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Research article

Pentoxifylline improves the survival of spermatogenic cells via oxidative stress suppression and upregulation of PI3K/AKT pathway in mouse model of testicular torsion-detorsion

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ABSTRACT

Testicular torsion-detorsion results in enhanced formation of free radicals which contribute to the pathophysiology of testicular tissue damage. Recent reports have identified protective role of pentoxifylline (PTX) against free radicals. Thus, we determined the protective effect of pentoxifylline against testicular damage in mouse model of testicular torsion-detorsion.

Twenty (6 weeks old) male mice were divided into 4 groups of 5 animals each namely: Control (sham operated group), T1 (Torsion-detosion + single dose 100 mg/kg PTX, T2 (torsion-detorsion + 20 mg/kg PTX for 2 weeks and T/D (torsion-detorsion only). Animals in T1, T2 and T/D groups underwent 2 h of testicular torsion with the left testes rotated 720° (clockwisely) followed by 30 min of detorsion. After detorsion, drug administration was done intraperitoneally. The left testes of all the animals were excised on the 35th day after torsion-detortion for histopathological and biochemical assay. Histomorphological analysis of the seminiferous tubules showed that there were significant increase ($P < 0.01$ or 0.05) in the mean seminiferous tubule diameter, Johnson score and germ cells of animals in Control and T1 compared to T2 and T/D with no significant difference ($P > 0.05$) in testes weight, sertoli, leydig and myoid cells in all groups. IHC results showed significant increase ($P < 0.01$ or 0.05) in id4 and scp3 protein markers in Control, T1 and T2 compared to T/D. Oxidative stress analysis revealed that Pentoxifylline significantly increased ($P < 0.01$ or 0.05) the level of SOD, catalase, mRNA expression of akt and pi3k genes but significantly suppress ($P < 0.01$ or 0.05) MDA and Caspase-3 level in Control, T1 and T2 compared to T/D. Pentoxifylline could be used as an adjunct therapy to surgery in the treatment of torsion-detorsion related testicular injury, However, Further studies are needed to evaluate the effects of pentoxifylline on testicular torsion.

1. Introduction

Testicular torsion is a urological emergency and a painful condition commonly affecting new born, children and adolescent boys that must be rapidly treated to avoid permanent damage to the testes [1]. It has been well established that the duration and degree of testicular torsion correspond with level of damage to the testes. In a study by Taneli et al., it was shown that prolonged torsion lead to testicular ischemia with high levels of oxidative stress in the ipsilateral testes associated with nitric oxide (NO) and hydrogen peroxide (H_2O_2) production, increased generation of lipid peroxide, isoprostane aggregation, decreased antioxidant enzymes level and increased rate of mitochondria-mediated apoptosis in the germ cell line [2]. Similarly, Deniz et al. reported that 60 min of testicular torsion-detorsion resulted to germ cell apoptosis [3]. Likewise, Lievano et al. revealed that 3 h of ischemia in experimental animals lead to a high level of oxidative stress, depleted

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testicular glutathione level and consequently, disruption of the process of spermatogenesis [4].

The equilibrium between the amount of reactive oxygen species (ROS) produced and eliminated is related to gamete cell stability and degree of damage. Thus, achieving this equilibrium state plays an important role in reproduction. Although oxidative stress is implicated in ischemia-reperfusion mediated testicular damage which may be supported by the sudden induction of lipid peroxidation and the concomitant suppression of endogenous antioxidant activities including superoxide dismutase (SOD), catalase and glutathione peroxidase [5], however, it has been shown that these free radicals have beneficial effects in sperm physiology such as sperm maturation and capacitation [6]. While torsion and detorsion resulted to ischemia and reperfusion injury respectively, detorsion is the main source of free radical formation and lipid peroxidation damage [7].

The inhibitor of differentiation (ID) family of helix-loop-helix proteins is a group of evolutionarily conserved molecules that play an important regulatory roles in organisms ranging from drosophila to humans. ID protein exists in four different isoforms (ID1–ID4) that are expressed preferentially in undifferentiated cell populations where they play diverse roles in fate determination by functioning as transcriptional repressors [8]. Examination of ID isoform expression in the mouse testis revealed that ID1 is expressed exclusively in spermatocytes, ID2 and ID3 expression is being localized to Sertoli cells [9] while ID4 expression was detected in the type-A spermatogonia population [9]. However, this population is heterogeneous, and whether expression is localized specifically to certain subpopulations of type-A spermatogonia or plays a role in germ cell development is unknown. In a study by Oatley et al., ID4 expression was observed exclusively in a single cell within seminiferous tubules, indicative of A_s spermatogonia in the germline [10]. It was found that Id4 protein expression is typically high in embryonic and adult stem/progenitor cells while the levels decrease as the cells differentiate [11]. Aileen and colleagues identified the level of ID4 as a determinant of stem cell or progenitor capacity in spermatogonia and as the main driver of transitions between the different functional states [12].

Homologues pairing, synapsing, recombination and segregation are essential processes that are mediated by a meiosis-specific structure known as the synaptonemal complex during prophase 1 of meiotic cell division [13]. Synaptonemal Complexes (SC) are zipper like structures that has two axial elements, one along each of the two homologous chromosomes. In mature SC, these homologous chromosome are connected by several transverse filaments along their length [14]. The synaptonemal complex protein 3 (SCP3) gene has about 10.5 kb located on 12q chromosome with nine exons (NM 153694 in GenBank). It is believed to be present in the core of the lateral element complex and function as a molecular framework to which other proteins attach. SCP3 is essential for regulating DNA binding to the chromatid axis, sister chromatid cohesion, synapsis, and recombination [15]. Recently, SCP3 has been shown to be involved in the monopolar attachment of sister kinetochores in meiosis I, and is not responsible for the maintenance of sister chromatid centromere cohesion during meiosis II [15]. In a study of SCP3 knockout mouse, it was found that SCP3 is required for fertility in both sexes and loss of SCP3 blocks synapsis initiation resulting to meiotic arrest [16].

Experimental animal model of testicular torsion-detorsion has been widely used to address the relative potential effect of different antioxidant agents in preventing oxidative stress induced testicular damage. So far, different studies have recorded significant protection of the testes for factors such as garlic extract [17], caffeic acid phenethyl ester (CAPE) [18], N-acetyl cysteine [19], pentoxifylline [20], erdostein [21], resveratrol [22], dexpathenol [23], L-carnitine [24] and propofol anaesthetic [25].

It is estimated that testicular torsion occurs annually in 1 of every 4000 males below 25 years of age and is known to be the main source of testicular injury and infertility in the USA [26]. Surgical intervention for the treatment of testicular torsion includes detorsion of the twisted testis to restore testicular blood flow after ischemia injury. However, many studies showed that detorsion further results to testicular reperfusion injury. Over the past few years, extensive investigatory studies have aimed to find effective treatment strategies and drugs to reduce, or even prevent testicular ischemial-reperfusion injury. However, apart from cooling of the scrotum, no other method has so far been successfully applied in clinical practice [27]. Although, many studies have attested to the effectiveness of pentoxifylline in managing oxidative stress related testicular disorders. For example, Yucesan et al. reported that Pentoxifylline attenuated reperfusion injury in experimental animal model of testicular torsion [28]. In another study, Chen et al. demonstrated that oral administration of pentoxifylline prevented pathological damage to the testes by suppressing oxidative stress [29]. Also, Yao et al. (2016) showed that PTX prevented against intermittent hypobaric hypoxia induced-oxidative stress in testicular tissue by maintaining redox homeostasis [29]. Furthermore, Salahshoor et al reported that pentoxifylline protected rats against Dimethyl nitrosamine (DMN) induced testicular damage and improved sperm motility and viability [30]. These studies, taken together, suggest the potential protective role of pentoxifylline against oxidative stress induced testicular damage. However, reports as therapeutic candidate for testicular torsion-detorsion is scanty. Testicular torsion-detorsion is known to impair or halt the process of spermatogenesis, and as such, studies on the cellular and molecular mechanisms of testicular damage for effective therapeutic strategies are warranted. However, there have been little investigatory effort to address this condition. Therefore, the aim of this study was to determine the antioxidant protective effect of pentoxifylline on spermatogenic cells survival, oxidative stress and germ cell apoptosis caused by testicular torsion-detorsion.

2. Materials and methods

2.1. Animals and experimental groups

A total number of 20 adult male NMRI mice aged 6 weeks (20–25g) were obtained from Pharmacy Faculty of Tehran University of Medical Sciences. Animals were preserved under standard condition of 12-hour light/12-hour darkness with a temperature range of 25 ± 3 °C and mean relative humidity of 50 ± 5%. Mice were maintained on commercial pellets ad libitum and had free access to water. All procedures were performed in accordance with approved guidelines for the use and care of animals by Tehran University of Medical Sciences. The protocol for this study was evaluated and approved by Institutional Research Ethics Committee, Office of the Vice Chancellor for Research Affairs, Tehran University of Medical Sciences, Tehran, Iran with ethical certificate ID, IR.TUMS.VCR.REC.1398.637.

Animals were acclimatized for a period of 2 weeks after which they were randomly allocated to four groups of 5 animals each: Control, T1, T2 and Torsion-detorsion (T/D) group.

2.2. Surgical procedure

Surgical procedures were performed on animals (8 weeks old) in T1, T2 and T/D groups through ilioinguinal incision according to Turner et al [31] under ketamine hydrochloride anesthesia (50 mg/kg, intraperitoneal). The left testes were accessed after skin shaving and preparation of the abdominal wall with 10% povidone-iodine solution. Torsion was created by twisting the left testes (720° in clockwise direction) and maintained by fixing it to the scrotum with a 5/0 atraumatic silk suture passing through the tunica albuginea and dartos. After 2 h of ischemia injury, the testis were detorted and replaced into the scrotum for 30 min of reperfusion. Animals in the control group underwent sham operation. After torsion-detorsion, animals in T1 group were treated with a single dose of 100 mg/kg pentoxifylline while animals in T2 received 25 mg/kg/day (IP) for 2 weeks. At the end of 5th week after torsion-detorsion, all animals were sacrificed by cervical dislocation and

orchiectomy of the left testes was performed. The left testes in all the animals were excised in sterile conditions. Testes were weighed and recorded and were then divided longitudinally into two halves: one for histopathological evaluation and the other part for assessment of oxidative stress status and apoptosis. Specimens for histopathological evaluation were immersed in formaldehyde fixative while specimen for other assays were placed in glass bottles with rubber caps, labeled and stored at -78 °C until assayed [28].

2.3. Histomorphological assessment of spermatogenesis

After 48 h, specimen fixed in formaldehyde were dehydrated in ascending grades of alcohol and then cleared in 2 changes of xylene. Thereafter, specimen were embedded with paraffin and thin sections (5μm) were obtained with microtome after which the sections were deparaffinized, and then gradually hydrated and stained with H&E. 50 round seminiferous tubule epithelium in each cross section were examined by a pathologist blinded to the experiment with the aid of an optical microscope (Olympus IX71) equipped with camera (Olympus E-30).

The tubules were then graded by the method of Johnson score [32] as follow: score 10 – complete spermatogenesis with normal regular tubules; score 9 – many sperms with presence of irregular germinal epithelium; score 8 – presence of few sperms; score 7 – seminiferous tubules with no sperm but many spermatids; score 6 – presence of few spermatids; score 5 – no sperm or spermatids; score 4 – presence of few spermatocytes; score 3 – presence of spermatogonia; score 2 – presence of sertoli cells; and score 1 – no cell present. Results were expressed as mean ± standard error of mean (SEM) for each group.

Furthermore, the number of different germ cells (spermatogonia, spermatocytes, round spermatids, elongated spermatids, sertoli, myoid and leydig cells in the seminiferous tubule epithelium were counted and the diameter of the seminiferous tubules measured with the aid of Image-J software.

2.4. Immunohistochemistry

To assess the expression of spermatogonia differentiation and spermatogenesis markers ((ID4 and SCP3 respectively), testes tissues fixed in formaldehyde solution were embedded in paraffin, and then sectioned at 5 μm. Thin sections were incubated with primary Rabbit polyclonal Anti-SCP3 antibody (ab15093, Abcam, 1/100/overnight) and anti-ID4 mouse antibody (PA5-26976, Invtrogen, US) and then secondary Goat Anti-Rabbit HRP conjugated antibody (ab6721, Abcam, 1/100/2 h) was added.

2.5. ELISA assay

2.5.1. SOD

Tissue superoxide dismutase (SOD) enzymatic activity was determined according to the method of Paoletti and Mocali, 1990 [33]. Briefly, SOD activity level was determined based on its ability to inhibit NADH oxidation in reaction mixture and conversion of superoxide anions (O_2^-) to H_2O_2 and molecular oxygen (O_2). SOD enzymatic activity was determined by decrease in absorbance at 340 nm during the reaction.

2.5.2. Catalase

Catalase activity was assayed by the method described by Aebi (1974) [34]. Briefly, 10 UL of 100% ethanol was mixed in 500 lL of sample, vortexed and incubated for 30 min in ice. 450 IL mixture of ethanol-tissue sample was then added to 50 lL of TritonX-100 (10% v/v) after which 100 IL aliquot of this sample was taken along with 2.8 mL of phosphate buffer (50 mM, pH. 7.00) for the catalase assay. The change in optical density was monitored at 240 nm immediately following the addition of 100 lL of the substrate H_2O_2 (60 mM) in the cuvette.

2.5.3. MDA

Malondialdehyde (MDA) accumulation in tissues is indicative of the extent of lipid peroxidation and is a marker of oxidative stress level. MDA is an end product of peroxidation of fatty acid in the lipid peroxidation process and the level was measured to determine oxidative damage in the testes caused by torsion-detorsion injury with a commercially available kit (Bioxytech MDA-586TM) according to the manufacturer's instructions. In brief, 2g of testis tissues homogenate was dissolved in 10ml phosphate buffer (20mM; P.H 7.4) and 375 mg of thiobarbituric acid (TBA) was dissolved in 2 ml HCl, mixed with 15% (100 ml) trichloroacetic acid (TCA), and then dissolved in 50 °C water bath. Testes tissue was homogenized in a solution of 5.1% KCl resulting in 10% homogenized mixture. Thereafter, 1 ml of the homogenized tissue mixture was mixed with 2 ml of TBA-TCA-HCl solution and then heated in boiling water for 45 min to produce a pink-orange solution. The solution was allowed to cool and then centrifuged at 1000 rpm for 10 min. The absorption (A) at 532 nm was read using a spectrophotometer (Biospect).

2.6. Quantitative real-time PCR

Expression of PI3K and AKT genes in PI3K/AKT anti-apoptotic signaling pathway were assessed by quantitative real-time PCR. Extraction of total RNA from the cells was performed using a Trizol reagent (Roche, Germany). 1 μg/mL DNase I (Fermentas, Germany) was added to prevent contamination with DNA. Then measurement of RNA concentration was carried out using a spectrophotometer (Eppendorf, Germany). Thereafter, the reverse transcription of 500 ng of the extracted RNA to complementary DNA (cDNA) was carried out by a cDNA synthesis Kit (PrimeScript™ RT Reagent Kit Fast, RR037A, TaKaRa Japan) according to the manufacturer's instruction. Evaluation of the PCR was done by using a thermocycler (Bio-Rad Laboratories) and a SYBR Green master mix (SYBR Premix Ex Taq II (Tli Plus), TaKaRa- RR820L), forward primer, reverse primer and 10 ng of template cDNA. Samples underwent an initial melting stage for 5 min at 95 °C followed by melting stage (40 cycles) for 5 s at 95 °C and synthesis for 30 s at 60 °C. Quality of PCR reactions was determined by a melting curve analysis with β-actin used as a reference gene. Cycle threshold (Ct) of the reference gene was subtracted from Ct of the target gene to obtain ΔCt after which ΔΔCt was calculated to measure the relative expression of the desired genes [35]. Sequences of the primers used are given in Table 1.

2.7. Western blot

Western blot was used to assess caspase-3 activity 5 weeks after testicular torsion-detorsion. Briefly, 100 mg of testicular tissue was homogenized in ice-cold lysis buffer containing 50mM tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 μl/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 30 μl/ml aprotinin and protease inhibitor cocktail (Roche Applied Science). Testes homogenate was centrifuged at 12000g for 20 min at 4 °C, and the supernatant collected. Then, 100 μg of supernatant protein was loaded onto each lane and electrophoresed on 10% SDS-PAGE gels after which the proteins were transferred onto nitrocellulose membranes for 1 h at room temperature and blocked with PBS containing 5% non-fat dried milk powder for 2 h. Membranes were washed using tris buffer which contain 1% Tween 20, and then probed with a monoclonal anti-casapase-3 antibody (1:1000; Abcam, St. Louis, MO, USA) overnight. Thereafter, a secondary anti-rabbit akp-linked antibody (1:10000; Abcam) was added for 1 h at room temperature and then the membranes were stained with BCIP/NBT. GAPDH served as a positive control for protein loading, and a high range molecular weight standard was used in determining the protein sizes. Immunoreactivity and signals were monitored by enhanced chemiluminescence [36] and the results were evaluated with the aid of NIH Image J software.

Table 1. Primer sequences.

Gene	Forward (5′-3′)	Reverse (3′-5′)	Amplicon length (bp)
B-actin	AGGCCAACCGTGAAAAGATG	ACCAGAGGCATACAGGGACAA	101
AKT	CACCTTTATCATCCGCTGCCT	TCGTCTCTTCTTCCTGCCTCTT	140
PI3K	GGAGAACCTATTGCGAGGGAAG	AGGGAGCTGTAGAGGTTGTAGG	171

2.8. Statistical analysis

Experimental data were analyzed by SPSS software (version 22.0 for Windows; SPSS Inc. Chicago, IL, USA) and Microsoft Excel for windows (version 2016). All results were expressed as means ± SEM. Statistical significant difference between groups were assessed by One-way analysis of variance. Multiple comparisons were made using the Tukey post hoc test and the differences were considered significant at $P < 0.05$. Graphs were plotted using GraphPad Prism (version 8.0.1, La Jolla, CA, USA).

3. Results

3.1. Histomorphological assessment of seminiferous tubules

Hematoxylin and eosin stained section of testicular tissues from all the groups were examined under microscope. Histological appearance of seminiferous tubules of animals in Control showed normal histoarchitecture of the seminiferous tubule epithelium with substantial amount of germ cells (Figure 1A). Spermatogona, spermatocytes, round spermatids, elongated spermatids, myoid, sertoli and leydig cells were all present in regular manner. Similarly, seminiferous tubules of animals in T1 group showed improved cellularity and regular arrangement of the epithelium (Figure 1B). In contrast, the seminiferous tubules of animals in torsion-detorsion (T/D) group appeared to have abnormal histomorphology with extensive distortion of germinal epithelium, very few germ cells and reduced diameter of the tubules albeit in the presence of significant amount of sertoli, leydig and myoid cells (Figure 1D). Animals in T2 group have seminiferous tubules histoarchitecture similar to T/D group characterized by distorted seminiferous tubule epithelium and few germ cells (Figure 1C).

The mean seminiferous tubules diameter (MSTD) in all the animal groups were measured and compared and the tubules were graded according to the mean Johnson score to determine the level of spermatogenesis in each group. Furthermore, the mean number of different germ cells were estimated and compared in all histological sections of the seminiferous tubules of all animal groups as presented in Table 2. Interestingly, we observed significant difference ($P < 0.01$ or 0.05) in the mean seminiferous tubules diameter, Jonson score and the number of different germ cells when T/D group is compared to Control and T1 animal groups with the exception of T2 group. However, there were no significant difference in the mean weight of the testes, number of sertoli, myoid and leydig cells in all groups.

3.2. Immunohistochemistry

Immunohistochemistry was performed to confirm the expression of ID4 and SCP3 proteins which are spermatogonia and spermatogenesis markers respectively (Figure 2 and Figure 3). We determined the percentage expression of these protein markers and the results are presented in Table 3. The mean ID4 and SCP3 protein expression in Control, T1 and T2 were significantly high ($P < 0.01$ or 0.05) when compared to T/D (Figure 4A and B).

3.3. Enzyme-linked immunosorbent assay (ELISA)

Elisa protocol was performed to assess the level of antioxidant markers (SOD and Catalase) and oxidative stress marker (MDA). SOD and Catalase activities in Control, T1 and T2 groups were significantly higher ($P < 0.01$) than T/D group. Surprisingly, these antioxidant markers were also increased significantly in T1 and T2 when compared to Control group (Figure 5A & B).

As expected, there was significantly high level ($P < 0.01$) of oxidative stress marker (MDA) in T/D group than all other groups. Interestingly, animals in T1 group showed significantly low ($P < 0.01$ or 0.05) MDA activity when compared to all other groups (Figure 5C). This implies an antioxidant effect of pentoxifylline against oxidative stress caused by testicular torsion-detorsion.

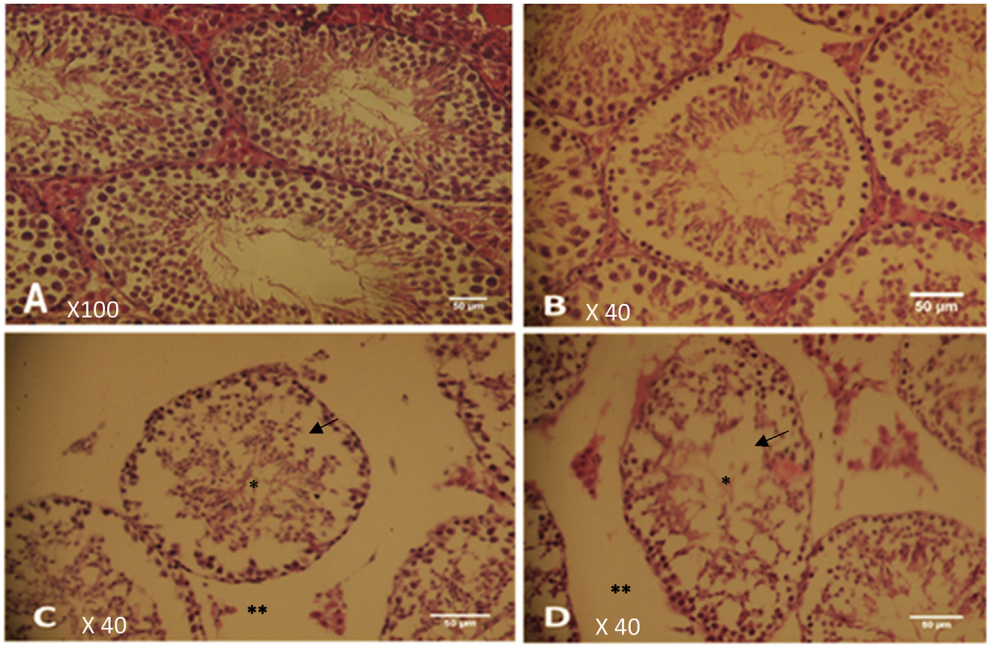

Figure 1. Light microscopy of H & E stained testicular tissue in experimental groups 5 weeks after torsion-detorsion. A: Control - normal testicular histology, B: T1 - increased cellularity with organized and improved tubular structure, C: T2 - Necrosis in epithelium (→), necrosis in interstitium (**) and decreased cellularity in tubules (*), D: T/D - Necrosis in epithelium (→), necrosis in interstitium (**) and decreased cellularity in tubules (*). (Full, non-unadjusted images are shown as supplementary material).

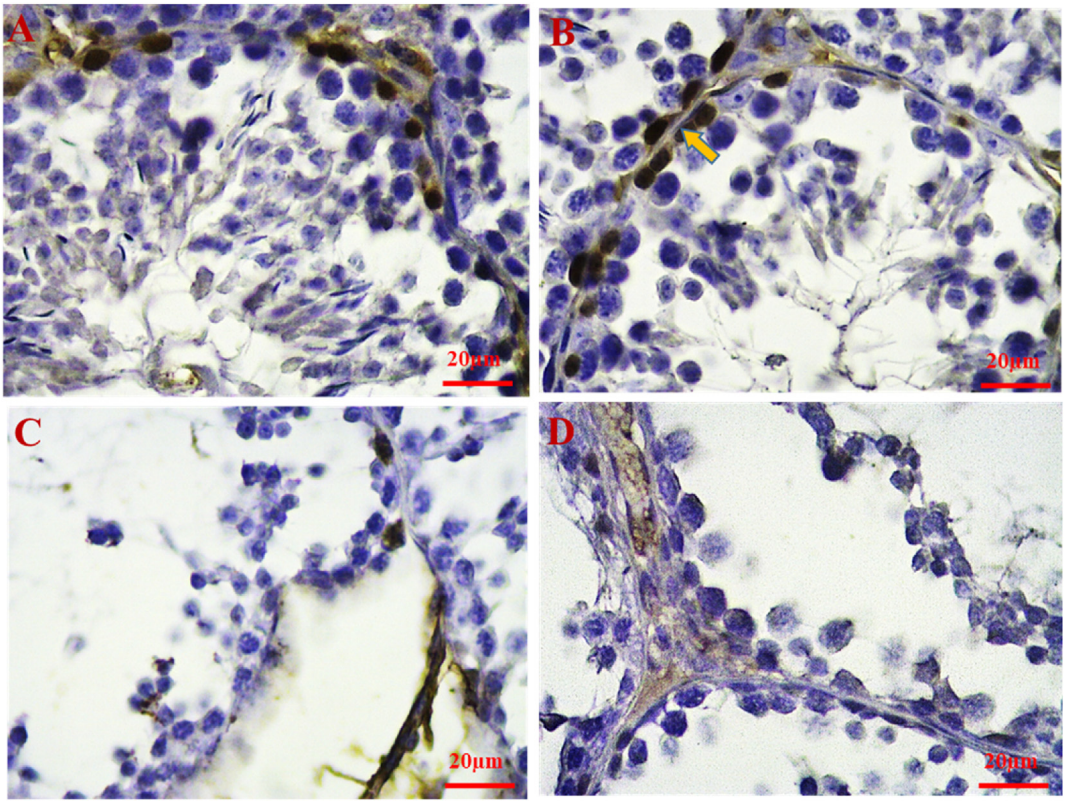

Figure 2. Immunohistochemistry staining for ID4 protein: A: Control with normal expression of ID4 protein in the seminiferous tubule, B: T1 (torsion-detorsion + single dose of 100 mg/kg PTX with significant expression of ID4 protein (yellow arrow)), C: T2 (torsion-detorsion + 25 mg/kg/day PTX for two weeks with low expression of ID4 protein, D: T/D (torsion-detorsion without any treatment and with significantly low expression of ID4 protein. (H & E, scale bar: 20 µm). (Full, non-unadjusted images are shown as supplementary material).

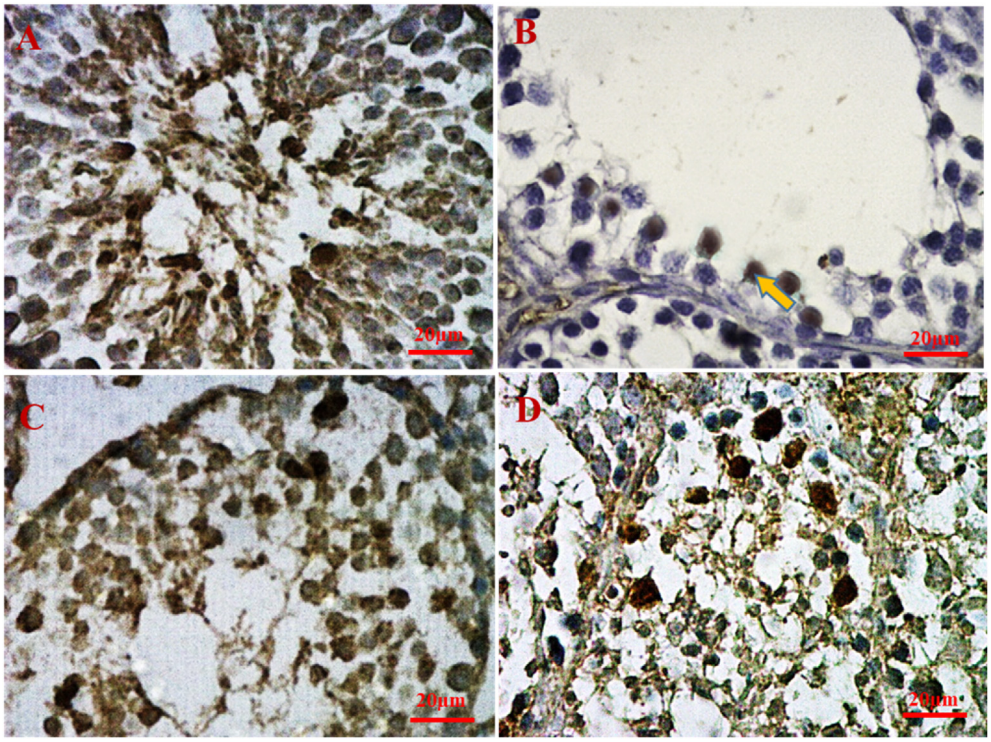

Figure 3. Immunohistochemistry staining for SCP3 protein; A: Control (with normal expression of ID4 protein in the seminiferous tubule), B: T1 (torsion-detorsion + single dose 100 mg/kg PTX with significant increase in the expression of scp3 protein (Yellow arrow)), C: T2 (torsion-detorsion + 25 mg/kg/day PTX for two weeks with significant increase in expression of sp3 protein, D: T/D (torsion-detorsion without any treatment and with significantly low expression of scp3 protein. (H & E, scale bar: 20 µm). (Full, non-unadjusted images are shown as supplementary material).

Table 2. Histomorphology of the seminiferous tubules 5 weeks after torsion-detorsion.

Parameters	T/D (Mean ± SEM)	T2 (Mean ± SEM)	T1 (Mean ± SEM)	CONTROL (Mean ± SEM)
Testes weight (g)	0.62 ± 0.09	0.61 ± 0.02$^{\#}$	0.58 ± 0.02$^{\#}$	0.63 ± 0.12$^{\#}$
MSTD (μM)	90.90 ± 1.34	91.45 ± 1.20$^{\#}$	103.95 ± 1.48^{a}	109.68 ± 1.97^{a}
Johnson score	4.28 ± 0.16	5.38 ± 0.21$^{\#}$	7.55 ± 0.20^{a}	8.33 ± 0.16^{a}
Spermatogonia	11.8 ± 0.98	13.85 ± 11.32$^{\#}$	20.45 ± 1.15^{a}	21.85 ± 1.45^{a}
Spermatocytes	20.55 ± 2.00	21.70 ± 1.72$^{\#}$	33.45 ± 2.7^{a}	41.45 ± 4.16^{a}
Round spermatids	15.85 ± 1.72	33.45 ± 3.14^{b}	65.15 ± 4.96^{a}	78.15 ± 5.45^{a}
Elongated spermatids	12.35 ± 2.17	15.25 ± 2.93$^{\#}$	18.55 ± 2.17$^{\#}$	29.60 ± 2.91^{a}
Sertoli cell	9.15 ± 0.99	9.05 ± 0.94$^{\#}$	9.85 ± 0.81$^{\#}$	10.65 ± 0.79$^{\#}$
Myoid cell count	10.95 ± 1.05	11.95 ± 1.01$^{\#}$	12.45 ± 0.97$^{\#}$	13.75 ± 1.21$^{\#}$
Leydig cell count	9.05 ± 0.95	9.9 ± 1.67$^{\#}$	9.50 ± 0.92$^{\#}$	10.6 ± 0.88$^{\#}$

Control: animals with sham operation, T1: Torsion-detorsion + single dose100 mg/kg PTX, T2: torsion-detorsion + 25 mg/kg PTX for 2 weeks, T/D: torsion-detorsion only, SEM: standard error of mean, MSTD: mean seminiferous tubular diameter. a = P < 0.01, b = P < 0.05 and # = P > 0.05 when compared with T/D.

Table 3. Immunohistochemistry for ID4 and SCP3 protein.

	Control ± (SEM)	T1 ± (SEM)	T2 ± (SEM)	T/D ± (SEM)
ID4	10.44 ± 0.87^{a}	10.20 ± 1.61$^{a,\#}$	7.51 ± 1.20$^{b,\#}$	3.47 ± 0.90
SCP3	52.84 ± 1.50^{a}	44.91 ± 0.87$^{a,\#}$	24.45 ± 2.45^{a}	8.17 ± 1.07

ID4: Inhibitor of DNA binding protein 4, SCP3: Synaptonemal complex protein 3, SEM: standard error of mean, T1: torsion-detorsion + single dose 100 mg/kg PTX, T2: torsion-detorsion + 25 mg/kg PTX for 2 weeks, T/D: torsion-detorsion only. a = P < 0.01 vs. T/D, b = P < 0.05 vs. T/D and # = P > 0.05 vs. control.

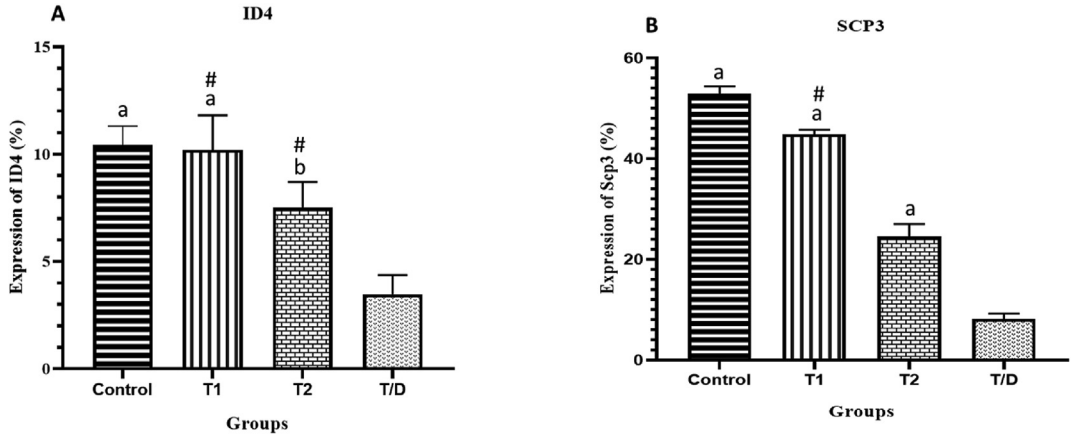

Figure 4. Percentage expression of spermatogonia and spermatogenesis marker (ID4 & SCP3) assessed at the end of 5th week after torsion-detorsion. A: Inhibitor of DNA binding protein 4 (ID4), B: Synaptonemal complex protein 3 (SCP3). Graphs are indicative of the percentage expression of the proteins. Data analyzed by Oneway Anova followed by Post Hoc Tukey tests. a = P < 0.01 vs. T/D, b = P < 0.05 vs. T/D, # = P > 0.05 vs. Control.

3.4. Real-time PCR analysis for AKT/PI3k mRNA

Relative mRNA expression of anti-apoptotic genes of AKT and PI3K were evaluated by real-time PCR at the end of 5th week after torsion-detorsion. The relative mRNA expression of AKT and PI3K in Control and treatment groups TI and T2 were significantly higher (P < 0.01) than T/D groups indicative of the anti-apoptotic effects of pentoxifylline against testicular torsion-detorsion induced apoptosis (Figure 6A & B).

3.5. Western blotting analysis for caspase-3

Evaluation of apoptotic marker of caspase-3 was done by Western blotting. There were significant high (P < 0.01 or 0.05) relative protein expression of caspase-3 in T/D group as compared to Control, T1 and T2 which confirmed the presence of more apoptotic germ cells in T/D animals. T1 and T2 showed no significant difference (P > 0.05) in their relative level of caspase-3 (Figure 6C).

4. Discussion

The present study demonstrated the protective role of pentoxifylline against impairment in the process of spermatogenesis and ultimately, testicular damage resulting from testicular torsion-detorsion. Based on our study, pentoxifylline restored germ cells, improved histoarchitecture of the seminiferous tubules, increased the expression of spermatogonia differentiation and spermatogenesis markers, reduce oxidative stress level and prevent testicular germ cell apoptosis caused by testicular torsion-detorsion in the ipsilateral testes.

Ischemia-reperfusion injury in testes commonly results to loss of germ cells and consequently leads to infertility. In our study, we showed that 720° testicular torsion for 2 h followed by detorsion significantly caused germ cell apoptosis evident by reduced number of spermatocytes, spermatogonia, round spermatids, elongated spermatids and noticeable distortion of the histoarchitecture of the seminiferous tubules which is in line with many past studies. For example, Kazemi-Darabadi et al. showed that ischemia-reperfusion (IR) injury to the testes caused infertility in

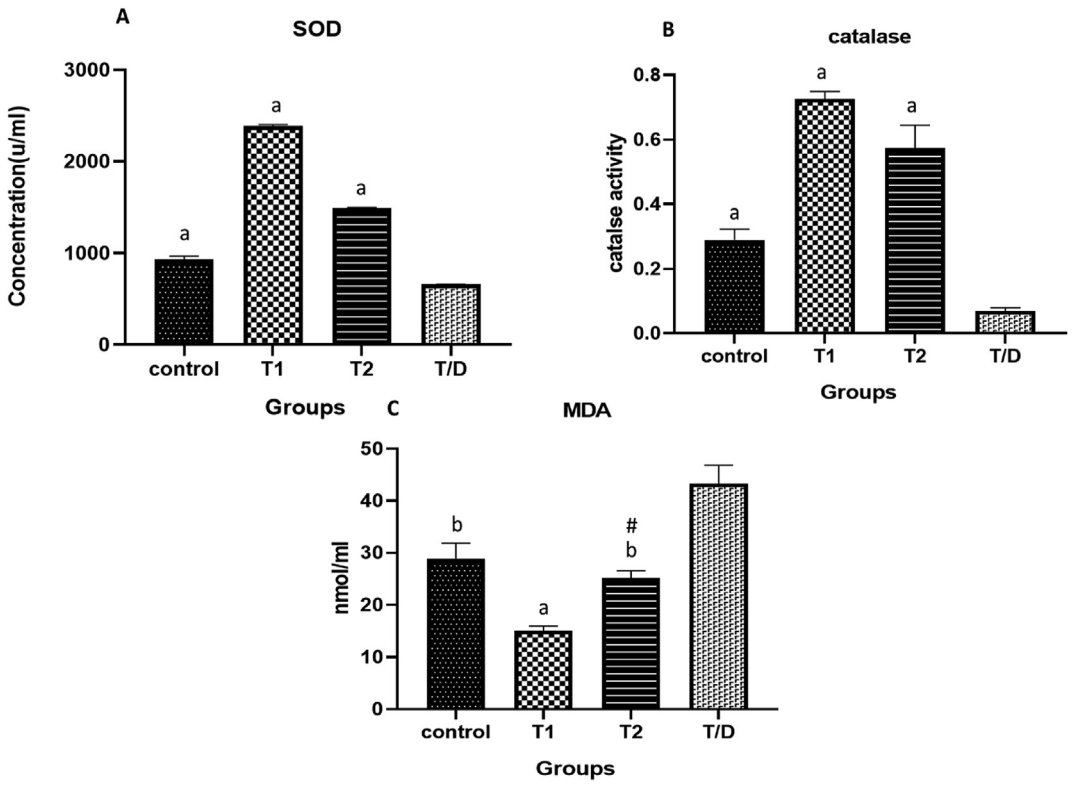

Figure 5. Enzyme-linked immunosorbent assay (ELISA) for the assessment of antioxidant and oxidative stress markers. A: SOD (Superoxide dismutase), B: Catalase, C: MDA (Malondialdehde). Control: animals with sham operation, T1: torsion-detorsion + single dose 100 mg/kg PTX, T2: torsion-detorsion + 25 mg/kg PTX for 2 weeks, T/D: Torsion-detorsion only. a = P < 0.01 vs. T/D, b = P < 0.05 vs. T/D, # = P > 0.05 vs. Control.

males [24]. In a similar study by Williamson et al. a rat model of testicular torsion-detorsion, they demonstrated that a 720° torsion induces ischemia and that an hour duration of this torsion followed by repair of torsion results to permanent loss of spermatogenesis [37].

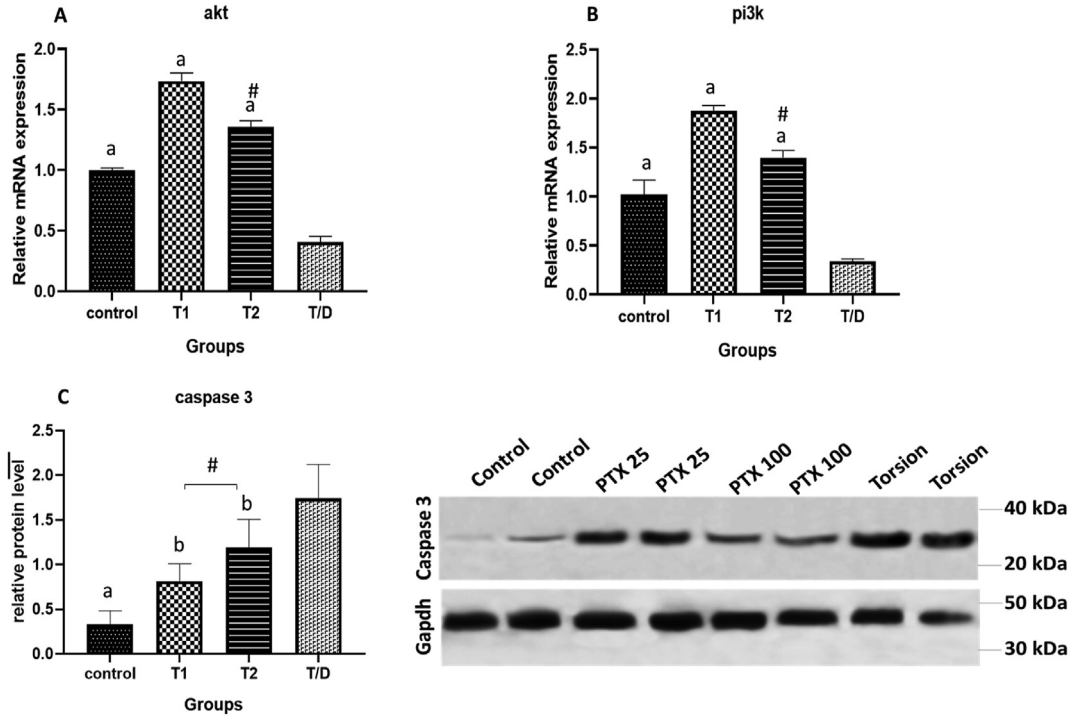

Figure 6. Relative mRNA expression of (A) akt (B) pi3k. Results were normalized firstly to β-actin and then to the control and (C) Western blot assay for caspase-3 five weeks after testicular torsion-detorsion. GAPDH served as internal control. Graph shows the ratio of normalization of the density of the apoptotic marker (caspase-3) to GAPDH. The results are presented as mean ± SEM, a = P < 0.01 vs. T/D, b = P < 0.05 vs. T/D, # = P > 0.05 vs. Control. (Full, non-unadjusted images are shown as supplementary material).

Likewise, Bozlu et al. reported that 720° torsion for 2 h resulted to irreversible loss of spermatogenesis in rats [38]. Also, Cosentino et al [39] studied the effect of changes in testicular blood flow on ipsilateral testis histology where they performed 720° spermatic cord torsion in periods of 1, 3, 5, 9, and 12 h and then removed the testes at the end of the ischemic period. They graded the testes in 4 histopathological categories and discovered histological damage to the testes, even after 1 h of torsion. Furthermore, Anderson et al. observed that unilateral testicular torsion seriously interfered with subsequent spermiogenesis in about half of the patients and produced borderline impairment in another 20% [40]. Elsewhere, In an experiment by Hagen et al., evidence of pathology in 57–88% of cases was observed in biopsies of contralateral testes after torsion characterized by tubular hyalinization, maturation arrest, germ cell degeneration, focal thickening of basement membranes and immature tubules. These abnormalities were present in patients with torsion duration of less than 24 h, suggesting that they are pre-existing rather than as a result of acute torsion. The changes in both testes may result from episodes of asymptomatic intermittent torsion [41]. Furthermore, another study showed that 48 h after blood flow restoration to the ischemic testis, bilateral histological alterations were observed, with remarkable tubular vacuolation and necrosis, as well as the loss of the maturation of germ cells [42]. The loss of germ cells and spermatogenesis observed in these studies correlate with our findings, albeit no significant changes in the mean weight of the testes, number of sertoli, myoid and leydig cells in line with the result of Baker et al. [43].

Many studies have reported the protective effect pentoxifylline on testicular tissue damage caused by torsion-detorsion. For example, a study conducted by Takhtfooladi et al. showed that administration of 40 mg/kg pentoxifylline significantly reduced testicular injury in a rat model of unilateral hindlimb ischemia/reperfusion injury [44]. In the PTX treated group, histopathological features such as edema, congestion, hemorrhage, and necrosis of the germinal cells were markedly less when compared to ischemia-reperfusion group. They showed that the reperfusion of the ischemia limb within 24 h lead to a systemic response indicated by the biochemical and histological impairment of the testis which was however, ameliorated by pentoxifylline treatment. Likewise, in this present study, we observed the protective effects of pentoxifylline in a single acute (100 mg/kg) and chronic dose (20 mg/kg for 2 weeks) against testicular damage in mouse model of 2-hour testicular torsion-detorsion capable of causing irreversible loss of spermatogenesis. Histomorphological analysis of the seminiferous tubules of animals in torsion-detorsion group by the end of 5th week after surgical torsion-detorsion revealed histological damage to the testes of animals with distortion of the tubules. This damage was significantly reduced by a single100 mg/kg pentoxifylline treatment evident by improved histopathological score, diameter of the seminiferous tubules and significant increase in the number of germ cells, although, this is not true for 25 mg/kg pentoxifylline treatment for 2 weeks where animals has testicular histopathological features characteristic of the testes of animal in torsion-detorsion group.

Testicular torsion-detorsion disrupts the process of spermatogenesis evident by the loss of protein markers which drive this critical process in male reproduction. For the first time, we observed for the presence or absence of ID4 and SCP3 protein which are well-known markers of spermatogonia differentiation and meiotic cell division respectively. These markers were observed five weeks after torsion-detorsion and we found significant low expression in the testes of animals that underwent only torsion-detorsion (T/D). However, 100 mg/kg PTX treatment, as well as 20 mg/kg PTX for 2 weeks significantly improved the expression of ID4 and SCP3 markers, indicative of a potential protective role of pentoxifylline in restoration of impaired spermatogenesis caused by testicular torsion-detorsion. However, further studies to ascertain the effectiveness of pentoxifylline in the treatment of spermatogenesis defects resulting from loss of makers at any of the stages of the process of spermatogenesis are warranted.

The testis is extremely sensitive to oxidative free radical injury [45]. The observed disturbances in sperm count, motility and morphology correlate with high levels of ROS in sperm, as previously reported in infertile patients [46]. Ayan et al. found that testicular torsion and repair has the features of a classical ischemia-reperfusion (IR) injury, and that ROS released from recruited PMNs is at least partially responsible for the germ cell-specific apoptosis observed after torsion repair [47]. Also, Takhtfooladi and colleagues showed that the main cause of tissue injury after ischemia-reperfusion is ROS [48]. Furthermore, Vigueras et al. observed that the main pathophysiologic response in testicular torsion is ischemia followed by reperfusion which generates ROS upon detorsion [49]. Filho et al. further confirmed that reperfusion injury plays a major role in testicular damage related to testicular torsion [45]. Ozmen and colleagues showed that after reperfusion injury, large amounts of oxygen-derived free radicals lead to tissue damage, and systemic complications resulting from an inflammatory response [50]. Therefore, tissue damage that occurs during reperfusion is more severe than that which occurs during ischemia [51]. . The inflammatory response that precede testicular damage is characterized by elevated level of pro-inflammatory cytokines, vascular adhesion molecules and testicular influx of neutrophil [52] and also, rapid generation of ROS [53]. Suppressing ROS generation is known to be an effective treatment strategy against ischemic-reperfusion-induced diseases such as cerebral stroke and myocardial infarction [54]. The ultimate result of a localized inflammatory response is the production of oxidative stress caused by excessive generation of ROS. This free radicals cause apoptosis of testicular germ cells. Under normal condition, this ROS production is regulated at physiological low level by endogenous antioxidant defense system in tissues [55] but in high condition of oxidative stress, such as reperfusion injury, these free radicals become abnormally high, capable of causing damage to tissues. However, Aguilar and team clarified that the degree of ischemia-reperfusion injury depends on the balance between the production of oxygen free radicals and antioxidant defenses [56] In this study, we showed that 2 h of testicular torsion followed by detorsion caused marked increase in lipid peroxidation damage indicated by significant high level of MDA in T/D group. However, pentoxifylline, a known antioxidant agent suppressed MDA level to a significantly low level in treatment animal groups (T1 and T2), albeit, more pronounced in T1 animals.

Although, oxygen-derived free radicals are a major cause of tissue injury after reperfusion, there exists an endogenous defence system to prevent against the sudden burst of free radical production in order to control damage in the ischemic tissue. Interestingly, it was shown that during I-R injury, the activities of several antioxidant enzymes increase. These enzymes include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) [57]. Pekcetin et al. identified SOD and glutathione peroxidase as the major enzymes that scavenge harmful ROS in male reproductive organs [58]. Generally, experimental studies of ischemia–reperfusion tissue injury have shown that antioxidants reduce short-term damage in testicular torsion [59]. Ayan et al. observed increased antioxidant enzyme (SOD, GSH-Px) activities and MDA levels in the tissues of ischemic testis [47]. Increased SOD and GSH-Px enzyme activities in the testicular tissues may indicate cellular oxidative stress, or, these enzymes may render compensatory mechanisms. SOD is one of the major intracellular enzymes that prevent tissue damage and can selectively and rapidly reduce superoxide anions (O_2^-) free radical to hydrogen peroxide (H_2O_2). Elshaari et al. reported that a long period of torsion results to ischemia and high oxidative stress in the ipsilateral testes [60] but with mild ischemia, tissue is able to counteract the oxidative stress situation through the action of antioxidant enzymes like SOD and CAT [45].

Theoretically, antioxidants play a dual role in ischemia-reperfusion in testicular injury. First, they limit the amount of damage by reducing the amount of free radicals produced by lipid peroxidation, and secondly, they neutralize inflammatory reactions mediated by ROS [61]. While there are no reports of therapeutic application in human, many studies have attested to the protective effect of several antioxidant agents in experimental animal models of testicular torsion and are proven to render remarkable benefits against testicular damage. For example, Bilge et al. demonstrated that ranolazine drug protected against testicular damage in 1-hr testicular torsion [62]. Likewise, Atilla and colleagues showed that zinc and melatonin administration partially protected against testicular injury resulting from 1-hour ischemia and 1-hour reperfusion [63]. Also, Belhan et al. found that chrysin protected the testes from oxidative and inflammatory markers in experimental testicular ischaemia/reperfusion injury in rats [64]. In a study by Turner et al., oxygen radical scavengers were infused into animals prior to the repair of torsion. Their testicular weight and daily sperm production were examined 30 days after surgery. The results revealed that ROS scavengers provided significant rescue of the testis function from being compromised [31]. Furthermore, Savas and colleagues reported that pentoxifylline has been used in ischemic intestine to improve blood flow and that this drug is unlikely to harm human because of its similarity to endogenous metabolite [65] while Takhtfooladi et al. reported that the activities of SOD and CAT in testicular tissues were decreased by ischemia/reperfusion, but administration of pentoxifylline increased the levels of these enzymes [66]. Accordingly, in our study, the lower level of antioxidant enzymes in T/D animals versus control group could be seen as a compromised enzymatic antioxidant defense system in testicular tissue which were significantly restored by pentoxiffyline treatment in T1 and T2 animal groups.

Apoptosis is a programmed cell death that involves multiple kinases and cysteine proteases known as caspases. It is a genetically controlled suicide, characterized by cleavage of DNA and does not produce inflammation [67]. Many studies showed that testicular ischemia-reperfusion leads to apoptosis of germ cells induced by ROS from neutrophils [68]. Activation of PI3K/AKT pathway is known to regulate apoptosis and promote the proliferation and survival of sperm [69]. AKT, a serine-threonine kinase, is an important downstream protein of PI3K. Inhibited AKT can activate downstream caspase-3 and initiate apoptotic process [70]. It was also shown that the PI3K/AKT/mTOR signal pathway can regulate cell cycle, protein synthesis and cell energy metabolism [71]. Caspases act in both pathways of apoptosis. Caspase-3, also known as the primary executioner caspase, is responsible for morphological changes such as chromatin condensation, DNA and membrane protein breakdown [72]. Lee et al. [73] reported increased expression of activated caspase-3 in testicular germ cells after varicocele creation. This study revealed that after testicular torsion-detorsion, the relative mRNA expression of PI3K and AKT protein were significantly lower in torsion-detorsion group compared to the control group, indicative of inhibition of anti-apoptic function of PI3K/AKT pathway. Torsion-detorsion also resulted to significant elevated level of caspase-3 in T/D, a mediator and a known marker of apoptosis. This agrees with the finding of Turner et al. that a 1-hour 720° testicular torsion in rat model resulted to germ cell-specific apoptosis as assessed by in situ TUNEL and by DNA laddering [31]. However, the signaling pathway associated with survival of cells under oxidative stress is attributed in part to upregulation of PI3K and AKT signaling pathways [74].

5. Conclusion

Our study showed that intraperitoneal administration of a single acute 100 mg/kg pentoxifylline dose restored impaired spermatogenesis caused by 2-hour 720° (clockwise) testicular torsion and detorsion by increased histopathological scores, increased diameter of seminiferous tubules and increase in the number of germ cell line whereas we observed no significant improvement with chronic 25 mg/kg of pentoxifylline administration for 2 weeks. However, in both treatment cases, pentoxifylline rendered significant protection against testicular damage via elevated levels of antioxidant enzymes (SOD and catalase), suppression of oxidative stress marker (MDA), increased expression of anti-apoptotic genes (akt and pi3k) and decreased expression of apoptotic marker (caspase-3). The free radical scavenging property and anti-inflammatory response of pentoxifylline are the likely mechanisms of its antioxidant properties and therefore, pentoxifylline drug could be used as an adjunct therapy to surgical repair of testicular injury resulting from testicular torsion-detorsion in human. However, we suggest further studies on the protective role of pentoxifylline against ischemia-reperfusion injury resulting from testicular torsion-detorsion on other subjects.

Declarations

Author contribution statement

Akanji Omotosho Dhulqarnain: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nasrin Takzaree, Golamreza Hassanzadeh, Tayebeh Rastegar: Conceived and designed the experiments.

Heidar Tooli, Mahsa Yaghobinejad, Somayeh Solhjoo: Contributed reagents, materials, analysis tools or data.

Mehrnoush Malekzadeh, Nasrin Khanmohammadi: Performed the experiment.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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