Remote organ injury induced by myocardial ischemia and reperfusion on reproductive organs, and protective effect of melatonin in male rats

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Objective: Myocardial ischemia and reperfusion (MI-R) leads to remote organ injury associated with oxidative stress. Melatonin is a well-known antioxidant and free-radical scavenger. This study was conducted to examine whether MI-R causes damage in the testes and sperm quality, and to investigate the possible protective effect of exogenous melatonin on these parameters in an in vivo rat model.
Design: Experimental study.
Setting: Experimental Research Center, Fırat University Medical School, Elazığ, Turkey.
Patient(s): Eight-week-old male Wistar rats (n = 18).
Intervention(s): To produce MI-R, a branch of the descending left coronary artery was occluded for 30 minutes, followed by 120-minute reperfusion. Melatonin (10 mg/kg) or vehicle was given 10 minutes before ischemia via the jugular vein.
Main Outcome Measure(s): Reproductive organ weights and epididymal sperm concentration, sperm motility, abnormal sperm rate, and testicular-tissue malondialdehyde (MDA) and glutathione (GSH) levels were examined after reperfusion.
Result(s): MI-R significantly decreased epididymal sperm motility, and increased the testes-tissue level of MDA, compared to the control group. Administration of melatonin reversed the harmful effects of MI-R significantly. However, MI-R did not change sperm concentration, GSH levels, and reproductive organ weights.
Conclusion(s): These findings indicate that MI-R leads to damage of testis tissue and sperm motility, and melatonin protects against MI-R-induced reproductive-organ injury. These results may also encourage the use of antioxidants to reduce remote organ injury in the testis after MI-R. (Fertil Steril® 2007;88:188–92. ©2007 by American Society for Reproductive Medicine.)

Key Words: Melatonin, myocardial ischemia and reperfusion, oxidative stress, sperm characteristics, remote organ

Cardiovascular disease is a leading cause of death worldwide, and remains one of the major killers in modern society. It can be initiated by multiple factors, including ischemia-and-reperfusion (I-R) injury (1). Myocardial ischemia and reperfusion (MI-R) represents a clinically relevant problem associated with thrombolysis, angioplasty, and coronary by-pass surgery (2). Several studies showed that MI-R leads to remote organ injury (3, 4), and that oxygen-based reactants may play a central role in this injury (5). Myocardial ischemia and reperfusion cause severe damage, as indicated by free radicals and loss of membrane phospholipids (6, 7).

Reactive oxygen species (ROS) are free radicals such as the hydroxyl radical (OH⁻) and the superoxide anion ($O_2 \cdot {}^-$),
or molecules such as hydrogen peroxide (H_2O_2). The production of ROS is a normal physiological event in various organs, including the testis. However, the overproduction of ROS stimulates DNA fragmentation and can be detrimental to sperm function, associated with peroxidative damage to the mitochondria and plasma membrane. In addition, spermatozoa are especially susceptible to peroxidative damage because of a high concentration of polyunsaturated fatty acids and low antioxidant capacity (8, 9).

Melatonin has been gaining acceptance as potent radical scavenger and antioxidant (10, 11). It was shown to inhibit MI-R-induced oxidative changes and reduce I-R-induced arrhythmias, mortality, and infarct size (11–13). On the other hand, the efficacy of melatonin on the oxidative changes in testis tissue and sperm characteristic due to MI-R has not been well-documented.

In this study, we aimed to investigate the effect of MI-R on remote organ injury in testis tissue, and the possible protective effects of the exogenous administration of melatonin after MI-R in an in vivo rat model. We evaluated

morphological changes in reproductive organs and sperm characteristics such as epididymal sperm concentration, motility, and abnormal sperm rate, in addition to levels of malondialdehyde (MDA), a stable metabolite of the free radical-mediated lipid peroxidation cascade which is widely used as a marker of oxidative stress, and glutathione (GSH), an important endogenous antioxidant whose levels are influenced by oxidative stress.

MATERIALS AND METHODS
Experimental Groups
Eighteen healthy, adult, male Wistar rats (weighing 250–300 g, 8 weeks old) were kept under standard laboratory conditions (12 hours light:12 hours dark, and $21°C \pm 2°C$). Experiments were performed between 9 AM–5 PM. This study was performed in accordance with the guidelines for animal research from the National Institutes of Health (Bethesda, Maryland), and was approved by our Local Committee on Animal Research.

The rats were divided into three groups of six rats each. The control group received isotonic saline; the MI-R group received melatonin vehicle; and the MI-R-plus-melatonin group received a single dose of melatonin. Melatonin (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and further diluted in saline to give a final concentration of 1%, and at a dose of 10 mg/kg was administered by IV injection 10 minutes before ischemia.

Ischemia and Reperfusion Procedure
Rats were anesthetized with intraperitoneal urethane (1.2–1.4 g/kg, Fluka Chemical, Buchs, Switzerland). The jugular vein and trachea were cannulated for drug administration and artificial respiration, respectively. Blood pressure and electrocardiography were monitored during I-R; the results are not described here because these parameters were used only to verify MI-R. The chest was opened by a left thoracotomy, followed by sectioning the fourth and fifth ribs, about 2 mm to the left of the sternum.

Positive-pressure artificial respiration was started immediately with room air, using a volume of 1.5 mL/100 g body weight at a heart rate of 60 beats/minute to maintain normal partial pressures of CO_2 (pCO_2) and O_2 (pO_2), and pH parameters. After the pericardium was incised, the heart was exteriorized by gentle pressure on the right side of the rib cage. A 6/0 silk suture attached to a 10-mm micropoint reverse-cutting needle was quickly placed under the left main coronary artery. The heart was then carefully replaced in the chest, and the animal was allowed to recover for 20 minutes. A small plastic snare was threaded through the ligature and placed in contact with the heart. The artery was then occluded by applying tension to the ligature, and reperfusion was achieved by releasing the tension. The artery was occluded for 30 minutes, and then reperfused for 120 minutes (13). The jugular vein was cannulated for drug administration.

As soon as reperfusion was completed, the animal was euthanized by cervical dislocation. The testes, epididymides, seminal vesicles, and prostate were removed and cleared of adhering connective tissue. Measurement of testis weight, length, and thickness was performed, and epididymal, seminal-vesicle, and prostatic weight were evaluated along with epididymal sperm concentration, sperm motility, and sperm morphology. Testis samples were stored at $-20°C$ until biochemical assays.

Epididymal Sperm Concentration, Motility, and Abnormal Sperm Rate
Spermatozoa in the right epididymis were counted by a modified method of Yokoi et al. (14). Briefly, the epididymis was minced with anatomical scissors in 5 mL of physiological saline, placed in a rocker for 10 minutes, and allowed to incubate at room temperature for 2 minutes. After incubation, 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 by use of a hemocytometer. Approximately 10 μL of the diluted sperm suspension were transferred to each counting chamber of the hemocytometer, and allowed to stand for 5 minutes. The cells that settled during this time were counted with the use of a light microscope at ×200 magnification.

Progressive motility was evaluated by an method described by Sönmez et al. (15). The fluid obtained from the left cauda epididymis with a pipette was diluted to 0.5 mL with Tris buffer solution. A slide was placed in a light microscope with a heated stage, an aliquot of this solution was placed on the slide, and the percentage of motility was evaluated visually at a magnification of ×400. Motility estimates were performed in three different fields from each sample. The mean of the three estimates was used as the final motility score. Samples for evaluation of motility were kept at 35°C. Slides were prepared with Indian ink for determination of the percentage of morphologically abnormal spermatozoa. In total, 300 sperm cells were counted on each slide under a light microscope at ×400 magnification (16).

Biochemical Assays
Testes tissue was homogenized in a Teflon-glass homogenizer with a buffer containing 1.5% potassium chloride, to obtain 1:10 (w/v) whole homogenate. Concentrations of MDA, as an index of lipid peroxidation (LPO), were measured in the homogenate. Then homogenates were centrifuged at 18,000 $\times$ g (4°C) for 30 minutes to determine reduced GSH levels. Concentrations of MDA were assayed according to a modified method of Ohkawa et al. (17), based on their reaction to thiobarbituric acid, and were expressed as nmol/mL. Tissue GSH concentrations were measured by a kinetic assay by means of a dithionitrobenzoic acid-recycling method described by Ellman (18), and were expressed as μmol/mL.

Sperm motility rates in control, MI-R plus vehicle, and MI-R plus melatonin groups. (**A** and **B**): The difference between the columns bearing different lower-case letters is statistically significant ($P<.01$).
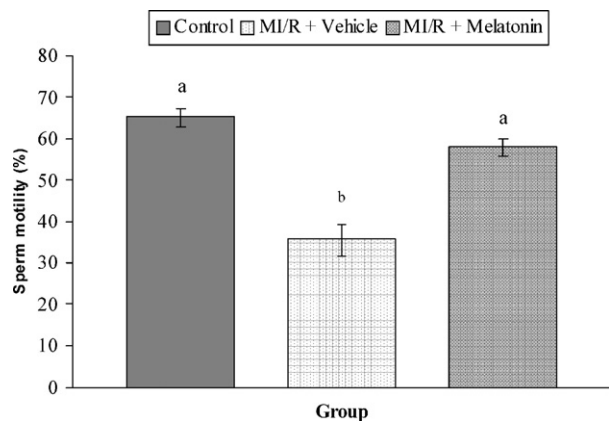
Sahna. Melatonin ischemia-and-reperfusion sperm interaction. Fertil Steril 2007.

Statistical Analyses

Data are presented as mean $\pm$ standard error of the mean (SEM). $P<.05$ was considered statistically significant. Data were analyzed by one-way analysis of variance and post hoc Duncan's test. We used the SPSS-PC program (version 10; SPSS, Inc., Chicago, IL) to determine differences among all groups.

RESULTS
Organ Weights and Dimensions

The values of testis weights and dimensions, and epididymis and accessory-gland weights in the control, MI-R, and MI-R plus melatonin groups were examined. However, no statistically significant differences were found among any groups relative to these parameters (data not shown).

Sperm Characteristics

The sperm-motility percentages of all groups of rats are shown in Figure 1. Sperm motility in the MI-R plus vehicle group was significantly ($P<.01$) lower than in the control group (35.5% $\pm$ 3.8% versus 65.0% $\pm$ 2.3%, respectively). However, reduced sperm motility obtained in MI-R plus vehicle group returned to its control group value in MI-R plus melatonin application group. Epididymal sperm concentration and abnormal sperm rates are shown in Figures 2 and 3, respectively. The observations of decrease in sperm concentration and increase in all abnormal sperm rates (i.e., head, tail, and total) in MI-R plus vehicle group were not statistically significant.

Epididymal sperm concentrations in control, MI-R plus vehicle, and MI-R plus melatonin groups. No difference was found among the groups.
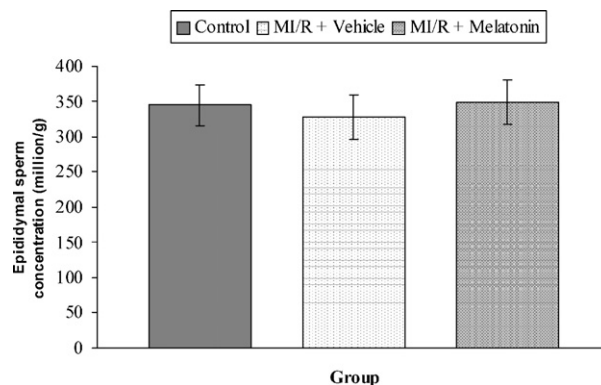
Sahna. Melatonin ischemia-and-reperfusion sperm interaction. Fertil Steril 2007.
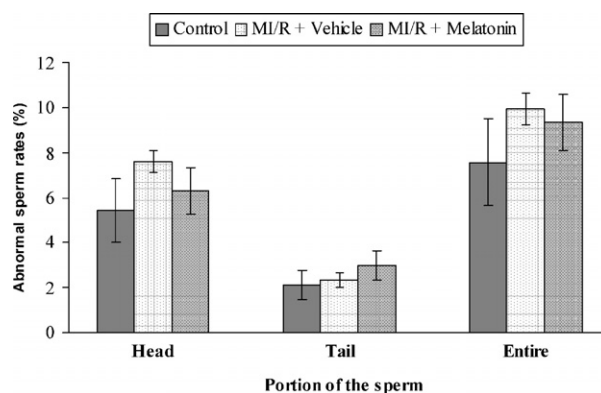
Testicular-Tissue Levels of MDA and GSH

The levels of MDA and GSH in testis tissue are given in Table 1. The MI-R was accompanied by a statistically significant ($P<.05$) increase in MDA level, whereas administration of melatonin significantly ($P<.05$) reduced this value. The level of GSH was not significantly affected by applications of MI-R plus vehicle and MI-R plus melatonin.

DISCUSSION

The present study shows that MI-R leads to lipid peroxidation in testicular tissue and a decrease in sperm motility.

Abnormal sperm rates in control, MI-R plus vehicle, and MI-R plus melatonin groups. No difference was observed among all groups in sperm head, sperm tail, and entire sperm abnormalities.

Sahna. Melatonin ischemia-and-reperfusion sperm interaction. Fertil Steril 2007.

TABLE 1

Testis-tissue levels of MDA and GSH in control, MI/R plus vehicle, and MI/R plus melatonin groups.

Group	MDA (nmol/mL)	GSH (μmol/mL)
Control	0.72 ± 0.23	2.34 ± 0.16
MI-R plus vehicle	1.34 ± 0.18[a]	3.66 ± 0.28
MI-R plus melatonin	0.88 ± 0.15[b]	3.06 ± 0.30

Note: Data are expressed as mean ± SEM.
[a] Significantly different from control group ($P<.05$).
[b] Significantly different from MI-R plus vehicle group ($P<.05$).

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Administration of melatonin to rats with MI-R attenuated lipid peroxidation, and repaired sperm motility.

The damage of sperm is the result of an improper balance between generation of ROS and scavenging activities. The scavenging potential in epididymal fluid is normally maintained by adequate levels of enzymatic and nonenzymatic (GSH) antioxidant mechanisms. The balance of this system is essential to eliminate ROS in the epididymis. The reduction in activities of antioxidant enzymes and the increase in ROS lead to damage in the membrane of spermatozoa (19).

Peroxidative damage induced by ROS is one of the major causes of defective sperm function. The sperm plasma membrane contains a high amount of polyunsaturated fatty acids, and so it is particularly susceptible to peroxidative damage. Lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa, and it is associated with a loss of motility and defective sperm (20, 21). In the present study, we found that MI-R negatively affected sperm motility, but not sperm concentration and abnormal sperm rate. This decrease in sperm motility may be explained by the lipid peroxidation mechanism that we have described.

Spermatogenesis occurs in an orderly fashion, with the spermatocytes (primary and secondary) being derived from the spermatogonia via mitotic division. Through meiotic division, the spermatids are formed from secondary spermatocytes; they contain a haploid number of chromosomes. During spermiogenesis, the mature spermatozoa are formed, and releases to the tubular lumen (22). As the period of application of MI-R in the present trial (3 hours) was limited, and the duration of spermatogenesis in the rat is approximately 48–52 days (23), any significant effect of I-R on spermatogenesis, sperm concentration, and abnormal sperm rates would not be observed.

It was recently reported that reperfusion of ischemic myocardium leads to an increase of oxidative stress (11, 13). At the onset of reperfusion, the mitochondrial respiratory rate markedly increases, and greater quantities of free radicals are generated. When these radicals exceed the capacity of the cellular intrinsic free-radical scavenging systems, toxic effects begin to damage the cells. The reactions caused by excessive radicals lead to lipid peroxidation of membranes (24, 25).

The beneficial effects of melatonin on MI-R-injury were demonstrated previously (12, 13). Recent studies pertaining to the efficacy of melatonin on remote organ injury demonstrated that melatonin was protective in renal (26) and cardiac (5) I-R injury. Eşrefoğlu et al. (5) showed that MI-R led to morphological testis damage and increased blood MDA and nitric oxide levels. To our knowledge, we are the first to demonstrate that MI-R causes an increase in levels of testis-tissue MDA and a decrease of sperm motility. In addition to this, the protective effect of melatonin on oxidative changes in testis tissue and sperm characteristics after MI-R was documented.

Gwayi and Bernard (27) reported that melatonin receptors were identified in the epididymis, and low-affinity melatonin-binding sites were described in spermatozoa. For this reason, it is possible that melatonin influences sperm motility when sperm transit through the epididymis.

In this study, we observed that melatonin positively ameliorated MI-R-induced damage on MDA level and sperm motility. These observations were most probably attributed to the potent free-radical scavenging ability of melatonin. Melatonin was demonstrated to scavenge OH^-, $O_2 \cdot{}^-$, singlet oxygen, the peroxyl radical, and the peroxynitrite anion. In addition, the antioxidant actions of melatonin probably derive from its stimulatory effect on superoxide dismutase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase, and its inhibitory action on nitric oxide synthase (4). Melatonin is an amphilic molecule (28, 29), which allows it to pass all biological barriers easily, and quickly penetrate into cells of the ischemic region and all subcellular compartments, where it scavenges damaging radicals. This is a clear advantage of melatonin over other antioxidants, which penetrate cells more slowly.

Glutathione is the major cellular sulfydryl which serves as both nucleophile and an effective reductant by interacting with numerous electrophilic and oxidizing compounds. It is important in the regulation of the cellular redox state, and a decline in its cellular level was considered indicative of oxidative stress (30). To our knowledge, after MI-R, the level of GSH in testis tissue has not been documented. Also, in this study, the GSH level of testis tissue was not significantly affected by MI-R.

In conclusion, these findings indicate that MI-R leads to damage of testis tissue and sperm motility, and melatonin protects against MI-R-induced reproductive-organ injury. These results may also encourage the use of antioxidants to reduce remote organ injury in the testis after MI-R.

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