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Antioxidant Effect of Melatonin in Systemic Circulation of Rats After Unilateral Testicular Torsion

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Aim: To investigate the antioxidant effects of melatonin in the systemic circulation of rats after unilateral testicular torsion.

Materials and Methods: After the approval of the study by the Local Ethical Committee, 30 male Wistar albino rats, 5.5–6 months old and weighing 250–300 g, were used in the study. The rats were randomly divided into three groups, each consisting of 10 rats. In group 1, the control group, the left testis was excised after sham operation. In group 2, the torsion group, torsion was created by rotating the left testis 720° in clockwise direction and maintained for 2 h. In group 3, the melatonin plus torsion/detorsion group, 50 mg/kg melatonin was given intraperitoneally 30 min before detorsion. After 2 h, the spermatic cord was detorsed to determine the blood levels of malondialdehyde (MDA), superoxide dismutase (SOD), protein carbonyl (PC), and nitric oxide (NO).

Results: Serum MDA, NO, SOD, and PC levels increased significantly in group 2 when compared with the sham operation group ($P < 0.001$). Administration of melatonin caused a significant decrease in lipid peroxidation and antioxidant enzyme activities when compared to the torsion group ($P < 0.05$).

Conclusions: Although this is an animal study, it can be suggested that melatonin may be used clinically as an antioxidant agent in the treatment of testicular torsion.

Key Words: Testis, torsion, melatonin, treatment

Ratlarda Tek Taraflı Testiküler Torsiyon Sonrası Melatoninin Sistemik Dolaşımdaki Antioksidan Etkisi

Amaç: Ratlarda, tek taraflı testiküler torsiyon sonrası, melatoninin sistemik dolaşımdaki antioksidan etkisini araştırmak.

Yöntem ve Gereç: Bu çalışmada, lokal etik kurul onayı alındıktan sonra ağırlıkları 250-300 gr arasında olan 5.5-6 aylık toplam 30 adet Wistar albino rat kullanıldı. Ratlar, her grupta 10 adet olacak şekilde 3 gruba randomize olarak ayrıldı. Grup 1 kontrol grubu idi ve bu grupta sol testis sham operasyonu sonrası alındı. İkinci grup torsiyon grubu olup, testis saat yönünde 720 derece döndürülerek bu şekilde 2 saat beklendi. Üçüncü grup, torsiyon/detorsiyon yapılmasına ilave olarak melatonin uygulanan gruptu. Bu gruba, intraperitoneal olarak detorsiyon öncesi 50 mg/kg melatonin verildi. İki saat sonra, detorsiyon yapılarak kan malondialdehit (MDA), superoksit dismutaz (SD), protein karbonil (PC) ve nitrik oksit (NO) seviyeleri ölçüldü.

Bulgular: Grup 2'de serum MDA, NO, SOD ve PC düzeyleri kontrol grubu ile karşılaştırıldığında anlamlı olarak daha yüksekti ($P < 0.001$). Torsiyon grubu ile kıyaslandığında melatonin verilmesinin anlamlı olarak antioksidan enzim ve lipid peroksidasyon ürünleri seviyesini kontrol grubu düzeylerine getirdiği görüldü ($P < 0.05$).

Sonuç: Bu sonuçlara göre, her ne kadar bu bir hayvan çalışması olsa da, melatoninin klinikte testiküler torsiyon tedavisinde kullanılabileceği söylenebilir.

Anahtar Sözcükler: Testis, torsion, melatonin, tedavi

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Introduction

Testicular torsion is the most common genital trauma of the adolescent boy and has been implicated in testicular injury, altered hormone production, subfertility and infertility (1). Although the basic pathological mechanism underlying testicular injury has not been completely understood, it has been shown that reactive oxygen species (ROS), formed during ischemia–reperfusion, play an important role in this process (1,2). Ischemia with consecutive reperfusion causes oxidative stress, which is characterized by an imbalance between ROS and the antioxidative defense system (1-3). In testicular torsion, surgical intervention is often necessary to reestablish blood flow; however, testicular atrophy may still ensue, depending on the degree of rotation and duration of the torsion (3). Previous studies using a rat model of testicular torsion have demonstrated that a 1-hour, 720° rotation of the testis followed by reperfusion results in the permanent loss of spermatogenesis (4). For these reasons, different therapeutic strategies have been investigated with the aim of reducing short- and long-term testis reperfusion damage. In agreement with the ischemia–reperfusion injury hypothesis and the role of free radicals in the disease process, numerous experimental animal studies have confirmed the efficacy of antioxidants in reducing the short-term damaging effect of torsion of the testis (1,2,5,6). Theoretically, antioxidants have a dual effect on testicular injury due to ischemia-reperfusion: they limit the development of damage by decreasing free radicals generated by lipid peroxidation and counteract ROS-mediated activation of inflammatory reaction (5,7,8).

Because of its antioxidant characteristics, melatonin has been used in many studies concerning ischemia–reperfusion injury. The aim of this study was to investigate the effect of melatonin on serum antioxidant enzyme levels in unilateral testicular reperfusion injury in rats.

Materials and Methods

The study was approved by the local ethical committee. A total of 30 male Wistar albino rats, 5.5–6 months old and weighing 250–300 g, were used in the study. The rats were randomly divided into three groups each consisting of 10 rats. The experimental animals were housed at 18-22 °C, under a 12 h light/12 h dark cycle and had free access to standard pellet diet for rats and to tap water *ad libitum* throughout the study. All surgical procedures were performed under xylazine/ketamine anesthesia in sterile conditions. All rats were sacrificed after the experimental procedures.

Group 1 (sham-operated control group) underwent a sham operation to determine basal values for biochemical evaluation. The testicle was brought out through the midline incision, a 4–0 silk suture was placed through the tunica albuginea, and the testicle was replaced into the scrotum with no additional intervention. Group 2 (ischemia and reperfusion) was designed to study the effects of testicular torsion on ipsilateral testicle. The tunica vaginalis was opened, and the right testis was delivered to the surgical field. The right testis was rotated 720° in a clockwise direction and maintained in this torsion position by fixing the testicle to the scrotum with a 4–0 silk suture. Group 3 (ischemia–melatonin treatment–reperfusion) was designed to determine the effect of melatonin on serum antioxidant levels after unilateral torsion. After 2 h, the spermatic cord was detorsed to determine the blood levels of malondialdehyde (MDA), superoxide dismutase (SOD), protein carbonyl (PC), and nitric oxide (NO). In this group, melatonin (50 mg/kg) was administered intraperitoneally (i.p.) in a single dose 30 min before detorsion was applied. Approximately 5 cc blood samples were obtained from the vena cava inferior of the rats.

Data were analyzed by a commercially available Statistical Package for Social Sciences (SPSS) program for Windows software. *P*-values <0.05 were regarded as statistically significant. Distribution of the groups was analyzed with the Kolmogorov–Smirnov one-sample test. One-way Analysis of Variance (ANOVA) test was performed and *post hoc* multiple comparisons were done with least-squares differences (LSD).

Biochemical Analyses

Blood samples were drawn into heparin-free tubes for biochemical analyses. After centrifugation (2000 x*g* for 15 min at +4 °C), serum samples were stored frozen at -70°C. Determinations of the following parameters were made in the serum samples using commercial chemicals supplied by Sigma (St. Louis, USA).

Serum antioxidant enzyme analysis

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to method of Sun et al. (9). The principle of the method is based on inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 ml of ethanol–chloroform mixture (5:3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the amount causing 50% inhibition in the NBT reduction rate. The SOD activity is expressed as U/mL. Glutathione peroxidase (GSH-Px) activity was measured by the method of Paglia and Valentine (10). The enzymatic reaction in the tube containing NADPH, reduced glutathione (GSH), 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. Activity was expressed as U/L.

Determination of thiobarbituric acid-reactive substance level

The tissue thiobarbituric acid-reactive substance (TBARS) level was determined by a method (Esterbaur and Cheeseman, 1990) based on reaction with thiobarbituric acid (TBA) at 90-100 ºC (11). In the TBA test reaction, MDA or MDA-like substances and TBA react to produce a pink pigment with an absorption maximum at 532 nm. The reaction was performed at PH 2-3 and 90 ºC for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein.

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. Results were expressed as µmol/L, according to the standard graphic prepared from measurements with a standard solution (1,1,3,3-tetramethoxypropane) (11).

Determination of tissue protein carbonyl (PC) content

The carbonyl contents were determined spectrophotometrically based on the reaction of carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone (12). 2,4-Dinitrophenylhydrazine was the reagent originally used for proteins subjected to metal–catalyzed oxidation. The results were given as nmol/ml.

NO determination

NO measurement is very difficult in biological specimens; therefore, tissue nitrite (NO2–) and nitrate (NO3–) were estimated as an index of NO production. Samples were initially deproteinized with Somogyi reagent (13). Total nitrite (nitrite + nitrate) was measured after conversion of nitrate to nitrite by copperized cadmium granules by a spectrophotometer at 545 nm. A standard curve was established with a set of serial dilutions (10^{-8}–10^{-3} mol/L) of sodium nitrite. Linear regression was carried out using the peak area from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as µmol/L.

Results

The levels of MDA, NO, and PC, which are lipid peroxidation products, were increased in the torsion group in comparison with the sham-operated group ($P < 0.05$). Melatonin treatment ameliorated antioxidant enzyme levels in serum after torsion/detorsion ($P < 0.05$). Similarly, antioxidant enzyme activities (SOD, GSHPx) were increased in the torsion group. Melatonin treatment caused decreased SOD and GSH-Px activities in comparison with the sham-operated control group ($P < 0.05$). The results of blood MDA, SOD, NO, PC, and GSH-Px values in both groups are shown in Table.

Discussion

Acute scrotum is a clinical syndrome generally caused by torsion of a testis, which was first described in 1810 (14). Spermatic cord torsion is characterized by tissue hypoxia and eventually by necrosis of germinal cells that gives rise to subfertility or infertility (1,3). A possible cause of the testicular injury due to torsion and detorsion is an ischemia–reperfusion injury attributed to oxygen free radicals (9). Excessive amounts of oxygen free radicals cause lipid peroxidation in the cellular and mitochondrial membranes. Peroxidation of the lipids in the membranes changes membrane permeability or disrupts membrane integrity and thus cell integrity (4,15). Mammalian testes are highly susceptible to

Table . Tissue GSH-Px and SOD activities and MDA, PC, and NO levels of both groups.

GROUPS (n=12)	SOD (U/ml)	GSH-Px (U/L)	MDA (µmol/L)	PC (nmol/ml)	NO (µmol/L)
1-SHAM	5.166 ± 0.553	723.289 ± 34.495	5.191 ± 0.518	256.808 ± 89.079	478.790 ± 77.454
2-I/R	7.280 ± 0.712	1132.233 ± 165.049	7.979 ± 0.968	485.471 ± 93.115	723.425 ± 126.861
3-I/R + MEL	5.606 ± 0.707	804.141 ± 109.650	4.891 ± 0.622	239.947 ± 77.292	492.111 ± 74.068
P-value					
1 vs 2	0.0001	0.0001	0.0001	0.0001	0.0001
1 vs 3	0.209 (ns)	0.215(ns)	0.442 (ns)	0.0699 (ns)	0.791 (ns)
2 vs 3	0.0001	0.0001	0.0001	0.0001	0.0001

GSH-Px: Glutathione peroxidase. SOD: Superoxide dismutase. MDA: Malondialdehyde. PC: Protein carbonyl. NO: Nitric oxide. MEL: Melatonin. ns: Non-significant.

oxidative stress. Like all cells living under aerobic conditions, spermatozoa produce ROS, mostly originating from normal metabolic activity. High concentrations of ROS play an important role in the pathophysiology of damage to human spermatozoa (16). Hence, oxidative stress has been shown to be a major cause of male infertility, and in a large proportion of infertile men, an elevation in the levels of seminal ROS activity was shown. However, spermatozoa and seminal plasma contain a battery of ROS scavengers, including enzymes such as SOD and catalase, and also a variety of substances with antioxidant activities (17,18).

During ischemia, ATP production decreases because of the limited oxygen availability. Intracellular Ca^{+2} concentration rises depending on the influx of Ca^{+2}, leading to proteolytic conversion of xanthine dehydrogenase to XO, which is a superoxide generator enzyme (20). During reperfusion, oxygen becomes abundant, and superoxide anions are generated by XO and the mitochondrial electron transport chain (20). In addition, ischemia activates the complement system and forms chemotactic factors, leading to a migration of polymorphonuclear leukocytes, which generate superoxide radicals, to the ischemic region after reperfusion (20). This situation promotes lipid peroxidation and testicular tissue damage. Under these conditions, antioxidant enzymes, such as SOD and GSH-Px, protect tissues from oxidative stress and oxidative damage (3,7,11). Excessive amount of free radicals will damage testicular tissue through peroxidation of lipids in the cell membranes (18,19). Parallel to this finding, in this study, blood levels of these enzymes in group 2 were increased compared with group 1, which shows ischemia/reperfusion injury. This observation is in agreement with previous studies (1,3,21,22). Surgical detorsion is currently the only available treatment and should be done as early as possible. In addition, various drugs, chemical substances, and physical methods have been used to protect testes against ischemia–reperfusion injury in experimental animals, and some of these have been found to be effective in preventing testicular damage, such as NG nitro-L-arginine methyl ester (a precursor of NO), polyethylene-glycol-superoxide dismutase, calcium channel blockers, *N*-acetyl cysteine, oxypurinol, erdosteine, caffeic acid phenethyl ester, inhibitors of poly (ADP-ribose) polymerase (PARP), and hypothermia (1-4,8,11,18,23).

Recent investigations on oxidative stress and ROS generation after testicular torsion suggest that pre-treatment with antioxidants can protect the testis against ROS insult (1,6). Melatonin is known to be a free radical scavenger and inhibits the peroxidation of membrane lipids (24,25). Its lipophilicity ensures that melatonin

rapidly enters cells and may accumulate in the nucleus. Presumably by binding to cell membrane receptors, melatonin decreases calcium and cAMP concentrations and increases antioxidant enzymes in cells (1,24). The protective effect of melatonin on lipid peroxidation has previously been shown in many investigations related to ischemia-reperfusion injury in other tissues and this protective effect was reported to arise from its action as a direct free radical scavenger (1,26-28). Because of its well-known antioxidant characteristics, we investigated its effects on the present experimental model. To our knowledge, melatonin has been used in experimental testis ischemia–reperfusion injury in only three studies (27,28). However, the blood level of the antioxidants was studied in only one investigation (28).

In our study, we observed that the level of blood MDA and NO were significantly increased in group 2. However, the enzyme levels return to nearly group 1 levels with melatonin administration. These results demonstrated that melatonin prevented the negative effect of antioxidants and lipid peroxidation products in serum after testicular torsion/detorsion, as evidenced by biochemical parameters. The decreased enzyme levels after melatonin administration were in agreement with previous studies (25-28).

From the findings of the present study, it can be suggested that even though this is an animal model, melatonin may be used clinically as an antioxidant agent in testicular torsion.

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