Curcumin Protects Against Ischemia/Reperfusion Injury in Rat Skeletal Muscle

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Background. Curcumin has been shown to decrease ischemia-reperfusion (I/R) injury in kidney or brain tissues. In this study, the effects of curcumin were evaluated in skeletal muscle during I/R injury.

Materials and Methods. Hind limb ischemia was induced by clamping the common femoral artery and vein. After 4 h ischemia, the clamp of the femoral vessels of animals was taken off and the animal underwent 2 h reperfusion. We measured plasma concentrations of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) using enzyme-linked immunosorbent assay (ELISA). The right gastrocnemius muscle was harvested and immediately stored at −30°C for the assessment of superoxide dismutase (SOD), catalase (CAT) activities, and measurement of glutathione (GSH), malondialdehyde (MDA), and protein oxidation (PO) levels. Curcumin (100 mg/kg), α-tocopherol, and normal saline (10 mL/kg) were administered intraperitoneally 1 h prior reperfusion.

Results. Plasma TNF-α or IL-1β levels increased significantly in I/R group. The plasma levels of these proinflammatory cytokines were reduced in curcumin group compared with the I/R group. Curcumin has more potent antioxidant activity than vitamin E in the skeletal muscle I/R.

Conclusion. In this study, protective effects of curcumin against skeletal muscle ischemia-reperfusion injury have been revealed. We underscore the necessity of human studies with curcumin that would be hypothetically beneficial preventing skeletal muscle I/R injury.

Key Words: ischemia-reperfusion injury; skeletal muscle; curcumin; reactive oxygen species.

INTRODUCTION

Ischemia and reperfusion (I/R) injury in skeletal muscle are unavoidable in many vascular and muscular traumas, diseases, and during a variety of time-consuming procedures or surgeries in the upper or lower extremities. Typical examples include limb amputation/replantation, transplantation, and free muscular-flap transfer. Ischemia reperfusion (I/R) injury is an influential factor that affects prognosis of cases subjected to reconstructive surgery, replantation surgery, and re-exploration. I/R injuries result in damage to endothelial and parenchymal cells, together with reactions of granulocytes and macrophages as well as humoral factors, including complement, coagulation factors, free radicals, and nitric oxide [1, 2]. I/R can activate macrophages and play a role in the release of cytokines which, in turn, influence leukocyte activation, transmigration, and target cell adhesion [3]. The enormous number of leukocytes that enter the extra-vascular space immediately after reperfusion release...
large quantities of reactive oxygen species (ROS) capable of overwhelming cell antioxidant defenses [4, 5]. ROS, including hydroxyl radical (OH), superoxide anion radical (O2•−), singlet oxygen (1O2), hydrogen peroxide (H2O2) and nitric oxide (NO) can cause cellular injury when they are generated excessively and hazardous to lipids, proteins, carbohydrates and nucleic acids [6–8]. Oxidative stress means an alteration in the delicate balance between free radicals and the scavenging capacity of antioxidant enzymes in favor of free radicals in the body systems [9]. ROS attack the polyunsaturated fatty acids in the membrane lipids, thereby lipid peroxidation results in loss of fluidity of the membranes and ruptures leading to release of cell [10]. I/R injury may extend beyond the ischemic area and damage the remote non-ischemic organs. For limiting or prevention of I/R injury, considerable effort has been made in developing new therapeutic strategies by the biotechnological and pharmaceutical industries. Many studies have been performed and various pharmacological and immunological agents have been used for this purpose [11–15].

The phytochemical curcumin (1,7-bis-[3-methoxy-4-hydroxyphenyl]-1,6-heptadiene-3,5-dione), a polyphenol derived from the root Curcuma longa, is a member of the ginger family. It has been used as a spice and food preservative for centuries. Curcumin has minimal side effects and has been administered safely in human subjects [16]. Curcumin’s medicinal properties include antioxidant, antimicrobial, antitumor, antiproliferative, and anti-inflammatory properties [17–24]. Toda et al. showed that curcumin is a strong antioxidant compared with vitamins C and E [25].

In this study, we aimed to evaluate the antioxidant effects of curcumin against skeletal muscle I/R injury in a rat model. We analyzed the antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), malondialdehyde (MDA), and protein oxidation (PO) levels in the muscle and plasma levels of various inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-β (IL-1β).

MATERIALS AND METHODS

Forty Wistar rats, weighing between 200 and 250 g, were used. The animals were housed in temperature (21 ± 2°C) and humidity (60% ± 5%) controlled rooms in which a 12/12 h light/dark cycle was maintained with free access to food and tap water. All the protocols were approved by the institutional animal ethics committee of the Canakkale Onsekiz Mart University.

Experimental Design and Functional Studies

The animals were randomly divided into four groups: group 1 (sham, n = 10), the animals in this group were sham operated with the exposure of the femoral artery, but were not subjected to any ischemia reperfusion. group 2 (I/R+saline, n = 10), animals were exposed to I/R, the rats in this group were subjected to 4 h of femoral pedicles occlusion followed by 2 h of reperfusion. Animals with I/R injury received saline (0.2 mL), with intraperitoneal injection 1 h before the reperfusion. Group 3 (curcumin-I/R, n = 10), animals were exposed to I/R, the rats in this group were subjected to 4 h of femoral pedicles occlusion followed by 2 h of reperfusion. Animals with I/R injury received curcumin (100 mg/kg) (Sigma, St. Louis, MO) with intraperitoneal injection 1 h before the reperfusion. Group 4 (vit E+I/R, n = 10), animals with I/R injury received α-tocopherol (Evigene-AKSU, Istanbul, Turkey) (10 mg/kg) with intraperitoneal injection 1 h before the reperfusion.

All surgical procedures were performed while the rats were under anesthesia with intraperitoneally administered 60 mg/kg ketamine and 10 mg/kg xylazine cocktail. Anesthesia was maintained by an additional half dose in every 90 min. Following induction of general anesthesia, the lower abdomen and groin were shaved, and sterile technique was employed in all surgical procedures. The animal was placed in a supine position. The right groin vessels were exposed via a transverse groin incision. The right femoral artery was isolated by clamping with an atraumatic microvascular clamp. After 4 h of ischemia, the microvascular clamp was removed. After removing the clamp, the lower extremity was inspected for restoration of blood flow. An ischemic insult was created in the right femoral artery for 4 h, followed by 2 h of reperfusion. Animals with I/R injury received either saline (0.2 mL ), curcumin (100 mg/kg) (Sigma, St. Louis, MO) or α-tocopherol (Evigene-AKSU) (10 mg/kg) with intraperitoneal injection 1 h before the reperfusion. The dose of curcumin was based on previous studies [26]. A heat lamp was used to maintain the body temperature at 37 ± 0.5°C during the experiment. At the end of the reperfusion period for biochemical assays blood was drawn from the inferior vena cava with heparinized syringes while still anesthetized. Blood samples were collected into polyethylene tubes and centrifuged (3500 × g for 30 min at 4°C) to separate platelet-poor plasma. We measured TNF-α and IL-1β in the plasma. Moreover, the right gastrocnemius muscle was harvested and immediately stored at −30°C for the assessment of SOD, CAT activities and measurement of GSH, MDA, and PO levels. Animals in the sham operation group underwent a surgical procedure similar to the other groups but the artery was not occluded.

Biochemical Assays

At the end of each experimental procedure, right gastrocnemius muscles were removed; the muscles were washed three times in cold isotonic saline (0.9% [vol/wt]). Then tissues were homogenized with cold Tris-HCl buffer (pH 7.4) to make a 10% homogenate. Glutathione measurements were taken using a modification of the Ellman procedure [27]. Briefly, after centrifugation at 2,000g for 10 min, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/ H2PO4−H2O2 solution. A 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg mL 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. Glutathione levels were calculated using an extinction coefficient of 1.36 × 105/M/cm. The results were expressed as nmol/100 mg protein in tissue samples. The total SOD activity was measured kinetically by a method described by Sun et al. [28]. The principle of the method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine/xanthine oxidase system as a superoxide generator [28, 29]. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was expressed as units/mg of tissue protein. Muscle tissue CAT (EC 1.11.1.16) activity was measured according to Aebi’s method [30]. The essential of the method was based on the determination of the rate constant k (s−1, k) of the H2O2 decomposition rate at 240 nm. Results were expressed as k (rate constant per gram protein; k g−1 protein).

The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously [31]. Lipid peroxidation is expressed in terms of
MDA equivalents using an extinction coefficient of $1.56 \times 105/M/cm$ and the results are expressed as nmol MDA/g tissue.

The protein content of tissue samples was determined by the Lowry et al. assay [32]. Oxidized protein (PO) was quantified using the interaction between dinitrophenylhydrazine (DNP) and the carbonyls to yield a chromophore that absorbs strongly at 360 nm. All samples were diluted to 2–4 mg/mL of protein with wash buffer and treated with 1% streptomycin. The carbonyl content was calculated assuming a molar extinction coefficient of 22,000.

Catalase (U/mg protein) 61.01
SOD (U/mg protein) 0.95

The MDA levels of the muscle tissue were expressed as nmol MDA/g of tissue protein.

Serum levels of TNF-α and IL-1β were quantified using enzyme-linked immunosorbent assay (ELISA) kits specific for the previously mentioned rat cytokines according to the manufacturer’s instructions and guidelines (Biosource Europe SA, Nivelles, Belgium). The total AOC in plasma was measured using colorimetric test system (ImAnOx, catalog no. KC5200; Immunodiagnostics AG, Benheim, Germany), according to the instructions provided by the manufacturer. The 8-hydroxy-2-deoxyguanosine (8-OHdG) content in the extracted DNA solution was determined by ELISA method (highly sensitive 8-OHdG ELISA kit; Japan Institute for the Control of Aging, Shizuoka, Japan). These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intra-assay precision, and small amount of plasma sample required to conduct the assay. The concentration of TNF-α and IL-1β in plasma were expressed as pg/mL.

**Statistical Analysis**

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). All data were expressed as mean ± SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. P values <0.05 were regarded as significant.

**RESULTS**

Results of oxidative stress markers in each group were shown in **Tables 1 and 2**. The GSH levels of the muscle tissue were as follows: sham group, 2.11 ± 0.18; control group, 0.94 ± 0.11; and curcumin treated group, 2.03 ± 0.12; and α-tocopherol treated group 1.83 ± 0.13. The muscle tissue GSH levels in the control group were significantly lower than the sham and curcumin-treated group and α-tocopherol treated group ($P < 0.001$ and $P < 0.01$, respectively). The difference between the sham and curcumin treated group and α-tocopherol treated group was significant ($P < 0.05$; see Table 1). The difference between the curcumin and α-tocopherol treated groups was also significant (Fig. 1). Ischemia-reperfusion injury to the muscle significantly decreased SOD activity muscle tissues compared with the sham group. SOD levels were significantly improved by curcumin-treated and α-tocopherol treated groups and there were no significant differences in the curcumin-treated and α-tocopherol treated groups (Fig. 2).

CAT activity in muscle tissue decreased markedly after reperfusion compared with the sham group. A significant decrease in the activity of CAT was observed in I/R group compared with curcumin treated group and α-tocopherol treated group. The difference between the curcumin and α-tocopherol treated groups was also significant (Fig. 3).

The MDA levels of the muscle tissue were as follows: sham group, 18.30 ± 2.59; control group, 44.67 ± 5.81; curcumin-treated group, 19.39 ± 3.09; and α-tocopherol treated group 23.84 ± 4.83. The tissue MDA levels in the control group were significantly higher than the sham and curcumin-treated and α-tocopherol treated groups. The difference between the sham and the curcumin and α-tocopherol treated groups was also significant ($P < 0.001$). The difference between the curcumin and α-tocopherol treated groups was also significant (Fig. 4). Carboxyl concentrations were 1.53 ± 0.17 nmol/mg proteins in sham group and 4.34 ± 0.51 nmol/mg protein in control group. In the curcumin treated group it was increased to 1.59 ± 0.16 nmol/mg protein ($P < 0.001$) and in α tocopherol treated rats it was increased to 2.21 ± 0.27 nmol/mg protein (Fig. 5).

The plasma levels of proinflammatory cytokines (TNF-α and IL-1β) in the control (ischemia reperfusion injury in the skeletal muscle) group were significantly higher ($P < 0.001$) than that of the control group, whereas the treatment of curcumin and α-tocopherol abolished these elevations significantly ($P < 0.01$). The differences of the plasma levels of the TNF-α and IL-1β between the curcumin and α-tocopherol treated groups was also significant (Fig. 6).

**TABLE 1**

Comparison of Effects of Ischemia/Reperfusion (I/R) on the Muscle GSH, SOD, CAT, MDA, PO Levels with the Curcumin + I/R, vitamin E + I/R, Control and Sham Groups

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R-SF control</th>
<th>I/R-curcumin</th>
<th>I/R-α-tocopherol</th>
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<tbody>
<tr>
<td>GSH (μmol/g)</td>
<td>2.11 ± 0.18</td>
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</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>0.95 ± 0.11</td>
<td>0.85 ± 0.06**</td>
<td>0.89 ± 0.10**</td>
<td>0.88 ± 0.05**</td>
</tr>
<tr>
<td>Catalase (U/mg protein)</td>
<td>61.01 ± 6.35</td>
<td>26.57 ± 5.26***</td>
<td>57.70 ± 5.38***</td>
<td>52.25 ± 6.44***</td>
</tr>
<tr>
<td>MDA (nmol/g)</td>
<td>18.30 ± 2.59</td>
<td>44.67 ± 5.81***</td>
<td>19.39 ± 3.09***</td>
<td>23.84 ± 4.83***</td>
</tr>
<tr>
<td>PO (nmol carbonyl/mg)</td>
<td>1.53 ± 0.17</td>
<td>4.34 ± 0.51***</td>
<td>1.59 ± 0.16***</td>
<td>2.21 ± 0.27***</td>
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* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the sham group.

† $P < 0.05$, † † $P < 0.01$, † † † $P < 0.001$ compared with the I/R group.
DISCUSSION

Restoration of blood flow is critical for reversing ischemia; paradoxically the insult to the tissues worsens [34]. After ischemia, tissues were shown to contain an accumulation of xanthine oxidase [35]. This enzyme uses molecular oxygen, which is available as the final electron acceptor. This reaction produces singlet oxygen molecules, which are extremely unstable. Using these molecules, secondary chemical reactions produce superoxide, hydrogen peroxide, and hydroxyl. ROS, particularly the hydroxyl radicals, interact with phospholipids, proteins, and nucleic acids leading to lipid peroxidation, which results in a loss of membrane integrity [34]. The post-ischemic endothelium is the main source of the superoxide radical. Indirectly, superoxide may be responsible for the production of the damaging hydroxyl radical. The tissue damage caused by the production of ROS can trigger several defense mechanisms. The first-line defense mechanism includes antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH). These enzymes catalyze the conversion of ROS into less reactive species [34, 36]. Under normal conditions, the damaging effects of superoxide are prevented by SOD, which converts superoxide into hydrogen peroxide [35, 37]. However, during reperfusion of ischemic tissues, these natural defenses may be overcome and hydrogen peroxide is converted into a hydroxyl radical, which in turn is capable of damaging a wide variety of biological molecules, including amino acids, membrane transport proteins, and nucleic acids [37, 38]. GSH is an important antioxidant, and the increased concentration of GSH in cells may be useful in the prevention of oxidative damage of endothelial cells [36]. GSH is part of the defensive strategy that cells and tissues use to combat oxidation. In the literature, there are several studies demonstrating that I/R in the tissue is associated with lipid peroxidation, which is an autocatalytic mechanism leading to oxidative destruction of cellular membranes [37]. Catalase, an oxidoreductase that catalyzes the conversion of hydrogen peroxide to water and oxygen, also can protect cells from damage induced by ischemia/reperfusion through scavenging ROS. GSH and SOD are the most important endogenous antioxidant defense mechanisms against oxidative stress [39, 40].

The most important damaging effect of free radicals on tissues is lipid peroxidation. The cell membrane is composed of fatty acids and phospholipids. Oxygen free radicals cause cellular injury by inducing lipid peroxidation, which results in functional and structural

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>I/R</th>
<th>I/R-curcumin</th>
<th>I/R-α-tocopherol</th>
</tr>
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<tbody>
<tr>
<td>TNF-α (pg/mL)</td>
<td>7.63 ± 1.14</td>
<td>33.98 ± 3.65***</td>
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</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>10.13 ± 1.46</td>
<td>32.69 ± 2.94***</td>
<td>12.89 ± 1.98</td>
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</table>

***P < 0.001 compared with the sham group.
††P < 0.001 compared with the I/R group.

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TABLE 2
Comparison of Effects of Ischemia/Reperfusion (I/R) on the Muscle TNF-α and IL-1β levels with the Curcumin-IR, vit e + I/R, Control and Sham Groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>I/R</th>
<th>I/R-curcumin</th>
<th>I/R-α-tocopherol</th>
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![FIG. 1. The effects of ischemia/reperfusion (I/R) and the treatment with curcumin, the treatment with α-tocopherol on the GSH levels. ***P < 0.001 compared with the control group. ††P < 0.01 compared with the IR group.](image1)

![FIG. 2. The effects of ischemia/reperfusion (I/R) and the treatment with curcumin, the treatment with α-tocopherol on the SOD levels. *P < 0.05 compared with the control group. **P < 0.01 compared with the IR group.](image2)
cell alterations [41]. Furthermore, ROS cause oxidation of protein structures and the protein damage can be revealed by the measurement of tissues protein carbonyl content. Malondialdehyde, a product of lipid peroxidation, is generated as a result of active oxygen radicals, which destroy unsaturated fatty acids in cell membranes [42].

ROS during ischemia and reperfusion to the skeletal muscle have a number of features, which include impaired muscle contractions, muscle necrosis, endothelial cell swelling, release of cellular proteins, and increased microvascular permeability to proteins [43, 44]. Many studies have suggested the beneficial effects of nutrients or other agents such as vitamin E [45], vitamin C [46], green tea extract [45], melatonin [47, 48], carbenoxolone [49], and green propolis [50, 51] in reducing or preventing cerebral or muscle injury during ischemia-reperfusion.

In this study, we employed a previously studied model of femoral vessels atraumatic clamping-induced skeletal muscle ischemia-reperfusion injury. Clamping-induced skeletal muscle ischemia-reperfusion injury is associated with free radical production and proinflammatory responses. Treatment with curcumin improves kidney,

**FIG. 3.** The effects of ischemia/reperfusion (I/R) and the treatment with curcumin, the treatment with α-tocopheral on the CAT levels. *P < 0.05, **P < 0.001 compared with the control group. **P < 0.01 compared with the IR group.

**FIG. 4.** The effects of ischemia/reperfusion (I/R) and the treatment with curcumin, the treatment with α-tocopheral on the MDA levels. *P < 0.05, **P < 0.001 compared with the control group. **P < 0.01 compared with the IR group.

**FIG. 5.** The effects of ischemia/reperfusion (I/R) and the treatment with curcumin, the treatment with α-tocopheral on the PO levels. +++P < 0.001 compared with the control group. ***P < 0.01 compared with the IR group.

**FIG. 6.** The effects of ischemia/reperfusion (I/R) and the treatment with curcumin, the treatment with α-tocopheral on the TNF-α and IL-1β levels. +++P < 0.001 compared with the control group. ***P < 0.01 compared with the IR group.
myocardium, pancreas, liver, lung mesentery ischemia-reperfusion injury. The aim of this study was to assess the hypothesis that curcumin attenuates skeletal muscle ischemia-reperfusion injury. Moreover, in the present study, we compared the effects of curcumin with a potent antioxidant, i.e., vitamin E. Curcumin is an effective scavenger for reactive oxygen radicals and reactive nitrogen species in vitro [52]. However, it has not been clear whether curcumin acts directly as an antioxidant in vivo. Curcumin has been shown to decrease the number of oxidative DNA adducts in malignant colorectal tissue [53]. In addition to direct antioxidant activity, curcumin may function indirectly as an antioxidant by inhibiting the activity of inflammatory enzymes or by enhancing the synthesis of glutathione, an important intracellular antioxidant. Chan et al. [18] reported that in vivo curcumin treatment produced 50%–70% reductions in iNOS mRNA expression in the liver of lipopolysaccharide (LPS)-injected mice. It has been reported that low concentrations of curcumin could inhibit NO production in activated macrophages, demonstrating that it significantly reduces the mRNA levels and 130-kDa inducible NOS protein expressed in activated macrophages [54]. Additionally, Shen et al. [54] reported that curcumin pretreatment protected the liver from warm I/R injury through multiple pathways, regulating expression and activity of multiple bioactive molecules and inhibiting cell apoptosis and neutrophil infiltration. Overexpression of antioxidant enzymes after curcumin pretreatment may play an essential role to protect the liver against warm I/R injury [53, 54]. The protective effects of curcumin on ischemia-reperfusion induced liver inflammation, capillary barrier dysfunction, tissue edema, and injury, in a similar manner to the steroid dexamethasone [55]. Curcumin reduces ischemia-reperfusion induced acute lung injury, probably through improvement of oxidative stress and nuclear factor-κB-mediated expression of inflammatory cytokines [53]. Curcumin, which has antioxidant and anti-inflammatory effects, significantly attenuated the inflammatory responses and airway hyper-reactivity induced by pancreatic I/R [56].

The administration of vitamin E prevents the muscles from oxidative stress, endothelial damage, intramuscular edema, and major muscle fiber damage [57, 58]. It has been demonstrated that vitamin E had a protective effect on oxidative muscle damage after a period of I/R in human. It was observed that MDA level and neutrophil infiltration in muscle fibers decreased in vitamin E-treated patients after a period of I/R [58]. Our results revealed that I/R injury led increases in MDA and PO levels in the skeletal muscle tissue. In our study, we showed that administration of curcumin significantly decreased tissue protein carbonyl levels as a marker of protein oxidation activities (PO) in skeletal muscle compared with I/R group. Curcumin reversed the increase of MDA levels to a considerable extent, thereby confirming its antioxidant role in I/R. Reactive oxygen species attack the polyunsaturated fatty acids in the membrane lipids and result in peroxidation, which may lead to disorganization of cell structure and function. This process results in an excess of free radicals, which can react with cellular lipids, proteins, and nucleic acids, leading to local injury and eventually organ dysfunction. It has been suggested that the protective effects of curcumin depend mainly on its antioxidant properties.

In this study, we also demonstrated the effect of curcumin on tissue GSH levels and SOD, CAT activity in a hind limb model of I/R injury. We found decreased SOD, CAT and GSH activities in I/R group compared to sham group. A significant decrease in the activity of SOD, CAT, and GSH was observed in I/R group compared with curcumin treatment group. Furthermore, plasma levels of the proinflammatory cytokines TNF-α and IL-1β were increased in I/R group compared with Sham group. Plasma levels of the proinflammatory cytokines TNF-α and IL-1β were reduced in curcumin group. Moreover, in the present study, we compared the effects of curcumin with one of the potent antioxidants; herewith curcumin has more potent antioxidant activity than vitamin E in the skeletal muscle I/R.

In conclusion, this study demonstrated that administration of curcumin might protect skeletal muscle from reperfusion injury to the extent that vitamin E did. Our data also revealed that this protective effect of curcumin is probably ascribed to its free radical scavenging activity. We underscore the necessity of human studies with curcumin that would be hypothetically beneficial in preventing skeletal muscle I/R injury, particularly during surgical interventions.

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