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Curcumin Ameliorates Ischemia-Induced Limb Injury Through Immunomodulation

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Statistical Analysis C
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Manuscript Preparation E
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Background: The prevalence of peripheral arterial disease (PAD) is increasing worldwide. Currently, there is no effective treatment for PAD. Curcumin is an ingredient of turmeric that has antioxidant, anti-inflammation, and anticancer properties. In the present study we investigated the potential effect of curcumin in protecting against ischemic limb injury.

Material/Methods: We used an established hindlimb ischemia mouse model in our study. Curcumin was administrated through intraperitoneal (I.P.) injection. Immunohistochemical staining and ELISA assays were performed. Treadmill training was used to evaluate skeletal muscle functions of animals.

Results: Our experiments using *in vivo* treadmill training showed that curcumin treatment improved the running capacity of animals after ischemic injury. Histological analysis revealed that curcumin treatment significantly reduced the skeletal muscle damage and fibrosis associated with ischemic injury. In order to determine the cellular and molecular mechanisms underlying curcumin-mediated tissue protection, immunohistochemical staining and ELISA assays were performed. The results showed that curcumin treatment led to less macrophage infiltration and less local inflammatory responses as demonstrated by decreasing TNF-α, IL-1, and IL-6 levels. Further immunofluorescent staining of tissue slides indicated that curcumin treatment inhibited the NF-κB signaling pathway. Finally, curcumin can inhibit NF-κB activation induced by LPS in macrophages.

Conclusions: Our study results show that curcumin treatment can ameliorate hindlimb injury following ischemic surgery, which suggests that curcumin could be used for PAD treatment.

MeSH Keywords: Anti-Inflammatory Agents • Curcumin • Peripheral Arterial Disease

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Background

Peripheral arterial disease (PAD) is a growing medical problem, especially in the aging population in developed countries [1]. PAD is estimated to develop in 500 to 1000 individuals per million persons per year. The prevalence of all stages of PAD in the general population is about 4.2% to 35%. In 4.3% to 9.6% of people with PAD, the disease will progress to critical limb ischemia (CLI), eventually resulting in amputation of the affected limb [1]. CLI is associated with surgery and hospitalization. Treatment with limb salvage often requires invasive revascularization procedures, which may not be suitable for a significant percentage of patients with prohibitive co-morbidities. Therefore, new therapeutic strategies that are less invasive and that are suitable for the majority of patients are needed [1]. It is very important to explore new strategies for treatment of ischemic limb.

Curcumin is a yellow-colored phenolic substance obtained from powdered rhizomes of the plant *C. longa* Linn. Curcumin has multiple pharmacological actions, such as anti-oxidant [2,3], anti-inflammatory [4], and anti-cancer [5,6] effects, which make it an ideal candidate for treatment of diseases caused by multiple factors. A number of investigations have suggested that administration of curcumin can reduce ischemic cerebral injury [7–9]. Previous studies have also demonstrated that curcumin improved outcome and attenuated focal cerebral ischemia injury [10,11]. However, little is known about the potential effects of curcumin in the treatment and protection of PAD and its underlying molecular mechanisms.

In the present study we established a hindlimb ischemia mouse model by permanent ligation of the left femoral artery. We performed animal exercise experiments to explore whether curcumin treatment can improve running capacity of the mice following surgery. Further pathological analysis was performed and demonstrated that administration of curcumin ameliorated ischemia-induced muscle damage and fibrosis. Moreover, we tested the molecular mechanisms underlying curcumin-mediated muscle protection and found that treatment with curcumin significantly reduced inflammatory responses following ischemic surgery. Finally, confocal imaging and biochemical assays demonstrated that the anti-inflammatory effect of curcumin occurs through suppression of macrophages by inhibiting the NF-κB signaling pathway. We demonstrated that the effects of curcumin in protecting ischemia hindlimb injury occur through immunomodulation. Our results suggest that curcumin may be an effective treatment for PAD.

Material and Methods

Hindlimb ischemia mouse model

Adult male C57BL/6J mice (10–12 weeks age, 20–25 g weight), provided by the Animal Facility, Shanghai Tongji University, were housed in the laboratory animal room and maintained at 25±1°C with 65±5% humidity on a 12-h light/dark cycle (lights on from 07:30 to 19:30) for at least 1 week before the start of the experiments. Animals were given free access to food and water. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experiments of Shanghai Tongji University. Animals were maintained and handled in accordance with the guidelines published by the Shanghai Experimental Animal Center of the Chinese Academy of Sciences.

Experimental groups and drug administration

Mice were randomly assigned to 3 groups: 1) a surgery group with curcumin treatment; 2) a surgery group with PBS treatment; and 3) a sham group without ligation, as a control group. Mice were anesthetized with intraperitoneal injection of 50 mg/kg sodium pentobarbital. For the surgery groups, the left femoral artery, great saphenous artery, iliac circumflex artery/vein, and muscular branch were doubly ligated with a 7-0 Prolene suture (Ethion, Somerville, NJ) to induce left hindlimb ischemia. After surgery, the mice were returned to the cages for recovery. For the sham group, all of the procedures were performed the same as in surgery groups except there was no ligation. Curcumin solution (4 mg/ml) was freshly prepared by dissolving curcumin in 0.5M NaOH, then immediately diluting it in PBS. One hour before the ligation surgery (ischemic injury), curcumin (100 mg/kg) was administrated by intraperitoneal (I.P.) injection. The same volume of NaOH and PBS was injected (I.P.) as a control.

Histological and biomedical analysis

Animals in the ischemia groups received either PBS (0.5 mL) or curcumin (100 mg/kg) (Sigma, St. Louis, MO) with intraperitoneal injection 1 h before the ligation. The dose of curcumin was based on previous studies [10–12]. The sham group was injected with the same volume of PBS. The animals were sacrificed at indicated time points. Part of the tibialis anterior (TA) muscle was harvested and cryopreserved in optimal cutting temperature (OCT) media compound (Bio-Optica). Frozen tissue sections were used for different histological and pathological examinations, such as hematoxylin and eosin (H&E) staining for histological evaluation and Masson's trichrome staining for fibrosis and muscle regeneration. The other part of the TA muscle was lysed for ELISA analysis of inflammatory factors and Western blot analysis.

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Immunohistochemical analysis

After deparaffinization, slides were incubated with target retrieval solution (DAKO, Carpentaria, CA). Staining was performed with the ImmunoCruz staining system (Santa Cruz, CA) according to the manufacturer's protocol. NF-κB antibody (Santa Cruz, CA) was used at 1:1000. Normal IgG was used as a negative control. The secondary antibody was used at 1:500. DAPI was used as a nuclear stain. Slides were viewed using a Nikon ECLIPSE TE2000-U inverted microscope connected to an RT Slider Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were acquired using SPOT software.

Western blot analysis

Muscle fibers were washed in PBS and homogenized in ice-cold lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerolphosphate, 5 mM EDTA, 1% Triton X-100, 200 mM sodium orthovanadate, 100 µg/ml phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 5 µg/mL pepstatin A, 10 µg/mL benzamidine, pH 7.4), using a Polytron tissue disrupter (Janke and Kunkel, Germany), at high speed (three 10-s pulses) on ice [13]. All homogenates were centrifuged at 400×g at 4°C for 5 min to remove cell debris. The supernatants were collected and protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Srl, Segrate, MI, Italy). For each sample, 25 µg of total protein was loaded and separated on 8% SDS polyacrylamide gels. Then blots were transferred to polyvinylidene difluoride membrane and blotted with primary antibody and horseradish peroxidase-conjugated secondary antibody. Peroxidase activity was developed with ECL kits (Pierce). Anti- NF-κB (ABcam, ab13594) and anti-GAPDH (ABcam, ab125147) antibodies were used in the experiments.

Treadmill exercise training

Mouse exercise was performed on a rodent treadmill (Columbus Instruments, Columbus, OH) as described in our previous study [14]. The treadmill exercise training was performed at 0, 7, and 14 days after surgery to evaluate the *in vivo* function of injured muscle. The mice were initially trained on the treadmill at a speed of 5 meter/min (m/min) for 3 min and the speed was then increased to 8.5 m/min. The training was performed until the mice were unable to keep pace. The running time for each mouse was recorded as a parameter for *in vivo* muscle function.

Bone marrow cells culture and isolation of macrophages

Bone marrow cells from tibiae and femora of donor mice were incubated for 7 days at 37°C in complete IMDM medium supplemented with 10 ng/ml of macrophage clone-stimulating factor (R&D systems, Abingdon, UK) to obtain adherent macrophages. Briefly, to obtain bone marrow (BM)-derived macrophages, hind legs of 2 mice were cleaned thoroughly, and bone marrow from the femurs and tibiae was flushed using ice-cold PBS. The collected bone marrow cells were pooled, washed once in ice-cold PBS, and resuspended in complete IMDM medium supplemented with 10 ng/ml of macrophage clone-stimulating factor (R&D systems, Abingdon, UK) and cultured for 8 days to differentiate macrophages. Macrophage was then induced by incubating adherent cells for 20 h at 37°C in complete IMDM medium supplemented with 1 ng/ml LPS (Santa Cruz Biotechnology, Inc., CA) in the presence or absence of curcumin (10 µM) according to previous reports [15–17]. Then, macrophages were cultured for 30, 60, and 90 min under the previously specified culture conditions. Macrophages were harvested at each specified time point. Total, cytoplasmic, and nuclear proteins of macrophages were isolated and analyzed as detailed above. ELISA was used to measure the level of inflammatory factors.

Results

Curcumin treatment enhances the recovery of muscle function after hindlimb ischemia

To examine the functional recovery of the injured hindlimb after curcumin treatment, we tested the running capacity of the mice by our treadmill exercise protocol [14]. As shown in Figure 1, the treatment with curcumin enhanced running capacity of injured mice as compared to PBS control mice, as evidenced by the increasing total running time on the treadmill.

Curcumin treatment improves morphological changes associated with hindlimb ischemia

The effect of curcumin on the recovery of running capacity in mice following ischemic injury prompted us to investigate the morphological chance of skeletal muscles. We performed histological examination on the skeletal muscle sections and found extensive muscle degeneration and pronounced interstitial fibrosis in the mice in the PBS-treated ischemic hindlimb group (Figure 2A) as compared to the sham operation group. Interestingly, remarkably reduced fibrosis and less muscle degeneration were observed in the curcumin-treated group (Figure 2A). When H&E and Masson's trichrome stainings were quantified, the mice in the curcumin-treated group exhibited significantly more muscle regeneration (enhanced fiber density) (Figure 2B) and less fibrosis (less collagen deposition) (Figure 2C).

Curcumin treatment reduces macrophage cell infiltration and local inflammation following hindlimb ischemia

Ischemia triggers a robust activation of resident and peripheral immune cells, which play an active role in the acute and

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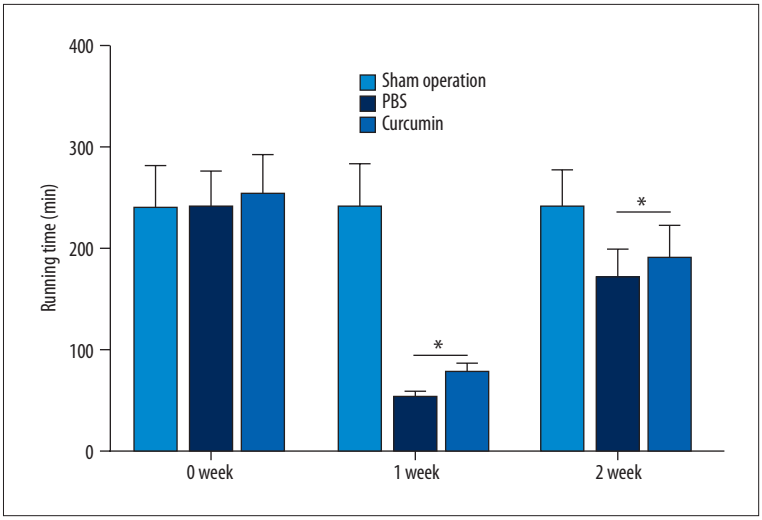

Figure 1. Curcumin treatment improves muscle function after ischemic injury. Animals were subjected to treadmill running test at indicated time points. Curcumin treatments significantly improved running ability of the animals after ischemic injury as compared to the PBS control group. Data are presented as mean ±SEM; n=10 per group; * p<0.05.

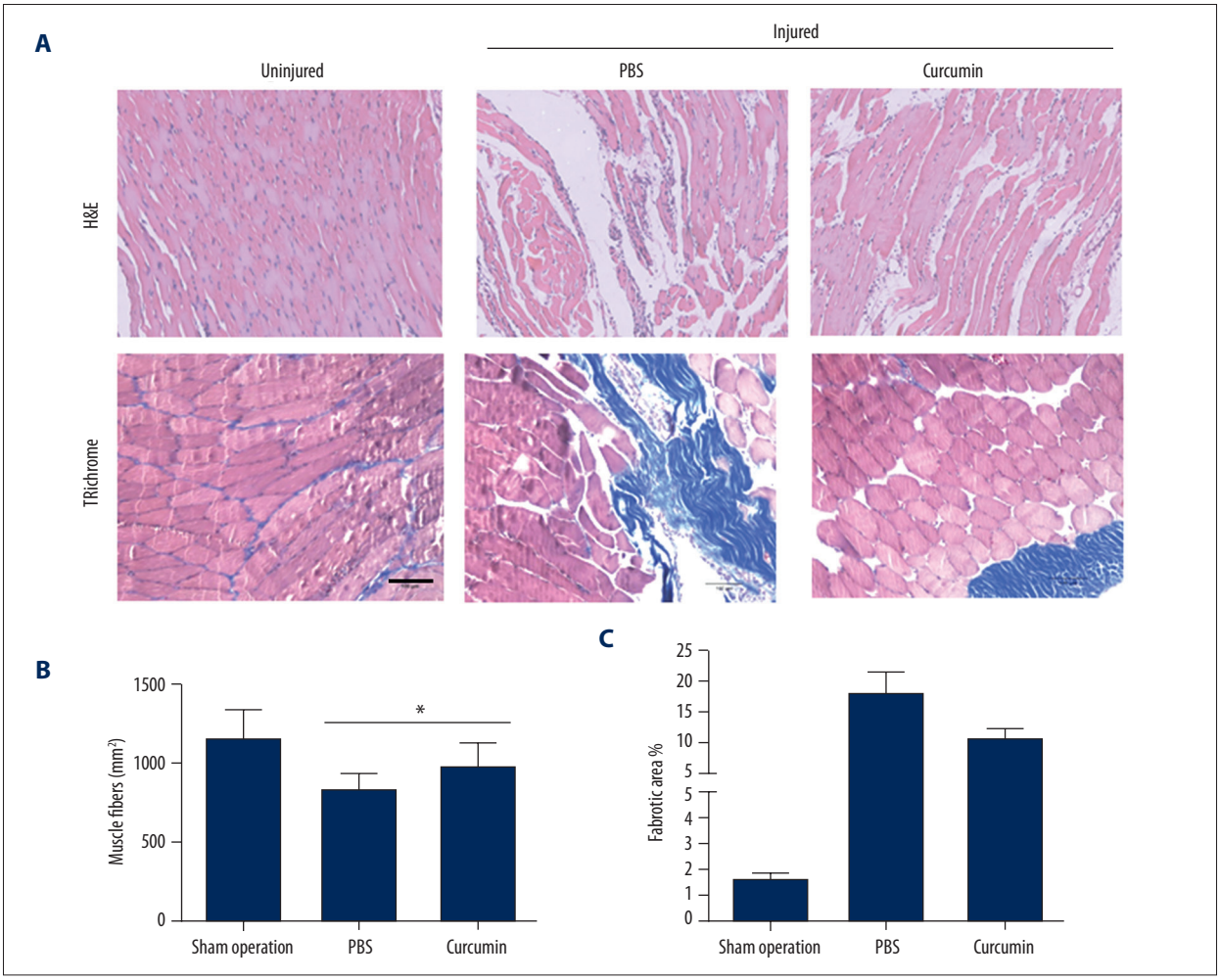

Figure 2. Curcumin treatment improves muscle morphology of hind limbs induced by ischemia. H&E staining and Masson's trichrome staining (**A**) of TA muscle sections. It shows less atrophy and less fibrosis in curcumin treatment skeletal muscle tissues as compared with PBS treatment. The muscle fiber number per mm^2 of the cross-section of TA muscle (**B**) and percentage of fibrosis (**C**) in different groups were quantified and summarized. Data are presented as mean ±SEM; n=10 per group, and a total of 30 sections were analyzed per parameter; * p<0.05. Scale bar: 20 μm.

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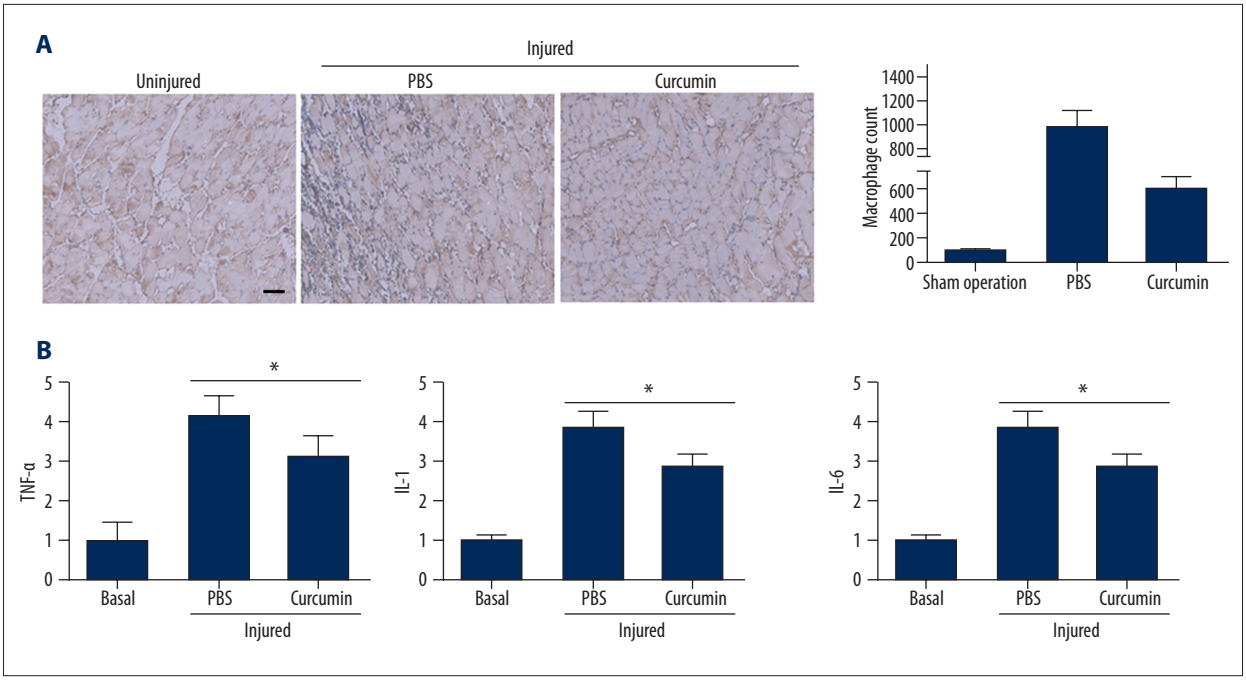

Figure 3. Curcumin treatment reduces macrophage infiltration and inflammatory responses following ischemic injury to hind limbs. (**A**) Macrophage infiltration was determined by immunohistochemical staining. The macrophages in ischemic tissue was significantly higher in the PBS-treated group (1399 ± 186 cells) than in the curcumin-treated group (946 ± 130 cells). A total of 27 sections were analyzed. (**B**) Inflammatory factors in ischemia tissues were determined by ELISA. Curcumin treatment can effectively inhibit levels of pro-inflammatory factors, TNF-α, IL-1β, and IL-6. Data are presented as mean ±SEM; * p<0.05. Scale bar: 20 μm.

chronic phases of injury, as well as in subsequent reorganization and repair processes. Numerous experimental studies have depicted the pivotal response of infiltrating monocyte-derived macrophages to the development of the ischemia injury.

To examine the effect of curcumin on inflammatory macrophage infiltration in the ischemic hindlimb muscle, we evaluated F4-80+ macrophages by immunohistochemistry. As shown in Figure 3A, curcumin treatment reduced macrophage infiltration in the ischemic muscle.

Curcumin regulates local inflammatory response through inhibiting the NF-κB-p65 pathway

The effect of curcumin on the production of local inflammatory cytokines in ischemic tissue was investigated by ELISA analysis. The results showed that curcumin treatment decreases the local levels of proinflammatory cytokines, TNF-α, IL-1β, and IL-6, which might explain why curcumin treatment reduced inflammatory response after ischemic injury to skeletal muscles (Figure 3B).

As NF-κB plays a central role in regulating the production of most inflammatory factors, such as IL-1β, IL-6 and TNF-α, we hypothesized that curcumin might play a role in regulating the NF-κB signaling pathway. We performed immunohistochemical analysis on muscle sections on uninjured controls and injured groups to determine the expression of NF-κB-p65, a subunit of NF-κB transcription complex, which plays a crucial role in inflammatory and immune responses. We found that the levels of p65 were obviously decreased with curcumin treatment in limb muscle tissue (Figure 4).

Curcumin suppresses LPS-induced NF-κB activation in macrophages

NF-κB is a ubiquitous transcription factor that regulates a number of genes involved in inflammation and immune response. In order to further define the molecular mechanisms underlying curcumin-mediated immunomodulation, we investigated the role of curcumin in regulation of NF-κB activation in macrophages. To activate NF-κB, LPS was added to culture media. Western blot analysis revealed that LPS treatment alone resulted in rapid and sustained p65 phosphorylation at serine 536 at 30 and 60 min time points, an indication of NF-κB activation. Interestingly, the presence of curcumin strongly inhibited LPS-induced NF-κB activation in macrophages at the 30 and 60 min time points (Figure 5). These results suggest that curcumin regulates inflammatory response following ischemic limb injury by inhibiting the NF-κB signaling pathway.

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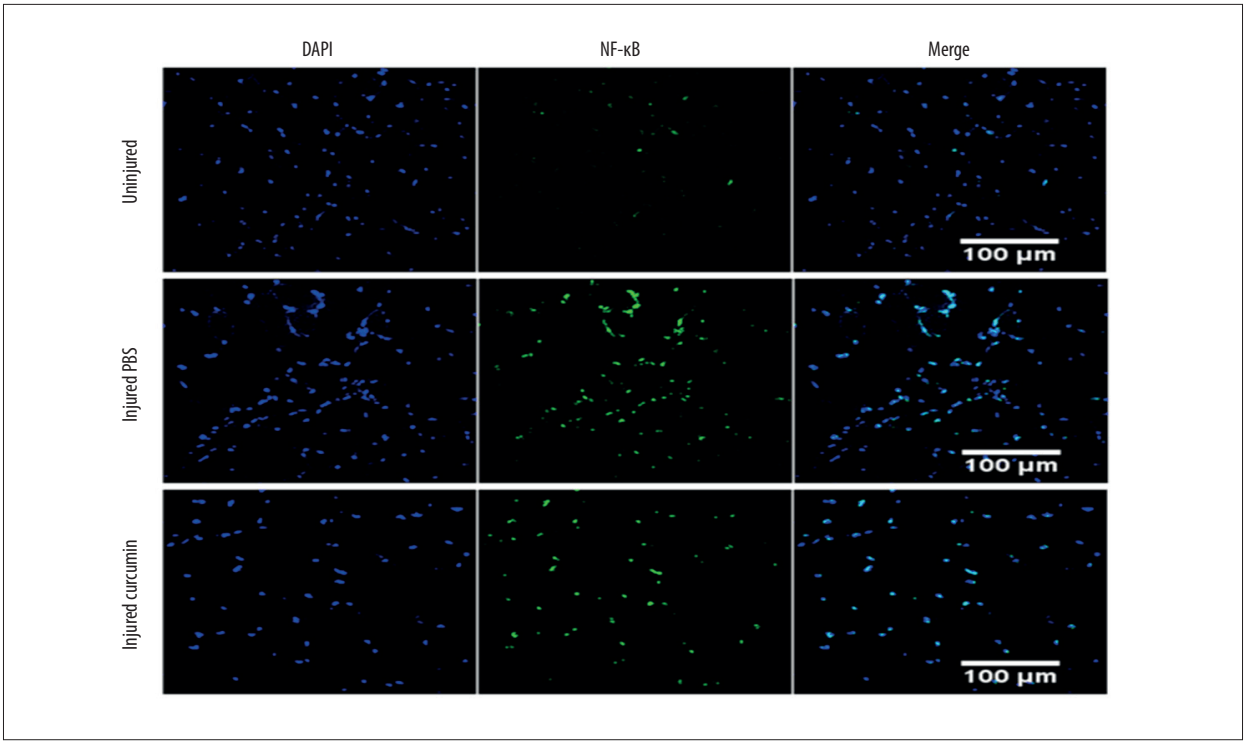

Figure 4. Curcumin treatment inhibits activation of NF-κB in ischemic tissue. (**A**) DAPI staining of nucleus. B. p65 staining in different groups of muscles. Increase in p65 expression was observed in the PBS group and treatment with curcumin decreased p65 expression in the muscle. (**B**) p65 fluorescent signal was quantified by ImageJ software. The results showed curcumin treatment significantly reduced p65 signaling. A total of 27 sections were analyzed. Data are presented as mean ±SEM; n=6 per group; ** p<0.01.

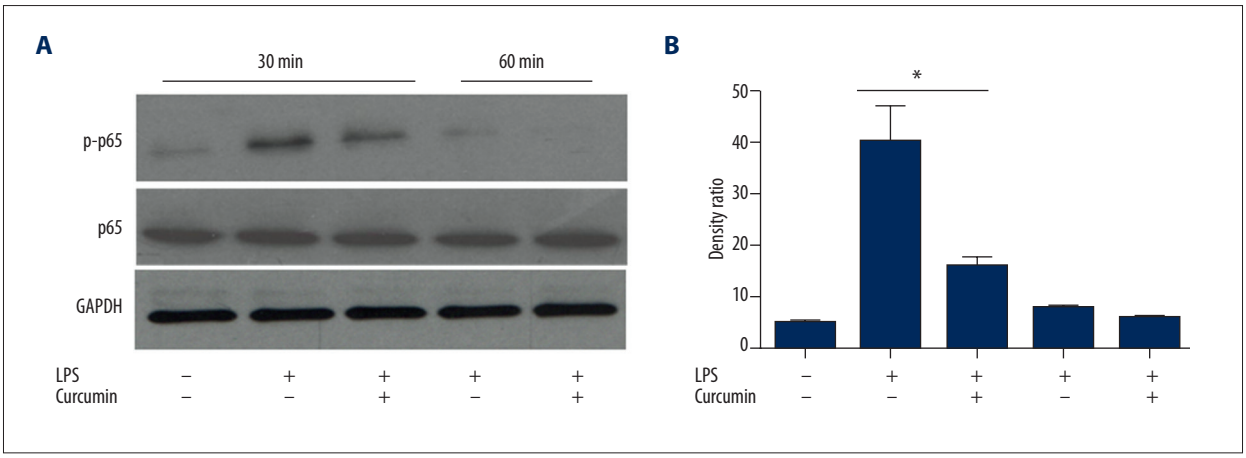

Figure 5. Curcumin inhibits activation of NF-κB in macrophages. (**A**) Western blot analysis shows that LPS induced activation of NF-κB signal in macrophages is inhibited by co-treatment of curcumin at different time points (30 min and 60 min). (**B**) Quantification of Western blot analysis by ImageJ software. Data are presented as mean ±SEM; n=6 per group; ** p<0.01.

Discussion

Previous studies have demonstrated that curcumin has a protective effect on ischemic disease *in vitro* and *in vivo* [11,18,19]. However, the effects of curcumin on limb ischemia have not yet been described. Curcumin is known to exert its anti-inflammatory activity in various cell lines. We thus hypothesized that curcumin treatment could ameliorate the local inflammatory immunological response. In this study, by using an established mouse model of hindlimb ischemia, we found that curcumin treatment is associated with reduced macrophage infiltration and an anti-inflammatory cytokine shift, as

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compared to the controls. The curcumin-mediated inflammatory modulation subsequently enhanced recovery of muscle function, as demonstrated by increased running capacity on the treadmill test. Taken together, results of our studies suggest that curcumin treatment can ameliorate ischemia-induced skeletal muscle injury by modulating local inflammatory response.

It has been demonstrated that the initial ischemic injury results in induction of cytokines and chemokines, which attracts numerous inflammatory cell types to the damaged region, and ultimately contributes to secondary tissues injury [20]. van Amerogen et al. found that macrophage depletion in the first week after ischemia injury in mice markedly increased mortality, impaired infarct healing, and accelerated adverse LV remodeling as shown by post-mortem histopathology [21]. Excessive activation of macrophages worsens the ischemia injury. In healthy muscle tissue, a small resident macrophage population exists. We found that within days after injury, macrophages were recruited at a high rate in the ischemic muscle [14]. Those that differentiate acquire M1-like properties, continue to express Ly-6C, and contribute to inflammation.

Inflammation plays a crucial role in the pathophysiology of ischemia disease by producing inflammatory mediators [22], such as IL-1,TNF-α, PGE2, NO, COX-2, and iNOS, which are important mediators implicated in the pathology of the ischemic tissues. Higher levels of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are found in human chronic wounds. Persistent inflammation inhibits the recovery process in ischemia. Thus, managing persistent inflammation is critical for treating ischemia disease. TNF-α and IL-1 are 2 well-studied cytokines involved in inflammatory responses to limb ischemia and they appear to aggravate ischemic damage [23]. Therapeutic intervention suppressing the proinflammatory cytokines has been proved to be effective [24]. In our current study, we observed that curcumin treatment decreases expression of various pro-inflammatory cytokines in ischemia limb tissues compared to controls, which correlated well with the recovery of muscle function. These results indicated that curcumin could be a candidate for the treatment of limb ischemia.

Previous studies have shown that, as a key regulator of inflammation, NF-κB is activated and contributes to ischemia-induced brain injury [25,26]. NF-κB also plays a central role in regulating the transcription of most inflammatory factors, such as IL-1β, IL-6, and TNF-α. We showed that curcumin treatment suppresses NF-κB activation in macrophages, which correlates with the down-regulation of pro-inflammatory cytokines. Using the ischemia mouse model, we showed that NF-κB was activated in ischemia, and curcumin was able to inhibit inflammatory processes through the NF-κB signaling pathway. These observations are consistent with previous findings of NF-κB-dependent regulation of the proinflammatory cytokines. These observations also correlate well with earlier reports in which curcumin displayed several *in vitro* anti-inflammatory capabilities [27].

We are aware that our study cannot test all the effects of curcumin in tissue protection. For example, curcumin has been reported to have an antioxidant effect in multiple disease models [28–32]. How this antioxidant activity of curcumin functions in our ischemia mouse model will be an interesting direction of our future research.

Conclusions

The results of our research suggest that curcumin treatment can ameliorate ischemia-induced skeletal muscle injury by modulating local inflammatory response. The compound may exert its anti-inflammation effect through inhibition of NF-κB signaling pathways. Curcumin could be a promising anti-inflammatory agent and therefore a candidate for treating limb ischemic diseases.

Disclosure statement

The authors declare no conflict of interest.

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