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## The antioxidant effect of melatonin in lung injury after aortic occlusion–reperfusion

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### Abstract

Acute aortic occlusion with subsequent ischemia-reperfusion of the lower extremities is well known to predispose to lung injury. Melatonin (MEL), a pineal hormone, is a free radical scavenger and an antioxidant. The purpose of this study was to assess the putative protective role of MEL in lung ischemia-reperfusion injury induced by aortic occlusion–reperfusion. Thirty-two rats were randomly allocated to four groups as follows: SHAM (Sham Laparotomy), SHAM + MEL, Aortic Ischemia Reperfusion (AIR) and AIR + MEL. Twenty mg/kg live weight MEL was given intraperitoneally 1 h prior to the experiment. An atraumatic microvascular clamp was placed across the infrarenal abdominal aorta (IAA) just after its origin from the aorta for 30 min. The microvascular clamp on IAA was removed and reperfused for 12 h. Lung tissues were assessed for malondialdehyde (MDA) level and myeloperoxidase (MPO) activity. MDA level and MPO activity, indicating the extent of lipid peroxidation and neutrophil infiltration of lung, respectively, significantly increased in AIR group when compared to SHAM and SHAM + MEL groups ( $P < 0.05$ ). Treating rat with MEL significantly decreased MDA levels as well as MPO activity in AIR + MEL group when compared to AIR group ( $P < 0.05$ ). In this study, exogenously administered MEL reduced lung injury after aortic occlusion reperfusion.

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**Keywords:** Melatonin; Ischemia; Reperfusion; Lung; Rats

### 1. Introduction

Temporary aortic occlusion, necessary in surgical procedures, is a condition that can lead to ischemia-reperfusion (IR) lesions with systemic alterations [1]. Acute visceral ischemia and subsequent reperfusion injury, which accompanies the surgical procedures of the aorta, are associated with high rates of morbidity and mortality. Importantly, postoperative lung injury is frequent after trauma and major vascular surgery [2]. Acute aortic occlusion with subsequent ischemia-reperfusion (IR) of the lower extremities is clearly described to be predisposed to lung injury [3,4].

Melatonin (MEL, *N*-acetyl-5-methoxytryptamine) is the main secretory product of pineal gland of all mammals including humans, but it is also produced in other organs [5].

Also, MEL has several abilities as an antioxidant, scavenging the hydroxyl radical and inhibiting the production of nitric oxide [6,7]. For the reduction of oxidative stress by several means, MEL scavenges hydrochlorous acid at a rate sufficient to protect catalase against inactivation by this molecule [8,9].

No studies have yet been conducted to test the effect of MEL against the lung injuries induced by AIR. MEL is expected to overcome the oxidative stress in order to reduce lung injury induced by AIR. Based on the antioxidant effect of MEL in many inflammatory processes, we aimed to investigate the putative protective effects of MEL against oxidative damage in lung induced by the occlusion–reperfusion of infrarenal abdominal aorta.

### 2. Materials and methods

Thirty-two male Wistar-Albino rats (200–250 g body wt.) obtained from Laboratory Animal Production Unit of

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Suleyman Demirel University were used in the study. They were kept in an environment of controlled temperature (24–26 °C), humidity (55–60%), and 12 h light and 12 h dark cycles, in individual wire-bottomed cages for 1 week before the start of experiment. A commercial balanced diet (Hasyem Ltd, Isparta-Turkey) and tap water were provided ad libitum. Food, but not water, was withdrawn 12 h prior to experiment. All animals received humane care, in compliance with institutional guidelines.

### 2.1. Aortic occlusion–reperfusion protocol

The rats were anaesthetised with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey) and were placed under a heating lamp. The skin was aseptically prepared and a midline laparotomy was performed. Ten milliliters of warm normal saline was instilled into the peritoneal cavity in order to maintain fluid balance. The abdominal aorta was exposed by gently deflecting the loops of intestine to the left with moist gauze swabs. An atraumatic microvascular clamp (vascu-statts II, midi straight 1001-532; Scanlan Int. St Paul, MN, USA) was then placed across the infrarenal abdominal aorta (IAA) for 30 min. The abdomen was then closed and the wound was covered with plastic wrap to minimize heat and fluid losses. The microvascular clamp on IAA was removed and reperused. Aortic occlusion and reperfusion were confirmed by the loss and reappearance of satisfactory pulsation in the distal aorta. Hence, no-reflow phenomenon was excluded. After 12 h, all animals were killed under anaesthesia, lungs were carefully removed en bloc from the thorax. The specimens were harvested and stored at –78 °C until biochemical assays. Time-matched, sham operated animals undergoing laparotomy and dissection of the IAA without occlusion served as controls for the experiment.

### 2.2. Melatonin treatment

MEL was purchased from Sigma Chemical Co. (St Louis, MO, USA). MEL was freshly dissolved in absolute ethanol and further dilutions were made in saline. The final concentration of ethanol was 1%. An equal volume of ethanol, as used in the MEL solution, was added to the saline solution. MEL was administered intraperitoneally at 20 mg/kg, 1 h prior to trial.

### 2.3. Experimental design

Thirty-two rats were randomly divided into four groups ( $n = 8$ , each): SHAM group; Sham laparotomy, SHAM + MEL group; Sham laparotomy and Melatonin, AIR group; Aortic Ischemia Reperfusion, and AIR + MEL group; Aortic Ischemia-Reperfusion and Melatonin. Rats received saline solution in both SHAM and AIR groups, while MEL was given to rats in both SHAM + MEL and AIR + MEL groups.

### 2.4. Biochemical analysis

The frozen tissue samples of lung were weighed and homogenised (Ultra Turrax T25, Germany) (1:10, w/v) in 100 mmol/l phosphate buffer (pH 7.4) containing 0.05% sodium azide in an ice bath. The homogenate was sonicated (Bandelin, Germany) for 30 s and centrifuged (5000 g for 10 min). The supernatant was frozen at –78 °C in aliquots until used for biochemical assays. The protein content of the supernatant was determined using the Lowry method [10].

### 2.5. Malondialdehyde assay

Malondialdehyde (MDA) levels, an indicator of free radical generation which increase at the end of the reperfusion, were estimated by the double heating method of Draper and Hadley [11]. The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of 100 g/l trichloroacetic acid solution was added to 0.5 ml supernatant in each centrifuge tube and the tubes were placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1000 g for 10 min and 2 ml of the supernatant was added to 1 ml of 6.7 g/l TBA solution in a test tube and the tube was placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient  $\epsilon = 1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ ) and is expressed as nanomoles per milligram of protein (nmol/mg protein).

### 2.6. Myeloperoxidase assay

Myeloperoxidase (MPO) activity, a sensitive index of tissue PMNL sequestration, was determined in the rat lung by using the peroxidase-catalysed,  $\text{H}_2\text{O}_2$ -dependent oxidation of tetramethylbenzidine as a measure of enzymatic activity [12]. Lung specimens were weighed as an 1 g and placed in a 9 ml 50 mM potassium phosphate buffer of pH 6 with 0.5% hexadecyltrimethylammonium bromide (Sigma, USA). The specimens were homogenised (Ultra-Turrax T25, Germany) for 20 s in an ice bath. The homogenate was sonicated (Bandelin Sonopuls UW 2070, Germany) for 30 s and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was assayed for MPO content spectrophotometrically by measuring the change in optical density at 460 nm over time. The assay buffer consisted of 50 mM potassium phosphate, pH 6.0 (50 ml), 0.83 ml  $\text{H}_2\text{O}_2$  (0.3% solution; Sigma, USA) and 8.34 mg *o*-dianisidine hydrochloride (Sigma, USA). The supernatant was mixed 1:80 (supernatant:assay buffer). MPO units are expressed as  $\Delta\text{A}/\text{min}/\text{g}$  tissue.

2.7. Statistics

Differences between groups were statistically analyzed by one-way ANOVA, and the differences between the means of groups were separated by least significant difference (LSD) test. All data were presented as mean ± standard error. Values of  $P < 0.05$  were regarded as significant. A computer program (SPSS 10.01, SPSS Inc. Chicago, IL, USA) was used for statistical analysis.

3. Results

**Malondialdehyde (MDA) levels.** Lung MDA levels (nmol/mg protein) in SHAM and SHAM + MEL groups were  $7.79 \pm 0.18$  and  $7.80 \pm 0.17$ , respectively (Fig. 1). MDA level was significantly high in AIR group,  $11.54 \pm 0.20$ , compared with SHAM and SHAM + MEL groups ( $P < 0.05$ ). Treatment with MEL significantly reduced MDA level in AIR + MEL group,  $9.65 \pm 0.14$ , compared with AIR group ( $P < 0.05$ ).

**Myeloperoxidase (MPO) activity.** Lung MPO ( $\Delta A/\text{min/g}$  tissue) activity in SHAM and SHAM + MEL groups were  $13.28 \pm 0.61$  and  $13.31 \pm 0.62$ , respectively (Fig. 2). MPO activity was found to be significantly increased in AIR group,  $25.57 \pm 0.50$ , compared to SHAM and SHAM + MEL groups ( $P < 0.05$ ). MEL treatment significantly

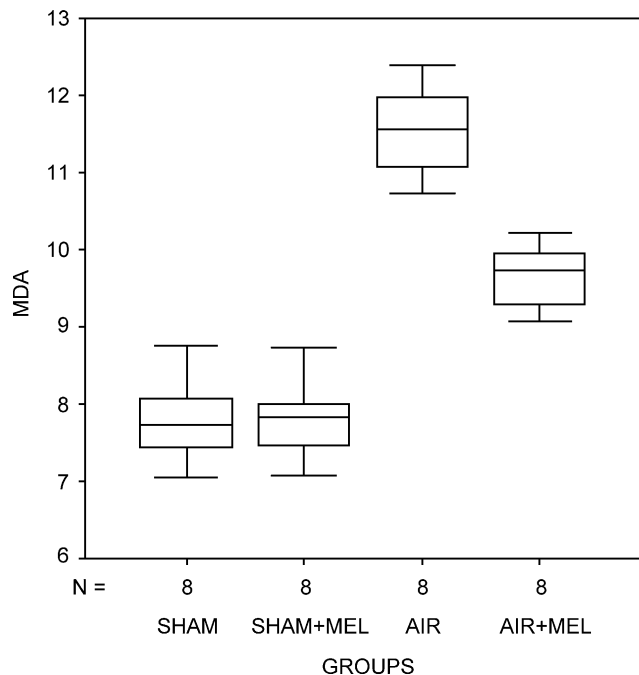


Fig. 1. MDA (nmol/mg protein) levels in the AIR group were significantly higher than the AIR + MEL group ( $P < 0.05$ ). In AIR + MEL group, the reductions in the level of MDA did not reach to the level of SHAM and SHAM + MEL groups. Boxplot of the MDA levels of each group. Horizontal bars represent the 90th, 75th, 50th (median), 25th, and 10th percentiles.

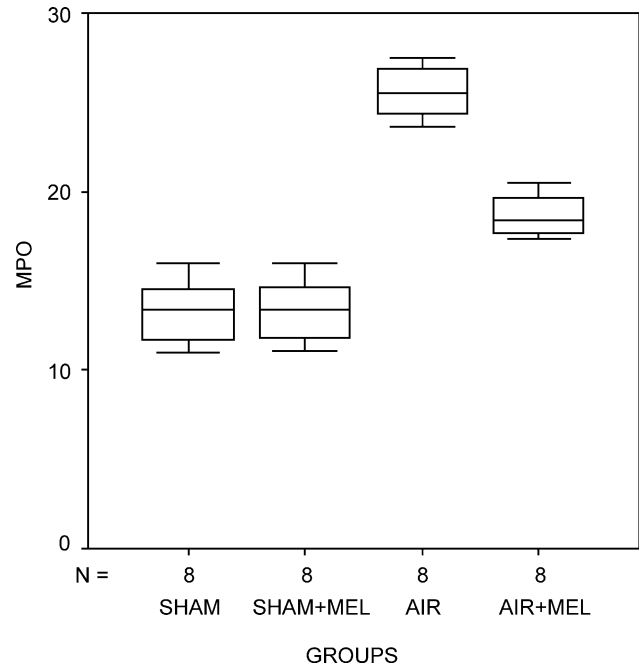


Fig. 2. MPO ( $\Delta A/\text{min/g}$  tissue) activity was significantly lower in the AIR + MEL group than the AIR group ( $P < 0.05$ ). In AIR + MEL group, the reductions in activity of MDA did not reach to the level of SHAM and SHAM + MEL groups. Boxplot of the MPO activity of each group. Horizontal bars represent the 90th, 75th, 50th (median), 25th, and 10th percentiles.

reduced MPO activity in AIR + MEL group,  $18.64 \pm 0.41$ , compared with AIR group ( $P < 0.05$ ).

4. Discussion

Aortic cross-clamping, as a surgical procedure, is routinely used for abdominal aortic surgery in the emergency and elective cases. Lung damage has been shown to occur following AIR. The ischaemic damage is resulted from a decrease in the blood flow to an organ. When restoring blood flow a more pronounced damage, so called reperfusion injury, occurs. In the development of IR injury, the enhanced generation of oxygen radicals has been suggested [13]. XO has been previously demonstrated to play a significant role in the etiology of remote lung injury in the rabbit model of hepatoenteric ischemia-reperfusion, and in other animal models as well [14]. It is well described that lung injury often occurs after hepatoenteric ischemia so as to XO releases due to reperfusion liver and intestines [15]. Furthermore, the effects of several pharmacological agents on ischemia-reperfusion (IR) injury have been investigated previously. Now, we attempted to examine the effect of MEL in lung injury induced by AIR. We determined a high level of MDA and increased MPO activity in the lung of AIR group, and that MEL significantly reduced MDA levels and MPO activity in lung induced by AIR.

In the present study, the level of MDA, an end product of lipid peroxidation, significantly increased in lung at the end of reperfusion in AIR group. However, MDA level was reduced in lung by MEL after aortic occlusion–reperfusion. Our results demonstrated that MEL significantly inhibits MDA elevations, but the extent of reduction in MDA level did not reach to the level in control groups (SHAM and SHAM + MEL), indicating that MEL could provide a protection for lung injury.

The tissue-associated MPO activity, known as an index of neutrophil infiltration, has been shown to be increased in the lung after AIR. MPO plays a fundamental role in oxidant production by neutrophils, and causes tissue damage [7]. In the present case, the treatment of rat with MEL significantly decreased MPO activity in AIR + MEL group when compared to AIR group. However, similar to level of MDA, the reductions in MPO activity did not reach to control levels.

In the past few years, the numerous free radical scavengers were used to reduce tissue damage and remove oxygen radical generated during IR tissue injury. Indeed, MEL is a direct free-radical scavenger and an indirect antioxidant [9]. While pineal gland derived MEL continues to be recognized as an important hormone in the regulation of seasonal reproduction in photoperiodical mammals as well as a regulator of circadian rhythms; it has also functions in the oxidative defense systems of organisms [9]. MEL's antioxidant actions 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 syntheses [9]. Reiter et al. found that MEL to stabilize the cell membranes and making more resistant to oxidative attack [6]. MEL was found to be effective in protecting cells, tissues, and organs from oxidative damage induced by a variety of free radical generating agents and processes, including IR and ionizing radiation [6].

In the current study, MEL could be given at 1 h prior to aortic cross-clamping. The aim of this procedure is relevant in the clinical setting of ruptured abdominal aortic aneurysm where there is a higher incidence of remote organ injury compared to elective aortic surgery. In this rat model, the results demonstrated that MEL protected the lung against aortic ischemia-reperfusion injury, which may be due to MEL's free radical scavenging activity and its ability to reduce neutrophil infiltration. One could speculate that treating the rat with higher doses of MEL than presently applied could progressively lower the tissue damage to the extent seen with control rats. Nevertheless, we have demonstrated that, in rat lung, MEL has an important ability to prevent lung damage due to AIR. However, further studies can be conducted to test the effects of various doses of MEL treatment in order to determine

the protection patterns of MEL against ischemia-reperfusion injury in lung.

## 5. Conclusions

We observed high levels of MDA and increased MPO activity in the non-treated AIR group, supporting the notion that lipid peroxidation and neutrophil infiltration occur during IR injury. Lipid peroxidation and neutrophil infiltration in the MEL-treated group was significantly lower than that of non-treated group, but the reductions in lung tissue damages by MEL treatment did not reach to the level of control rats. We believe that this study indicated the 'potential' of attenuating lung injury, although this will require further studies to show improvement in the morphology and function of the injured lungs.

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