

## Original Article

# Anthocyanins protected hearts against ischemic injury by reducing MMP-2 activity via Akt/P38 pathways

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**Abstract:** Growing evidences suggest that there are close associations between anthocyanins and cardiac protection. However, little is known about the detailed roles of anthocyanins in regulating extracellular matrix (ECM) remodeling. Incubation of primary cultured fibroblasts with anthocyanins reduced both intracellular collagen expression and extracellular collagen secretion. Down-regulation of collagen production was also shown in infarcted cardiac tissues after permanent coronary artery ligation in mice treated with anthocyanins. The phosphorylation levels of Akt and/or P-38 were significantly increased by anthocyanins supplementation in primary cultured fibroblasts. Gelatin zymography analysis of matrix metalloproteinase-2 (MMP-2) activity in conditioned medium collected from fibroblasts demonstrated that anthocyanins treatment significantly reduced MMP-2 activity. These results demonstrated that anthocyanins play a role in mediating myocardial ECM remodeling and that the Akt/P-38 pathways mediate these protective effects on hearts.

**Keywords:** Extra cellular remodeling, MMPs, anthocyanins, infracted heart, fibroblasts

## Introduction

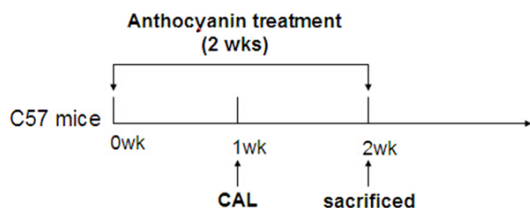
Extracellular matrix (ECM) is no longer considered as a passive support structure. Instead, cardiac ECM is capable of fundamentally altering cardiomyocytes specificity and behaviors. Fibroblasts are the major contributors to the interstitial fibrosis that control the production and degradation of ECM components, especially collagen networks. 85% of the ECM proteins are constituted by collagens [1], which have a triple helix of polypeptide chains and globular domains, and comprise a family of proteins of at least 19 genetically distinct types, among which type I and type III constitute two-thirds. Up-regulated interstitial collagens account for the reduction of capillary density and oxygen diffusion, reduced contractility of cardiomyocytes, and increased structural rigidity of the left ventricular [2]. It was reported that infarcted myocardium was completely replaced with fibroblast accompanied by an increased deposition of collagen, and then replaced by connective tissue within 21 days after coronary artery ligation in rats. Thus, remodeling in the non-infarcted left ventricle is associated with the

development of myocardial interstitial fibrosis with rearrangement of extracellular matrix architecture.

Although numerous enzymes are able to degrade individual ECM components, proteolytic process of ECM is primarily under the control of specific MMPs and their inhibitors (i.e., TIMPs) [3]. Until now, little information is known about the specific targeting of ECM components except some drugs (i.e abciximab, which is used to treat acute coronary syndrome) that target the major cellular receptors for ECM, integrins. Integrins are transmembrane heterodimeric glycoproteins composed of one  $\alpha$  and one  $\beta$  subunit, which usually recognize only relative short peptide motifs in a molecule.

Anthocyanins, as one group of flavonoids, are widely distributed amongst blue and purple fruits and vegetables (e.g. berries and grapes), which have been shown to have various protective properties mainly due to their antioxidant capacity. The antioxidant properties of anthocyanins are determined by the number and position of hydroxyl group as substituents.

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**Figure 1.** Workflow of CAL and anthocyanin treatment. CAL: permanent coronary artery ligation surgery. Anthocyanins: 10 mg/kg per day, for two weeks.

Growing evidences suggest that Anthocyanins are able to elicit beneficial effect on heart via attenuation of oxidative stress [4], inflammatory disorder [5], and atherosclerosis [6]. For example, Skemiene *et al.* [7] found that anthocyanins attenuated ischemic injury by blocking caspase activation through reducing cytosolic cytochrome c. The relationship between anthocyanins and cardiac remodeling has seldom been investigated. One study reported that alcohol-free red wine extract (AFRW) was rich in 14 types of polyphenols and 4 types of anthocyanins. Feeding with AFRW for 8 weeks significantly decreased the amount of collagen type III in remodeling heart [8]. However, the detailed molecular mechanism mediating anthocyanins on collagen expression remained unknown, and the direct effect of anthocyanins on MMPs activity has not yet been determined.

In this study, using both animal and cell models, we aimed to investigate the effect of anthocyanins on cardiac ECM remodeling and the underlying mechanisms. It was found that anthocyanins significantly reduced expression and secretion of collagens, and the activities of MMPs, which was correlated with Akt/p38 pathways.

### Materials and methods

#### Reagents

Malvidin-3-O-glucoside (malvidin, a kind of anthocyanins) was purchased from Indofine Chemical Co. (Hillsborough, NJ). The antibodies of Collagen, Akt, and p38 MAPK were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Rabbit anti-type I or III collagen, and MT-MP1 antibodies were purchased from Abcam (Cambridge, MA).

#### Animals

All the animal experimental procedures were approved by the Committee on the Use of Live

Animals of Hebei Medical University, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

#### Permanent coronary artery ligation surgery and anthocyanins treatment

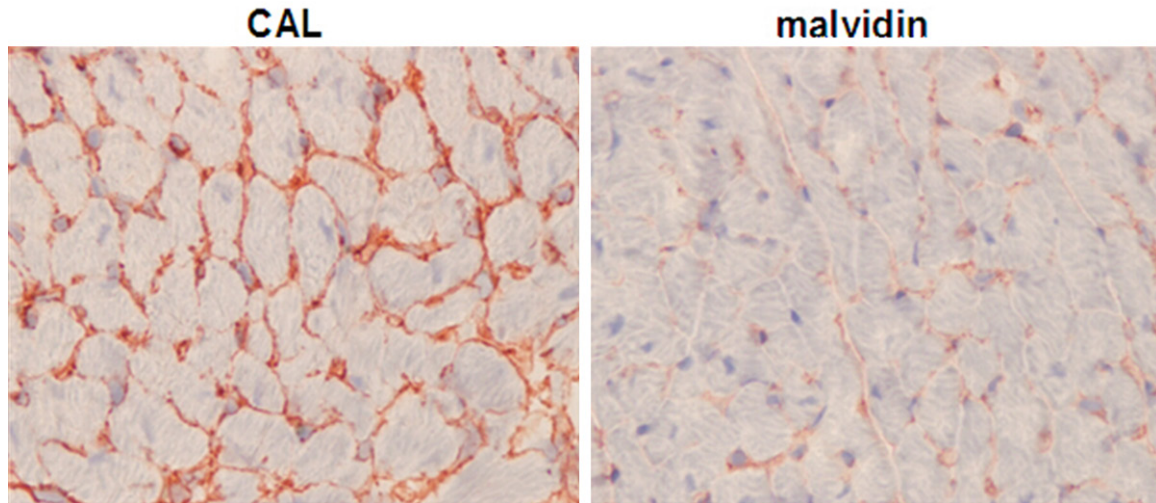
6-8 wk C57BL/6J mice were administered with anthocyanins orally (10 mg/kg per day) [9], as shown in **Figure 1**. After one week's treatment, the mice were subjected to permanent coronary artery ligation (CAL) surgery, as previously reported [10], in order to induce cardiac extra cellular matrix (ECM) remodeling. Briefly, mice were intraperitoneally anesthetized with a combination of Ketamine (90 mg/kg) and Xylazine (10 mg/kg). The mice were ventilated with oxygen and 2% isoflurane by oral endotracheal intubation. A left thoracotomy was performed for exposing the left anterior descending coronary artery (LAD). The LAD was ligated with 8-0 silk suture permanently. The mice were sacrificed for the following experiment at 7-d after the surgery. After surgery, the mice were still given with anthocyanins for another one week. Then the mice were sacrificed for serum and heart tissues collection. The left ventricles were divided in two parts: infarcted area, non-infarcted area. The tissue homogenates prepared from non-infarcted area were used in the following studies.

#### Immunohistochemistry staining of collagen

The immunohistochemistry staining of type I collagen was conducted as described elsewhere [11]. Cryostat sections (5 to 7  $\mu$ m) were washed 3 times with PBS and then blocked with 2% BSA solution for 60 minutes. Slides were incubated with the collagen I antibody (1:500 dilution) for overnight at 4°C. Slides were washed 3 times with BSA solution and incubated for 1 hour at RT with goat anti-rabbit secondary Ab. The images were captured by Olympus microscope (Japan).

#### Primary cardiac fibroblast culture

Neonatal cardiac fibroblasts (CFs) were isolated from 3-4 day old Wistar rats as previously described [12]. Briefly, excised hearts were digested with 0.15% trypsin for 1 h and neutralized with DMEM containing 10% FBS. The digested cells were incubated in cell culture incu-



**Figure 2.** Anthocyanins reduced cardiac collagen deposition after myocardial infarction. The degree of cardiac fibrosis was accessed by staining type I collagen using immunohistochemistry method. CAL: wild type mice subjected to left anterior descending coronary artery ligation. malvidin: the CAL mice were treated with anthocyanins for 4 weeks. Representative images from each group are shown. Magnification, 400 $\times$ .

bator for 1 h and then the cardiomyocytes in suspension were removed. The remaining attached cells in plates were CFs. After CFs were passaged twice and grown to 100% confluence, they were starved with serum-free DMEM for at least 3 hours and then treated with anthocyanins.

#### *Picosirius Red staining*

Conditioned medium from CFs was concentrated and analyzed as previously described [13]. Briefly, concentrated conditioned medium samples (50 mg) were loaded into 96-well plates and allowed to evaporate for 48 hr at 37 $^{\circ}$ C in 5% CO<sub>2</sub>. Following staining with 0.1% Picosirius Red for 1 hr and washing with 10 mM HCl, the stain was solubilized with 0.1 M NaOH. The OD of samples and collagen standard were then measured by spectrophotometry at an absorbance of 540 nm.

#### *Western blotting*

Protein samples from CFs were prepared according to previously mentioned methods [14]. Primary anti-phospho-Akt, anti-phospho-p38 MAPK, anti-total-Akt, anti-total p38 MAPK, anti-type I and III collagen were used at 1:1000 dilutions, and appropriate HRP-conjugated secondary antibodies were used at 1:10,000 dilutions. The signals of immune complexes were visualized using enhanced chemiluminescence

(ECL) system PLUS or ADVANCE. Quantitative analysis was performed after densitometric scanning by Bio Imaging Analyzer LAS 3000 (FUJIFILM, Tokyo, Japan).

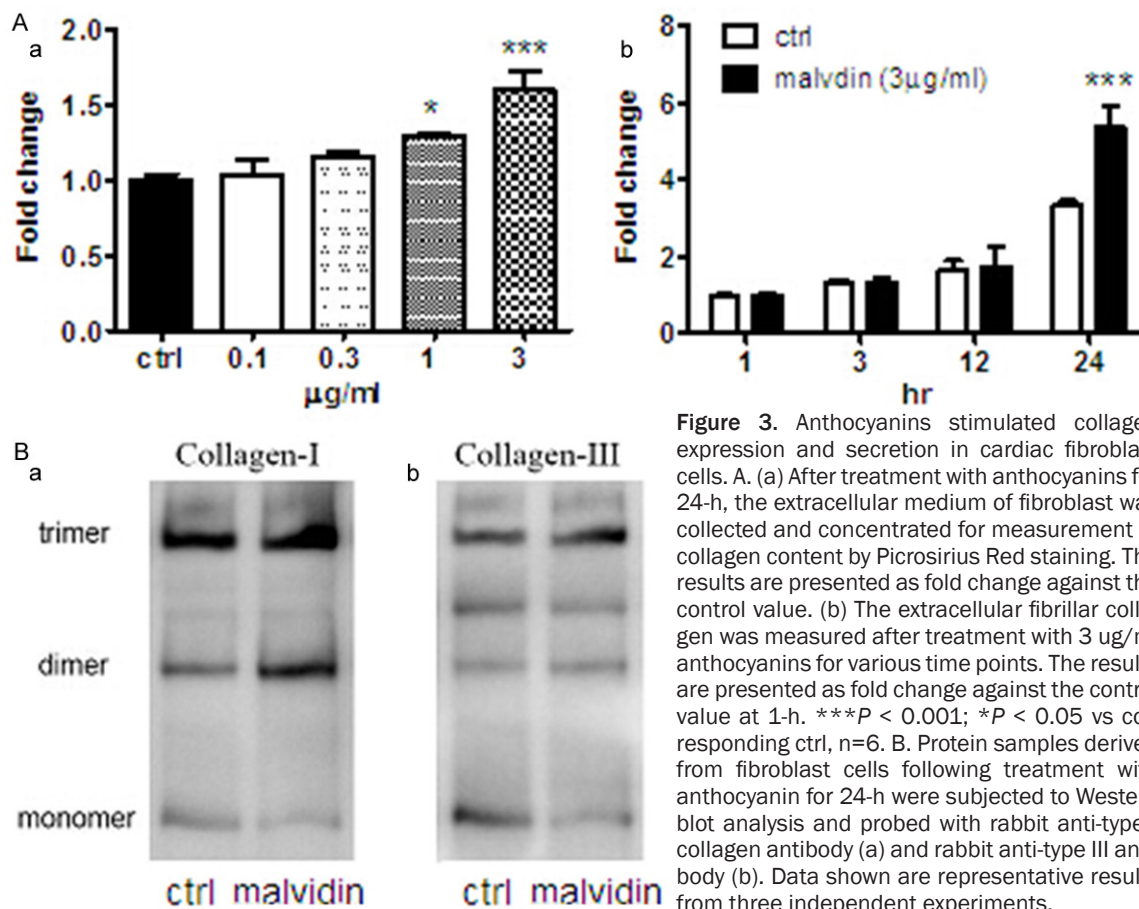
#### *Gelatin zymography*

The conditioned culture medium collected from CFs was concentrated using Amicon filter (Millipore, Ontario, Canada) and protein content of the culture medium was measured by BCA method. Aliquots of culture media containing 25  $\mu$ g of protein were then resolved on a 10% SDS polyacrylamide gel containing 0.1% gelatin. The gel was renatured in 2.5% Triton X-100 buffer for 1 h, followed by washing 4 times in ddH<sub>2</sub>O. The gel was then incubated for 20–72 h at 37 $^{\circ}$ C with gentle shaking in 0.05 M Tris-HCl (pH 7.5) with 5 mM CaCl<sub>2</sub> to allow activation of MMPs. The activation was stopped by incubation the gel with 100 mM EDTA for 15 min. Gels were then stained for 30 min in 0.5% Coomassie blue R-250, and destained in 40% methanol, 10% acetic acid to highlight protease activity of MMPs. Protease activity was quantified using densitometric scanning and Image J software (NIH).

#### *Data analysis*

All results were derived from at least three sets of repeated experiments. The statistical calculations were performed by Student's t-test or

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**Figure 3.** Anthocyanins stimulated collagen expression and secretion in cardiac fibroblast cells. A. (a) After treatment with anthocyanins for 24-h, the extracellular medium of fibroblast was collected and concentrated for measurement of collagen content by Picrosirius Red staining. The results are presented as fold change against the control value. (b) The extracellular fibrillar collagen was measured after treatment with 3  $\mu\text{g/ml}$  anthocyanins for various time points. The results are presented as fold change against the control value at 1-h. \*\*\* $P < 0.001$ ; \* $P < 0.05$  vs corresponding ctrl,  $n=6$ . B. Protein samples derived from fibroblast cells following treatment with anthocyanin for 24-h were subjected to Western blot analysis and probed with rabbit anti-type I collagen antibody (a) and rabbit anti-type III antibody (b). Data shown are representative results from three independent experiments.

ANOVA followed by Tukey multiple comparisons using Prism version 5 (GraphPad Software, San Diego, CA, USA). All values were presented as means  $\pm$  SEM. In all statistical comparisons, a  $P$  value less than 0.05 was used to indicate significant differences.

### Results

#### *Supplementation with anthocyanins attenuated interstitial collagen deposition in heart*

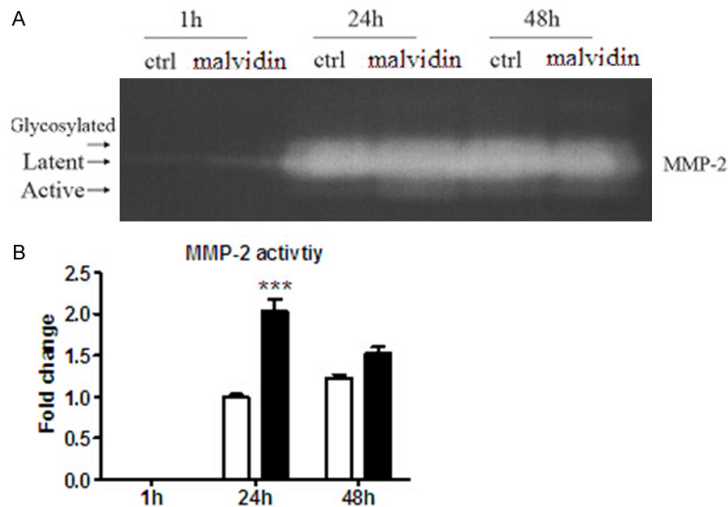
In order to examine whether anthocyanin elicited an effect on extra cellular matrix (ECM) remodeling in heart, the mice were subjected to CAL. After intragastrically feeding with anthocyanin for two weeks, the mice were sacrificed for heart tissues collection. The non-infarcted sections of left ventricle were subjected to type I collagen staining for assessment of degree of cardiac fibrosis. As shown in **Figure 2**, interstitial collagen deposition was significantly reduced after two week's treatment with anthocyanins.

#### *Anthocyanins reduced fibrillar collagen expression and secretion in primary cultured fibroblasts*

Next, the effect of anthocyanins on regulation of both extracellular and intracellular collagen production in fibroblasts was studied. Using Picrosirius Red staining method, it was found that extracellular secretion of collagen was stimulated by 3  $\mu\text{g/ml}$  of anthocyanins at 24-h **Figure 3Aa**. Furthermore, dose dependent experiments were investigated as shown in **Figure 3Ab**, indicating that treatment with 1 or 3  $\mu\text{g/ml}$  of anthocyanin for 24 h significantly increased the accumulation of fibrillar collagen in conditioned medium by  $\sim 1.30$ - or  $\sim 1.59$ -fold, respectively.

To examine intracellular collagen expression, we used western blotting to measure alterations of collagen-I and -III expression following anthocyanins administration. It was shown that treatment with anthocyanin for 24-h could significantly increased multimerization of colla-

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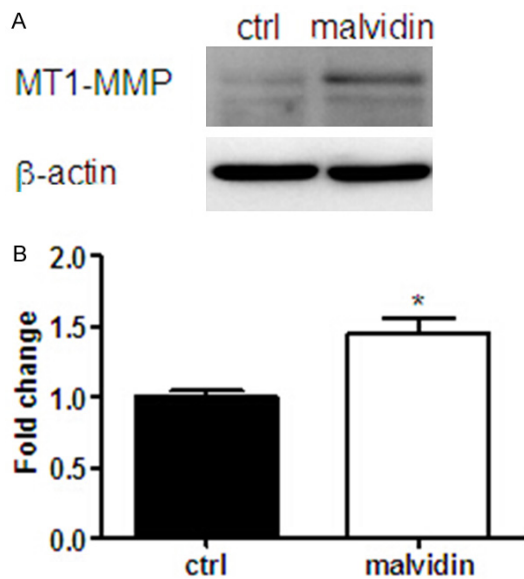
**Figure 4.** Gelatin zymographic analysis of the effect of anthocyanins on MMPs activity. Through the analysis of conditioned medium obtained from CFs, it was demonstrated that anthocyanins increased MMP-2 activity at 24-h post treatment. A. Representative zymography gel images of MMP-2 (glycosylated form corresponding to ~72 kDa, latent form corresponding to ~68 kDa and active form corresponding to ~62 kDa) are shown. B. Proteolytic activity was measured by densitometric scanning and quantitative analysis of the active form of MMP2 for n=6 experiments performed under the conditions indicated. The results are presented as fold change against the control value at 24-h. \*\*\* $P < 0.001$  vs ctrl, n=6.

gen-I and -III in primary cultured cardiac fibroblast **Figure 3B**.

*Anthocyanins increased MMPs proteolytic activity in primary cultured fibroblasts*

Using gelatin zymography, the effect of anthocyanins on MMPs activity in primary cultured fibroblasts was investigated. After treatment with anthocyanins for specific time, the conditioned medium was collected and concentrated. 25  $\mu$ l of concentrated medium was loaded into gelatin contained SDS-PAGE and the bands of MMPs were visualized by staining the gels with coomassie blue. It was found that MMP-2 activity was significantly increased following 3  $\mu$ g/ml anthocyanins treatment for 24 h by 2.03 fold, while 48-h treatment showed no effect **Figure 4**.

*Anthocyanins increased MMP-2 proteolytic activity via up-regulation of MT1-MMP*



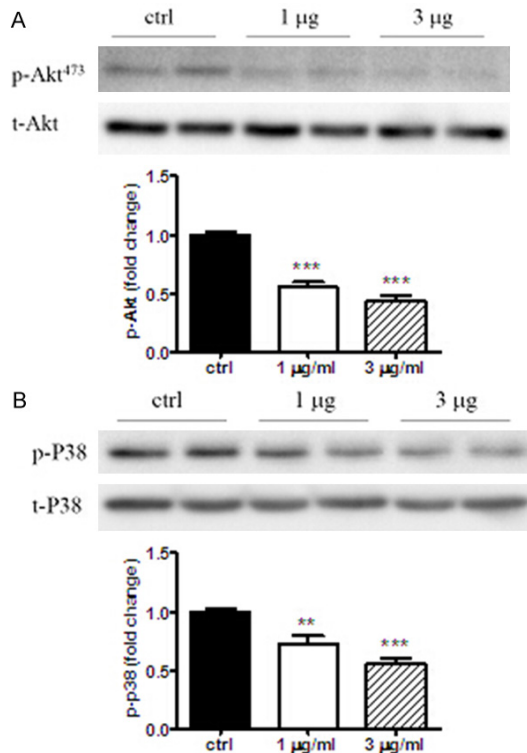
**Figure 5.** Anthocyanins increased intracellular MT1-MMP expression level in CFs. A. The protein expression level of MT1-MMP following anthocyanin (3  $\mu$ g/ml) treatment for 24-h was checked by Western blot. B. The bands corresponding to ~65 kDa were measured by densitometric scanning and the results were normalized by  $\beta$ -actin values and presented as fold change against control values. \* $P < 0.05$  vs ctrl, n=6.

The effect of anthocyanins on MT1-MMP, a membrane bound MMP isoform and a known activator of MMP-2, was also investigated. As shown in **Figure 5A**, treatment with anthocyanins increased MT1-MMP expression level by 1.45-fold, which suggested that anthocyanins stimulated MMP-2 proteolytic activity via up-regulation of MT1-MMP.

*Anthocyanins enhanced Akt and/or p38 phosphorylation levels in fibroblast cells*

In order to elucidate what the molecular mechanism underlying the regulation of anthocyanin on extra cellular remodeling, the level of p-Akt or P38 in cultured fibroblast cells were investigated by Western blot. As shown in **Figure 6A**, both 1 and 3  $\mu$ g/ml of anthocyanins could significantly decreased p-Akt level at 24-h, by 45% and 57% respectively. Similarly, it was shown that anthocyanin with the dose of 1 and 3  $\mu$ g/ml attenuated p-p38 levels by 28% and 45%.

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**Figure 6.** Anthocyanins reduced p-Akt and p-p38 levels in cardiac fibroblast cells. The phosphorylation level of Akt (A) or p38 (B) induced by anthocyanins treatment for 24-h was checked by Western blot. The corresponding bands were measured by densitometric scanning and the results were normalized by t-Akt or t-p38 values. All the results are presented as fold change against ctrl values. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$  vs ctrl.  $n=6$ .

### Discussion

Although most literatures supported that anthocyanins may protect the heart against ischemic injury by mechanisms related to their antioxidant activities, the detailed molecular mechanism is still unknown. One of the novel findings of our study was that anthocyanins play a role in regulating of cardiac ECM remodeling, which was demonstrated both *in vivo* and *in vitro* models.

Anthocyanins, either single active ingredient (i.e., cyaniding 3-O-beta-glucopyranoside) [15], or fruit/plant extracts [8], have been implicated to reduced collagen synthesis in various tissues. On the other hand, anthocyanins were also demonstrated to play a role in reducing the activities of MMP-2 [16] or -9 [17] in cancer cells metastasis. However, we have no idea whether anthocyanins elicit a regulative effect on cardiac ECM remodeling, induced by CAL.

It has been reported that Type III procollagen mRNA levels were already increased at day 2 after myocardial infarction (MI), whereas type I procollagen mRNA followed this response at day 4 after MI. This increase was sustained for at least 21 days in the infarcted left ventricle for type III procollagen mRNA, whereas type 1 procollagen mRNA levels were keeping elevating till 90 days after MI [18]. In the present study, we found that the protein expression level of collagen III was dramatically enhanced on Day 7 after CAL, which could be attenuated by anthocyanins treatment. In primary cultured CFs, anthocyanins treatment significantly reduced MMP-2 activity, which might be mediated by MT1-MP.

p38 is one sub-family of mitogen-activated protein kinases (MAPKs) and activated by inflammatory cytokines, osmotic stress and apoptotic signals [19]. p38 has been shown to be involved in the regulation of MMPs, but its effect on MMPs is still contradicting. For example, IL-1 $\alpha$  upregulated the expression of both MMP-9 and -13 in liver myofibroblast cell line, and the induction of MMP-13 but not MMP-9 was in p38 dependent manner [20]. Similarly, TNF $\alpha$  and TGF $\beta$  induced expression of MMP-13 and MMP-1 was abolished by p38 inhibitor, SB20-3580 [21]. On the other hand, another study showed that p38 alpha could inhibit ERK1/2 with a subsequent suppression of MMP-1 [22]. Activation of p38 beta2 could inhibit platelet-derived growth factor-BB-mediated MMP activation [23]. Tumor suppressor gene product, retinoblastoma, negatively regulates p38 activation, leading to decreased MMP-1 secretion [24]. The above results might imply that p38 regulates MMP-1 gene expression bidirectionally depending on the stimuli. Our results showed that anthocyanins significantly reduced phosphorylation level of p38, thereby inhibiting MMP-2 activity.

The relationship between Akt pathway and collagen expression is quite consistent. Hubchak *et al.* found that TGF- $\beta$  stimulated collagen-I expression in mesangial cell through a PI3K/Akt mechanism [25]. Exposure of lung fibroblasts to Bleomycin (a known inducer of fibrosis) resulted in a rapid activation of PI3K/Akt and a parallel increase in collagen production [26]. In adult cardiac fibroblasts, PI3K/Akt and Smad pathways were involved in TGF- $\beta$  induced lysyl oxidase (a key enzyme to form mature collagens) gene, protein and activity up-regulation

[27]. These data are in line with ours, which showing that anthocyanins significantly reduced the levels of p-p38 and collagens.

The effect of Akt pathway on MMPs expression or activity remains nearly unknown. Blockade of PI3Ky resulted in increased levels of cAMP and expression of MMPs, including MMP2 [28]. Inhibition of Akt by various strategies led to down-regulation of collagen-I production but up-regulation of MMP1 in human dermal fibroblasts [29]. In this regard, it might be possible that anthocyanins induced dephosphorylation of Akt will result in activation of MMP-2 and -9. Additional studies are required further to address these potential mechanisms.

In summary, in myocardial infarction model induced by permanent coronary artery ligation, we demonstrated for the first time that anthocyanins attenuated ECM remodeling by reducing collagens expression and secretion, and MMP activity, which might be mediated by Akt/p38 MAPK pathways. Supplementation with anthocyanins has a therapeutic effect on ischemic heart via modulating of ECM remodeling.

### Disclosure of conflict of interest

None.

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