Erdosteine protects rat testis tissue from hypoxic injury by reducing apoptotic cell death

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Summary
The purpose of this study was to examine the effects of hypobaric hypoxia on testis morphology and the effects of erdosteine on testis tissue. Caspase-3 and hypoxia-inducible factor 1α expressions were detected by immunohistochemistry. Adult male Wistar rats were placed in a hypobaric hypoxic chamber. Rats in the erdosteine group were exposed to the same conditions and treated orally with erdosteine (20 mg kg⁻¹ daily) at the same time from the first day of hypoxic exposure for 2 weeks. The normoxia group was evaluated as the control. The hypoxia group showed decreased height of spermatogenic epithelium in some seminiferous tubules, vacuolisation in spermatogenic epithelial cells, deterioration and gaps in the basal membrane and an increase in blood vessels in the interstitial area. The erdosteine group showed amelioration of both epithelial cell vacuolisation and basal membrane deterioration. Numbers of hypoxia-inducible factor 1α–immunostained Sertoli and Leydig cells were significantly higher in the hypoxia group than in the erdosteine group. The number of seminiferous tubules with caspase-3–immunostained germ cells was highest in the hypoxia group and decreased in the erdosteine and normoxia groups respectively. Based on these observations, erdosteine protects testis tissue from hypoxic injury by reducing apoptotic cell death.

Introduction
Haemoglobin is known to carry less oxygen at high altitudes because the partial inspired pressure of oxygen and the amount of oxygen available for diffusion into the bloodstream decrease (Verratti et al., 2008). This effect causes hypoxia, meaning low oxygen in the body, often specified as tissue hypoxia. Exposure to high altitudes may be acute (e.g. in mountain climbers, tourists and/or people engaging in sport) or chronic and continuous (e.g. in native inhabitants of high-altitude regions) (Farias et al., 2005a; Liao et al., 2010). Hypoxia results in physiological adaptations or leads to pathological responses (Hwang et al., 2009). Alterations caused by hypoxia are known to occur in different parts of the body, such as the respiratory (Uzun et al., 2006), cardiovascular and endocrine systems (Foster et al., 2007). Hypoxia has also been suggested to reduce fertility (Farias et al., 2005a; Cikutovic et al., 2009; Hwang et al., 2009; Gat et al., 2010a,b). The negative influence of hypoxia has been demonstrated using seminological parameters, such as reduced motility and an increase in abnormal or immature spermatozoa (Verratti et al., 2008; Hartley et al., 2009). Histological examination of rat testes after hypoxic exposure has shown the changes in testicular morphology and loss of spermatogenic cells (Farias et al., 2005a; Hartley et al., 2009; Paul et al., 2009; Liao et al., 2010). In a study of nine azoospermic men, Gat et al. (2010a) suggested that hypoxia due to varicoceles led to progressive deterioration in sperm production, which may deteriorate fertility to infertility.

Oxidative stress is a consequence of an imbalance between the production of reactive oxygen species (ROS) and the body’s antioxidant defence mechanisms (Makker et al., 2009). A high excess of ROS generated from endogenous sources, such as mitochondria, in response to inflammatory conditions, upregulation of various enzymes or environmental factors (smoking, radiation or industrial
pollution) may damage macromolecules, such as lipids, proteins and DNA, and induce neurological disorders, atherosclerosis, diabetic complications or ageing (Vaya, 2013).

Adaptation to hypoxia and oxidative stress is regulated by hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor comprising an oxygen-regulated \( \alpha \) subunit and a constitutively expressed \( \beta \) subunit. HIF-1\( \alpha \), the predominant subunit in the testis, may mediate proapoptotic or antiapoptotic responses in the ischaemic testis depending on the duration and extent of ischaemia and hypoxia (Powell et al., 2002; Palladino et al., 2011).

Reactive oxygen species (ROS) synthesis is induced by exposure to hypoxia, and these molecules play a very important role in the pathogenicity of testicular and sperm damage (Hartley et al., 2009). In many cell types, hypoxia and oxidative stress have been shown to trigger apoptosis and cell cycle arrest. Oxidative stress has recently been shown to cause depletion of germ cells, and germ cell death was associated with an increase in the expression of cleaved caspase-3 (Paul et al., 2009). Antioxidants have a widespread effect in andrology. They have been shown to decrease the DNA fragmentation induced by oxidative stress. The addition of vitamin E or C to sperm preparation media during density gradient separation using Percoll protected spermatozoa from DNA damage. Many new antioxidants that can reduce oxidative stress and improve sperm quality are now available, but a major concern in their usage is the lack of scientific evidence of their effectiveness (Makker et al., 2009). Existing evidence supports the use of systemic antioxidants, as well as antioxidants, in sperm preparation techniques.

The occurrence of spontaneous germ cell death in mammalian testes has been known for a long time and has been proposed to serve as a control of germ cell populations. In spontaneous and injury-associated germ cell deaths, apoptosis appears to occur (Anton, 2003). Several agents or conditions, such as ischaemia/reperfusion (Bergh et al., 2001), reduced intratesticular testosterone concentration (Kim et al., 2001), direct testicular cooling (Zhang et al., 2004) and flutamide (Omezzine et al., 2003), etoposide (Stumpp et al., 2004), cimetidine (Sasso-Cerri, 2009) and ethanol (Koh & Kim, 2006) exposure, have been reported to increase apoptotic germ cell death. Apoptosis plays a major role in the pathogenesis of male factor infertility (Moustafa et al., 2004; Said et al., 2004; Makker et al., 2009), and caspase-3 is the major executioner protease-mediating apoptotic cell death (Moustafa et al., 2004; Makker et al., 2009).

Erdosteine is a drug that was first developed for the treatment of chronic obstructive bronchitis (Dechant & Noble, 1996) and has antioxidant activity (Moretti & Marchioni, 2007). It contains two blocked sulphhydryl groups that are released following its metabolic process. The reducing potential of these sulphhydryl groups accounts for the free radical scavenging and antioxidant activity of erdosteine (Fadilügli et al., 2003a; Yagmurca et al., 2003). Several lines of experimental evidence support the protective effect of erdosteine in acute injury induced by a variety of pharmacological or noxious agents, mediated by products of oxidative stress (Uzun et al., 2006; Moretti & Marchioni, 2007). Erdosteine is known to decrease the production of radicals with xanthine oxidase and has scavenger effects on ROS, and its protective effect on testicular tissue has been shown in different studies (Fadülıglü et al., 2003b; Koc et al., 2005).

The purpose of the present study was to examine the protective effects of erdosteine on testis tissue exposed to hypobaric hypoxia. We investigated the effect of erdosteine on caspase-3 and HIF-1\( \alpha \) expressions.

**Materials and methods**

**Animals and hypoxic exposure**

The Animal Care Ethics Committee of Canakkale Onsekiz Mart University School of Medicine, Canakkale, Turkey, approved this study (B.30.2.ÇAU.0.05.06-050.04-012). A hypobaric chamber (COMMAT, Ankara, Turkey) was used to simulate hypobaric hypoxic conditions at high altitudes. This chamber has been designed to study small laboratory animals at hypoxic condition for long periods by reducing barometric pressure using vacuum pumps. The pressure was controlled with a manometer. Thus, the fall in the partial pressure can give rise to hypobaric hypoxia. In this study, rats were place in this chamber and first exposed to 560 mmHg pressure for 5 min; then, the vacuum was increased, and the rats were exposed to 380 mmHg (0.5 atm). The experiments were continued for 2 weeks under 380 mmHg pressure. Ninety-day-old adult male Wistar rats (240–275 g, \( n = 23 \)) were separated into three groups. Rats in the first group (\( n = 8 \)) were placed in the chamber as a hypoxic group. Rats in the second group (\( n = 8 \)) were also exposed to hypobaric hypoxia for 2 weeks. During these 2 weeks, they were treated orally with the antioxidant erdosteine (Ilisan, Istanbul, Turkey) 20 mg kg\(^{-1}\) daily from the first day of hypoxic exposure. Erdosteine was dissolved in equimolar amounts of sodium bicarbonate (NaHCO\(_3\)) in distilled water. Rats in the third group (\( n = 7 \)) served as the control group and were housed under normobaric, normoxic conditions. The rats were housed at 24 °C on a 12 h dark/light cycle, fed standard rat chow and given free access to water. The experiment was ended on day
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14. The rats were anaesthetised with ether before sacrificing, and the right testes were collected for histopathological studies and immunohistochemical staining evaluation.

Light microscopic examination (histopathology)
At the time of sacrifice, testes were collected for histopathological and immunohistochemical studies. The right testes of rats were fixed in Bouin’s fixative solution for approximately 24 h at room temperature. The testes were dehydrated with gradually increased concentrations of alcohol before being embedded in paraffin. Sections of 5 μm thickness were cut and mounted on glass slides. The sections were stained with haematoxylin and eosin (H&E) for routine light microscopic evaluation and with Gomori’s trichrome to evaluate the presence of peritubular fibrosis according to the thickness of collagen deposition surrounding the tubules. Transverse sections of seminiferous tubules were examined, and histological changes were evaluated. Under light microscopy, histopathological findings were scored in terms of the decrease in height of the seminiferous epithelium, vacuolisation in epithelial cells, deterioration and gaps in the basal membrane, increase in blood vessels, oedema in the interstitial space and peritubular fibrosis.

Semiquantitative evaluation
Histopathological evaluation was performed with the method modified from Cosentino et al. (1986). For histopathological evaluation of testes, 50 randomly selected seminiferous tubules and interstitial spaces between them were blindly observed by one investigator. Evaluations were performed for each specimen under ×40 magnification in four testis sections. All tubular sections in each section of the testis tissue were evaluated systematically, and each was scored on a scale ranging from 0 to 3. The mean score was then calculated as the score for each sample. The height of the seminiferous epithelium was scored as follows: normal histology (0), hypospermatogenesis (1), spermatocytic arrest (2) or Sertoli cells only (3); Vacuolisation in epithelial cells and deterioration and gaps in the basal membrane were scored on a scale ranging from 0 to 3 as follows: 0–5 vacuoles and gaps (0), 5–50 vacuoles and gaps (1), 50–100 vacuoles and gaps (2) or >100 vacuoles and gaps (3). Peritubular fibrosis was scored on a scale ranging from 0 (absent or minimal) to 1 (evident).

Immunohistochemical examination
Paraffine sections of 3 μm thickness were mounted onto poly-L-lysine-covered glass slides. The sections were deparaffinised and rehydrated by passing through two changes of xylol (15 min each); two changes of 100%, then 96% and 70% ethyl alcohol; and two changes of redistilled sterile water for 15 min each. Slides were heated in a high-temperature microwave oven for antigen retrieval while immersed in citrate-buffered saline (0.01 M citrate buffer [pH 6.0]). After cooling at room temperature for 20 min, the tissues on the glass slides were circled with a hydrophobic PAP pen. After washing the slides with distilled water and phosphate-buffered saline (PBS), hydrogen peroxide (3% H₂O₂ and methanol for 30 min) was added dropwise. After washing with PBS (pH 7.4), the tissues were incubated overnight with primary antibody, HIF-1α (H1α 67, 1 : 100 dilution; sc-53 546 mouse monoclonal antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or caspase-3 (CPP32, 1 : 500 dilution; rabbit polyclonal antibody; Diagnostic BioSystems, Pleasanton, CA, USA). After rewashing with PBS and incubating with biotinylated polyvalent immunoglobulin G (ready-to-use; Invitrogen, Paisley, Scotland, UK) for 15 min, sections were rinsed with PBS (2 × 5 min) and incubated with a streptavidin–horseradish peroxidase complex (ready-to-use; Invitrogen) for 15 min. Following rinsing with PBS (2 × 5 min), sections were incubated with substrate chromogen solution freshly prepared by dissolving in 1 mg 3,3′-diaminobenzidine (DAB; Invitrogen) for 15 min. Finally, the slides were counterstained with Mayer’s haematoxylin for 45 s. For dehydration, each slide was soaked in graded alcohol (e.g. 75–80–96–2 × 100%), cleared twice with xylene and coverslipped with mounting medium. Negative controls for all groups were performed by replacing the primary antisera with PBS.

Immunostaining evaluation for caspase-3 and HIF-1α
Quantitative analysis
Quantitative analysis of HIF-1α immunostaining of Sertoli and Leydig cells and caspase-3 immunostaining of seminiferous tubules was performed using AXIOVISION microscopy software [AXIOVISION Software Release 4.8.1 (Zeiss, Germany)].

Seminiferous tubules
The number of seminiferous tubules with caspase-3–immunostained germ cells was counted in 50 randomly selected seminiferous tubules in one section, and statistical analysis was performed using the average value.

Leydig cells
The total number of HIF-1α–positive Leydig cells was counted in four testis sections per animal. One hundred Leydig cells for each section were evaluated. For quantita-
tive evaluation, the numbers of cells that displayed no staining, only cytoplasmic staining (C) and cytoplasmic + nuclear staining (C + N) were counted, and statistical analysis was performed using average values.

**Sertoli cells**

All HIF-1α-immunostained Sertoli cells were counted in 50 randomly selected seminiferous tubules in one section visualised at ×40 magnification, and statistical analysis was performed using the average value.

Hypoxia-inducible factor (HIF-1) α immunostaining and caspase-3 immunostaining were scored as none (0), weak (1), moderate (2) or intense (3), according to differences in staining intensity.

A single investigator who was unaware of the animals’ group assignments performed the immunohistochemical and light microscopy evaluations (Axioskop 40 Microscope, AxioCamICc3; Carl Zeiss, Göttingen, Germany).

**Statistical analysis**

Data analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). The chi-squared test was used to evaluate the significance of differences in histopathological scores among the hypoxia, erdosteine and normoxia groups. Immunohistochemical data were analysed using the Kruskal–Wallis nonparametric test for multiple comparisons (P < 0.05 was considered statistically significant), followed by evaluation with the Mann–Whitney U-test for multiple comparisons. Resulting P-values were corrected according to the Bonferroni method (P = 0.016).

**Results**

**Histopathological results**

The height of the seminiferous epithelium showed severe (score 3) thinning in the seminiferous tubule epithelium (33% of cases) in the hypoxia group (Fig. 1) as a consequence of the depletion of cellular elements in this epithelium. No reduction in epithelial thickness was observed in the erdosteine group, but no statistically significant difference was present between the hypoxia and erdosteine groups (P = 0.057). Scores in the normoxia and erdosteine groups were 0 (100% of cases). The erdos-
tein group showed diminution in the vacuoles and deterioration and gaps in the basal membrane compared with the hypoxia group (P = 0.001, P = 0.0001, and P = 0.0001 respectively; Fig. 1). The normoxic control group showed none of these morphological differentiations. The hypoxia group showed a significant increase in vascularity compared with the control group (P = 0.007), but no difference in comparison with the erdosteine group. Oedema in the interstitial area was observed in some cases in the hypoxia group but was not seen in the other groups. No statistically significant difference in oedema was observed among groups (P = 0.057). Peritubular fibrosis was scored as 0 in all groups.

Immunohistochemical results

Caspase-3 immunostaining
C + N immunostaining was observed in meiotic and post-meiotic germ cells in some seminiferous tubules in all groups (P < 0.016). The counts of caspase-3–positive seminiferous tubules were made using AxiO Vision 4.7 microscopy software. The count of seminiferous tubules with caspase-3–immunostained germ cells was highest in the hypoxia group (mean, 36.37 ± 0.90) and lowest in the normoxia group (mean, 11.71 ± 1.28; Fig. 2), and this difference was significant (P = 0.0001). The erdosteine group showed a reduction in the number of seminiferous tubules with immunostained germ cells (mean, 21.00 ± 1.56) compared with the hypoxia group (P = 0.0001; Table 1).

The intensity of caspase-3 immunostaining in the seminiferous tubules was scored as 3 in the hypoxia group, 2 in the erdosteine group and 1 in the normoxia group.

No immunostaining in Sertoli cells was observed in any group.

In the normoxia group, immunostaining of Leydig cells was observed in the cytoplasm, whereas it was observed in the cytoplasm and nuclei in the hypoxia and erdosteine groups. These two groups showed no statistically significant difference.

HIF-1α immunostaining
Normoxic testes showed no HIF-1α immunostaining in any cell type. No HIF-1α immunostaining was observed.

![Fig. 2 Caspase-3 immunostaining: It was observed in meiotic and post-meiotic germ cells in some of seminiferous tubules in all of the three groups. The number of seminiferous tubules having Caspase-3 immunostained germ cells was higher in hypoxia group. Cytoplasmic immunostaining and nuclear immunostaining of Leydig cells in interstitial space (*) were observed in hypoxia and erdosteine groups. Scale bars: 200 μm, 100 μm, 50 μm. N, normoxia group; H, hypoxia group; E, erdosteine group.](image-url)
in germ cells of spermatogenic epithelium in any group (Fig. 3). The count of HIF-1α-immunostained Sertoli cells was significantly higher in the hypoxia group (mean, 25.75 ± 5.23) than in the erdosteine group (mean, 10.00 ± 2.12). Most Leydig cells in the hypoxic group showed C+N (mean, 74.37 ± 8.63) or C (mean, 25.62 ± 8.63) immunostaining. Some Leydig cells in the erdosteine group showed C+N (mean, 33.75 ± 11.94) or C (mean, 35.62 ± 7.75) immunostaining. The count of C+N-immunostained Leydig cells was significantly higher in the hypoxia group than in the erdosteine group (P = 0.028; Fig. 3, Table 2).

The intensity of HIF-1α immunostaining in Sertoli and Leydig cells was scored as 3 in the hypoxia group, 2 in the erdosteine group and 0 in the normoxia group.

Negative controls for all groups were evaluated by replacing the primary antisera with PBS (Fig. 4).

### Discussion

The results of this study suggest that germ cell death occurs via apoptosis, whether spontaneously during normal spermatogenesis or triggered by different stimuli (Blanco-Rodrı´guez & Martı´nez-Garcı´a, 1998). Studies of testis morphology by different stimuli have observed damage in different cell types. Increased caspase-3 expression was reported in germ cells in azoospermic smokers (Kilic et al., 2009), in Leydig cells and round spermatids after the surgical removal of bilateral superior and inferior spermatic nerves (Huo et al., 2010), in spermatocytes after short photoperiod exposure (Morales et al., 2007) and in primary spermatocytes and round spermatids after cooling treatment (Zhang et al., 2004). We observed caspase-3-immunostained meiotic and post-meiotic germ cells in all groups in our study. The normoxia group showed the lowest count of spontaneous apoptosis, and the hypoxia group showed the highest count of hypoxia-triggered apoptosis. Liao et al. (2010) reported that apoptotic germ cells increased in rats after exposure to hypoxia, and most TUNEL-positive germ cells were spermatogonia and primary spermatocytes. In a heat stress study, Paul et al. (2009) suggested that many caspase-3-positive cells were located close to the basement membrane and were likely to be spermatogonia. On the contrary, we did not observe caspase-3 expression in spermatogonia in any group, but

### Table 1

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<th>The number of seminiferous tubules having Caspase-3–immunostained meiotic and post-meiotic germ cells</th>
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<tbody>
<tr>
<td>Hypoxia (n = 8)</td>
<td>36.37 ± 0.90*</td>
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<tr>
<td>Erdosteine (n = 8)</td>
<td>21.00 ± 1.56**</td>
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<tr>
<td>Normoxia (n = 7)</td>
<td>11.71 ± 1.28***</td>
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<td>P</td>
<td>0.0001</td>
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*P = 0.0001, compared with erdosteine group and normoxia group.

**P = 0.0001, compared with hypoxia group.

***P = 0.001, compared with erdosteine group.

Data are expressed as the mean ± SE. Kruskal–Wallis variance analysis was used.
we observed it in meiotic and post-meiotic germ cells of some seminiferous tubules.

We observed more vacuolisation in epithelial cells and deterioration and gaps in the basal membrane in the hypoxia group than in the other groups. Vascularity was significantly increased in the hypoxia group. These results are consistent with the findings of Farias et al. (2005a,b, 2008). The normoxic group did not show these morphological features. A hypoxia study by Cikutovic et al. (2009) revealed a significant decrease in the diameter of seminiferous tubules and the thickness of the spermatogenic epithelium in rats exposed to intermittent chronic hypobaric hypoxia. Similarly, we observed a decrease in the height of seminiferous epithelium in testis tissues of rats exposed to hypoxia in our study. These data suggest that hypobaric hypoxia inhibits spermatogenesis in rats.

Hypoxia-inducible factor 1 (HIF-1) plays a master regulatory role in the cellular response to hypoxia (Powell et al., 2002). Several studies have attempted to localise HIF-1α in testicular tissue. HIF-1α protein has been shown to be localised in the cytoplasm under normoxic conditions; however, the exposure to hypoxia or heat stress results in reduction in proteosome-dependent degradation, and HIF-1α translocates to the nucleus (Firth et al., 1995; Forsythe et al., 1996; Paul et al., 2009). Immunoblot and immunocytochemical experiments have demonstrated that Leydig cells are the major source of HIF-1α in normoxic and hypoxic testes, and HIF-1α was not detected in spermatogenic cells or Sertoli cells (Palladino et al., 2011). We observed HIF-1α immunostaining in Sertoli and Leydig cells in the hypoxia and erdosteine groups. Most Leydig cells in the hypoxia group showed HIF-1α immunostaining in the cytoplasm and nucleus. The erdosteine group showed a decrease in HIF-1α immunostaining in both of these cells.

Table 2. Count of hypoxia-inducible factor 1 (HIF-1) α immunostaining of Leydig cells/100 Leydig cells and Sertoli cells/50 seminiferous tubules

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<th>Immunohistochemical HIF-1α immunostaining</th>
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<td>Sertoli cells</td>
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<tr>
<td>Hypoxia (n = 8)</td>
<td>25.75 ± 5.23*</td>
</tr>
<tr>
<td>Erdosteine (n = 8)</td>
<td>10.00 ± 2.12</td>
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<tr>
<td>P</td>
<td>0.021</td>
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*P = 0.028, compared with erdosteine group. There was significant difference statistically.

**P = 0.021, compared with erdosteine group. There was significant difference statistically.

Data are expressed as the mean ± SE HIF-1α immunostaining was not detected in the normoxic testis.

C + N, cytoplasmic + nuclear staining; C, cytoplasmic staining; Mann–Whitney U-test was used.

In certain circumstances and cell types, HIF-1 promotes apoptosis in the presence of hypoxia (Malhotra et al., 2008). In a varicocele study in rats, Wang et al. (2010) recently demonstrated a relationship between hypoxia and germ cell apoptosis in rat testes. They suggested that HIF-1α is an useful factor in predicting the degree of germ cell apoptosis in rat testes.
The testis has its own antioxidants, which have been suggested to be induced in response to thermal stress despite the presence of insufficient amounts to compensate for increased levels of ROS (Paul et al., 2009). Several studies have been performed to reveal the protective effects of antioxidants on testicular tissue. Protective effects of molsidomine against ischaemia/reperfusion injury (Dokucu et al., 2009) and melatonin against damage caused by intermittent hypobaric hypoxia (Hartley et al., 2009) have been shown. Several lines of experimental evidence support the protective effect of erdosteine on different organs. Two studies have investigated the effect of erdosteine on testicular tissue. Oktar et al. (2010) detected this effect by biochemical methods. They suggested that erdosteine could effectively protect testes from methotrexate-induced toxicity (Oktar et al., 2010). Koc et al. (2005) used biochemical methods and light microscopic observations to demonstrate that erdosteine administration significantly reduced the histological damage associated with torsion/detorsion of the spermatic cord.

Our study is the first to examine the effect of erdosteine on hypoxic testes by the combined evaluation of caspase-3 and HIF-1α expressions. The role of HIF-1α in apoptosis in hypoxic testis tissue remains unclear. We report that erdosteine protects testis tissue from hypoxic injury by reducing the number of seminiferous tubules with caspase-3-immunostained germ cells. Sertoli cells play a role in meiosis, and Leydig cells play a role in the spermatogenic process. HIF-1α activity in Sertoli and Leydig cells may damage the spermatogenic process. HIF-1α activity in Sertoli cells may promote caspase-3 activation in meiotic and post-meiotic germ cells so that Sertoli cells have a role in germ cells meiosis. We suggest that additional studies should be performed to explain the relationship between HIF-1α and caspase-3 activities.

References


