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Investigating the protective effect of melatonin on liver injury related to myocardial ischemia-reperfusion

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A Study Design
B Data Collection
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Summary

Background: An animal model of myocardial ischemia-reperfusion (MI/R) was used to test the hypothesis that free radicals released with MI/R have hazardous effects on liver through remote organ injury.

Material/Methods: Twenty-one rats were divided into three groups: sham-treated, MI/R, and MI/R+melatonin. To produce MI/R, a branch of the left coronary artery was occluded for 30 min followed by two hours of reperfusion. Melatonin or vehicle was given 10 min before ischemia. At the end of the study, liver tissue was obtained for biochemical determination. The false discovery rate (FDR) is explained and was used for multiple comparisons of the groups' means.

Results: Compared with the sham group, MI/R significantly decreased glutathione (GSH) content and increased levels of nitric oxide (NO) and malondialdehyde (MDA). Melatonin administration significantly increased GSH levels and decreased the levels of NO and MDA compared with the MI/R group.

Conclusions: Melatonin could prevent liver damage due to its strong antioxidant and free radical scavenger effects. Therefore, melatonin may have beneficial effects on remote organ injury such as MI/R-induced liver disorders.

key words: **false discovery rate • liver injury • melatonin • myocardial ischemia-reperfusion • rat**

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BACKGROUND

One of the important remote organ injuries in myocardial ischemia-reperfusion (MI/R) may be liver injury, which is believed to be a consequence of free-radical generation in the liver [1,2]. The incidence of liver injury is increasing worldwide. Viral infection, alcohol or drug toxicity, and many other factors, including ischemia-reperfusion, can cause damage to hepatocytes. Therefore, these factors may cause inflammatory and oxidant reactions in the liver [3–6].

It is possible that MI/R injury can lead to remote organ injury, including liver damage [7]. Based on this relationship, Esrefoglu et al. [8] recently reported that MI/R induces severe testicular damage and that antioxidant agents, especially melatonin, have protective effects on testicular injury after MI/R-induced remote organ injury [8]. To our knowledge there are few studies [8,9] related to MI/R-induced remote organ injury. Also, no study has yet been carried out on MI/R-induced liver injury and treatment with melatonin in the literature.

In the current study, an animal model of MI/R was used to test the hypothesis that free radicals released with MI/R have effects on other remote organ systems, such as the liver. Antioxidants may have a beneficial effect on MI/R-induced liver injury. Therefore melatonin, a highly effective antioxidant and free-radical scavenger [10], could prevent liver damage resulting from MI/R in rats. To test this hypothesis we examined various oxidative stress markers such as malondialdehyde (MDA) and nitric oxide (NO) production and reduced glutathione (GSH) content in sham, MI/R, and MI/R + melatonin groups.

MATERIAL AND METHODS

Myocardial ischemia-reperfusion procedure

Male Wistar rats weighing 250–350 g were anesthetized with urethane (1.2–1.4 g/kg) administered intraperitoneally (i.p.). The jugular vein and trachea were cannulated for drug administration and artificial respiration. Systemic blood pressure (BP) was monitored from the carotid artery by a Harvard model 50-8952 transducer and displayed on a Harvard Universal pen-recorder together with a standard 1-lead electrocardiogram (ECG).

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 rate of 60 strokes/min to maintain normal PCO_2, PO_2, and pH parameters.

After the pericardium was incised, the heart was exteriorized by a 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 min. Any animal in which this procedure produced arrhythmias or a sustained decrease in BP to less than 70 mm Hg was discarded.

A small plastic snare was threaded through the ligature and placed in contact with the heart. The artery could then be occluded by applying tension to the ligature (30 min), and reperfusion was achieved by releasing the tension (120 min) [11]. However, after the above procedure, the coronary artery was not occluded or reperfused in the sham rats. All rats were sacrificed and the liver tissues were quickly removed after 150 min.

Experimental groups and other procedures

Twenty-one Male Wistar rats were divided into three equal groups: sham-treated (group 1), MI/R (group 2), and MI/R+melatonin (group 3). Vehicle or melatonin (10 mg/kg) was administered by intravenous injection 10 min before ischemia via the jugular vein. Melatonin (Sigma, St. Louis, MO, USA) was dissolved in ethanol and further diluted in saline to give a final concentration of 1%. All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Local Committee on Animal Research.

Evaluation of blood pressure

Blood pressure was measured from the carotid artery before and during 10, 20, and 30 min of occlusion and 30, 60, and 120 min of reperfusion.

Biochemical determination

A total of 200 mg of liver tissue was homogenized in ice-cold 150 mM KCl for determination of MDA. The MDA content of the homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid-reactive substances (TBARS) [12]. Results were expressed as nmol/g tissue. GSH was determined by the spectrophotometric method based on the use of Ellman's reagent. Results were expressed as nmol/g tissue [13]. Since tissue nitrite (NO_2^-) and nitrate (NO_3^-) levels can be used to estimate NO production, we measured the concentration of these stable NO oxidative metabolites in the homogenate according to methods described elsewhere. Quantification of NO_2^- and NO_3^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by the reaction of NO_2^- with a mixture of naphthlethylenediamine and sulfanilamide. Results are expressed as nmol/g tissue [14].

Statistical analysis

Levels of GSH, NO, and MDA were analyzed by one-way analysis of variance (ANOVA). Differences were considered significant when $p<0.05$. After obtaining a significant ANOVA F test, the false discovery rate (FDR), a versatile, simple, and powerful statistical method, was used for multiple comparisons of the groups. Since this method is not included in most statistics software packages, we can employ this method manually as follows:

1. Sort the k (the number of comparisons) comparisons by decreasing magnitude of P_i (the significance level associated with comparison i).
2. Estimate the critical significance level, i.e. d_i:
$$d_i = \left(\frac{i}{k}\right) f_{DR};\ i=k,\ k-1,\ \ldots,\ 1.$$
Here, f_{DR} is the false discovery rate.
3. If $P_i \leq d_i$, reject the null hypotheses associated with the remaining i comparisons.

Table 1. Effects of melatonin administration and myocardial ischemia reperfusion (MI/R) on the levels of GSH, MDA, and NO in liver.

Group		GSH (nmol/g)	MDA (nmol/g)	NO (nmol/g)
Sham	(n=7)	956.25±30.24*	107.80±21.30*	291.45±54.81*
MI/R	(n=7)	782.67±37.69**	156.82±24.71**	402.01±33.36**
MI/R + melatonin	(n=7)	945.98±81.60	113.77±12.09	297.47±53.01

* vs. the MI/R group; ** vs. the MI/R+melatonin group; statistical results are based on the false discovery rate (FDR). Values are given as the mean ±standard deviation (*SD*).

Table 2. Results of the false discovery rate.

i	H_o	P_i (GSH)	P_i (MDA)	P_i (NO)	d_i
3	$\mu_1=\mu_3$	0.760	0.531	0.838	0.050
2	$\mu_1=\mu_2$	0.001$^-$	0.002	0.001	0.047
1	$\mu_2=\mu_3$	0.001$^-$	0.001	0.001	0.044

P_i – achieved significance level; d_i – critical significance level; a value of 0.001$^-$ refers to $P_i<0.001$; μ_1 – mean of the sham group; μ_2 – mean of the MI/R group; μ_3 – mean of the MI/R + melatonin group.

Briefly, the null hypothesis H_0: $\mu_i=\mu_j$, $i\neq j$ is tested using the *t* test for each comparison. Then the three steps described above are applied and the decision for each comparison is made according to step 3 [15–17].

Results

Descriptive statistics of GSH, MDA, and NO for each group are given in Table 1. Based on the results of FDR, compared with sham group, MI/R significantly decreased GSH content and increased levels of NO and MDA in the liver tissue (956.25±30.24, 291.45±54.8, 107.80±21.30; 782.67±37.69, 402.01±33.36, 156.82±24.71, respectively). Melatonin administration significantly increased GSH levels and decreased the levels of NO and MDA (945.98±81.60, 297.47±53.01, 113.77±12.09, respectively) compared with the MI/R group.

When evaluating the results of rat carotid artery pressure, melatonin did not significantly change BP compared with the sham and MI/R groups; therefore the data was not shown.

As can be seen in Table 2, owing to $P_1<d_1$ and $P_2<d_2$ for i=1 and 2, null hypotheses 1 and 2 were rejected. Therefore, the means of groups 1 and 2, and groups 2 and 3 were significantly different for GSH, NO and MDA. In this situation, we took FDR f_{DR} =0.05 equal to the family error rate α_{FW}. The detailed results of FDR are described in Table 2.

Discussion

In the pathophysiology of MI/R-induced end-organ injury, all authors agree with the significance of oxidant/antioxidant status. For instance, Parlakpinar et al. [18] reported that MI/R plays a causal role in kidney injury and aminoguanidine (a specific iNOS inhibitor) exerts renal-protective effects, probably by inhibiting NO production and antioxidant activities. Ozer et al. [11] noted that MI/R plays a critical role in kidney injury through overproduction of oxygen radicals or insufficient antioxidant system, and caffeic acid phenethyl ester (CAPE) exerts renal-protective effects probably by its radical scavenging and antioxidant activities. Also recently, Esrefoglu et al. [8] investigated the role of oxidative injury on testicular damage following myocardial I/R injury and the effects of the antioxidant agents melatonin and CAPE on testicular injury. They demonstrated that MI/R induces severe testicular damage, antioxidant agents, especially melatonin, have protective effects on testicular injury after myocardial I/R, and that oxygen-based reactants may play a central role in remote organ injury. Similarly more recently, Sahna et al. [19] reported that MI/R leads to remote organ injury associated with oxidative stress. The findings of their study indicated that MI/R leads to damage of testicular tissue and sperm motility and may also suggest the use of antioxidants to reduce remote organ injury in the testis after MI/R, and that melatonin protects against MI/R-induced reproductive-organ injury. Ince et al. [20] studied the short-term cardio-protective effect of CAPE in an I/R rat heart model and demonstrated that CAPE exerts cardioprotective effects in short-term I/R of the rat heart. Several of the studies on MI/R and end-organ injury mentioned above revealed that melatonin, CAPE, or aminoguanidine etc. (i.e. antioxidant agents) have protective effects on kidney injury, testicular injury, reproductive-organ injury, and other possible ischemia-reperfusion-induced injuries. Based on the results of the current study and several other studies on MI/R and end-organ injury, melatonin administration may have significant protective effects on liver injury owing to its being a potent free-radical scavenger and a well-known antioxidant.

Related with the previous MI/R and end-organ injury studies, in the current study we investigated the possible protective effect of melatonin, a strongly antioxidant free-radical scavenger and a hormone derived from the pineal gland [21], against MI/R-induced liver damage as a remote injury in a rat model. Based on the present results, MI/R significantly increased NO and MDA production and decreased GSH content in the liver. Melatonin ameliorated the NO and MDA production and increased GSH level caused by ischemia/reperfusion. Melatonin thus has a protective role in liver injury due to MI/R which is independent of an effect on the BP. The protective effect of melatonin was independent of its hemodynamic effects on

MI/R injury. Melatonin's protective effects against MDA, a stable metabolite of free radical-mediated lipid peroxidation, may also involve its ability *in vivo* to stimulate antioxidative enzymes which remove oxygen- and nitrogen-based reactants. Also, melatonin increases the intracellular GSH concentration by stimulating the rate-limiting enzyme in its synthesis [22]. It is well established that GSH is an important endogenous antioxidant whose levels are affected by oxidative stress.

Sahna et al. [23] recently reported that melatonin, a major hormone produced by the pineal gland, plays an important role in various physiological processes, including regulation of circadian and endocrine rhythms, aging, stimulation of immune functions, and also cardio-protective effects. Parallel to our findings, Esrefoglu et al. [8] reported that MI/R injury leads to testicular damage as remote organ injury and melatonin ameliorated NO and GSH content by free-radical scavenging and antioxidant effects.

Melatonin is also known to increase tissue mRNA levels for superoxide dismutase (SOD), the enzyme which dismutases O_2^- to H_2O_2, and of catalase, which metabolically removes H_2O_2 from intracellular environment, further reducing the H_2O_2 levels and.OH generation [24]. Therefore it prevents the production of $ONOO^-$, a toxic oxygen metabolite produced during acute reperfusion of ischemic tissue. It has been reported that the combination of NO and O_2^- is effective in the damage and forms $ONOO^-$ in the tissue during ischemia reperfusion [9].

In the interpretation of the results, we used the FDR, which may be the best practical solution to the problem of multiple comparisons and much more powerful than comparable procedures which control the traditional familywise error rate [17,25], for multiple comparisons of the groups.

CONCLUSIONS

The present study demonstrated that excessive generation of NO in the liver during MI/R is capable of triggering the detrimental machinery of NO. Although a specific NO inhibitor was not investigated in this study, in our previous study [26] we used aminoguanidine (a specific iNOS inhibitor) in the same model and it ameliorated MI/R injury related with NO. Parallel to this result, in the current study melatonin reduced NO levels. Therefore, in accordance with the same beneficial results, it seems that reduction of liver injury by melatonin may be due to inhibiting excessive production of NO or $ONOO^-$ formation and by melatonin's free-radical scavenger effects. The reduction in MI/R-related changes in the liver homogenates is a predominantly regional, hepatic effect or a systemic effect of the well-known scavenging potential of melatonin. Taken together, the results of the current study would suggest that MI/R induces remote organ injury in the liver and that this injury might be mediated by oxygen radicals released with MI/R.

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