diet-fed mice. Mol Cell Biochem 387, 123–134, 2014.

Guneli E, Tugyan K, Ozturk H, Gumustekin M, Cilaker S, Uysal N. Effect of melatonin on testicular damage in streptozotocin-induced diabetes rats. Eur Surg Res 40, 354–360, 2008.

Hsia Y, Neubert AC, Rani F, Viner RM, Hindmarsh PC, Wong IC. An increase in the prevalence of type 1 and 2 diabetes in children and adolescents: results from prescription data from a UK general practice database. Br J Clin Pharmacol 67, 242–249, 2009.

Jalali M, Hassanipour M, Hajizadeh M, Khanamani Falahati Pour S, Khoshdel A, Roustai F, Rezai M, Mahmoodi M. Troxerutin chronic treatment protects against fructose-induced metabolic syndrome in male rats. IJML 4, 201–210, 2017.

Jangir RN, Jain GC. Diabetes mellitus induced impairment of male reproductive functions: a review. Curr Diabetes Rev 10, 147–157, 2014.

Kanter M, Aktas C, Erboga M. Protective effects of quercetin against apoptosis and oxidative stress in streptozotocin-induced diabetic rat testis. Food Chem Toxicol 50, 719–725, 2012.

Keyhanmanesh R, Hamidian G, Alipour MR, Ranjbar M, Oghbaei H. Protective effects of sodium nitrate against testicular apoptosis and spermatogenesis impairments in streptozotocin-induced diabetic male rats. Life Sci 211, 63–73, 2018.

Keyhanmanesh R, Hamidian G, Alipour MR, Oghbaei H. Beneficial treatment effects of dietary nitrate supplementation on testicular injury in Streptozotocin-induced diabetic male rats. Reprod Biomed Online 39, 357–371, 2019.

Kheirollahi A, Abbaszadeh A, Anbari K, Rostami B, Ahangari A, Hasanvand A, Gholami M. Troxerutin protect sperm, seminiferous epithelium and pituitary-gonadal axis from torsion-detorsion injury: An experimental study. Int J Reprod Biomed 16, 315–322, 2018.

Kianifard D, Sadrkhanlou RA, Hasanzadeh S. The ultrastructural changes of the Sertoli and Leydig cells following streptozotocin induced diabetes. Iran J Basic Med Sci 15, 623–635, 2012.

King AJ. The use of animal models in diabetes research. Br J Pharmacol 166, 877–894, 2012.

Kushwaha S, Jena GB. Enalapril reduces germ cell toxicity in streptozotocin-induced diabetic rat: investigation on possible mechanisms. Naunyn Schmiedebergs Arch Pharmacol 385, 111–124, 2012.

La Vignera S, Condorelli RA, Di Mauro M, Lo Presti D, Mongioi LM, Russo G, Calogero AE. Reproductive function in male patients with type 1 diabetes mellitus. Andrology 3, 1082–1087, 2015.

Lu YX, Zhang Q, Li J, Sun YX, Wang LY, Cheng WM, Hu XY. Antidiabetic effects of total flavonoids from Litsea Coreana leve on fat-fed, streptozotocin-induced type 2 diabetic rats. Am J Chin Med 38, 713–725, 2010.

Mokhtari B, Badalzadeh R, Alihemmati A, Mohammadi M. Phosphorylation of GSK-3β and reduction of apoptosis as targets of troxerutin effect on reperfusion injury of diabetic myocardium. Eur J Pharmacol 765, 316–321, 2015.

Muller D, Jones PM, Persaud SJ. Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic β-cells. FEBS Lett 580, 6977–6980, 2006.

Naderi R, Mohaddes G, Mohammadi M, Ghaznavi R, Ghyasi R, Vatankhah AM. Voluntary exercise protects heart from oxidative stress in diabetic rats. Adv Pharm Bull 5, 231–236, 2015.

Oghbaei H, Asl NA, Sheikhzadeh F. Can regular moderate exercise lead to changes in miRNA-146a and its adapter proteins in the kidney of streptozotocin-induced diabetic male rats? Endocr Regul 51, 145–152, 2017.

Oghbaei H, Alipour MR, Hamidian G, Ahmadi M, Ghorbanzadeh V, Keyhanmanesh R. Two months sodium nitrate supplementation alleviates testicular injury in streptozotocin-induced diabetic male rats. Exp Physiol 103, 1603–1617, 2018.

Oghbaei H, Alipour MR, Mohaddes G, Hamidian GR, Keyhanmanesh R. Evaluation of ameliorative effect of sodium nitrate in experimental model of streptozotocin-induced diabetic neuropathy in male rats. Endocr Regul 53, 14–25, 2019.

Panat NA, Maurya DK, Ghaskadbi SS, Sandur SK. Troxerutin, a plant flavonoid, protects cells against oxidative stress-induced cell death through radical scavenging mechanism. Food Chem 194, 32–45, 2016.

Pari L, Monisha P, Mohamed Jalaludeen A. Beneficial role of diosgenin on oxidative stress in aorta of streptozotocin induced diabetic rats. Eur J Pharmacol 691, 143–150, 2012.

Pourmemar E, Majdi A, Haramshahi M, Talebi M, Karimi P, Sadigh-Eteghad S. Intranasal cerebrolysin attenuates learning and memory impairments in D-galactose-induced senescence in mice. Exp Gerontol 87, 16–22, 2017.

Sampath S, Karundevi B. Effect of troxerutin on insulin signaling molecules in the gastrocnemius muscle of high fat and sucrose-induced type-2 diabetic adult male rat. Mol Cell Biochem 395, 11–27, 2014.

Shrilatha B, Muralidhara. Occurrence of oxidative impairments, response of antioxidant defences and associated biochemical perturbations in male reproductive milieu in the Streptozotocin-diabetic rat. Int J Androl 30, 508–518, 2007.

Soudamani S, Malini T, Balasubramanian K. Effects of streptozotocin-diabetes and insulin replacement on the epididymis of prepubertal rats: histological and histomorphometric studies. Endocr Res 31, 81–98, 2005.

Wu XH, Yang SH, Duan DY, Cheng HH, Bao YT, Zhang Y. Anti-apoptotic effect of insulin in the control of cell death and neurologic deficit after acute spinal cord injury in rats. J Neurotrauma 24, 1502–1512, 2007.

Yaghoubi A, Shahedi A, Akbari H, Nematollahi-Mahani SN. Do insulin replacement and omega3 protect the male reproductive function of the streptozotocin-induced diabetic mice? J Nutr Metab 2017, 6102985, 2017.

Yu Y, Zheng G. Troxerutin protects against diabetic cardiomyopathy through NF-κB/AKT/IRS1 in a rat model of type 2 diabetes. Mol Med Rep 15, 3473–3478, 2017.

Zhang ZF, Shan Q, Zhuang J, Zhang YQ, Wang X, Fan SH, Lu J, Wu DM, Hu B, Zheng YL. Troxerutin inhibits 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47)-induced hepatocyte apoptosis by restoring proteasome function. Toxicol Lett 233, 246–257, 2015.

Zhang S, Yuan L, Zhang L, Li C, Li J. Prophylactic use of Troxerutin can delay the development of diabetic cognitive dysfunction and improve the expression of Nrf2 in the hippocampus on STZ diabetic rats. Behav Neurol 2018, 8678539, 2018.

Zhao Y, Tan Y, Dai J, Li B, Guo L, Cui J, Wang G, Shi X, Zhang X, Mellen N, Li W. Exacerbation of diabetes-induced testicular apoptosis by zinc deficiency is most likely associated with oxidative stress, p38 MAPK activation, and p53 activation in mice. Toxicol Lett 200, 100–106, 2011.