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An animal model of ischemic priapism and the effects of melatonin on antioxidant enzymes and oxidative injury parameters in rat penis

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Abstract This experimental study was designed to produce ischemia–reperfusion (I/R) injury in rat corpus cavernosum by inducing 1 h of priapism and investigating the effects of melatonin on the levels of oxidative injury parameters. Twenty-one adult male rats were randomly divided into three groups as follows; sham operated, control group (Group C): only penectomy was performed and blood (3 ml) drawn from vena cava inferior (VCI), ischemia and reperfusion group (Group I/R); priapism (1 h) + ½ h reperfusion + penectomy + blood from VCI, melatonin treatment group (Group I/R + M); priapism (1 h) + melatonin (½ h before reperfusion, 50 mg/kg, ip) + ½ h reperfusion + penectomy + blood from VCI. Priapism was induced with a vacuum erection device (cut tip of 2-cc syringe) and a rubber band was placed at the base of the erect penis. In two groups, excluding Group C, penectomies were performed after 1 h of ischemic priapism and ½ h reperfusion for biochemical analysis of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and protein carbonyl (PC) in the tissues. In all groups, about 3 ml blood was drawn from VCI to study the same parameters in systemic circulation. The results were compared statistically using one-way analysis of variance (ANOVA). As a result, in biochemical examination of penile tissues, there were significant increase in SOD, CAT activities and MDA levels in I/R group in comparison with group C ($P < 0.05$). With melatonin treatment, these levels were decreased closer to control levels ($P < 0.05$). The changes in PC levels were insignificant in penile tissues of all groups ($P > 0.05$). Analysis of serum in all rats revealed that the activity of SOD and the levels of MDA, NO and PC were increased in I/R group when compared with control group but with multiple comparisons only the increases in SOD activity and NO level were significant ($P < 0.05$). Decrease in the activity of SOD and the levels of NO and PC were significant after melatonin administration in serum of all groups ($P < 0.05$). The results of this study showed that experimentally induced priapism caused oxidative injury in cavernosal tissues of rats, and treatment with melatonin alleviated these effects. From the result of this experimental study, it can be extrapolated that melatonin may be used as an antioxidant agent in the treatment of ischemic priapism in the future urology practice.

Keywords Ischemic priapism · Antioxidants · Reperfusion injury · Melatonin

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Introduction

Priapism is a term that is modified from the Greek God "Priapus" and describes an emergent condition with prolonged erection lasting more than 4–6 h without sexual stimulation [1]. Priapism can be divided into high-flow and low-flow states. Ischemic priapism (IP) represents the low-flow state, which is the more common form of priapism and is usually idiopathic. Its association with venous outflow obstruction and venous stasis results in significant tissue hypoxia, acidosis and ultimately ischemia and fibrosis occurs in cavernosal smooth muscle function, which leads to severe permanent erectile dysfunction [2].

Ischemia–reperfusion (I/R) injury is a complex phenomenon that causes destruction in both local and remote tissues [3]. Although reperfusion of ischemic tissue is necessary for reparative mechanisms in the body, it has been shown that reperfusion worsens the injury that is caused by ischemia via release of reactive oxygen species (ROS) to the systemic circulation. Oxidative injury develops when there is excessive production of ROS and/or free radicals, which exceeds the natural antioxidant defence mechanisms in the body [4, 5]. The oxidative stress can produce important destructive effects in tissues by causing alterations in the cell membranes, which lead to irreversible cellular damage. Eventually, oxidative damage causes increase in tissue levels of malondialdehyde (MDA) and protein carbonyl (PC), which are the end products of lipid peroxidation and protein oxidation, respectively [6].

Various antioxidant agents have been used previously to show their antioxidative effects on IR injury in many organ systems. Melatonin (*N*-acetyl-5-methoxytryptamine) is one of the most powerful endogenous antioxidant agents [7]. It is the main secretory product of the pineal gland and secreted in a circadian rhythm [8, 9]. Melatonin exerts antioxidative effects both directly via scavenging ROS and indirectly by stimulating several antioxidative enzymes, such as superoxide dismutase (SOD) and catalase (CAT). Another effect of melatonin is to inhibit the synthesis of nitric oxide synthase (NOS), which is a pro-oxidative enzyme [10, 11].

Induction of ischemic priapism and reversal of priapism in animals may resemble the type of I/R injury as in other organ systems. Thus, this experimental study was conducted to determine the oxidative effects of priapism on cavernosal tissues of rats and to evaluate the antioxidant effects of melatonin by using biochemical parameters of oxidative injury. To our knowledge, this is the first experimental study in the literature that investigated the effects of melatonin in a rat priapism model.

Materials and methods

Animals

The protocol of the experimental study was approved by the local ethical committee. Twenty-one adult male Wistar-Albino rats weighing between 250 and 300 g were used in the study. They were housed in solid plastic cages as three rats for each cage at a constant temperature of 21°C. The rats were allowed to eat standard rodent chow and water ad libitum.

Experimental design

All operations were performed under sterile conditions. The animals were anesthetized with 1-shot ketamine injection (50 mg/kg, ip). Priapism was accomplished with the method described by Sanli et al. [12]. The tip of a 5-cc syringe was cut to accommodate the device to the base of the flaccid penis, so a vacuum erection device was created. Before application of vacuum to the penis, a constriction band, which was cut from 16 Fr foley catheter in 2-mm slices, was loaded around the tip of the vacuum erection device. Then the tip of the syringe was placed to the base of the penis and withdrawn gently to induce erection in the rat penis. After induction of erection in sufficient grade, the constriction band was placed to the base of the penis by slipping off the syringe (Fig. 1).

The animals were divided into three groups with each containing seven rats. In the control group (Group C), only penectomy was performed, and 3 ml blood was withdrawn from inferior vena cava to determine the basal levels of SOD, CAT, MDA, PC and nitric oxide (NO) in cavernosal tissues and systemic circulation of rats. In the second group (Group I/R), after 1 h of ischemic priapism, the band was removed from the base of the penis and the penile tissue was allowed to reperfusion for an half (½) h. Then penectomy was performed, and blood

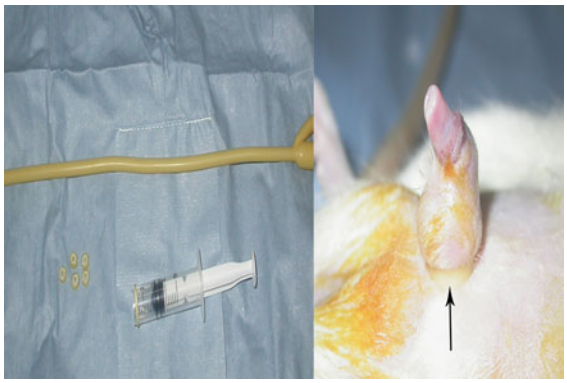


Fig. 1 The special devices produced for this experiment and the constriction band applied to the base of the penis (*arrow*)

samples were taken to study the same parameters. In the third group (Group I/R + M), during IP period and ½ h before reperfusion of penis, 50 mg/kg melatonin (Sigma, St. Louis, MO, USA) was injected intraperitoneally. After the same period of reperfusion (½ h), penectomy was performed and blood was withdrawn to evaluate the effects of melatonin.

Biochemical analyses

Blood samples of all rats were withdrawn from VCI with syringes at the end of the reperfusion period while the animals were still anesthetized. Blood samples were collected in the polyethylene tubes and centrifuged (3,500×*g* for 30 min at 4°C) to separate the serum. Penectomy materials were stored at −70°C until the analysis. Penile tissues were homogenized in five volumes of ice-cold tris–HCl buffer (50 mM, pH 7.4) containing 0.50 ml/l Triton x-100. Then homogenization procedure (homogenizer: IKA Ultra-Turrax t 25 Basic, Germany) was carried out for 2 min at 13,000 rpm. All procedures were performed at 4°C. Homogenate, supernatant and extracted samples were prepared and the following determinations were made on the samples using commercial chemicals supplied by Sigma (St. Louis, USA). Protein measurements were made in the samples according to the method explained elsewhere [13].

Serum and penile tissue antioxidant enzyme analysis

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. [14]. 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 was expressed as U/ml for serum and as U/mg protein for penile tissue. Catalase activity was determined according to Aebi's method [15]. The principle of the method was based on determination of the rate constant k (s^{-1}) of the H_2O_2 decomposition at 240 nm. Tissue enzyme activity was expressed as k/g protein.

Determination of tissue PC

The carbonyl contents were determined spectrophotometrically (Cintra 10 E, Austria) based on reaction of carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone [16]. The results were given as nmol/mg protein for serum and as nmol/ml for penile tissue.

Determination of MDA level

The tissue thiobarbituric acid-reactive substance level was determined by a method based on reaction with thiobarbituric acid (TBA) at 90–100°C [17]. 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 2–3 pH and 90°C 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 for serum and as nmol/g wet tissue for penile tissue, according to the standard graph prepared from measurements with a standard solution (1, 1, 3, 3-tetramethoxypropane).

NO determination

Nitric oxide measurement is very difficult in biologic specimens; therefore, tissue nitrite (NO^{2-}) and nitrate

(NO^{3-}) were estimated as an index of NO production [18]. Samples were initially deproteinized with Somogyi reagent. 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. Serum NO results were expressed as μmol/l.

Statistical analysis

Because the distribution of continuous variables were normal, according to Kolmogorov–Smirnov normality test, one-way analysis of variance (ANOVA) was used to compare the biochemical and pathological data among three groups (for multiple comparison Sheffe and Tamhane T2 test was used). The continuous variables were presented as the mean and standard deviation. A P value <0.05 was considered significant. Analyses were performed using SPSS statistical software (PASW v18 SPSS Inc., Chicago, IL).

Results

The SOD and CAT activities increased significantly after I/R period. But the SOD and CAT activities in penile tissue was brought closer to the levels in the Group C with administration of melatonin. The MDA levels in penile tissues increased significantly in Group I/R ($P < 0.05$). Melatonin administration caused a significant decline in the MDA levels in Group I/R + M compared with the Group I/R ($P < 0.05$). Melatonin treatment increased PC in penile tissues when compared to Group I/R and Group C. Multiple comparisons between groups were not significant when PC level was concerned ($P > 0.05$). (Table 1).

The results of biochemical assays of serum are presented in Table 2. The SOD activity in serum was increased in Group I/R ($P < 0.05$), but melatonin administration decreased SOD activity significantly in Group I/R + M ($P < 0.05$). The significant increase in serum MDA levels were also reversed closer to control levels by melatonin administration ($P < 0.05$). Also, the significant increase in NO levels was decreased by melatonin treatment ($P < 0.05$). Multiple comparisons of the differences between all groups were statistically significant when these three parameters were concerned, as well. Serum PC level

Table 1 Biochemical analysis results and comparisons in penile tissues of all groups

	Groups (n:7, for each group)	Mean ± SD	Min–Max	*P*
SOD (U/mg protein)	Group C	0.16 ± 0.03	0.11–0.19	<0.001*
	Group I/R	0.25 ± 0.07	0.11–0.33	
	Group I/R + M	0.12 ± 0.03	0.05–1.15	
CAT (k/g protein)	Group C	0.15 ± 0.05	0.07–0.18	<0.001*
	Group I/R	0.35 ± 0.11	0.26–0.51	
	Group I/R + M	0.17 ± 0.05	0.11–0.23	
MDA (nmol/g wet tissue)	Group C	20.92 ± 11.70	0.82–37.61	<0.001*
	Group I/R	77.27 ± 8.81	63.23–90.88	
	Group I/R + M	29.91 ± 14.61	22.44–62.66	
PC (nmol/mgprot)	Group C	3.05 ± 0.86	1.33–3.98	0.537
	Group I/R	2.77 ± 1.15	0.71–4.25	
	Group I/R + M	3.35 ± 0.83	2.42–4.22	

SD Standard deviation, *SOD* superoxide dismutase, *CAT* catalase, *MDA* malondialdehyde, *PC* protein carbonyl

* According to multiple comparison test results, there were statistical significances between Group I/R and Group C and between Group I/R and Group I/R + M

Table 2 The biochemical analysis results of all groups in serum and comparisons between the groups

	Groups (n:7, for each group)	Mean ± SD	Min–Max	*P*
SOD (U/ml)	Group C	9.10 ± 1.48	6.99–10.80	0.006*
	Group I/R	11.77 ± 1.01	10.11–12.94	
	Group I/R + M	9.68 ± 1.69	7.24–12.22	
MDA (μmol/l)	Group C	1.17 ± 1.51	0.95–1.37	0.155
	Group I/R	1.49 ± 0.42	1.05–2.05	
	Group I/R + M	1.40 ± 0.27	1.09–1.9	
NO (μmol/l)	Group C	333.61 ± 111.03	209.73–556.86	<0.001*
	Group I/R	1,375.66 ± 339.52	926.67–1,800.94	
	Group I/R + M	452.26 ± 158.02	277.53–706.93	
PC (nmol/ml)	Group C	823.01 ± 311.15	478.91–1,422.55	0.009**
	Group I/R	1,278.77 ± 436.82	669.82–1,882.91	
	Group I/R + M	713.77 ± 138.79	608.73–1,012.36	

SD Standard deviation, *SOD* superoxide dismutase, *MDA* malondialdehyde, *NO* nitric oxide, *PC* protein carbonyl

* According to multiple comparison test results, there were statistical significances between Group I/R and Group C and between Group I/R and Group I/R + M

** There was statistical significance between Group I/R and Group I/R + M

increased in Group I/R, but melatonin treatment decreased the level of PC in serum ($P < 0.05$).

Discussion

Priapism is defined as prolonged penile erection that is maintained in the absence of sexual stimulation and persists despite orgasm. Ischemic priapism is a true urologic emergency that results from an imbalance of the vasoconstrictive and vasorelaxatory mechanisms leading to the formation of a closed compartment syndrome in the penis, and this syndrome is biochemically characterized by hypoxia, hypercapnia, and acidosis [19, 20]. Prolonged corporal smooth muscle exposure to these conditions results in irreversible damage to erectile tissue with subsequent corporal fibrosis. Although eventual successful detumescence can be achieved after medical intervention or shunt surgery, still there is a high incidence of erectile dysfunction (ED) in over half of these men and in 90% if the priapism episode lasts more than 24 h [21, 22]. Hypoxia that is seen during priapism causes inhibition of oxidative phosphorylation with loss of high-energy phosphates, necessary for the homeostasis of calcium and the contractile mechanism of the smooth muscle.

The ROS, also called as oxygen-free radicals, have been implicated in numerous disease states, such as cancer, arthritis, cardiovascular diseases, and connective tissue disorders [3]. They are highly reactive molecules produced in the cell either as part, or as end-products of biochemical reactions. The ROS production increases in ischemic tissue in IR injury. Under normal conditions, most of ROS scavenging potential in the various organs is maintained by adequate levels of antioxidant enzymes [23]. In this study, the production of ROS in excess amounts caused increase in activities of SOD and CAT enzymes to scavenge the deleterious effects of ROS. An increase in the activity of SOD in serum was also noted. During the process of oxidative stress, an oxidative damage to mitochondrial DNA occurs, which is a well-known phenomenon that occurs in all aerobic cells rich in mitochondria as are the penile tissues. Major target of highly reactive ROS are membrane lipids, initiating the self-perpetuating process of lipid peroxidation, which disrupts the functional state and integrity of the membrane [24, 25]. Re-establishing corporal blood flow during the management of IP is associated with reperfusion injury in the ischemic tissues. This drastic increase in corporal oxygen tension generates ROS, which may cause tissue damage. The continuous ROS production

increases the destructive effects, and end products of tissue destruction are released into the circulation. NO is an important messenger molecule involved in many beneficial and detrimental physiologic and pathologic processes within the body [26]. Depending on the vessel density of the penis, NO levels in serum increases in IR conditions, as it was seen in this study. The counteraction between NO and ROS causes production of peroxynitrites, which lead to the increase in harmful effects of free radicals. Melatonin therapy also brought the levels of NO closer to control levels in this study.

During these IR processes, destructive events occur in cavernosal tissue to some extent. These events had been studied in scarce number of studies. One of these is the study of Muneer et al. [20] in which study they demonstrated that prolonged low-flow priapism led to smooth muscle dysfunction and fibrosis within the corpus cavernosum. In an another experimental study, Sener et al. [27] showed that reoxygenation of ischemic corporal tissue increased lipid peroxidation. In present study, an ischemic condition was produced in the rat corpus cavernosum. It was observed that after an hour of IP and a half-hour reperfusion period, the serum levels of SOD and NO increased significantly in priapism group when compared to the levels in sham group. The end products of tissue destruction process such as MDA and PC were also increased in IP group. Similar results have been reported in many studies in IR models [27, 28].

The main strategy in prevention of oxidative stress is the usage of antioxidant agents. In literature, it has been shown that various antioxidant agents have been used in different conditions to prevent the effects of oxidative stress. As an antioxidant agent, the effect of melatonin on oxidative stress has been shown in numerous in vivo and in vitro studies previously [27–30]. The free radical scavenger functions of melatonin in prevention of I/R injury and attenuation of the effects of reperfusion injury has been investigated in many organs such as liver, heart, kidney, testis, intestines. But the antioxidative effects of melatonin in corpus cavernosum after such an I/R method,
i.e., constriction of the base of the penis, has not been studied yet. In a similar study of Sener et al., the effect of melatonin on I/R injury of corpus cavernosum was investigated with a different model of I/R by occlusion of abdominal aorta with a midline laparotomy incision. Due to its antioxidant and anti-inflammatory properties, melatonin has been shown to have beneficial results and a significant protective effect in various experimental models of I/R injury [27]. Because of its high liposolubility property, melatonin penetrates all cellular membranes easily and shows its all protective effects [28] Different dose regimens and administration schemes of melatonin have been used in experimental studies. Dose regimen varied between 2.5 and 100 mg/kg and applications from one shot to daily or weekly multiple shots [29]. In most of the animal studies, melatonin has been used in a dose of multiple 10 mg/kg daily or weekly injections. But due to the nature of ischemic priapism as being acute condition, in this current study we used one shot 50 mg/kg, ip. dose of melatonin, as was the dose in some other studies [7, 30].

Administration of an antioxidant agent to prevent acute and chronic effects of I/R injury in cavernosal tissue is a new idea that led us to execute such an experimental study. The results of this study showed that experimentally induced priapism caused oxidative stress in cavernosal tissues of rats. All of the oxidative parameters were restored to nearby sham-group levels both in tissue and blood specimens by administration of melatonin. The changes in all of these parameters after melatonin administration were in agreement with previous studies [31–33]. These results demonstrated the beneficial effects of melatonin in I/R injury and lipid peroxidation, as evidenced by changes in biochemical parameters. We hope that this preliminary study will enlighten the researchers with ED to construct more comprehensive studies on the effects of ischemia and reperfusion in cavernosal tissue. Thus, the usage of antioxidant agents in such conditions and clinical implications of antioxidant administration can be improved in the future.

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