EXPERIMENTAL STUDY

Melatonin as a shield against skeletal muscle damage: A study on ischemia-reperfusion injury

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ABSTRACT

BACKGROUND: We evaluated the protective effects of melatonin against skeletal muscle ischemia-reperfusion injury, a significant cause of skeletal muscle damage. Ischemia-reperfusion (I/R) injury occurs due to a temporary restriction of blood flow (ischemia) followed by its restoration (reperfusion), triggering oxidative stress, inflammation, and cell death. Although current treatments are limited, melatonin's antioxidant and anti-inflammatory properties suggest potential benefits.

METHODS: We studied 30 male mice divided into five groups: control, melatonin control, I/R, melatonin + I/R, and dimethyl sulfoxide control. After the designated treatments, we assessed muscle tissue for antioxidant capacity (total antioxidant status [TAS]), oxidative stress markers (total oxidative status [TOS] and malondialdehyde [MDA]), inflammation (myeloperoxidase [MPO]), and cell death (terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL] assay and histological analysis).

RESULTS: Melatonin significantly increased antioxidant capacity (TAS) compared to all other groups. Conversely, oxidative stress (TOS) was significantly lower in the melatonin + I/R group compared to the I/R group alone. Histological analysis revealed greater necrosis, edema, inflammation, and cell death in the I/R group compared to others. Interestingly, the melatonin + I/R group exhibited significantly less damage than the I/R group, highlighting melatonin's protective effect.

CONCLUSION: This study demonstrates that exogenous melatonin effectively reduces oxidative stress, inflammation, and cell death in skeletal muscle tissue subjected to I/R injury. These findings suggest that melatonin may be a promising therapeutic agent for mitigating I/R-induced complications in skeletal muscle injury.

Keywords: Skeletal muscle; ischemia-reperfusion; melatonin; mouse.

INTRODUCTION

Skeletal muscle, a vital tissue responsible for locomotion and maintaining posture, is susceptible to damage following ischemia-reperfusion (I/R) injury. This condition occurs when blood flow is restricted to a region (ischemia) and subsequently restored (reperfusion), triggering a cascade of events that lead to oxidative stress, inflammation, and ultimately, cell death. The resulting tissue damage can have significant functional consequences, particularly affecting individuals undergoing surgical procedures, trauma, or organ transplantation.[1-3]

Melatonin, a hormone secreted by the pineal gland, has emerged as a promising therapeutic candidate for alleviating I/R injury in various organs. Its diverse biological functions, including potent antioxidant and anti-inflammatory properties, position it as a potential protector against the harmful effects of reperfusion.[4,5]

The antioxidant capacity of melatonin is well-established. It directly scavenges free radicals, which are harmful byproducts of oxygen metabolism, and activates endogenous antioxidant enzymes such as glutathione peroxidase and superoxide dis-

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mutase, thereby strengthening the cellular defense system. Furthermore, melatonin exhibits a synergistic effect with other antioxidants, such as vitamin E and glutathione, enhancing their protective capabilities.[6-8]

Beyond its antioxidant properties, melatonin modulates inflammatory pathways that play a key role in I/R injury. It suppresses the production of pro-inflammatory substances and cytokines, reduces the inflammatory response, and supports tissue repair. Additionally, its immunomodulatory effects may prevent excessive immune cell activity, thereby mitigating further tissue damage by regulating the immune system's response.[7,8]

While numerous studies have demonstrated melatonin's protective effects in other organ systems, such as the heart and brain, its role in skeletal muscle I/R injury remains relatively unexplored. Although existing studies suggest its potential benefits, a comprehensive understanding of its mechanisms and effectiveness in this context is lacking.[9-12]

Understanding and harnessing the protective potential of melatonin in skeletal muscle I/R injury could have significant clinical implications. It may offer a novel therapeutic approach for individuals undergoing procedures or experiencing conditions that involve reperfusion injury, potentially improving their outcomes and reducing functional impairment. This study contributes to bridging the knowledge gap and paving the way for further research into the therapeutic potential of melatonin in this setting.[4,13]

This study aims to investigate the protective potential of melatonin against I/R-induced damage in skeletal muscle. We hypothesize that melatonin administration, either before or after I/R injury, will attenuate oxidative stress, inflammation, and tissue damage in a murine model. By evaluating various parameters, including markers of oxidative stress, inflammatory mediators, and tissue integrity, we seek to elucidate the mechanisms underlying melatonin's protective effects and assess its potential therapeutic value in mitigating I/R injury in skeletal muscle.

MATERIALS AND METHODS

Chemicals and Experimental Protocol

The melatonin used in this study was commercially obtained from Sigma-Aldrich, with no sponsorship or affiliation with the company. All experimental procedures adhered to established guidelines for the ethical treatment of laboratory animals, including the European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes, the 3Rs principles (reduction, replacement, refinement), and the Canadian Council on Animal Care (CCAC) guidelines. The study was conducted at the Gazi University Experimental Animal Research Center (GÜDAM) and received prior approval from the Gazi University Animal Experiments Local Ethics Committee (approval code: G.Ü.ET-20.005).

Study Animals and Experimental Groups

The study utilized thirty adult male Swiss albino mice, aged 6-8 months and weighing 45-65 grams each. They were housed in standard cages, with three animals per cage, for one week before the experiment. The room environment was carefully controlled, maintaining a temperature range of 21-24°C and a humidity level of 50%. A 12-hour light/dark cycle was automatically regulated. Throughout the study, the mice had unrestricted access to standard pellet food and fresh drinking water.

After the acclimation period, the animals were randomly assigned to five groups of six mice each. These groups were designated as follows:

- **Mouse Control Group:** Served as the baseline control, receiving no treatment.
- **Melatonin Control Group:** Received only melatonin to assess its independent effects.
- **Mouse Ischemia-Reperfusion Group:** Underwent the I/R procedure without any additional intervention.
- **Melatonin + IR Group:** Received both melatonin administration and the I/R procedure to evaluate the protective effects of melatonin.
- **DMSO Control Group:** Received a vehicle control, dimethyl sulfoxide (DMSO), to account for any potential solvent effects.

Technical Procedure

Anesthesia and Initial Preparation

Prior to surgery, all mice received general anesthesia using a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg). Additionally, an intravenous bolus of heparin (100 IU/kg) was administered 30 minutes before the procedure in all groups. Afterward, the abdominal area was shaved, disinfected with an antiseptic solution, and the surgical field was thoroughly prepared. Throughout the surgery, the mice were maintained in a supine position under a heating lamp to ensure optimal comfort and body temperature.

Experimental Groups and Procedures

Mouse Control Group (Gr Control, n=6): Underwent a midline laparotomy followed by immediate closure without any further intervention, serving as the baseline for comparisons.

Melatonin Group (Gr Melatonin, n=6): Mice received a single intraperitoneal injection of melatonin (10 mg/kg) before laparotomy. No ischemia-reperfusion (I/R) procedure was performed in this group.

Ischemia-Reperfusion Group (Gr IR, n=6): These mice underwent the full I/R procedure without receiving melatonin. Following laparotomy, a microvascular clamp was placed on the abdominal aorta for 120 minutes to induce ischemia.

The clamp was then removed, allowing reperfusion for another 120 minutes.

Melatonin + Ischemia-Reperfusion Group (Gr Melatonin-IR, n=6): This group underwent the same I/R procedure as the previous group but additionally received a single intraperitoneal injection of melatonin (10 mg/kg) one hour before ischemia.

DMSO Control Group (Gr DMSO, n=6): This group served as a control for the DMSO solvent used to dissolve melatonin. They received intraperitoneal injections of DMSO at a dose equivalent to the melatonin groups (50 mg melatonin dissolved in 1 mL DMSO) but without the I/R procedure. Following the injection, they underwent a 120-minute waiting period, followed by laparotomy and closure.

Tissue Sampling

Two hours after completing the respective procedures in each group, lower extremity skeletal muscle samples (gastrocnemius muscle) were collected from all mice under anesthesia.

Muscle Tissue Homogenization for Analysis

Following dissection, individual hind leg muscle tissues were labeled and stored at -80°C until analysis of their total antioxidant status, total oxidant status, and oxidative stress index. Tissue samples were weighed while frozen on a precision scale to ensure accuracy. Liquid nitrogen was then used to rapidly pulverize the frozen tissues in a pre-chilled porcelain bowl. The powdered tissue was transferred to a homogenization tube, and a 140 mM potassium chloride (KCl) solution was added at a 1:10 dilution per gram of tissue. To prevent heat generation during processing, the tube was immersed in an ice-filled beaker for two minutes before and after homogenization at 50 revolutions per minute (rpm). The resulting homogenate was transferred to labeled Eppendorf tubes, covered with paraffin, and centrifuged at 3,000 rpm for 10 minutes. Following centrifugation, the supernatant was collected in separate Eppendorf tubes for subsequent analysis of total oxidative status (TOS) and total antioxidant status (TAS).[14,15]

Automated Measurement of Total Antioxidant and Oxidant Status

Both total antioxidant status and total oxidant status were determined using the Relassay® kit on a Mindray BS300 device, following a fully automated process.

TAS Measurement:

1. A total of 300 µL of Reagent 1 (measurement buffer) and 18 µL of the sample were mixed in a cuvette.
2. After 30 seconds, the first absorbance reading was taken at 660 nm.
3. Then, 45 µL of Reagent 2 (colored 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid [ABTS]) was added, and the mixture was incubated for five minutes.
4. A second absorbance reading was taken at 660 nm.
5. Trolox solution (1 mmol/L) was used as a standard, replacing the sample in steps 1-4.
6. All absorbance measurements were performed in triplicate, and their averages were calculated.
7. The change in absorbance (ΔAbs) was determined by subtracting the first reading (A1) from the second (A2).
8. TAS levels were calculated using the formula provided in the kit and expressed as mmol Trolox Eq/L.

TOS Measurement:

1. A total of 300 µL of Reagent 1 and 45 µL of the sample were mixed in a cuvette by the automated device.
2. After 30 seconds, the first absorbance reading was taken at 530 nm.
3. Then, 15 µL of Reagent 2 (pro-chromogenic solution) was added, and the mixture was incubated for five minutes.
4. A second absorbance reading was taken at 530 nm.
5. Hydrogen peroxide (H2O2) standard solution (10 µmol/L) was used for calibration.
6. All absorbance measurements were performed in triplicate, and their averages were calculated.
7. ΔAbs was determined by subtracting the first reading (A1) from the second (A2).
8. TOS levels were calculated using the formula provided in the kit and expressed as mmol HO Eq/L.

Oxidative Stress Index (OSI) Calculation: To quantify the overall oxidative stress level, the OSI was calculated as the ratio of total oxidant status to total antioxidant status. This formula integrates both pro-oxidant and antioxidant activities into a single value:

OSI (arbitrary unit) = TOS (µmol H_2O_2 equivalent/L) / TAS (µmol Trolox equivalent/L).

Before calculation, TAS values were converted to µmol/L units for consistency with TOS values. This ratio provides a snapshot of the oxidative balance within the sample, with higher OSI values indicating a greater pro-oxidant burden and potential oxidative stress.[16-18]

Assaying Myeloperoxidase Activity: Chlorination and Peroxidation

Myeloperoxidase (MPO) is an enzyme with diverse catalytic abilities. Its primary role is the generation of hypochlorous acid (HClO), a potent oxidant, from hydrogen peroxide and chloride ions. Additionally, MPO functions as a peroxidase,

oxidizing various substrates using hydrogen peroxide. Assessing these two catalytic activities provides valuable insights into MPO function.

To quantitatively measure MPO activity, commercial kits such as the Relassay Myeloperoxidase Chlorination and Peroxidation Activity Assay Kits offer convenient and accurate colorimetric approaches.

Chlorination Activity: MPO catalyzes the conversion of taurine and chloride ions into a chlorine-containing intermediate, taurine chloramine. This intermediate reacts with a chromophore, resulting in a color change that allows for absorbance measurement. MPO activity is directly proportional to the rate of color change and is expressed in units based on the amount of substrate consumed per minute.

Peroxidation Activity: MPO oxidizes o-dianisidine using hydrogen peroxide, generating a colored product with absorbance measurable at 412 nm. The increasing absorbance reflects the reaction's progress, enabling the kinetic assessment of MPO activity. This kit is adaptable for both manual and automated analysis. These assays serve as valuable tools for investigating MPO's role in various biological processes and its potential involvement in disease states.[19]

Measuring Tissue Damage with Malondialdehyde (MDA)

Malondialdehyde is a key marker of lipid peroxidation, a process associated with cellular damage. This study employed a well-established method to quantify MDA levels in tissue samples.

Thiobarbituric Acid (TBA) Test:

1. **Reaction:** Tissue homogenate was incubated with TBA at high temperatures (90-100°C), triggering a reaction between MDA and TBA. This reaction produces a pink pigment with peak absorption at 532 nm.
2. **Protein Removal:** To isolate the colored product, trichloroacetic acid was added to precipitate proteins, which were then removed by centrifugation.
3. **MDA Quantification:** The remaining supernatant, containing the MDA-TBA adduct, was reacted with additional TBA in boiling water.
4. **Absorbance Measurement:** After cooling, the absorbance at 532 nm was measured, and the MDA level was calculated based on a standard curve and expressed as nanomoles per gram of wet tissue.

This method provides a reliable and sensitive assessment of lipid peroxidation, offering insights into tissue damage within the context of the study.[20,21]

Tissue Preparation and Histopathological Analysis

Tissue Fixation and Processing: Upon collection, tissue samples were promptly immersed in 10% neutral buffered formalin for 48 hours to ensure proper fixation. Following fixation, standard histopathological processing steps were meticulously followed. The processed tissues were then embedded in paraffin blocks for further analysis.

Histological Staining and Evaluation: Thin sections (5 microns) were obtained from the paraffin blocks and stained with hematoxylin-eosin (H&E) for detailed microscopic examination. The stained sections were graded according to a predefined scoring system based on the presence and severity of necrosis, edema, and inflammatory cell infiltration. The grading scale ranged from 0 (no damage) to 3 (severe damage). An Olympus CX43 microscope equipped with a camera was used for the comprehensive evaluation of the stained sections.[4,22]

Apoptosis Assessment: To determine the extent of programmed cell death (apoptosis), the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method was employed on 4-micron sections obtained from the paraffin blocks. A commercially available TUNEL kit (ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit) was used for this analysis. Under 400× magnification, five randomly chosen regions of each section were carefully examined. Cells with brown or black staining were considered TUNEL-positive, indicating apoptosis.[23] The apoptotic index (AI) was calculated for each section as the percentage of TUNEL-positive cells relative to the total number of counted cells using the formula:

Apoptotic Index (AI) = (Number of positive cells / Total number of cells counted) x 100.

Analyzing the Data: A Statistical Perspective

To summarize the data, we calculated the mean and standard deviation values for each measurement. However, not all data sets followed a normal distribution. To assess normality, we applied the Shapiro-Wilk test to measurements of necrosis, edema, inflammatory cell infiltration, TUNEL, TAS, TOS, OSI, MPO, and MDA across the study groups. Since the test results indicated non-normal distributions for most measurements (e.g., necrosis, edema, etc.) and given the small group sizes (n=6), we opted for non-parametric statistical tests. To compare the five different groups regarding these measurements, the Kruskal-Wallis test was used. This test identifies significant differences among the groups. Further analysis using the Mann-Whitney U test pinpointed specific group pairs with statistically significant differences for each measurement. These significant pairwise comparisons are presented in Tables 1 and 2. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS 22.0 software package.

Table 1. Comparison of total antioxidant status (TAS), total oxidant status (TOS), oxidative stress ındex (OSI), myeloperoxidase (MPO), and malondialdehyde measurements (MDA) measurements among groups

TEST	Mouse Control (1) X±SD	Melatonin (2) X±SD	Mouse IR (3) X±SD	Melatonin + IR (4) X±SD	DMSO (5) X±SD	p	Difference
TAS (mmol/L)	0.39±0.13	0.57±0.09	0.41±0.12	0.40±0.25	0.39±0.10	0.01*	1-2 (p=0.01)*
							2-3 (p=0.01)*
							2-4 (p=0.01)*
							2-5 (p=0.01)*
TOS (µmol/L)	3.46±1.76	3.27±0.48	6.67±2.5	5.03±0.50	3.48±0.96	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							3-5 (p=0.01)*
							1-4 (p=0.02)*
							2-4 (p=0.02)*
							5-4 (p=0.02)*
OSI	0.89±0.61	0.57±0.20	1.62±2.91	1.25±0.80	0.89±0.23	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							4-3 (p=0.01)*
							5-3 (p=0.01)*
MPO (U/L)	59.42±18.1	58.71±27.58	179.79±45.95	150.34±24.75	57.49±13.72	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							3-5 (p=0.01)*
							1-4 (p=0.01)*
							2-4 (p=0.01)*
							5-4 (p=0.01)*
MDA	0.41±0.13	0.41±0.17	1.11±0.25	0.93±0.15	0.37±0.04	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							3-5 (p=0.01)*
							1-4 (p=0.01)*
							2-4 (p=0.01)*
							5-4 (p=0.01)*

-Kruskal Wallis test; -Difference test; -Mann-Whitney U test. *Indicates a statistically significant difference.
Notes: Data are presented as mean ± standard deviation (SD). The Kruskal-Wallis test was used for multiple-group comparisons, followed by the Mann-Whitney U test for pairwise comparisons. Statistical significance was defined as a p-value of less than 0.05.
Abbreviations: IR: Ischemia-Reperfusion; DMSO: Dimethyl Sulfoxide; TAS: Total Antioxidant Status; TOS: Total Oxidant Status; OSI: Oxidative Stress Index; MPO: Myeloperoxidase; MDA: Malondialdehyde.

RESULTS

This section presents the study's findings, exploring the differences among experimental groups in terms of antioxidant status, oxidative stress, and tissue damage.

Antioxidant Defense and Oxidative Stress

Total Antioxidant Status: Significant differences in TAS measurements were observed among the study groups (p=0.01). Pairwise comparisons demonstrated that the Melatonin Control Group consistently exhibited significantly higher TAS levels compared to all other groups (p=0.01, p<0.05).

Total Oxidant Status: Significant differences in TOS measurements were found among the study groups (p=0.01). Post-hoc analysis revealed that the Mouse IR Group and the Melatonin + IR Group exhibited significantly higher TOS levels than the other groups (p<0.05). However, when comparing the Mouse IR Group and the Melatonin + IR Group directly, no significant difference in TOS levels was observed (p>0.05).

Oxidative Stress Index: Significant differences in OSI measurements were found among the study groups (p=0.01). Pairwise comparisons revealed that the Mouse IR Group consistently exhibited significantly higher OSI levels compared to all other groups (p=0.01). Specifically, the OSI levels of the

Table 2. Examination of necrosis, edema, inflammatory cell infiltration, and TUNEL staining among groups

Test	Mouse Control (1) X±SD	Melatonin (2) X±SD	Mouse IR (3) X±SD	Melatonin + IR (4) X±SD	DMSO (5) X±SD	p	Difference
NECROSIS (0-4)	0.17±0.41	0.17±0.41	2.67±0.52	1.97±0.52	0.23±0.51	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							3-5 (p=0.01)*
							1-4 (p=0.01)*
							2-4 (p=0.01)*
							5-4 (p=0.01)*
							3-4 (p=0.03)*
Edema (0-3)	0.00±0.00	0.33±0.52	2.5±0.55	1.95±0.55	0.17±0.41	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							3-5 (p=0.01)*
							1-4 (p=0.01)*
							2-4 (p=0.01)*
							5-4 (p=0.01)*
							3-4 (p=0.02)*
Inflammatory Cell Infiltration (0-5)	0.00±0.00	0.34±0.52	2.60±0.52	2.07±0.52	0.18±0.40	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							3-5 (p=0.01)*
							1-4 (p=0.01)*
							2-4 (p=0.01)*
							5-4 (p=0.01)*
							3-4 (p=0.02)*
TUNEL-Positive Cells + (%)	7.00±1.41	9.00±1.41	34.67±1.21	31.5±2.59	6.67±1.63	0.01*	1-3 (p=0.01)*
							2-3 (p=0.01)*
							3-5 (p=0.01)*
							1-4 (p=0.01)*
							2-4 (p=0.01)*
							5-4 (p=0.01)*
							3-4 (p=0.04)*

-Kruskal-Wallis test; -Difference test; -Mann-Whitney U test. *Indicates a statistically significant difference.

Notes: The table presents quantitative data on tissue injury markers (necrosis, edema) and inflammatory responses (inflammatory cell infiltration) across different experimental groups. Apoptotic cell death was assessed using TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining. Due to the non-normal distribution of the data, nonparametric tests (Kruskal-Wallis and Mann-Whitney U test) were used for group comparisons. Statistical significance was set at $p<0.05$.

Abbreviations: IR: Ischemia-Reperfusion; DMSO: Dimethyl Sulfoxide; TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling.

Mouse IR Group were statistically higher than those of the Melatonin + IR Group (p=0.01).

Myeloperoxidase Activity: Significant differences in MPO measurements were found among the study groups (p=0.01). Post-hoc analysis revealed that the Mouse IR Group and the Melatonin + IR Group exhibited significantly higher MPO levels compared to the other groups ($p<0.05$). Similar to TOS, MPO activity was elevated in both the Mouse IR Group and the Melatonin + IR Group, potentially contributing to increased oxidant production. However, no significant difference was observed between these two groups ($p>0.05$).

Malondialdehyde Levels: Significant differences in MDA measurements were observed among the study groups (p=0.01). Post-hoc analysis revealed that the Mouse IR Group and the Melatonin + IR Group exhibited significantly higher MDA levels than the other groups ($p<0.05$). Both the Mouse IR Group and the Melatonin + IR Group displayed significantly elevated MDA levels, indicating lipid damage caused by oxidative stress. However, similar to other markers, these two groups did not significantly differ from each other.

Tissue Damage Assessment:

- **Necrosis, Edema, and Inflammatory Cell Infiltration:** These key indicators of tissue damage were significantly elevated in both the Mouse IR Group and the Melatonin + IR Group compared to the other groups. Notably, the Mouse IR Group consistently exhibited more severe damage than the Melatonin + IR Group, suggesting the potential of melatonin to mitigate tissue injury.
- **Cell Death (TUNEL):** Similar to other tissue damage markers, TUNEL measurements, reflecting cell death, were significantly higher in both the Mouse IR Group and the Melatonin + IR Group compared to the other groups. Once again, the Mouse IR Group displayed higher levels of cell death than the Melatonin + IR Group, supporting melatonin's potential protective effect.

Visual Confirmation: Figures 1 and 2 provide visual representations of these findings. Figure 1 depicts H&E stained sections, where arrows highlight areas of necrosis, edema, and inflammatory cell infiltration. Figure 2 shows TUNEL staining, specifically marking apoptotic cell nuclei. Comparing these images across the study groups reinforces the quantitative conclusions (Figures 1 and 2).

The study revealed that ischemia-reperfusion injury led to increased oxidative stress and tissue damage in the examined groups. While melatonin did not completely prevent these effects, it consistently demonstrated a protective role, particularly in reducing tissue damage and cell death compared to the Mouse IR Group alone. These findings suggest that melatonin may serve as a promising strategy for mitigating injury caused by ischemia-reperfusion.

DISCUSSION

This study explores the potential of melatonin, a widely distributed molecule with diverse biological functions, as a therapeutic shield against the detrimental effects of ischemia-reperfusion injury in a mouse hindlimb muscle model. The findings provide compelling evidence of its multifaceted protective role, paving the way for further investigation and potential clinical applications.[24,25]

Ischemia-reperfusion injury, characterized by the deprivation of blood flow followed by its restoration, triggers a cascade of harmful events within tissues. The sudden influx of oxygen upon reperfusion initiates a surge of free radicals, leading to oxidative stress, lipid peroxidation, and cellular damage.[26] This study highlights melatonin's potential to counteract this oxidative assault. The significant increase in total antioxidant status observed in the melatonin-treated group suggests its ability to enhance the body's natural defense mechanisms against free radicals. This finding aligns with previous research demonstrating melatonin's capacity to directly scavenge free radicals and enhance antioxidant enzyme activity.[27] Furthermore, the observed decrease in oxidative stress index reinforces this concept, indicating that melatonin effectively protects tissues from the harmful consequences of oxidative stress.[28]

Previous studies have demonstrated a significant increase in lipid peroxidation, as measured by malondialdehyde, in skeletal muscle following reperfusion. Melatonin has been shown to mitigate this oxidative stress by reducing MDA levels.[4,29,30] While our current findings did not reach statistical significance for myeloperoxidase or MDA, the numerical trend toward lower values in the Melatonin Group suggests its potential to

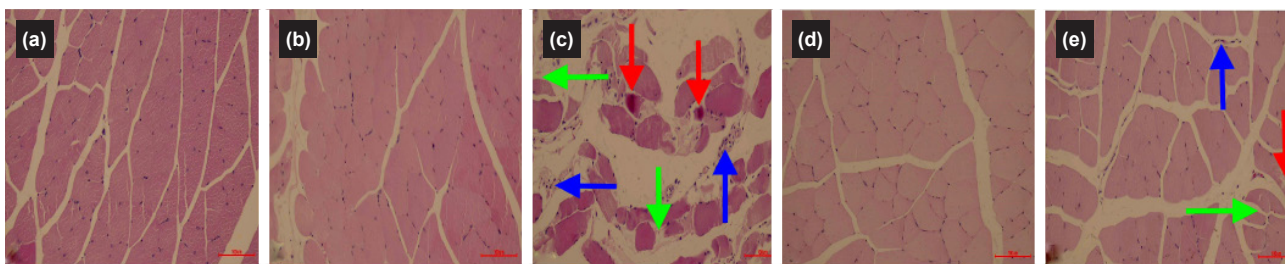


Figure 1. Hematoxylin-eosin (H&E) stained images of histological sections from the experimental groups. A, B, C, D, and E correspond to the Control, Dimethyl Sulfoxide (DMSO), Ischemia-Reperfusion, Melatonin, and Melatonin + Ischemia-Reperfusion groups, respectively. The images highlight areas of necrosis (red arrow), edema (green arrow), and inflammatory cell infiltration (blue arrow) (Magnification: x200).

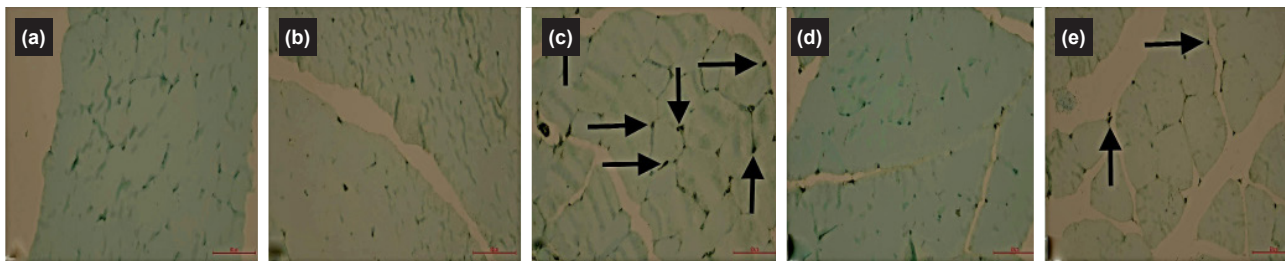


Figure 2. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) stained images were used to evaluate apoptosis in the experimental groups. A, B, C, D, and E correspond to the Control, DMSO, Ischemia-Reperfusion, Melatonin, and Melatonin + Ischemia-Reperfusion groups, respectively. In the images, arrows indicate TUNEL-positive (+) apoptotic cell nuclei (Magnification: x400).

attenuate both lipid peroxidation and inflammatory cell infiltration. This hypothesis is further supported by histopathological evidence of reduced inflammation in the melatonin-treated group. Although further research with larger sample sizes is needed, these results suggest that melatonin may exert anti-inflammatory effects beyond its direct antioxidant properties. This aligns with previous studies highlighting its ability to modulate immune cell function and inflammatory pathways, potentially through mechanisms such as suppressing pro-inflammatory cytokines and promoting anti-inflammatory mediators.[31-34]

The mechanisms underlying melatonin's protective effects are multifaceted and warrant further investigation. Its antioxidant properties are likely mediated by direct free radical scavenging and modulation of antioxidant enzyme activity. Previous studies have demonstrated that melatonin can reduce apoptosis by mitigating mitochondrial dysfunction. Our immunohistochemical analysis corroborates these findings, indicating that melatonin decreased apoptosis in our experimental model.[35,36] Additionally, its anti-inflammatory effects may involve the suppression of pro-inflammatory pathways, modulation of immune cell function, and regulation of inflammatory mediators. The present study serves as a foundation for future research to further elucidate the complex mechanisms by which melatonin protects tissues from ischemia-reperfusion injury.[27]

The findings presented here highlight the potential for translating melatonin's protective properties into clinical practice. Its widespread availability, well-tolerated profile, and ease of administration make it an attractive candidate for therapeutic intervention. However, several crucial questions remain. Determining the optimal dosage, timing of administration, and target patient population is essential for maximizing efficacy while minimizing potential side effects. Further research assessing its safety and efficacy in larger clinical trials is necessary before widespread clinical implementation.

Limitations and Future Directions

This study acknowledges the limitation of a relatively small sample size, which may have contributed to the lack of statistical significance for certain parameters. Future studies with larger sample sizes and more robust statistical analyses are essential to confirm the observed trends and strengthen the conclusions. Additionally, further investigations into the optimal dosage and timing of melatonin administration, potential combination therapies with other established treatments for reperfusion injury, and long-term safety and efficacy assessments are crucial for clinical translation.

CONCLUSION

While this study focused on a mouse hindlimb muscle model, the potential therapeutic applications of melatonin extend far beyond this specific context. The underlying mechanisms of ischemia-reperfusion injury and other oxidative stress-related pathologies share similarities across various tissues and organs. Therefore, the observed protective effects of melatonin in this study warrant further investigation in other models, such as myocardial infarction and stroke models, to evaluate its generalizability and therapeutic potential in a broader range of clinical settings. Furthermore, exploring the potential of melatonin in combination with other established therapeutic strategies could further enhance its efficacy and expand its applicability in medical practice.

Ethics Committee Approval: The study was approved by the Gazi University Research Ethics Committee (no: G.Ü.ET-20.005, date: 03.01.2020).

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DENEYSEL ÇALIŞMA - *ÖZ*

İskelet kası hasarına karşı bir koruyucu olarak melatonin: Bir iskemi-reperfüzyon hasarı çalışması

AMAÇ: İskemi-reperfüzyon (İ/R) hasarı, dokulara kan akışının geçici olarak kesintiye uğraması (iskemi) ve ardından yeniden başlaması (reperfüzyon) sonucu oluşan ve oksidatif stres, inflamasyon ve hücre ölümüne yol açan bir patolojik süreçtir. Mevcut tedavi seçenekleri sınırlıdır, ancak melatoninin güçlü antioksidan ve anti-inflamatuvar özellikleri, İ/R hasarında potansiyel bir terapötik ajan olarak öne çıkarmaktadır. Bu çalışmada, iskelet kası İ/R hasarına karşı melatoninin koruyucu etkileri araştırıldı.
GEREÇ VE YÖNTEM: Kontrol, melatonin kontrol, İ/R, melatonin+İ/R ve dimetil sülfoksit (DMSO) kontrol olmak üzere beş gruba ayrılmış 30 erkek fare üzerinde çalıştık. Alt ekstemite iskelet kasında iskemi-reperfüzyon prosedürü, kontrol grupları ve melatonin ile tedaviden sonra yapılan İ/R prosedürlerinden sonra, kas dokusunda antioksidan kapasite (TAS), oksidatif stres belirteçleri (TOS ve MDA), inflamasyon (MPO) ve hücre ölümü (TUNEL ve histoloji) değerlendirildi.
BULGULAR: Melatonin, diğer tüm gruplara kıyasla antioksidan kapasiteyi (TAS) önemli ölçüde artırdı. Melatonin uygulanan İ/R grubunda, oksidatif stres (TOS) seviyeleri, melatonin uygulanmayan İ/R grubuna göre belirgin şekilde daha düşük bulundu. Histolojik analizde, İ/R grubunda diğerlerine kıyasla daha fazla nekroz, ödem, inflamasyon ve hücre ölümü olduğu ortaya koyuldu. İlginç bir şekilde, melatonin+İ/R grubu, İ/R grubuna göre önemli ölçüde daha az hasara sahipti ve bu da melatoninin koruyucu etkisini vurgulamaktadır.
SONUÇ: Bu çalışma, eksojen olarak verilen melatoninin, İ/R hasarına maruz kalan iskelet kası dokusunda oksidatif stresi, inflamasyonu ve hücre ölümünü etkili bir şekilde azalttığını göstermektedir. Bu sonuçlar, melatoninin iskelet kası hasarında İ/R kaynaklı komplikasyonları azaltmak için umut verici bir terapötik ajan olabileceğini düşündürmektedir.

Anahtar sözcükler: İskelet kası; iskemi-reperfüzyon; melatonin; fare.

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