Life Sciences 222 (2019) 203–211

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Treatment with estrogen receptor agonist ERβ improves torsion-induced oxidative testis injury in rats

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ARTICLE INFO

Keywords:
Testicular torsion
Estrogen
Infertility
Sperm
Apoptosis
Oxidative injury

ABSTRACT

Aims: The purpose of the present study was to investigate the potential antioxidant, anti-apoptotic and sperm function-preserving effects of estrogen, estrogen receptor (ER)α and ERβ agonists in a rat model of testis torsion-detorsion (T/D).

Main methods: Under anesthesia, 6–8-week-old male Sprague-Dawley rats underwent sham-operation or testicular torsion by fixing left testis rotated at 720° for 2 h. After detorsion, rats were treated with ERα agonist (1 mg/kg/day, subcutaneously, sc) or ERβ agonist (1 mg/kg/day, sc) or estradiol (E_2, 1 mg/kg/day, in drinking water) or vehicle on the following two days. On the third day, testicular blood-flow was recorded and then left testes were extracted for molecular and histochemical analysis.

Key findings: The findings showed that reduced testicular blood-flow following torsion was partially restored on the 3rd day of detorsion, while treatments with either of the ER agonists or E_2 returned blood flow fully back to the control levels. When the testis-torsioned rats were given ERβ agonist during the detorsion period, tubular injury was lessened, sperm count and motility were increased, while the production of reactive oxygen metabolites and apoptosis in the testis tissues were totally suppressed. Although a down-regulated expression of androgen receptor (AR) along with a reduction in serum testosterone level was observed in the vehicle-treated T/D group, all three treatments up-regulated the expressions of AR and its mRNA, while ERα agonist and E_2 suppressed the testosterone level.

Significance: ERβ receptor activation during the post-ischemic period may be beneficial in protection against torsion-related oxidant testicular injury and infertility.

1. Introduction

Testicular torsion can be seen in all age groups with a higher incidence in early childhood and puberty, nearly in 3.8 per 100,000 males younger than 18 years old [1,2]. Torsion causes an ischemic injury of the testis due to disrupted blood supply whereas reperfusion further exacerbates the severity of injury [3]. During ischemic injury and reperfusion, generation of reactive oxygen species induces several signal molecules and leads to progressive structural and functional impairment in the ipsilateral testis [4], which may cause gonadal apoptosis and infarct [5,6]. Since irreversible testis injury is unavoidable upon a prolonged ischemic period, an immediate surgical intervention is vital for preserving the reproductive functions [7]. In addition to conventional surgical therapy, use of antioxidants during the critical post-ischemic period could have a protective action in ameliorating the severity of testicular torsion, thereby could prevent the occurrence of infertility. Several antioxidants have been evaluated for the initial treatment of testis torsion in animal models [8–10]. Natural estrogens were found to be approximately 2.5-fold more potent than vitamin C and E in scavenging free radicals [11]. Estrogen also exhibits additional anti-inflammatory effects by inhibiting pro-inflammatory gene expression [12], inducing anti-apoptotic genes [13] and by inhibiting apoptotic proteins [14], which suggest that estrogen could be regarded as a potent agent in alleviating post-ischemic injury of torsioned testis.

The important role of estrogen was documented in the normal functioning of male reproductive system through its impact on

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https://doi.org/10.1016/j.lfs.2019.02.056
Received 5 February 2019; Received in revised form 25 February 2019; Accepted 26 February 2019
Available online 27 February 2019
0024-3205/

hypothalamus-hypophysis-gonadal (HPG) axis, Leydig cells, Sertoli cells, germ cells and epididymis. Release of gonadotrophins via the estrogen feedback on HPG axis [15], development of efferent ductules and epididymis, regulation of Leydig cell maturation and descent of testes are modulated by estrogen. While Sertoli cells are the major source of estrogen in pre-puberty, in adulthood Leydig cells become responsible from estrogen production through the activity of aromatase enzyme [16,17]. Estrogen regulates proliferation, growth and function of Sertoli cells and has a major role in controlling the apoptosis of germ cells and acrosome biogenesis [18,19]. In males, estrogen receptor (ER) α is highly expressed in the Leydig cells of the testes, epididymis and prostate stroma, while ERβ is expressed in the epithelial cells of prostate, endothelial cells and testes [20–22]. In neonatal period, ERα signal is needed for the growth of Sertoli cells [23], while absence of ERα in the adulthood causes dysfunction of efferent ductules and excess liquid accumulation in testes, and results in decreased epididymal sperm count [24,25]. ERα has a main role in regulating spermiogenesis, while ERβ regulates spermatocyte apoptosis and spermiation [26].

The protective effects of estrogen and ER agonists through their antioxidant and anti-inflammatory properties have been previously demonstrated in several inflammation models [27,28]. However, the effects of estrogen and ER agonists on a testicular injury model were not evaluated before. Thus, the aim of the current study was to investigate the possible antioxidant, anti-apoptotic and sperm function-preserving effects of estrogen, ERα and ERβ in a rat model of testis torsion-detorsion.

2. Materials and methods

2.1. Animals

Forty male Sprague Dawley rats (180–230 g), supplied by the Marmara University (MU) Animal Center (DEHAMER), were housed in an air-conditioned room with light and dark cycles of 12 h, relative humidity (65–70%) and constant temperature (22 ± 2 °C). The rats had free access to food and water. The experiments were performed in compliance with the Turkish law on the use of animals in experiments, and the principles and guidelines developed by the New York Academy of Sciences were followed. All experimental procedures were approved by the MU Animal Care and Use Committee (approval code: 078.2016.mar).

2.2. Surgery and experimental design

In sterile conditions and under general anesthesia with the intraperitoneal injection of ketamine (100 mg/kg) and xylazine (1 mg/kg) combination, tunica vaginalis of the left testes was dissected with a longitudinal surgical incision. Testis torsion was applied by rotating the testis 720° clockwise around its axis along spermatic cord, and the twisted testis was fixed in place with a silk suture [10]. The testis was covered with a sterile gauze pad and kept moist with normal saline until the end of 2-hour torsion period, then detorsion was applied by untwisting the testis. In the sham-operated group, the anesthetized rats underwent scrotal incision, but their left testes were not rotated. Following the suturing of the testicular incision, all rats were returned to their home cages.

Starting immediately after the surgical closure, rats with torsion and detorsion (T/D; $n = 32$) were randomly divided and treated daily with either vehicle (sunflower oil, 1 ml/kg/day; subcutaneously, sc) or ERα agonist propyl pyrazole-triol (PPT; 1 mg/kg/day, sc; Tocris) or ERβ agonist diarylpropionitrile (DPN; 1 mg/kg/day, sc; Tocris), while the sham-operated group (n = 8) received vehicle. In another T/D group, estradiol (1 mg/kg/day, Bayer Turk) was administered in the drinking water in the following two days. All rats were also treated with an analgesic (0.1 mg/kg/day, sc; Perfalgan, Bristol Myers Squibb) and an antibiotic (0.1 mg/kg/day, sc; ciprofloxacin, Sandoz) immediately after the surgery and during the following two days. The rationale in selecting the doses of estradiol and agonists was based on our previous study [27,28].

At the end of the third day, following the testicular blood flow recording, rats were euthanized with intracardiac blood withdrawal and left testes were removed. Testosterone levels were measured in serum samples by Enzyme-Linked Immuno Sorbent Assay (ELISA), according to the manufacturer's guidelines of the commercial kit (Sun Red, Shanghai). Caudal epididymis of each rat was placed in a transport medium for light microscopic examination of semen. Half of each testis was put in formaldehyde solution for histopathologic evaluation, while the other half was stored at -20 °C for biochemical analyses.

2.3. Blood flow measurement

Under ketamine-xylazine anesthesia, blood flow was measured using a laser Doppler device (PeriFlux System 5000, PERIMED, Sweden, 2010) and PeriSoft 2.5.5 program. The probe (Probe 307) was placed on the surface of the left testis of both the T/D and sham-operated rats [10], and 3 measurements were taken from each animal as before torsion, 1 h after detorsion and before euthanasia.

2.4. Assessment of epididymal sperm parameters

For semen analysis, epididymis samples of all rats in each group were dissected in Earle's Balanced Salts Solution (Sigma, USA) and supernatants were discarded following a 5-min sedimentation period. Using the gradient method, pellets were first centrifuged at 1800 rpm for 18 min, then supernatants were discarded, and diluted with a sperm-washing medium (SAGE, UK) to centrifuge at 2000 rpm for 10 min and supernatants were removed again. After adding a fertilization medium (SAGE, UK) to the pellets, Macler Counting Chamber (Sefi Medical Instruments, Haifa, Israel) was used to count the sperms and to define their motility rate under a photomicroscope (Olympus BX51, Tokyo, Japan). All the sperms in 100 squares were counted and multiplied by a million. Ethanol was used to both fix and dehydrate the cells on the smear slides, which were then stained with Diff-Quick kit (Medion Diagnostics, Grafelfing, Germany). In each preparation, 100 spermatozoa were selected and examined at 100× immersion oil objective of the photomicroscope to evaluate their morphologies [10].

2.5. Measurement of malondialdehyde and glutathione levels

In testis samples, malondialdehyde (MDA) levels were measured as products of lipid peroxidation by monitoring the formation of thiobarbituric acid chromogenic reagent, while glutathione (GSH) levels were determined with a spectrophotometric measurement based on the modified Ellman procedure [29,30].

2.6. Measurement of myeloperoxidase activity and superoxide dismutase activity

In order to quantify accumulation of polymorphonuclear leukocytes to the tissues, tissue myeloperoxidase (MPO) activity was utilized, while superoxide dismutase (SOD) activity was assessed as an endogenous antioxidant. Using spectrophotometric (Schimadzu UV-02 model spectrophotometer, at 460 nm) methods, MPO activity was determined by the measurement of H_2O_2-dependent oxidation of o-dianizidine 2HCl [31]. Similarly, superoxide dismutase (SOD) activity in the tissue was determined by a spectrophotometric method using a standard curve obtained by bovine SOD (Sigma–Aldrich; S-2515-3000 U) as a reference [32].

2.7. Chemiluminescence assay

Generation of reactive oxygen metabolites (ROM) was

quantitatively measured using a luminometer (Junior LB 9509, EG&G Berthold, Germany) with the addition of the probes lucigenin (bis-*N*-methylacridiniumnitrate, Sigma) or luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) to the testis tissue samples [33]. Luminol specifically detects hydroxyl radical, hydrogen peroxide and hypochlorite radicals, while lucigenin selectively indicates the presence of superoxide radicals. The results are expressed in relative light units (rlu) per mg of tissue.

2.8. Western blot analyses for protein expression

Testis tissue samples were homogenized using RIPA cell lysis buffer (89900, Thermo), and the protein concentrations were determined by the BCA protein assay (Thermo Scientific). Tissue samples were denaturated with Laemli buffer and 25 µg protein was loaded to each well. The samples were resolved by 4–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membrane, which was then blocked with 3% BSA (Capricorn Scientific, BSA-1T) in Tris buffered saline (TBS). The membrane was washed twice in TBS containing Tween-20 (0.1%) and incubated overnight with primary antibodies (1:500 dilution anti-caspase-3 and cleaved anti-caspase-3 sc-7272, anti-AR sc-816, anti-B-actin sc-47778, Santa Cruz Biotechnology, Heidelberg, Germany) and washed with TBS containing Tween-20 (0.1%). The membrane was washed and then incubated with horseradish peroxidase conjugated secondary antibody (anti-mouse; sc-2060 or anti-rabbit; sc-2004, Santa Cruz Biotechnology) for 2 h. The blots were developed with chemiluminescence reagents (sc2048, Santa Cruz Biotechnology, Texas, USA) and analysed with chemiluminescent imaging systems (Syngene, Cambridge, UK). Data were analysed using "Image J Programme Optical Density Analysis Software" (NIH) by measuring each band three times for quantification. Signals were normalized with respect to B-actin.

2.9. qPCR analysis for gene expression

Using the PureLink® RNA Mini Kit (Invitrogen, K1560-02), RNA was first isolated from the testis tissues and this isolation was done according to the kit procedure. Then, cDNA was synthesized from isolated RNA and used in accordance with the kit procedure using the commercially purchased High-Capacity cDNA Reverse Transcription Kit (ABI-4387406). The obtained cDNAs were subjected to qPCR using specific primers designed to measure gene expression levels. QRT-PCR was performed using the Power SYBR Green Supermix (ABI-4368577, Applied Biosytem) on the StepOne Plus System. At the end of the amplification cycles, a melting temperature analysis was carried out through a slow increase in temperature (0.1 °C/s) up to 95 °C. The primers used were as follows: GAPDH Forward: 5′-GTGGATATTGTTG CCATC-3′; GAPDH Reverse: 5′-ACTCATACAGCACCTCA-3′; AR Forward: 5′-GGGGCAATTCGACCATATCTG-3′; AR Reverse: 5′-CCCTTT GGCGTAACCTCCCTT-3′. It was made using Step One Plus System Software. An Applied Biosystem program was used for the analysis and the Ct value was calculated according to the PCR reaction curve and relative quantitative data were analysed by the $2^{-\Delta\Delta Ct}$ method. The results are expressed as normalized ratios.

2.10. Measurement of tissue caspase-3 activity

In order to determine the tissue levels of caspase-3 activity, testis tissue samples were homogenized with cell lysis buffer and centrifuged for 10 min at 9000 rpm at 4 °C. Using a commercial kit (Calbiochem, USA) and following the manufacturer's instructions, supernatant was used for measuring caspase-3 activity as a marker of apoptosis in tissues.

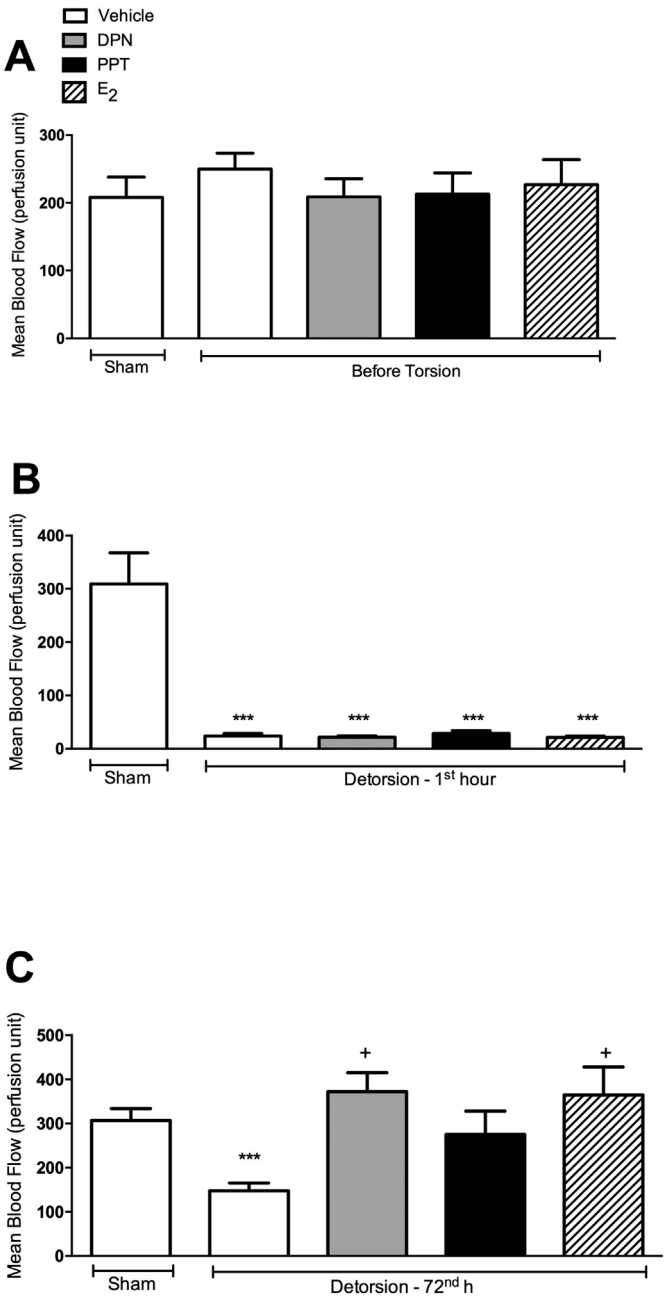

Fig. 1. Mean blood flow measured by laser Doppler in the rat testes. Measurements were taken from each animal before torsion (A), at 1 h after detorsion (B) and at 72nd hour after detorsion (C). ***$p < .001$ compared to sham-vehicle group; $^{+}p < .05$ compared to torsion-vehicle group.

2.11. Histopathologic preparation and analysis

Torsion-applied left testes were fixed in 10% formaldehyde solution, dehydrated with ascending ethanol series and then cleared with toluene. At room temperature, testes were embedded and blocked in paraffin that was incubated overnight in a 60 °C incubator. Thereafter hematoxylin and eosin (H&E) staining was applied to approximately 5 µm-thick paraffin sections in midline area of the testis. In each section of testes tissues, at least 20 seminiferous tubules were evaluated microscopically at ×200 magnification. After randomly selecting the first seminiferous tubule, the rest were observed by moving the section clockwise. Histopathological scoring was made by using the modified Johnsen scoring method as follows: *10: Full spermatogenesis; 9: Many late spermatids, but a slightly impaired spermatogenesis with disorganized*

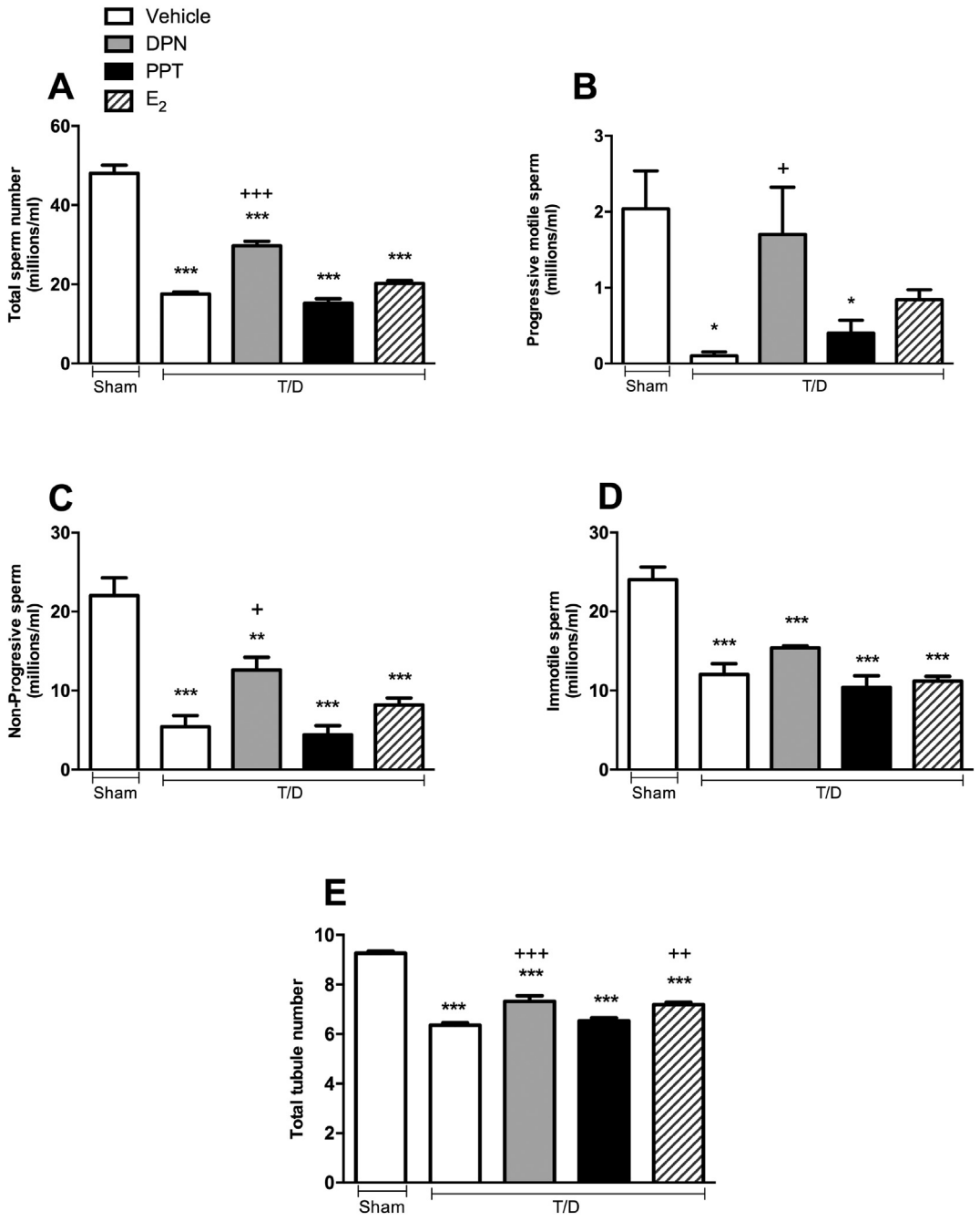

Fig. 2. Total sperm numbers (A), progressive (B), non-progressive (C) and immobile (D) sperm and total tubule (E) numbers in the sham-operated and torsion-detorsion (T/D) groups. *$p < .05$, **$p < .01$, ***$p < .001$ compared to sham-vehicle group; $^{+}p < .05$, $p < .01$, $^{+++}p < .001$ compared to T/D-vehicle group.

epithelium; 8: A few late spermatids and more than 5 spermatozoa/tubule; 7: Many early spermatids, but no late spermatids or spermatozoa; 6: A few early spermatids, but no spermatozoa or late spermatids; 5: Many spermatocytes, but no spermatozoa or spermatids; 4: A few spermatocytes, but no spermatozoa or spermatids; 3: Only spermatogonia; 2: Only Sertoli cells, but no germinal cells; 1: No seminiferous epithelium at all [34,35].

2.12. Statistical analysis

Statistical analysis was done by GraphPad Prism 6.3 (GraphPad Software, San Diego; CA; USA). All data are expressed as means ± S.E.M. One-Way ANOVA and Student's *t*-test were used in analysis of data. $p < .05$ was considered as statistically significant.

3. Results

Testicular basal blood flow levels were similar in all the experimental groups (Fig. 1). As expected, blood flows were dramatically depressed at the first hour of detorsion period as compared to the sham-operated group ($p < .001$), confirming that T/D model was achieved. At the 72-h of reperfusion period, blood flow was still impaired in the vehicle-treated T/D group ($p < .001$). However, testicular blood flows of PPT, DPN- or E_2-treated T/D groups were not different as compared to that of the sham-operated group, while blood flows in the ERβ agonist DPN- or E_2-treated T/D groups were statistically higher than that was measured in the vehicle-treated group ($p < .05$).

In torsion-applied groups, total sperm count was significantly reduced with respect to sham group ($p < .001$), and this reduction was

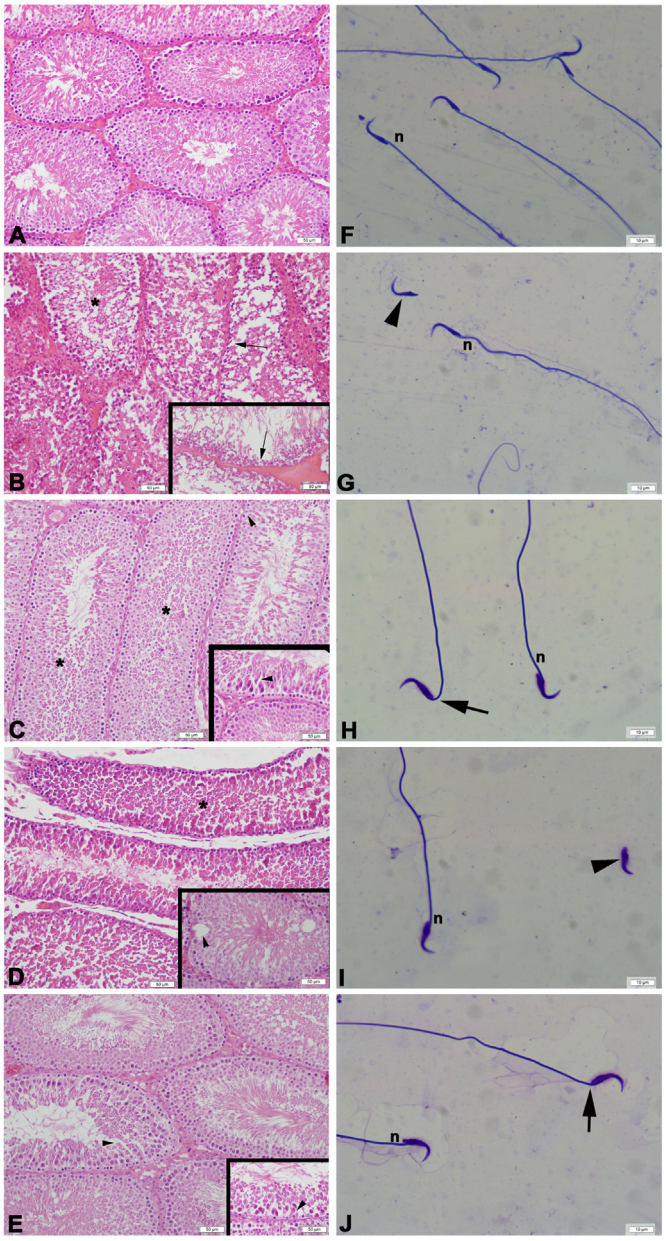

Fig. 3. Representative photomicrographs in experimental groups. Normal testis morphology with seminiferous tubules in sham-operated control group (A); decreased cell line (arrow), disorganized epithelium and immature germinal cells in lumen (*) in left testes of torsion-applied group (B); dilatation (arrowhead) between the seminiferous tubules, immature cells (*) in the lumen of seminiferous tubules in E_2- (C), PPT- (D) or DPN- (E) treated and torsion-applied groups. Normal spermatozoa (n) and abnormal spermatozoa with absence of tail (arrowhead) and neck damage (arrow) in the sham-operated control (F), torsion-applied (G) and E_2- (H), PPT- (I) or DPN- (J) treated and torsion-applied groups. A–E: H&E staining, F–J: Diff Quick staining, Scale bar: A–E: 50 μm; F–J: 10 μm.

also evident in progressively motile ($p < .05$), non-progressively motile ($p < .001$) and non-motile ($p < .001$) sperm counts (Figs. 2 and 3). Treatment with ERβ agonist elevated motile sperm counts ($p < .05$) as well as the total number of sperms ($p < .001$) that were reduced by T/D. Microscopic analysis revealed normal testes morphology with regular seminiferous tubules and spermatogenic cells in the sham-operated control group (Fig. 3). However, in the testes of vehicle-treated T/D group, most of the seminiferous tubules were degenerated with vacuole formation in spermatogenic cells and many immature cells were present in the lumen, while spermatogenic germ cells were decreased in number. Despite a few number of normal germinal epithelia with spermatids and spermatozoa, some of the seminiferous tubules did not have spermatozoa or spermatids at all. On the other hand, in E_2- or ERβ agonist-treated T/D groups, most of the seminiferous tubules demonstrated a quite regular germinal epithelium with spermatogonia, spermatocytes, spermatids and spermatozoa, but some tubules showed a dilatation between the spermatogenic cells. In contrast, in the testes of T/D group treated with ERα agonist, many immature cells were observed in the tubular lumen, spermatogenic germ cells were reduced, while degeneration and vacuolization were observed in the germinal epithelia of some seminiferous tubules; but many tubules still showed a normal germinal epithelium with spermatids and spermatozoa (Fig. 3). In addition, total number of seminiferous tubules in all the T/D groups was less than that of the sham-operated control ($p < .001$; Fig. 2). However, these reductions in the number of tubules were significantly reversed in both the ERβ agonist- ($p < .001$) and E_2- ($p < .01$) treated T/D groups. Epididymal spermatozoa evaluation in all torsion-applied groups showed many spermatozoa with absence of tail or abnormal spermatozoa with neck and tail defects, while the number of abnormal spermatozoa was decreased in the testes of E_2- or DPN- or PPT-treated T/D groups (Fig. 3F–G).

Levels of MDA, which is the major catabolic product of lipid peroxidation, were found to be higher in the testis tissues of the vehicle-treated T/D group than those in the control tissues ($p < .05$), but the elevations in E_2- and PPT-treated T/D groups were not statistically significant (Fig. 4). On the other hand, testicular MDA level of the group injected with the ERβ agonist was significantly lower than of the vehicle-treated T/D group ($p < .05$). MPO activity, which shows neutrophil infiltration to the inflamed tissue, was increased in the testes of all T/D groups, as compared to that of the sham-operated group ($p < .05$; Fig. 4). Luminol- and lucigenin-enhanced chemiluminescence levels in the testes were elevated in vehicle- or PPT-treated T/D groups, indicating elevated generation of OH, H_2O_2, HOCl radicals and O_2^- radicals, respectively ($p < .05$–0.001; Fig. 4). However, in the T/D groups treated with either ERβ agonist or 17β estradiol, these elevations were abolished ($p < .01$–0.001). Testicular SOD levels demonstrated a tendency to increase in T/D groups as compared to sham-operated control group, suggesting a compensatory elevation in the tissue antioxidant content, while a statistical significance was reached only in the ERα agonist PPT-treated group that has demonstrated the highest levels of oxidative response ($p < .05$; Fig. 4).

As an indicator of apoptosis, caspase-3 activity in the testis tissue was elevated in the vehicle-treated T/D group with respect to sham-operated group ($p < .001$), while treatment with either estradiol or ER agonists abolished the apoptotic activity in the torsioned testes ($p < .001$; Fig. 5). In parallel, expressions of caspase-3 and cleaved caspase-3 were also elevated by T/D injury ($p < .001$; Fig. 5). However, caspase-3 and cleaved caspase-3 expressions were significantly depressed by either of the treatments ($p < .01$–0.001). On the other hand, expressions of androgen receptor (AR) protein and AR mRNA were decreased in the torsion-detorsioned testes ($p < .05$). Treatment with estradiol or either of the ER agonists abolished T/D-induced reductions and further up-regulated testicular AR and mRNA expressions ($p < .05$–0.001; Fig. 5). Serum testosterone levels were also diminished in T/D groups, but these reductions have become statistically significant in PPT or E_2 administered groups ($p < .001$ and $p < .01$), but not in vehicle- or ERβ agonist-treated T/D groups.

4. Discussion

The present findings revealed that decreased testicular blood flow following torsion was partially recovered on the 3rd day of detorsion, while treatments with either of the ER agonists or E_2 returned blood flow fully back to the control levels. When the testis-torsioned rats were treated with ERβ agonist DPN during the detorsion period, tubular

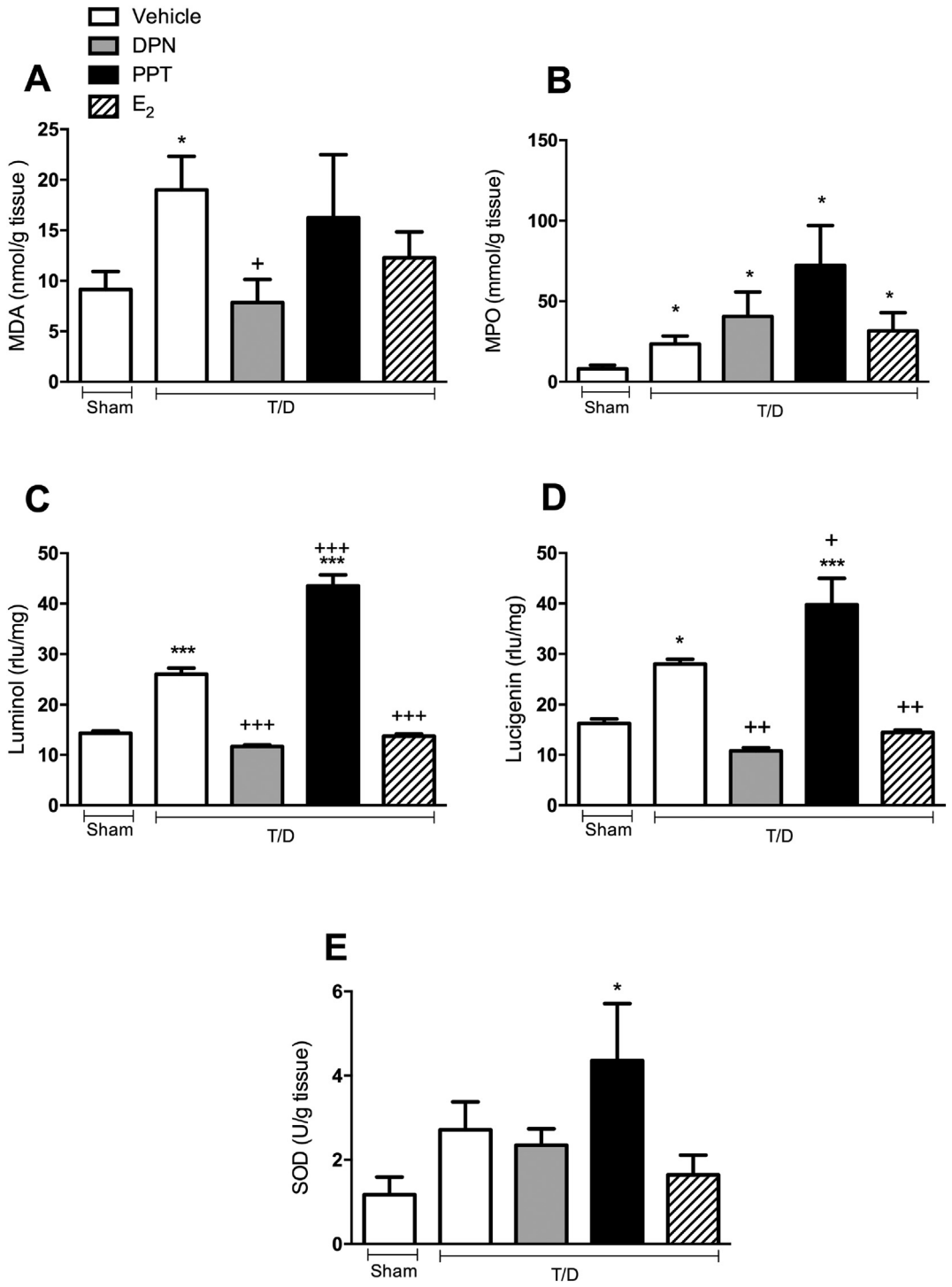

Fig. 4. Malondialdehyde levels (A), myeloperoxidase activity (B), luminol (C) and lucigenin (D) chemiluminescence (CL) and superoxide dismutase (E) levels in the testicular tissues of sham-operated and vehicle-treated, E_2-, PPT- or DPN-treated torsion-detorsion (T/D) groups. *p < .05, ***p < .001 compared to sham-vehicle group; $^{+}$p < .05, ^{++}p < .01, $^{+++}$p < .001 compared to T/D-vehicle group.

injury was decreased, sperm count and motility were increased, while the production of ROMs and apoptosis in the testis tissues were totally suppressed. On the other hand, ERα agonist PPT treatment had no effect on the reduced sperm number or motility and even accelerated oxidative injury, but it was equally efficient as DPN and E_2 in depressing torsion-induced testicular apoptosis. Although a down-regulated expression of AR along with a reduction in serum testosterone level was observed in the vehicle-treated T/D group, all three treatments up-regulated the expressions of AR and its mRNA, while ERα agonist and E_2 further suppressed the testosterone level.

In addition to gonadotropin hormones and testosterone, E_2 was also proven to be involved in the proliferation and differentiation of germ cells into mature spermatids, and hence it is accepted to have an important role in testicular functions [36]. In the adult testis, local production of E_2 is dependent mainly on the germ cells, Sertoli cells and Leydig cells through the conversion of testosterone to E_2 by the enzyme aromatase cytochrome P450 [37], thereby administration of aromatase inhibitors has resulted in a reduced number of germ cells [38]. Estrogen

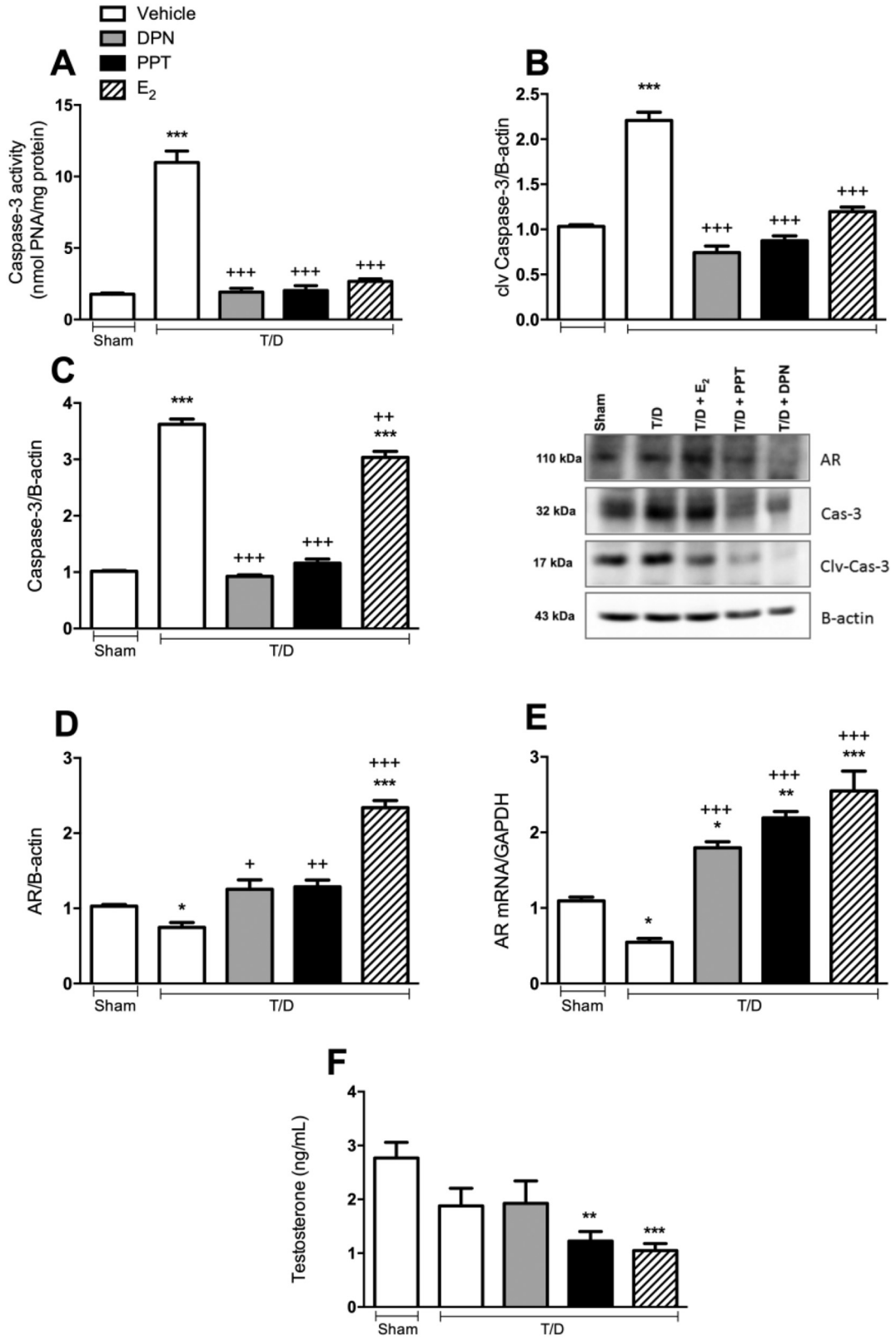

Fig. 5. Caspase-3 activity (A) of testis tissues in the experimental groups. Western blot results of apoptotic markers clv-caspase-3 (B), caspase-3 (C), androgen receptor (AR) (D), AR gene expression (E) in the testis tissues and serum testosterone levels (F) in the sham-operated and torsion-detorsion (T/D) groups. Presented blots represent images of three repetitive experiments. *$p < .05$, **$p < .01$, ***$p < .001$ compared to sham-vehicle group; $^{+}p < .05$, $^{++}p < .01$, $^{+++}p < .001$ compared to T/D-vehicle group.

receptors, which regulate transcription of specific target genes in many tissues, are also expressed in the male reproductive system [39]. E_2 was demonstrated to be critical for the survival of the germ cells in the human seminiferous epithelium, where both ERα and ERβ receptors were strongly expressed, mediating the development and regulation of the germ cells [40–42]. Moreover, gene expressions of both nuclear estrogen receptors were indicated to be essential for spermatogenesis and fertility in rats [41]. However, the modulatory effects of estrogen in controlling the proliferative and/or apoptotic events in testis are believed to be due to a combination of ER-mediated genomic and rapid non-genomic actions via the membrane receptors [43]. In the current study, ischemia-reperfusion-induced testicular injury and disrupted spermatogenesis were reduced mainly by the ERβ agonist and partly by E_2. In accordance with these results, treatment with dehydroepiandrosterone, which is an adrenal gland-derived sex steroid precursor, was shown to protect against oxidative injury caused by testis torsion [44], but the protective effects of estrogen and its agonists were not evaluated before.

Although immediate surgical intervention is worthwhile in preventing the occurrence of complications, testicular torsion may consequently result in testicular dysfunction, atrophy, poor ejaculate quality and infertility in nearly one-fourth of the patients [7,45]. Since experimental studies have demonstrated that testicular dysfunction following T/D-induced ischemia/reperfusion injury appears to involve the activation of inflammatory and apoptotic pathways [46,47], research has focused on evaluating the efficiency of several antioxidant agents in alleviating testicular injury induced with T/D [48,49]. It was previously reported that testicular MDA and 8-hydroxy-2′-deoxyguanosine levels, as well as the pro-inflammatory cytokines have started to increase at the earlier periods of reperfusion [10,50,51] and were sustained for several days [52]. In the present study, testicular levels of MDA and ROMs measured on the 3rd day of reperfusion were greater than the control levels, indicating that the oxidative injury was progressing. However, treatment with ERβ agonist or E_2 during this post-ischemic period abolished oxidative damage. On the other hand, T/D-induced increase in neutrophil recruitment as evidenced by elevated MPO activity was not altered by any of the treatments, which appear to operate without any impact on neutrophil-induced oxidant injury. SOD, which plays an important role against oxidative stress, is available in the cytosol and mitochondria of the testis tissue, while extracellular SOD is also derived from Sertoli and germ cells [49]. In the current study, testicular SOD level showed a tendency to increase in the T/D group and was the highest in the PPT-given group that had the highest oxidative injury, suggesting a compensatory elevation in response to oxidative injury. In parallel with the oxidative injury, T/D resulted in increased activity and expression of both caspase-3 and cleaved caspase-3, while both ER agonists and E_2 were effective in alleviating testicular apoptosis. It is well known that T/D in the testis causes rupture of the Sertoli cell membranes, resulting in apoptotic cytochrome C release into the cytosol and DNA breakage, which lead to reduction in the number and viability of the germ cells, primarily spermatogonia and spermatocytes [53,54]. Without any torsion, even fixing the testicle in the scrotum has significantly reduced that the density of seminiferous epithelium, seminiferous tubule length, and tubular width in rats [55]. The current data also indicated a decrease in the number of seminiferous tubules in the T/D group, while administration of ERβ agonist or E_2 has increased their numbers. Taken together, results suggest that mainly the ERβ agonist, and to some extent E_2 and ERα agonist protected the seminiferous tubules from reperfusion injury by their anti-apoptotic activity. In contrast to diminished release of ROMs along with increased sperm quantity and motility provided by ERβ agonist and E_2 treatments, PPT further exaggerated the generation of ROMs, while the disruption in spermatogenesis was not improved. Thus, these data further implicate that ERβ agonist and E_2 support the maintenance of tubular epithelial function during T/D-induced testicular damage by limiting the oxidative stress.

In a previous study, we have demonstrated that T/D-induced impairment in testicular functions was accompanied by an elevated AR expression when measured at the end of 2-h detorsion period [10]. In contrast, the present data implicate that the early elevation in AR as a reaction to T/D-induced inflammatory challenge and depressed spermatogenesis was then replaced by the down-regulation of AR expression on the 3rd day. Moreover, testicular injury-induced suppression in AR expression was reversed by a 3-day treatment with either of the ER agonists or E_2. Studies have shown that decreased testosterone production and deterioration in spermatogenesis are the consequences of testicular inflammation [56–58]. Ikebuaso et al. [59] have reported that serum testosterone level, sperm count and mobility were decreased in the T/D rats. Although the ERβ agonist DPN did not alter the serum testosterone levels, ERα agonist PPT and E_2 further suppressed the level of testosterone in conjunction with the elevations in AR expressions, demonstrating a feedback control. On the other hand, treatment with ERα- and ERβ-selective agonists as well as ERβ antagonist for 60 days was shown to decrease testosterone production and fertility in healthy adult male rats [60]. Considering together, it appears that estrogen signaling, despite its negative effects upon over-activation, plays significant roles in maintaining male fertility and improves torsion-disturbed spermatogenesis and spermiation, where ERβ receptor activity has a distinct effect.

5. Conclusion

In conclusion, estradiol and ERβ agonist supported the reversal of impaired testicular blood flow, increased androgen receptor expression and alleviated T/D-induced oxidant injury, apoptosis and tubular damage, while the ERβ agonist had an additional advantageous effect on sperm count and motility. The results of our study suggest that ERβ receptor activation during the post-ischemic period may be beneficial in protection against torsion-related oxidant testicular injury and infertility.

Acknowledgements

The authors would like to thank to Hakim Ahmetoğlu for the technical support. The study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] V.J. Sharp, K. Kieran, A.M. Arlen, Testicular torsion: diagnosis, evaluation, and management, Am. Fam. Physician 88 (12) (2013).
[2] Zhao, L.C., et al., Pediatric testicular torsion epidemiology using a national database: incidence, risk of orchiectomy and possible measures toward improving the quality of care. J. Urol., 2011. 186(5): p. 2009–2013.
[3] Cuzzocrea, S., et al., Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol. Rev., 2001. 53(1): p. 135–159.
[4] Wilhelm Filho, D., et al., Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia–reperfusion injury. Mol. Asp. Med., 2004. 25(1–2): p. 199–210.
[5] Cay, A., et al., The effects of N-acetylcysteine on antioxidant enzyme activities in experimental testicular torsion. J. Surg. Res., 2006. 131(2): p. 199–203.
[6] H.M. Prillaman, T.T. Turner, Rescue of testicular function after acute experimental torsion, J. Urol. 157 (1) (1997) 340–345.
[7] J.B. Anderson, R.C. Williamson, The fate of the human testes following unilateral torsion of the spermatic cord, Br. J. Urol. 58 (6) (1986) 698–704.
[8] . DaJusta, D.G., et al., Contemporary review of testicular torsion: new concepts, emerging technologies and potential therapeutics. J. Pediatr. Urol., 2013. 9(6 Pt A): p. 723–30.
[9] Kar, A., et al., Experimental unilateral spermatic cord torsion: the effect of poly-polymerase enzyme inhibitor on histopathological and biochemical changes in the early and late periods in the ipsilateral and contralateral testicles. Urology, 2010. 76(2): p. 507 e1–5.
[10] Tamer, S.A., et al., Nesfatin-1 ameliorates testicular injury and supports gonadal function in rats induced with testis torsion. Peptides, 2018. 107: p. 1–9.
[11] Ruiz-Larrea, M.B., et al., Antioxidant activities of estrogens against aqueous and lipophilic radicals; differences between phenol and catechol estrogens. Chem. Phys. Lipids, 2000. 105(2): p. 179–88.

[12] Ghisletti, S., et al., 17beta-estradiol inhibits inflammatory gene expression by controlling NF-kappaB intracellular localization. Mol. Cell. Biol., 2005. 25(8): p. 2957–68.
[13] Alkayed, N.J., et al., Estrogen and Bcl-2: gene induction and effect of transgene in experimental stroke. J. Neurosci., 2001. 21(19): p. 7543–50.
[14] Stoltzner, S.E., et al., Estrogen regulates bcl-x expression in rat hippocampus. Neuroreport, 2001. 12(13): p. 2797–800.
[15] P.J. Shughrue, M.V. Lane, I. Merchenthaler, Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system, J. Comp. Neurol. 388 (4) (1997) 507–525.
[16] Rommerts, F.F., et al., Development and cellular localization of rat testicular aromatase activity. J. Reprod. Fertil., 1982. 65(2): p. 281–8.
[17] van der Molen, H.J., et al., Testicular oestrogens. J. Endocrinol., 1981. 89 Suppl: (p. 33P-46P).
[18] O'Donnell, L., et al., Estrogen and spermatogenesis. Endocr. Rev., 2001. 22(3): p. 289–318.
[19] A. Revelli, M. Massobrio, J. Tesarik, Nongenomic actions of steroid hormones in reproductive tissues, Endocr. Rev. 19 (1) (1998) 3–17.
[20] Couse, J.F., et al., Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology, 1997. 138(11): p. 4613–21.
[21] T. Iguchi, Developmental pattern of estrogen receptor expression in male mouse genitalorgans, Mol. Androl. 3 (1991) 109–120.
[22] Jefferson, W.N., et al., Expression of estrogen receptor beta is developmentally regulated in reproductive tissues of male and female mice. Biol. Reprod., 2000. 62(2): p. 310–7.
[23] Li, H., et al., Regulation of rat testis gonocyte proliferation by platelet-derived growth factor and estradiol: identification of signaling mechanisms involved. Endocrinology, 1997. 138(3): p. 1289–98.
[24] Hess, R.A., et al., A role for oestrogens in the male reproductive system. Nature, 1997. 390(6659): p. 509–12.
[25] Sinkevicius, K.W., et al., Estrogen-dependent and -independent estrogen receptor-alpha signaling separately regulate male fertility. Endocrinology, 2009. 150(6): p. 2898–905.
[26] Walczak-Jedrzejowska, R., et al., During seminiferous tubule maturation testosterone and synergistic action of FSH with estradiol support germ cell survival while estradiol alone has pro-apoptotic effect. Folia Histochem. Cytobiol., 2007. 45 Suppl 1: p. S59–64.
[27] Kumral, Z.N., et al., Estrogen alleviates acetic acid-induced gastric or colonic damage via both ERalpha- and ERbeta-mediated and direct antioxidant mechanisms in rats. Inflammation, 2014. 37(3): p. 694–705.
[28] Ozdemir Kumral, Z.N., et al., Estrogen receptor agonists alleviate cardiac and renal oxidative injury in rats with renovascular hypertension. Clin. Exp. Hypertens., 2016. 38(6): p. 500–9.
[29] Şen, L.S., et al., Treatment with either obestatin or ghrelin attenuates mesenteric ischemia–reperfusion-induced oxidative injury of the ileum and the remote organ lung. Peptides, 2015. 71: p. 8–19.
[30] Aykaç, G., et al., The effect of chronic ethanol ingestion on hepatic lipid peroxide, glutathione, glutathione peroxidase and glutathione transferase in rats. Toxicology, 1985. 36(1): p. 71–76.
[31] Solmaz, A., et al., Nesfatin-1 improves oxidative skin injury in normoglycemic or hyperglycemic rats. Peptides, 2016. 78: p. 1–10.
[32] Mylroie, A.A., et al., Erythrocyte superoxide dismutase activity and other parameters of copper status in rats ingesting lead acetate. Toxicol. Appl. Pharmacol., 1986. 82(3): p. 512–520.
[33] Krol, W., et al., Anti-oxidant property of ethanolic extract of propolis (EEP) as evaluated by inhibiting the chemiluminescence oxidation of luminol. Biochem. Int., 1990. 21(4): p. 593–597.
[34] S.G. Johnsen, Testicular biopsy score count–a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males, Hormone Research in Paediatrics 1 (1) (1970) 2–25.
[35] Gozen, A., et al., Protective activity of ischemic preconditioning on rat testicular ischemia: effects of Y-27632 and 5-hydroxydecanoic acid. J. Pediatr. Surg., 2013. 48(7): p. 1565–1572.
[36] Carreau, S., et al., Estrogens and male reproduction: a new concept. Braz. J. Med. Biol. Res., 2007. 40(6): p. 761–768.
[37] . Carreau, S., et al., Sources of oestrogen in the testis and reproductive tract of the male. Int. J. Androl., 1999. 22(4): p. 211–223.
[38] Shetty, G., et al., Effect of long-term treatment with aromatase inhibitor on testicular function of adult male bonnet monkeys (M. radiata). Steroids, 1998. 63(7–8): p. 414–420.
[39] Paterni, I., et al., Estrogen receptors alpha (ERα) and beta (ERβ): subtype-selective ligands and clinical potential. Steroids, 2014. 90: p. 13–29.
[40] Van Pelt, A.M., et al., Ontogeny of estrogen receptor-β expression in rat testis. Endocrinology, 1999. 140(1): p. 478–483.
[41] Bois, C., et al., Age-and cell-related gene expression of aromatase and estrogen receptors in the rat testis. J. Mol. Endocrinol., 2010. 45(3): p. 147–159.
[42] Pentikäinen, V., et al., Estradiol acts as a germ cell survival factor in the human testis in vitro. The Journal of Clinical Endocrinology & Metabolism, 2000. 85(5): p. 2057–2067.
[43] Chimento, A., et al., Role of estrogen receptors and G protein-coupled estrogen receptor in regulation of hypothalamus–pituitary–testis axis and spermatogenesis. Front. Endocrinol., 2014. 5: p. 1.
[44] Aksoy, H., et al., Dehydroepiandrosterone treatment attenuates reperfusion injury after testicular torsion and detorsion in rats. J. Pediatr. Surg., 2007. 42(10): p. 1740–1744.
[45] T. Krarup, The testes after torsion, Br. J. Urol. 50 (1) (1978) 43–46.
[46] T.T. Turner, H.J. Bang, J.L. Lysiak, The molecular pathology of experimental testicular torsion suggests adjunct therapy to surgical repair, J. Urol. 172 (6) (2004) 2574–2578.
[47] Lysiak, J.J., et al., Ischemia-reperfusion of the murine testis stimulates the expression of proinflammatory cytokines and activation of c-jun N-terminal kinase in a pathway to E-selectin expression. Biol. Reprod., 2003. 69(1): p. 202–210.
[48] Arena, S., et al., Medical perspective in testicular ischemia-reperfusion injury. Experimental and therapeutic medicine, 2017. 13(5): p. 2115–2122.
[49] R.J. Aitken, S.D. Roman, Antioxidant systems and oxidative stress in the testes, Oxidative Med. Cell. Longev. 1 (1) (2008) 15–24.
[50] Polat, E.C., et al., The investigation of the protective effects of nimesulide on experimental testicular ischemia-reperfusion injury in rats. Revista internacional de andrologia, 2018.
[51] Abat, D., et al., Beneficial effects of rolipram, a phosphodiesterase 4 specific inhibitor, on testicular torsion-detorsion injury in rats. J. Pediatr. Surg., 2018.
[52] Unal, D., et al., Protective effects of trimetazidine on testicular ischemia-reperfusion injury in rats. Urol. Int., 2007. 78(4): p. 356–362.
[53] Lysiak, J.J., et al., Essential role of neutrophils in germ cell-specific apoptosis following ischemia/reperfusion injury of the mouse testis. Biol. Reprod., 2001. 65(3): p. 718–725.
[54] Chaki, S., et al., Apoptosis and cell removal in the cryptorchid rat testis. Apoptosis, 2005. 10(2): p. 395–405.
[55] Ribeiro, C.T., et al., Effects of testicular transfixation on seminiferous tubule morphology and sperm parameters of prepubertal, pubertal, and adult rats. Theriogenology, 2015. 84(7): p. 1142–1148.
[56] R.P. Amann, The cycle of the seminiferous epithelium in humans: a need to revisit? J. Androl. 29 (5) (2008) 469–487.
[57] K. Del Punta, E.H. Charreau, O.P. Pignataro, Nitric oxide inhibits Leydig cell steroidogenesis, Endocrinology 137 (12) (1996) 5337–5343.
[58] Kostic, T., et al., The involvement of nitric oxide in stress-impaired testicular steroidogenesis. Eur. J. Pharmacol., 1998. 346(2–3): p. 267–73.
[59] Ikebuaso, A.D., et al., Experimental testicular torsion in a rat model: effects of treatment with Pausinystalia macroceras on testis functions. J Reprod Infertil, 2012. 13(4): p. 218–24.
[60] Dumasia, K., et al., Effect of estrogen receptor-subtype-specific ligands on fertility in adult male rats. J. Endocrinol., 2015. 225(3): p. 169–80.