

Original Article

Garlic antagonizes skeletal muscle ischemia reperfusion injury through regulating inflammation, apoptosis and desmin expression in adult male rats

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Abstract: Background: Skeletal muscle injuries with subsequent bleeding is common cause of death on both sports and battle grounds. Application and removal of tourniquet is fast intervention to control hemorrhage resulting ischemia reperfusion (IR) injury. The effect of IR in skeletal muscle is far more severe compared to other body tissues because of the devastating systemic complication. Garlic has beneficial effects in IR of various organs. However, using garlic in IR of skeletal muscle is deficient. Goals: To investigate the possible protective effect of garlic in rat model of hind limb IR and its possible mechanisms of action. Methods: Fifty adult male rats divided into five groups; C: control, IR: ischemia/reperfusion group subjected to 2 hours ischemia followed by 2 hours reperfusion (2/2 hr IR) and three garlic treated groups; G1+IR: 24 hr before I/R, G2+IR: 30 min before IR and G3+IR: immediately before reperfusion. We measured wet to dry weight ratio (W/D) of gastrocnemius muscle, serum creatine kinase (CK), Interleukin 1 β (IL-1 β), Interleukin-10 (IL-10), gastrocnemius caspase-3 and desmin expression and histopathological damage score. Results: Garlic treatment caused significant decrease in W/D, serum CK, IL-1 β , caspase-3 expression and significant increase in IL-10 as well as desmin expression when compared to IR group. Garlic ameliorated IR-induced histopathological damage and significantly reduced the apoptosis score. Better results obtained with earlier administration before IR. Conclusion: Garlic protected against IR-induced skeletal muscle damage through reducing inflammation, apoptosis score and elevating desmin expression. We recommend the earlier use of garlic as prophylactic natural medicine in skeletal muscle IR.

Keywords: Ischemia reperfusion (IR), skeletal muscle, garlic, interleukin 1 β (IL-1 β), interleukin-10 (IL-10), creatine kinase (CK), caspase 3, desmin

Introduction

Ischemia reperfusion injury is considered as major clinical problem following acute coronary occlusion, organ transplantation, septic conditions and limb injury [1]. The damaging effect of IR in skeletal muscle is far more severe compared to other body tissues because of the devastating systemic complication [2]. Severe muscle exercise, crush injuries, compartment syndrome, hypothermia or shock states are among the most common causes of muscle damage leading to leakage of creatine kinase (CK), myoglobin, troponin and other enzymes that are considered as serum markers of muscle injury [3]. Muscle edema resulting from IR

considered as an important cause of compartment syndrome, morbidity and mortality [4]. Wet to dry weight ratio of the muscle is considered as good indicator of muscle edema [5]. Ischemia caused mitochondrial damage, pump failure, electrolyte imbalance, disturbed enzyme activity, increased water inflow and cell swelling [6]. Reperfusion caused retention of reactive oxygen species, impaired function of the endothelium, inflammation, release of cytokines that promote cell damage and apoptosis [7]. Oxidative stress promoted mitochondrial damage, leakage of proteolytic enzymes, toxins and apoptotic factors like caspase into the cell ending in cell death [8]. Improper function of desmin, a necessary protein for structural

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integrity of the muscle, was associated with abnormal mitochondrial distribution, number, morphology and function [9].

Garlic had long been known historically as flavoring substance, traditional medicine and food that improve health both physically and mentally [10]. Previous studies reported that garlic has protective effect against IR in various organs including heart, brain, kidneys, liver and lungs [11-15]. However, up to our knowledge, the use of garlic in IR of skeletal muscle was deficient. The goal of the current study is to test the possible protective effect of garlic in rat model of hind limb IR through measuring the inflammatory cytokines, serum CK, desmin expression, apoptosis and damage score.

Materials and methods

Chemicals and natural extract

Aqueous garlic extract prepared and given at dose (500 mg/Kg IP); Urethane Cat. No. U 285-7 was purchased from Aldrich chemical company, Inc. (Craftsmen in Chemistry Milwaukee Wis 53233 USA); Creatine phosphokinase (CK) Cat. No. MBSLR 10 purchased from LAB-CARE DIAGNOSTICS (INDIA) (PVT. LTD. C1 Type, Shed No. 3225, Chemical Zone, GIDC Sarigam, SARIGAM - 396 155 Dist. Valsad, INDIA); Rat IL-1 beta ELISA kit, Cat. No. K0331212 and Rat IL-10 ELISA Kit, Cat. No. K0332134, both purchased from KOMA BIOTECH INC. (19F, IS BIZ Tower, Sunyudo Station 1cha, Yangpyeong-ro 21 gil 26, Yeongdeungpo-gu, Seoul 07207, Korea); Primary antibody against caspase-3 for immunohistochemistry (rabbit polyclonal 1:200; ThermoFisher Scientific, CPP32) and desmin (mouse monoclonal 1:200; Agilent Dako, clone D33).

Preparation of fresh aqueous garlic extract

Fifty grams of fresh garlic bulbs bought from local grocery shop, peeled, cut, homogenized with seventy ml normal saline for ten minutes in a blender at thirty second bursts and filtered three times through cheesecloth. The filtrate centrifuged at two thousand round per minute for ten minutes yielding clear supernatant that was diluted to a hundred ml of normal saline and stored at minus twenty degrees Celsius for later use. This final concentration of garlic was

500 mg/ml based on the weight of the starting material (50 g/100 ml) [16].

Animal grouping and experimental approach

Ethical approval: The protocol was approved by the Local Experimental Ethical Committee at Deanship of Scientific Research of Assiut University, Assiut, Egypt. The investigators understand the ethical principles that their work complies with this animal ethics checklist. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Animal groups: A total of fifty male Albino Wistar rats (180-250 gm body weight) obtained from Animal Core Facility, Faculty of Medicine, Assiut University, Assiut, Egypt and kept for 1 week before start of study for acclimatization. Animals were kept in metal cages as three rats per cage, allowed food and water ad libitum under 12 hours light/dark cycle and temperature 25°C.

Rats were divided into three groups; control (C) group ten animals; ischemia/reperfusion (IR) group ten animals; ischemia/reperfusion and garlic (G+IR) 30 animals. The last group subdivided into three groups ten animals each; (G1+IR), (G2+IR) and (G3+IR) as follows; C group: served as control; IR group: animals were subjected to 2 hours (hr) ischemia by application of a rubber band tourniquet around the groin of both hind limbs as high as possible [16] followed by 2 hr reperfusion (2/2 hr IR); G1+IR group: rats were injected I.P. by aqueous garlic extract (500 mg/kg) 24 hours before ischemia. Then, they were subjected to 2/2 hr I/R using the same method of IR group; G2+IR group: was injected I.P. by garlic extract (500 mg/kg) 30 minutes before ischemia. Then, they were subjected to 2/2 hr IR using the same method of IR group; G3+IR group: rats were subjected to 2/2 hr IR using the same method of IR group and were injected I.P. by garlic extract (500 mg/kg) immediately before reperfusion [18].

Anesthesia

All rats were anesthetized using urethane prepared by dissolving the powder (25 gm dissolved in 100 ml distilled water) and injected I.P. at a dose of 600 mg/kg before starting IR

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and maintained on anesthesia during the whole period of the experiment [19].

Samples collection

At the end of the 2 hours reperfusion scarification was done by cervical dislocation and blood samples were collected in non-heparinized tubes, left to clot at room temperature then were centrifuged at 3000 rpm for 10 minutes, sera were separated and divided into small aliquots and kept frozen at -20°C for further biochemical analysis. The right gastrocnemius muscles were dissected out from its origin and insertion for wet/dry weight ratio. The left gastrocnemius muscle was dissected out, washed three times in cold phosphate buffer saline (PBS), and were kept in 10% formalin for further histopathological and immunohistochemical study processing.

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

Gastrocnemius specimens were weighed immediately after dissection to obtain the 'wet' weight then were dried in at 80°C ventilated oven for 48 hr and reweighed again until reaching constant weight to obtain 'dry' weight. The W/D was then calculated to compare muscle edema between the groups by using the following equation: $[(\text{wet weight} - \text{dry weight}) / \text{wet weight} * 100]$ [5].

Estimation of serum creatine kinase (CK)

CK catalyzes the reaction between creatine phosphate and ADP, giving creatine and ATP. The ATP and glucose are converted to ADP and glucose-6-phosphate by hexokinase. Glucose-6-phosphate dehydrogenase (G-6-PHD) oxidizes glucose-6-phosphate and reduces nicotinamide adenine dinucleotide (NAD) to NADH. The rate of NADH formation is determined photometrically at 340 nm and is directly proportional to the CK activity in the sample following manufacturer instruction and using Auto Biochemistry analyzer (Robonite Prietest-touch-India).

Determination of serum inflammatory markers

Determination of serum proinflammatory interleukin 1 beta (IL-1 β) using rat IL-1 β ELISA kit and interleukin-10 (IL-10) using Rat IL-10 ELISA

Kit both from KOMA BIOTECH INC and following manufacturer's instruction. Absorbance was read using Automated ELIZA plate reader (Robonite-Readwell-India).

Histopathology and immunohistochemistry

Gastrocnemius muscle samples from four animals per group were fixed in 10% of neutral buffered formalin. After proper fixation, the specimens were dehydrated in aggraded series of ethanol, cleared in xylene, embedded in paraplast and sectioned at 5 μ m thick sections. Further processing of sections either for Hematoxylin and Eosin (H&E) or immunohistochemistry.

Immunohistochemistry

Sections were analyzed by immunohistochemistry for caspase-3 (rabbit polyclonal 1:200) and desmin (mouse monoclonal 1:200). Briefly after deparaffinization and hydration, endogenous peroxidase blocked with 0.9% hydrogen peroxide. Background blocking was performed with normal goat and donkey serum. The tissue sections were incubated with primary antibody (caspase-3 and desmin) either overnight at 4°C or one hour at room temperature in PBS (PH 7.4). The slides were washed with PBS followed by incubation with biotinylated secondary antibody (goat anti-rabbit and donkey anti-mouse 1:2000 respectively) for 30 minutes at room temperature. The detection system used was avidin-biotin complex (ABC) staining kit. The peroxidase activity was revealed in 0.03% 3, 3-diaminobenzidine tetra-dihydrochloride (DAB; Sigma) that was used as a chromogen, rinsed with tap water for 5 min, and counterstained with 0.1% methylene blue. Negative controls were prepared by incubating additional sections in all solutions except primary antibody. Sections washed in water, dehydrated through graded ethanol, cleared in xylene, and mounted with DPX and photographed using light microscope (Leica Q500MC).

Quantification of caspase 3 and desmin expression in different groups

Sections of gastrocnemius muscle corresponding to four animals from each group were used for calculation. The average percentage of immuno-stained area of myocyte cytoplasm for caspase 3 or desmin calculated in 5 fields per

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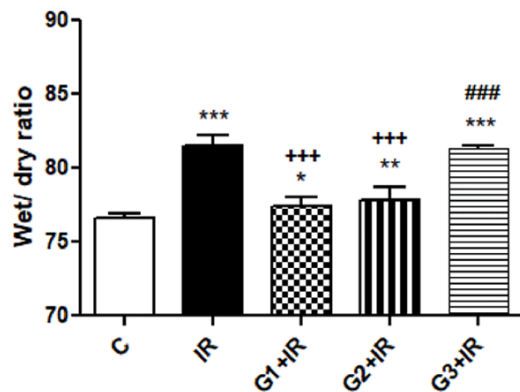


Figure 1. Effect of garlic on wet to dry weight ratio (W/D) of rat gastrocnemius muscle in different treated groups. C: control group; IR: ischemia reperfusion group; G1+IR: garlic given 24 hours before IR; G2+IR: garlic given 30 minutes before IR; G3+IR: garlic given immediately before reperfusion; One Way ANOVA with Bonferroni posthoc test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ### $P < 0.001$, (*) significance difference of C group vs any other group; (+) IR group vs garlic treated groups; (#) G3+IR vs G1+IR or G2+IR; $n = 10$ in each group.

rat (20 fields per group) using image J software and expressed as percentages in relation to the non-stained area.

Histopathology

Sections were stained with Hematoxylin and Eosin (H&E) for demonstration of the general histological structure of the examined gastrocnemius muscle and examined with a light microscope (Leica Q500MC). The histologic damage in each muscle sample was evaluated quantitatively in eight fields per rat and four rats per group using the following parameters; muscle fiber degeneration, inflammatory cell infiltration, hemorrhage, disorganization and intermuscular spaces (0 as normal; 1 as mild; 2 as moderate; and 3 as severe). Total damage score was estimated by summation [20].

Statistical analysis

GraphPad Prism 6.07 (GraphPad Software Inc., La Jolla, CA, USA) was used for data analysis. Data were presented as mean \pm SD. Data were compared among the three groups using One Way ANOVA Non-Parametric with Bonferroni Multiple Comparison as post hoc test as appropriate. A (P) value of less than 0.05 was considered to represent a statistically significant difference. The average percent of immuno-posi-

tive cells is calculated using Image J (IJ-1.46r software).

Results

Effect of garlic on wet to dry weight ratio (W/D) of gastrocnemius muscle in hind limb ischemia reperfusion

The current work showed that I/R caused significant increase in W/D of rat gastrocnemius muscle in IR group when compared to C group ($81.53 \pm 0.68\%$ vs $76.64 \pm 0.26\%$, $P < 0.001$). Garlic caused significant decrease in W/D when given 24 hrs (G1+IR; $77.42 \pm 0.57\%$ $P < 0.001$) and 30 min. (G2+IR; $77.77 \pm 0.94\%$, $P < 0.001$) before I/R in comparison with IR group. However, garlic failed to significantly decrease the W/D weight ratio when given immediately before IR (G3+IR; $81.34 \pm 0.22\%$, $P > 0.05$) when compared to IR. We found significant decrease in W/D weight ratio in G1+IR and G2+IR groups when compared with G3+IR group. However, there was insignificant difference in W/D weight ratio between G1+IR and G2+IR (Figure 1).

Effect of garlic on serum creatine kinase (CK) level in hind limb ischemia reperfusion

The current study demonstrated significant increase of serum CK level in IR group (925.5 ± 73.15 IU/L, $P < 0.001$) when compared to that of C group (46.50 ± 4.403 IU/L). Garlic administration caused significant decrease in serum CK level when administered 24 hr and 30 min before IR in G1+IR group (273.3 ± 47.24 IU/L, $P < 0.001$) and G2+IR group (546.3 ± 60.49 IU/L, $P < 0.001$) respectively in comparison with IR group. Garlic caused insignificant difference in serum CK in G3+IR group (884.9 ± 81.49 IU/L, $P > 0.05$) when compared to IR group. There were significant decrease in serum CK in G1+IR and G2+IR when compared to G3+IR ($P < 0.001$). Moreover, there was a significant decrease in serum CK level in G1+IR when compared with G2+IR group ($P < 0.001$) (Figure 2).

Effect of garlic on serum inflammatory cytokines in hind limb ischemia reperfusion (IR)

Effect of garlic on serum interleukin1 beta (IL-1 β) in the studied groups: The current study demonstrated significant increase of IL-1 β in IR group (191.2 ± 6.73 IU/L, $P < 0.001$) when com-

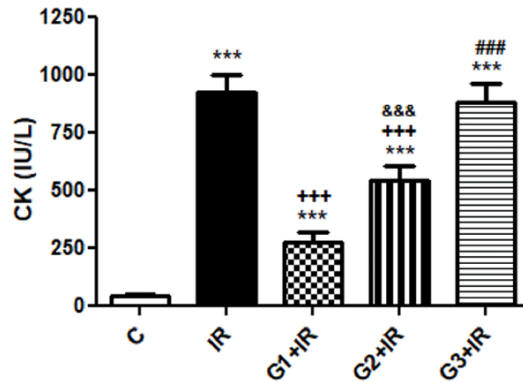


Figure 2. Effect of garlic on serum creatine kinase (CK) in the studied groups. C: control group; IR: ischemia reperfusion group; G1+IR: garlic given 24 hours before IR; G2+IR: garlic given 30 minutes before reperfusion; G3+IR: garlic given immediately before IR; CK: creatine kinase; IU/L: international unit per liter; One Way ANOVA with Bonferroni posthoc test; *** $P < 0.001$, +++ $P < 0.001$, ### $P < 0.001$, &&& $P < 0.001$; (*) significance difference of C group vs any other group; (+) IR group vs garlic treated groups; (#) G3+IR vs G1+IR or G2+IR; (&) P value of G2+IR vs G1+IR; $n = 10$ in each group.

pared to that of C group (45.60 ± 1.78 IU/L). Garlic administration caused significant decrease of IL-1 β when injected 24 hr before IR in G1+IR group (89.80 ± 4.49 IU/L, $P < 0.001$), 30 min before IR in G2+IR group (153.2 ± 6.29 IU/L, $P < 0.001$) and immediately before the reperfusion in G3+IR group (184.2 ± 4.42 IU/L, $P < 0.05$) when compared to IR group. There were significant decrease in serum IL-1 β level in G1+IR and G2+IR when compared to G3+IR ($P < 0.001$). Moreover, there was a significant decrease in serum IL-1 β level in G1+IR when compared with G2+IR ($P < 0.001$) (Figure 3A).

Effect of garlic on serum interleukin10 (IL-10) in the studied groups

The results of the current study showed significant rise of serum IL-10 level in IR group (65.90 ± 3.07 IU/L, $P < 0.001$) when compared to that of C group (31.40 ± 3.06 IU/L). Further significant rise in IL-10 with administration of garlic in G1+IR group (129.0 ± 8.52 IU/L, $P < 0.001$) and G2+IR group (112.7 ± 4.00 IU/L, $P < 0.001$) if compared with IR group. Insignificant difference in IL-10 was found in G3+IR group (68.40 ± 2.07 IU/L, $P > 0.05$) when compared with IR group. There were significant increase in serum IL-10 level in G1+IR and G2+IR when compared

to G3+IR ($P < 0.001$). Moreover, there was a significant increase in serum IL-10 level in G1+IR when compared with G2+IR ($P < 0.001$) (Figure 3B).

Effect of garlic on caspase-3 expression in hind limb ischemia reperfusion (IR)

Sections of the control group showed no detectable immunoreactivity for activated caspase-3 in the gastrocnemius muscle sections (Figure 4A). In contrast, caspase-3 immunoreactivity was very strongly apparent in the cytoplasm of gastrocnemius muscle sections from IR group (Figure 4B). The garlic treated groups showed positive immunoreactivity for caspase-3 with different degrees (Figure 4C-E); weak in G1+IR group (Figure 4C), moderate in G2+IR group (Figure 4D) and strong in G3+IR group (Figure 4E).

Quantification of mean caspase 3 expression (apoptosis score) in studied groups

The current work demonstrated significant increase in the mean density of caspase 3 immunostaining in IR group (64.49 ± 2.87 ; $P < 0.001$) compared to control (19.68 ± 4.49). Garlic administration caused significant decrease in caspase 3 immunostaining in G1+IR (35.91 ± 3.35 , $P < 0.001$), G2+IR (45.57 ± 3.45 , $P < 0.001$) and G3+IR (60.82 ± 3.24 , $P < 0.001$) compared to IR group. There was significant difference between G1+IR compared to both G2+IR and G3+IR groups ($P < 0.001$). Moreover, we found significant difference between G2+IR and G3+IR ($P < 0.001$) (Figure 4F).

Effect of garlic on desmin expression in hind limb ischemia reperfusion (IR)

A strong desmin expression was observed in the gastrocnemius muscle in the control group (Figure 5A). In contrast, IR group showed weak or no desmin immunoreaction with loss of lateral alignment of myofibrils and nuclear displacement in desmin-negative areas (Figure 5B). Garlic treated groups showed positive immunostaining with varying degrees. (Figure 5C-E); strong in G1+IR group with almost normal alignment of skeletal muscle fibers with visible striations and multiple elongated peripheral nuclei (Figure 5C), moderate in G2+IR group with better striations, better alignment of myofibrils and peripherally located nuclei

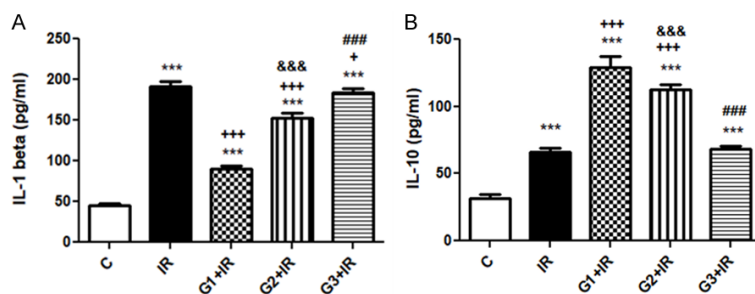


Figure 3. Effect of garlic on serum interleukin1 beta (IL-1 β) (A) and interleukin 10 (IL-10) (B) in the studied groups. C: control group; IR: ischemia reperfusion group; G1+IR: garlic given 24 hours before IR; G2+IR: garlic given 30 minutes before IR; G3+IR: garlic given immediately before reperfusion; IL-1 β : Interleukin-1 beta; pg/ml: picogram per milliliter; One Way ANOVA and Bonferroni posthoc test; *** $P < 0.001$, * $P < 0.05$, *** $P < 0.001$, ### $P < 0.001$, &&& $P < 0.001$; (*) significance difference of C group vs any other group; (+) IR group vs garlic treated groups; (#) G3+IR vs G1+IR or G2+IR; (&) G2+IR vs G1+IR; n=10 in each group.

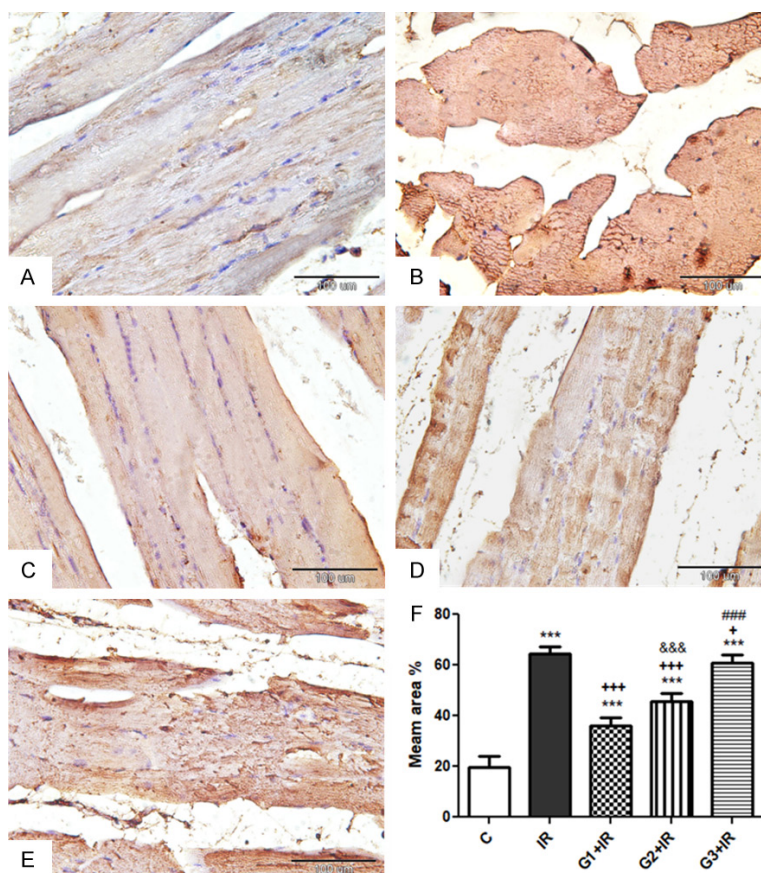


Figure 4. Photomicrographs of caspase-3 immunostaining of rat gastrocnemius muscle (A-E); Apoptosis score (F). (A) Control group; (B) IR group; (C) G1+IR group; (D) G2+IR group; (E) G3+IR group; (F) Mean area immunostained with caspase 3 \pm SD; C: control group; IR: ischemia reperfusion; G1+IR: garlic given 24 hours before IR; G2+IR: garlic given 30 minutes before IR; G3+IR: garlic given immediately before reperfusion; One Way ANOVA and Bonferroni posthoc test; *** $P < 0.001$, * $P < 0.05$, *** $P < 0.001$, ### $P < 0.001$, &&& $P < 0.001$; (*) significance difference of C group vs any other group; (+) IR group vs garlic treated groups; (#) G3+IR vs G1+IR or G2+IR; (&) G2+IR vs G1+IR; Original magnification 40X; n=4 in each group.

(Figure 5D) and weak in G3+IR group with loss of lateral alignment of myofibrils and nuclear displacement in desmin-negative areas (Figure 5E).

Quantification of mean desmin expression of gastrocnemius muscle of studied groups

Strong immunostaining of desmin observed in skeletal muscle of control group. Significant decrease in desmin expression denoted by marked decrease in immunostaining of cytoplasm of IR group (12.62 ± 3.68 ; $P < 0.001$) compared to C group (55.35 ± 5.43). Significant increase in desmin immunostaining observed in G1+IR group with mean density (44.00 ± 3.21 , $P < 0.001$), G2+IR group with mean density (33.04 ± 3.43 , $P < 0.001$) and G3+IR group with mean density (16.80 ± 5.23 , $P < 0.01$) compared to IR group. Significant difference between G1+IR and G2+IR was found ($P < 0.001$). Moreover, significant difference was found in G1+IR or G2+IR if compared to G3+IR ($P < 0.001$) (Figure 5F).

Effect of garlic on gastrocnemius muscle histopathology in hind limb ischemia reperfusion (IR)

Longitudinal sections of C group showed normal muscle architecture; long striated muscle fibers with numerous elongated peripheral nuclei, intact sarcolemma, non-fragmented sarcoplasm and fine connective tissue between muscle fibers (Figure 6A). IR group showed structural disorganization; wavy myofibrils, loss of striation, fragmentation of sarcoplasm, inflammatory cell infiltration, hemorrhage and wi-

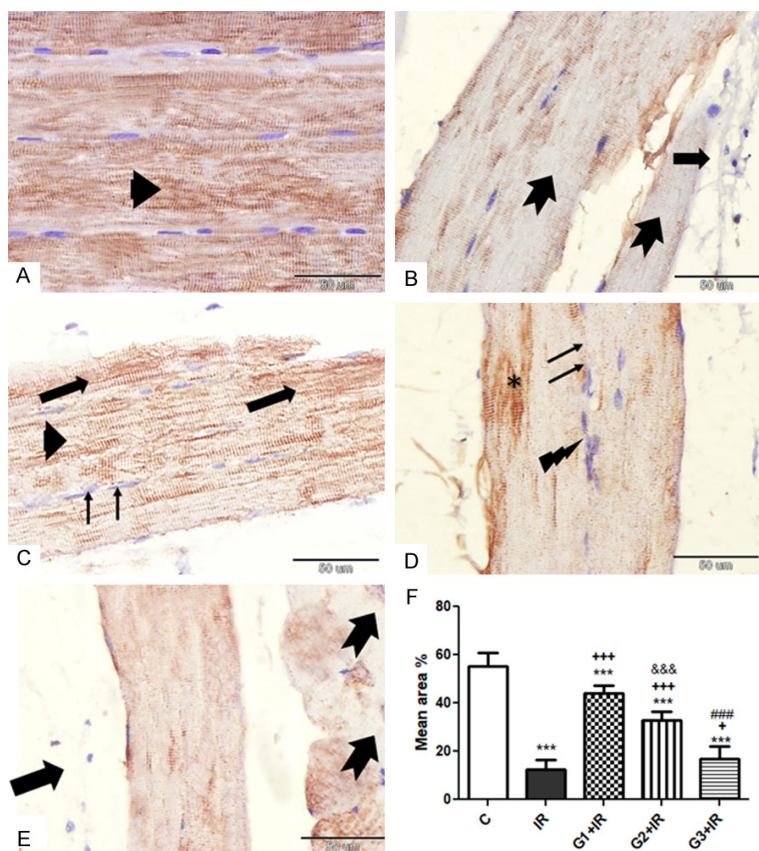


Figure 5. Photomicrographs of desmin immunostaining of rat gastrocnemius muscle. (A-E) Longitudinal muscle sections; (F) Mean desmin immunostained area in the studied groups; (A) Control group showing: strong desmin expression (arrow head) and apparent striations; (B) IR group showing weak or no expression (bifid arrow), loss of lateral alignment of myofibrils and nuclear displacement (thick arrow) in desmin-negative areas; (C-E) Rats treated with garlic plus IR, (C) G1+IR showing: increased desmin expression (arrow head), almost normal alignment of skeletal muscle fibers with visible striations (thick arrow) with multiple elongated peripheral nuclei (thin arrow); (D) G2+IR showing increased desmin expression with better striations (asterisk), better alignment of myofibrils (thin arrows), peripherally located nuclei (lightening bolt); (E) G3+IR showing areas weak or no expression (bifid arrow), loss of lateral alignment of myofibrils and myocytes and nuclear displacement (thick arrow) in desmin-negative areas; (F) Mean area \pm SD; C: control group; IR: ischemia reperfusion group; G1+IR: garlic given 24 hours before IR; G2+IR: garlic given 30 minutes before IR; G3+IR: garlic given immediately before reperfusion; One Way ANOVA and Bonferroni posthoc test; *** $P < 0.001$, * $P < 0.05$, +++ $P < 0.001$, ### $P < 0.001$, &&& $P < 0.001$; (*) significance difference of C group vs any other group; (+) IR group vs garlic treated groups; (#) G3+IR vs G1+IR or G2+IR; (&) G2+IR vs G1+IR; original magnification 40X; $n = 4$ in each group.

de separation between the muscle fibers (**Table 1; Figure 6B**). Garlic administration 24 hrs before ischemia reperfusion (G1+IR) caused marked improvement of the skeletal muscle architecture with visible striations, multiple elongated peripheral nuclei, almost normal appearance of intercellular spaces, less inflammatory cellular infiltration and less hemorrhage

(**Table 1; Figure 6C**). However, garlic administration half an hour before ischemia (G2+IR) and immediately before the reperfusion (G3+IR) showed less improvement compared to G1+IR group. G2+IR group showed slight narrowing of intercellular spaces between the muscle fibers, lower inflammatory cells and hemorrhage than both IR and immediately garlic treatment group (**Table 1; Figure 6D**). G3+IR group showed wide intercellular spaces between the muscle fibers, degeneration, hemorrhage and high inflammatory cells infiltration (**Table 1; Figure 6E**).

The histopathological total damage score of gastrocnemius muscle in studied groups

We found significant higher total damage score in IR group when compared to C group ($P < 0.001$). Garlic administration caused significant improvement in total damage score of G1+IR group ($P < 0.001$) and G2+IR group ($P < 0.001$) and G3+IR ($P < 0.05$) if compared to IR group. Total damage score was significantly higher in G3+IR ($P < 0.001$) and G2+IR ($P < 0.01$) compared to G1+IR (**Figure 6F**).

Discussion

Garlic has many beneficial effects on health and protect against a variety of diseases. Up to our knowledge, garlic

effects on skeletal muscle IR and possible pathophysiological mechanisms involved have not yet been evident. Therefore, understanding those mechanisms may provide a novel preventive as well as therapeutic opportunities. The present study demonstrated significant rise in W/D of gastrocnemius muscle in IR group compared to their controls. Previous studies

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Table 1. Histopathology damage score in the studied groups

Groups	C	IR	G1+IR	G2+IR	G3+IR
Deg	0.09 ± 0.39	2.38 ± 0.87	0.47 ± 0.51	1.50 ± 0.80	2.06 ± 0.95
<i>P</i> value		***	+++	***, +++, &&&	***, &&&, #
Inf	0.03 ± 0.18	2.16 ± 0.77	1.16 ± 0.63	1.53 ± 0.67	2.00 ± 0.78
<i>P</i> value		***	***, +++	***, ++	***, &&&
Hem	0.06 ± 0.35	1.56 ± 1.13	0.75 ± 0.76	0.97 ± 0.74	1.50 ± 0.76
<i>P</i> value		***	**, +++	***, +	***, &&
Dis	0.09 ± 0.30	2.41 ± 0.80	0.50 ± 0.57	1.72 ± 0.73	2.06 ± 0.91
<i>P</i> value		***	+++	***, ++, &&&	***, &&&
Spaces	0.06 ± 0.25	2.03 ± 0.90	0.41 ± 0.56	1.00 ± 0.98	1.50 ± 1.05
<i>P</i> value		***	+++	***, +++, &	***, &&&
TDS	0.33 ± 1.47	10.54 ± 4.47	3.29 ± 3.03	6.72 ± 4.38	7.78 ± 4.6
<i>P</i> value		***	*, +++	***, +++, &&	***, +, &&&, ###

Data represent means ± standard deviation; Deg: degeneration; Inf: inflammatory cell infiltrate; Hem: hemorrhage; Dis: disorganization; Spaces: inbetween myofibrils; TDS: total damage score; C: control group; IR: ischemia reperfusion group; G1+IR: garlic administration 24 hours before IR; G2+IR: garlic administration 30 minutes before IR; G3+IR: garlic administration immediately before reperfusion; (0= No; 1= mild; 2= moderate; 3= severe); ANOVA and Bonferroni posthoc test; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ significant difference versus C group; +: $P < 0.05$; ++: $P < 0.01$; +++: $P < 0.001$ significant difference versus IR group; &: $P < 0.05$; &&: $P < 0.01$; &&&: $P < 0.001$ significant difference versus G1+IR group; #: $P < 0.05$; ###: $P < 0.001$ significant difference versus G2+IR group; n=4 in each group; damage score evaluated from 8 visual fields from each rat.

showed that exposure to 3/2 hr IR [21] or 2/24 hr IR [22] caused significant rise in W/D of rat gastrocnemius muscle. The current work showed that garlic extract administration succeeded to reduce gastrocnemius W/D in G1+IR and G2+IR compared to IR group. In agreement with this result, previous study demonstrated that administration of allicin decreased W/D in rat lung injected by lipopolysaccharide [23]. The IR-induced rise in W/D was attributed to lipid peroxidation, loss of vascular integrity, increased vascular permeability leading to edema of the skeletal muscle that was further proved by the histopathology of the muscle [24]. We demonstrated that garlic administration especially 24 hr and 30 min before IR significantly reduced in myofibril degeneration, inflammatory cellular infiltration, hemorrhage, disorganization and space between myofibrils indicating reduced edema. Taken together, we may speculate that garlic significantly reduced edema through maintaining vascular, myofibril integrity and reduced local muscle inflammation.

The current work showed significant rise in serum total CK level after 2/2 hr IR compared to their controls. In line with us, previous studies demonstrated significant rise of total serum CK levels induced by IR of skeletal muscle [21, 22, 25, 26]. Those studies used different thera-

peutic methods that protect against IR-induced inflammatory muscle injury and rise in serum CK such as salvianolic acid; colchicine; iloprost plus antioxidant N-acetyl cysteine and tramadol respectively. This study demonstrated the protective effect of garlic against skeletal muscle IR-induced rise of serum CK when administered 24 hours and 30 minutes before the reperfusion. In line with us, previous studies reported that garlic administration at 250 mg/Kg orally for 30 days before IR alone [27] or with preconditioning [28], or with Ramipril, hypotensive drug, caused significant decrease in serum CK and protected the myocardium against IR [29].

The present study showed that IR of the hind limbs caused significant rise in serum IL-1 β . This result is supported by previous studies who found rise of IL-1 β in rat model of skeletal muscle IR [24, 25]. In the present work, garlic counterbalances the IR-induced elevation of IL-1 β . In line with this result, one study reported that treatment with garlic was beneficial to spinal cord IR by reducing oxidative stress, IL-1 and TNF- α [30]. In addition, another study demonstrated higher anti-inflammatory effect of fresh raw aqueous compared to aged black garlic extract [31]. They reported decreased IL-1 β , IL-6, nitric oxide and prostaglandin E2 levels in lipopolysaccharide-induced inflammation of

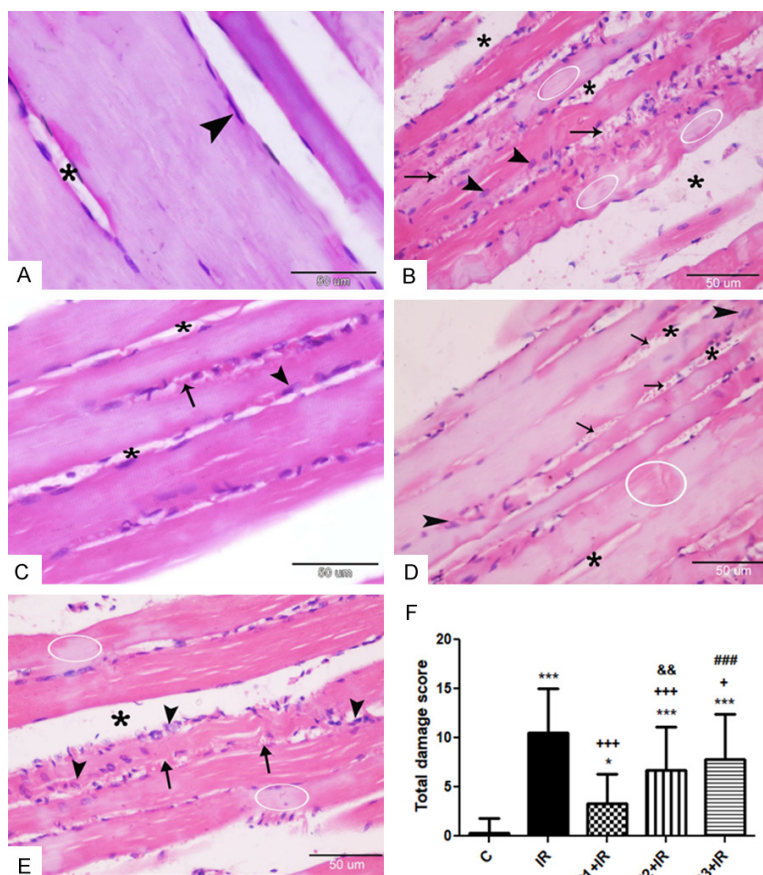


Figure 6. Photomicrographs of longitudinal sections of rat gastrocnemius muscle (A-E) and total damage score (F). (A) Control group; (B) IR group; (C) G1+IR group; (D) G2+IR group; (E) G3+IR group; nuclei (large arrow head), intercellular spaces (asterisk), loss of striation and degenerative areas of muscle fiber (circle), inflammatory cells infiltrations (small arrow head) and hemorrhage between the muscle fibers (arrow); (F) Total damage score \pm SD; C: control group; IR: ischemia reperfusion; G1+IR: garlic given 24 hours before IR; G2+IR: garlic given 30 minutes before IR; G3+IR: garlic given immediately before reperfusion; One Way ANOVA and Bonferroni posthoc test; * $P < 0.05$, *** $P < 0.001$, * $P < 0.05$, *** $P < 0.001$, ### $P < 0.001$, && $P < 0.01$; (*) significance difference of C group vs any other group; (+) IR group vs garlic treated groups; (##) G3+IR vs G1+IR; (&) G2+IR vs G1+IR; H&E original magnification 40X; $n = 4$ in each group.

RAW264.7 cell culture. It was reported that hind limb IR not only locally affect skeletal muscle but also affect distant organs such as liver evidenced by increase in biomarker levels and histological changes of liver tissue [32]. Therefore, we suggest that garlic-induced reduction of IL-1 β may block the systemic complication following hind limb IR.

The present work showed significant elevation of IL-10 serum level after exposure of the rat to IR. In agreement with us, several previous studies found significant elevation of IL-10 in rat

model of limb IR [33] and myocardial IR [34] and renal IR [35]. We found that garlic enhanced the rise of serum IL-10 level in G1+IR and G2+IR groups compared to control and IR groups. In agreement with this result, previous study showed rise of IL-10 induced by one month of garlic administration in rat model of renal IR [13]. Moreover, aged garlic extract [AGE] upregulated IL-10 in peripheral blood monocytes which in turn caused down regulation of proinflammatory cytokines IL-1 β and IL-12 by negative feedback mechanism in murine model of sporotrichosis [36]. Furthermore, garlic inhibited T helper cells causing decreased TNF- α , IL-6, IL-8 and IL-1 β and increased IL-10 production in inflammatory bowel disease [37]. Taken together, we may speculate that garlic administration induced rise of IL-10 in skeletal muscle IR that may have inhibitory effect on IL-1 β production thus reducing inflammation and helping recovery.

In the current study, we found that the expression of caspase-3 was significantly increased in gastrocnemius muscle of IR group compared to controls. This result is supported by previous studies

who reported significant rise in caspase-3 and apoptosis score induced by skeletal muscle IR [20, 24, 30]. We demonstrated that administration of garlic significantly decreased caspase-3 expression and improved gastrocnemius apoptosis score in garlic treated groups compared to IR group. In line with this result, recent study found that treatment with aged garlic extract orally 15 days before the spinal cord IR was enough to reduce caspase-3 [30]. Another study showed that allicin administration attenuated lung, kidney and intestine IR-induced damage and apoptosis via decrease the p38

mitogen-activated protein kinase signal transduction pathway [38]. In addition, it was reported that allicin administration at the beginning of reperfusion improved pulmonary blood flow and inhibited apoptosis [15]. In contrast, it was reported that allicin induced apoptosis in several tumor cell lines [39]. It was found that high concentration of allicin induced apoptosis while low concentration was organ protective and exerted anti-apoptotic, anti-caspase-3 effects [40, 41]. The apparent contradiction in the previous studies may be explained on the base of the different concentration used or being in vivo or in vitro tissue culture studies.

The present study showed degeneration, disorganization of the myofibril, wide spaces between myofibrils indicating edema, inflammatory cell infiltration and hemorrhage in IR group. In agreement with our study, several previous histopathological studies confirmed myofibril structural damage induced by IR [20, 21, 24]. Desmin, the largest intermediate filament located near Z bands, is important for maintenance of myocyte structure, chemical signaling and effective force transmission [42]. We found significant reduction in desmin expression in IR group compared to control group. In agreement with us, previous studies showed significant decrease in desmin expression in myocardial IR model [43]. In the current study garlic significantly increased desmin expression in all studied groups compared IR group. However, administration of garlic 24 hr before IR was more effective in increasing desmin expression compared to 30 min before IR or immediately before reperfusion. Moreover, we found that garlic administration protected against IR-induced muscle degeneration, disorganization, edema, inflammatory cell infiltration and hemorrhage particularly in G1+IR group. It caused significant reduction in total damage score in all the three studied groups compared to IR group. Significant difference between the three groups observed that was in favor of earlier treatment with garlic. In line with us, it was demonstrated that chronic garlic treatment protected against oxidative stress, mitochondria damage, disruption of Z bands, reduced focal loss of myofibrils, edema and inflammation in rat model of myocardial IR [44]. Most previous studies that used garlic in rodent models of IR reported reduced edema, congestion, hemorrhage in other tissues such as spinal cord [30], renal [18], cere-

bral [45] and hepatic tissues [46]. Taken together, we may suggest that prophylactic administration of garlic-induced upregulation of desmin expression, downregulation of caspase expression, reduced inflammatory changes and edema that played an important role in keeping structural integrity of SKM myofibrils.

Conclusion

The present study showed that garlic treatment 24 hours and half an hour before IR significantly decreased W/D weight ratio, serum CK, IL-1 β and significantly increased IL-10 when compared to IR group. Moreover, prophylactic garlic treatment ameliorated the IR-induced histopathological damage to various degrees, significantly elevated desmin expression and reduced apoptosis score. We suggested that prophylactic garlic treatment provide novel way to protect against local and systemic complication IR of the skeletal muscle. We recommend the earlier administration of garlic to give the optimum results in expected limb injury whether accidental or surgical.

Disclosure of conflict of interest

None.

Abbreviations

IR, Ischemia reperfusion; 2/2 hr IR, 2 hours ischemia followed by 2 hours reperfusion; W/D, Wet to dry weight ratio; IL-1 β , Interleukin 1 β ; IL-10, Interleukin-10; CK, Creatine kinase.

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References

- [1] Wu MY, Yianga GT, Liaoc WT, Tsaie AP, Cheng YL, Cheng PW, Li CY, Li CJ. Current mechanistic concepts in ischemia and reperfusion injury. *Cell Physiol Biochem* 2018; 46: 1650-1667.
- [2] Gillani S, Cao J, Suzuki T, Hak DJ. The effect of ischemia reperfusion injury on skeletal muscle. *Injury* 2012; 43: 670-675.
- [3] Brancaccio P, Lippi G, Maffulli N. Biochemical markers of muscular damage. *Clin Chem Lab Med* 2010; 48: 757-767.

- [4] Walters TJ, Mase VJ Jr, Roe JL, Dubick MA, Christy RJ. Poloxamer-188 reduces muscular edema after tourniquet-induced ischemia-reperfusion injury in rats. *J Trauma* 2011; 70: 1192-7.
- [5] Garbaisz D, Turocz Z, Aranyi P, Fulop A, Rosero O, Hermes E, Ferencz A, Lotz G, Harsanyi L, Szijarto A. Attenuation of skeletal muscle and renal injury to the lower limb following ischemia-reperfusion using mPTP inhibitor NIM-811. *PLoS One* 2014; 9: e101067.
- [6] Hoehna RS, Seitz AP, Jernigana PL, Gulbinsa E, Edwardsa MJ. Ischemia/reperfusion injury alters sphingolipid metabolism in the gut. *Cell Physiol Biochem* 2016; 39: 1262-1270.
- [7] Ornellas FM, Ornellas DS, Martini SV, Castiglione RC, Ventura GM, Rocco PR, Gutflen B, de Souza SA, Takiya CM, Morales MM. Bone marrow-derived mononuclear cell therapy accelerates renal ischemia-reperfusion injury recovery by modulating inflammatory, antioxidant and apoptotic related molecules. *Cell Physiol Biochem* 2017; 41: 1736-1752.
- [8] Tran TP, Tu H, Liu J, Muelleman RL, Li YL. Mitochondria-derived superoxide links to tourniquet-induced apoptosis in mouse skeletal muscle. *PLoS One* 2012; 7: e43410.
- [9] Brocca L, Pellegrino MA, Desaphy JF, Pierno S, Camerino DC, Bottinelli R. Is oxidative stress a cause or consequence of disuse muscle atrophy in mice? A proteomic approach in hindlimb-unloaded mice. *Exp Physiol* 2010; 95: 331-350.
- [10] Rahman K, Lowe GM. Garlic and cardiovascular disease: a critical review. *J Nutr* 2006; 136 Suppl: 736S-740S.
- [11] Czompa A, Szoke K, Prokisch J, Gyongyosi A, Bak I, Balla G, Tosaki A, Lekli I. Aged [black] versus raw garlic against ischemia/reperfusion-induced cardiac complications. *Int J Mol Sci* 2018; 19: 1017.
- [12] Kong X, Gong S, Su L, Li C, Kong Y. Neuroprotective effects of allicin on ischemia-reperfusion brain injury. *Oncotarget* 2017; 8: 104492-104507.
- [13] Ali SI, Alhusseini NF, Atteia HH, Idris RA, Hasan RA. Renoprotective effect of a combination of garlic and telmisartan against ischemia/reperfusion-induced kidney injury in obese rats. *Free Radic Res* 2016; 50: 966-86.
- [14] Shaik IH, George JM, Thekkumkara TJ, Mehvar R. Protective effects of diallyl sulfide, a garlic constituent, on the warm hepatic ischemia-reperfusion injury in a rat model. *Pharm Res* 2008; 25: 2231-42.
- [15] Batirel HF, Naka Y, Kayano K, Okada K, Vural K, Pinsky DJ, Oz MC. Intravenous allicin improves pulmonary blood flow after ischemia-reperfusion injury in rats. *J Cardiovasc Surg (Torino)* 2002; 43: 175-9.
- [16] Thomson M, Al-Amin ZM, Al-Qattan KK, Shaban LH, Ali M. Anti-diabetic and hypolipidaemic properties of garlic (*Allium sativum*) in streptozotocin-induced diabetic rats. *Int J Diabetes & Metabolism* 2007; 15: 108-115.
- [17] Pottecher J, Kindo M, Chamaraux-Tran TN, Charles AL, Lejay A, Kemmel V, Vogel T, Chakfe N, Zoll J, Diemunsch P, Geny B. Skeletal muscle ischemia-reperfusion injury and cyclosporine A in the aging rat. *Fundam Clin Pharmacol* 2016; 30: 216-225.
- [18] Bagheri F, Gol A, Dabiri S, Javadi A. Preventive effect of garlic juice on renal reperfusion injury. *Iran J Kidney Dis* 2011; 5: 194-200.
- [19] Schneider MP, Hughes FM Jr, Engmann AK, Purves JT, Kasper H, Tedaldi M, Spruill LS, Gullo M, Schwab ME, Kessler TM. A novel urodynamic model for lower urinary tract assessment in awake rats. *BJU Int* 2015; 115: 8-15.
- [20] Güneysu E, Koçman AE, Özatik O, Ovali C, Can B, Alataş İÖ, Sevin MB. The effects of iloprost and alprostadil on ischemia-reperfusion injury in preventing inflammation, tissue degeneration, and apoptosis in rat skeletal muscle. *Turk J Med Sci* 2017; 47: 1028-1036.
- [21] Wang L, Shan Y, Chen L, Lin B, Xiong X, Lin L, Jin L. Colchicine protects rat skeletal muscle from ischemia/reperfusion injury by suppressing oxidative stress and inflammation. *Iran J Basic Med Sci* 2016; 19: 670-5.
- [22] Takhtfooladi HA, Takhtfooladi MA, Karimi P, Asl HA, Mobarakeh SZ. Influence of tramadol on ischemia-reperfusion injury of rats' skeletal muscle. *Int J Surg* 2014; 12: 963-8.
- [23] Zheng YL, Cai WW, Yan GZ, Xu YZ, Zhang M. Allicin protects against lipopolysaccharide-induced acute lung injury by up-regulation of claudin-4. *Trop J Pharm Res* 2014; 13: 1063-1069.
- [24] Zong H, Li X, Lin H, Hou C, Ma F. Lipoxin A4 pretreatment mitigates skeletal muscle ischemia-reperfusion injury in rats. *Am J Transl Res* 2017; 9: 1139-1150.
- [25] Xiang Y, Ye S, Cai C, Chen J, Zhao X, Zhu N, Zeng C. Salvianolic acid attenuates limb ischemia/reperfusion injury in skeletal muscle of rats. *Biomed Pharmacother* 2018; 97: 551-556.
- [26] Tiryakioglu O, Erkok K, Tuncer B, Uysal O, Altin HF, Gunes T, Aydin S. The effect of iloprost and N-acetylcysteine on skeletal muscle injury in an acute aortic ischemia-reperfusion model: an experimental study. *Biomed Res Int* 2015; 2015: 453748.
- [27] Asdaq SM, Inamdar MN, Asad M. Pharmacodynamic interaction of garlic with propranolol in ischemia-reperfusion induced myocardial damage. *Pak J Pharm Sci* 2010; 23: 42-7.
- [28] Bhatti R, Singh K, Ishar MP, Singh J. The effect of *Allium sativum* on ischemic preconditioning

- and ischemia reperfusion induced cardiac injury. *Indian J Pharmacol* 2008; 40: 261-5.
- [29] Kumar SV, Asdaq SMB. Impact of garlic on cardioprotective potential of ramipril on ischemia-reperfusion induced myocardial dysfunction in rats. *Int J Complement Alt Med* 2017; 5: 00151.
- [30] Cemil B, Gokce EC, Kahveci R, Gokce A, Aksoy N, Sargon MF, Erdogan B, Kosem B. Aged garlic extract attenuates neuronal injury in a rat model of spinal cord ischemia/reperfusion injury. *J Med Food* 2016; 19: 601-6.
- [31] Jeong YY, Ryu JH, Shin JH, Kang MJ, Kang JR, Han J, Kang D. Comparison of anti-oxidant and anti-inflammatory effects between fresh and aged black garlic extracts. *Molecules* 2016; 21: 430.
- [32] Sagara G, Ogikubo O, Takeyama H, Tada T, Suzuki N, Okamoto H, Otsuka T. Comparison of effects of ischemia period in rat limb ischemia/reperfusion injury. *Nagoya Med J* 2009; 50: 83-91.
- [33] Wang T, Zhou YT, Chen XN, Zhu AX. Putative role of ischemic postconditioning in a rat model of limb ischemia and reperfusion: involvement of hypoxia-inducible factor-1 α expression. *Braz J Med Biol Res* 2014; 47: 738-45.
- [34] Aravinthan A, Kim JH, Antonisamy P, Kang CW, Choi J, Kim NS, Kim JH. Ginseng total saponin attenuates myocardial injury via anti-oxidative and anti-inflammatory properties. *J Ginseng Res* 2015; 39: 206-12.
- [35] El Morsy EM, Ahmed MA, Ahmed AA. Attenuation of renal ischemia/reperfusion injury by açai extract preconditioning in a rat model. *Life Sci* 2015; 123: 35-42.
- [36] Buriana LP, Sacramento LVS, Carlosb IZ. Fungal infection control by garlic extracts [*Allium sativum* L.] and modulation of peritoneal macrophages activity in murine model of sporotrichosis. *Braz J Biol* 2017; 77: 848-855.
- [37] Hodge G, Hodge S, Han P. *Allium sativum* [garlic] suppresses leukocyte inflammatory cytokine production in vitro: potential therapeutic use in the treatment of inflammatory bowel disease. *Cytometry* 2002; 48: 209-15.
- [38] Zhang Y, Yao HP, Huang FF, Wu W, Gao Y, Chen ZB, Liang ZY, Liang TB. Allicin, a major component of garlic, inhibits apoptosis in vital organs in rats with trauma/hemorrhagic shock. *Crit Care Med* 2008; 36: 3226-32.
- [39] Park SY, Cho SJ, Kwon HC, Lee KR, Rhee DK, Pyo S. Caspase-independent cell death by allicin in human epithelial carcinoma cells: involvement of PKA. *Cancer Lett* 2005; 224: 123-32.
- [40] Cho SJ, Rhee DK, Pyo S. Allicin, a major component of garlic, inhibits apoptosis of macrophage in a depleted nutritional state. *Nutrition* 2006; 22: 1177-84.
- [41] Miron T, Mironchik M, Mirelman D, Wilchek M, Rabinkov A. Inhibition of tumor growth by a novel approach: in situ allicin generation using targeted alliinase delivery. *Mol Cancer Ther* 2003; 2: 1295-301.
- [42] Marzuca-Nassr GN, Vitzel KF, Mancilla-Solorza E, Márquez JL. Sarcomere structure: the importance of desmin protein in muscle atrophy. *Int J Morphol* 2018; 36: 576-583.
- [43] Yuan J, Mo H, Luo J, Zhao S, Liang S, Jiang Y, Zhang M. PPAR α activation alleviates damage to the cytoskeleton during acute myocardial ischemia/reperfusion in rats. *Mol Med Rep* 2018; 17: 7218-7226.
- [44] Banerjee SK, Dinda AK, Manchanda SC, Maulik SK. Chronic garlic administration protects rat heart against oxidative stress induced by ischemic reperfusion injury. *BMC Pharmacol* 2002; 2: 16.
- [45] Colín-González AL, Ortiz-Plata A, Villeda-Hernández J, Barrera D, Molina-Jijón E, Pedraza-Chaverrí J, Maldonado PD. Aged garlic extract attenuates cerebral damage and cyclooxygenase-2 induction after ischemia and reperfusion in rats. *Plant Foods Hum Nutr* 2011; 66: 348-54.
- [46] Sener G, Sehirli O, Ipçi Y, Ercan F, Sirvanci S, Gedik N, Yeğen BC. Aqueous garlic extract alleviates ischaemia-reperfusion-induced oxidative hepatic injury in rats. *J Pharm Pharmacol* 2005; 57: 145-50.