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# Alterations in the Erythrocyte Antioxidant Enzyme Levels in Experimental Sepsis: Role of Melatonin

## (Deneysel Sepsiste Eritrosit Antioksidan Enzim Seviyelerindeki Değişiklikler: Melatoninin Rolü)

[1]Mükerrem Betül Yerer,
[1]Sami Aydoğan,
[2]Recep Saraymen

[1]Department of Physiology, Medical Faculty, University of Erciyes, Kayseri, Turkey

[2]Department of Biochemistry, Medical Faculty, University of Erciyes, Kayseri, Turkey

**Yazışma Adresi**
(Correspondence Address)

M. Betül Yerer,

Department of Physiology, Medical Faculty, University of Erciyes, 38039, Kocasinan, Kayseri/Turkey.
E-mail: eczbetul@yahoo.com
Tel: +90 352 437 4938 – 23310

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**ABSTRACT**

The organisms possess antioxidant defense systems, including antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), to deal with the reactive oxygen species generated in physiological and pathophysiological situations. Melatonin was recently shown to act as a putative antioxidant in the pathology of many diseases. The aim of this study was to investigate the effects of melatonin on antioxidant enzyme activities in septic rats. Swiss albino male rats weighing 250-300 g were randomly divided into 4 groups comprising of 10 animals in each group. The first group was the control group. The second group was given melatonin (10 mg/kg) and third group was given only lipopolysaccharide (LPS, 10 mg/kg) by i.p. injection. The fourth group received melatonin (10 mg/kg, i.p.) after 4 hours than the LPS administration. All the rats in 4 groups were killed at the end of the 6th hour after the first injection. The erythrocytes isolated from the whole blood were used to evaluate the GSH-Px, SOD and CAT activities. As a result, GSH-Px, SOD were significantly stimulated in sepsis with LPS treatment where CAT activities were decreased. Furthermore, GSH-Px and SOD activities were triggered significantly by melatonin administration, whereas the CAT activity reduced. These findings reveal that melatonin stimulates the antioxidant enzyme activities in the erythrocytes of septic rats, but further information is required to find out its inhibitory effects on the CAT activity.

**Key Words:** Sepsis, Melatonin, Glutathione Peroxidase (GSH-Px), Superoxide Dismutase (SOD), Catalase (CAT)

**ÖZET**

Fizyolojik ve patofizyolojik olaylarda gelişen oksidatif hasara karşı organizmalar, glutatyon peroksidaz (GSH-Px), süperoksit dismutaz (SOD) ve katalaz (CAT) gibi antioksidan enzimleri de içeren antioksidan savunma sistemine sahiptir. Melatoninin birçok hastalığın patolojisinde önemli koruyucu etkilerinin olduğu gösterilmiştir. Bu çalışmanın amacı sepsis oluşturulmuş ratlarda melatoninin antioksidan etkilerinin incelenmesidir. Çalışmada 250-300g ağırlığında Swiss albino erkek sıçanlar her bir grupta 10'ar adet olmak üzere 4 farklı gruba ayrılmıştır. İlk grup kontrol grubu olup, ikinci gruba sadece melatonin (10mg/kg, i.p.), üçüncü gruba sadece Lipopolisakkarit (LPS; 10 mg/kg, i.p.) uygulanmıştır. Dördüncü gruba LPS uygulanmasından 4 saat sonra melatonin (10mg/kg, i.p.) uygulanmıştır. Bütün gruplardaki sıçanlar ilk uygulamayı takiben 6. saatte dekapite edilmiştir. Gruplardan alınan kan örneklerinde GSH-Px, SOD ve CAT enzim aktiviteleri ölçülmüştür. Sonuç olarak; GSH-Px ve SOD aktiviteleri LPS ile indüklenen sepsiste önemli derecede artarken, CAT aktivitesi azalmıştır. Bunun da ötesinde, GSH-Px ve SOD aktiviteleri de melatonin uygulaması ile tetiklenmiş, ancak CAT aktivitesi benzer şekilde azalmıştır. Bu veriler, septik sıçanlarda melatoninin antioksidan savunma sistemini tetiklediğini göstermektedir, ancak CAT aktivitesi üzerindeki inhibitör etkilerinin ortaya konması için daha fazla bilgiye gerek vardır.

**Anahtar Kelimeler:** Sepsis, Melatonin, Glutatyon Peroksidaz, Süperoksit Dismutaz, Katalaz

## INTRODUCTION

It is indispensable for aerobic organisms to generate low levels of reactive oxygen species in many biochemical processes, including intracellular signaling in the cell differentiation and cell progression or the arrest of growth, apoptosis (1), immunity (2), and defense against microorganisms (3). These organisms possess antioxidant defense systems, including some antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), to deal with the reactive oxygen species that are generated in response to both external and internal stimuli (2). In contrast, high doses and/or inadequate removal of reactive oxygen species result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules (4,5).

There is a balance between the activities of these antioxidants and the levels of reactive oxygen species that is essential for the survival of organisms and their health. An unbalanced production of reactive oxygen species also plays an important role in the pathogenesis of number of diseases such as ischemia/reperfusion injury, atherosclerosis, neurodegenerative disease, cancer and sepsis (2,6). LPS has shown to induce the cascade of mediators leading to septic shock and their overproduction is associated with elevated body temperature, hypotension, tachycardia, tachypnea, leukopenia, end-organ dysfunction, and death (7)

Since melatonin was shown to be a putative biological antioxidant by its radical scavenging activity and antioxidant enzyme activity stimulating functions (8,9), it was of interest to determine how melatonin would affect the antioxidant enzyme activities in the pathophysiology of severe sepsis.

## MATERIALS AND METHODS

Male Swiss albino rats weighing 250-300g were purchased from the Experimental and Clinical Research Center of University of Erciyes, Kayseri, Turkey and kept under a 10/14 hr night-dark cycle at 21 ±1°C with free access to food and tap water. Rats were randomly divided into 4 groups comprising of 10 animals in each group. The first group was the control group. The second group was given melatonin (10 mg/kg) and third group was given only LPS (10 mg/kg) by i.p. injection. The fourth group received melatonin (10 mg/kg, i.p.) after 4 hours than the LPS administration. All the rats in 4 groups were killed at the end of the 6$^{th}$ hour after the first injection. The local ethics committee of the University of Erciyes has approved the study protocol.

Cellular GSH-Px activity was measured using the method described by Paglia and Valentine (10). In this assay, oxidized glutathione is reduced to glutathione by the enzyme glutathione reductase, which oxidizes NADPH to $NADP^+$ in the catalytic cycle. The change in absorbance at 340 nm resulting from the oxidation of NADPH is the basis for the quantification of cellular GSH-Px activity. Immediately after diluting the erythrocytes with water in a ratio of 1/5, hemolysates were mixed with concentrated Drabkins' solution (1:1). The final hemolysate was mixed with 0.05 M phosphate buffer (including 5mM EDTA) and 8.4 mM NADPH reagent containing 0.15 M GSH, 100 U/ml GSSG-Rd and 112.5 mM $NaN_3$. Kinetic spectrophotometric analyses were commenced by the addition of 0.1 ml 2.2 mM $H_2O_2$ at 340 nm. The rate of decrease in $A_{340}$ per min was determined by averaging the rate of changes for 1 min intervals in 4 minutes. One unit of GSH-Px activity is defined as the enzyme level to catalyze 1 micromoles of NADPH oxidation per minute.

Cellular SOD activity was measured using the modified method of Sun et al (11). The changes in the levels of oxidized NBT as a result of xanthine/xanthine oxidase system were determined. Kinetic spectrophotometric analysis was started by the addition of 167 U/l xanthine oxidase and limited by adding 0.8M $CuCl_2$ and analyzed at 560 nm and the enzyme activities were examined as U/g Hb. One unit of SOD activity was defined as the enzyme level which inhibits the NBT reaction rate 50%in 1 minute.

Cellular CAT activity was determined by measuring the decomposition of $H_2O_2$ by the method of Aebi (12). The kinetic analysis was initiated by adding $H_2O_2$ to the diluted hemolysate and the rate of decomposition per min was determined at 240 nm. One unit of CAT activity was defined as the enzyme level to reduce the $H_2O_2$ levels to 50%in 100 seconds at 25°C.

The medians of GSH-Px, SOD and CAT of the groups were analyzed by the help of Kruskal Wallis one way analysis of variance on ranks and all the pair wise multiple comparison procedures were analyzed by Dunn's method. The data were analyzed with the help of a computer software, Sigma Stat for Windows Version 3.10 (Copy write 2004 Systat Software). All the data are expressed as the medians ±SD and the number of animals are indicated by n.

## RESULTS

The GSH-Px, SOD and CAT activities obtained from the control and the septic groups with or without melatonin are revealed in the Figures seperately.

GSH-Px activity in untreated animals was 45.26 ±6.81 (n = 10) nmol/gHb, while in LPS treated animals it was significantly increased to 76.41 ±7.67 (n = 10) nmol/gHb, indicating that the GSH-Px enzyme activity increases in severe sepsis. The induction in the glutathione peroxidase activity was significant in septic rats treated with melatonin (92.94 ±10.41 nmol/g Hb), compared to the control and melatonin groups (Figure 1).

SOD activity was slightly increased when treated with melatonin alone, but this increase was not statistically significant. However, there was a significant increase

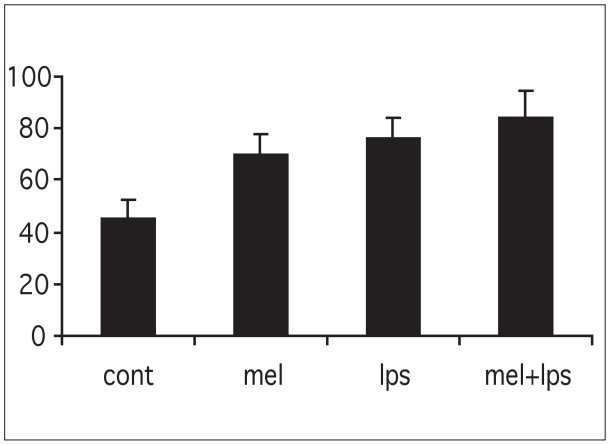


**Figure 1.** Alterations in the GSH-Px activity. cont: control group; mel: melatonin-treated group; lps: LPS-treated septic group; mel + lps: melatonin treated septic group. Different symbols indicate statistically significant differences by Kruskal Wallis and Dunn's tests. As a result of the Dunn's test; "lps" and "mel+lps" groups are statistically different from "control" group and "mel+lps" group is statistically different from "mel" group. However, there are not any differences between "mel and control", "mel and lps", "lps and lps+mel" since they are indicated with the same symbol as an identification of the Dunn's test.

in the SOD enzyme activity (3509.65 ±319.61 U/gHb) in the septic rats. The SOD activity was higher in the melatonin treated septic group (3078.39 ±143.87 U/gHb) when compared to the control group (2372.31 ±135.18 U/gHb).On the other hand, there was a slight but not a significant decrease in SOD activity in melatonin treated septic rats when compared to the septic group (Figure 2).

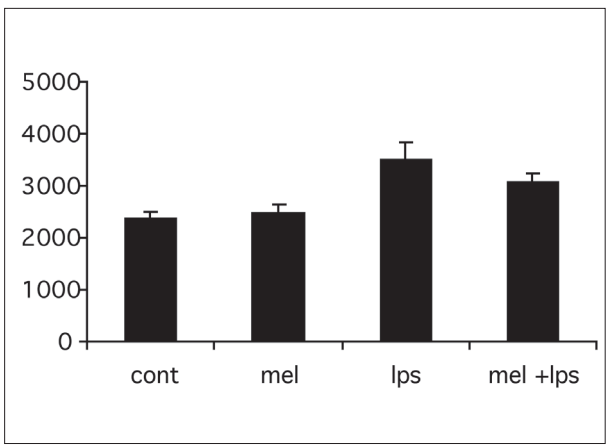


**Figure 2.** Alterations in the SOD activity of the control and the experimental groups. cont: control group; mel: melatonin-treated group; lps: LPS-treated septic group; mel + lps: melatonin treated septic group. Different symbols indicate statistically significant differences by Kruskal Wallis and Dunn's tests. As a result of the Dunn's test; "lps" and "lps+mel" groups are statistically different from "cont" and "mel" groups. However, there are not any difference between "control and mel", and "lps and mel+lps" since they are indicated with the same symbol as an identification of the Dunn's test.

Among animals which recieved either melatonin or melatonin + LPS, relatively low levels of enzymatic activity for CAT were seen in the latter. In addition, melatonin treated septic rats possessed a significantly reduced CAT activity when compared to the septic group (Figure 3).

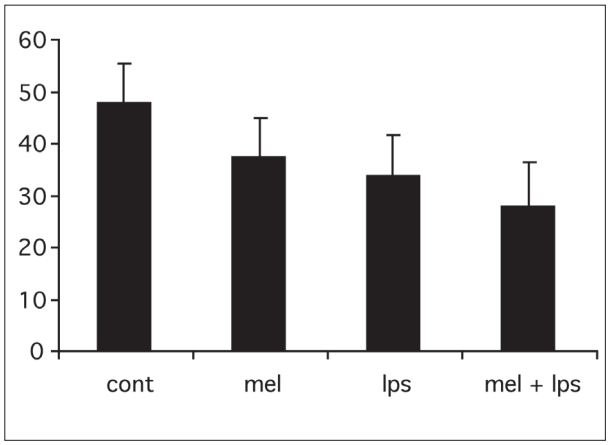


**Figure 2.** CAT activity in the control and the experimental groups. cont: control group; mel: melatonin-treated group; lps: LPS-treated septic group; mel + lps: melatonin treated septic group. Different symbols indicate statistically significant differences by Kruskal Wallis and Dunn's tests. As a result of the Dunn's test; "lps" and "mel+lps" groups are statistically different from the "control" group. However, there are not any significant differences between "mel", "lps" and "mel+lps" groups " since they are indicated with the same symbol as an identification of the Dunn's test.

## DISCUSSION

Melatonin's direct antioxidant activity (13,14) and its stimulatory effect on the antioxidant enzyme activities (15,16) may have clinical implications for the treatment of different pathological conditions. The evidence that melatonin increases the gene expression for both GSH-Px and SOD suggests a potential mechanism of action at the DNA level in different tissues however the mechanism by which melatonin stimulates GSH-Px and SOD activity in erythrocytes still remains unknown (15).

Antolin et al (15) reported that daily melatonin administration resulted in highly increased mRNA levels of copper-zinc SOD and manganase SOD in female Syrian hamster harderian gland. A recent study demonstrated that melatonin increases the mRNA levels not only of GSH-Px, but also of copper-zinc SOD and manganase SOD in the rat brain cortex (17). These stimulatory effects were observed after both acute and chronic melatonin treatment.

In the present study it is revealed that antioxidant SOD activity is elevated in sepsis within the erythrocytes, while it increased only slightly when melatonin was given alone. When compared to the control group, the SOD activity was higher in the melatonin treated septic group, indicating that melatonin stimulates the SOD enzyme activity in septic rats.On the other hand, there was

a slight but not a significant decrease in SOD activity in melatonin treated septic rats when compared to the septic group (Figure 2) and these results were compatible with our previous studies for the control group (18).

A stimulatory effect of melatonin on brain GSH-Px activity also has been reported by Barlow-Walden et al (19), who reported that exogenously administered melatonin (500 μg/kg) cause 2-fold rise in GSH-Px activity within 30 minutes in the brain of mature female rats. That study also demonstrated that brain GSH-Px activity is significantly higher at night than day time and is correlated with high night-time tissue melatonin levels.

In this study, it is suggested that melatonin has a potent GSH-Px stimulating effect in response to the free radical damage in severe sepsis. The elevated GSH-Px enzyme activity reveals that its activity increases in response to the free radical damage in severe sepsis. Also, melatonin injection caused an increase in GSH-Px activity alone but this increase was not statistically significant. One of the most important point to notice is that, the induction in the glutathione peroxidase activity was significant in septic rats treated with melatonin, compared to the control and septic groups, indicating its potent antioxidative role in sepsis (Figure 1) and these results were compatible with our previous studies for the control group (18).

Melatonin has been found to reduce significantly LPS-induced free radical damage in a variety of organs (20). According to the report by Fjaerli et al (21), melatonin exerts its effects through the amplififcation of the activation of T-lymphocytes in the septic shock. Melatonin was also recently identified as a potent therapeutic agent in connection with septic shock (22). It is well-known that the generation of free radical species and nitric oxide promote lipid peroxidation and subsequent tissue injury if they are formed in excessive amounts. During sepsis both nitric oxide synthesis and free radical production are substantially enhanced in a variety of tissues, effects which favor the development of lipid peroxidation. One of our previous research has shown that melatonin is a better inihibitor than L-Nitromethylarginin (L-NAME), one of the well-known nitric oxide inhibitors, for nitric oxide. Thereby, we suggested that sepsis-induced oxidative damage to the erythrocytes can be inhibited by melatonin by two different mechanisms, i.e reducing the NO formation and preventing the membrane lipids from peroxidation (23).

However, there is no information concerning with the relationship between the antioxidant effects of melatonin and antioxidant enzyme activity in severe sepsis. The present study showed elevated activities of SOD and GSH-Px, whereas CAT activity seemed to diminish with melatonin administration. The marked increase in the activity of GSH-Px in septic rats was due to adequate antioxidant status in these septic animals. Melatonin protects the erythrocytes from oxidative damage through free radical scavenging mechanism (24) and maintains the structural component of the cell membrane (24,25).

In the present study, in contrary to other antioxidant enzymes, CAT was shown to reduce with melatonin administration (Figure 3) and these results were compatible with our previous studies for the control group (18). Tan and et al. has found valuable data indicating that melatonin is a $H_2O_2$ scavenger and this process is thought to be a potentially new metabolic pathway of melatonin biotransformation. Although there are no further investigations about the effects of melatonin on CAT activity, we suggest that this should be due to the competitive inhibition of melatonin in $H_2O_2$ scavenging with CAT enzyme, but further investigations are required to improve this phenomena.


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