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

Xiao-Liang Chen[1*], Xiang-Ru Liu[1*], Yong-Xiang Fang[1], Lang Xu[2]

[1]Department of Cardiovascular, The First People's Hospital of Chenzhou, Chenzhou 423000, Hunan, China; [2]Department of Pathology, Medical College, Wuhan University of Science and Technology, Wuhan 430065, Hubei, China. *Equal contributors.

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 inhibitionand activated caspase 3 enzyme and bax/bax3 signaling pathways. Curcumin reduced significantly decreased apoptosis andsize of myocardial infarct in the I/R model of male albino rats. Taking all ou 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 tremendousimbalance 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 usedto tissue damage and ischemic diseases. Jennings et al. [10] have reported the reperfusion following thrombosis leads to theformation of ROS and induce tissue damage. Yellon and Hausenloy [11] have reported that myocardial ischemia and reperfusion could act as a fundamental role in thepathogenesis 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
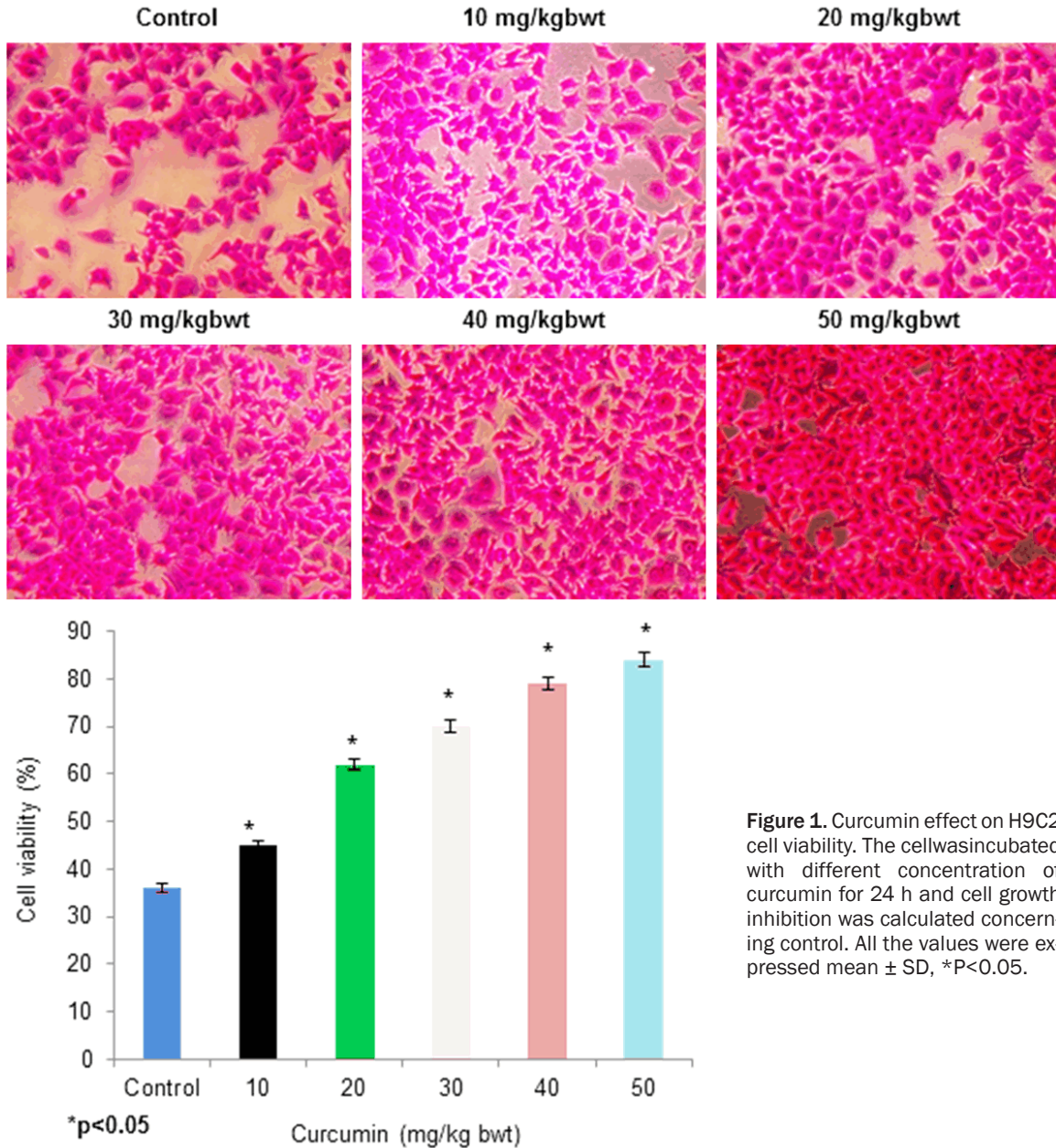
Figure 1. Curcumin effect on H9C2 cell viability. The cellwasincubated with different concentration of curcumin for 24 h and cell growth inhibition was calculated concerning control. All the values were expressed mean ± SD, *P<0.05.

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-reperfusiondamage at in vivo level.

Materials and methods

Materials

Curcumin, dimethyl sulphoxide (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 animalwas kept in polypropylene cages under standard conditions (T: 25 ± 0.5°C, RH: 61 ± 4% and a photoperiod of 12 h/ day).

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 regarding control

MDA (nmol/mg of protein)	Control	12.0 ± 0.2
	10 mg/kg bwt	10.1 ± 0.1
	20 mg/kg bwt	8.30 ± 0.1
	30 mg/kg bwt	6.10 ± 0.1
	40 mg/kg bwt	4.50 ± 0.15
	50 mg/kg bwt	2.90 ± 2.9
SOD (Units/mg of protein)	Control	0.130 ± 0.001
	10 mg/kg bwt	0.142 ± 0.002
	20 mg/kg bwt	0.160 ± 0.002
	30 mg/kg bwt	0.177 ± 0.003
	40 mg/kg bwt	0.210 ± 0.003
	50 mg/kg bwt	0.290 ± 0.002
Catalase (Units/mg of protein)	Control	1.50 ± 0.02
	10 mg/kg bwt	2.00 ±0.06
	20 mg/kg bwt	2.10 ± 0.10
	30 mg/kg bwt	2.30 ± 0.10
	40 mg/kg bwt	2.50 ± 0.10
	50 mg/kg bwt	3.00 ± 0.10

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 cellwas pretreated 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 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. MDA was determined by according to Muthuraman et al. [15] in H9C2 cells. MDA is thefinal product of membrane lipid peroxidation. A 0.1 ml super-natant 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 andindicated in nmol/mg protein.

Determination antioxidantenzyme activities

H9C2 cell was cultured in a 6-well plate at $2×10^4$ cells/well of density. The cellwas 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 a6-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 byadministration of 5% chloral hydrate solution. The ratwas kept lateral position and tubewas insertedinto 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 occlusionin rats lasted 45 minutes. Chest of each animalwas closed, and therats were removed from thesetup and placed warm. Following the self-breath recovery, the ventilator was removed.
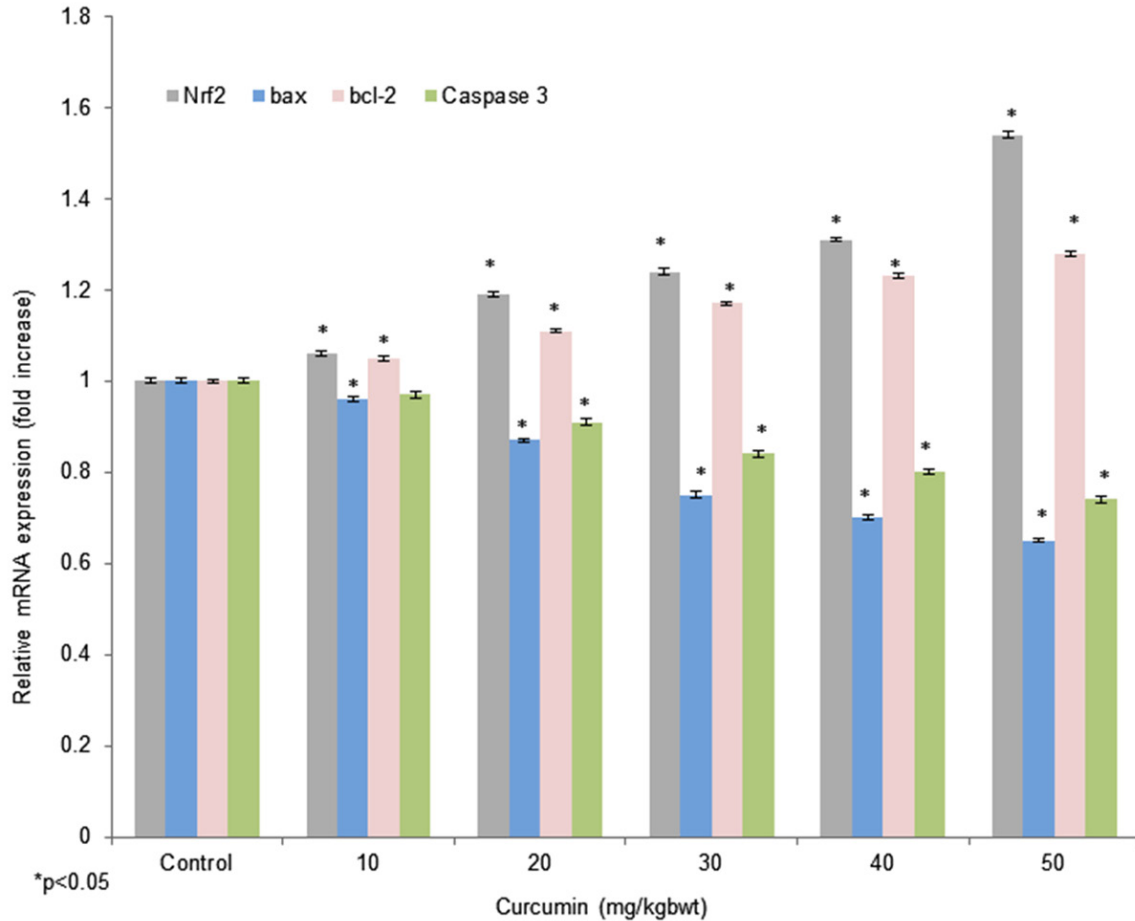
Figure 2. Curcumin effect on mRNA expression. The cellwasincubated with different dose of curcumin for 24 h, and mRNA expression of Nrf2, bax, bcl-2 and caspase 3 were determined concerning control. All the values were expressed mean ± SD, *P<0.05.

Myocardial infarct size measurement

Myocardial infarct size of rats was measured using standard method [17]. The coronary artery was tiedagain, 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 thearea at the risk which is stainedas red. The non-ischemic myocardia of rats were stained as blue. The infarcted rat heart stained aspalecolor. 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 tissueswere fixed in formaldehyde solution. Tissues were cross-sectioned as 4 μm and deparaffinized.

Sections were treated with 4% H_2O_2 for 15 minutes. Staining was carried out, and the image was obtained under aconfocal microscope (Nikon, NIKON A1R/A1, Japan).

Results

Effect of curcumin on cell viability

Oxidative stress affects cell viability and leads to cell death. We have investigated the curcumin effect on cellgrowth inhibition under TBHP induced theoxidative condition. TBHP is more stable and suitable compound to stimulate ROS than H_2O_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
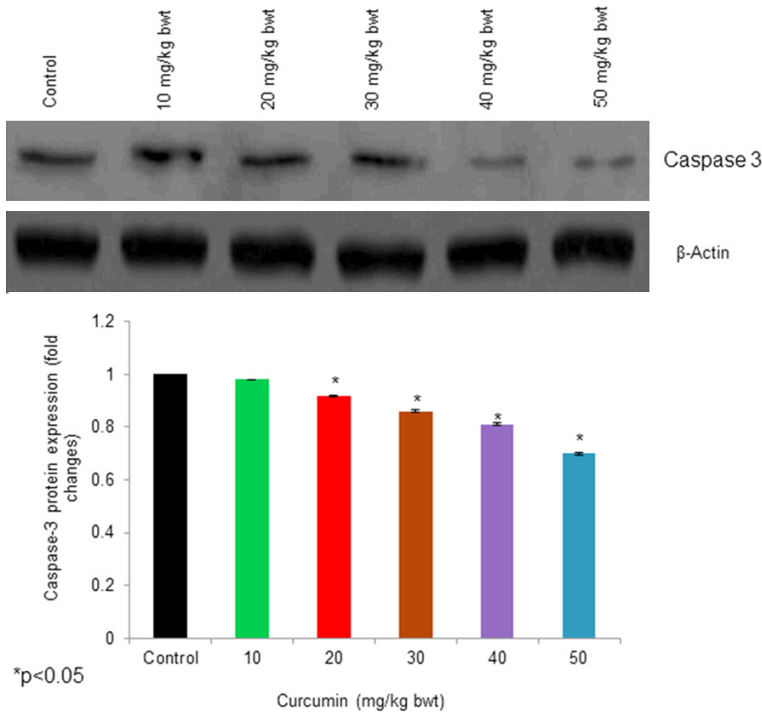
Figure 3. Curcumin effect on protein expression. The cellwas incubated with different dose of curcumin for 24 h, and protein expression of caspase 3 was determinedconcerning control. All the values were expressed mean ± SD, *P<0.05.

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_2O_2 induced theoxidative condition. Cell was treated with curcumin (10, 20, 30, 40 & 50 mg/kg bwt) for 4 h. Then cells were treated with H_2O_2 for 24 h. H_2O_2 significantly increased lipid peroxidation. However, curcumin pretreatment protected cells from lipid peroxidation (**Table 1**).

Effect of curcumin on SOD activity

Increased membrane fatty acid oxidation produces oxidativestress that affects cell viability and leads to cell growth inhibition. We investigated the curcumin effect on SOD activity

under H_2O_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_2O_2 for 24 h. H_2O_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_2O_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_2O_2 for 24 h. H_2O_2 significantly reduced catalase activity. However, curcumin pretreatment retained catalase activity in a dose-dependent manner (**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 ratwas treated with curcumin (50 mg/kg/day) for 15 consecutive days
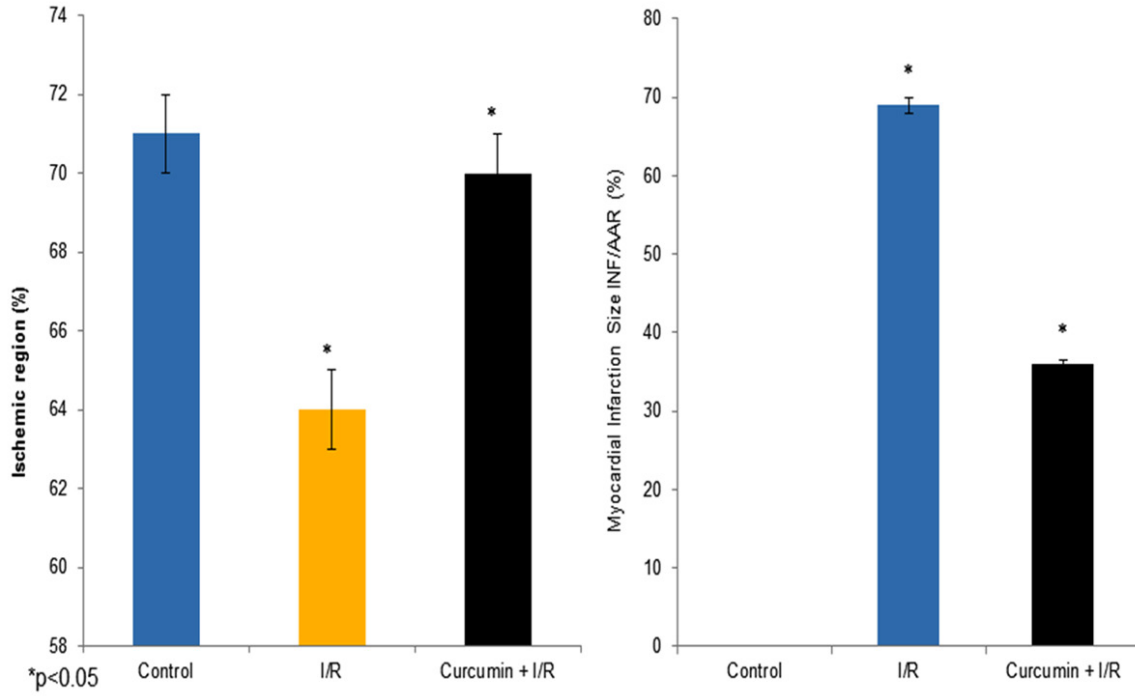
Figure 4. Curcumin effect on myocardial infarct size and ischemic region. Male albino rats were administered curcumin for 24 h, and percentage of ischemic region and myocardial infarct size were determinedconcerning control. All the values were expressed mean ± SD, *P<0.05.
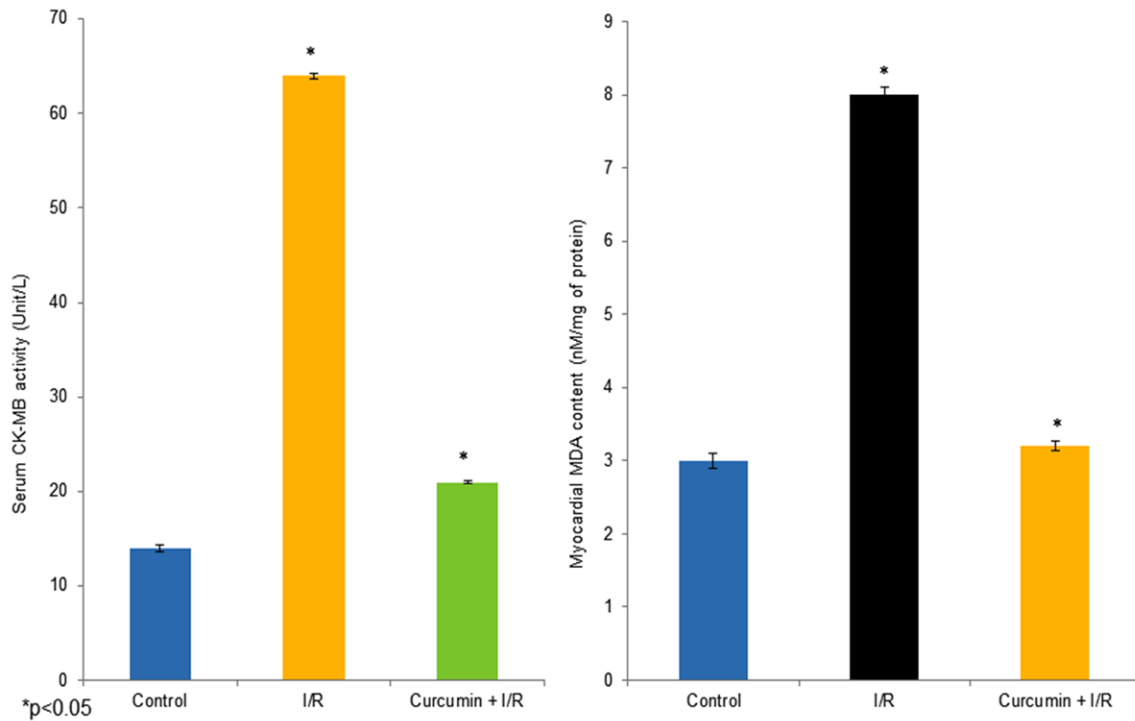

Figure 5. Curcumin effect on serum creatine kinase and myocardial MDA content. Male albino rats were administered with curcumin for 24 h, and percentage of serum creatine kinase and myocardial MDA content was determinedconcerning control. All the values were expressed mean ± SD, *P<0.05.
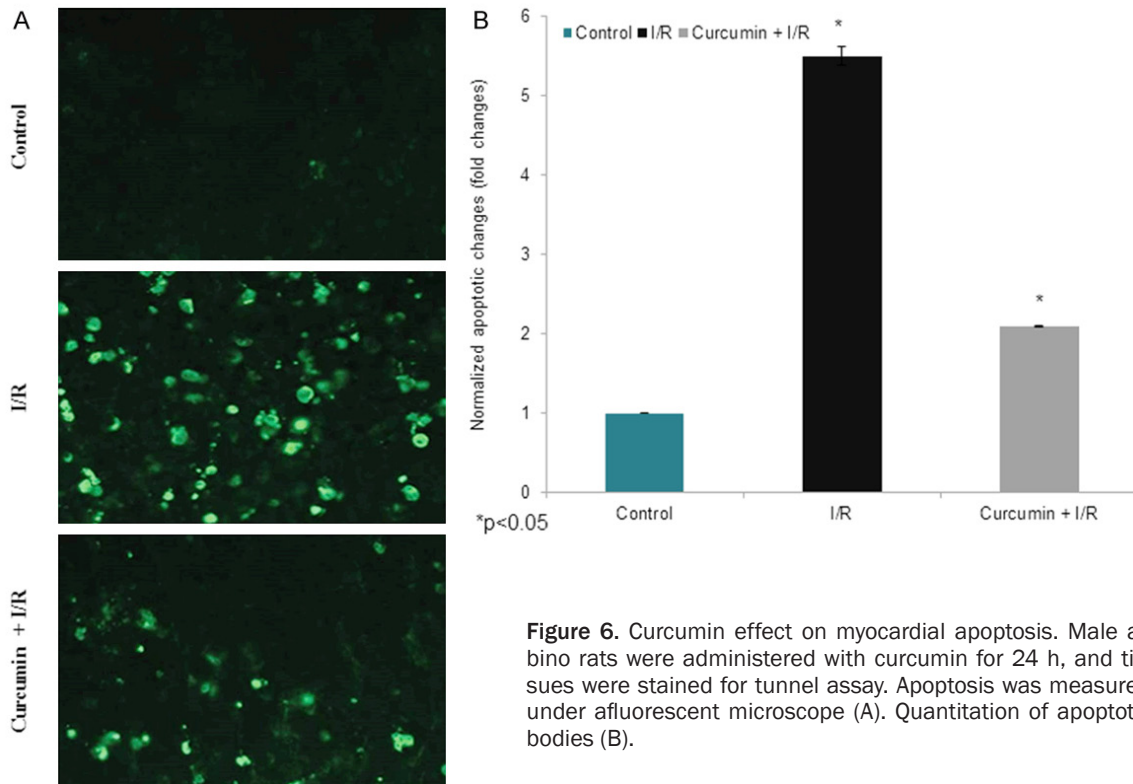
Figure 6. Curcumin effect on myocardial apoptosis. Male albino rats were administered with curcumin for 24 h, and tissues were stained for tunnel assay. Apoptosis was measured under afluorescent microscope (A). Quantitation of apoptotic bodies (B).

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-protectiverole in myocardial ischemia-reperfusion of male albino rats. A study ofcurcumin derivatives showed anti-inflammatory and antioxidant effect [18]. Curcumin reduced H_2O_2 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 thekey approach to limit themyocardial damage.

We have investigated the effect of curcumin on antioxidant markers and its efficiency for decreasingcellular 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 inischemia-reperfusiondamagein 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 thenucleus and thereby acts

against cellular oxidative stress [20]. Nrf2 play acritical role in the defense system inmyocardial ischemia-reperfusionof male albino rats. Calvert et al. [21] have reported the Nrf2 deficient ratsexhibited increasedcellular oxidative stress and myocardialinjury during ischemia-reperfusion. Therefore, Nrf2 pathway is apotential 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 theantiapoptotic effect. Curcumin pretreatment reduced TBHP-induced caspase-3 and bax mRNA expression and enhanced the antiapoptotic bcl-2 mRNA expression. In vitro effects of curcumin directly translated for the cardioprotection against ischemia-reperfusion at 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 there sult of the down streamaction of antioxidant defense. Kannan and Jain [23] have reported the ROS, and oxidative stress could play a keyrole apoptosis.

Yellon and Hausenloy [11] have reported the increased ROS level trigger calcium and endoplasmic stress that leads to lose of membrane potential of mitochondria and caspase 3 activations. Curcumin prevents cells from apoptosis by inhibiting caspase 3 and bax activation and curcumin activate 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, theanti-oxidative defense is anappropriate target to prevent ischemia-reperfusion damage. Kim et al. [25] have reported that curcumin reduced myocardial ischemic reperfusion damage. Curcumin treatment reduced H_2O_2 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 potentialagent, and it could be akey pharmacological approach to limit cardiac ischemic injury in male albino rats.

Disclosure of conflict of interest

None.

Address correspondence to: Lang Xu, Department of Pathology, Medical College, Wuhan University of Science and Technology, 947 Heping Avenue, Qingshan District, Wuhan 430065, Hubei, China. Tel: 0086-027-68893395; Fax: 0086-027-68893-395; E-mail: xulangxlx@hotmail.com

References

[1] Manolova Y, Deneva V, Antonov L, Momekova D, Lambov N. The effect of the water on the curcumin tautomerism: A quantitative approach. Spectrochimica Acta 2014; 132: 815-820.

[2] Dutta S, Padhye S, Priyadarsini KI, Newton C. Antioxidant and antiproliferative activity of curcumin semicarbazone. Bioorg Med Chem Lett 2005; 15: 2738-44.

[3] Weber WM, Hunsaker LA, Abcouwer SF, Deck LM, Vander Jagt DL. Anti-oxidant activities of curcumin and related enones. Bioorg Med Chem 2005; 13: 3811-20.

[4] Lim CS, Jin DQ, Mok H, Oh SJ, Lee JU, Hwang JK, Ha I, Han JS. Antioxidant and anti-inflammatory activities ofxanthorrhizol in hippocampal neurons and primary cultured microglia. J Neurosci Res 2005; 82: 831-8.

[5] Biswas SK, McClure D, Jimenez LA, Megson IL, Rahman I. Curcumin induces glutathione biosynthesis and inhibits NF-kappaB activation and interleukin-8 release in alveolar epithelial cells: Mechanism of free radical scavenging activity. Antioxid Redox Signal 2005; 7: 32-41.

[6] Kala C, Ali Syed S, Abid M, Rajpoot S, Khan NA. Protection against FCA induced oxidative stress-inducedDNA damage in a model of arthritis and in thevitro anti-arthritic potential of costus species rhizome extract. International Journal of Pharmacognosy and Phytochemical Research 2015; 7: 383-389.

[7] Lauderback CM, Hackett JM, Huang FF, Keller JN, Szweda LI,Markesbery WR, Butterfield DA. The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the

Alzheimer's disease brain: The role of Abeta1-42. J Neurochem 2001; 78: 413-6.

[8] Buja LM. Myocardial ischemia and reperfusion injury. Cardiovasc Pathol 2005; 14: 170-175.

[9] Reimer KA, Ideker RE. Myocardial ischemia and infarction: anatomic and biochemical substrates for ischemic cell death and ventricular arrhythmias. Hum Pathol 1987; 18: 462-475.

[10] Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. Arch Pathol 1960; 70: 68-78.

[11] Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med 2007; 357: 1121-1135.

[12] Marczin N, El-Habashi N, Hoare GS, Bundy RE, Yacoub M. Antioxidants in myocardial ischemia-reperfusion injury: therapeutic potential and basic mechanisms. Arch Biochem Biophys 2003; 420: 222-236.

[13] Hung LM, Chen JK, Huang SS, Lee RS, Su MJ. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovasc Res 2000; 47: 549-555.

[14] Muthuraman P, Enkhtaivan G, Bhupendra M, Chandrasekaran M, Rafi N, Kim DH. Investigation of the role of aspartame in apoptosis process in Hela cells. Saudi Journal of Biological Sciences 2015.

[15] Muthuraman P, Muthuviveganandavel V, Kim DH. Cytotoxicity of zinc oxide nanoparticles on antioxidant enzyme activities and mRNA expression in the cocultured C2C12 and 3T3-L1 cells. Appl Biochem Biotechnol 2015; 175: 1270-1280.

[16] Muthuraman P, Ramkumar K, Kim DH. Analysis of thedose-dependent effect of zinc oxide nanoparticles on the oxidative stress and antioxidant enzyme activity in adipocytes. Appl Biochem Biotechnol 2014; 174: 2851-63.

[17] Yang Y, Duan W, Lin Y, Yi W, Liang Z, Yan J, Wang N, Deng C, Zhang S, Li Y, Chen W, Yu S, Yi D, Jin Z. SIRT1 activation by curcumin pretreatment attenuates mitochondrial oxidative damage induced by myocardial ischemia-reperfusion injury. Free Radic Biol Med 2013; 65: 667-679.

[18] Liu Z, Tang L, Zou P, Zhang Y, Wang Z, Fang Q, Jiang L, Chen G, Xu Z, Zhang H, Liang G. Synthesis and biological evaluation of alkylated and prenylated mono-carbonyl analogs of curcumin as anti-inflammatory agents. Eur J Med Chem 2014; 74: 671-682.

[19] Baird L, Dinkova-Kostova AT. The cytoprotective role of the Keap1-Nrf2 pathway. Arch Toxicol 2011; 85: 241-272.

[20] Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 2009; 47: 1304-1309.

[21] Calvert JW, Jha S, Gundewar S, Elrod JW, Ramachandran A, Pattillo CB, Kevil CG, Lefer DJ. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. Circ Res 2009; 105: 365-374.

[22] Shehzad A, Lee YS. Molecular mechanisms of curcumin action: signal transduction. Biofactors 2013; 39: 27-36.

[23] Kannan K, Jain SK. Oxidative stress and apoptosis. Pathophysiology 2000; 7: 153-163.

[24] Tian H, Zhang B, Di J, Jiang G, Chen F, Li H, Li L, Pei D, Zheng J. Keap1: one stone kills three birds Nrf2, IKKbeta, and Bcl-2/Bcl-xL. Cancer Lett 2012; 325: 26-34.

[25] Kim YS, Kwon JS, Cho YK, Jeong MH, Cho JG, Park JC, Kang JC, Ahn Y. Curcumin reduces the cardiac ischemia-reperfusion injury: involvement of the tolllike receptor 2 in cardiomyocytes. J Nutr Biochem 2012; 23: 1514-1523.