Original Article

Melatonin reduces rhabdomyolysis-induced acute renal failure in rats

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Abstract: Rhabdomyolysis is one cause of acute renal failure, and melatonin could attenuate organ damage with its antioxidant activity and anti-inflammatory effects. In this study, we aimed to investigate the possible effects of melatonin on glycerol-induced rhabdomyolysis and acute renal failure in rats. After an intramuscular injection of 10 mL/kg 50% glycerol in rats, rhabdomyolysis was induced. Ten minutes later, the rats were administered an intravenous injection of melatonin (10 mg/kg in 0.5 mL normal saline). Blood urea nitrogen (BUN), creatinine (Cre), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and creatine phosphokinase (CPK) were measured at 0, 1, 3, 6, 12, 18, 24, and 48 h. The rats were sacrificed 48 h after the glycerol injection, and the kidneys were removed immediately for pathological and immunohistochemistry (IHC) examinations. The results showed that glycerol-induced rhabdomyolysis significantly increased blood BUN and Cre levels; induced severe histopathologic damage in the kidneys. In the kidneys, increased expression of nuclear factor-κB (NF-κB) and inducible nitric oxide synthase (iNOS) and decreased expression of E-cadherin were detected using IHC. Treatment with melatonin decreased serum BUN and Cre levels; suppressed NF-κB and iNOS expression; decreased renal tissue injury scores; and preserved E-cadherin expression in rhabdomyolysis-induced acute renal failure. The beneficial effects of melatonin were observed as it helped protect the kidneys from glycerol-induced rhabdomyolysis in rats.

Keywords: Acute renal failure, melatonin, rhabdomyolysis

Introduction

Rhabdomyolysis is characterized by the leakage of muscle cell contents, including electrolytes, myoglobin, and other sarcoplasmic proteins (e.g., creatine phosphokinase [CPK], glutamic oxaloacetic transaminase [GOT], and glutamic pyruvic transaminase [GPT]) into the circulatory system [1-3]. It ranges in severity from an asymptomatic elevation of CPK levels in blood to severe life-threatening cases associated with acute renal failure [1]. Released heme proteins from rhabdomyolysis-generated free-radical formation, scavenged nitric oxide, and activated endothelin receptors produce a synergistic effect on renal vasoconstriction and induce intraluminal cast formation that causes myoglobinuric acute renal failure [4, 5].

Melatonin, the major product of the pineal gland, exerts their pleiotropic effects through antioxidant activity and anti-inflammatory effects [6-8]. A study noted that melatonin could help protect the kidneys from oxidative damage secondary to mercuric chloride in rats [9]. Melatonin can protect the kidneys from inflammation by improving the course of chronic renal failure in rats with renal mass reduction [10]. Our previous study also noted treatment with melatonin suppresses the release of serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and ameliorates hemorrhagic shock-induced renal failure in rats [11]. In the present study, we investigated the effects of melatonin on blood biochemistry, renal histopathology, E-cadherin, inducible nitric oxide synthase (iNOS), and nuclear factor-κB (NF-κB)
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**Materials and methods**

**Preparation of animals**

Twenty-four male Sprague-Dawley rats weighing 280-300 g were purchased from the National Animal Center. The rats were housed in our animal center under a controlled environment at a temperature of 22 ± 1°C with a 12-h light/dark cycle. Food and water were provided ad libitum. The experimental protocol was approved by the Animal Usage Regulation Committee of Tzu Chi Hospital. The animals were anesthetized with ether inhalation for about 10 minutes. During the period of anesthesia, polyethylene catheters (PE-50) were inserted into the femoral artery for collecting blood samples and the femoral vein for intravenous administration of drugs or fluids. The operation was completed within 15 minutes, and the wound was as small as possible (<0.5 cm²). After the operation, the animal was placed in a conscious rat metabolic cage (Shingshieying Instruments, Hualien, Taiwan). The rats awakened soon after the operation, and rhabdomyolysis was induced 24 h later while the rats were in a conscious state [11-13].

**Experimental design**

The animals were randomly divided into 3 groups. In the vehicle group (n = 8), rats were intramuscularly injected in each hind leg with 10 mL/kg normal saline and then were immediately intravenously administered melatonin (10 mg/kg; Sigma Chemical, St. Louis, MO, USA) in a 0.5-mL normal saline injection [11]. Rats in the glycerol group (n = 8) were intramuscularly injected in each hind leg with 50% glycerol (10 mL/kg; Sigma Chemical, St. Louis, MO, USA) and then were immediately intramuscularly administered a 0.5-mL normal saline injection [12, 13]. In the glycerol/melatonin group (n = 8), rats were intramuscularly injected in each hind leg with 50% glycerol (10 mL/kg) and then were intravenously administered melatonin (10 mg/kg; Sigma Chemical, St. Louis, MO, USA) in a 0.5-mL normal saline injection after rhabdomyolysis. Rats were sacrificed by decapitation 48 h after glycerol administration.

**Blood sample analyses**

To measure BUN, Cre, GOT, GPT, and CPK, 0.5-mL blood samples were obtained before rhabdomyolysis and 1, 3, 6, 12, 24, 48 h after glycerol administration. Blood samples were immediately centrifuged at 3000 g for 10 min. The serum was decanted and stored at 4°C. Biochemical examinations were performed within 1 h of specimen collection. Serum BUN, Cre, GOT, GPT, and CPK levels were measured using an autoanalyzer (COBAS Integra 800, Roche Diagnostics, Basel, Switzerland) [11-13].

**Histological examination**

The kidneys were removed immediately after sacrifice. Tissue specimens were fixed overnight in 4% buffered formaldehyde, processed using standard methods, and stained with hematoxylin and eosin (H&E). The observer who performed the tissue analyses was blinded to which specimen groups the rats belonged. Renal tissue injury was scored by estimating the percentage of tubules in the cortex or outer medulla that had epithelial necrosis, luminal necrotic debris, or tubular dilation, as well as the development of heme casts, as follows: 0, none; 1. <5%; 2. 5% to <25%; 3. 25% to 75%; and 4. >75% [12, 13]. All evaluations were made according to 5 fields per section and 5 sections per kidney.

**Immunohistochemistry**

For immunohistochemistry, 4-μm serial sections were deparaffinized, rehydrated, and incubated with different mouse monoclonal antibodies at 4°C overnight according to the manufacturer’s directions. Antigen retrieval was used for E-cadherin, nuclear factor-kB/P65 (NF-kB p65), and iNOS. Dilutions were 1 in 100 for E-cadherin, NF-kB/p65, and iNOS (Neomarkers, Lab Vision Corporation, Fremont, California, USA). After incubation, tissue sections were covered with biotinylated goat anti-mouse polyvalent secondary antibody (1:100 dilution) and incubated at room temperature for 30 min. After washing, the slides were incubated in peroxidase-conjugated streptavidin-biotin complex (Dako, Copenhagen, Denmark) for 10 min. Cells positive for E-cadherin, NF-kB/p65, and iNOS were semi-quantituated using immunohistochemistry (IHC) performed on par-
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Figure 1. Change in hemoglobin (A), creatine phosphokinase (CPK) (B), glutamic oxaloacetic transaminase (GOT) (C), glutamic pyruvic transaminase (GPT) (D), blood urea nitrogen (BUN) (E) and creatinine (Cre) (F) after rhabdomyolysis-induced acute renal failure in rats. *P<0.05 for the Glycerol group compared with the Vehicle group. #P<0.05 for the Glycerol + Melatonin group compared with the Glycerol group.
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affin-embedded tissue sections and counted in 10 high-power fields (× 200) per section; data are expressed as the percentage of positivity in the total area examined [12, 13]. All scoring was performed in a blinded manner on coded slides.

Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons among different groups at corresponding time points were made by repeated measures of two-way analysis of variance, followed by a post hoc test (Bonferroni’s method). Histological scores were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test. A p value of <0.05 was considered significant.

Results

Compared with the vehicle group, hemoglobin was increased at 1 and 3 h and further decreased 12, 18, 24, and 48 h after rhabdomyolysis (*P<0.05; Figure 1A). Compared with the glycerol group, treatment with melatonin did not significantly increase hemoglobin after induction of rhabdomyolysis (Figure 1A). Serum CPK peaked at 6 h and increased significantly 1, 3, 6, 12, 18, 24, and 48 h after glycerol injection, as compared with the corresponding values in the vehicle group (*P<0.05; Figure 1B). Serum CPK values in the glycerol/melatonin group were significantly lower at 3, 6, 12, 18, 24, and 48 h after rhabdomyolysis, as compared with the corresponding values in the glycerol group (*P<0.05; Figure 1B).

Serum GOT peaked 24 h after rhabdomyolysis (Figure 1C). Compared with the vehicle group, serum GOT values increased significantly at 1, 3, 6, 12, 18, 24, and 48 h after glycerol injection (*P<0.05; Figure 1C). Serum GOT values in the glycerol/melatonin group were significantly lower 3, 6, 12, 18, 24, and 48 h after rhabdo-
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myolysis, as compared with the corresponding values in the glycerol group ("P<0.05; Figure 1C). Serum GPT values increased significantly 1, 3, 6, 12, 18, 24, and 48 h after glycerol injection, as compared with the corresponding values in the vehicle group ("P<0.05; Figure 1D). GPT values in the glycerol/melatonin group were significantly lower 3, 6, 12, 18, 24, and 48 h after rhabdomyolysis, as compared with the corresponding values in the glycerol group ("P<0.05; Figure 1D).

Serum BUN increased significantly 1, 3, 6, 12, 18, 24, and 48 h after glycerol injection, as compared with the corresponding values in the vehicle group ("P<0.05; Figure 1E). Serum BUN values in the glycerol/melatonin group were significantly lower 3, 6, 12, 18, 24, and 48 h after rhabdomyolysis, as compared with the corresponding values in the glycerol group ("P<0.05; Figure 1E). Serum Cre increased significantly 3, 6, 12, 18, 24, and 48 h after glycerol injection, as compared with the corresponding values in the vehicle group ("P<0.05; Figure 1F). Compared with the glycerol group, serum Cre values were significantly lower in the glycerol/melatonin group 3, 6, 12, 18, 24, and 48 h after rhabdomyolysis ("P<0.05; Figure 1F).

In the rat kidneys in the vehicle group, we found no epithelial necrosis or heme casts (Figure 4C). The kidneys obtained from the glycerol group demonstrated moderate epithelial necrosis, tubular dilation, and several heme casts after rhabdomyolysis (Figure 2A). The renal tubules from the rats in the glycerol/melatonin group demonstrated mild epithelial necrosis and tubular dilation (Figure 2B). The renal tissue injury scores significantly increased after glycerol injection, as compared with the corresponding scores in the vehicle group at 48 h.
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Compared with the glycerol group, the renal tissue injury scores in the glycerol/melatonin group were significantly lower after rhabdomyolysis (*P<0.05; Figure 2D).

In the vehicle group, IHC staining of the kidney sections for E-cadherin indicated a normal quantity and distribution of E-cadherin (Figure 3C). After the glycerol injection and onset of rhabdomyolysis, IHC for E-cadherin showed a great E-cadherin deficiency in the renal tubule cells (Figure 3A). In the glycerol/melatonin group, IHC showed preservation of E-cadherin in the renal tubule cells after glycerol injection (Figure 3B). The percentage of E-cadherin-positive cells in the renal tubules was significantly lower in the glycerol group, as compared with the vehicle group (**P<0.05; Figure 3D). After rhabdomyolysis, the glycerol/melatonin group had significantly greater E-cadherin positivity in the renal tubular cells, as compared with the glycerol group (**P<0.05; Figure 3D).

The renal tubule cells in the vehicle group showed mildly increased iNOS expression (Figure 4C). After the glycerol injection and onset of rhabdomyolysis, IHC for iNOS showed increased iNOS in the glycerol group (Figure 4A). The glycerol/melatonin group demonstrated less iNOS-positive renal tubule cells after rhabdomyolysis (Figure 4B). Semi-quantification of iNOS-positive cells showed significantly greater numbers in the glycerol group than the vehicle group (**P<0.05; Figure 4D). The glycerol/melatonin group demonstrated significantly fewer iNOS-positive cells than the glycerol group (**P<0.05; Figure 4D).

There were few NF-κB-positive renal tubule cells in the vehicle group (Figure 5C). After the glycerol injection and onset of rhabdomyolysis,
IHC staining for NF-κB showed increased NF-κB in the renal tubular cells of rats in the glycerol group (Figure 5A). The glycerol/melatonin group also demonstrated little NF-κB in the renal tubule cells after rhabdomyolysis (Figure 5B). NF-κB positive cells in the kidneys were significantly higher in the glycerol group than the vehicle group (*P<0.05; Figure 5D). The glycerol/melatonin group demonstrated significantly fewer NF-κB-positive cells in the kidneys than the glycerol group after rhabdomyolysis (*P<0.05; Figure 5D).

Discussion

In this study, it was found that treatment with melatonin reduced serum GOT, GPT, and CPK elevation after glycerol-induced rhabdomyolysis and ameliorated rhabdomyolysis-induced acute renal failure accompanied by decreasing renal tissue NF-κB and iNOS production after rhabdomyolysis in rats.

Rhabdomyolysis is a potentially life-threatening syndrome characterized by the breakdown of skeletal muscle resulting in the subsequent release of intracellular contents into the circulatory system [1, 3, 14]. These cell contents include enzymes such as CPK and GOT, heme pigment myoglobin, and electrolytes [1-3]. Excess myoglobin may cause renal tubular obstruction, direct nephrotoxicity, and acute renal failure [1, 2]. Acute renal failure develops in up to 15% of patients and is associated with high morbidity and mortality [15]. Our results showed that posttreatment with melatonin reduced rhabdomyolysis-induced serum BUN, Cre, GOT, GPT, and CPK elevation and also alleviated histopathologic changes in the kidneys after a glycerol injection in rats.
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After rhabdomyolysis, released muscle heme proteins are metabolized by the kidney, generating free-radicals, scavenging nitric oxide, and activating endothelin receptors, which produces a synergistic effect on renal vasoconstriction and induces intraluminal cast formation causing myoglobinuric acute renal failure [4, 5]. Moreover, the loss of cell adhesion due to reduced levels of E-cadherin weakens the junctions between cells, allowing filtrate to leak back into the renal interstitium, which impairs renal function [16]. Many studies over the past 4 decades have demonstrated that the accumulation of myoglobin in the kidney is the main mechanism leading to kidney injury. However, some discussion exists regarding the mechanism mediating this oxidative injury [17]. Melatonin had antioxidant activity that maintains mitochondrial function with oxidative stress [18]. Melatonin scavenges nitric oxide (NO); suppresses the activity of its rate-limiting enzyme, nitric oxide synthase (NOS); and as an indirect antioxidant, stimulates gene expression and activity of superoxide dismutase activity, thereby inducing the rapid conversion of O₂ to less toxic hydrogen peroxide (H₂O₂) [7]. Catalase and glutathione peroxidase enzymes are also stimulated by melatonin [6, 7]. Melatonin stimulates γ-glutamyl cysteine synthase, thereby increasing the level of reduced glutathione (GSH) [7]. Melatonin also has anti-inflammatory effects by suppressing the release of TNF-α and IL-6 production after hemorrhagic shock in rats [11]. Similarly, melatonin ameliorated gastric mucosal injury by decreasing TNF-α and IL-6 activity in gastric tissue during the early stages of acute necrotizing pancreatitis in rats [19]. Melatonin therapy decreased the expressions of Toll-like receptor 4, myeloid differentiation primary response protein, and NF-κB p65 and increased inhibition of NF-κB (IkB) expression per a histological and immunoblot analysis in the local vasculature of high-fat-fed rabbits that induced vascular endothelial dysfunction and typical atherosclerotic plaque formation [20]. Melatonin suppresses visfatin-induced iNOS upregulation in macrophages by inhibiting signal transducers and activators of the transcription 3 and NF-κB pathways [21]. Additionally, melatonin inhibited lipopolysaccharide-stimulated TNF-α, IL-1β, IL-6, IL-8, and IL-10 production in Raw264.7 cells through a mechanism involving the attenuation of NF-κB activation [22]. Our results show that iNOS activity had decreased in the kidneys after melatonin treatment in rhabdomyolysis-induced acute renal failure. Furthermore, posttreatment with melatonin decreased NF-κB activity in the kidneys after rhabdomyolysis, as compared with the glycerol group. These results indicate that melatonin is associated with the suppression of NF-κB and protection against rhabdomyolysis-induced acute renal failure in rats.

After being treated with melatonin, rats with renal ablation showed decreased plasma level of malondialdehyde and p65 NF-κB positive inflammatory cells and renal interstitial cells as well as amelioration of renal function deterioration and tubulointerstitial damages [10]. In rats with heatstroke associated multiple organ failure, melatonin administration was demonstrated to reduce plasma index of toxic oxidizing radicals, attenuate plasma inflammatory cytokines like tumor necrosis factor α, interleukine 6, and improve renal function as well as survival time [23]. In the study of ischemia-reperfusion injury, Chang et al. showed that rats treated with melatonin revealed decreased serum levels of BUN, Cre, protein expressions of inflammatory markers like iNOS, and protein expressions indicative of integrity of podocyte like E-cadherin and P-cadherin [24]. Hrenak et al. demonstrated that melatonin, as well as angiotensin converting enzyme inhibitor and angiotensin receptor blocker, could prevent increase in oxidative stress and loss of glomeruli in doxorubicin-induced nephrotoxicity [25]. Taken these studies together, it indicates that melatonin, which possess anti-oxidant effects and as free radical scavenger, could have renoprotective effects in both acute renal failure and chronic kidney disease.

Moreover, melatonin was demonstrated to stimulate the activity of arginase while inhibiting the concentration of nitric oxide in the kidney tissues of a glycerol-induced rhabdomyolysis rat model [26]. Another similar model of glycerol-induced myoglobinuric acute renal failure revealed that melatonin could decrease the level of renal cortical tubular necrosis and decrease the renal concentration of malondialdehyde through a glutathione-independent pathway [27]. In agreements with these studies showing beneficial against oxidative stress and inflammation of melatonin, our study showed...
that melatonin alleviated histopathological damages arising in the kidney and preserved E-cadherin-positive renal tubule cells, in addition to the demonstration of decreasing renal iNOS activity after rhabdomyolysis induced acute renal failure.

In conclusion, posttreatment with melatonin in a rat model of rhabdomyolysis demonstrated decreased BUN, Cre, GOT, GPT, and CPK levels; less renal tissue damage; suppressed NF-κB and iNOS activity; and preserved E-cadherin expression in the kidneys. Although additional studies are required to elucidate the detailed mechanisms of antioxidant activity and anti-inflammatory effects between melatonin and rhabdomyolysis, our study demonstrated that melatonin has beneficial effects and could protect the kidneys of rats from myoglobinuric acute renal failure.

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Disclosure of conflict of interest

None.

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