

## Colchicine protects rat skeletal muscle from ischemia/reperfusion injury by suppressing oxidative stress and inflammation

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### ABSTRACT

**Objective(s):** Neutrophils play an important role in ischemia/reperfusion (IR) induced skeletal muscle injury. Microtubules are required for neutrophil activation in response to various stimuli. This study aimed to investigate the effects of colchicine, a microtubule-disrupting agent, on skeletal muscle IR injury in a rat hindlimb ischemia model.

**Materials and Methods:** Twenty-one Sprague-Dawley rats were randomly allocated into three groups: IR group, colchicine treated-IR (CO) group and sham operation (SM) group. Rats of both the IR and CO groups were subjected to 3 hr of ischemia by clamping the right femoral artery followed by 2 hr of reperfusion. Colchicine (1 mg/kg) was administered intraperitoneally prior to hindlimb ischemia in the CO group. After 2 hr of reperfusion, we measured superoxide dismutase (SOD) and myeloperoxidase (MPO) activities, and malondialdehyde (MDA), tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  levels in the muscle samples. Plasma creatinine kinase (CK) and lactate dehydrogenase (LDH) levels were measured. We also evaluated the histological damage score and wet/dry weight (W/D) ratio.

**Results:** The histological damage score, W/D ratio, MPO activity, MDA, TNF- $\alpha$  and IL-1 $\beta$  levels in muscle tissues were significantly increased, SOD activity was decreased, and plasma CK and LDH levels were remarkably elevated in both the IR and CO groups compared to the SM group ( $P < 0.05$ ). Colchicine treatment significantly reduced muscle damage and edema, oxidative stress and levels of the inflammatory parameters in the CO group compared to the IR group ( $P < 0.05$ ).

**Conclusion:** Colchicine attenuates IR-induced skeletal muscle injury in rats.

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### Introduction

A timely and effective restoration of blood flow, i.e. reperfusion, is essential for ischemic limb recovery, but reperfusion can also aggravate the initial ischemia-induced injury, which is known as ischemia/reperfusion (IR) injury (1). IR injury not only exacerbates the damage of the local ischemic tissues, but also causes remote organ injury or even multiple organ dysfunction syndromes (2-4). The pathogenesis of IR injury is complex and multifactorial, involving an intrinsic intracellular injury process during the ischemic phase and an induced inflammatory response during the reperfusion phase. Importantly, neutrophils were found to play an important role in IR injury (5-7).

Microtubules are filamentous intracellular structures that are responsible for a variety of movements in all eukaryotic cells, and the microtubule network plays a crucial role in the development and maintenance of cellular polarity.

Therefore, compromising microtubule function in neutrophils is theoretically effective in inhibiting neutrophil migration and limiting neutrophil-dependent injuries (8). Colchicine, a microtubule-disrupting agent, has an anti-inflammatory feature and is currently used in the treatment of inflammation-linked diseases including acute gout, Behcet's disease, Mediterranean fever, recurrent pericarditis, and secondary amyloidosis (9-11). Mechanistically, colchicine was shown to limit neutrophil-mediated inflammation and decrease the levels of inflammatory mediators in several settings (12-16). However, whether colchicine can ameliorate or prevent IR-induced skeletal muscle injury remains unknown.

The present study aimed to investigate if colchicine could protect skeletal muscle against IR-induced injury in a rat ischemic hindlimb model. We also aimed to study the associated molecular basis of colchicine's protective mechanism. We hypothesized

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that colchicine attenuates skeletal muscle damage in response to IR via suppressing oxidative stress and inflammatory response. Our findings point to a potential clinical application of colchicine in treatment of IR injury.

## Materials and Methods

### Group allocation

This study was approved by the Animal Care and Use Committee of Wenzhou Medical University, and all protocols were in accordance with the Guidelines of the National Institutes of Health for Care and Use of Laboratory Animals. Twenty-one male adult Sprague-Dawley rats weighing 250-300 g were obtained from the Animal Center of Wenzhou Medical University and randomly allocated into three groups: IR group, colchicine treated-IR (CO) group, and sham operation (SM) group (7 rats per group). The rats in both the IR and CO groups were subjected to 3 hr of ischemia followed by 2 hr of reperfusion in the right hindlimb. Colchicine (1 mg/kg, Sigma Chemical, St. Louis, MO, USA) or an equal volume of saline was intraperitoneally administered immediately before induction of hindlimb ischemia in the CO or IR group, respectively. The rats in the SM group underwent sham operation.

### Experimental protocol

The rats were deprived of food but not water for 12 hr prior to the surgical procedure. Rats were administered an intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg diazepam and placed in a supine position on a heating pad to maintain body temperature at 36-37 °C. The right femoral artery was then isolated and clamped, and after limb exsanguination, a rubber band was applied on the right greater trochanter for 3 hr to ensure interruption of arterial blood supply to the hindlimb in both the IR and CO groups. After 3 hr of ischemia, the arterial clamps and rubber bands were removed and an additional 2 hr were allowed for reperfusion before sampling. In the SM group, the femoral artery was exposed but not occluded, and the rubber band was in place without being inflated. At the end of reperfusion, rats were euthanized, and the gastrocnemius muscles and blood samples from abdominal aortas were collected for subsequent analysis.

### Histological examination

Muscle tissue samples were placed in 10% buffered formaldehyde for 48 hr, dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin wax. 5 µm thick sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope by a pathologist who was blinded to the experimental groups. The histological damage score was used to

assess the extent of skeletal muscle injury. Muscle fiber degeneration, disorganization, and inflammatory cell infiltration were scored using previously described methods (17).

### Measurement of wet/dry weight (W/D) ratio of muscle tissues

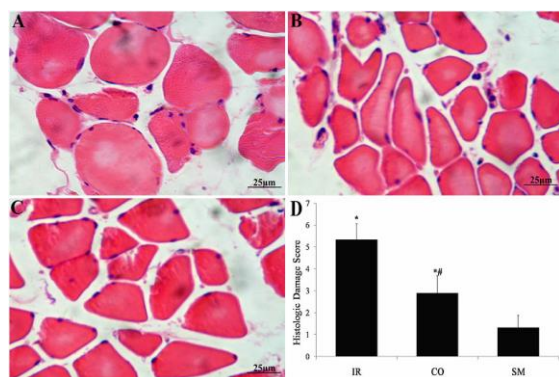
Gastrocnemius specimens were weighed immediately after euthanasia to obtain the 'wet' weight, then dried in an 80°C ventilated oven for 48 hr and weighed again to obtain 'dry' weight. The wet/dry weight (W/D) ratio was then calculated to compare muscle edema between the three groups.

### Determination of oxidative stress and inflammatory parameters in muscle tissues

Muscle tissues were cut into small pieces, homogenized in ice-cold Tris-HCl buffer solution (pH 7.4, 0.2 mmol/l) and then centrifuged at 3,500 ×g for 60 min to obtain supernatant for the subsequent analyses. Superoxide dismutase (SOD) and myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels in the supernatants were measured with a spectrophotometer using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). MDA levels were measured at wavelength 532 nm using the thiobarbituric acid reaction method. Quantification of thiobarbituric acid reactive substances (TBARS) was established comparing the absorption of 1, 1, 3, 3-tetraethoxypropane. The xanthine method employs the xanthine-oxidase system to produce superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to form a purple formazan at wavelength of 550 nm, and one unit of SOD was described as the amount causing 50% inhibition of this reaction at 37 °C and expressed as U/mg total tissue protein. MPO activity was measured using o-dianisidine, as the chromogenic substrate, and H<sub>2</sub>O<sub>2</sub> to initiate the reaction. MPO activity was calculated based on light absorbance at 460 nm. Levels of inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β in injured muscles were measured using commercially available enzyme-linked immunosorbent assay kits (Westang Biotechnology Co. Ltd, Shanghai, China) according to manufacturer's instructions.

### Detection of circulating creatinine kinase and lactate dehydrogenase levels

Blood samples obtained from the abdominal aortas were centrifuged to separate plasma. Sample were then stored at -20 °C for subsequent analysis of creatinine kinase (CK) and lactate dehydrogenase (LDH) levels using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).



**Figure 1.** Representative images ( $\times 400$ ) of H&E staining of skeletal muscle tissues: A, IR group; B, colchicine treatment-IR (CO) group; C, sham operation (SM) group. D: Histological damage scores of muscle tissues in three groups. \*  $P < 0.05$  compared to SM; #  $P < 0.05$  compared to IR

### Statistics analyses

Statistical analyses were performed with SPSS version 13.0 software. Continuous and normally distributed data were presented as mean  $\pm$  standard deviation, and the one-way analysis of variance (ANOVA) followed by the *post-hoc Bonferroni* method were used to compare differences among groups. A  $P$ -value  $< 0.05$  was considered statistically significant.

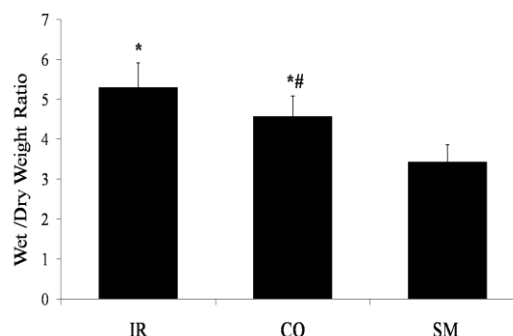
## Results

### Colchicine significantly reduced skeletal muscle damage by IR

First, we used microscopic examination and histological damage scoring system to evaluate the degree of IR-induced muscle injury in the SM, IR and CO groups. Skeletal muscle cell edema, muscle fiber degeneration, necrosis, sarcoplasm dissolution, interstitial vessel hemorrhage, and neutrophil infiltration were commonly observed in skeletal muscles subjected to IR injury in both the IR and CO groups but not in the SM group (Figure 1A - C). However, colchicine treatment significantly reduced this damage, which was accompanied by a decrease in structural damage and scattered neutrophil infiltration (Figure 1, compare A and B). Figure 1D shows higher histological damage scores in both the IR and CO groups compared to the SM group ( $P < 0.05$  vs SM); however, the CO group exhibited a significantly reduced damage score compared to the IR group ( $P < 0.05$  vs. IR).

### Colchicine significantly diminished the IR-induced edema in skeletal muscles

W/D ratio was used as an indicator of edema in gastrocnemius muscles. As shown in Figure 2, IR-induced injury led to a significant increase in muscle edema, as evidenced by higher W/D ratios in the IR and CO groups compared to the SM group ( $P < 0.05$  vs SM); however, colchicine treatment greatly decreased the W/D ratio in the IR-injured



**Figure 2.** The degrees of muscle edema in the IR group, colchicine treated-IR (CO) group and sham operation (SM) group. \*  $P < 0.05$  compared to SM; #  $P < 0.05$  compared to IR

muscles compared to the IR group ( $P < 0.05$  vs. IR).

### Colchicine attenuated the IR-elicited oxidative stress and inflammatory response in skeletal muscles

Next, we evaluated oxidative stress in the IR-damaged skeletal muscle. We measured MPO activity, which represents the number of infiltrated neutrophils, SOD activity, MDA levels, and the inflammatory markers TNF- $\alpha$  and IL-1 $\beta$ . As expected, IR significantly increased MPO activity as well as MDA, TNF- $\alpha$  and IL-1 $\beta$  levels in muscle tissues, but decreased SOD activity in both the IR and CO groups compared to the SM group ( $P < 0.05$  vs. SM; Figure 3). However, colchicine treatment greatly improved MPO activity, MDA, TNF- $\alpha$  and IL-1 $\beta$  levels, and SOD activity compared to the IR group ( $P < 0.05$  vs. IR; Figure 3).

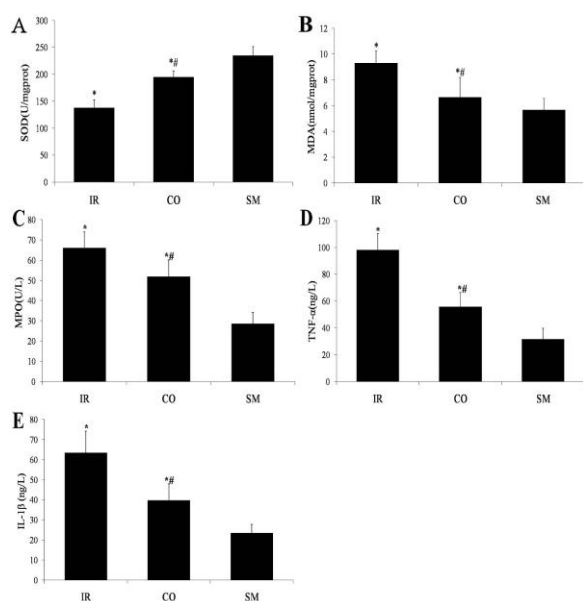
### Colchicine reduced the levels of circulating CK and LDH in IR-injured rats

Plasma CK and LDH levels are considered systemic biomarkers of muscle injury. As shown in Figure 4, plasma CK and LDH levels in both the IR and CO groups were significantly increased compared to the SM group ( $P < 0.05$  vs. SM). As expected, colchicine treatment significantly lowered the levels of circulating CK and LDH compared to the IR group ( $P < 0.05$  vs. IR).

## Discussion

Our study showed that the gastrocnemius muscle was damaged following IR, as evidenced by pathological changes, increased edema and systemic muscle injury biomarkers. Furthermore, our study also revealed that treatment with colchicine prior to ischemia significantly attenuated IR-induced skeletal muscle damage.

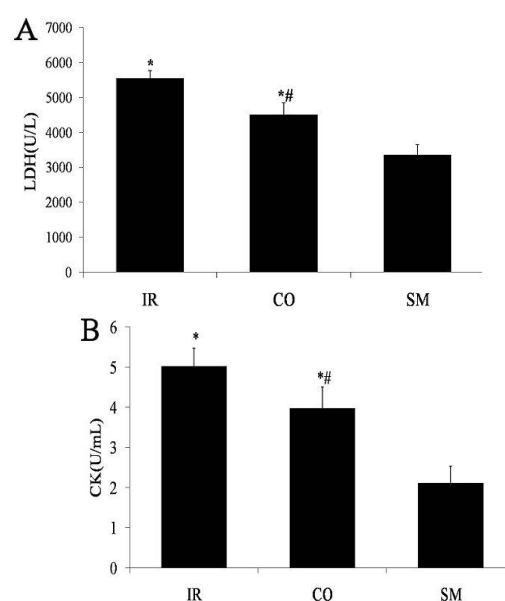
Our study showed an increase in MPO activity in the muscle tissues subjected to IR. MPO, a heme protein, is most abundantly expressed in neutrophils, and increased activity is therefore an indicator of neutrophil activation. Local accumulation of neutrophils is a characteristic



**Figure 3.** Measurement of superoxide dismutase (SOD) activity (A), malondialdehyde (DMA) level (B), myeloperoxidase (MPO) activity (C), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) level (D) and interleukin-1 $\beta$  (IL-1 $\beta$ ) level (E) in skeletal muscle tissues of rats from the three groups. \*  $P < 0.05$  compared to SM; #  $P < 0.05$  compared to IR

feature of IR-induced injury, and neutrophil depletion decreases oxidative stress and attenuates IR-induced skeletal muscle injury (6, 7). Recruited and activated neutrophils are associated with abundant production of reactive oxygen species (ROS) as well as proinflammatory cytokines including TNF- $\alpha$  and IL-1 $\beta$  (1, 18-20). ROS is increased during the early stage of reperfusion and induces expression of adhesive molecules on the microvessel endothelium and facilitates transendothelial migration of leukocytes, which in turn causes further production of ROS (20, 21). The interaction between ROS and membrane lipids leads to an increase in membrane permeability, consequently causing cell death (17, 22, 23). MDA is one of the most commonly used biomarkers of lipid peroxidation and reflects the extent of cellular oxidative injury, while SOD is an important enzyme that catalyzes the conversion of superoxide radical into hydrogen peroxide. As demonstrated in our study, the increased MDA level and decreased SOD activity in the muscle tissues after IR suggests that increased oxidative stress might play a role in IR-induced skeletal muscle injury, which is consistent with previous findings (24, 25).

Increased levels of proinflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , are also implicated in muscle injury induced by IR (4). In agreement with these findings, we observed significant increases in TNF- $\alpha$  and IL-1 $\beta$  levels in the injured



**Figure 4.** The Circulating levels of biomarkers lactate dehydrogenase (LDH, A) and creatinine kinase (CK, B) in rats from the IR group, colchicine treated-IR (CO) group and sham operation (SM) group. \*  $P < 0.05$  compared to SM; #  $P < 0.05$  compared to IR

muscle tissues. TNF- $\alpha$  has been shown to activate polymorphonuclear neutrophils and trigger neutrophil infiltration. Moreover, TNF- $\alpha$  induces chemotactic factors and adhesion molecules in vascular endothelial cells and facilitates leukocytes infiltration, which contributes to excessive inflammatory processes (26, 27). Similarly, IL-1 $\beta$  up-regulates endothelial cell adhesion molecules and induces endothelial cells to release IL-8 which also contributes to excessive inflammatory responses (28). Conversely, polyclonal antibodies against TNF- $\alpha$  and IL-1, or soluble TNF- $\alpha$  receptor and IL-1 receptor antagonists, reduce neutrophil chemotaxis and sequestration and provide significant protection against IR-induced limb injury (4).

Colchicine, a microtubule polymerization inhibitor, has been shown to mitigate the IR injury in the intestine (29), myocardium (30), cerebral tissue (31) and ovarian tissue (32). Mechanistically, 1) by inhibiting mechanical properties of neutrophils, including locomotion and deformability, which are crucial for neutrophil extravasation in response to inflammatory stimuli, colchicine decreases the release of inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , from activated neutrophils (29); 2) colchicine inhibits the generation of leukotriene B4 (LTB4), which acts as a potent stimulus for neutrophil activation, including neutrophil chemotaxis, aggregation and enzyme release (33); 3) colchicine directly modulates TNF- $\alpha$  function (34, 35); 4) Colchicine depresses neutrophil degranulation, which is closely related to the secretory response and oxidative outbreak (36, 37),

and colchicine has been shown to attenuating lipid peroxidation and stabilizing membranes (38). Consistent with these observations, our study demonstrated that 1 mg/kg colchicine in rats significantly decreased MDA, TNF- $\alpha$  and IL-1 $\beta$  levels, and increased SOD activity in IR-injured muscle tissues, indicating that colchicine treatment decreases levels of lipid peroxidation and inflammatory cytokines. However, further studies are needed to: 1) determine the dose-effect relationship and the functional interactions of colchicine with other agents, 2) evaluate the efficacy of colchicine treatment in IR injury in a clinical setting, and 3) investigate the effects of colchicine on remote organ dysfunction following IR injury in skeletal muscle.

### Conclusion

IR leads to skeletal muscle damage, and colchicine treatment prior to ischemia attenuates IR-triggered skeletal muscle injury in part by suppressing the inflammatory response and oxidative stress. Therefore, colchicine may serve as a potential alternative strategy to prevent or treat skeletal muscle injury induced by IR.

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