Abstract. – Background: Testicular torsion due to oxidative stress results in infertility and testicular damage which can be preventable an important health problem worldwide.

Aim: The purpose of the present study was to investigate the changes of malondialdehyde (MDA), total antioxidant status (TAS), total oxidant status (TOS) levels; histopathological alterations; morphology, concentration and motilities of the sperm in post ischemic reperfused (I/R) testis tissue.

Materials and Methods: Forty adult male Wistar rats were carried out and were randomized to five groups; (1) Control group, (2) Ipsilateral left testis ischemia, (3) Melatonin plus ipsilateral left testis ischemia, (4) Contralateral right testis ischemia, 5. Melatonin plus contralateral right testis ischemia. After 1 h ischemia and 24 h perfusion; MDA, TAS and TOS levels were measured, histopathological alterations were determined using by Johnsen’s score (JS) and sperm morphology, concentration, motility were examined.

Results: MDA, TAS and TOS levels of the testis tissue did not change in all groups (p > 0.05 for all). JS was decreased in I/R group and melatonin treatment reversed histopathological changes and increased JS both in ipsilateral and contralateral testis. Abnormal sperm rate significantly increased in I/R group and melatonin administration changed abnormal sperm rate to normal.

Conclusions: As a result, the present study demonstrated that testicular damage occurs following I/R without an increase of MDA, TAS and TOS levels. Our results also suggested that melatonin is a potent antioxidant agent in preventing testicular I/R injury, as shown by increased JS and changed abnormal sperm rate.

Key Words:
Ischemia-reperfusion, Melatonin, Testis, Oxidative stress, Johnsen’s score.

Introduction
Testicular torsion is a surgical emergency. Late presentation or failure to diagnose and correctly manage this condition leads to testicular injury and subfertility. Testicular injury following torsion has both ischemic and reperfusion components. The reperfusion component typically involves the generation of reactive oxygen species (ROS) with the return of blood flow following a period of ischemia. ROS causes damage to DNA, impairment of protein function and peroxidation of lipids. Mammalian spermatozoa are very rich in polyunsaturated fatty acids and they are very susceptible to attack by ROS.

Melatonin, which is secreted by the pineal gland, has been shown recently to be a potent free radical scavenger. It has both a lipophilic and hydrophilic compound. Thus, it freely permeates all morphophysiological barriers of cells in any organ. Melatonin was detected in the reproductive sys-
tems of different species, so it seems reasonable to assume that melatonin exerts its actions through direct interaction with the steroidogenic cells of the reproductive organ9,10.

Melatonin, as well as mentioned above, has been shown to be beneficial effects on ischemic reperfused (I/R) induced oxidative damage on testes in several studies. Besides, several antioxidant enzymes have been studied in these reports10-17. Beyond these studies, the purpose of the present work was to investigate the changes of malondialdehyde (MDA) which is widely used as an indicator of oxidative stress, total antioxidant status (TAS) and total oxidant status (TOS) levels in post ischemic reperfused tissue, and to determine whether protective effect of melatonin is related to modulation expression of these changes. Moreover, histopathological alterations by using Johnsen’s score (JS) parameters, sperm morphology, concentration and motilities were determined.

Materials and Methods

Animals

Forty adult male Wistar rats, weighing 200-300 g, were used. Animals were housed under continuous observation in appropriate cages in a quiet temperature (21±2°C) and humidity (60±5%) controlled room in which a 12-12 h light-dark cycle was maintained. The animals were housed five per cage, and fed with commercial standard diet and water ad libitum, but were made to fast overnight before surgery. All experiments in this research were performed in accordance with the “Principles of Laboratory Animal Care” (NIH publication No. 86-23, revised 1984) and were approved by the Committee on Animal Research at Harran University, Sanliurfa.

Rats were randomly assigned to five groups each containing eight rats as follows:

1. Control group
2. Ipsilateral left testis ischemia (ILT)
3. Melatonin plus ipsilateral left testis ischemia (IMLT)
4. Contralateral right testis ischemia (IRT)
5. Melatonin plus contralateral right testis ischemia (IMRT)

Surgery and Experimental Protocol

Under anesthesia by intraperitoneal injection of 75 mg/kg ketamine hydrochloride and 8 mg/kg xylazine, an abdominal incision was made. The testicular artery and vein of the left testis were occluded with a vascular clamp for 1 h, after this process the clamp was removed and the organ was allowed to reperfusion 24 h. The rats were treated with either melatonin (10 mg/kg, i.p.) at 10 min prior to ischemia. Melatonin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in ethanol and then diluted in saline (0.09% NaCl wt/vol) to give a final alcohol concentration of 1% ethanol. For histopathologic evaluation and biochemical analysis were analyzed in all groups at 24th hour.

Biochemical Analysis

The rats were sacrificed at 24 h of reperfusion for evaluation of biochemical parameters. The left and right testis tissue samples stored at -70°C for measurement of TAS, TOS and MDA level activities. The testes were weighed, tissue homogenate were prepared as described below. The following determinations were made on the samples using commercial chemicals supplied by Sigma (St. Louis, MO, USA). The testis tissues were homogenized for 3 min at 16,000 rpm in four volumes of ice-cold 0.2 mM Tris-HCl buffer (pH 7.4) using a homogenizer (Ultra-Turrax T25 basic homogenizer, IKA Laborttechnik, Staufen, Germany)18.

Determination of Total Antioxidant Capacity

Plasma TAS levels were determined using a novel automated measurement method, developed by Erel19. In this method, hydroxyl radical, which is the most potent radical, is produced via Fenton reaction. In the classical Fenton reaction, the hydroxyl radical is produced by mixing of ferrous ion solution and hydrogen peroxide solution. In the most recently developed assay by Erel, same reaction is used. In the assay, ferrous ion solution, which is present in the Reagent 1, is mixed by hydrogen peroxide, which is present in the reagent 2. The sequential produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. In this assay, antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has got excellent precision values, which are lower than 3%. The results are expressed as mmol Trolox equiv/l.

Determination of Total Oxidant Status

Plasma TOS levels were determined using a novel automated measurement method, devel-
oped by Erel\textsuperscript{20}. In this method, oxidants present in the sample oxidize the ferrous ion-oxygenidansidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically (V-530; Jasco\textsuperscript{8}, Tokyo, Japan), is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H\textsubscript{2}O\textsubscript{2} Equiv./L).

**Determination of Malondialdehyde (MDA) Level**

The tissue MDA level was determined by a method\textsuperscript{21} based on the reaction with thiobarbituric acid (TBA). In the TBA test reaction, MDA or MDA-like substances and TBA react with the production of a pink pigment with a maximum absorption at 532 nm. The reaction was performed at pH 2-3 at 90°C for 15 min. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid for protein precipitation. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed as nmol per g wet tissue.

**Epididymal Sperm Concentration and Motility**

Spermatozoa in the epididymis were counted by a modified method of Yokoi et al\textsuperscript{22}. Briefly, the epididymis was minced with anatomical scissors in 5 ml of physiological saline, placed in a rocker for 10 min, and incubated at room temperature for 2 min. The supernatant fluid was diluted 1:100 with a solution containing 5 g sodium bicarbonate, 1 ml formalin (35%), and 25 mg eosin per 100 ml of distilled water. Total sperm number was determined with a hemocytometer. Approximately 10 µl of the diluted sperm suspension was transferred to each counting chamber and was allowed to stand for 5 min for counting under a light microscope at 200 × magnification. Sperm progressive motility was evaluated by an earlier method described by Sonmez et al\textsuperscript{23}. For this purpose, fluid was obtained from the caudal epididymis with a pipette and diluted to 2 ml with Tris buffer solution. The system was pre-warmed (35°C) and percentage of motility was evaluated visually at 400 × magnification. Motility estimations were performed from three different fields in each sample. The mean was used as the final motility score. The method by Evans and Maxwell\textsuperscript{24} was used for determination of the percentage of morphologically abnormal spermatozoa after adapting the method for use in rats. According to this method, slides were prepared with India ink. A total of 300 sperm cells were counted on each slide under light microscope at 400 × magnification.

**Histological Analysis**

The rats were sacrificed at 24 h of reperfusion for determination of sperm concentration, motility, abnormal sperm rate and histopathologic evaluation. For light microscopic evaluation, tests were fixed in 10% neutral buffered formalin, processed routinely by automatic tissue processor and embedded in paraffin wax. Four mm sections were stained with hematoxylin-eosin (H-E) before investigation under light microscopy (Olympus BX-51 TF, Tokyo, Japan). Histological findings in seminiferous tubuli were evaluated according to Johnsen’s scoring system\textsuperscript{25}. Tubuli in 10 consecutive 400 X field areas were scored and mean values were determined. The Johnsen score is based on the premise that with testicular damage there is successive disappearance of the most mature cell type, with progressive degeneration of germinal epithelium, with the disappearance of spermatozoa and spermatids, then spermatocytes and finally Sertoli cells, in that order. A score of 1 to 10 was given to each tubule according to the maturity of the germ cells: A score of 1 indicated no seminiferous epithelial cells and tubular sclerosis. A score of 2 indicated no germ cells, only Sertoli cells. A score of 3 indicated spermatogonia only. A score of 4 indicated no spermatids, few spermatocytes, and arrest of spermatogenesis at the primary spermatocyte stage. A score of 5 indicated no spermatids and many spermatocytes. A score of 6 indicated no late spermatids, few early spermatids, arrest of spermatogenesis at the spermatid stage, and disturbance of spermatid differentiation. A score of 7 indicated no late spermatids and many early spermatids. A score of 8 indicated few late spermatids. A score of 9 indicated many late spermatids and disorganized tubular epithelium. A score of 10 indicated full spermatogenesis.
Effects of melatonin on testis ischemia-reperfusion injury in rats

Table I. Comparison of biochemical parameters of groups at 24th hour.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>ILT</th>
<th>IMLT</th>
<th>p α</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA, µmol/g protein</td>
<td>8.20 ± 0.25</td>
<td>8.10 ± 0.18</td>
<td>8.30 ± 0.25</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>TAS, mmol Trolox/Equiv./g protein</td>
<td>3.70 ± 0.33</td>
<td>3.70 ± 0.21</td>
<td>3.60 ± 0.65</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>TOS, □mol H2O2 Equiv./g protein</td>
<td>21.90 ± 1.78</td>
<td>18.30 ± 0.39</td>
<td>18.50 ± 0.33</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD. MDA: malondialdehyde; TAS: total antioxidant status; TOS: total oxidant status; C: Control group; ILT: Ipsilateral left testis; IMLT: Melatonin plus ipsilateral left testis. By one way Anova test.

Table II. Comparison of Johnsen’s scores among groups at 24th hour.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>ILT</th>
<th>IMLT</th>
<th>IRT</th>
<th>IMRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnsen’s score</td>
<td>8.2 ± 0.20</td>
<td>6.3 ± 0.20*</td>
<td>7.3 ± 0.09**</td>
<td>7.1 ± 0.21***</td>
<td>8.1 ± 0.18*</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD. C: Control group; ILT: Ipsilateral left testis; IMLT: Melatonin plus ipsilateral left testis; IRT: Contralateral right testis; IMRT: Melatonin plus contralateral right testis; *p < 0.05 vs. control group. **p < 0.05 vs. only ischemic groups. ***p < 0.05 shows the difference between ipsilateral and contralateral ischemic groups (7.30 ± 0.09; 8.1 ± 0.18, respectively) (Figures 2, 3). In I/R group, sperm intensity and motility rates did not change. However, abnormal sperm rate significantly increased. Melatonin administration changed abnormal sperm rate to normal (Table III).

Statistical Analysis

All statistical analyses were performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). Distribution of the samples in the groups was analyzed with one sample Kolmogorov-Smirnov test. The data were expressed as arithmetic means and standard deviations. One way Anova test was used to assess the differences among groups. Two-sided p < 0.05 was considered statistically significant.

Results

The ipsilateral and contralateral testis MDA, TAS and TOS levels are presented on Table I. In I/R group, MDA, TAS and TOS levels of the testis tissue were similar to control group in both ipsilateral and contralateral testis (p > 0.05 for all). Besides, melatonin did not change the MDA, TAS and TOS levels of the tissue in both I/R and control groups (p > 0.05 for all).

JS of all groups were shown on Table II. The greatest significant histopathological scores observed were in ipsilateral and contralateral testes of I/R group (6.30 ± 0.20; 7.10 ± 0.21, respectively). The JSs between two groups were statistically significant when compared with each other. Extensive disorganization, sloughing and loss of maturation of germ cells were observed in ipsilateral testes of I/R group according to Johnsen’s scoring system (Figure 1). Melatonin treatment significantly reduced these changes both in ipsilateral and contralateral ischemic groups (7.30 ± 0.09; 8.1 ± 0.18, respectively) (Figures 2, 3). In I/R group, sperm intensity and motility rates did not change. However, abnormal sperm rate significantly increased. Melatonin administration changed abnormal sperm rate to normal (Table III).

Discussion

This study showed that, (1) MDA, TAS and TOS levels of the testis tissue did not change in all groups, (2) JS was decreased in I/R group, (3) melatonin treatment reversed histopathological changes.

Table II. Comparison of Johnsen’s scores among groups at 24th hour.

Figure 1. Extensive disorganization, sloughing and loss of maturation of germ cells in ipsilateral testes of I/R group (H&E × 200).
changes and increased JS both in ipsilateral and contralateral testis, (4) abnormal sperm rate significantly increased in I/R group and (5) melatonin administration changed abnormal sperm rate to normal.

Testicular torsion results in infertility and testicular damage. It has been shown that one hour of minimum time causes testicular damage after experimental testicular torsion in the rat. Although the basic pathological mechanism underlying testicular injury has not been completely understood, it has been shown that oxidative reperfusion injury is thought to be a central mechanism of the cellular damage affecting all organs and tissues after ischemia. The generation of oxygen-derived free radicals has been suggested to be significantly responsible for I/R injury in various organs including the heart, liver, stomach, kidney, brain, and testis. The lipid peroxidation, indicating the presence of enhanced ROS due to I/R injury has been shown to increase after testicular torsion and detorsion.

In recent years, several antioxidant agents have been used to prevent I/R-induced tissue damage in experimental testicular torsion such as superoxide dismutase, catalase, calcium channel blockers, oxypurinol, allopurinol and melatonin. Melatonin is well known as a protector against ROS and has been shown to have protective effects on I/R of various organs including testis. Thus, melatonin protects lipid, protein and DNA against oxidative damage. It also stimulates testis growth and increases the secretion of testosterone and affects angiogenesis positively together by ameliorating testicular injury. However, in the present study MDA, TAS and TOS levels were not significantly increased in ipsilateral and contralateral testes and melatonin did not change the levels of them.

Defective sperm function is the most prevalent cause of male infertility, and it is difficult to treat. The ROS production by a sperm is a normal physiological process and ROS is produced by a variety of semen components, including immotile or morphologically abnormal spermatozoa, leukocytes, and morphologically normal but functionally abnormal spermatozoa. In previous related human studies it has been shown that oxidative stress is associated with a reduction in sperm motility, viability and defects in sperm-oocyte fusion. Yurtcu et al. have used a number of antioxidant agents to prevent I/R-induced tissue damage in experimental testicular torsion such as superoxide dismutase, catalase, calcium channel blockers, oxypurinol, allopurinol and melatonin. Melatonin is well known as a protector against ROS and has been shown to have protective effects on I/R of various organs including testis. Thus, melatonin protects lipid, protein and DNA against oxidative damage. It also stimulates testis growth and increases the secretion of testosterone and affects angiogenesis positively together by ameliorating testicular injury. However, in the present study MDA, TAS and TOS levels were not significantly increased in ipsilateral and contralateral testes and melatonin did not change the levels of them.

Defective sperm function is the most prevalent cause of male infertility, and it is difficult to treat. The ROS production by a sperm is a normal physiological process and ROS is produced by a variety of semen components, including immotile or morphologically abnormal spermatozoa, leukocytes, and morphologically normal but functionally abnormal spermatozoa. In previous related human studies it has been shown that oxidative stress is associated with a reduction in sperm motility, viability and defects in sperm-oocyte fusion.

Table III. Morphologic comparisons of sperms between ILT, IMLT and the control group.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>ILT group</th>
<th>IMLT group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of abnormal head, %</td>
<td>5.30 ± 0.86a</td>
<td>8.10 ± 1.48a</td>
<td>9.10 ± 1.90a</td>
</tr>
<tr>
<td>Rate of abnormal tail, %</td>
<td>12.70 ± 2.06b</td>
<td>11.00 ± 1.49</td>
<td>7.40 ± 1.37ab</td>
</tr>
<tr>
<td>Rate of abnormal sperm, %</td>
<td>18.10 ± 2.13b</td>
<td>19.10 ± 1.71a</td>
<td>16.50 ± 2.76b</td>
</tr>
</tbody>
</table>

Variables were expressed as mean ± SD. ILT: Ipsilateral left testis; IMLT: Melatonin plus ipsilateral left testis. *Difference from the control (p < 0.05), aDifference from the IR group.
ported that melatonin prevents testicular I/R injury, as shown by increased JS. In the present work, JS was decreased in I/R group and melatonin treatment reversed histopathological changes and increased JS both in ipsilateral and contralateral testes. Also melatonin treatment changed abnormal sperm rate to normal. The decrease of histopathologic score in contralateral testis may be also an evidence for the damage in contralateral testis after I/R. This study may support the other investigations, which has been shown that ipsilateral testicular torsion causes a decrease not only in the ipsilateral testis but also in the contralateral nontorted testicular perfusion.

**Conclusion**

Although findings of the present study have demonstrated the testicular damage occurs following I/R without a change of MDA, TAS and TOS levels; melatonin may be a potent agent in preventing testicular I-R injury, as shown by increased JS and changed abnormal sperm rate. Repairing the harmful effects of oxidative stress on reproductive tissues with antioxidant agents might have great therapeutic potential in the future because of improvements in knowledge regarding the role of oxidative stress in infertility. Even though this is an animal model, melatonin may clinically be used as an antioxidant agent in human testicular torsion.

**Acknowledgements**

This study was supported by a grant from The Scientific and Technical Research Council of Turkey (Tübitak) (SBAG-1045237).

**References**


