

Neovascularization of Ischemic Myocardium by Newly Isolated Tannins Prevents Cardiomyocyte Apoptosis and Improves Cardiac Function

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During remodeling progress post myocardial infarction, the contribution of neoangiogenesis to the infarct-bed capillary is insufficient to support the greater demands of the hypertrophied but viable myocardium resulting in further ischemic injury to the viable cardiomyocytes at risk. Here we reported the bio-assay-guided identification and isolation of angiogenic tannins (angio-T) from *Geum japonicum* that induced rapid revascularization of infarcted myocardium and promoted survival potential of the viable cardiomyocytes at risk after myocardial infarction. Our results demonstrated that angio-T displayed potent dual effects on up-regulating expression of angiogenic factors, which would contribute to the early revascularization and protection of the cardiomyocytes against further ischemic injury, and inducing antiapoptotic protein expression, which inhibited apoptotic death of cardiomyocytes in the infarcted hearts and limited infarct size. Echocardiographic studies demonstrated that angio-T-induced therapeutic effects on acute infarcted myocardium were accompanied by significant functional improvement by 2 days after infarction. This improvement was sustained for 14 days. These therapeutic properties of angio-T to induce early reconstitution of a blood supply network, prevent apoptotic death of cardiomyocytes at risk, and improve heart function post infarction appear entirely novel and may provide a new dimension for therapeutic angiogenesis medicine for the treatment of ischemic heart diseases.

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INTRODUCTION

Ischemic heart diseases remain the leading cause of morbidity and mortality in most countries. Ischemia of myocardium induces myocardial infarction and results in an irreversible loss of myocardium. During remodeling progress post infarction, the neoangiogenesis, or revascularization, to the infarcted heart tissues in the whole infarct zone, including central infarct area and the infarct border zone, is insufficient to keep pace with the tissue growth required for contractile compensation and is unable to support the greater demands of the hy-

perrophied but viable myocardium, especially the myocardium along the border zone of the infarct, that is, the cardiomyocytes at risk. The relative lack of oxygen and nutrients to the hypertrophied myocytes might be an important etiological factor in the death of otherwise viable myocardium, resulting in progressive infarct extension and fibrous replacement. Therefore, the most direct way to rescue the cardiac myocytes at risk is to establish a new blood supply at an early stage in the whole infarcted area that would allow circulating stem cells, nutrients, and growth factors, in addition

to oxygen, to be delivered to the infarct zone. Restoration of coronary blood flow by rapid angiogenesis may offer a direct and effective therapeutic modality to intractable ischemic heart diseases.

Although therapeutic angiogenesis has been studied intensively as an alternative treatment for ischemic vascular diseases using growth factors such as VEGF, aFGF, bFGF or PDGF, these factors take a period of approximately 3 days to 3 weeks to act (1–8), while myocardial necrosis in the acute severe coronary occlusion area occurs very rapidly within a matter of hours (4,9–10). The consequence is that fibrous tissue grows rapidly despite the relative ischemic condition, which replaces the infarcted heart tissues and also blocks the space for any newly-regenerated myocyte replacement. Up to now, there is no pharmaceutical or therapeutic method available that can

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promote early reconstitution of the damaged coronary vasculature with newly-formed vessels.

Recently, during the course of screening for angiogenic reagents from Chinese herbal medicine, an alcoholic extract of whole plant of *Geum japonicum* (GJ) has been identified from approximately 300 selected herbs that showed potent dual actions on stimulating rapid growth of new vessels (< 48h) and myogenesis in animal models with severely damaged skeletal muscles. A few bio-active triterpenoids, which could stimulate the activation and differentiation of myogenic precursor cells leading to efficient myofiber regeneration and muscle repair, were isolated (11), and here we reported 3 other tannins (angio-T) were isolated from GJ which showed significant healing effect on infarcted myocardium by stimulating early growth of new blood vessels and preventing apoptosis of myocardium at risk adjacent to the infarct zone, blocking further extension of the infarct compared with the larger infarction and fibrous scar tissue replacement in the controls.

MATERIAL AND METHODS

Bio-assay and Isolation of the Angio-T

GJ collected from Guizhou Province of China in August was dried and percolated with methanol at room temperature for 7 days. The extract was then dried under reduced pressure to yield a powder residue. The dried powder was suspended in H₂O and successively partitioned with hexane, EtOAc, and n-BuOH respectively. All hexane, EtOAc and n-BuOH soluble fractions were filtered and evaporated under reduced pressure at 30-50°C, yielding 3 different fractions, which were tested for their ability to stimulate angiogenesis in cell culture and myocardial infarction (MI) animal models. The soluble fraction, which could significantly enhance the proliferation of human coronary artery endothelial cells (HCAECs, Clonetics, Inc., East Rutherford, NJ) and stimulate rapid neovascularization in the infarct zone of MI ani-

mal models, was applied on a column of Sephadex LH-20 equilibrated with 10% methanol and eluted with increasing concentration of methanol in water. All the fractions were tested for their angiogenic effects with cell culture systems. The most effective fraction was used to test its healing effects on a MI animal model. The structures of the active compounds contained in this active fraction were determined by NMR analysis.

In Vitro Bioassay for Angiogenic Activities

Primary human coronary artery endothelial cell line (HCAECs) was purchased from Clonetics. The cells were cultured in endothelial basal medium-2 (Clonetics), which consisted of 500 mL endothelial cell basal medium, 5 ng human recombinant epidermal growth factor, 0.5 mg hydrocortisone, 25 mg gentamycin, 50 µg amphotericin B, 6 mg bovine brain extract, and 25 mL FBS, as described previously (12). The 3rd passages of HCAECs were used in this study. The cells were incubated with the test samples of grade concentration for 24 h and then were pulse-labeled with 10 µM BrdU for 4 h using the Labeling and Detection Kit (Roche) according to the manufacturer's instructions. The equivalent volume of 5% DMSO was used for the control. After labeling, the cells were rinsed with PBS, fixed in 100% methanol and prepared for immunolocalization of BrdU as described (11). The percentage of BrdU-labeled nuclei was determined in 8 random microscope fields per culture and the values for each culture represent the mean of 8 high power fields (× 40) per culture, which was further averaged with 8 parallel cultures for each sampling point and are normalized with respect to the total number of 4', 6'-diamino-2-phenylindole dihydrochloride (DAPI, 0.1 µg/µL in PBS) (Santa Cruz Biotechnology).

Animals, Surgical Procedures

Male 250-300 g Sprague-Dawley (SD) rats used in this study were approved by

the Animal Experimentation Ethics Committee (AEEC), the Chinese University of Hong Kong. Briefly, after proper anesthesia, a left thoracotomy was performed, the pericardium was opened and the left anterior descending (LAD) coronary artery was ligated. Angio-T dissolved in 5% DMSO (0.1 mL, containing 0.3 mg angio-T) was injected into the distal myocardium (the presumed ischemic region) of the ligated artery immediately after the ligation (test group, n = 45). An equivalent amount of 5% DMSO was injected to the corresponding location of the rats as the control group (n = 45). Fifteen rats of each group were killed on day 2, day 7, and day 14 post-infarct for morphological and functional assessment. For sham group (n = 6), left thoracotomy was performed and the pericardium was opened but with no LAD ligation. Rats without any treatment were set as normal control (n = 6).

Measurement of Neovascularization in the Infarct Zone

On days 2 and 7 post-infarction, the hearts of experimental rats were removed and cut from apex to base in 3 transverse slices. The slices were fixed in formalin and embedded in paraffin. Vascular density was determined from histology sections by counting the number of vessels within the infarct zone using a light microscope under high power field (HPF) (× 40). Eight random and non-overlapping HPF within the infarct field were used for counting all vessels in each section of all angio-T treated hearts and control hearts. The number of vessels in each HPF was averaged and expressed as the number of vessels per HPF. Vascular counts were performed by 2 investigators in a blinded fashion.

Measurement of Myocyte Apoptosis by TUNEL Assay of Paraffin Tissue Sections

We used the TUNEL assay for in situ detection of apoptosis at the single-cell level (13). Rat myocardial infarction tissue sections were obtained from both the test group and the control group on

day 7 post-infarction. After general deparaffinization and rehydration, tissues were digested with Proteinase K (Dako) for 15 min and incubated with TdT (Roche) and Biotin-16-dUTP (Roche) for 60 min at 37°C. After incubation with SP-HRP (Roche) for 20 min, the TUNEL staining was visualized with DAB (Dako), which stained the nuclei with DNA fragmentation brown. Tissue sections were examined microscopically at high power field ($\times 40$) and at least 100 cells were counted in a minimum of 10 HPF. The number of the apoptotic myocytes per HPF was termed the apoptotic index.

Estimation of the Myocardial Infarction

The hearts of experimental rats killed on day 14 were removed and sectioned from apex to base in 3 transverse slices and embedded in paraffin. Thin sections (5 μm thickness) were cut from each slide and stained with H&E staining and Masson's trichrome (Sigma, USA), which labels collagen blue and myocardium red. These sections from all slices were projected onto a screen for computer-assisted planimetry (Image J 1.34S, Wayne Rasband, National Institutes of Health, USA). The endocardial and epicardial circumferences as well as the length of the scar were measured for each slice. The infarcted portion of the left ventricle was calculated from these measurements and the ratio of scar length to ventricular circumference of the endocardium and epicardium of the slices was expressed as a percentage to define the infarct size (13-15).

Echocardiography Assessment of Myocardial Function

In all, 132 SD rats received baseline echocardiography before the experimental procedure. Echocardiography was recorded under controlled anesthesia using an S10-MHz phased-array transducer and GE VingMed Vivid 7 system. M-mode tracing and 2-dimensional (2D) echocardiography images were recorded from the parasternal long and short axis

views. Short axis view was at the papillary muscle level. Left ventricular end-systolic and end-diastolic dimensions, as well as systolic and diastolic wall thicknesses, were measured from the M-mode tracings by using the leading-edge convention of the American Society of Echocardiography. For each M-mode measurement, at least 3 consecutive cardiac cycles were sampled. Left ventricular ejection fraction (LVEF) and fractional shortening (FS) were derived from LV cross-sectional area in 2D short axis view: $\text{LVEF}(\%) = [(\text{LVDA}^3 - \text{LVSA}^3) / \text{LVDA}^3] \times 100$; $\text{FS}(\%) = [(\text{LVDA} - \text{LVSA}) / \text{LVDA}] \times 100$ (16), where LVDA and LVSA correspond to LV areas in diastole and in systole. Standard formulas were used for echocardiographic calculations.

Rat Signal Transduction Pathway Finder Microarray

Total RNA of infarcted myocardial tissue with and without angio-T treatment were isolated with Qiagen RNeasy Mini Kit (Catalog Number 74104, Qiagen, Germany) following the instruction of the manual, dissolved in 20-30 μL RNase-free water and stored at -80°C . The integrity of the ribosomal RNA and DNA contamination was checked routinely using formaldehyde denaturing RNA gel electrophoresis (1.2%) before proceeding with the further array analysis. Protein contamination and concentration of the total RNA was assessed by determining the ratio OD260:280 spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany).

cDNA was synthesized and labeled with Biotin-16-UTP (Roche, Cat. No. 11388908001) following the User Manual of the TrueLabeling-AMPTM Linear RNA Amplification Kit (Cat. No. GA-010, SuperArray). The cRNA targets were further purified before hybridization with SuperArray ArrayGrade cRNA Cleanup Kit (Cat. No. GA-012). Parallel Oligo GEArray Rat Angiogenesis SuperArray (Cat. No. ORN-014, SuperArray). The Oligo GEArray Rat Signal Transduction PathwayFinder Microarray profiles the expression of 113 genes repre-

sentative of the 18 signal transduction pathway, such as Jak-stat pathway, NF κ B Pathway, Wnt Pathway and so on. The membranes were hybridized parallelly with 10 μg of each biotin-labeled cRNA probe at 60°C overnight with continuous agitation. Then they were washed with 2X SSC, 1% SDS for 15 min at 60°C , followed by washing with 0.1X SSC, 0.5% SDS for exactly 15 min at 60°C . The signals were detected by CDP-Star chemiluminescent substrate according to the manual (Chemiluminescent Detection Kit, Cat No D-01, SuperArray BioScience Corporation). X-ray films were exposed for 2 min, 5 min, and 10 min. The films were scanned, the images saved in TIFF format and further analyzed by Quantity One Software. Differentially-regulated genes were defined when the differences between the 2 samples were two-fold or more. The differentially-expressed genes were confirmed by RT-PCR.

RT-PCR Analysis

A small slice from the above prepared infarcted myocardial tissue was put into liquid nitrogen immediately after incision and stored at -80°C . Total RNA was isolated with Qiagen RNeasy Mini Kit (Qiagen, Germany) as mentioned above.

Two μg of total RNA was reverse-transcribed with oligo(dT) and M-MLV reverse transcriptase (Invitrogen) at 37°C for 1 h; 0.5 μL of the reverse-transcribed material was amplified with Taq DNA polymerase. The primer pairs used are shown in Table 1. For PCR, 30 cycles were used at 95°C for 30 s, 56 to 60°C for 30 s, and 72°C for 45 s. Rat GAPDH was amplified as an internal reference. Seven μL of each amplification mixture was subjected to electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The signal intensity was quantified by a computerized medical image-processing system (Kodak 1D). The ratio of target genes to GAPDH was used to express the relative level of targeted genes.

Table 1. Primer sets used in semiquantitative RT-PCR

Name	mRNA	Primer sequence, 5' to 3'	Length, bp
GAPDH	BC059110	Forward: ATG ACA TCA AGA AGG TGG TG Reverse: CAT ACC AGG AAA TGA GCT TC	177
VEGF-b	NM_053549	Forward: AAC TCA TGG GTA ATG TGG TC Reverse: ACT GCT CGG GTA CTG GAT	152
VEGF-c	NM_053653	Forward: CTG GCG TGT TCC TTG CTC Reverse: GCT CCT CCA GGT CTT TGC	151
IL4R	X69903	Forward: TGT GGA GCA ACC CAT ACC Reverse: CTT GGG TTC CGT GTA GGT	127
IRF1	NM_012591	Forward: TTC AGG CTA TTC CTT GTG C Reverse: ACC TCA GAA CGC CAA TCC	275

Western Blot Analysis

About 50mg of the above-prepared infarcted myocardial tissue was ground to powder in liquid nitrogen. One mL lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 200 mM NaF, 20 mM sodium pyrophosphate, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 200 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) was added to the powder and put on ice for 30 min, followed by centrifuge of 10000g for 30 min. The supernatant was collected and quantified by Bio-Rad DC protein assay kit (Bio-Rad). Equal amounts (10 μ g) of total protein were size-fractionated by SDS-PAGE and transferred to PVDF membranes (Amersham, USA). The blots were blocked with phosphate-buffered saline plus 0.1% (vol/vol) Tween 20 (PBST) containing 5% (wt/vol) milk powder (PBSTM) for 30 min at room temperature and probed for 60 min with specific primary antibodies against rat phospho-Akt1 (mouse, Santa Cruz), rat Bcl-2 (mouse, Sigma-Aldrich) and rat VEGF (rabbit, Santa Cruz), all diluted to 1:800 in PBSTM. β -actin (rabbit, Santa Cruz) was used as internal reference. After washing extensively in PBST, the blots were probed by alkaline phosphatase-conjugated secondary antibodies (Amersham Biosciences) (1/1000 dilution in PBSTM, 60 min), extensively washed with PBST, and developed by NBT/BCIP. The membranes were scanned using a Bio-Rad GS-800 Calibrated Densitometer.

The signal intensity was quantified by the software Quantity One (BioRad). The ratio of target proteins to β -actin was used to express the relative level of the targeted proteins.

Biostatistics

All morphometric data were collected blindly. Results are presented as mean \pm SD computed from the average measurements obtained from each heart. Statistical significance for comparison between 2 measurements was determined using the unpaired two-tailed Student t test. Values of $P < 0.05$ were considered to be significant.

RESULTS

Isolation of Angio-T from *Geum japonicum*

In the present study, we have identified the methanol extract made of GJ, which has been used as a diuretic and an astringent in China and Japan for hundreds of years (17), showing potent effects on stimulating rapid growth of new vessels (< 24h) and regeneration of muscles in a rat model with skeletal muscle injury (11). Approximately 50 grams of dried powder were produced from methanol extraction of 1 kg dried whole plant. The dried powder derived from methanol extract was suspended in H₂O and partitioned successively with hexane, ethylacetate, and n-butanol which produced 3 different fractions. The tests of these 3 partitioned fractions for their specific angiogenic activities demonstrated that n-butanol fraction displayed the highest activities in enhancing the proliferation of human coronary artery endothelial cells (HCAECs) in cell culture systems (Figure 1A), and early revascularization in infarcted myocardium in a rat MI model (unpublished observation). The n-butanol fraction was further separated

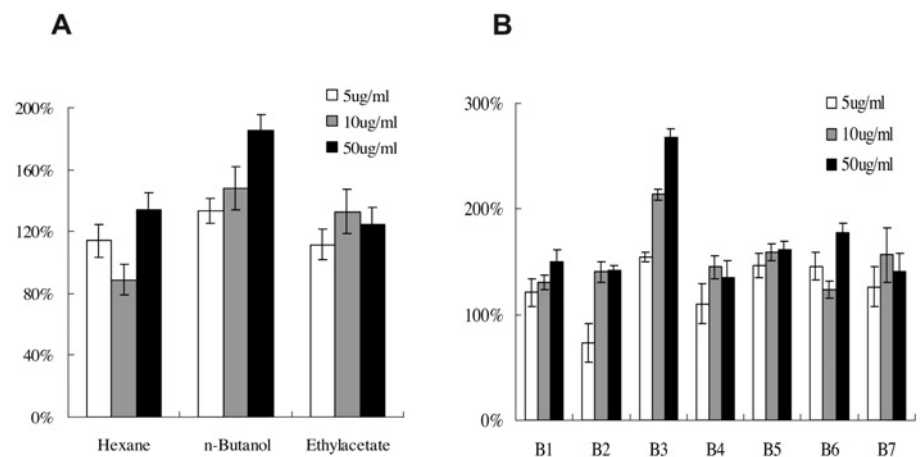


Figure 1. Isolation of the active compounds by bio-assay-guided fractionation strategy. (A) It was shown that the n-butanol fraction displayed a dose-dependent and the highest activities in enhancing the proliferation of human coronary artery endothelial cells (HCAECs). (B) Among the 7 separated fractions from n-Butanol extract, fraction 3 displayed most potent and dose-response effect on promoting the proliferation of HCAECs in vitro.

by a liquid column chromatography and 7 fractions were resolved. Assays for angiogenic activity of all resolved fractions demonstrated that fraction 3 displayed a potent effect on promoting the proliferation of HCAECs *in vitro* (Figure 1B), and on stimulating revascularization in infarcted myocardium *in vivo*. Structural studies of fraction 3 by NMR spectroscopy method demonstrated that the major compounds of fraction 3 are 5-desgalloylstachyurin, Gemin A, and tellimagrandin II termed as *angio-T*, which had been reported to have potent anticoagulant activity by significantly prolonging the clotting of rabbit plasma (18).

Angio-T-mediated Revascularization in Infarcted Myocardium

Histology studies revealed that numerous newly-formed vessels, some of which were filled with blood cells and some of which were still at the early stage of the vessel regeneration development and displayed as a lumen-like structure without filling of blood cells, were found in the whole infarct zone, including the central areas and the border zones of the infarction on day 2 post infarction (Figure 2A,G). The capillary density in the whole infarct zone of the *angio-T* treated myocardium averaged 18 (18 ± 3.9) filling with blood cells and 8 (8 ± 2.8) lumen-like structures per HPF derived from 8 randomly selected view fields of each slide and 15 slides in total from 15 *angio-T* treated hearts on d 2. In contrast, fewer blood vessels (5 ± 2.1 per HPF) with inflammatory cell infiltration were observed in the whole infarct zone in the control myocardium on d 2 post MI (Figure 2B,G). On day 7 post MI the newly formed blood vessels filled with blood cells remained (11 ± 3.6) in the whole infarct zone. However, the lumen-like structures in *angio-T* treated hearts were almost undetectable (Figure 2C,G). By contrast, the main feature was fibrous tissue replacement of the infarcted myocardium with only a few blood vessels (3 ± 1.2) in the control MI, 7 days post infarction (Figure 2D,G). RT-PCR and Western blot analysis demonstrated that

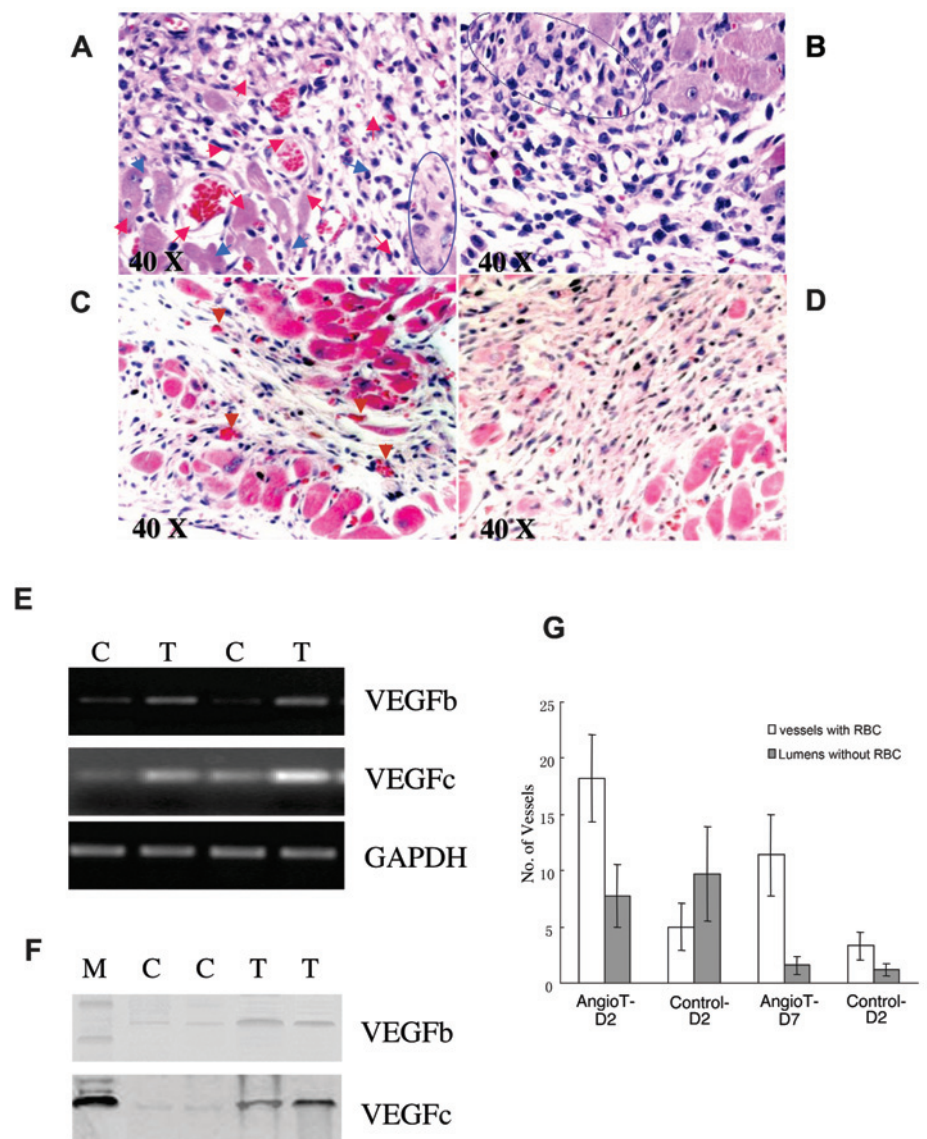


Figure 2. Early neovascularization of the infarcted myocardium following *angio-T* treatment. (A) Two days after LAD ligation and *angio-T* injection, numerous newly-formed vessels, some of which were filled with blood cells (red arrowheads) and some of which displayed as a lumen-like structure without filling of blood cells (blue arrowheads), were found in the whole infarct zone. (B) In contrast, only a few vessels with inflammatory cell infiltration were observed in the whole infarct zone in the control hearts on day 2 post MI. (C) Seven days after LAD ligation and *angio-T* injection, many functional vessels filled with blood cells were observed in the whole infarct areas. (D) The main feature was fibrous tissue replacement of the infarcted myocardium with only a few blood vessels in non-treated hearts on 7-day post-infarction. (E&F) RT-PCR and Western blot analysis demonstrated significantly up-regulated gene expressions of VEGFb and VEGFc in the *angio-T* treated heart tissues compared with their expressions in non-treated control myocardium.

the *angio-T*-induced revascularization within 24 h in infarcted myocardium was concomitantly accompanied with the up-regulated gene expressions of VEGFb

and VEGFc in the corresponding heart tissues. The expressions of VEGFb and VEGFc in *angio-T*-treated myocardium were increased to 2.4 and 1.6 folds re-

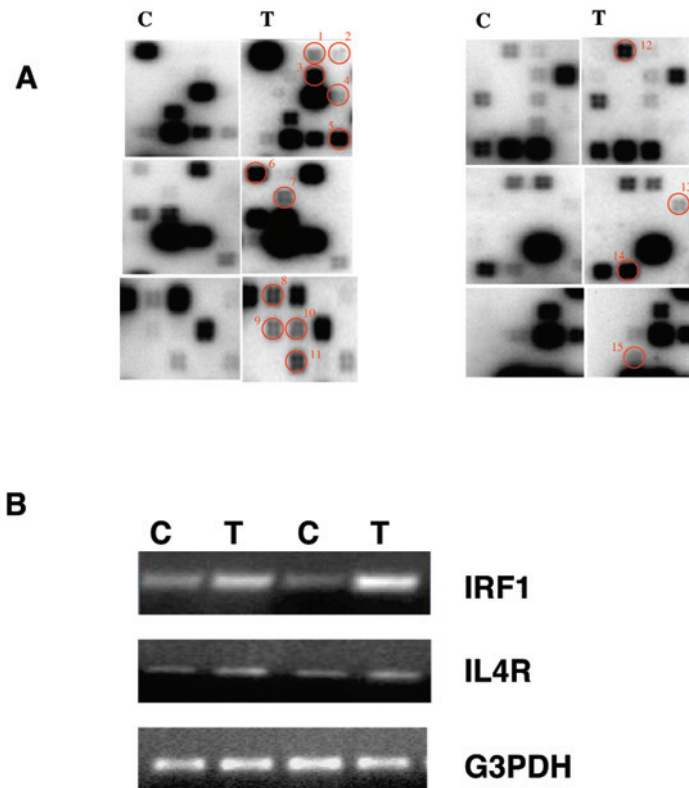


Figure 3. Gene expression profiling analysis of signal transduction pathway. (A) 15 genes were found to be up-regulated in the angio-T treated heart tissues compared with those in non-treated control myocardium. Each circle marked by number 1-15 stands for: BAX, BCL2, BMP4, CDKN1b, FASN, CCL20, CDKN2a, ICAM1, IL4R, IRF1, MDM2, ODC1, PRKCE, TRIM25 and GADD45a respectively (Table 2). (B) RT-PCR analysis demonstrated significantly up-regulated gene expression of IL4R and IRF1 in the angio-T treated heart tissues.

spectively compared with their expressions in non-treated myocardium of control group (Figure 2E,F).

To investigate the molecular process of the angiogenesis induced by angio-T, we adopted the Oligo GEArray Rat Signal Transduction PathwayFinder™ Microarray which profiled the expression of 113 genes representative of the 18 signal transduction pathways. There were 15 genes activated or up-regulated in the angio-T-treated infarcted myocardial tissue compared with the control (Figure 3A, Table 2). Among these 15 genes, there were 6 genes well documented to contribute to the revascularization in the infarcted myocardia including Tripartite motif protein 25 (estrogen pathway), Interleukin 4 receptor and Inter-

feron regulatory factor 1 (Jak-Stat pathway), Chemokine ligand 20, B-cell leukemia/lymphoma 2 and Intercellular adhesion molecule 1 (NFκB pathway) (19–21). The 6 genes were further analyzed by RT-PCR, 2 of which were confirmed to be over expressed in the angio-T treated infarcted myocardial tissue (Figure 3B).

Angio-T-enhanced Survival Potential and Reduction of Infarct Size

Seven days after LAD ligation, the myocytes at risk along the peri-infarct rim of DMSO controls showed distorted and irregular shapes compared with the myocytes at the distal part of the heart. By contrast, the myocytes at the peri-infarct rim of angio-T treated hearts showed a

regular shape. TUNEL staining showed that approximately 3-fold lower numbers of apoptotic myocytes were detected in angio-T treated left ventricle myocardium compared with DMSO controls (per high power field: 1.70 ± 0.18 versus 5.04 ± 0.75 , $P < 0.001$, $n = 15$; Figure 4a). These differences were particularly evident within the peri-infarct rim, where irregularly shaped myocytes in the control hearts had the highest number of apoptotic nuclei. Most of the apoptotic nuclei were observed at the peri-infarct rim rather than the myocytes distal to the infarct zone. Interestingly, significantly higher density of capillaries surrounded by the myocytes with much less apoptotic nuclei were found in the infarct zone of angio-T-treated hearts. By contrast, significantly less density of capillaries but more apoptotic nuclei were observed in the non-treated hearts in control group. Together, these results indicated that the angiogenesis induced by angio-T treatment prevented an extending pro-apoptotic process in both myocytes and endothelial cells, enhanced survival of the viable myocytes and endothelial cells within the peri-infarct zone and in consequence improved myocardial function. To explore whether the angio-T induced anti-apoptotic effect on the viable myocytes at risk was backed by expressions of anti-apoptotic proteins, Western blot analyses were performed. It was demonstrated that the angio-T-induced prevention of extending pro-apoptotic process of heart tissue at risk were concomitantly accompanied by increased gene expressions of key survival factors in that the expressions of Akt1 and Bcl2 were increased to 3.3 and 2.8 folds respectively compared with the heart tissues at risk in control group (Figure 4B).

To investigate whether the increased survival potential of the viable myocytes and endothelial cells within the peri-infarct zone induced by angio-T would result in reduced infarct size, we measured the infarct sizes of different groups. The mean proportion of collagenous deposition or scar tissue/left ventricular myocardium (as defined by Masson's Trichrome stain) was $27.44\% \pm$

Table 2. Differential up-regulation genes of specific signal transduction pathways

ID	Name	Description	Pathway involved
1	BAX	Bcl2-associated X protein	P53 pathway
2	BCL2	B-cell leukemia/lymphoma 2	1. Survival pathway a. PI3 kinase/AKT b. Jak/Src c. NF- κ B 2. Estrogen pathway 3. Phospholipase C pathway
3	BMP4	Bone morphogenetic protein 4	Hedgehog pathway
4	CDKN1b	Cyclin-dependent kinase inhibitor 1B	TGF- β pathway
5	FASN	Fatty acid synthase	Insulin pathway
6	CCL20	Chemokine (C-C motif) ligand 20	NF- κ B pathway
7	CDKN2a	Cyclin dependent kinase inhibitor 2A	TGF- β pathway
8	ICAM1	Intercellular adhesion molecule 1	1. NF- κ B pathway 2. Phospholipase C pathway
9	IL4R	Interleukin-4 receptor	Jak-Stat pathway
10	IRF1	Interferon regulatory factor 1	Jak-Stat pathway
11	MDM2	Similar to mdm2 gene product	P53 pathway
12	ODC1	Ornithine decarboxylase 1	Calcium and protein kinase C pathway
13	PRKCE	Protein kinase C ϵ	Calcium and protein kinase C pathway
14	TRIM25	Tripartite motif protein 25	Estrogen pathway
15	GADD45a	Growth arrest and DNA-damage-inducible 45 α	P53 pathway

7.34% in rats treated by angio-T compared with $39.53\% \pm 5.97\%$ for those in control group 14-day post infarction (Figure 4C), indicating that angio-T-enhanced survival potential of both myocytes and endothelial cells significantly increased the mass of viable myocardium within the anterior free wall of left ventricles.

Echocardiography Assessment of Myocardial Function

We compared the effects of the angio-T treatment on myocardial function of rats following MI. By 48 h post-infarction, left ventricular ejection fraction (LVEF) in angio-T treated group had significantly improved (57.38 ± 2.41) by 11% compared with that (51.39 ± 2.27) in control hearts ($P < 0.001$, $n = 15$), although they were both reduced compared with sham group (76.07 ± 2.97 , sham vs. angio-T/control group $P < 0.05/P < 0.001$, $n = 15$). FS in angio-T treated group was signifi-

cantly higher than in control group (30.77 ± 1.67 vs. 23.92 ± 1.36 , $P < 0.05$, $n = 15$) and left ventricular end-diastolic dimension in angio-T treated hearts was smaller. These improvements were maintained to 1-month post infarction (Figure 5). After 14 days' follow-up, LVEF in angio-T treated group was still significantly higher than that in control group (59.60 ± 3.44 vs. 52.30 ± 2.18 , $P < 0.05$, $n = 15$) as well as FS was significantly higher in angio-T treated group compared with control group (29.06 ± 2.32 vs. 23.49 ± 1.21 , $P < 0.05$, $n = 15$).

DISCUSSION

The major new findings of this study were that (1) the bio-active angiogenic tannins (angio-T) were identified and isolated from GJ; (2) angio-T up-regulated the expressions of angiogenic factors (VEGFb and VEGFc) that probably contributed to the early revascularization (< 24 h) in infarcted myocardium; and

(3) angio-T also induced anti-apoptotic protein expression and inhibited apoptotic death of cardiomyocytes in the infarcted hearts, which may contribute to the increased survival potential of the viable myocytes at risk post MI preventing the progressive extending of further ischemic injury and limiting infarct size. The present findings demonstrated that angio-T plays a key role in promoting early revascularization and reducing infarct size leading to significant functional improvement in acute MI. Following infarction, the viable myocardial tissue bordering the infarct zone is significantly hypertrophied. Although neoangiogenesis/revascularization within the infarcted heart tissues appeared to be an integral component of the remodeling process, under normal circumstances the growth of the capillary network cannot keep pace with tissue growth and is unable to support the greater demands of the hypertrophied but viable myocardia, which subsequently undergo apoptosis due to inadequate oxygenation and nutrient supply (13,22). Therefore, discovery of a small molecule which could be used clinically to increase survival potential of viable cardiomyocytes is of great interest. Clinical administration of such a therapeutic may decrease further ischemic injury.

Angio-T-induced expression of anti-apoptotic proteins (Akt1 and Bcl2), demonstrated by Western blots with antibodies specific to phospho-Akt1 and Bcl2, suggested the increased survival potential of the viable myocytes at risk. This was further confirmed by significantly reduced apoptotic nuclei in infarct zone compared with those in control hearts. The smaller sizes of the infarcts in angio-T treated hearts may also have been due to the consequence of the increased survival and reduced apoptotic death of the cardiomyocytes at risk. Akt and Bcl2 are believed to be important general mediators of variety survival signals that are required for cell survival (23,24) by targeting apoptotic family members Ced-9/Bcl-2, Ced-3/caspases and IKK- α /IKK- β . Therefore, up-

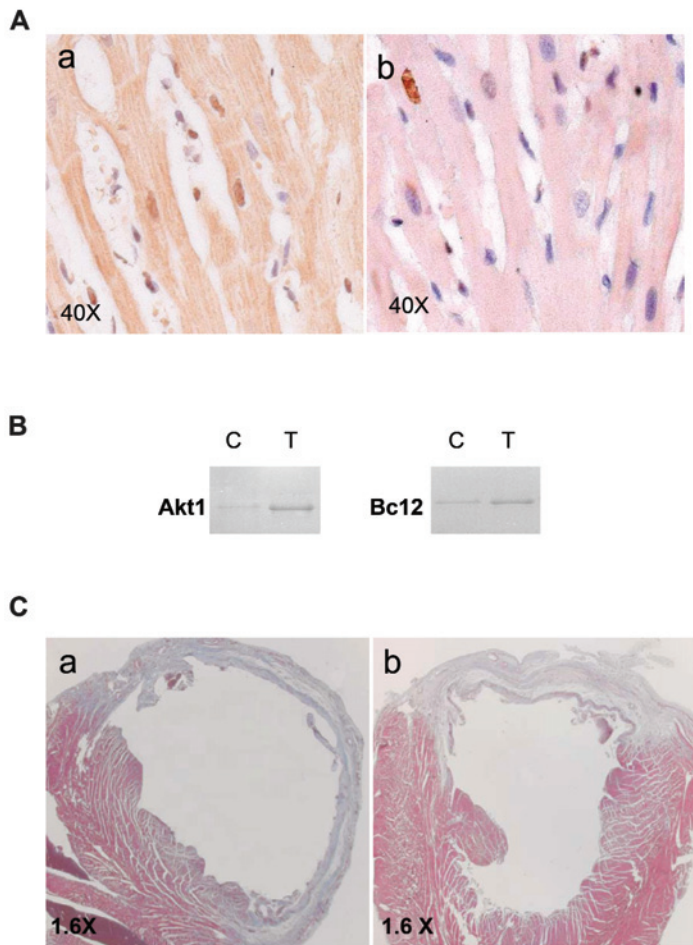


Figure 4. Angio-T-enhanced survival potential and reduction of infarct size. (A) Seven days after LAD ligation, the irregular myocytes at the peri-infarct rim had more apoptotic nuclei in the control (a). In contrast, the myocytes were regular and few apoptotic nuclei were evident in the peri-infarct rim of rats in angio-T-treated hearts. (B) Western Blot analysis showed the increased gene expressions of phospho-Akt1 and Bcl2 respectively upon angio-T treatment compared with their expressions in control hearts. (C) The trichrome staining of the rat myocardium at 2-week post infarct. There was extensive loss of anterior free wall myocardial mass, with collagen deposition and scar formation extending almost through the entire free wall of left ventricular (a). In contrast, trichrome stain of rat myocardium at 2-week post infarction in angio-T treated rats showed significantly reduced infarct size and increased mass of viable myocardium within the anterior wall (b).

regulation of Akt1 and Bcl2 are indications of the increased survival potential of the cardiomyocytes.

The early revascularization in infarcted myocardia within 24 h observed in the angio-T treated MI group in this study was probably induced by the up-regulated expressions of angiogenic factors-VEGFb and VEGFc, as was confirmed by Western blots with specific

antibodies against VEGF. The Jak-Stat signal transduction pathway in the viable cardiomyocytes bordering the infarct was activated upon angio-T treatment, which has been shown to induce various angiogenic factors besides anti-apoptotic proteins (25). It was also reported that G-CSF induced cardiac expression of angiogenic factors in vitro and in vivo appeared to be mediated by

cardiac Stat3 activation (25,26). Thus, activation of Jak-Stat pathway in the angio-T treated hearts may also promote angiogenesis and protect against endothelial apoptosis by producing angiogenic factors, resulting in revascularization in infarcted myocardia and prevention of further ischemic death of the cardiomyocytes at risk. The activation of the angiogenesis enhancing pathway may induce the production of various angiogenic factors which make efficiently revascularize in infarcted myocardia, as was demonstrated in our myocardial infarction studies. Where the newly formed vessels could be found as early as 24 h post infarction and many newly formed blood vessels and capillaries filled with blood cells were observed in the whole infarct areas. The density of the newly formed vessels in the whole infarct zones of the angio-T treated myocardia was on average 3-4 fold more than that observed in the controls.

The exact mechanisms, by which the angio-T can induce expressions of anti-apoptotic proteins and angiogenic factors in infarcted myocardia and therefore leading to the early revascularization, anti-apoptotic death of viable myocardium, reduction of infarct size and significantly improved functional performances of the infarcted hearts, are not clear. However, as was demonstrated in our RT-PCR, Western blots and initial signal transduction pathway profiling experiments, the genes involved in angiogenesis, cell survival and Jak-stat signal transduction pathways, which were silent or low-level expressed in non-treated myocardia, were activated or up-regulated upon angio-T treatment. These activated or up-regulated functionally-associated-genes may contribute to the decreased apoptotic death or increased survival potential of the viable myocytes and early revascularization in infarcted myocardium.

The properties of angio-T to up-regulate the expressions of VEGFb and VEGFc resulting in early reconstitution of blood supply network, induce the expression of anti-apoptotic proteins-Akt1

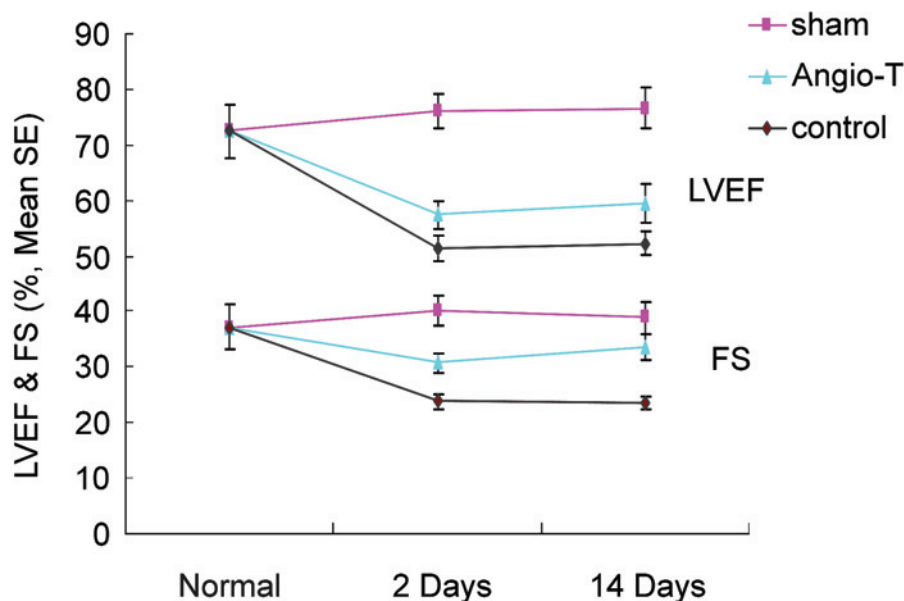


Figure 5. The effects of the angio-T treatment on myocardial function of rats following MI. LVEF in angio-T treated hearts had significantly improved compared with that in control hearts on day 2 ($P < 0.001$) and day 14 ($P < 0.001$) post infarction. FS in angio-T treated hearts was significantly higher than that in control group on day 2 ($P < 0.05$) and day 14 ($P < 0.001$) post infarction.

and Bcl2 preventing apoptotic death of cardiomyocytes at risk, which was accompanied by significant functional improvement, appear entirely novel. It can provide a new dimension for the emerging field of therapeutic angiogenesis medicine for the treatment of ischemic heart diseases.

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