E-Selectin expression in the mouse testis after experimental testicular torsion (ischemia/reperfusion)

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ABSTRACT
Germ cell-specific apoptosis occurs after ischemia/reperfusion of the testis and is dependent on E-selectin expression. The aim of the study was to determine differences in E-selectin expression in testes tissues of control, sham and treatment groups after ischemia/reperfusion in mice. Mice were subjected to 720˚ testicular torsion for 1 h or 2 h duration (ischemia) followed by detorsion (reperfusion). After 2 h of reperfusion, the testes were fixed in Bouin fixative and immunohistochemical analysis performed for E-Selectin expression. E-selectin expression increased in the ischemic testis and contralateral testis after 2 h of reperfusion in mice. This increase in E-selectin expression may confirm that E-selectins play a key role in mediating of apoptosis in germ cells after ischemia/reperfusion. Thus, the blockage of E-selectins might be a strategy for rescue of post-ischemic testes.

Key words: E-selectin, ischemia, reperfusion, mouse, testis

Introduction
Tissue ischemia-reperfusion (IR) may result in complex pathological changes in the affected organ. Ischemia-reperfusion injuries have been reported in some organs, such as the brain (LIPTON, 1999), kidney (RABB and POSTLER, 1998), heart (KAJSTURA et al., 1996), intestine (YOSHIKAWA et al., 1990), liver (PERALTA et al., 2002) and testis (TURNER et al., 1997). Activation of neutrophils (CONNOLLY et al., 1996), proinflammatory cytokine production (MITSUI et al., 1999), mitochondrial dysfunction (JASSEM et al.,...
M. Selcuk et al.: E-selectin expression after testicular torsion

2002), generation of reactive oxygen species (LI and JACKSON, 2002) and changes in intracellular calcium homeostasis (AMBROSIO and TRITTO, 1999) commonly occur in IR injuries. Most of these events usually cause organ dysfunction due to apoptosis of cells in the affected organ (TURNER et al., 1997; YIN et al., 1997).

Testicular torsion (torsion of spermatic cord) culminates in tissue ischemia due to the impedence of blood flow to the testis. In humans, testicular torsion is a medical emergency that usually requires surgical intervention to allow reperfusion of the affected testis (CASA et al., 1980; WILLIAMSON, 1985). It has been mentioned that even after reperfusion of the affected testis, testicular atrophy may be the outcome (LYSIAK et al., 2000a). Spermatogonia in the second and third stage of the seminiferous tubule epithelial cycle and spermatocytes are predominant apoptotic cells (LYSIAK et al., 2000b). LYSIAK et al. (2001) state that germ cell-specific apoptosis is determined after IR of the murine testis, and this apoptosis is exactly related to the neutrophil recruitment to subtunical venules. Furthermore, endothelial cell adhesion molecules (particularly E-selectin) have a key role in mediating this pathology (LYSIAK et al. 2001).

It has been mentioned that microvascular blood flow in the affected testis cannot return to normal after repair of torsion within a short time, and this may also cause the standing impairment of spermatogenesis (BECKER et al., 1997). Testicular blood flow displays vasomotion (TURNER et al., 1997) which is the rhythmic dilation and constriction of precapillary sphincters. That is periodical variations in blood flow through the capillaries. Vasomotion regulates local vascular resistance, the exchange of nutrients, oxygen, carbon dioxide and fluids between the vascular system and interstitial space (RENKIN, 1984). Testicular blood flow is completely blocked by experimental testicular torsion. The mean blood flow returns to the pretorsion rate within minutes of negating the torsion, but vasomotion does not occur until 1 h after repair of torsion (BECKER et al. 1997).

The degree of fertility loss with testis torsion depends on the duration of testicular ischemia and afterwards the injury of the contralateral testis. The contralateral testicular injury after unilateral IR and the mechanism of the injury of the contralateral testis still cannot be fully explained (COSENTINO et al., 1985). SUKHOTNIK et al. (2008) report that apoptosis in germ cells occurred for 24 h after IR and testicular ischemia duration has increased the extent of apoptosis. SUKHOTNIK et al. (2007) state that germ cell apoptosis has increased in the contralateral testis 96 h after IR. Germ cell apoptosis plays important roles in various physiological processes during the development of fetal and adult testicular tissue, and it is essential for normal spermatogenesis. However, it is considered that there is a critical balance between germ cells and Sertoli cells for assuring cellular homeostatis (SAID et al., 2004).
E-selectin, expressed in the activated endothelial cell, is an adhesion molecule. Selectins are responsible for rolling leukocytes on endothelial cells during the chemotaxis of leukocytes from the bloodstream into the tissues, in the initiation of inflammation. Leukocytes hold on to the endothelial surface, followed by association with the ligand of a selectin, and a low affinity linkage occurs. The linked leukocyte begins rolling along the surface of the endothelial cell (direction of blood flow) via new selectin-mediated links (SHARAR et al., 1995; HARARI et al., 1999).

The aim of the study was to determine the differences in the level of E-selectin expression in testis tissues of control, sham and experimental groups after experimental testicular torsion (ischemia/reperfusion) in mice.

**Materials and methods**

**Experimental testicular torsion.** A total of 25 male adult mice (C57BL/6) were used in the study. For experimental testicular torsion, the mice were randomly divided into 5 groups (n = 5 for each): C) control group (nonoperated and without torsion), Sh1) sham 1 group (without torsion/ 1 h), Sh2) sham 2 group (without torsion/ 2 h, T1) treatment 1 group (with 720° torsion/ 1 h), T2) treatment 2 group (with 720° torsion/ 2 h). The work was approved by the Local Ethical Committee at Ondokuz Mayis University.

Testicular torsion in the experimental groups (720° torsion/ 1 h and 720° torsion/ 2 h) was performed according to LYSIAK et al. (2001). The mice were anesthetized with an intra peritoneal (IP) injection of a mixture of 6 mg /100 g of ketamine and 0.5 mg/100g of xylazine. Under aseptic conditions the testis was exteriorized through a low ventral midline incision, the gubernaculum was incised and the testis was released from the epididymo-testicular membrane. The testis was rotated 720° and left in torsion (ischemia) for a duration of 1 h (T1) and 2 h (T2) during which time it remained in the abdomen with a closed incision. After 1 h and 2 h of torsion, the incision was reopened and the testis was counter-rotated to the natural position (reperfusion) and reinserted into the scrotum and then the incision was closed. After 2 h of reperfusions, the testes were fixed in Bouin fixative for determining E-selectin expression.

The sham-operated groups were treated identically as the IR groups (T1 and T2) without testes torsion. The testes of mice in C (nonoperated and without torsion) were fixed with Bouin fixative for determining E-selectin expression.

Contralateral testes not rotated for torsion of mice in groups were collected and fixed with Bouin fixative for determining E-selectin expression.

**Immunohistochemical analysis for E-selectin.** Briefly, testes tissues, fixed with Bouin fixative for 12 h, were trimmed and then kept in Bouin fixative for postfixation. Tissue samples were embedded in paraffin by routine methods. Sections were cut in 5 μm thickness and were stained with hematoxyline-eosin (HE). A combination of the avidin-
biotin-peroxidase method (Zymed, Histostain Plus Kit, California, USA) was performed to identify the E-selectin localized to the endothelium of venules, according to the manufacturers’ protocols. A rabbit anti-mouse E-selectin antibody (E-selectin polyclonal antibody, dilution 1:50, BioVision, cat no 3631-100, USA) was used as the primary antibody. Tissue samples for negative controls were treated with phosphate-buffered saline (PBS-pH 7.4), while primary antibodies were used for tissue samples of the control. The sections were deparaffinized, dehydrated in graded alcohol and microwave-pretreated in citrate buffer (pH 6) at 600 W for 20 min. The specimens were incubated in hydrogen peroxide (0.3% hydrogen peroxide in methanol) for 7 min followed by incubation with serum protein blocking solution for 10 min. Thereafter, samples were incubated overnight at +4 °C with E-selectin primary antibody. The sections were incubated with a biotinylated secondary antibody and then treated with streptavidin peroxidase conjugate. After all processes, the sections were bathed in PBS twice for 5 min, with the exception of the serum protein blocking phase. Finally, the sections were stained with 3-amino-9-ethylcarbazole (Zymed AEC RED substrat kit, USA) for 10 min under a microscope. Counter-staining was performed with Gill’s hematoxylene and mounted with an aqueous mounting medium (Shandon Immu-mount, USA). All sections were examined under a light microscope (Nikon Eclipse E600W). For each group, the number of stained vessels was counted. The E-selectin index was calculated per total of ten subtunical venules (SUKHOTNIK et al., 2008).

Statistics. The right tailed t test was used for the comparison of traits observed from the left and right testes. The generalized linear model procedure (GENMOD) fitted with Poisson distribution and linked to log function for the error terms was executed for each testis measurement in the treatment groups in SAS (2009). To compare the differences among the groups, the means were analyzed with third order orthogonal polinomials.

Results

Testis histopathology.

Ischemic testis. Histopathologically, degenerative and necrotic changes were detected in the spermatogenic cells of some seminiferous tubules in the T1. In this group, late spermatid (spermatozoon) numbers were significantly decreased. However, spermatozoa were not seen in the tubuli. Occasionally, giant cells were detected in the lumen of some tubuli. Cytoplasm of some spermatogenic cells were vacuolized and/or with presence of desquamated spermatogenic cells in some tubuli.

Seminiferous tubuli were dilated and spermatogenic cells showed either necrosis or mild degeneration, characterized by cytoplasmic vacuolization and desquamation in some tubular lumens in the T2. Furthermore, spermatozoon production decreased in some tubuli (Fig.1a).
No pathological change was observed in C, Sh1 and Sh2.

Contralateral testis. Seminiferous tubuli were markedly dilated and late spermatid forms reduced in number in the T1. A greater number of undamaged spermatids in the contralateral testis were detected compared to those in the ischemic testis. However, mild degeneration, necrosis and desquamation were observed in spermatogenic cells. The cytoplasm of some spermatogenic cells shown vacuolization and giant cell formations were also noted sporadically.

Seminiferous tubuli were dilated and spermatogenic cells were either necrotic or mildly degenerated, and characterized by cytoplasmic vacuolization and desquamation in some tubular lumens in the T2. Furthermore, there was a cessation of spermatozoon production in some tubuli (Fig. 1b).

Fig. 1. Necrosis (arrowhead) and vacuolization (arrow) in the spermatogenic cells of seminiferous tubules. a: ischemic testis treatment 2, H&E. b: controlateral testis treatment 2, H&E.

Fig. 2. a: E-selectin negative reaction in the endothelial cells in control testis, ABC. b: E-selectin positive reaction in the endothelial cells of testis in treatment 2 (arrow), ABC.
No pathological changes were seen in C, Sh1 and Sh2 groups.

**E-selectin expression. Ischemic testis.** E-selectin expression in C, Sh1, Sh2, T1 and T2 were 0.2 ± 0.2, 0.8 ± 0.3, 1.0 ± 0.3, 3.0 ± 0.7 and 2.2 ± 0.8 respectively. Testicular IR culminated in an increase (P<0.01) in E-selectin expression in T1 compared to C, Sh1 and Sh2. However, E-selectin expression of T1 was similar to that of T2 (Table 1).

Table 1. E-selectin expression in the ischemic testis and the contralateral testis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ischemic testis**</th>
<th>Contralateral testis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>$\bar{X} \pm S_{\bar{X}}$</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0.2 ± 0.2a</td>
</tr>
<tr>
<td>Sh1</td>
<td>5</td>
<td>0.8 ± 0.3bc</td>
</tr>
<tr>
<td>Sh2</td>
<td>5</td>
<td>1.0 ± 0.3bc</td>
</tr>
<tr>
<td>T1</td>
<td>5</td>
<td>3.0 ± 0.7a</td>
</tr>
<tr>
<td>T2</td>
<td>5</td>
<td>2.2 ± 0.8ab</td>
</tr>
</tbody>
</table>

C: Control, Sh1: Sham 1, Sh2: Sham 2, T1: Treatment 1, T2: Treatment 2; * P<0.05; ** P<0.01; a,b,c, means with the same letter in columns are not significantly different; NS, Not significant

E-selectin expression in the ischemic testis of the T1 increased (P<0.01) after 1 h of reperfusion compared with that of C, Sh1 and Sh2. E-selectin expression in T2 was similar to that of T1, Sh1 and Sh2 (Fig. 2a).

**Contralateral testis.** E-selectin expressions in C, Sh1, Sh2, T1 and T2 were 0.2 ± 0.2, 1.0 ± 0.3, 0.8 ± 0.3, 2.6 ± 0.9 and 1.6 ± 0.4, respectively. Testicular IR increased (P<0.05) E-selectin expression in T1 compared to C, Sh1 and Sh2. However, E-selectin expression of T1 was similar to that of T2 (Table 1).

E-selectin expression in the contralateral testis of the T1 increased (P<0.05) after 1 h of reperfusion, compared with that of C, Sh1 and Sh2. E-selectin expression in T2 was similar to that of T1, C, Sh1 and Sh2 (Fig. 2b).

**Discussion**

Selectins mediate neutrophil migration to inflammatory sites during postischemic inflammatory response, thus they also mediate adhesive interactions between leukocytes and endothelial cells required for recruitment of leukocytes to inflammatory sites. According to the results of some recent studies, germ cell apoptosis in ischemic testis results in reduction of daily sperm production, and this is linked to the recruitment of neutrophils to subtunical venules (TURNER et al., 1997) which is regulated by E-selectin (LYSIK et al., 2003). SUKHOTNIK et al. (2008) reported that testicular ischemia for 90 min in rats caused an increase of E-selectin expression in the ischemic testis at 1 h
after reperfusion. Although maximum E-selectin expression in the ischemic testis was determined at 6 h after reperfusion, E-selectin expression gradually decreased at 21 h, 48 h and 72 h after reperfusion. In the present study, E-selectin expression in the ischemic testis at 2 h after reperfusion in the mice subjected to 1 h ischemia inducing torsion (T1) was higher (P<0.01) than those of C, Sh1 and Sh2. E-selectin expression in the ischemic testis at 2 h after reperfusion in the mice subjected to 2 h ischemia inducing torsion (T2) was similar to that of T1.

The results of the present study for E-selectin expression at 2 h after reperfusion of the T1 and T2 were numerically higher than that of SUKHOTNIK et al. (2008) who reported E-selectin expression for 1 h after reperfusion in rats. The differences in the results of the studies were attributed to different animal material used in the studies and IR duration.

SUKHOTNIK et al. (2008) reported that E-selectin expression in the contralateral testis increased markedly after 1 h of reperfusion and then remained unchanged within the first 24 h of reperfusion, and decreased after 48 h and 72 h of reperfusion. The E-selectin expression mechanism in the contralateral testis remains poorly understood. It is commonly accepted that tumor necrosis factor-α and interleukin-1β activate the stress-related kinase metabolic pathway to E-selectin expression in the ischemic and contralateral testis, and neutrophil recruitment. Although cytokine activation in the ischemic testis occurs immediately at the onset of ischemia, the effects of proinflammatory cytokines on E-selectin expression in the contralateral testis may occur after they enter into circulation, therefore, this effect is observed later compared to the ischemic testis (SUKHOTNIK et al., 2008). In the present study, E-selectin expression in the contralateral testis was lower after 1 h and 2 h of IR than that in the ischemic testis. This data was consistent with the result of SUKHOTNIK et al. (2008).

720° testicular torsion for 1 h in rats caused loss of spermatogenesis, despite the normal functions of the Leydig and Sertoli cells. After IR, the return of blood flow (LYSIK, 2000), and the normal functions of leydig (BAKER and TURNER, 1995) and sertoli (TURNER and MILLER, 1997) cells, permanent loss of spermatogenesis was observed in rats. It was reported that this loss of spermatogenesis was related to germ cell specific apoptosis (TURNER et al., 1997; LYSIAK et al., 2000). The results of the present study for degenerative changes in spermatogenic cells and dramatic reduction in spermatids were consistent with those of LYSIAK et al. (2000), BAKER and TURNER, (1995) and TURNER and MILLER (1997).

In conclusion, E-selectin expression increased in the ischemic testis and contralateral testis after 2 h of reperfusion in mice compared to the C, Sh1 and Sh2. This increase in E-selectin expression may confirm that E-selectins play a key role in the formation of apoptosis in germ cells after I/R. Thus, the blockage of E-selectins might be a strategy for the rescue of post-ischemic testes.
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M. Selcuk et al.: E-selectin expression after testicular torsion


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SAŽETAK

Apoptoza germinativnih stanica javlja se nakon ishemije i reperfuzije sjemenika, a ovisna je o ekspresiji E-selektina. Cilj ovog istraživanja bio je odrediti razlike u ekspresiji E-selektina u tkivu sjemenika kontrolne, placebo i pokusne skupine nakon ishemije/reperfuzije u miševa. Miševi su bili podvrgnuti torziji sjemenika od 7200 tijekom jednog ili dva sata (ishemija), nakon čega je slijedila detorzija (reperfuzija). Nakon dva sata reperfuzije tkivo sjemenika bilo je fiksirano u Bouinovom fiksativu i pretraženo imunohistokemijski na ekspresiju E-selektina. Ekspresija E-selektina povećala se u ishemičnim sjemenika nakon dva sata reperfuzije. Može se reći da povećanje ekspresije E-selektina potvrđuje njihovu ključnu ulogu u nastanku apoptoze germinativnih stanica nakon ishemije/reperfuzije pa bi blokada E-selektina mogla biti od važnosti za spašavanje sjemenika nakon ishemije.

Ključne riječi: E-selektin, ishemija, reperfuzija, miš, sjemenik