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Melatonin Attenuates Ischemia/Reperfusion Injury of Rat Flap through the Rip3-Mlkl-Mptp Necroptotic Pathway

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Abstract

In this study, we investigated the protective effects of melatonin on Ischemia/Reperfusion (I/R) injury model in rat flaps and its possible mechanism. In total, 60 male Sprague-Dawley rats were randomly divided into three groups: Sham operation group (SH group), normal saline group and melatonin group. After I/R treatment, rats in the operation group were treated with normal saline and melatonin. The flap survival rate was calculated on the 7th day after operation. Immunohistochemistry and RT-qPCR were used to detect the expression of RIP3, MLKL and MPTP. Compared with the normal saline group, the flap survival area of in the SH group and the melatonin group was larger. The expression of RIP3, MLKL and MPTP were highly expressed in the normal saline group, which was significantly decreased in the melatonin group. In addition, the expression of necrosis factor in the SH group and the melatonin group were similar and significantly lower than that in the normal saline group, suggesting that the protective effect of melatonin on skin I/R necrosis in rats may be associated with the necrotic pathway.

Keywords: Flap; Ischemia/reperfusion; Melatonin

Introduction

Flap transplantation is the most classical and important method for tissue defect repair caused by trauma, tumor resection and congenital malformations in clinical practice. Perforator flaps were first described by Koshima in 1989 [1] and have been widely used in the reconstruction of soft tissue defects. Due to the diversity and flexibility of the design, the free perforator flap has greatly replaced the traditional flap. Despite improvements in microsurgical techniques and equipment, flap necrosis remains a major surgical complication. Lipid Peroxides (LPO), Reactive Oxygen Species (ROS) and etc., as well as severe inflammatory reactions secondary to flap Ischemia/Reperfusion (I/R) injury are extremely harmful to patients, which are considered to be the key factors causing flap necrosis [2].

A variety of factors are involved in flap I/R injury, including activation of reactive oxygen free radicals, release of inflammatory factors and apoptosis. In recent years, necroptosis, as an important alternative of cell death, has been increasingly considered as an important process in the cellular life cycle. Necroptosis is a special type of cell necrosis, different from apoptosis, autophagy and pyroptosis, which is regulated by specific molecular mechanisms and is associated with a series of human diseases including I/R injury. Necroptosis plays an important role in I/R injury of heart [3], brain [4], liver [5] and kidney [6]. A study by Liu H et al. [7] has shown that necroptosis was present in a rat abdominal I/R injury flap model.

Receptor-Interacting Protein (RIP) kinases are members of the serine/threonine kinase family, which are considered as key enzymes regulating necroptosis [8], especially RIP1 and RIP3. The interaction between RIP1 and RIP3 is essential for necroptosis [9]. After the formation of Necrosome, RIP3 recruits a specific substrate protein, Mixed Lineage Kinase Domain-like protein (MLKL), and phosphorylates its 375 threonine and 358 serine. MLKL phosphorylation is the marker of necroptosis onset. Phosphorylated MLKL would switch from monomer to oligomer and translocate to cell membrane and organelle membranes [10]. On these membrane structures, oligomerized MLKL can be combined with phosphoinositide and cardiolipin, which can subsequently destroy the membrane integrity and form penetrating pore channels on these membrane structures. Pore channels may cause: 1. Excessive exudation of sodium ions and excessive permeability of calcium ions, causing changes of intracellular osmotic pressure and subsequent cell swelling. 2. Cell membrane ruptures and release of cell contents. In addition, MLKL can also combine necrosome with Phosphoglycerate Mutase 5 (PGAM5). The downstream execution event of necroptosis is the opening of RIPK3-

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activated PGAM5 and MPTP [11].

Melatonin is one of the main secretory products of the pineal body [12], and has been used to treat cardiac IR injury in several clinical trials [13,14], showing that the final range of myocardial infarction is attenuated in patients, and the supplementation of melatonin in the cardiac IR environment can maintain microvascular perfusion and reduce endothelial death. In 2018, Zhu H et al. [15] have shown that necroptosis is a new approach causing reperfusion-mediated microvascular injury. Meanwhile, melatonin treatment can inhibit the RIP3-PGAM5-CypD-MPTP cascade, thereby reducing necroptosis and exerting an important protective effect on myocardial I/R injury. Based on these findings, relieving endothelial necroptosis by melatonin supplementation would be considered as the effective way to reduce microvascular injury caused by reperfusion. However, there are no studies investigating the regulatory mechanism of melatonin on necroptosis of rat flap I/R injury. The present study was designed to investigate whether melatonin can reverse IR injury in rat flaps by inhibiting necroptosis and normalizing the RIP3-MLKL-MPTP pathway.

Materials and Methods

Animals and groups

In total, 60 6-8-week-old male Sprague-Dawley rats (weight: 280 gm to 320 gm) were purchased and maintained in at 22°C to 25°C with sufficient food and water. All procedures were performed strictly according to the "Guidelines for the Care and Use of Laboratory Animals" of National Institutes of Health. This study was conducted in accordance with the requirements of the Ethics Committee of Experimental Animal Rights Protection of our hospital. Rats were randomly divided into three groups before operation: Sham operation (SH) group (N=20), normal saline group (N=20), melatonin group (N=20).

Melatonin treatment

According to pre-experiment results, animals in the melatonin group were intraperitoneally injected with melatonin (20 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) immediately after I/R. Meanwhile, rats in the normal saline group were abdominally injected with the same volume of normal saline with the same method.

Surgical operation

A model of flap I/R injury was established according to the protocol introduced by Zhao G et al. [2]. In brief, rats were intraperitoneally anesthetized with 10%, 1 ml/300 gm chloral hydrate. A 6 cm × 9 cm rectangular flap was drawn on the abdomen, followed by good disinfection. The skin was cut along the medial border of the flap, followed by separation of the subcutaneous tissue close to the mesial layer. The flap was gradually lifted and the three-vessel pedicle was separated, the arteria thoracodorsalis perforator and intercostal artery perforator were ligated, and arteria circumflex ilium profunda perforator was retained and completely separated. After lifting the flap, the left arteria circumflex ilium profunda perforator was clamped for 3 h in rats from the normal saline and melatonin groups, followed by placing of a 0.1 mm-thick silicone sheet between the flap and the recipient bed to prevent revascularization. Reperfusion was started by removing the clamps and confirmed by pulse recovery of angioplerosis.

Survival rate of flap

The flap survival was grossly observed and recorded 12 h, 3 h, 5 h, and 7 days after reperfusion, including the flap color, tissue elasticity, texture, skin and hair growth, infection, necrosis, and etc. On the seventh day after operation, rats were anesthetized and high-quality photographs were obtained using a digital camera and imported into Image-Pro Plus v6.0 software to calculate the percentage of flap survival area. The flap survival rate was calculated according to the following formula:

Flap survival rate = flap survival area/total flap area × 100%

The criteria of flap necrosis were as follows: The flap color was black, the tissue was hard, shrunk, dry necrosis, inelastic, and no blood outflow when cutting.

Flap perfusion volume

On the seventh day after operation, rats were fixed in the supine position after anesthesia, followed by observation of flap blood flow by a laser Doppler perfusion imager. Briefly, the laser probe was placed on the upper part of the flap area, followed by setting of the corresponding observation range, including the flap and surrounding normal skin. After measuring the skin blood flow volume in the setting area, LDF automatically reported the skin blood flow data and different color images within the measurement range. Red represented the maximum perfusion of the flap, black represented the smallest or no blood perfusion, and yellow and blue were between red and black. Each measurement time exceeded 1 min, and the average blood flow was recorded as perfusion unit (pu). The laboratory temperature was maintained around 25°C throughout the measurement.

Seven days after reperfusion, rats were sacrificed by high-dose anesthesia. Afterwards, one section of flap tissue (1 × 1 cm^2) was resected from the proximal flap vascular axis for Hematoxylin-Eosin (H&E) staining, Immunohistochemistry (IHC) and RNA extraction.

H&E staining

Samples were embedded in paraffin, sectioned, and mounted on glass slides. The sections were subjected to HE staining and histological examination.

IHC

Paraffin-embedded sections were dewaxed, rehydrated, and incubated with 3% H2O2 for 10 min to block endogenous peroxidase. Afterwards, the sections were heated at 95°C in citrate buffer for 15 min for antigen retrieval, followed by incubation with normal goat serum at 37°C for 30 min to block non-specific staining. The sections were placed in a humidified chamber and were incubated with anti-RIP3 (1: 200, Abcam, Cambridge, UK), anti-MLKL (1:200, Abcam, Cambridge, UK), and anti-MPTP (1:100, Abcam, Cambridge, UK) at 37°C for 2 h. The sections were then rinsed with PBS, stabilized by DAB and counterstained by hematoxylin. Brown indicated the presence of antibody binding to the antigen and was detected by a light microscope with a computer-controlled digital camera and imaging software.

RNA extraction and RT-qPCR

Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Siegen, Dusseldorf, Germany) according to the manufacturer's instructions. The concentration of the extracted RNA was determined by a UV spectrophotometer (Thermo, Waltham, MA, USA), followed by observation of RNA integrity by electrophoresis on a 1% agarose gel. Afterwards, 1 μg of total RNA was reversely transcribed into cDNA using ProtoScript-M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Lpswich, MMA, USA) and anchored oligo

(dT) primers [d (T) 23VN]. A real-time quantitative PCR system (Agilent, Santa Clara, CA, USA) was used for RT-qPCR. The specific primer sequences of RIP3, MLKL, and MPTP used in this study were shown in Table 1. The expression of gene of interest was normalized to the expression level of the β-*actin* gene as the internal control. The condition for real-time PCR was as follows: UDG pretreatment at 50°C for 2 min, initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15s, annealing at TM for 1 min, and extension at 72°C for 30s.

Statistical analysis

All data were shown as mean ± SD in this study. One-way Analysis of Variance (ANOVA) along with LSD-t test was used to determine statistical significance. After statistical processing, $P<0.05$ indicated statistical significance. SPSS24.0 software was used for statistical analysis.

Results

Survival rate of flaps

On the third day after operation, the necrotic flap was inelastic, brown, gray, or black. Instead, the survived tissue was pink and elastic. On the seventh day after operation, the statistical analysis was shown in Figure 1A. The survival rate of flap in the SH group was 92.684 ± 2.532%, which was the highest among the three groups. Compared with the normal saline group (23.457 ± 1.564%), the survival rates of flaps in the SH group and melatonin group (80.543 ± 2.175%) were significantly improved (normal saline group *vs.* SH, $P<0.001$; normal saline group *vs.* melatonin, $P<0.001$; Figure 1A).

Flap perfusion

On the seventh day after operation, the average blood perfusion volume in the SH group was (197.612 ± 15.240) PU. The average blood perfusion volume of the melatonin group and the normal saline group was (148.531 ± 9.753) PU and (61.743 ± 8.562) PU, respectively. The significant differences among tissues suggested that melatonin might be able to relieve inflammation (Figure 1B).

HE staining

HE staining was used to evaluate ischemic injury by assessing inflammatory infiltration (Figure 2). As a result, inflammatory infiltration was observed in all three groups. However, the number of inflammatory cells in the skin tissue of the normal saline group was the largest, indicating that melatonin may be able to attenuate the inflammatory response.

Expression of RIP3, MLKL and MPTP proteins after I/R injury

IHC assay was used determine the expression of target proteins, including RIP3, MLKL and MPTP, which have been validated as key proteins to be involved in necroptosis. IHC assay showed that the protein expression of RIP3, MLKL and MPTP in the normal saline group were higher than that in the SH group and melatonin group (Figure 3). The percentage of positive staining cells by immunofluorescence was shown in Table 2. The expression of RIP3, MLKL and MPTP was significantly different between the melatonin group and the normal saline group ($P<0.001$).

mRNA expression of RIP3, MLKL and MPTP

Seven days after I/R, the mRNA levels of RIP3, MLKL and MPTP in Melatonin group were 0.745 ± 0.013, 0.656 ± 0.021 and 0.821 ± 0.023, respectively, compared with the normal saline group.

Discussion

In this study, we investigated the protective effect of melatonin on I/R injury in a rat model. Intraperitoneal injection of melatonin after flap I/R injury could significantly improve flap survival rate and inhibit necroptosis in flap cells after I/R injury. In addition, IHC assay and RT-qPCR analysis indicated that the anti-necroptosis effects of melatonin might be associated with the decreased levels of RIP3, MLKL and MPTP. Therefore, melatonin is a potential novel type of anti-necroptosis drug to relieve flap I/R injury.

Accumulative evidence has indicated that necroptosis plays a vital role in the pathogenesis of multiple types of diseases and tissue injury,

Table 1: Sequences of primers used for quantitative real-time PCR.

Target gene	ID	Forward	Reverse
RIP3	246240	CGTGGAGCAGTGTGGAACAG	GGCTCAGAACTCCAGCATG
MLKL	690743	TAGTCCTGAGGGCAGCTAGA	CTGCTGATGTTTCGGTGGAG
MPTP	114114	TGATGCCTGTGGGCTAATGA	GTTCCTGACCACCATCTCCA

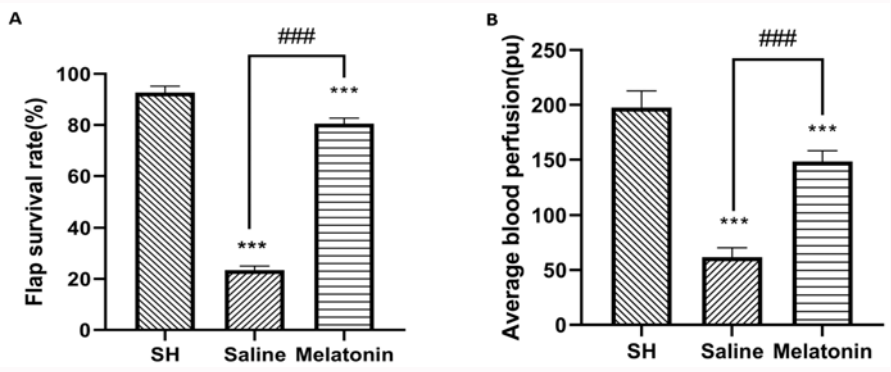

Figure 1: Flap survival rate and blood perfusion seven days of I/R injury. A. Survival rate of the total flap area. The survival rates of flaps in the SH group and the melatonin group were significantly higher than that in the normal saline group; B. The average blood perfusion volume of the whole flap: The average blood perfusion volume of the total flaps in the melatonin group was higher than that in the normal saline group. The mean value was shown as means ± SD (n=15/group; *** compared with SH group $P<0.001$; comparison of the Melatonin group and Saline group, ### $P<0.001$).

Table 2: Percentage of positive cells by immunofluorescence staining.

Factor	Percentage of positive cells			P* value
	SH	Saline	Melatonin	
RIP3	0.5322 ± 0.0312	0.8958 ± 0.0251	0.6723 ± 0.0215	<0.001
MLKL	0.1832 ± 0.0262	0.7864 ± 0.0241	0.4568 ± 0.0153	<0.001
MPTP	0.1659 ± 0.0135	0.8754 ± 0.0214	0.3762 ± 0.0142	<0.001

* Saline *vs.* Melatonin

The percentage of immunofluorescent positive cells in each group. The percentage of RIP3, MLKL and MPTP positive cells was the highest in the normal saline group. Data were shown as Means ± SD

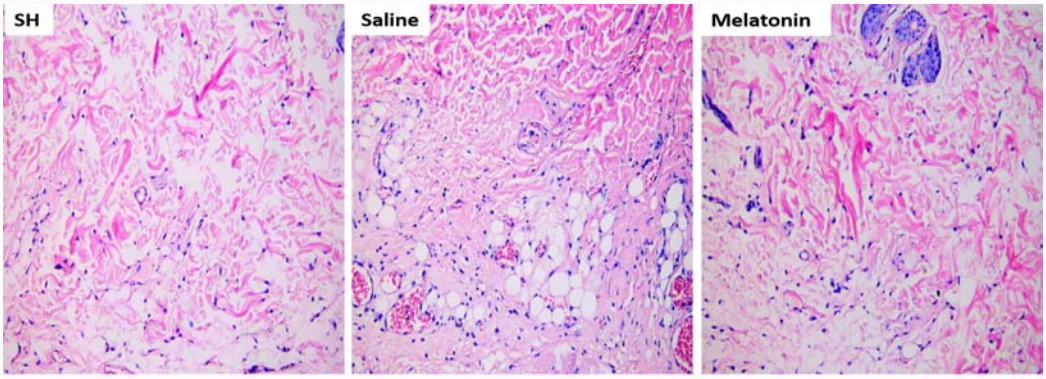

Figure 2: H&E staining 72 hours after I/R injury. The tissue morphology (400x) of flaps in each group by HE staining. There were fewer infiltrating cells in the SH and melatonin groups than those in the normal saline group.

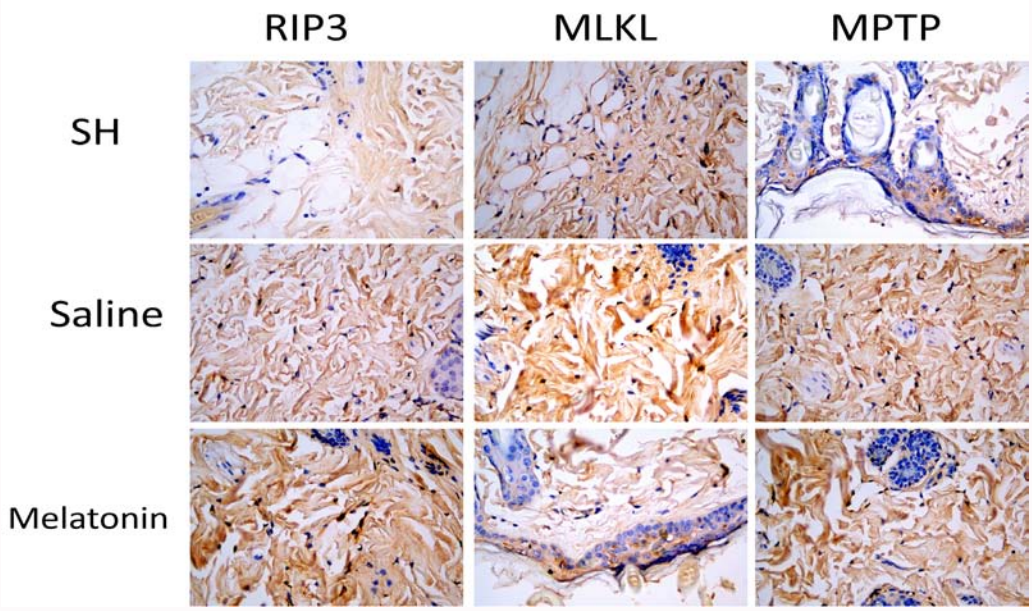

Figure 3: Representative IHC image (400x) of RIP3, MLKL and MPTP staining in the skin tissues of the above three groups. Brown staining indicated areas of positive expression, and the color intensity represented the expression level of protein of interest. The expression level of RIP3, MLKL and MPTP in the skin tissue of rats in the melatonin group was lower than that in the normal saline group.

such as brain, heart, lung, and kidney [3-6]. Therefore, necroptosis has become an exciting new research field in biomedicine. Recent studies have shown that inhibition of necroptosis can rescue flap I-R injury [7]. Numerous studies have clarified the complex mechanism of necroptosis triggered by certain factors (including I/R injury) [8-10]. After inhibiting caspase-8 activity, RIP3 and RIP1 interact through their respective isotype interaction motifs [16], and this domain is located at the C-terminus of RIP1. RIP3 subsequently undergoes autophosphorylation at serine 227 [17], leading to the recruitment of the pseudokinase MLKL [18], which further interacts with RIP1/RIP3 to form a dead body. The dead body binds to PGAM5, and subsequently recruits and activates mitochondrial fission factor, eventually leading to mitochondrial fragmentation and cell death [19].

Based on the above-described theory, in this study, we mainly focus on the application of melatonin to reveal the protective effect of melatonin on I/R injury of rat flaps by inhibiting necroptosis. According to our results, melatonin exerts a protective effect on I/R injury of the flap, which is characterized by decreased ischemic area

and a decreased cellular necroptosis. Compared with the normal saline group, the survived area and blood perfusion are significantly increased, while the expression of RIP3, MLKL and MPTP is significantly decreased in the melatonin group, suggesting that the protective effect of melatonin is associated with the inhibition of the necroptosis signal transduction. In this study, we establish a new theory to explain the mechanism of melatonin in treating flap I/R injury, which provides a new perspective for methods of alleviating I/R injury in flap transplantation and for developing novel therapeutic strategies. In terms of the limitations of this study, the specific molecular mechanism of the protective role of melatonin in necroptosis has not been completely clarified in this study, and we will explore its specific mechanism in future studies.

Conclusion

In this study, we show that melatonin can inhibit skin I/R injury and improve the survival rate of flaps by attenuating necroptosis. In addition, we also provide new evidence that the anti-necroptosis effect of melatonin may be associated with the regulation of the expression of RIP3, MLKL and MPTP. These results might help elucidate the therapeutic mechanism of melatonin.

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