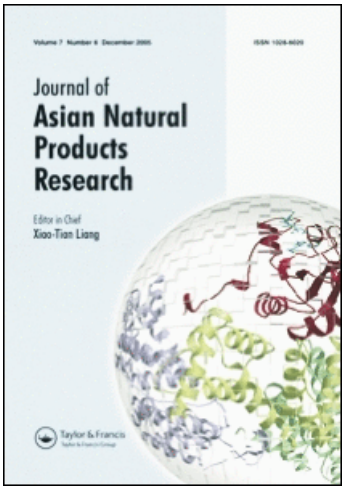

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Effect of astragaloside on cardiomyocyte apoptosis in murine coxsackievirus B_3 myocarditis

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Astragaloside is the major component of *Astragalus membranaceus*, one of the Chinese medical herbs, and has several pharmacological actions on cardiovascular system, including positive inotropic, anti-arrhythmia and anti-oxidant activities. We have investigated the effect of astragaloside on cardiomyocyte apoptosis and expression of apoptosis-associated genes in mice with coxsackievirus B_3 (CVB_3)-induced myocarditis, the former of which has been detected by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay and the latter determined by using a focused microarray. Results showed that cardiomyocyte apoptosis and expression of various apoptosis associated genes are inhibited after treatment with astragaloside. The anti-apoptotic activity of astragaloside may contribute to the improvement of clinical outcomes in treating myocarditis with pharmaceutics of *Astragalus membranaceus*.

Keywords: Astragaloside; Apoptosis; Coxsackievirus B (CVB); Myocarditis; Microarray

1. Introduction

The pharmaceutics of *Astragalus membranaceus*, one of the Chinese medical herbs, have been clinically administrated to treat viral myocarditis for a couple of decades in China, the beneficial effects of which have been verified by many experimental and clinical investigations. One of the active components of *Astragalus membranaceus*, astragaloside (figure 1), has positive inotropic, anti-arrhythmia and anti-oxidant activities [1–3]. In addition, astragaloside upregulated receptors of interferon (IFN)-α and IFN-γ dose-dependently as we recently reported [4]. In the present study, we investigated its effect on cardiomyocyte apoptosis and the expression of relevant genes in mice with coxsackievirus B_3 (CVB_3)-induced myocarditis by using a focused cDNA microarrays containing 96 known apoptosis associated genes.

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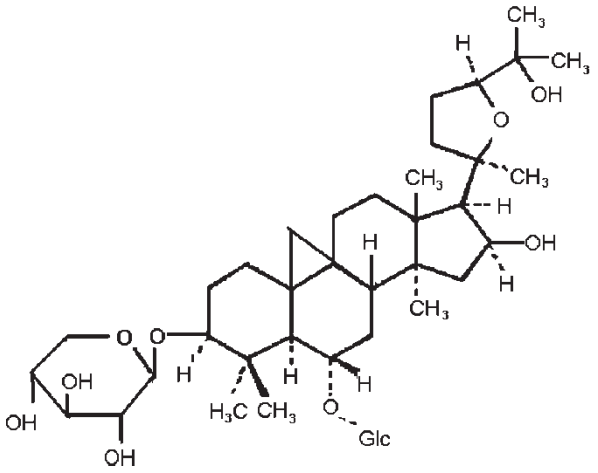


Figure 1. Chemical structure of astragaloside.

2. Results and discussion

2.1 Astragaloside inhibited cardiomyocyte apoptosis in myocarditic mice

In the present study, we established an animal model of myocarditis by utilizing 32 BALB/C mice infected with coxsackievirus B_3; the uninfected controls ($n = 16$) were also prepared. Half of the infected mice were orally administrated with astragaloside at a dose of $0.6\,mg\,kg^{-1}$ Daily for a week, the remaining half and the controls were treated with saline. By terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay, no TUNEL-positive cells were seen in controls (figure 2A) and notable apoptotic events occurred in myocardium, particularly in inflammatory lesions, of untreated myocarditis mice (figure 2B), while in astragaloside-treated mice, cardiomyocyte apoptosis was minimal (figure 2C), indicating that astragaloside inhibited apoptosis *in vivo*. Cardiomyocyte apoptosis plays important roles in the pathogenesis of CVB_3 myocarditis and the development from myocarditis to end-stage dilated cardiomyopathy (DCM), which

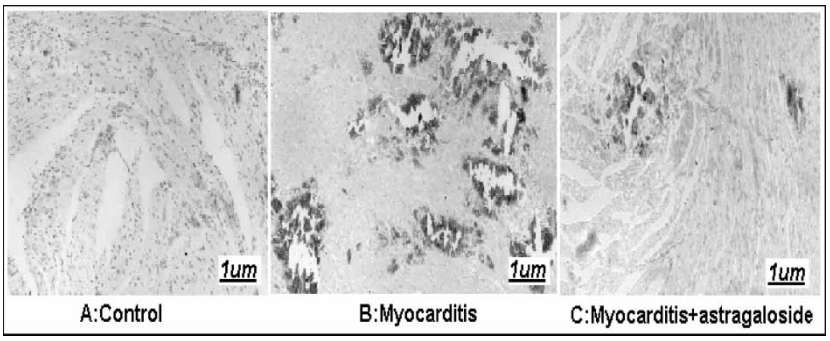


Figure 2. Cardiomyocyte apoptosis in each group of mice by TUNEL assay (magnification × 200). No TUNEL-positive cells were seen in control (A); notable apoptotic events were presented in myocarditis mice heart (B); in astragaloside-treated myocarditis mice, minimal cardiomyocyte apoptosis was observed (C).

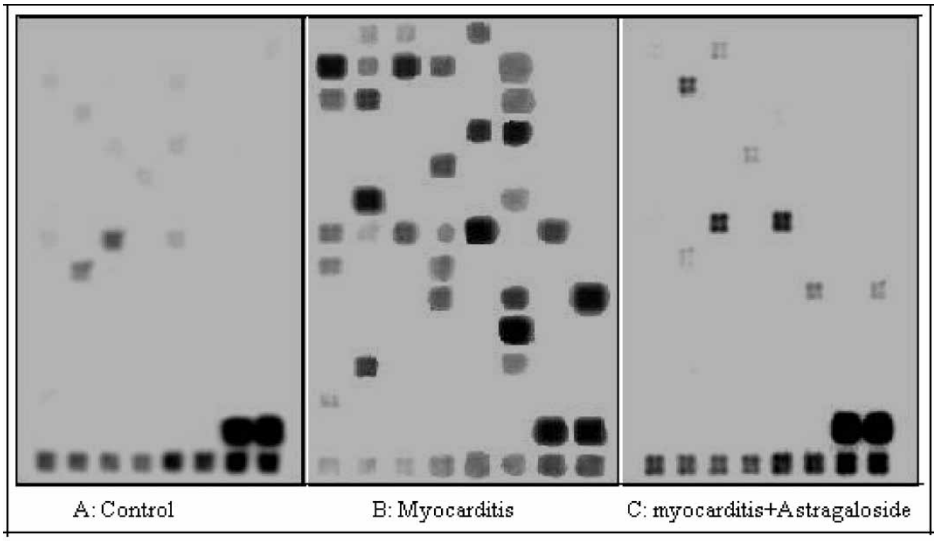
requires heart transplantation [5]. CVB_3-associated myocardial damage involves apoptosis [6]. Inhibition of cardiomyocyte apoptosis by astragaloside may contribute to the improvement of clinical outcomes in treating myocarditis with pharmaceutics of *Astragalus membranaceus*.

2.2 Effect of astragaloside on expression of apoptosis associated genes in experimental acute CVB_3 myocarditis

We compared expression of apoptosis-associated genes in heart tissues of control, untreated and astragaloside-treated myocarditic mice by GEArray™ Q series Mouse Apoptosis Gene Array (SuperArray Inc., Bethesda, MD), which was designed to profile the expression of 96 key genes involving in apoptosis. Through a side-by-side hybridization, the expression profile of these 96 genes in different groups of mice was determined and compared simultaneously (figure 3). Several genes involved in onset and regulation of apoptosis were highly expressed in heart of myocarditic mice in comparison with control (table 1). The balance between the pro-apoptotic and anti-apoptotic genes was broken in CVB_3 infected myocardium; the former was in an advantageous position so that it mediates apoptotic cell death. Among these selected genes, the tumor necrosis factor ligands family members, TNF-b, CD27-L and HVEM-L, may play a role in the cell-mediated myocardial damage in patients with acute myocarditis and dilated cardiomyopathy as in murine viral myocarditis [7]; Bax may be associated with the apoptosis process in CVB3-induced myocarditis [8]. Functions of other highly expressed genes listed in table 1 and relevant proteins in the pathogenesis of viral myocarditis need further investigation.

Astragaloside inhibited most of the upregulated genes mentioned above except for Arc, DFFT40 and CARD, and upregulated certain p53 and ATM pathway members' genes such as p21Waf1, chk2 (RAD53) and Rpa (table 1 and figure 3C). Most of them function in onset and/or regulation of apoptosis through governing two major pathways, the death receptor and the mitochondria pathways, for caspase activation. Myd88, FADD and certain TNF receptor family members such as TNF-R1, DR3 and DR6 contain a conserved 'death domain (DD)', and can mediate apoptosis by activating various intracellular signaling pathways such as the c-Jun N-terminal kinase (JNK) cascade and the nuclear factor-kB (NF-kB) pathway [9–11]. LTbR, lacking a DD, may execute cell death through a mechanism of cytotoxicity by some members of the TNF-R family, such as TNF-R1 [11]. Caspase 9 is the apical caspase for onset of apoptosis. The pro-apoptotic (Bax, bok/mtd, hrk, bik, and Nip3) and anti-apoptotic (bcl-2 and bfl-1) bcl-2 family members govern the mitochondria-dependent pathway for apoptosis [10]. Overexpression of TRAF1 demonstrates suppression of both the death receptors-dependent and mitochondria-dependent pathways [12,13]. Briefly, astragaloside inhibited most upregulated genes in CVB_3 infected myocardium and seems to renovate the broken balance between pro-apoptotic and anti-apoptotic genes mentioned above.

In conclusion, the present study confirmed the involvement of apoptosis in acute CVB_3 myocarditis. Astragaloside inhibited cardiomyocyte apoptosis and expression of certain apoptosis-associated genes in myocardium of CVB_3 infected mice, which may provide a novel beneficial effect in treating viral myocarditis and contribute to explaining the improvement of clinic outcomes in treatment of viral myocarditis with pharmaceutics of *Astragalus membranaceus*.



	A	B	C	D	E	F	G	H
1	Apaf1	April	Arc	ASC	ATM	bad	bak	Bar-like
2	bax	CARD	bcl-2	bfl-1	bcl-x	Bcl-10	Bcl-w	Bid
3	hrk	bik	bim	NAIP1	NAIP2	NAIP5	IAP1	IAP2
4	X-linked IAP	survivin	Bruce	blk	Nip3	Bok/mtd	Casper	Casp1
5	Casp11	Casp12	Casp14	Casp2	Casp3	Casp6	Casp7	Casp8
6	Flash	Casp9	p21Waf1	chk1	CIDE-A	CIDE-B	CRADD	DAPkinase
7	DFFA	DFF40	DR6	FADD	gadd45	Hus1	TNFb	LT-b
8	LtbR	Mcl-1	Mdm2	Myd88	Nop30-like	RAD53	RIP	Rpa
9	TANK	TNFa	DR5	TNFRSF11A	OPG	DR3	TNFR2	TNFR1
10	OX40	CD40	Fas	CD27	CD30	4-1BB	Trail	TNFSF11
11	TNFSF12	HVEM-L	OX40L	CD40L	FasL	CD27L	CD30L	4-1BBL
12	TRAF1	TRAF2	TRAF3	TRAF4	TRAF5	TRAF6	TRIP	P53
13	PUC18	PUC18	PUC18	Blank	Blank	Blank	GAPDH	GAPDH
14	cyclophlinA	cyclophlinA	cyclophlinA	cyclophlinA	RPL13A	RPL13A	beta-actin	beta-actin
Gene template : Array layout table with gene name and position information								

Figure 3. SuperArray analysis of apoptosis-associated genes in murine heart tissues. Total RNA was isolated from hearts of each group of mice ($n = 5$ each), and a 5 μg pool of RNAs was converted into cDNA by using biotinylated dUTP. Hybridized products were detected by using avidin-alkaline phosphatase and a chemiluminescent substrate. Upper part: (A) expression in control; (B) expression in CVB_3 myocarditis mice; (C) expression in CVB_3 myocarditis mice after Astragaloside treatment. Lower part: Gene template. Cyclophlin A (14A to 14D), RPL13a (14E to 14F), GAPDH and β-actin served as internal controls (spots 13G to 13H and 14G to 14H, respectively). Bacterial plasmid pUC18 (97 to 99) served as a negative control.

3. Experimental

3.1 Experimental model of viral myocarditis and tissue processing

Forty eight 4-week-old inbred male BALB/C mice (body weight 12–16 g; obtained from the Department of Laboratory Animals, Fudan University. License No: SCYK Shanghai 2002-0002) were kept under standard conditions for 3 days, and then randomized to 3 groups ($n = 16$ each). One group was injected ip with Eagle's minimal essential medium (EMEM) solution and served as controls, the other two groups were inoculated ip with 0.1 ml 10^9

Table 1. Apoptosis-associated genes with high expression in myocardium of each group of mice ($n = 5$ each).

Group	*A: Control*	*B: Myocarditis ($Ratio_{B/A} \geq 3$)*[a]	*C: Myocarditis + astragaloside ($Ratio_{C/A} \geq 3$)*[b]
TNF ligand family		TNF-b, CD27L(CD70), TNFSF14 (HVEM-L)	
TNF-R family	DR6	TNFR1, LTbR, Tnfrsf9 (4-1BB), TNFRSF11A, DR3 (Apo3), April, DR6	
Bcl-2 family	blk, bax, bik, bcl-x, bok/mtd, mcl-1	Bcl-2, CARD, bfl-1, Bcl10/HuE10, Bax, bok/mtd, hrk, bik, Nip3,	CARD, blk,
Caspase family	Casp2	Casp2, Casp9 (Mch6)	
IAP family		NAIP5	
TRAF family		TRAF1	
CARD family	Bcl10	Arc, Bcl10 /HuE10,	Arc
Death domain family		FADD, Myd88,	
Death effector domain family		FADD	
CIDE domain family	DFFA	DFFA, DFF40 (CAD), CIDE-B	CIDE-A, DFF40 (CAD),
p53 and ATM pathway	Gadd45	gadd45, ATM,	p21Waf1, chk2 (RAD53), Rpa

[a] $Ratio_{B/A}$ = Average integrated intensity of genes in group B/Average integrated intensity of genes in group A.
[b] $Ratio_{C/A}$ = Average integrated intensity of genes in group C/Average integrated intensity of genes in group A.

$TCID_{50}$ (50% tissue culture infective dose) coxsackievirus B_3 (Nancy strain) diluted in EMEM solution. Half of the infected mice were treated with astragaloside (purity: 83.7%; supplied by China National Institute for Control of Pharmaceutical and Biological Products, NICPBP) ig daily at a dose of 0.6 mg kg^{-1}, the remaining half and controls were treated with an equal volume of normal saline for 7 days. Hearts of all surviving mice, sacrificed on day 14 postinoculation, were quickly removed and sectioned into two portions. One portion, which included the right and left ventricles, was frozen in liquid nitrogen and then stored at -70°C until use. The other portion, the mid-ventricular section, was fixed in 10% neutral buffered formalin for subsequent histopathological examination and TUNEL assay.

3.2 Histopathological examination

Formalin-fixed specimens from each group were cleared and embedded in paraffin wax, cut into 4 μm thick sections, mounted on glass slides and stained with hematoxylin-eosin. The tissue preparations were then examined for the degree of myocardial cellular damage and infiltration of inflammatory cells, or tissue injury.

3.3 TUNEL assay

TUNEL assays were performed to evaluate the fragmented DNA by using an ApoTag kit (Oncer Inc., Gaithersburg MD) as previously described [14]. Formalin-fixed paraffin sections (4 μm) were rehydrated by sequential incubation with xylene, 100, 95, and 70% ethanol, distilled H_2O, and PBS for 5 min each. After protease K (Roche) digestion for 30 min at room temperature and quenching of endogenous peroxidase using 0.3% H_2O_2 solution for 15 min at room temperature, tissue sections were incubated with the enzyme TdT, reaction buffer with Co^{2+} cations, and digoxigenin-labeled nucleotides. As a negative control, the TdT

enzyme was omitted from the protocol. Single positive cells were detected after color reaction with TBL-streptavidin-horseradish peroxidase detection solution. The slides were counterstained with eosin.

3.4 Total RNA and mRNA preparation

After determination of the animal model with histopathological methods, total RNA was prepared from heart samples of each group of mice ($n = 5$ each) using TRIZOL reagent (Invitrogen/Life Technologies Inc., Grand Island, NY) subsequent cleanup was carried out with an RNeasy Maxi kit (Qiagen, Inc., Germany) according to the manufacturer's instructions. The quality of total RNA was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in an Ultrospec III spectrophotometer (Pharmacia). The A_{260}:A_{280} ratio of samples was 1.9–2.1. mRNA was isolated using an Oligotex mRNA Midi purification kit (Qiagen, Inc.).

3.5 Synthesis of cDNA probes

Total RNA was used as a template for biotinylated probe synthesis using a GEArray™ Q series Kit (SuperArray). Total RNA (5 μg) was annealed with GEAprimer Mix at 70°C for 3 min and cooled to 42°C. The RNA was then labeled with a room temperature cocktail consisting of Nonrad-GEAlabeling Buffer (SuperArray), biotin-16-dUTP (Promega), RNase inhibitor (Roche), and reverse transcriptase (Promega) at 42°C for 60 min. The reaction was stopped, denatured, and neutralized by specific solutions provided by SuperArray. The resulting cDNA probe was ready to be used for hybridization.

3.6 Hybridization and chemiluminescent detection

GEArray™ Q series membrane (SuperArray, Inc.) was prehybridized with GEAhyb Hybridization Solution (SuperArray, Inc.) containing denatured sheared salmon sperm DNA (100 μg of DNA per ml; Invitrogen/Life Technologies,) at 60°C for 1.5 h and hybridized in the hybridization solution containing denatured cDNA probe of the samples at 60°C overnight. After washing the membrane twice with 5 ml of washing solution 1 (300 mM sodium chloride, 30 mM sodium citrate, and 1% SDS) and twice with 5 ml of washing solution 2 (15 mM sodium chloride, 1.5 mM sodium citrate, and 0.5% SDS) for 15 min each at 60°C, the membrane was blocked in 1.5 ml of GEAblocking solution Q for 40 min at room temperature and incubated with diluted alkaline phosphatase-conjugated streptavidin (1:7,500 dilution) in buffer F for 30 min at room temperature. After the membrane was washed in a washing buffer (4 ×) and rinsed in a rinsing solution, the membrane was incubated with CDP-Star chemiluminescent substrate and exposed to X-ray film. All experiments were performed in triplicate, signals were quantitated by scanning the film using a ScanJet scanner (Canon), and the intensity of the spots was analyzed by using the Image-Pro Plus software (Media Cybernetics) and a GEArrayAnalyzer (SuperArray, Inc.). Cyclophlin A, ribosomal protein L13a (RPL13a) (23 kDa highly basic protein), β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as positive controls, and bacterial plasmid (pUC18) was used as a negative control.

3.7 Comparison of genes expression among 3 groups of mice

Through a side-by-side hybridization, the expression profile of 96 apoptosis associated genes was determined simultaneously. By using paired membranes containing equal amounts of each probe, levels of mRNA expression of matched samples were semiquantitatively compared by using a laser densitometer to determine the average integrated intensity of each dot. Average integrated intensities of apoptosis-associated genes dots were compared with the average integrated intensity of two GAPDH dots for each paired hybridization experiment. Genes that appeared more than 2 times in 3 Superarray hybridization analyses were selected as highly expressed genes; genes with average integrated intensities of dots more than 3 times in comparison with that of control are listed in table 1.

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References

[1] X.T. Shen, S.P. Zhou, Q. Sha, M.F. Jiang, X.J. Liu, R.H. Dai. *Acta Pharmacol. Sin.*, **14**, 95 (1993).
[2] A.L. Miller. *Altern. Med. Rev.*, **3**, 422 (1998).
[3] D.D. Hermann. *Am. J. Cardiovasc. Drugs*, **2**, 173 (2002).
[4] Z.C. Zhang, S.J. Li, Y.Z. Yang, H.Z. Chen. *Chin. J. New Drugs Clin. Rem.*, **22**, 515 (2003).
[5] C. Kawai. *Circulation*, **99**, 1091 (1999).
[6] A. Saraste, A. Arola, T. Vuorinen, V. Kyto, M. Kallajoki, K. Pulkki, L.M. Voipio-Pulkki, T. Hyypia. *Cardiovasc. Pathol.*, **12**, 255 (2003).
[7] Y. Seko, S. Ishiyama, T. Nishikawa, *et al. Cardiovasc. Pathol.*, **11**, 166 (2002).
[8] C.H. Joo, H.N. Hong, E.O. Kim, J.O. Im, S.Y. Yoon, J.S. Ye, M.S. Moon, D. Kim, H. Lee, Y.K. Kim. *Intervirology*, **46**, 135 (2003).
[9] K. Burns, F. Martinon, C. Esslinger, H. Pahl, P. Schneider, J.L. Bodmer, F. Di Marco, L. French, J. Tschopp. *J. Biol. Chem.*, **273**, 12205 (1998).
[10] J.C. Reed. *Am. J. Pathol.*, **157**, 1415 (2000).
[11] M. Grell, G. Zimmermann, E. Gottfried, C.M. Chen, U. Grunwald, D.C.S. Huang, Y.H. Wu Lee, H. Durkop, H. Englmann, P. Scheurich, H. Wajant, A. Strasser. *EMBO J.*, **18**, 3034 (1999).
[12] G.H. Cheng, H. Dadgostar. *J. Biol. Chem.*, **273**, 24775 (1998).
[13] E. Leo, L. Quinn, Q.L. Deveraux, C. Buchholtz, K. Welsh, S. Matsuzawa, H.R. Stennicke, G.S. Salvesen, J.C. Reed. *J. Biol. Chem.*, **276**, 8087 (2001).
[14] R.L. Debiasi, C. Edelstein, B. Sheery, K. Tyler. *J. Virol.*, **75**, 351 (2001).