Original Article
Cardioprotective role of curcumin in myocardial ischemia-reperfusion of male albino rats

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Abstract: Our present study investigates the antioxidant potential of curcumin against the myocardial ischemia-reperfusion (I/R) model of male albino rats. Reduction or alleviation of oxidative stress is a key factor to reduce ischemia-reperfusion based heart injury. Curcumin is well known natural agent and play a vital role in cardiac I/R damage. Myocardial I/R rat models were administrated curcumin utilized for in vivoinvestigation. H9C2 cell was used for the investigations. H9C2 cell viability, lipid peroxidation (MDA), superoxide dismutase (SOD) and catalase level was measured. Curcumin showed excellent antioxidant activity in the in vitro studies. Curcumin significantly inhibited oxidative stress-induced cell growth inhibition and activated caspase 3 enzyme and bax/bax3 signaling pathways. Curcumin reduced significantly decreased apoptosis and size of myocardial infarct in the I/R model of male albino rats. Taking all our data together, it is concluded that curcumin is splendid antioxidant compound and significantly limits myocardial ischemia-reperfusion damage.

Keywords: Rats, curcumin, caspase 3, reperfusion, antioxidant

Introduction
Curcumin is the principal curcuminoid of turmeric and belongs to a member of the ginger family. Turmerics exist as desmethoxycurcumin and bis-desmethoxycurcumin. The yellow color of turmeric due to natural phenols. Curcumin exists in various tautomeric forms, such as 1,3-diketo shape, and two equivalent enol forms. The keto form is energetically less stable than enol form [1]. Curcumin is known to have antioxidant and anti-inflammatory potential [2-5]. Curcumin reported as promising compound against cerebral ischemia, and cerebral vasospasmin subarachnoidhemorrhage-induced rats. A tremendous imbalance between reactive oxygen species (ROS) and a cellular system’s capacity to detoxify the less reactive intermediates will reflect oxidative stress. Changes in the normal redox state of cells could affect normal cell physiology via the generation of free radicals and peroxides. Those radicals affect DNA, proteins and lipids. Oxidative stress is known to cause strand breaks in DNA [6]. Oxidative stress is known to damage and protein misfolding such as glutamate transporters [7].

Buja [8] have reported that the occurrence of myocardial ischemia during the impairment of coronary blood flow rate to the myocardium due to several reasons such as atherosclerosis, vascular spasm, and thrombosis. Reimer and Ideker [9] have reported that the imbalance between oxygen supply and demand due to ischemia leads to cardiac damage and necrosis. Reperfusion is defined as recovering of blood flow, which is used to tissue damage and ischemic diseases. Jennings et al. [10] have reported the reperfusion following thrombosis leads to the formation of ROS and induce tissue damage. Yellon and Hausenloy [11] have reported that myocardial ischemia and reperfusion could act as a fundamental role in the pathogenesis of myocardial infarction. Marczin et al. [12] have reported that the reduction of ROS level is a crucial strategy for limiting ischemic reperfusion damage. Hung et al. [13] have reported the antioxidants are essential for the reduction of myocardial ischemic reperfusion...
Curcumin and myocardial ischemia-reperfusion damage. Our experimental results suggested that curcumin was a potent compound to reduce TBHP-induced apoptosis at in vitro level and protect against myocardial ischemia-reperfusion damage at in vivo level.

**Materials and methods**

**Materials**

Curcumin, dimethyl sulfoxide (DMSO), sulforhodamine B (SRB) have purchased from Sigma. Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin-streptomycin (antibiotics), trypsin-EDTA and fetal bovine serum (FBS) were purchased from Sigma-Aldrich. Primers were synthesized from Santa Cruz Biotechnology, Inc. (Delaware Avenue, California, USA).

**Animals**

Healthy male albino Wistar strain rats purchased from the animal house, Shanghai, China, weighing (160-180 g) was selected for the study. The animal was kept in polypropylene cages under standard conditions (T: 25 ± 0.5°C, RH: 61 ± 4% and a photoperiod of 12 h/day).

![Figure 1. Curcumin effect on H9C2 cell viability. The cell was incubated with different concentration of curcumin for 24 h and cell growth inhibition was calculated concerning control. All the values were expressed mean ± SD, *P<0.05.](image-url)
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Table 1. Curcumin effect on lipid peroxidation, SOD and catalase activity. The cell was incubated with different concentration of curcumin for 24 h and lipid peroxidation was determined according to control

<table>
<thead>
<tr>
<th>MDA (nmol/mg of protein)</th>
<th>Control</th>
<th>10 mg/kg bwt</th>
<th>20 mg/kg bwt</th>
<th>30 mg/kg bwt</th>
<th>40 mg/kg bwt</th>
<th>50 mg/kg bwt</th>
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<tbody>
<tr>
<td>Control</td>
<td>12.0 ± 0.2</td>
<td>10.1 ± 0.1</td>
<td>8.30 ± 0.1</td>
<td>6.10 ± 0.1</td>
<td>4.50 ± 0.15</td>
<td>2.90 ± 2.9</td>
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<tr>
<th>SOD (Units/mg of protein)</th>
<th>Control</th>
<th>10 mg/kg bwt</th>
<th>20 mg/kg bwt</th>
<th>30 mg/kg bwt</th>
<th>40 mg/kg bwt</th>
<th>50 mg/kg bwt</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.130 ± 0.001</td>
<td>0.142 ± 0.002</td>
<td>0.160 ± 0.002</td>
<td>0.177 ± 0.003</td>
<td>0.210 ± 0.003</td>
<td>0.290 ± 0.002</td>
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<tr>
<th>Catalase (Units/mg of protein)</th>
<th>Control</th>
<th>10 mg/kg bwt</th>
<th>20 mg/kg bwt</th>
<th>30 mg/kg bwt</th>
<th>40 mg/kg bwt</th>
<th>50 mg/kg bwt</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.50 ± 0.02</td>
<td>2.00 ± 0.06</td>
<td>2.10 ± 0.10</td>
<td>2.30 ± 0.10</td>
<td>2.50 ± 0.10</td>
<td>3.00 ± 0.10</td>
</tr>
</tbody>
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All the values were expressed mean ± SD, *P<0.05.

Cell culture

H9C2 cells were obtained from the ATCC (University Boulevard, Manassas, VA 20110 USA). H9C2 cell was maintained in growth medium provided with 1% antibiotics and 10% FBS. The cell was grown in a CO_2 incubator at 5% CO_2 and 37°C.

SRB assay

H9C2 cell was seeded in 96-well plates at 4000 cells/well of density. The cell was pre-treated with curcumin (10, 20, 30, 40 and 50 mg/kg bwt) for 4 h and treated with tertbutylhydroperoxide (TBHP) at 200 μM for 24 h. SRB assay determined viable cell numbers and cell death [14].

Determination of malondialdehyde (MDA)

H9C2 cell was cultured in a 6-well plate at 2×10^4 cells/well of density. The cell was pre-treated with curcumin (10, 20, 30, 40 and 50 mg/kg bwt) for 4 h and stimulated with hydrogen peroxide (H_2O_2) at 200 μM for 24 h. MDA was determined by according to Muthuraman et al. [15] in H9C2 cells. MDA is the final product of membrane lipid peroxidation. A 0.1 ml supernatant and 1.9 ml of sodium phosphate buffer incubated for 1 h. Centrifuged following precipitation and the supernatant was collected. 1 ml of TBA was added and heated for 15 minutes. MDA content was measured at 532 nm and indicated in nmol/mg protein.

Determination antioxidant enzyme activities

H9C2 cell was cultured in a 6-well plate at 2×10^4 cells/well of density. The cell was pretreated with curcumin (10, 20, 30, 40 and 50 mg/kg bwt) for 4 h and stimulated with hydrogen peroxide (H_2O_2) at 200 μM for 24 h. SOD and catalase enzyme activities were assayed [15].

qPCR and Western blot analysis

H9C2 cell was cultured in a 6-well plate at 2×10^4 cells/well of density. The cell was pretreated with curcumin (10, 20, 30, 40 and 50 mg/kg bwt) for 4 h and stimulated with TBHP at 200 μM for 24 h. RNA was isolated and qPCR was carried out using primers specific of Nrf2, caspase 3, bax and bcl-2 [16]. All primers were synthesized from Invitrogen (Invitrogen, China). Caspase-3 protein expression was determined using Western blot analysis. SDS-PAGE was carried for cell homogenates, and then Western blot analysis was performed using caspase-3 monoclonal antibody and HRP-conjugated secondary antibody.

Ischemia and reperfusion model

Curcumin (50 mg/kg bwt) was given by gavage to the male albino rats for consecutive 15 days before myocardial ischemia. Following 2 h of the curcumin administration, the animal was anesthetized by administration of 5% chloral hydrate solution. The rat was kept lateral position and tube was inserted into the trachea. Ventilation was provided at a rate of 105 cycles/minute. Chest of each animal was opened with uppermargin of the third rib. The coronary artery occlusion in rats lasted 45 minutes. Chest of each animal was closed, and the rats were removed from the setup and placed warm. Following the self-breath recovery, the ventilator was removed.
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Myocardial infarct size measurement

Myocardial infarct size of rats was measured using standard method [17]. The coronary artery was tied again, and dye was administered into the ventricle. The myocardium was removed immediately from animals, and the section was prepared. In left ventricle, viable tissue was known as the area at the risk which is stained as red. The non-ischemic myocardia of rats were stained as blue. The infarcted rat heart stained as pale color. The areas at risk and myocardial infarction were determined.

TUNEL assay

The heart tissue was from the male albino rats following reperfusion (4 h). Heart tissues were fixed in formaldehyde solution. Tissues were cross-sectioned as 4 μm and deparaffinized.

Results

Effect of curcumin on cell viability

Oxidative stress affects cell viability and leads to cell death. We have investigated the curcumin effect on cell growth inhibition under TBHP induced oxidative condition. TBHP is more stable and suitable compound to stimulate ROS than H$_2$O$_2$. Cell was treated with curcumin (10, 20, 30, 40 & 50 mg/kg bwt) for 4 h. Then cell was treated with TBHP for 24 h. TBHP significantly reduced cell proliferation. However, curcumin pretreatment protected cells from cell growth inhibition (Figure 1). Also, TBHP
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Induced apoptotic morphology modification such as nuclear condensation, cell shrinkage, and fragmentation. However, curcumin pretreatment protected cells from these morphology modifications.

**Effect of curcumin on lipid peroxidation**

Increased membrane fatty acid oxidation produces oxidativestress that affects cell viability and leads to cell growth inhibition. We investigated the curcumin effect on lipid peroxidation under H$_2$O$_2$ induced oxidative condition. Cell was treated with curcumin (10, 20, 30, 40 & 50 mg/kg bwt) for 4 h. Then cell was treated with H$_2$O$_2$ for 24 h. H$_2$O$_2$ significantly reduced SOD activity. However, curcumin pretreatment retained SOD activity (Table 1).

**Curcumin inhibits apoptosis through activation of Nrf2**

We determined the effect of curcumin on prevention cell apoptosis through the Nrf2 signaling pathway. TBHP incubation did not alter Nrf2 expression, whereas the incubation of curcuminenhanced Nrf2 in a concentration-dependent manner. Curcumin reduced TBHP-induced signaling alteration including pro-apoptotic overexpression of bax and antiapoptotic bcl-2 reduction. Also, curcumin inhibited caspase-3 mRNA and protein expression in TBHP incubated cells in a dose-dependent manner (Figures 2, 3).

**Curcumin limits ischemia/reperfusion injury**

An antioxidant is believed to be suitable for the reduction of heart injury in I/R. Thus, we determined the curcumin effect on cardiac ischemia model. The male albino rat was treated with curcumin (50 mg/kg/day) for 15 consecutive days under H$_2$O$_2$ induced oxidative condition. Cell was treated with curcumin (10, 20, 30, 40 & 50 mg/kg bwt) for 4 h. Then cell was treated with H$_2$O$_2$ for 24 h. H$_2$O$_2$ significantly reduced SOD activity. However, curcumin pretreatment retained SOD activity (Table 1).

**Effect of curcumin on catalase activity**

Increased membrane fatty acid oxidation produces oxidativestress that affects cell viability and leads to cell growth inhibition. We investigated the curcumin effect on catalase activity under H$_2$O$_2$ induced oxidative condition. Cells were pretreated with curcumin (10, 20, 30, 40 & 50 mg/kg bwt) for 4 h. Then cells were treated with H$_2$O$_2$ for 24 h. H$_2$O$_2$ significantly reduced catalase activity. However, curcumin pretreatment retained catalase activity in a dose-dependent manner (Table 1).

**Curcumin effect on protein expression.** The cell was incubated with different dose of curcumin for 24 h, and protein expression of caspase 3 was determined concerning control. All the values were expressed mean ± SD, *P<0.05.

![Curcumin effect on protein expression](image.png)
Figure 4. Curcumin effect on myocardial infarct size and ischemic region. Male albino rats were administered curcumin for 24 h, and percentage of ischemic region and myocardial infarct size were determined concerning control. All the values were expressed mean ± SD, *P<0.05.

Figure 5. Curcumin effect on serum creatine kinase and myocardial MDA content. Male albino rats were administered with curcumin for 24 h, and percentage of serum creatine kinase and myocardial MDA content was determined concerning control. All the values were expressed mean ± SD, *P<0.05.
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and allowed for 45 minutes. Curcumin is highly safe in clinical trials usually used at a dose level in animals. Curcumin pretreatment before to ischemic incident reduced myocardial infarct size. Also, curcumin inhibited the creatinekinase-MB level in the male albino rats and reduced MDA content in the rat myocardium (Figures 4, 5).

Curcumin reduces apoptosis in the rat myocardium

We studied the molecular mechanism of curcumin on cardiac ischemia model. Curcumin reduced apoptosis which is induced by I/R. TUNEL assay was used to measure the level of apoptosis in control treated cells (Figure 6).

Discussion

We studied the curcumin's antioxidant and cardio-protective role in myocardial ischemia-reperfusion of male albino rats. A study of curcumin derivatives showed anti-inflammatory and antioxidant effect [18]. Curcumin reduced H₂O₂ induced lipid peroxidation (MDA) and promoted catalase and SOD activity. Also, curcumin significantly prevented cell apoptosis in the ischemia/reperfusion model of male albino rats. Increased oxidative stress induces myocardial injury in ischemia-reperfusion and antioxidants are the key approach to limit the myocardial damage.

We have investigated the effect of curcumin on antioxidant markers and its efficiency for decreasing cellular oxidative stress and limiting the myocardial apoptosis and necrosis in ischemia-reperfusion of male albino rats. Curcumin significantly reduced creatine kinase in serum and inhibited apoptosis and myocardial infarct size. This indicates the effective utility of curcumin in ischemia-reperfusion damage in male albino rats. Antioxidant and antiapoptotic effect of curcumin are fundamental for its cardio-protective effect. Commonly, the antioxidant activity of curcumin is Nrf2 mediated pathway.

Nrf2 is considered as a key biochemical oxidative stress sensor [19]. Nrf2 is present in the cytosol that is retained by Keap1-CUL3 ubiquitin E3 ligase enzyme system. It acts as a potential mediator and inhibitor of Nrf2 degradation. Oxidative stress initiates Keap1-Nrf2 ligase complex system dissociation, which leads Nrf2 translocation to the nucleus and thereby acts
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against cellular oxidative stress [20]. Nrf2 plays a critical role in the defense system in myocardial ischemia-reperfusion of male albino rats. Calvert et al. [21] have reported that Nrf2 deficient rats exhibited increased cellular oxidative stress and myocardial injury during ischemia-reperfusion. Therefore, Nrf2 pathway is a potential target for the antioxidant therapy against myocardial ischemic reperfusion damage. Shehzad and Lee, [22] have reported that curcumin is a very good agonist for Nrf2 pathway. Curcumin treatment before ischemic reperfusion increased Nrf2 expression, and myocardial infarct size was reduced.

Our experimental results demonstrate that curcumin increases the resistance capacity of cardiomyocyte against increased oxidative stress and myocardial ischemic reperfusion damage. In addition to the above effects, curcumin exhibits the anti-apoptotic effect. Curcumin pretreatment reduced TBHP-induced caspase-3 and bax mRNA expression and enhanced the anti-apoptotic bcl-2 mRNA expression. In vitro effects of curcumin directly translated for the cardioprotection against ischemia-reperfusion at the in vivo level. Curcumin significantly reduced cell growth inhibition in the region of ischemic myocardium of male albino rats. Anti-apoptotic effect of curcumin could be the result of the downstream action of antioxidant defense. Kannan and Jain [23] have reported the ROS, and oxidative stress could play a key role in apoptosis.

Yellon and Hausenloy [11] have reported the increased ROS level triggers calcium and endoplasmic stress that leads to the loss of membrane potential of mitochondria and caspase 3 activations. Curcumin prevents cells from apoptosis by inhibiting caspase 3 and bax activation and curcumin activates anti-apoptotic effect through Nrf2. Tian et al. [24] have reported the increased bax expression leading apoptosis. Oxidative stress is critical for myocardial function and survival of cardiomyocyte. Therefore, the anti-oxidative defense is an appropriate target to prevent ischemia-reperfusion damage. Kim et al. [25] have reported that curcumin reduced myocardial ischemic reperfusion damage. Curcumin treatment reduced H₂O₂ induced caspase 3 and bax activation.

Conclusion

In conclusion, curcumin acts as a potent anti-oxidative agent and sound source and bioavailability. It acts as a Nrf2 activator. Therefore, curcumin could be considered as a potential agent, and it could be a key pharmacological approach to limit cardiac ischemic injury in male albino rats.

Disclosure of conflict of interest

None.

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