

RESEARCH PAPER

Curcumin promotes cardiac repair and ameliorates cardiac dysfunction following myocardial infarction

Ning-Ping Wang¹, Zhang-Feng Wang¹, Stephanie Tootle¹, Tiji Philip¹ and Zhi-Qing Zhao^{1,2}

¹Cardiovascular Research Laboratory, Mercer University School of Medicine, Savannah, GA, USA, and ²Department of Physiology, Shanxi Medical University, Taiyuan, Shanxi, China

Correspondence

Zhi-Qing Zhao, Department of Basic Biomedical Sciences, Mercer University School of Medicine, Savannah, GA 31404, USA.
E-mail: zhao_z@mercer.edu

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BACKGROUND AND PURPOSE

Curcumin, the natural yellow pigment extracted from the rhizomes of the plant *curcuma longa*, has been demonstrated to exhibit a variety of potent beneficial effects, acting as an antioxidant, anti-inflammatory and anti-fibrotic. In this study we tested the hypothesis that curcumin attenuates maladaptive cardiac repair and improves cardiac function after ischaemia and reperfusion by reducing degradation of extracellular matrix (ECM) and inhibiting synthesis of collagens via TGF β /Smad-mediated signalling pathway.

EXPERIMENTAL APPROACH

Sprague-Dawley rats were subjected to 45 min of ischaemia followed by 7, 21 and 42 days of reperfusion respectively. Curcumin was fed orally at a dose of 150 mg·kg⁻¹·day⁻¹ only during reperfusion.

KEY RESULTS

Curcumin reduced the level of malondialdehyde, inhibited activity of MMPs, preserved ECM from degradation and attenuated collagen deposition, as it reduced the extent of collagen-rich scar and increased mass of viable myocardium. In addition to reducing collagen synthesis and fibrosis in the ischaemic/reperfused myocardium, curcumin significantly down-regulated the expression of TGF β 1 and phospho-Smad2/3, and up-regulated Smad7 and also increased the population of α -smooth muscle actin expressing myofibroblasts within the infarcted myocardium relative to the control. Echocardiography showed it significantly improved left ventricular end-diastolic volume, stroke volume and ejection fraction. The wall thickness of the infarcted middle anterior septum in the curcumin group was also greater than that in the control group.

CONCLUSION AND IMPLICATIONS

Dietary curcumin is effective at inhibiting maladaptive cardiac repair and preserving cardiac function after ischaemia and reperfusion. Curcumin has potential as a treatment for patients who have had a heart attack.

Abbreviations

ECM, extracellular matrix; EF, ejection fraction; FS, fraction shortening; LCA, left coronary artery; LV, left ventricle; MDA, malondialdehyde; MI, myocardial infarction; α -SMA, α -smooth muscle actin

Introduction

Death and disability after myocardial infarction (MI) continue to be a major public health problem (Ferdinandy *et al.*, 2007;

Liem *et al.*, 2007). The current paradigm in the treatment of MI patients is to open the infarct-related artery through either percutaneous coronary intervention or coronary artery bypass graft surgery. However, the restoration of

blood flow to an ischaemic myocardium may paradoxically exaggerate tissue injury and initiate a maladaptive tissue repair process (Vinten-Johansen *et al.*, 2005; Prasad *et al.*, 2009). During the early stages of MI, the degradation of extracellular matrix (ECM) by regional oxidative stress-activated MMPs results in progressive infarct expansion, ventricular wall thinning and chamber dilatation (Spinale *et al.*, 2002; Tessone *et al.*, 2005). This is followed by healing of the infarct area during which fibroblasts proliferate and deposit collagen to form a reparative fibrotic and non-contractile scar. These changes further result in the left ventricular global dilatation, cardiac dysfunction and heart failure (Brown *et al.*, 2005).

It is generally agreed that, initially, the degraded pre-existing collagenous network leads to ventricular wall thinning and dilatation, while newly synthesized collagen results in scar tissue formation and left ventricle (LV) diastolic dysfunction (Brower *et al.*, 2006). In this regard, activation of MMPs has been associated with degradation of the extracellular matrix (ECM), and TGF β 1/Smads signalling pathway has been suggested to play a major role in mediating collagen synthesis and scar tissue formation in the infarcted myocardium during cardiac repair (Spinale *et al.*, 2002; Brower *et al.*, 2006).

The increasing incidence of heart failure resulting from maladaptive cardiac repair after MI has stimulated investigative efforts to develop pharmacological strategies for protecting against tissue injury (Gallagher *et al.*, 2007). However, few of the drugs that inhibit scar tissue formation, promote cardiac repair and thereby improve cardiac function after reperfusion have gained clinical acceptance for a routine treatment of MI patients (Downey and Cohen, 2009).

Curcumin (diferuloylmethane) is the natural yellow pigment extracted from the rhizomes of the plant *curcuma longa* (Ghosh *et al.*, 2010). In several studies, curcumin has been demonstrated to exhibit a variety of potent beneficial effects such as antioxidant, anti-inflammatory and anti-fibrotic (Venkatesan, 1998; Ghosh *et al.*, 2009; 2010; Smith *et al.*, 2010). Although it has previously been shown that curcumin inhibits the development of myocardial cell hypertrophy and hypertension-induced heart failure by attenuating p300 histone acetyltransferase activity (Morimoto *et al.*, 2008), it is not known whether curcumin, when it is fed on a daily basis after ischaemia followed by reperfusion, promotes cardiac repair and improves cardiac function by modulating ECM degradation and collagen synthesis.

Therefore, we tested the hypothesis that curcumin inhibits the fibrotic process and promotes cardiac function in a rat model of ischaemia/reperfusion-induced heart failure. Specifically, the effects of curcumin on oxidative stress, MMP activity, ECM degradation and TGF- β 1/Smad-mediated collagen synthesis were examined. Our results revealed that curcumin improves cardiac function and this is associated with a mechanism of action that balances ECM degradation and collagen synthesis in the infarcted myocardium.

Methods

Surgical preparation of animals

Male Sprague-Dawley rats weighing 400–450 g were anaesthetized with an initial i.p. injection of a mixture of ketamine

(90 mg·kg⁻¹) and zylaxine (10 mg·kg⁻¹). If re-dosing was required during surgery, ketamine alone was given. The animals were intubated and mechanically ventilated with oxygen-enriched room air using a rodent respirator. Procedures used on the first surgical day were performed under sterile conditions. The chest was opened by a left thoracotomy through the fourth intercostal space. After pericardiectomy, a 6-0 proline ligature was placed under the left coronary artery (LCA) where it emerged from beneath the left atrium, and the ends of the tie were threaded through a small plastic tube to form a snare for reversible LCA occlusion. After ischaemia, this proline ligature was kept in the chest for re-ligation at the end of the study. The body temperature was maintained constant at 37°C by a heating pad.

All animals received humane care in compliance with 'The Guide for the Care of Use of Laboratory Animals' published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996) and the study protocol was approved by the Institutional Animal Care and Use Committee of Mercer University. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Experimental protocol

The rats were randomly assigned to one of four groups (Figure 1): (i) control ($n = 8$ for each time point): no intervention was given at reperfusion; (ii) curcumin treatment ($n = 8$

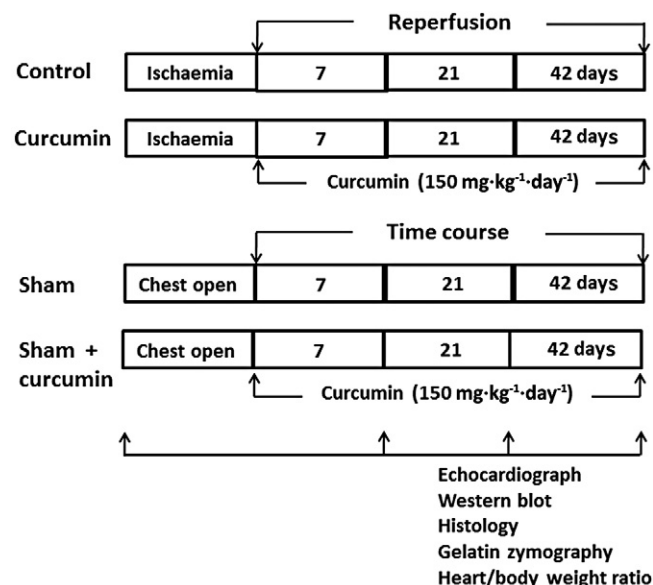


Figure 1

Experimental study group. In the control group ($n = 8$ for each time point), 45 min coronary occlusion was followed by 7, 21 and 42 days of reperfusion, respectively; in the curcumin group ($n = 8$ for each time point), curcumin was fed daily during the entire period of reperfusion; in the sham and sham + curcumin group ($n = 6$ for each time point, respectively), the chest was opened without coronary occlusion and curcumin was fed during the reperfusion. At each observational point, the heart was removed for histological analysis after cardiac function was measured.

for each time point): curcumin (Sigma Chemical Co., St. Louis, MO, USA) was orally fed at a dose of $150 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (in peanut paste) during reperfusion. The rats in the control and curcumin groups were subjected to 45 min of LCA occlusion followed by 7, 21 and 42 days of reperfusion, respectively; (iii) sham control ($n = 6$ for each time point): the ligature was placed under LCA without occlusion and the observation period lasted 42 days; (iv) sham + curcumin ($n = 6$ for each time point): no LCA occlusion was conducted and curcumin was fed at the same dose as that in the curcumin group during the experiment. At the end of the surgical operation, the incisions were closed in layers. The chest and endotracheal tubes were removed after the spontaneous breathing was recovered.

Cardiac function and wall thickness by echocardiography

Two-dimensional (2D) guided M-mode ultrasound system (Acuson Sequoia, Siemens Medical Solutions USA, Inc., Mountain View, CA, USA) was used to assess LV systolic and diastolic function as we previously reported (Zhao *et al.*, 2010). In brief, 2D images from a short-axis view of LV at the level of the papillary muscles were obtained using a 15 MHz linear transducer. LV % fraction shortening (FS) and ejection fraction (EF) were calculated. Pulsed-wave Doppler spectra of mitral inflow from the apical four-chamber view was used to assess LV diastolic flow characteristics by calculating a ratio of early diastolic filling (E-wave) velocity and atrial filling (A-wave) velocity. The 2D images, which were frozen at the end of diastole, were used to measure LV volume, interventricular septum and posterior wall thickness. The echocardiography was performed before opening the chest (baseline) and during the time course of the experiment in all groups. All measurements were averaged over three consecutive cardiac cycles.

Myocardial tissue preparation

At the end of each experiment, the LCA was re-ligated and 2 mL of 20% unisperse blue dye was injected into the carotid vein to outline the non-ischaemic and area at risk myocardium. The animal was killed and the heart was removed. After LV wet weight to body weight ratio was determined, infarct size was determined using triphenyl tetrazolium chloride staining as reported previously (Mykytenko *et al.*, 2008) and calculated as a percentage of the area at risk myocardium. In other rats, a piece of transverse slice including the non-ischaemic and ischaemic zones was initially fixed in 10% phosphate-buffered formalin solution for 24 h, and then embedded in paraffin for further histological analysis. Cryosections (4 μm thick) of the tissue blocks for immunohistochemical staining were obtained using a Microtome cryostat (Leica RM2135, Meyer Instruments, Houston, TX, USA) as previously reported (Zhao *et al.*, 2010). For Western blot analysis, the non-ischaemic and ischaemic zones were immediately frozen in liquid nitrogen and kept at -70°C until use.

Determination of lipid peroxidation

Malondialdehyde (MDA), a stable metabolite of the reactive oxygen species (ROS)-mediated lipid peroxidation cascade, was measured as previously reported (Sun *et al.*, 2005). In

brief, 100 mg of heart tissue from the non-ischaemic and ischaemic zones was minced in ice-cold Tris-HCl buffer and homogenized using a Teflon pestle. After centrifugation, the resulting supernatant fraction was used to determine the level of MDA using a lipid peroxidation assay kit (Calbiochem, San Diego, CA, USA). The absorbance of the supernatant was determined by a spectrophotometer (BioTek, Inc, Winooski, VT, USA) at 586 nm. The concentration of MDA was calculated using standard curves and is expressed as $\mu\text{M}\cdot\text{g}^{-1}$ protein.

Activity of MMP by gelatin zymography

Gelatin zymography was performed as described previously (Tessone *et al.*, 2005). In brief, freshly frozen ischaemic tissue samples were homogenized in lysis buffer and gelatin (Sigma Chemical Co.) was used as a substrate. Samples (20 μg) and zymography standard (7 ng) of purified human MMP-9 and MMP-2 (Chemicon Inc., Temecula, CA, USA) were directly loaded onto standard polyacrylamide gels. After electrophoresis, the gels were soaked in Triton X-100, rinsed in water and incubated in zymogram development buffer (Bio-Rad). After incubation, the gels were stained in Coomassie blue R-250 and de-stained in methanol and acetic acid until clear bands were displayed. Molecular sizes of bands displaying enzymatic activity were characterized by comparison with purified standard MMP-9 or MMP-2. Zymographic activity was quantified using a digital image analyser (ImageJ, NIH).

Collagen deposition by Masson's trichrome staining

Masson's trichrome staining was used to evaluate collagen deposition within the scar muscle as previously reported (Zhao *et al.*, 2010). In brief, the paraffin slides were deparaffinized, hydrated with distilled water and stained by Masson's trichrome method. The staining protocols produced collagen blue, nuclei black and viable muscle fibre red. Eight transmural sections in each heart including the infarct scar and the non-infarct myocardium were averaged to determine the collagen-rich area, calculated as a percentage of the area at risk myocardium (ImageJ, NIH).

Expression of TGF β 1, collagen and Smads by Western blotting

Western blotting was performed as described previously (Zhao *et al.*, 2010). In brief, the freshly frozen non-ischaemic and ischaemic tissue samples were homogenized in lysis buffer and protein concentration was measured by the DC Protein Assay. The protein was then boiled and loaded onto gradient SDS-polyacrylamide gel using Mini Protean II Dual Stab Cell (Bio-Rad). Membranes were subsequently exposed to the following antibodies: a mouse monoclonal anti-TGF β 1 (Abcam, Inc., Cambridge, MA, USA), the goat polyclonal anti-collagen I, IV and a mouse monoclonal anti-collagen III (Sigma), the rabbit monoclonal phospho-Smad2 and Smad3 (Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-Smad4 and Smad7 (Santa Cruz Biotech, Santa Cruz, CA, USA) respectively. Bound antibody was detected by horseradish peroxidase conjugated anti-rabbit IgG. The membrane was incubated with chemiluminescence substrate and exposed to an X-ray film. The scanned images were imported into the

ImageJ. Actin was used as a standard of protein-loading control for normalizing bands at different time points. The final results are calculated as the ratio of intensity of each band divided by actin intensity.

Detection of fibroblast differentiation with immunohistochemistry

Immunohistochemical staining of tissue sections was performed as described previously (Zhao *et al.*, 2003). In brief, paraffin-embedded blocks were deparaffinized in xylene and dehydrated in graded ethanol. The transverse paraffin sections were stained using a monoclonal antibody against α -smooth muscle actin (α -SMA) (Sigma Chemical Co.). The slides were washed in PBS, incubated with an anti-mouse IgG and stained using the ABC-red and substrate kit (Vector Laboratories, Burlingame, CA, USA). The quality of the assay was controlled by either elimination of the primary antibody or incubation of the tissue with a non-immune IgG. Data were analysed using computed-assisted morphometry (ImageJ, NIH). Differentiation of fibroblasts is reported as mean number of α -SMA-expressing myofibroblasts from eight randomized high-powered fields (Zhao *et al.*, 2010).

Statistical analysis

All data are presented as the mean $\pm$ SEM. A one-way ANOVA followed by Student–Newman–Keul's *post hoc* test was used to analyse group differences in the intensity of TGF β 1, collagens, Smads and the percentage of collagen-rich area within the area at risk myocardium. Echocardiographic data were analysed by one-way repeated measures ANOVA followed by *post hoc* analysis with Student–Newman–Keul's test for multiple comparisons by SigmaStat (Systat Software Inc., Point Richmond, CA, USA). Statistical significance was set at a value of $P < 0.05$.

Results

A total of 93 rats were initially included in this study. Two rats in the control group and one rat in the curcumin group died immediately due to bleeding from an injured coronary artery. Two rats in the control group and two rats in the curcumin group died 1 day after the chest was closed due to a large area of infarction. One rat in the control group and one rat in the curcumin group were excluded because no evidence of ischaemic zone was found after coronary ligation. The final 84 rats were included for the histological examination and echocardiography. No animals died during the 42 days of observation. The curcumin dose selected was primarily based on a previous study (Ghosh *et al.*, 2010). It has been reported that curcumin concentration after oral administration would reach a plateau in blood at 6 h, and remain at a significantly high level even at 24 h (Suresh and Srinivasan, 2010).

Curcumin treatment during reperfusion ameliorated oxidative stress

Detection of MDA has been used as an indicator of lipid peroxidation from damaged tissue stimulated by ROS. The

profile of MDA levels measured from the lysed myocardium is presented in Figure 2A. MDA was detectable during the time course of the experiment in the sham and sham plus curcumin groups, but no significant difference among the groups was found, and, therefore, data were pooled. In addition, there was no significant difference in the MDA level in the non-ischaemic area among all the groups compared to the level seen in the sham group. Ischaemia/reperfusion resulted in a significant increase in MDA level during reperfusion relative to the sham control. Treatment with curcumin significantly decreased the levels of MDA during the course of reperfusion relative to the corresponding points in the control group.

Curcumin treatment during reperfusion inhibited activity of MMPs

The activity of MMP-9 and MMP-2 on gelatin zymogram SDS-PAGE gel is illustrated in Figure 2B; proMMP-9 was not found in the sham and sham plus curcumin groups, but detected in the control and curcumin groups throughout the experiment. Consistent with a significant increase in expression of proMMP-9 at day 7, MMP-9 was significantly higher in the control group, but no MMP-9 was detected during 21 and 42 days. Curcumin treatment inhibited both proMMP-9 and MMP-9. ProMMP-2 was expressed in the sham and sham plus curcumin groups as well as in the non-ischaemic zones in the control and curcumin groups, but no group difference was detected. Consistent with enhanced proMMP-2, there was a significant increase in the expression of MMP-2 during the course of the experiment, suggesting that reperfusion triggers a constant activation of MMP2. However, both proMMP-2 and MMP-2 were significantly inhibited by daily administration of curcumin.

Curcumin treatment during reperfusion reduced infarct size and collagen deposition

No group difference in the area at risk myocardium was found, average $38 \pm 6\%$ in the control and $39 \pm 4\%$ in the curcumin group ($P > 0.05$) respectively. At 3 days after curcumin treatment, infarct size was significantly reduced relative to the control ($45 \pm 6\%$ vs. $58 \pm 8\%$ of area at risk myocardium, $P < 0.05$, $n = 6$ for each group). The collagen-rich area was evaluated using Masson's trichrome staining. As shown in Figure 3, no newly synthesized collagen was detected in the non-ischaemic zones in the control and curcumin groups or in the sham and sham plus curcumin groups throughout the experiment. However, in the control animals, the collagen positive area was increased after 7 days of reperfusion, as determined by the size of collagen-rich area within the area at risk myocardium. The collagen-rich scar tissue was extended through the LV wall from endomyocardium to epimyocardium after 42 days of reperfusion. However, at this stage, the hearts treated with curcumin exhibited a significant reduction in the collagen-rich area within the area at risk myocardium, and more viable myocardium was detected, suggesting less degradation of pre-existing ECM. The ischaemic/reperfused myocardium appeared more organized and circumscribed, consistent with inhibition of MMPs by curcumin.

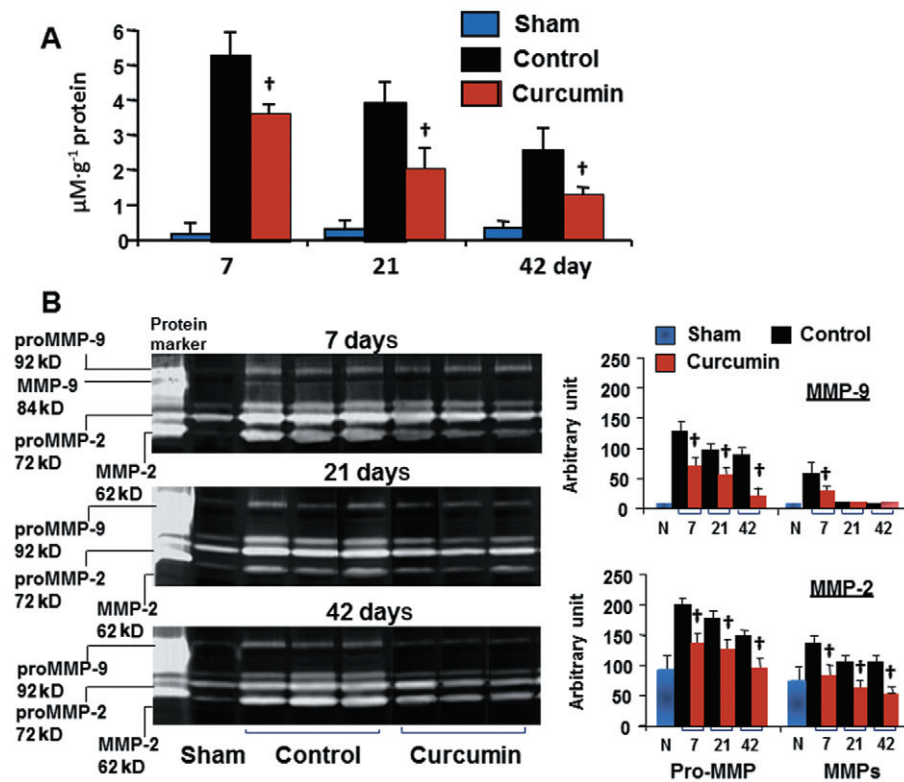


Figure 2

Tissue level of MDA (A) and activity of MMPs (B). MMP-9 was expressed at the 92-kD band (pro-form) and the 84-kD band (active form); MMP-2 was evident at the 72-kD (pro-form) and the 62-kD band (active form). Curcumin significantly reduced MDA levels during the course of the experiment, along with inhibition of both pro- and active forms of MMP-9 and MMP-2 as measured by an arbitrary unit. $n = 8$, values are mean $\pm$ SEM. $^{\dagger}P < 0.05$ versus control.

Curcumin treatment during reperfusion reduced collagen synthesis

To further confirm the results demonstrated by Masson's trichrome staining, we measured the expression of collagen type I, III and IV in the infarcted tissue using Western blotting as shown in Figure 4. Collagen types I and III, but not collagen type IV, were expressed in the non-ischaemic zones in the control and curcumin as well as in both sham groups, but no significant difference among the groups was detected. However, the synthesis of these collagens was significantly increased at day 7, and continuously maintained at higher levels after 42 days of reperfusion relative to those in the sham group. The hearts treated with curcumin showed a significant reduction in synthesis of collagens relative to the control group at all time points measured. These data are consistent with the findings showing a reduction in fibrotic tissue in the infarcted myocardium identified by Masson's trichrome staining.

Curcumin treatment during reperfusion inhibited the expression of TGF β 1 and phosphorylation of Smads

To demonstrate whether inhibition of collagen synthesis by curcumin is associated with TGF β 1-Smads signalling pathways, we measured TGF β 1 expression and Smads phosphor-

ylation from the transmural tissue samples obtained from the infarcted myocardium using Western blotting assay. As shown in Figure 4, TGF β 1 was expressed in the sham and sham plus curcumin groups as well as in the non-ischaemic myocardium in the control and curcumin groups, but no significant difference among the groups was found. Ischaemia/reperfusion caused a constant increase in TGF β 1 expression during the course of the experiment, which was significantly attenuated by administration of curcumin. As shown in Figure 5, the phospho-Smad2 and Smad3 were expressed at a relatively lower level in the non-ischaemic zone and no significant difference was found between the sham, the sham plus curcumin, the control and the curcumin groups. Ischaemia/reperfusion caused a significant change in the phosphorylation of Smad2 and Smad3 at day 7, and they remained in an activated state until 42 days of reperfusion, consistent with an enhanced expression of Smad4. However, expression of Smad7 was down-regulated during the course of the experiment. Daily administration of curcumin enhanced the expression of Smad7 and attenuated the phosphorylation of Smad2 and Smad3 as well as the expression of Smad4, suggesting that Smad7 inhibits the phosphorylation of Smad2 and Smad3. These data are consistent with the significant reduction in collagen synthesis/deposition in the infarcted myocardium detected by Western blotting assay and Masson's trichrome staining.

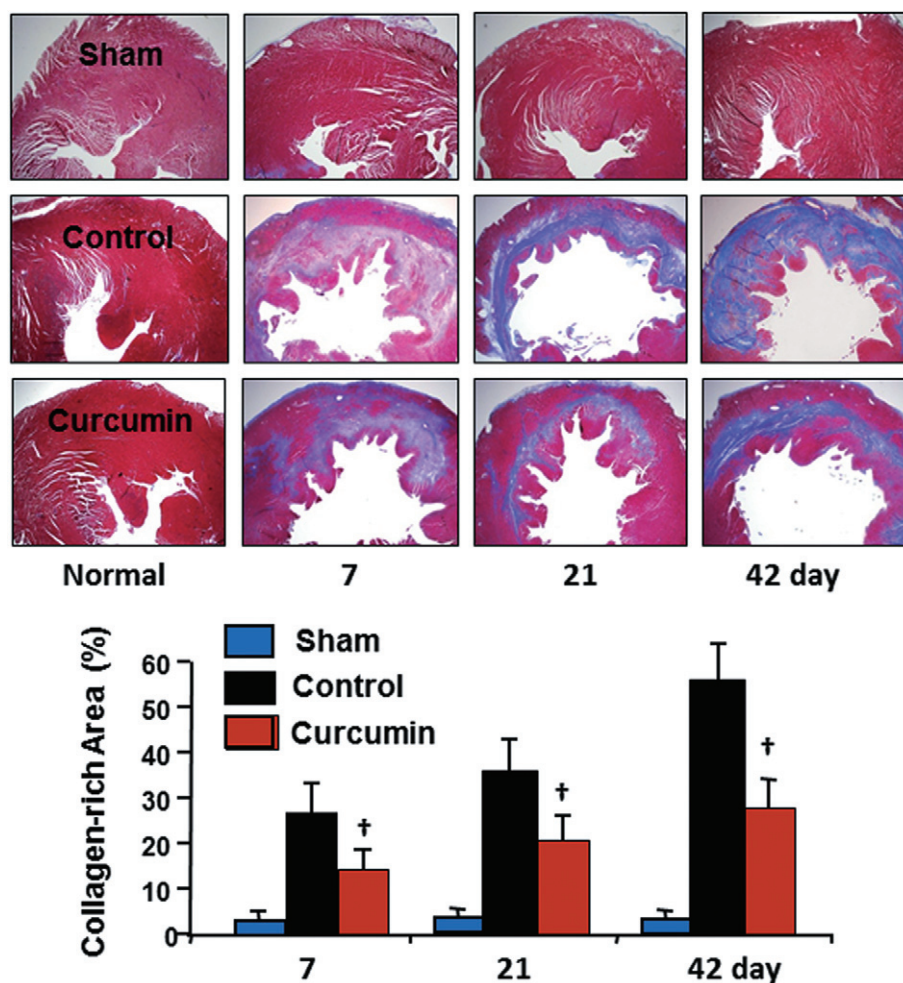


Figure 3

Collagen deposition in the myocardium. The scope of collagen deposition was measured by collagen-rich area calculated as the percentage of the area at risk myocardium. There was extensive loss of myocardial mass in the control group over time. Curcumin treatment significantly reduced collagen deposition with increased mass of viable myocardium within the LV anterior wall. No collagen deposition was detected in either of the sham groups. $n = 8$, values are mean $\pm$ SEM. $\dagger P < 0.05$ versus control.

Curcumin treatment during reperfusion inhibited the differentiation of fibroblasts in the myocardium

The differentiation of fibroblasts was identified as the accumulation of α -SMA-expressing myofibroblasts by immunohistochemical analysis. No α -SMA-expressing myofibroblasts were detected in either of the sham groups. A few α -SMA-expressing myofibroblasts were present only in vascular smooth muscle cells in the non-ischaemic myocardium in the control and curcumin groups. As shown in Figure 6, α -SMA-expressing myofibroblasts were significantly increased after 7 days of reperfusion in the control group. The majority of α -SMA-expressing myofibroblasts had aligned with the host myocardial fibres and along the ischaemic border and scar zones. After 42 days of reperfusion, the number of α -SMA-expressing myofibroblasts in these zones was still significantly higher in the control group relative to the non-ischaemic zone and sham control. However, the increase in the number of α -SMA-expressing myofibroblasts accumulated

in the infarcted zone in the curcumin group was significantly reduced during the entire period of reperfusion, suggesting that treatment with curcumin inhibits differentiation of fibroblasts.

Curcumin treatment during reperfusion enhanced cardiac repair

The LV wet weight to body weight ratio, LV end-diastolic volume and wall thickness were used to assess cardiac repair with curcumin treatment. As a sign of chronic heart failure, the heart : body weight ratio in the control group was significantly increased relative to the sham group ($0.39 \pm 0.01\%$ vs. 0.24 ± 0.02 in Sham, $P < 0.05$) at day 42. Oral administration of curcumin for 42 days resulted in a significant reduction in this calculated ratio ($0.26 \pm 0.02\%$, $P < 0.05$ vs. control), suggesting a less marked hypertrophic response to ischaemia/reperfusion injury. As shown in Figure 7, the LV end-diastolic volume was not altered during experimental periods in either sham group (data were pooled). However, ischaemia/

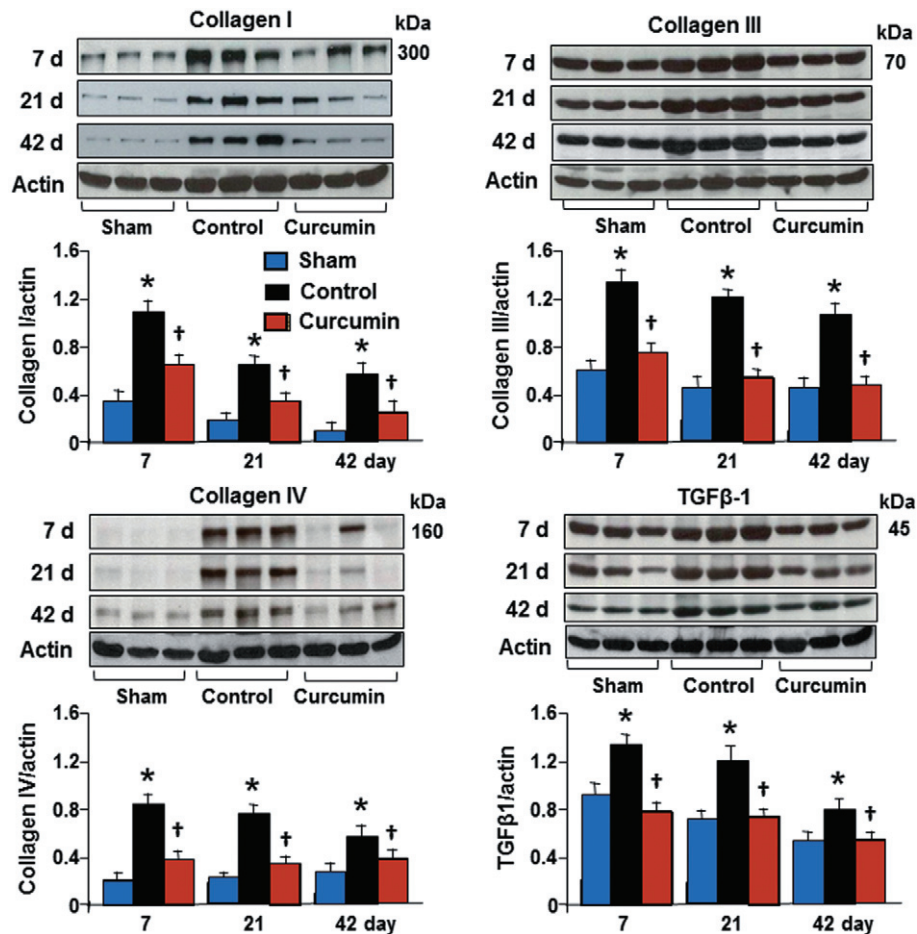


Figure 4

Levels of collagen I, III, IV and TGFβ1 protein. There was a significant increase in levels of collagen I, III IV and TGFβ1 protein relative to the sham and non-infarcted zone in the control group. Curcumin treatment significantly attenuated the changes in these proteins during the course of the experiment, as assessed by reduced intensity of bands shown at the bottom of each panel. All bands were normalized by actin as illustrated at 42 days. $n = 8$, values are mean $\pm$ SEM. * $P < 0.05$ versus sham; † $P < 0.05$ versus control.

reperfusion caused a significant increase in the LV end-diastolic volume during the course of the experiment, when compared with baseline values in the sham and control groups. Furthermore, the wall thickness of the infarct middle anterior septum was significantly reduced relative to the posterior septum in the control group. Consistent with a reduction in the heart : body ratio in the curcumin group, the hearts treated with curcumin had smaller LV end-diastolic volume and greater wall thickness as measured from echocardiographic images.

Curcumin treatment during reperfusion improved cardiac systolic and diastolic function

Echocardiographic results of cardiac systolic and diastolic function among the groups are summarized in Table 1. No significant difference was found in echocardiographic baseline values in both the sham groups (data were pooled), control and curcumin groups. LVSD, LVSS, LVDd and LVDs

were significantly higher during 21 and 42 days of reperfusion in the control group. In addition, the indices of systolic function, that is, the FS, EF and stroke volume, were significantly lower compared to the baseline value, suggesting cardiac contractile dysfunction (Figure 8A). Oral administration of curcumin over 42-day periods improved contractile function. EF values were increased by $25 \pm 6\%$ in the curcumin group compared to the control group at day 42.

The peak E-wave velocity/peak A-wave velocity ratio was selected to access diastolic function through the pulsed-wave Doppler recordings of mitral inflow. As shown in Figure 8B, ischaemia/reperfusion increased both early diastolic filling (E-wave) velocity and atrial filling (A-wave) velocity, and therefore resulted in a significant reduction in the E : A ratio relative to baseline values over 42-day periods in the control animals. However, this change in the E : A ratio was significantly attenuated by oral administration of curcumin. No change in the E : A ratio was found in either of the sham groups during the observation periods.

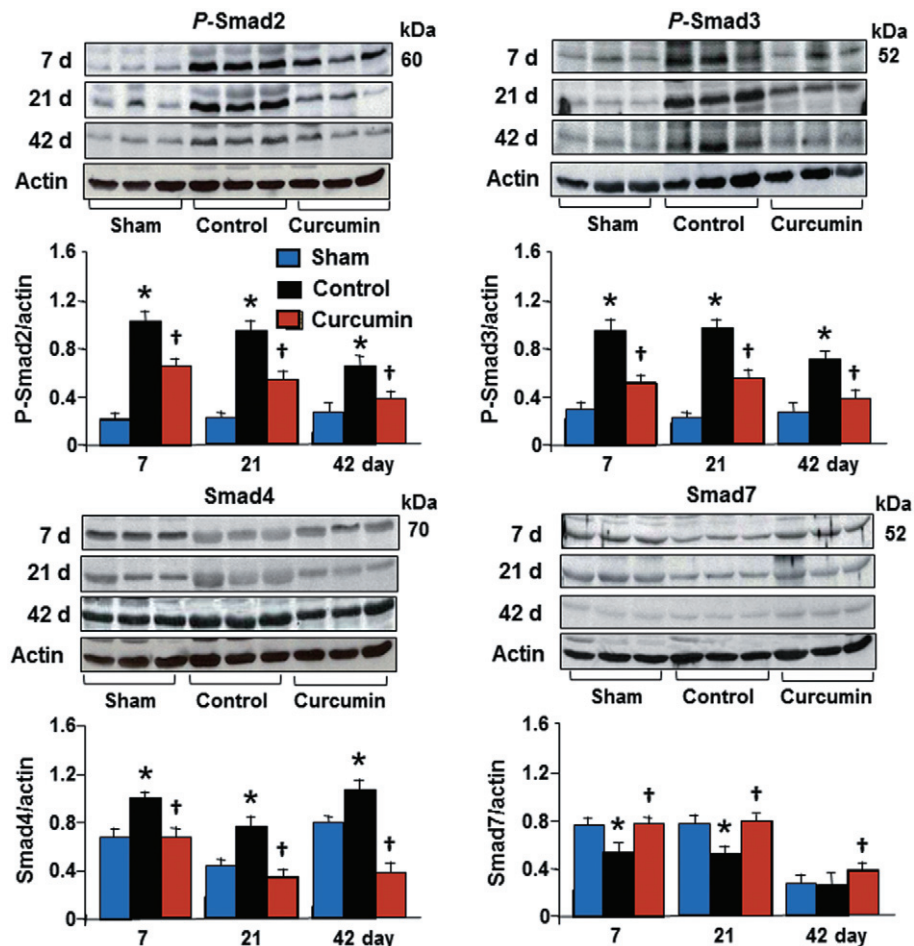


Figure 5

Expression of Smads protein. No significant changes in the total levels of Smad2 and Smad3 were detected (data not shown). However, relative to the sham and non-infarcted zone, ischaemia/reperfusion induced a significant increase in levels of phospho-Smad2, Smad3 and Smad4, and a significant reduction in Smad7 during the course of the experiment in the control group. Curcumin treatment significantly attenuated changes in these proteins as assessed by reduced intensity of bands; shown at the bottom of each panel. All bands were normalized to actin as illustrated at 42 days. $n = 8$, values are mean $\pm$ SEM. * $P < 0.05$ versus sham; † $P < 0.05$ versus control.

Discussion

The major new results of the present study demonstrated that oral administration of curcumin after ischaemia reduces tissue levels of MDA, protects the existing ECM from degradation by attenuating the activity of MMPs and attenuates collagen synthesis via the TGF β 1/Smads-mediated signalling pathways as well as inhibits accumulation of α -SMA-expressing myofibroblasts, indicative of a reduction in fibroblast differentiation. Consistent with these beneficial effects of curcumin on cardiac repair, cardiac function was improved, LV dilatation was reduced and wall thickness was increased. These results support the concept that curcumin could be an effective therapeutic candidate against ischaemia/reperfusion-induced heart failure (Srivastava and Mehta, 2009).

The preservation of original ECM after MI is one of the most important factors to retain tissue integrity and prevent maladaptive cardiac repair (Spinale *et al.*, 2002). MMPs are

members of a family of zinc endopeptidases and capable of cleaving all ECM components, resulting in degradation of collagens and formation of new fibrotic tissue in the infarcted myocardium. MMPs are synthesized as pro-enzymes and are usually activated by proteolytic cleavage of an inhibitory propeptide domain (Cheung *et al.*, 2000). Although the cell types that synthesize and secrete MMPs are not yet clear, the stimulation of neutrophils, fibroblasts, endothelial cells and vascular smooth muscle cells by reactive oxygen species generated during reperfusion has been proposed to activate MMPs (Cheung *et al.*, 2000; Falk *et al.*, 2002). In the present study, proMMP-9 was detected during the entire period of the experiment, but its 84 kDa-activated form was only displayed at day 7, consistent with the highest tissue level of MDA at this time point. In contrast, both proMMP-2 along with a 62 kDa active form, MMP-2, were detected over the time of reperfusion. Daily curcumin treatment significantly reduced both the 84 kDa MMP-9 and 62 kDa MMP-2. This inhibition of MMPs with curcumin is potentially mediated through its

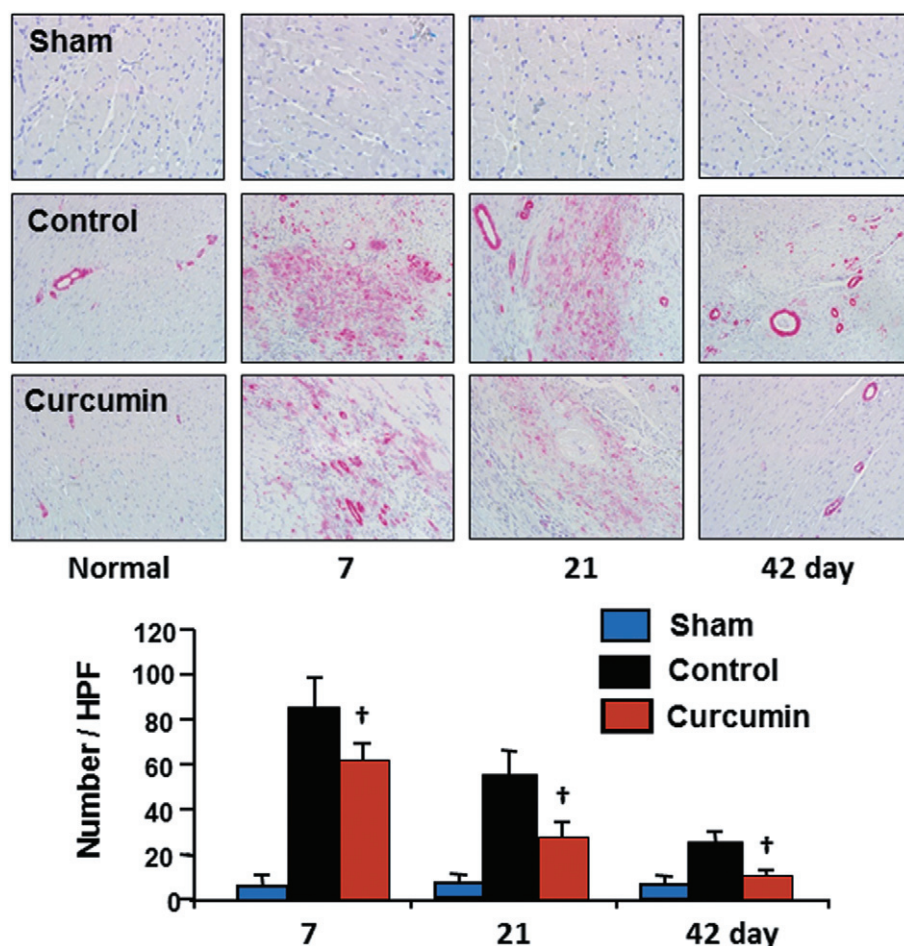


Figure 6

Differentiation of fibroblasts to myofibroblasts. No α -SMA expressing myofibroblasts were detected in the non-ischaemic zone in the control group or in either of the sham groups. Ischaemia/reperfusion caused a significant increase in the number of α -SMA expressing myofibroblasts accumulated during reperfusion, which subsequently declined to day 42 in the control group. However, this increase in accumulation of α -SMA expressing myofibroblasts was significantly reduced by curcumin relative to each time point during reperfusion in the control group. $n = 8$, values are mean $\pm$ SEM. * $P < 0.05$ versus sham; $^{\dagger}P < 0.05$ versus control.

potent antioxidant action, as demonstrated by a significant reduction in tissue MDA level.

Cardiac ECM turnover is primarily determined by the balance between collagen deposition and degradation. If collagen deposition predominates, the fibrosis can predispose the heart to undergo maladaptive tissue repair and failure. If collagen degradation predominates, the LV wall thinning can lead to aneurysm and rupture (Spinale *et al.*, 2002; Tessone *et al.*, 2005). Cardiac fibroblasts are known to synthesize and release collagen I, III, IV and many other ECM-related proteins (Brown *et al.*, 2005). Differentiation of fibroblasts to myofibroblasts, characterized by the expression of α -SMA, is a key event in maladaptive cardiac repair. The simultaneous localization of myofibroblasts and of an excess of tissue collagens in the present study suggests myofibroblasts are a tissue source of extra collagen.

In the normal myocardium, types I and III collagen represent most of the newly synthesized collagen, and are the predominant fibrillar components of ECM with a small pro-

portion of type IV collagen, a key component of the basement membrane (Cleutjens *et al.*, 1995; Brown *et al.*, 2005). We found that type I and type III collagens were expressed in the non-ischaemic myocardium by Western blotting, but their expression occurred predominantly in the infarcted zone at day 7 and remained elevated for 42 days, which is the time frame that the necrotic myocytes are entirely replaced by fibrous tissue (Sun, 2009). Type IV collagen was not detected in the non-infarcted zone but increased dramatically during the course of the experiment. Coincident with the reduction in collagen deposition identified by Masson's trichrome staining, the current study demonstrated that daily curcumin treatment significantly down-regulated the expression of types I, III and IV collagen. Although we cannot outline the exact time frame for collagen degradation and synthesis after ischaemia, previous studies have demonstrated that rapid collagen breakdown occurs during the early stages of reperfusion (Takahashi *et al.*, 1990; 1999). We postulate that curcumin protects the ECM from degradation by

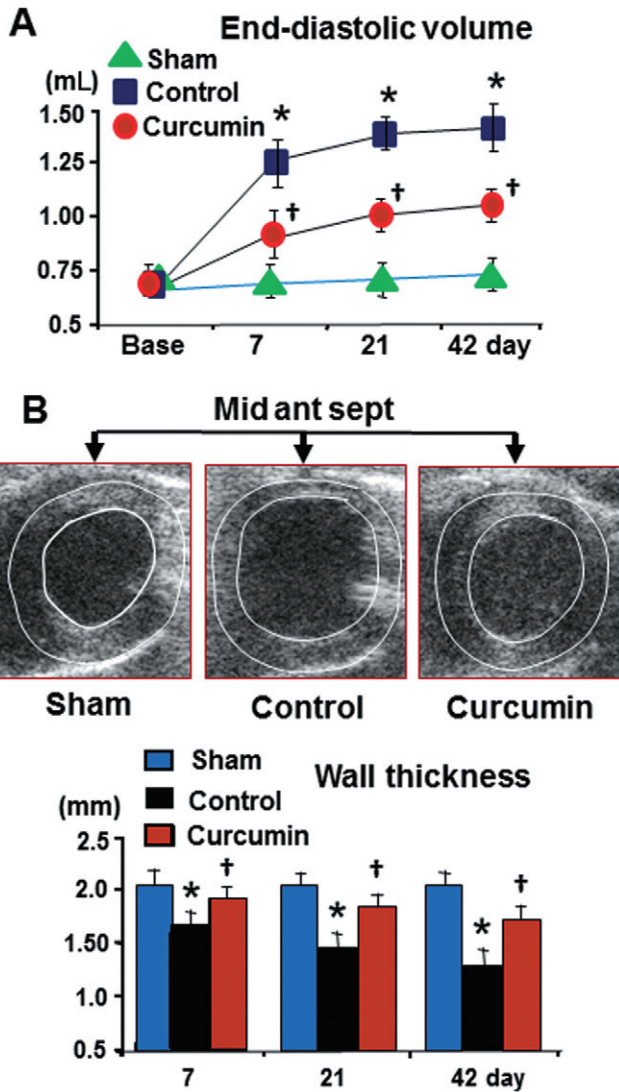


Figure 7

LV end-diastolic volume (LVEDV) and wall thickness. Relative to the sham control, ischaemia/reperfusion caused a significant increase in LVEDV (A) and reduced wall thickness (B) over time in the control group. Curcumin treatment significantly reduced the LVEDV and increased the wall thickness in the infarcted anterior and septum relative to the control. $n=8$, values are mean $\pm$ SEM. * $P < 0.05$ versus normal; † $P < 0.05$ versus control.

attenuating MMPs during the early phases of reperfusion and reduces the fibrotic tissue by constantly inhibiting collagen synthesis over time.

The TGF β 1/Smad pathway stimulated by oxidative stress has been reported to be primarily involved in the stimulation of collagen synthesis in the heart (Sakata *et al.*, 2008; Yuan and Jing, 2010; Euler-Taimor and Heger, 2006). We tested the hypothesis that inhibition of collagen by curcumin is associated with changes in the TGF β 1/Smad signalling pathway. Smads family can be categorized into three distinct proteins that function downstream from the TGF β 1 receptor: receptor-activated Smads (Smad1, Smad2, Smad3, Smad5 and Smad8), common mediator Smads (Smad4 and Smad10) and inhibi-

Table 1

Echocardiographic data

Index	Sham			Control			Curcumin		
	Baseline	7	21	Baseline	7	21	Baseline	7	21
LVDd (mm)	2.0 $\pm$ 0.3	1.9 $\pm$ 0.4	1.9 $\pm$ 0.4	2.0 $\pm$ 0.3	1.6 $\pm$ 0.4	1.4 $\pm$ 0.3	1.9 $\pm$ 0.2	1.8 $\pm$ 0.2	1.7 $\pm$ 0.2†
LVVs (mm)	2.4 $\pm$ 0.2	2.4 $\pm$ 0.3	2.3 $\pm$ 0.6	2.5 $\pm$ 0.5	1.5 $\pm$ 0.4	1.6 $\pm$ 0.2*	2.4 $\pm$ 0.2	2.2 $\pm$ 0.4†	2.1 $\pm$ 0.1†
LVDd (mm)	7.2 $\pm$ 0.8	7.3 $\pm$ 0.7	7.5 $\pm$ 0.6	6.8 $\pm$ 0.8	7.2 $\pm$ 0.7	9.0 $\pm$ 0.7*	7.4 $\pm$ 0.5	7.2 $\pm$ 0.7	7.3 $\pm$ 0.7
LVDs (mm)	5.0 $\pm$ 0.9	5.2 $\pm$ 0.8	5.2 $\pm$ 0.4	4.9 $\pm$ 0.9	5.6 $\pm$ 0.8	7.2 $\pm$ 0.5*	5.0 $\pm$ 0.8	4.9 $\pm$ 0.7	5.5 $\pm$ 0.2†
LVPWd (mm)	1.9 $\pm$ 0.1	2.0 $\pm$ 0.3	2.0 $\pm$ 0.2	2.0 $\pm$ 0.1	1.9 $\pm$ 0.2	2.0 $\pm$ 0.2	2.0 $\pm$ 0.1	1.9 $\pm$ 0.2	2.1 $\pm$ 0.3
LVPWs (mm)	2.3 $\pm$ 0.1	2.2 $\pm$ 0.1	2.3 $\pm$ 0.1	2.3 $\pm$ 0.1	2.2 $\pm$ 0.1	2.1 $\pm$ 0.1	2.2 $\pm$ 0.1	2.1 $\pm$ 0.1	2.2 $\pm$ 0.1
Ant-sep (mm)	2.1 $\pm$ 0.2	2.0 $\pm$ 0.2	2.0 $\pm$ 0.3	2.1 $\pm$ 0.2	1.6 $\pm$ 0.2*	1.5 $\pm$ 0.5*	2.1 $\pm$ 0.1	1.8 $\pm$ 0.2†	1.7 $\pm$ 0.5†
Post-sep (mm)	2.0 $\pm$ 0.1	1.9 $\pm$ 0.3	2.0 $\pm$ 0.1	2.1 $\pm$ 0.2	2.0 $\pm$ 0.2	1.9 $\pm$ 0.5	1.9 $\pm$ 0.2	2.0 $\pm$ 0.2	1.9 $\pm$ 0.3

Ant-sep, middle anterior septum; LVDd or LVDs, left ventricle volume in diastole or systole; LVPWd or LVPWs, left ventricular posterior wall in diastole or systole; LVVs or LVVs, interventricular septum thickness in diastole or systole; Post-sep, middle posterior septum.

Values are mean $\pm$ SEM.

* $P < 0.05$ versus baseline values.

† $P < 0.05$ versus control group.

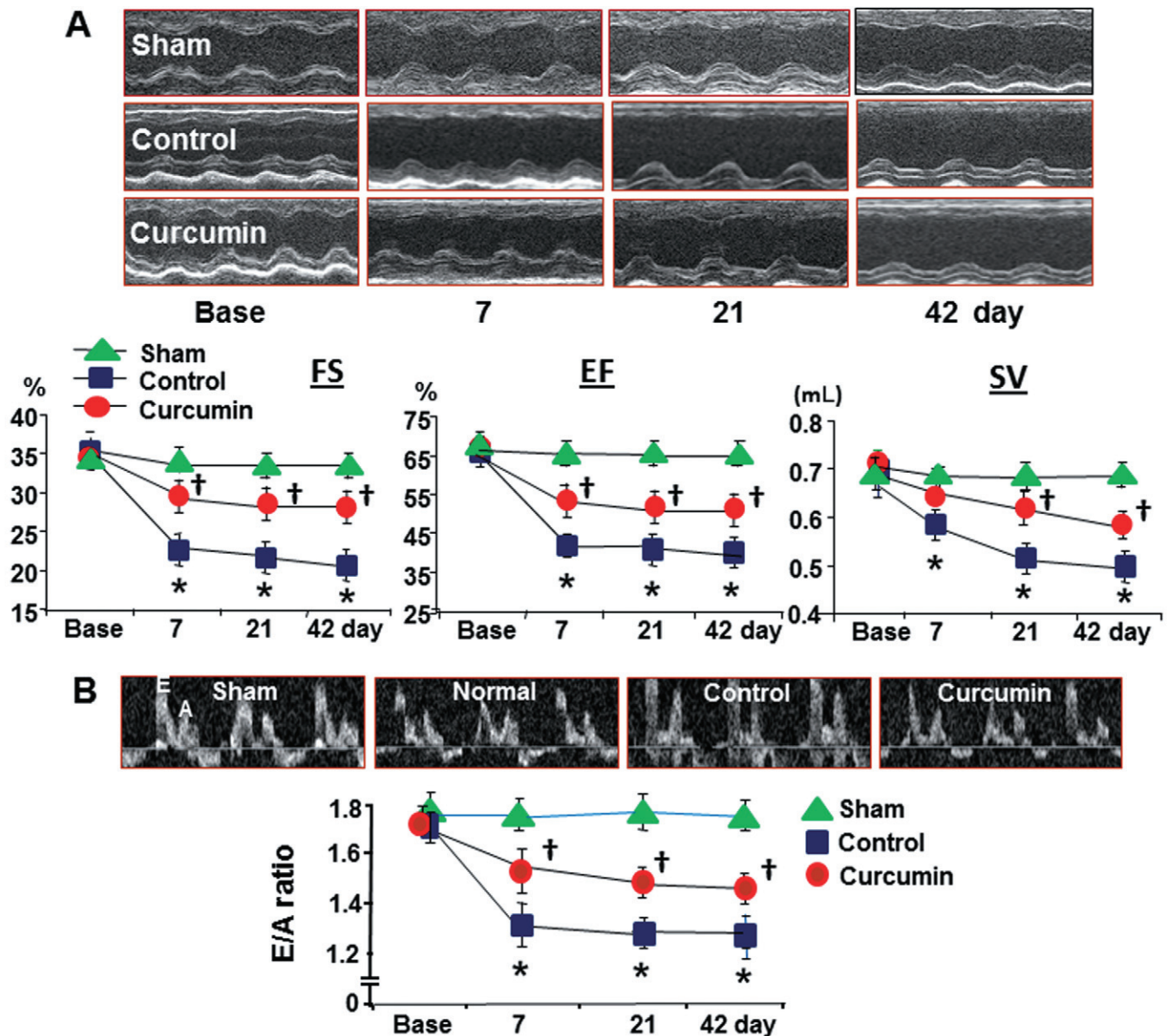


Figure 8

LV systolic and diastolic function. Ischaemia/reperfusion caused a significant reduction in cardiac systolic function measured by echocardiography (A) over the 42 days of reperfusion. Relative to these values in the control group, curcumin treatment significantly preserved cardiac systolic function; measured as FS, fraction shortening; SV, stroke volume; EF, ejection fraction. Cardiac diastolic function was measured by a pulsed-wave Doppler spectra of mitral inflow from the apical four-chamber view, that is, a ratio of early diastolic filling (E-wave) velocity and atrial filling (A-wave) velocity (panel B). Relative to the control, curcumin significantly preserved the E : A ratio over time. $n = 8$, values are mean $\pm$ SEM. * $P < 0.05$ versus baseline values in the sham and control group; † $P < 0.05$ versus control.

tory Smads (Smad6 and Smad7). Upon activation of the cellular surface receptor by TGF β 1 in the heart, Smad2 forms a complex with Smad3 and Smad4. This complex then translocates from the cytoplasm into the nucleus, resulting in an alteration in the expression of TGF β 1-targeted collagen genes. The inhibitory Smad6 and Smad7 function as inhibitors of TGF β 1 signalling by preventing Smad2/3 phosphorylation and disrupting Smad complex formation (Yuan and Jing, 2010; Euler-Taimor and Heger, 2006; Bujak and Frangogiannis, 2007). The administration of exogenous Smad7 (adeno-

viral delivery) has previously been demonstrated to inhibit TGF β 1/Smad2/3-mediated collagen synthesis and fibrosis in *in vivo* and *in vitro* models (Wang *et al.*, 2007). In the current study, we found that changes in the phosphorylation of Smad2/3 and down-regulation of Smad7 occur primarily during the first 21 days, suggesting they have a role in mediating collagen synthesis in the early stages of reperfusion. Given the fact that down-regulation of Smad7 is associated with enhanced expression of phosphorylated Smad2/3 (Wang *et al.*, 2007), inhibition of phospho-Smad2/3 with cur-

cumin was primarily associated with an up-regulation of Smad7. The data obtained in the present study are in agreement with the paradigm of suppression of TGF β 1/Smad pathway in mediating ECM production by curcumin in cultured fibroblasts, as reported recently (Gaedeke *et al.*, 2004; Hsu *et al.*, 2010; Hu *et al.*, 2010). α -SMA-expressing myofibroblasts are thought to arise from the transdifferentiation of fibroblasts in response to TGF β 1 stimulation. At the site of damaged myocardium, myofibroblasts are responsible for the collagen secretion and contraction/realignment of the nascent collagen fibre (Dobazewski *et al.*, 2010). In the present study, the accumulation of α -SMA expressing myofibroblasts was significantly attenuated by curcumin, in parallel with an inhibition in expression of TGF β 1, suggesting a role for TGF β 1 in fibroblast-to-myofibroblast transformation during periods of final fibrotic tissue formation.

Adequate replacement of necrotic tissue by granulation and scarless tissue is suggested to be beneficial because it can prevent the most common complications that occur during the healing of myocardial injury, such as wall thinning, chamber dilatation and cardiac dysfunction (Rohde *et al.*, 1999; Payne *et al.*, 2007). Current data confirm this hypothesis, because the faster healing and less scar formation induced by curcumin reduced wall thinning and inhibited ventricular lumen dilatation. Along with an increase in ventricular wall thickness and a reduction in LV end-diastolic volume at 42 days of reperfusion, curcumin significantly improved cardiac systolic and diastolic function compared with the control animals.

In summary, the current study demonstrates that daily treatment with curcumin during reperfusion protects against maladaptive tissue repair and improves cardiac function after ischaemia. Cardioprotection with curcumin was associated with attenuation of lipid peroxidation and active MMPs, inhibition of the TGF β 1-Smad signalling pathway and differentiation of fibroblasts and modulations in the balance between collagen degradation and synthesis. Through all these beneficial effects, curcumin could maintain the ECM integrity and stability responsible for cardiac recovery. Since curcumin has clinically been demonstrated as a safe food additive, it might be used as a potential add-on therapeutic drug to other conventional therapies, such as statins, β -blockers or ACE inhibitors for the treatment of patients after a heart attack.

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Conflict of interest

No conflicts of interest are declared by the authors.

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