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The protective role of erdosteine on testicular tissue after testicular torsion and detorsion

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Abstract

Testicular torsion and detorsion are important clinical problems for infertile man and oxidative stress may have a role in this clinical situation. The aim of this study was to investigate the protective role of erdosteine, an antioxidant, on unilateral testicular reperfusion injury in rats. The rats were divided into four groups including seven rats in each group: control, torsion, torsion/detorsion and torsion/detorsion+erdosteine. Rats, except the sham operation group, were subjected to left unilateral torsion (720° rotation in the clockwise direction) without including the epididymis. The experiments were finished after sham operation time for control, 120 min torsion for torsion group and 120 min torsion and 240 min detorsion for torsion/detorsion groups. Bilateral orchiectomy was performed for all groups of rats. The ipsilateral and controlateral testis were divided into two pieces to analyse biochemical parameters and to investigate the light microscopic view.

Malondialdehyde level of ipsilateral testis was increased in torsion and torsion/detorsion groups in comparison with the other groups ($p < 0.05$). Erdosteine treatment ameliorated lipid peroxidation after torsion/detorsion in ipsilateral testis ($p < 0.05$). Also, xanthine oxidase activity of ipsilateral testis was increased in torsion/detorsion group in comparison with the others ($p < 0.05$). Nitric oxide (NO) level of ipsilateral testis was higher in all experimental groups than sham operated control group ($p < 0.05$). Also, NO level of torsion group was increased in comparison with detorsion groups ($p < 0.05$). Erdosteine treatment caused increased glutathione peroxidase activity in comparison with torsion and torsion/detorsion groups and catalase activity in comparison with the other groups in ipsilateral testis ($p < 0.05$). Superoxide dismutase activity of ipsilateral testis was higher in torsion/detorsion and torsion/detorsion+erdosteine groups than control and torsion groups ($p < 0.05$). The biochemical parameters were not affected in controlateral testis in all groups. Torsion, torsion/detorsion and torsion/detorsion+erdosteine groups showed ipsilateral testicular damage in the histological examination, but the specimens from torsion/detorsion had a significantly greater histological injury than those from the other groups ($p < 0.05$). Control rats showed normal seminiferous tubule morphology. Rats in torsion group had slight-to-moderate disruption of the seminiferous epithelium. Rats in torsion/detorsion group displayed moderate-to-severe disruption of the seminiferous epithelium. In all animals from torsion/detorsion+erdosteine group, the testicular tissues were affected with slight-to-moderate degenerative changes of the seminiferous epithelium. Administration of erdosteine resulted in a significantly reduced histological damage associated with torsion of the spermatic cord compared with torsion/detorsion. In all groups, the contralateral testes were histologically normal.

In conclusion, the results clearly displayed that erdosteine treatment may have a protective role on testicular torsion/detorsion injury. (Mol Cell Biochem **280:** 193–199, 2005)

Key words: testicular torsion/detorsion, oxidative stress, erdosteine, antioxidant, light microscopy

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Introduction

One of the most common genital traumas of adolescent boy is testicular torsion involved in testicular injury, altered hormone production, subfertility and infertility. The testicular injury is depends on the duration and degree of torsion [1]. Events occurring during testicular torsion and detorsion are representative of an ischemia-reperfusion type of injury observed in other organs [2]. It was demonstrated that reactive oxygen species (ROS) increase in the areas of ischemia and reperfusion, which are responsible for ischemia-reperfusion injury. Tissue ischemia causes tissue hypoxia and leads to a complex cascade of events resulting in an injury. After reperfusion and re-oxygenation, the imbalance between restoration of oxygen supply and mitochondrial respiratory function results in the massive generation of superoxide anion ($O_2^{\bullet -}$) in mitochondria. On the other hand, ischaemia results in the accumulation of purines (hypoxanthine and xanthine) from the catabolism of ATP and the substantial proteolytic conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO), which is a common generator of $O_2^{\bullet -}$ [3]. The reduction of dioxygen to $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$) is believed to cause the actual injury induced by re-oxygenation injury. The major intracellular antioxidant enzymes, Cu/Zn-superoxide dismutase (Cu/Zn-SOD) in the cytoplasm and Mn-SOD in the mitochondria, rapidly and specifically reduce $O_2^{\bullet -}$ to H_2O_2. The other endogenous antioxidant enzymes, glutathione peroxidase (GSH-Px) and catalase (CAT), act to detoxify H_2O_2 to water [4, 5].

Nitric oxide (NO), particularly high iNOS activity, is another source of injury during reperfusion. The high levels of NO may further participate in oxidative damage through peroxynitrite formation by reacting with $O_2^{\bullet -}$. Peroxynitrite is a potent and aggressive cellular oxidant and causes the formation of 3-nitro-L-tyrosine [6].

Erdosteine [*N*-(carboxymethylthioacetyl)-homocysteine thiolactone], a mucolytic agent, contains two blocked sulphydril groups which are released following its metabolic process. The reducing potential of these sulphydril groups account for free radicals scavenging and antioxidant activity of erdosteine [7]. It was demonstrated that erdosteine prevented lipid peroxidation in renal tissue induced by ischemia-reperfusion of kidney [8].

The aim of this study was to investigate the effects of erdosteine on testis torsion/detorsion injury via oxidant/antioxidant system and the light microscopic evaluation of both ipsilateral and controlateral testis tissues.

Materials and methods

Male Wistar Albino rats were used in the experiments. The animals were housed in quiet rooms with 12:12-h light–dark cycle (7 am to 7 pm) and the experiments were performed in accordance with "Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 85-23, 1985".

Twenty-eight rats were divided into four groups: control untreated sham operated rats ($n = 7$); torsion ($n = 7$); torsion/detorsion ($n = 7$) and torsion/detorsion+erdosteine ($n = 7$) groups. The rats in the torsion group were subjected to left unilateral testicular torsion (720° rotation in the clockwise direction) without including the epididymis under urethane (i.p. 1.2 g/kg) anaesthesia for 120 min. The rats in the torsion/detorsion group were subjected to 120 min torsion then 240 min detorsion. Sham operations were performed through standard ilioinguinal incisions to the control rats and the left testicle was fixed to the scrotum by a silk suture through the tunica albuginea. In the sham operation control group, the testes were brought through the incisions and replaced, and a silk suture placed through the tunica albuginea. After all the surgical procedures and applications, the incision was closed with silk suture. Erdosteine was applied 50 mg/kg/day for 2 days before experiments to the torsion/detrosion+erdosteine group. After experimental procedures, all rats were sacrificed and bilateral orchidectomy were performed and rapidly sectioned vertically into two pieces for microscopic examination and biochemical analyses. The testicular tissue was stored at −70 °C until biochemical analyses.

Biochemical analysis

After weighing the tissue, homogenate, supernatant and extracted samples were prepared as described elsewhere [5] and the following determinations were made on the samples using commercial chemicals supplied by Sigma (St. Louis, USA). Protein measurements were analysed in homogenate, supernatant and extracted samples according to the method explained elsewhere [9]. The tissue homogenate was used for nitric oxide (NO) and malondialdehyde (MDA) levels. The tissue supernatant was used for analysis of catalase (CAT), glutathione peroxidase (GSH-Px) and xanthine oxidase (XO). Superoxide dismutase (SOD) activity was assessed in the extracted sample, ethanol phase of the lyzate after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged.

The tissue MDA level was determined by a method [10] based on the reaction with thiobarbituric acid (TBA) at 90–100 °C. In the TBA test reaction, MDA or MDA-like substances and TBA react with the production of a pink pigment having an absorption maximum at 532 nm. Nitric oxide has a half-life of only a few seconds, because it is readily oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) which serves as index parameters of NO production. The method for plasma nitrite and nitrate levels was based on the Griess

reaction [11]. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite+nitrate) was measured by spectrophotometry at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules.

The principle assay of CAT activity was determined according to Aebi's method at 240 nm [12]. Glutathione peroxidase activity was measured by the method of Paglia and Valentine [13]. The enzymatic reaction in the tube, which is containing following items: NADPH, reduced glutathione, sodium azide, and glutathione reductase, was initiated by addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer. Tissue XO activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm, according to Prajda and Weber's method [14].

Total (Cu/Zn and Mn) SOD activity was determined according to the method of Sun *et al.* [15]. The principle of the method is based on the inhibition of nitroblue-tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate.

Histological evaluation

Both ipsilateral and contralateral testes of all animals were fixed in 10% neutral buffered formalin and embedded in paraffin. The paraffin blocks were cut in 6 μm thick. The sections were stained with H&E and examined under the light microscope. The histological evaluation was done in a blind, randomly numbered fashion without any knowledge of which testis was experimentally torsed or treated. A four-level grading scale similar to that of Cosentino *et al.* was used to quantify histological injury [16]. Grade 1 showed normal testicular architecture with orderly arrangement of germinal cell. Grade 2 (slight effect) injuries showed less orderly, noncohosive germinal cell and closely packed seminiferous tubules. Grade 3 (moderate effect) injuries exhibited disordered, sloughed germinal cell with shrunken, pyknotic nuclei and less distinct seminiferous tubule border. Grade 4 (severe effect) injuries defined seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells. Additionally, the status of the testicular tissue was also evaluated in terms of hemorrhage, edema and vascular congestion.

Statistical analysis

Data were analysed by using a commercially available statistics software package (SPSS® for Windows v. 9.0, Chicago, USA). One-way ANOVA test was performed and Post Hoc multiple comparisons were done with LSD. Results were presented as means ± S.E.M. $p < 0.05$ were regarded as statistically significant.

Results

Biochemical results

The results were shown in Tables 1 and 2. The MDA level of ipsilateral testis tissue was increased in the torsion and torsion/detorsion groups in comparsion with control and torsion/detorsion+erdosteine groups ($p < 0.05$). Rats in torsion/detorsion group had higher MDA level of ipsilateral testis than in torsion group, too ($p < 0.05$).

Table 1. Malondialdehyde (MDA) and nitric oxide (NO) levels with xanthine oxidase (XO) activities in all groups

	MDA (nmol/g wet tissue)		XO (U/g protein)		NO (μmol/g wet tissue)	
Groups ($n = 7$ per groups)	Ipsilateral (left)	Controlateral (right)	Ipsilateral (left)	Controlateral (right)	Ipsilateral (left)	Controlateral (right)
I – Control	14.878 ± 4.031	14.467 ± 4.216	0.619 ± 0.098	0.621 ± 0.104	0.217 ± 0.089	0.207 ± 0.090
II – Torsion	27.447 ± 4.802	14.201 ± 6.611	0.629 ± 0.114	0.602 ± 0.267	0.798 ± 0.354	0.252 ± 0.185
III – Torsion/Detorsion	43.310 ± 9.776	15.996 ± 6.024	1.050 ± 0.287	0.624 ± 0.209	0.450 ± 0.127	0.225 ± 0.111
IV – Torsion/Detorsion+Erdosteine	18.935 ± 6.320	15.191 ± 8.349	0.676 ± 0.109	0.614 ± 0.305	0.444 ± 0.143	0.214 ± 0.137
p						
I–II	0.002	N.S.	N.S.	N.S.	0.0001	N.S.
I–III	0.0001	N.S.	0.0001	N.S.	0.045	N.S.
I–IV	N.S.	N.S.	N.S.	N.S.	0.050	N.S.
II–III	0.0001	N.S.	0.0001	N.S.	0.004	N.S.
II–IV	0.024	N.S.	N.S.	N.S.	0.004	N.S.
III–IV	0.0001	N.S.	0.0001	N.S.	N.S.	N.S.

N.S.: non significant.

Table 2. Glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) activities of the all groups

	GSH-Px (U/g protein)		CAT (k/g protein)		SOD (U/mg protein)	
Groups ($n = 7$ per groups)	Ipsilateral	Controlateral	Ipsilateral	Controlateral	Ipsilateral	Controlateral
I – Control	0.748 ± 0.118	0.751 ± 0.167	0.108 ± 0.021	0.106 ± 0.025	0.361 ± 0.054	0.324 ± 0.084
II – Torsion	0.589 ± 0.202	0.701 ± 0.134	0.100 ± 0.034	0.102 ± 0.022	0.387 ± 0.060	0.322 ± 0.117
III – Torsion/detorsion	0.561 ± 0.231	0.740 ± 0.305	0.072 ± 0.023	0.112 ± 0.024	0.503 ± 0.092	0.318 ± 0.084
IV – Torsion/detorsion+erdosteine	0.981 ± 0.234	0.719 ± 0.381	0.161 ± 0.047	0.115 ± 0.049	0.498 ± 0.111	0.333 ± 0.105
p						
I–II	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
I–III	N.S.	N.S.	N.S.	N.S.	0.004	N.S.
I–IV	N.S.	N.S.	0.006	N.S.	0.005	N.S.
II–III	N.S.	N.S.	N.S.	N.S.	0.015	N.S.
II–IV	0.001	N.S.	0.002	N.S.	0.019	N.S.
III–IV	0.001	N.S.	0.0001	N.S.	N.S.	N.S.

N.S.: non-significant.

MDA level of ipsilateral testis was not significant in control and torsion/detorsion+erdosteine groups. Xanthine oxidase activity of torsion/detrosion group was significantly increased in comparison with other groups in ipsilateral testis ($p < 0.05$). The NO level of ipsilateral testis was higher in torsion group than other groups ($p < 0.05$). Also, NO level of ipsilateral testis was increased in torsion/detorsion and torsion/detorsion+erdositene groups in comparison with control group ($p < 0.05$). The MDA and NO levels with XO activity of all groups' controlateral testis were not significantly different each other.

The GSH-Px activity of ipsilateral testis was increased in torsion/detorsion+erdosteine group in comparison with torsion and torsion/detorsion groups ($p < 0.05$). The CAT activity of torsion/detorsion+erdosteine group was significantly higher than other groups in ipsilateral testis ($p < 0.05$). The SOD activity of ipsilateral was increased in torsion and torsion/detorsion+erdosteine groups in comparison with control and torsion groups ($p < 0.05$). The SOD, CAT and GSH-Px activities were not significantly different in controlateral testis of all groups.

Table 3. Histologic evaluation of the groups

Groups	Ipsilateral (left)	Controlateral (right)
I – Control	1.000 ± 0.000	1.000 ± 0.000
II – Torsion	2.471 ± 0.112	1.000 ± 0.000
III – Torsion/detorsion	3.271 ± 0.101	1.000 ± 0.000
IV – Torsion/detorsion+erdosteine	2.700 ± 0.151	1.000 ± 0.000
p		
I–II	0.000	N.S.
I–III	0.000	N.S.
I–IV	0.000	N.S.
II–III	0.000	N.S.
II–IV	N.S	N.S.
III–IV	0.001	N.S.

N.S.: non-significant.

Histological results

Light microscopic scoring results were summarized in Table 3. Torsion, torsion/detorsion and torsion/detorsion+erdosteine groups showed testicular damage in the histological examination of ipsilateral testis, but the specimens from torsion/detorsion had a significantly greater histological injury than those from the other groups (3.271 ± 0.101, $p < 0.05$).

Figures 1–4 illustrates sections from testes of animals from sham-operated control, torsion, torsion/detorsion and torsion/detorsion+erdosteine groups. The control rats showed normal seminiferous tubule morphology (Fig. 1). Seminiferous tubules, germ cells, sertoli and leydig cells appear complete, without infiltration and hemorrhagic sign (1.000 ± 0.000, $p < 0.001$). Rats that received a 720° testicular torsion for 2 h (torsion group) had slight-to-moderate disruption of the seminiferous epithelium (Fig. 2). Light microscopy showed that torsion of the left testis resulted in interstitial space dilatation and edema (2.471 ± 0.112; $p < 0.001$). Rats that underwent 720° testicular torsion for 2 h and detorsion for 4 h (torsion/detorsion group) displayed moderate-to-severe disruption of the seminiferous epithelium (Fig. 3). The tests in this group showed also disordered, sloughed germinal cell with shrunken, pyknotic nuclei, interstitial space dilatation and presence of edema and hemorrhage (3.271 ± 0.101, $p < 0.001$). In

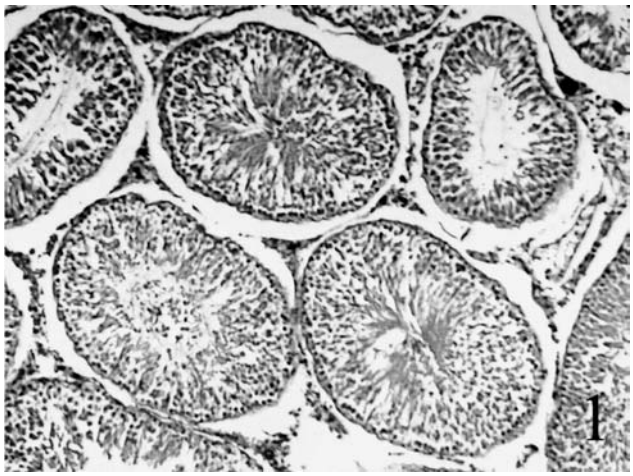

Fig. 1. Control group shows normal seminiferous tubule morphology. Note the orderly arrangement of germinal cells (H&E, original magnification, ×150).

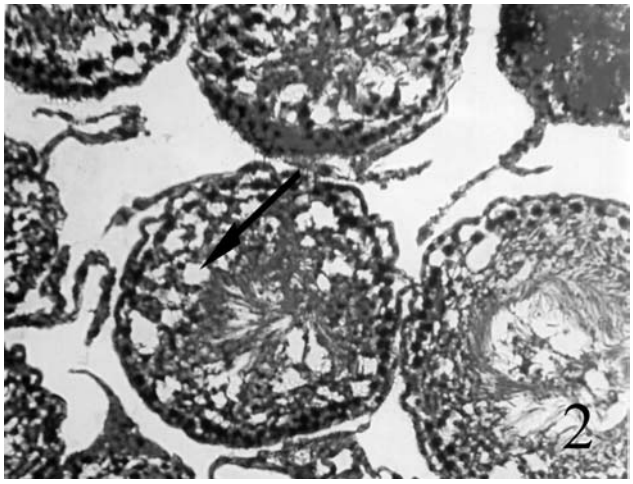

Fig. 2. Torsion group. Testis shows interstitial space dilatation (*arrow*) (H&E, original magnification, ×230).

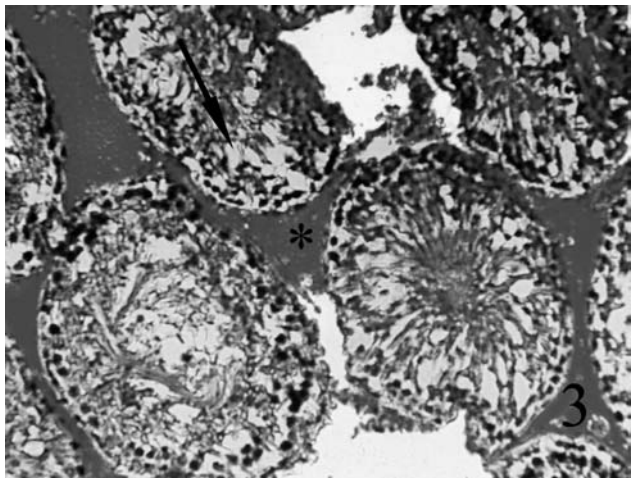

Fig. 3. Torsion/detorsion group. Interstitial space dilatation (*arrow*), presence of edema and hemorrhage (*asterisk*) are seen in the testis tissue (H&E, original magnification, ×230).

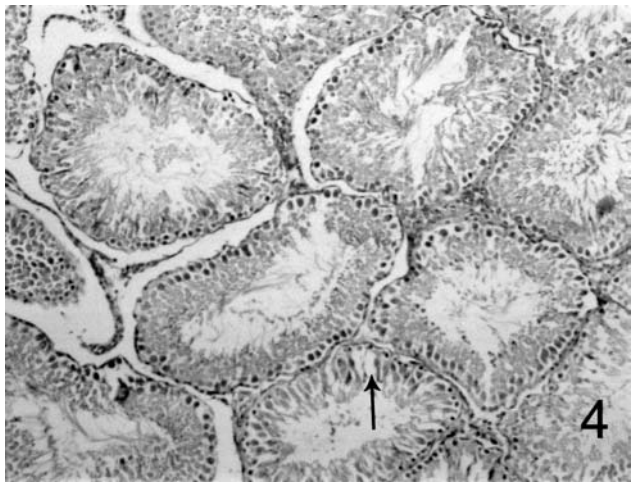

Fig. 4. Torsion/detorsion+erdosteine group. Treatment with erdosteine resulted in almost normal seminiferous tubule morphology. Note arrow indicates interstitial space dilatation (H&E, original magnification, ×150).

all animals from torsion/detorsion+erdosteine group, the testicular tissues were affected with slight-to-moderate degenerative changes of the seminiferous epithelium (Fig. 4). Histological grade of this group was significantly lower than that of torsion/detorsion group ($P < 0.001$). But no significant difference was detected between testes of torsion/detorsion+erdosteine and torsion groups ($P > 0.05$). Administration of erdosteine resulted in a significantly reduced histological damage associated with torsion/detorsion of the spermatic cord in comparison with torsion/detorsion (2.700 ± 0.151; $p < 0.001$). In all groups, the contralateral testes were histologically normal.

Discussion

The present study indicated that ischemia itself and ischemia-reperfusion caused injury to testis tissue. Previous investigations of experimental torsion to 0.5-h 720 ° torsion of the rat testis showed no effect on ipsilateral testis weight or cauda epididymal sperm concentrations, but increasing the time of torsion to 1 h caused a progressive loss in testis weight and caused a loss of spermatogenesis [17]. Thus, we formed a 2-h testicular torsion model rotating left testis 720 ° to constitute a slight-to-moderate testicular damage. Similarly, Romeo *et al.* have made a total occlusion (3 h) of the left testis with

a 720 ° twisting of the spermatic cord and followed by 4 h of reperfusion [18]. They have found that the left testis torsion-detorsion caused interstitial space dilation, edema and hemorrhage. Their findings are consistent with our results.

The lipid peroxidation is one of the important indications of tissue injury due to oxidative stress. There are many types of ROS which exist in the cellular environment under normal condition. Superoxide anion, H_2O_2, OH are thought to be much more important ROS but $^{\bullet}OH$ is considered as the most active and toxic radical belonging to this group of ROS [3]. Toxic products of the reaction catalysed by XO including $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ are generated in quantities that overwhelm the capacity of endogenous free radical scavengers and inflict significant injury on cellular organism. However, the protective role of antioxidant enzymes such as SOD, CAT and GSH-Px against free radical attack is in balance under normal condition. Superoxide dismutase is a potent protective enzyme that can selectively scavenge the $O_2^{\bullet -}$ into H_2O_2. Glutathione peroxidase and CAT catalyse the conversion of H_2O_2 to H_2O. This balance is disturbed under high oxidative stress such as reperfusion injury [18]. In the present study, it was shown that lipid peroxidation was occurred in unilateral testicular tissue after both ischemia and ischemia/reperfusion procedures. Our results confirmed those of Uz *et al.* that testicular torsion and torsion/detorsion resulted in high lipid peroxidation in testis [3]. The present study indicated that one source of $O_2^{\bullet -}$ may be XO system during reperfusion period. The XO activity was increased in reperfused rats in this study. Also, our study showed that SOD activity was increased in torsion/detorsion groups. This result may be due to necessity of conversion of high produced $O_2^{\bullet -}$ into H_2O_2.

Our results indicated that testicular torsion and torsion/detorsion induced significant changes in NO levels. High NO level was detected in ischemic and reperfused groups in the present study. Our study showed similar results with Koltuksuz *et al.* [19]. A decrease in NO level after detorsion period might due to consumption of NO by $O_2^{\bullet -}$. Superoxide anion and NO react to form a third free radical, peroxynitrite.

Erdosteine, which is a mucolytic agent, is a molecule containing two sulphur atoms, one of which is blocked in the aliphatic side-chain and the other is enclosed in the heterocyclic ring [20]. Erdosteine, owing to the presence of two sulphydryl group in its metabolites, can act as a free oxygen radical scavenger and this component of the mechanism of action is likely to be involved in the testicular injuries induced by ischemia/reperfusion. Fadillioglu *et al.* demonstrated that erdosteine prevented lipid peroxidation in cardiac tissue [21]. Also, other studies showed protective effects of erdosteine in various tissues against oxidative injury [7, 22]. The protective effect of erdosteine on testicular tissue was observed in the present study. Erdosteine treatment resulted in prevention of lipid peroxidation via decreased production of radicals with XO and scavenger effects on ROS. Erdosteine treatment caused high antioxidant enzyme activity such as GSH-Px and CAT. It showed that erdosteine prevent cardiac lipid peroxidation with high antioxidant enzyme activity [21]. The decrease in XO activity also prevents ROS production. The present study confirmed those of Fadillioglu et al. that erdosteine treatment resulted in decrease in XO activity [23].

Bozlu *et al.* have demonstrated that the histological parameters of the contralateral testis did not show any statistically significant differences among their groups, which is confirmed by our observations [24]. Several studies have claimed that contralateral testis is not affected by unilateral torsion while other favoured the opposite view. The contralateral testicular damage after unilateral testicular torsion can be explained by an autoimmune response [25, 26]. Cosentino *et al.* suggested that the critical period within which to reduce spermatic cord torsion so as to prevent contralateral testicular damage is less than 3 h [27]. In our study, the time period that we used was shorter than 3 h (2-h torsion model), so the torsion time was not enough to stimulate autoimmune mechanisms for contralateral testicular alterations to occur. It is still controversial that unilateral torsion/detorsion has an adverse effect on the contralateral testis. Some authors believe that testicular torsion causes a negative effect on the contralateral testis tissue, some not. Different hypotheses have been put forward to try to explain this phenomenon. Andiran *et al.* indicated a reflex mechanism that can be responsible for reduced contralateral testicular blood flow, thus being responsible for tissue hypoxia [28]. However, our results demonstrated that testicular torsion did not affect the controlateral testicular tissue. We found no difference in MDA and NO levels, XO, SOD, GSH-Px and CAT activities of any contralateral testis between groups. Uz *et al.* also showed that there was no significant difference in lipid peroxidation levels of controlateral testis between groups [3]. Ozkan *et al.* did not found significant differences between MDA levels of controlateral testis tissue of the groups, either [29]. It was demonstrated by another study that contralateral testicular damage was resulted in 5 weeks later after unilateral testicular torsion by decreased serum inhibin B levels, which was accepted as a marker of spermatogenesis; however, it was not shown histopathologically [30]. In the present study, our results indicated that the time period after testicular torsion/detorsion was too short for immunologic response. The period of ischemia and reperfusion is important factor for detrimental effect on controlateral testis.

In recent years, several agents have been used to prevent ischemia/reperfusion damage in experimental testicular torsion such as a superoxide dismutase (SOD), catalase, calcium channel blokers, oxypurinal, and allupurinol. In addition to these agents, here we report for the first time that erdosteine ameliorated the testicular damage after testicular torsion. It is well known that erdosteine decreases production of radicals with XO and scavenger effects on ROS, which may underlie

how erdosteine ameliorated the histological testicular damage after 2 h of torsion in the current study. This might provide a potential therapeutic approach for post ischemic testicular damage.

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