Research Article

Effect of Melatonin on Erythrocyte Deformability in Mice With Ischemia-Reperfusion Injury in Skeletal Muscle

Hakan Kartal[*]**, Faruk Metin Comu**

Department of Cardiovascular Surgery, Gulhane Education and Research Hospital, Ankara, Turkey

[*]**Corresponding Authors:** Hakan Kartal, Department of Cardiovascular Surgery, Gulhane Education and Research Hospital, Ankara, Turkey, E-mail: hakhankartal@gmail.com

Abstract

Aim: Erythrocyte deformability is the deformation movement that erythrocytes perform while passing through the capillary vessel in the microvascular area. Erythrocyte deformability is vital in microvascular circulation. The aim of this study was to investigate how melatonin affects the ability of erythrocytes to deform in mice who underwent ischemia-reperfusion in the lower limb.

Materials and methods: In the study, 30 male Swiss breed albino mice weighing 45-65 g were used. Mice were randomly divided into 5 groups, (Control group (C), melatonin control group (M), Dimethyl Sulfoxide (DMSO) group, ischemia-reperfusion (IR) group, melatonin - IR group (M+IR)), 6 being in each group.

All groups were administered 100 IU/kg intravenous heparin bolus 30 minutes before the procedure. Lower limb ischemia (2 hours) and reperfusion (2 hours) protocols were applied to the IR and M+IR groups by clamping the main femoral artery. Melatonin was administered to M and M+IR groups 1 hour before the experimental procedure (10mg / kg intraperitoneal). After the experiment protocol mice were sacrificed by the intracardiac blood collection method. From the blood samples taken, erythrocytes were extracted from heparinized whole blood samples. Erythrocytes were passed through the filtration system and pressure changes were measured to investigate the erythrocyte deformation index.

Results: Control group, melatonin group, and DMSO group's deformability levels are observed to be at

levels lower than or similar to melatonin group + IR and ischemia-reperfusion group. In addition, melatonin group + IR was found to be at a lower level than ischemia-reperfusion group in terms of deformability.

Conclusion: Unless there is an event having adverse effects on erythrocytes, melatonin has no effect on the deformability index. However, it is observed that melatonin positively affects deformability in cases that adversely affect the erythrocyte deformability, such as ischemia-reperfusion injury.

Keywords: Melatonin; Ischemia-reperfusion injury; Erythrocyte deformability

1. Introduction

Acute limb ischemia is defined as a sudden decrease in limb perfusion that potentially threatens the viability of the limb [1]. Blood reperfusion is required to restore the metabolic function of ischemic tissue; however, the reperfusion process often causes increased reactive oxygen species (ROS) and neutrophil infiltration and also induces inflammatory reactions that can cause local and distant tissue damage [2]. Ischemıa reperfusıon damage ıs not only ıschemıc tıssue; It damages blood [3], heart [4], lungs [5] and many tissues.

The first target of ischemia and subsequent reperfusion injury is microvascular circulation [6]. All these processes that occur during or after ischemia-reperfusion can cause changes in red blood cell deformability [7].

The ability of the erythrocytes to deform is the ability to change the shape and return to the previous form.

The ability of erythrocytes (about 7.2 μm in diameter) to pass through capillaries as small as 3-4 μm in diameter is one of the most important determinants for erythrocytes' survival in circulation. This is also very important for microcirculatory flow and to supply enough oxygen to the tissues [8].

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous hormone produced mainly by the pineal gland in the brain [9]. Since erythrocytes are in close contact with all hormones, including melatonin, it is estimated that melatonin may affect the functions of erythrocytes, such as deformability [10].

2. Materials and Methods

2.1 Animals and chemicals

The study was conducted at Gazi University Laboratory Animal Breeding and Experimental Researches Center (GÜDAM) with the permission obtained from Gazi University Animal Experiments Local Ethics Committee with the code number G.Ü.ET-19.047. Melatonin used in the study was provided from Sigma-Aldrich company without any institution or company support. Blood samples were analyzed in the physiology laboratory of Kırıkkale University. All procedures were carried out in accordance with the Standards for the Care and Use of Laboratory Animals.

In the study, 30 male Swiss breed albino mice weighing 45-65 g were used. Mice were randomly divided into 5 groups (Control group (C), melatonin control group (M), Dimethyl Sulfoxide group (DMSO), ischemia-reperfusion (IR) group, melatonin + ischemia-reperfusion group (M + IR)), 6 being in each group.

2.2 Technical procedure

At the beginning of the experiment, anesthesia was achieved by intramuscular injection by administering 50 mg/kg dose of ketamine hydrochloride (Ketalar® vial, Parke-Davis, USA) and 10 mg/kg xylazine hydrochloride (Alfazyne, 2%, Ege Vet). The procedure was performed under a heat lamp with the mice in the supine position. Anticoagulation with heparin was applied intraperitoneally to all groups (100 u/ kg). In order for heparin not to cause differentiation in the study results, the same amount was also applied intraperitoneally to the groups without ischemia-reperfusion. A longitudinal skin incision was performed on the inguinal region. Common-superficial and profundal femoral artery were explored. An atraumatic microvascular clamp was placed in the common femoral artery. Following the 120-minute ischemic period, the microvascular clamp on the common femoral artery was removed and reperfusion was achieved for 120 minutes. The same time (240 minutes) was awaited by applying an inguinal incision on the mice forming the control group, but IR was not generated in these groups. Mice were sacrificed by intracardiac blood collection method.

Control group group (Gr C, n = 6): After heparin application, only an inguinal incision was performed and closed without ischemia in this group of mice, and four hours after the procedure, intracardiac blood samples were taken from the mice under anesthesia.

Melatonin group group (Gr M, n = 6): In this group, 10 mg/kg dose of melatonin was administered intraperitoneally, without ischemia. Only an inguinal incision was performed and closed. Four hours after the procedure, intracardiac blood samples were taken from the mice under anesthesia.

DMSO group group (Gr DMSO, n = 6): After heparin administration, DMSO as much as the DMSO amount given to the melatonin groups (50 mg melatonin dissolved in 1 ml DMSO) was administered intraperitoneally without ischemia to this group of mice in order to examine the effects of DMSO, in which melatonin was dissolved, on erythrocytes. After that only inguinal incision was made and closed without ischemia. Four hours after the procedure, intracardiac blood samples were taken from the mice under anesthesia.

İschemia-reperfusion group group (Gr IR, n = 6): After heparin administration, an inguinal incision was made, without melatonin injection, on the mice in this group. Atraumatic microvascular clamp was placed on the main femoral artery, and after 120 minutes of ischemia and 120 minutes of reperfusion, intracardiac blood samples were taken from the mice under anesthesia.

Melatonin ischemia-reperfusion group group (Gr M+IR, n = 6): After heparin administration, melatonin was administered intraperitoneally at a dose of 10 mg/kg 1 hour before application of ischemia in this group of mice. An inguinal incision was performed. Atraumatic microvascular clamp was placed on the main femoral artery, and after 120 minutes of ischemia and 120 minutes of reperfusion, intracardiac blood samples were taken from the mice under anesthesia.

2.3 Deformability measurements

Erythrocyte deformability indexes were determined in Kırıkkale University Faculty of Medicine Physiology Laboratory, which is isolated against external factors such as heat and electricity. A constant flow filtrometer system was used to measure erythrocyte

deformability. In the system, calibration was achieved before use in the pressure ranges 0.5-4 cmH$_2$O. Within 2 hours, measurement procedures were started in the blood samples taken. Erythrocyte packages were formed by washing the heparinized blood 3 times in PBS (Phosphate-buffered saline) buffer. Then, erythrocytes brought to room temperature (25°C) were resuspended in PBS buffer as Htc: 5% and 10ml samples were prepared. Nucleopore polycarbonate filters (Nuclepore Polycarbonate filter, Merck-Whatman) with a diameter of 25mm and a pore size of 5 μm were used in the system. Separate filters were used for each study. 1.5 ml/min. constant flow rate (infusion pump, Biopac Systems Inc. - Commat, Ltd.) was achieved, and the resulting filtration pressure was measured in cmH 2 O. Actual pressure changes were monitored by being transferred to the computer via Data Acquisition System (Data Acquisition System Biopac systems Inc. USA). Two measurements were made for each sample and the mean was used. Many variables such as measured pressure values and time to reach that level were recorded and analyzed.

P L: Erythrocyte Suspension Filtration Pressure

P T: Buffer Solution Filtration Pressure

Relative pressure values were calculated; Rrel: P Erythrocyte /P Buffer

The relative pressure values obtained give us insight about the deformability of erythrocytes. An increase in that value indicates a negative effect on erythrocyte deformability.

2.4 Statistical method

Shapiro-Wilk test was performed to examine the normality levels of deformability measurements. According to the test result, since the distribution of the deformability measurement was not parametric and the sample numbers within the groups were low, analyses were made with non-parametric test approaches. In the analysis of the data, descriptive statistics are presented with mean, standard deviation values. For the study groups, the Mann- Whitney U test was used. In the study, p values less than 0.05 were considered statistically significant. All analyses were made using SPSS 22.0 software package.

3. Results

In our study, there was no difference in terms of deformability index between the Control group and the Melatonin group (p = 0.95). There was no difference in terms of the deformability index between Control group and DMSO group (p = 0.95). Deformability index levels were different between control group and ischemia-reperfusion group, and ischemia-reperfusion group had higher deformability index levels (p = 0.01). In addition, deformability index levels were different between control group and melatonin group + IR, and melatonin group + IR had higher deformability index levels (p = 0.01).

In the study, there was no difference between the melatonin group and the DMSO group in terms of the deformability index (p = 0.97). Deformability index levels were different between melatonin group and ischemia-reperfusion group, ischemia-reperfusion group had higher deformability index levels (p = 0.01). In the study, deformability index levels were different between melatonin group and melatonin group + IR, and melatonin group + IR had higher deformability index levels (p = 0.01).

		Group Melatonin	p
		X±s.s	
		1,78±0,19	**0,95**
		Group DMSO	**p**
		X±s.s	
Deformability index	**Group Control** X±s.s 1,76±0,19	1,79±0,19	**0,91**
		Group Ischemia Reperfusion	**p**
		X±s.s	
		2,96±0,19	**0,01***
		Group Melatonin + IR	**p**
		X±s.s	
		2,21±0,22	**0,01***

** Mann-Whitney U test was made. * significant difference p <0.05

Table 1: Melatonin group, DMSO group, İschemia-reperfusion group and Melatonin group + IR 's comparison with the Control group

		Group DMSO	p
		X±s.s	
		1,79±0,19	**0,97**
		Group Ischemia Reperfusion	**p**
Deformability index	**Group Melatonin** X±s.s	X±s.s	
		2,96±0,19	**0,01***
		Group Melatonin + IR	**p**
		X±s.s	
		2,21±0,22	**0,01***

** Mann-Whitney U test was made. * significant difference p <0.05

Table 2: Comparison of DMSO group, ischemia-reperfusion group and melatonin group + IR 's group with melatonin group

Deformability index	Group DMSO X±s.s 1,79±0,19	Group Ischemia Reperfusion	p
		X±s.s	
		2,96±0,19	0,01
		Group Melatonin+IR	p
		X±s.s	
		2,21±0,22	0,01

** Mann-Whitney U test was made. * significant difference p <0.05

Table 3: Comparison of ischemia-reperfusion group and melatonin group + IR with DMSO group

In the study, deformability index levels were different between DMSO group and ischemia-reperfusion group, and ischemia-reperfusion group had higher deformability index levels (p = 0.01). Deformability index levels were different between DMSO group and melatonin group + IR, and melatonin group + IR had higher deformability index levels (p = 0.01).

Deformability index	Group Ischemia Reperfusion	Melatonin+IR	p
	X±s.s	X±s.s	
	2,96±0,19	2,21±0,22	0,03*

** Mann-Whitney U test was made. * significant difference p <0.05

Table 4: Comparison of ischemia-reperfusion group and melatonin group + IR

In the study, deformability index levels were different between melatonin group + IR and ischemia-reperfusion group, and ischemia-reperfusion group had higher deformability index levels (p = 0.01).

Generally, deformability index levels of the control group, melatonin group, and DMSO group were similar to each other and at lower levels than the melatonin group + IR and ischemia-reperfusion group. In addition, in terms of the deformability index, the melatonin group + IR was at a lower level than the ischemia-reperfusion group.
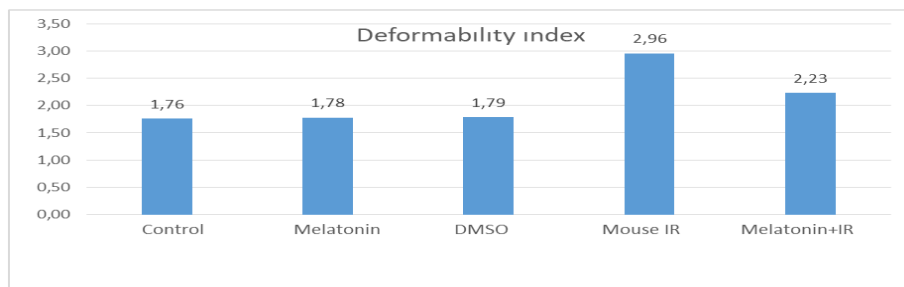

Figure 1: Study Groups and Deformability indexes

4. Discussion

The effects of the ischemia-reperfusion injury have been previously studied on many organs and tissues. It has been shown to affect many organs and tissues, including the brain, heart, liver, lung, kidney, skeletal muscles, testicular tissue, and endothelial tissue, and causes severe morbidity and mortality worldwide [11-18].

Impairment of the erythrocyte deformability feature may lead to increased blood viscosity, obstruction in microvessels, impaired perfusion, and ischemia. Although the underlying mechanisms are partially explained, ischemia-reperfusion injury affects blood and plasma viscosity, erythrocyte deformability, and aggregation [19,20]. Reduced deformability of erythrocytes leads to microcirculatory irregularities such as occlusion in the capillary vessels and decreased oxygenation for the whole body [21]. Therefore, the deformability feature of erythrocytes, which are the main effect area of ischemia-reperfusion injury and play the most important role in the microvascular bed, also play an important role in protecting against reperfusion injury.

The hormone melatonin, which acts according to circadian rhythm and is secreted by the pineal gland in the dark, plays a role in the regulation of many physiological functions in the body [22-24]. Endogenously produced melatonin provides a strong defense against ischemia-reperfusion injury as well as being protective against lipid peroxidation due to oxidative damage [25-28].

In our study, the deformability index of erythrocytes was observed to increase in the ischemia-reperfusion group compared to the control groups (control group, melatonin group, DMSO group). This is proof that ischemia-reperfusion affects erythrocytes negatively and disrupts microvascular circulation.

There was no significant difference between the melatonin group and the control group deformability indexes. In a previous study, it has been observed that the increase and decrease in the plasma levels, between day and night, of melatonin, which has a circadian rhythm, does not have any effect on the deformability index of erythrocytes [7]. However, in another study, it was shown that melatonin corrected the erythrocyte deformability, which had deteriorated due to oxidative stress [29]. When evaluated together with the results of our study, melatonin alone does not provide additional benefit unless there is a condition that impairs erythrocyte deformability.

The fact that the DMSO group has a similar deformability index compared to the control group shows that DMSO, the solvent of melatonin, does not negatively affect deformability. In addition, the deformability index in the ischemia-reperfusion group was significantly higher than the melatonin + IR group. This indicates that melatonin positively affects the erythrocyte deformability that deteriorates due to reperfusion injury.

Our study aiming to investigate these controversial effects of melatonin on erythrocytes, regarding which there is no consensus in previous studies, shows that if there is no event negatively affecting erythrocytes, melatonin does not affect deformability index, but indeed positively does so in cases negatively affecting erythrocyte deformability, such as ischemia-reperfusion injury.

More *in vivo* and clinical studies are needed in the future to better understand the effects of melatonin on erythrocytes.

Limitations

Statistical analysis showed us that more animals should be used in the study groups. We consider that keeping the number of animals higher in future studies may help overcome these statistical problems.

Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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