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Cardioprotective effects of tanshinone IIA on myocardial ischemia injury in rats

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Received October 16, 2008, accepted November 14, 2008

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Pharmazie 64: 332–336 (2009) *doi: 10.1691/ph.2009.8771*

Tanshinone IIA (Tan IIA), a derivative of phenanthrenequinone isolated from *Salvia miltiorrhiza*, has been widely used for treating cardiovascular diseases in China. In the present study, we assessed the effect of Tan IIA on cardiac function, vascular endothelial growth factor (VEGF) expression and angiogenesis on models of myocardial infarction (MI) in rats. The results demonstrated that TanIIA elicited a significantly cardioprotective effect by improving heart function, reducing infarct size, and increasing survival rate in MI rat. Our results offer, for the first time, further insight into Tan IIA promoting angiogenesis and up-regulating VEGF expression in MI rats due to the enhancement of hypoxia-inducible factor 1α mRNA expression, and provide a novel target for Tan IIA in the prevention and treatment of myocardial ischemia injury.

1. Introduction

Many types of drugs are used for treating patients with Myocardial infarction (MI), including medicines aimed to reduce the peripheral resistance, such as nitroglycerin, propranolol, captopril, diltiazem, etc, and molecules which tend to increase the inotropy of myocardium, as isoprotenerol and dopamine. If medical treatment is inadequate, invasive revascularization procedures that improve coronary perfusion, say, Percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass graft (CABG) are considered (Kleiman et al. 2003). But the long-term success of angioplasty remains limited by the high occurrence of restenosis (Sheppard et al. 2003). In recent years, some newly developed strategies for curing myocardial ischemia injury are using the molecules which can stimulate myocardial angiogenesis, such as the vascular endothelial growth factor (VEGF) and the fibroblast growth factors (FGFs). But the method is not mature, and it has been reported that a patient died after administration of large amounts of adenoviral vector into the hepatic artery (Hollon 2000; Patterson et al. 2000; Yockman et al. 2008). To sum up, the ongoing therapeutic method exists in different degrees of defect. However, in the past decades, there has been a great increase in the use of herbs and its extracts in the treatment of the disease (Wang et al. 2007). Herb mixtures have also been being paid attention for their effective synergy effects and few side-effects.

Danshen, a herbal drug which derived from the dried root or rhizome of *Salviae miltiorrhizae* Bge has been widely used in China and many other Asian countries. Previous studys showed that Danshen has remarkable therapeutic efficacy in cardiovascular diseases such as angina pectoris, MI (Ji et al. 2003) and stroke. As one of the key components of Danshen, Tashinone IIA (Tan IIA) is widely used for treating cardiovascular diseases in China. It can dilate coronary arteries, increase coronary flow, and protect the myocardium against ischaemia. Recent studys suggested that Tan IIA can protect cardiac myocyte *in vitro* and *in vivo*, due to its antioxidative and anti-apoptosis properties (Fu et al. 2007; Gao et al. 2008). In this study, our aim was to investigate the effect of Tan IIA on cardiac function, vascular endothelial growth factor (VEGF) expression and angiogenesis in MI rats.

2. Investigations and results

2.1. Effect of Tan IIA on cardic function

To assess the protective effect of Tan IIA on cardic function, hemodynamic measurements were performed two weeks after coronary ligation in rats. As our results showed, the Tan IIA treatment group had a significant higher LV systolic pressure (LVSP) and a significant lower LV end-diastolic pressure (LVEDP) compared with MI rats. LV maximum dP/dt was significantly higher and LV minimum dp/dt tended to be lower in the Tan IIA treatment group as well. Administration of Tan IIA significantly improved ventricular contractility reflected by the increase in LV + dp/dtmax and LVsystolic pressure (Table 1).

2.2. Effect of Tan IIA on infarct size

Tan IIA treatment decreased the infarct size two weeks after MI in rats. Quantitative analysis demonstrated that cardiac infarct size was significantly smaller in the MI + Tan IIA than in the MI group: $37.5 \pm 6.2\%$ vs. $26.8 \pm 5.6\%$ ($p < 0.05$; Fig. 1C).

Table 1: Effects of TanIIA on haemodynamic, histopathological in rats with myocardial ischemia

	Sham	MI	MI + Tan IIA
Haemodynamic data			
LVP (mmHg)	139.8 ± 16.7	$111.5 \pm 9.3^{\#\#}$	$131.5 \pm 13.0^{*}$
LVEDP (mmHg)	4.39 ± 0.86	$6.4 \pm 0.7^{\#\#}$	$4.84 \pm 1.83^{*}$
LV + dp/dtmax (mmHg · s^{-1})	12198 ± 2164	$8105 \pm 1550^{\#\#}$	$11431 \pm 2541^{*}$
LV-dp/dtmin (mmHg · s^{-1})	-8364 ± 764	$-4772 \pm 947^{\#\#}$	-6297 ± 1323
Histopathological data			
LV/BW(mg/g)	2.52 ± 0.10	$2.84 \pm 0.07^{\#\#}$	$2.67 \pm 0.16^{*}$

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. MI group

2.3. Effect of Tan IIA on enzymic antioxidants in the serum

Oxidative stress has been documented in patients early and late after MI. In our study, a significant rise in the level of lipid peroxidation was observed in the serum of the MI group (12.72 ± 2.50 nmol/ml, $p < 0.05$) as compared with the sham group (10.48 ± 1.56 nmol/ml), and that was paralleled by a significant declined in the level of the activity of GSH-Px and anti-peroxidation enzyme (SOD). We found that administration of Tan IIA significantly increased the levels of SOD and GSH-Px in serum of rat with MI, and decreased MDA (10.19 ± 0.64 nmol/ml, $p < 0.05$) content in serum as well (Table 1).

2.4. Effect of Tan IIA on angiogenesis and VEGF expression

Angiogenesis limits damage to ischemic by recovering tissue blood flow. To investigate the effect of Tan IIA on angiogenesis in rats with myocardial ischemia, the number of vessels on HE staining of sections was counted. Two weeks after coronary ligation, numerous blood vessels were observed in the border zone of the MI + Tan IIA group (Fig. 2). The capillary density in the peri-infarct area of the Tan IIA treated myocardium was 8 (± 4) per high-power field ($400 \times$ magnification), However, fewer blood vessels (≈ 2 per HPF) were observed in the MI group.
VEGF is one of the key angiogenic growth factors which elicits a strong angiogenic response by affecting the survival, proliferation, and migration of vascular tissue. It is thought that elevation of VEGF may cause the development of collateral circulation and contribute to both minimize and heal the infarction site (Roy et al. 2003). Therefore, we detected the expression of VEGF in the heart by immunohistochemistry. As shown in Fig. 3, the VEGF expression was remarkably increased in the MI + Tan IIA group compared with the MI group in the border area. Unfortunately, the expressions of VEGF mRNA were too weak to be quantified.

2.5. Effect of Tan IIA on HIF-1α mRNA expression

The transcriptional activation of VEGF mRNA is mediated by a transcriptional activator called hypoxia inducible transcription factor (HIF-1) and its biological activity depends directly on the amount of HIF-1α subunits. Therefore, we explored the expression of HIF-1α mRNA in rats with MI. Our data showed that, in the peri-infarct

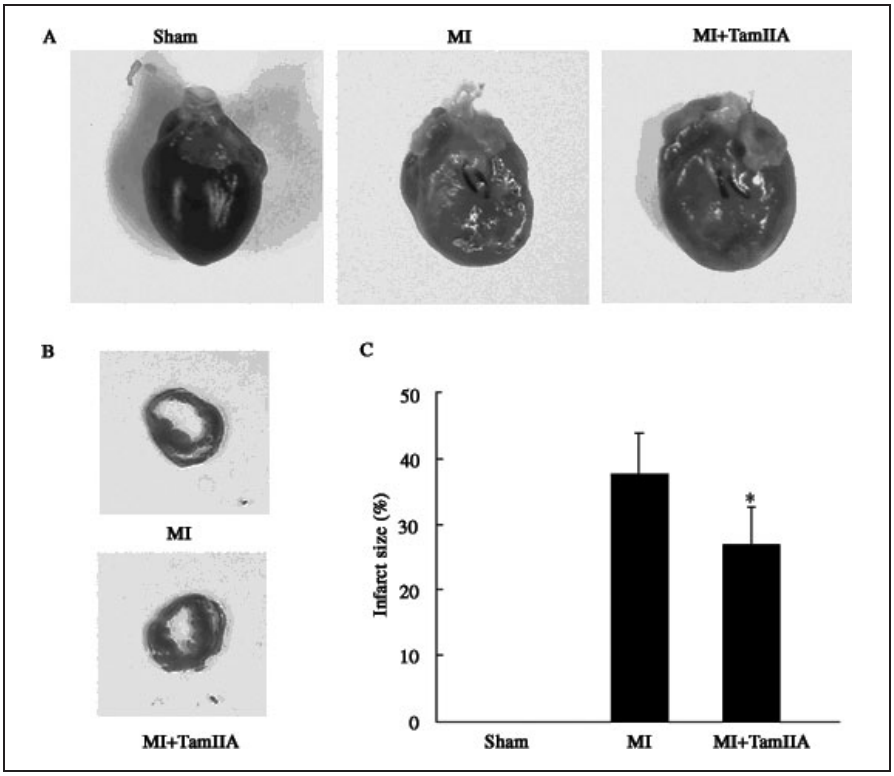

Fig. 1:
Effect of Tan IIA on myocardial infarct size two weeks after MI (A) Macroscopic view of the hearts. (B) Representative Masson's trichrome-stained myocardial sections from MI and MI + Tan IIA groups. (C) quantitative analysis demonstrating that Tan IIA groups significantly decreased infarct size. Values are means ± SD $^{*}p < 0.05$ vs. MI group

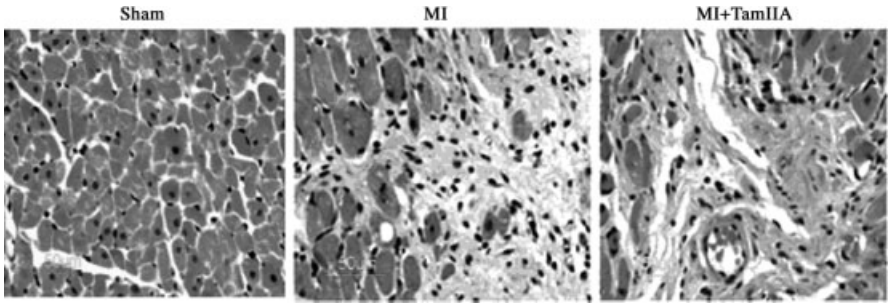

Fig. 2:
Effect of Tan IIA on blood capillaries in border zone of heart sections, stained with hematoxylin and eosin

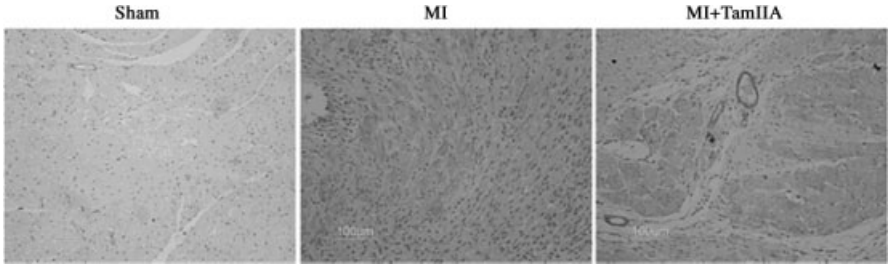

Fig. 3:
Effect of Tan IIA on VEGF expression examined by immunohistochemistry in rat two weeks after myocardial infarction. The brown part indicated the newly formed VEGF

Table 2: Effect of tanIIA on on enzymic antioxidants in the serum

	Sham	MI	MI + Tan IIA
SOD (U/ml)	208.65 ± 13.47	184.46 ± 23.83$^{\#}$	222.76 ± 37.78*
MDA (nmol/ml)	10.48 ± 1.56	12.72 ± 2.50$^{\#}$	10.19 ± 0.64*
GSH-Px (U/ml)	9498.40 ± 1968.21	7324.64 ± 909.64$^{\#}$	8650.52 ± 1214.68*

$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs. sham group; * $p < 0.05$, ** $p < 0.01$ vs. MI group

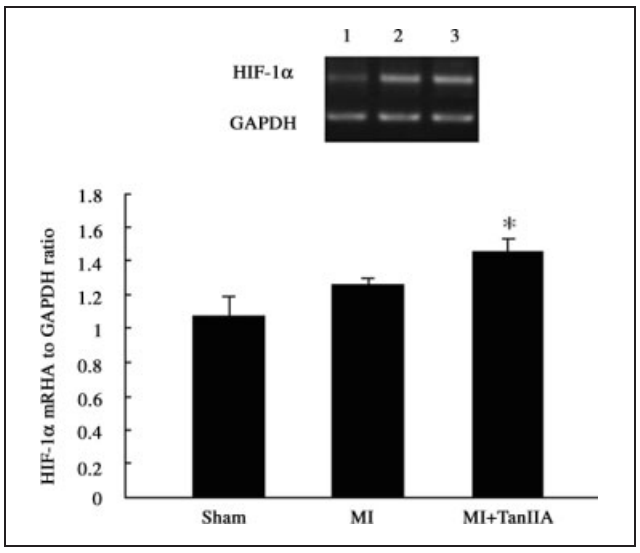

Fig. 4: Effect of Tan IIA on HIF-1αmRNA expression in the border LV area in rat two weeks after MI

area, HIF-1α mRNA expression was significantly higher in the MI + Tan IIA group than in the MI group ($n = 3$, $p < 0.05$) (Fig. 4). The expression of HIF-1α mRNA tended to increase in MI group compared with sham-operated group at the different degree.

3. Discussion

Myocardial damage which resulted from MI afflicts nearly 15 million people worldwide. MI is a complex clinical syndrome with poor prognosis, and its development is a long-term and complex process involving many factors, such as the sympathetic nervous system, renin-angiotensin system, reactive oxygen species (ROS), apoptosis (Krijnen et al. 2002). Despite considerably scientific data on the biochemical and molecular characteristics of MI, the precise molecular mechanisms responsible for MI still remain unclear. However, it is known to all that: diminution of infarct size, improvement of heart function and microcirculation, and prevention of ventricular remodeling are important end-points in the treatment of cardiovascular disorders post-infarction.

Tan IIA, a derivative of phenanthrenequinone isolated from *Salvia miltiorrhiza* Bge, has been widely used for treating cardiovascular diseases in China. It can dilate coronary arteries, increase coronary flow, and protect the myocardium against ischaemia (Adams et al. 2006). Previous studies showed that Tan IIA could protect cardiac myocytes *in vitro* and *in vivo* due to its antioxidative and anti-apoptosis properties (Yang et al. 2008). In the present study, we demonstrated that Tan IIA elicited a significant cardioprotective effect by improving heart function, reducing infarct size, and increaseing survival rate in MI rats. Furthermore, we demonstrated for the first time that Tan IIA could promote angiogenesis and up-regulate VEGF expression in MI rats, which may be due to the enhancement of HIF-1α mRNA expression.

Angiogenesis means sprouting, bridging, intussusception, and/or enlargement of capillaries from the pre-existing ones (Yla-Herttuala et al. 2007). This tightly regulated process involves the degradation of extracellular matrix combined with migration and sprouting endothelial cells (ECs) from preexisting capillaries. VEGF is one of the key angiogenic growth factors and stimulates migration, proliferation, and tube formation of ECs primarily through the VEGF receptor type2 (VEGR2, KDR/Flk1) (Ushio-Fukai 2007). VEGF is also a potent survival factor for ECs during physiological and tumor angiogenesis, and has been shown to induce the expression of antiapoptotic proteins in the ECs (Friehs et al. 2006; Gerber et al. 1998).

Early animal studies were promising, demonstrating that recombinant VEGF administered intravenously enhanced collateral formation in ischemic tissues (Banai et al. 1994; Takeshita et al. 1994). In a word, VEGF elicits a strong angiogenic response by affecting the survival, proliferation, and migration of vascular tissue, thereby restores blood flow and limits damage to ischemic tissue (Cross et al. 2003). In the present study, we found that Tan IIA augmented VEGF expression in border area of non-ischemic rat myocardium following acute MI (AMI) (Fig. 4), which contributed to the increase of blood vessel density and myocardial repair after AMI (Fig. 3).

VEGF expression is potently up-regulated in response to a low-oxygen, or hypoxic environment. This response seems to depend on hypoxia regulated/responsive element/enhancer sequences in the 5′ and 3′ regions of the VEGF-A gene. A transcriptional activator, HIF-1, can bind to the enhancer sequences of the VEGF-A gene, facilitate transcription and RNA stability, and result in VEGF up-regulation (Chi et al. 2004; Loor et al. 2008). HIF-1 is a heterodimer, consisting of the HIF-1α and HIF-1β subunits, both are basic-helix-loop-helix per-aryl hydrocarbon receptor nuclear translocator-sim proteins. HIF-1β is constitutively expressed. However, HIF-1α is degraded under normoxic conditions by ubiquitination, stable under hypoxic conditions (Hoeben et al. 2004). In the present study, we found that Tan IIA augmented HIF-1α mRNA expression in border area of the nonischemic rat myocardium following AMI, which may be associated with the increase of VEGF expression and angiogenesis.

Additionally, we found that Tan IIA increased the levels of SOD and GSH-Px in serum of rats with MI, and decreased MDA content in serum as well, and the results were consistent with previous studies (Fu et al. 2007). ECs generate reactive oxygen species (ROS) which plays an important role in physiological and pathophysiological responses (so-called "redox signaling") (Maulik 2002), depending on their intracellular concentrations. Physiological concentrations of ROS are involved in signaling to mediate ECs migration, growth, and differentiation. But excess amounts of ROS such as $O_2^{\bullet -}$ and H_2O_2 have deleterious effects on cells and contribute to various cardiovascular diseases including hypertension, heart failure, atherosclerosis, and diabetes (Egashira 2002). They cause oxidative damage of macromolecules, membranes, and DNA usually indirectly through the generation of more toxic (reactive) radicals such as $ONOO^{\bullet -}$ and $^{\bullet}OH$ (Li et al. 2004). As a natural antioxidant, Tan IIA provides its cytoprotective effect through inhibition of ROS (mainly H_2O_2) production in human aortic smooth muscle cells (Zhang et al. 2007). It has a cytoprotective effect on H_2O_2 induced human umbilical vein endothelial cell (ECV-304 cell) damage via anti-oxidative action (Lin et al. 2006). In our study, Tan IIA increased the levels of SOD and GSH-Px in serum of rat with MI, and decreased MDA content in serum as well. These results suggested that the cardioprotective and angiogenic effects of Tan IIA may be associated with an increased capability in scavenging oxygen free radicals and preventing lipid peroxidation in both cardiac myocytes and ECs.

In summary, the present studies demonstrated that Tan IIA exerts beneficial cardioprotective effects, such as improving cardiac function, reducing infarct size and angiogenesis. It was further proved that Tan IIA augmented VEGF expression in border area of nonischemic rat myocardium following AMI, which contributes to increase of blood vessel density and myocardial repair after AMI. In addition, the data suggested that Tan IIA might up-regulate the VEGF expression by increasing the HIF-1α mRNA expression; Meanwhile, Tan IIA may preserve ECs from oxidative stress through anti-oxidantal effects which indirectly promote angiogenesis.

4. Experimental

4.1. Animals

Male sprague–Dawley rats weighing 200–220 g (aged 10 weeks) were purchased from the Laboratory Animal Institute of Zhejiang Academy of Medical Science, China. Rats were placed in constant conditions at a temperature of 23 ± 3°, humidity of 60 ± 5%,and on a 12 h light/dark cycle. Rats had free access to a standard diet and drinking water. Experiments were performed according to the standards established by the Guide for the Care and Use of Laboratory Animals of Zhejiang University, and approved by the local ethics committee. The whole laboratory procedure was carried out under the permission and surveillance of the ethics committee.

4.2. Drugs

Tan IIA used in the study was obtained from Laboratory of Plant Resource and Phytochemistry of Zhejiang Univerity, China.

4.3. Experimental MI

MI was produced in rats by ligation of the left anterior descending coronary artery for two weeks. The surgical procedure was performed as described previously (Segers et al. 2007). Rats were anesthetized by urethane (1.2 g/kg, i.p.), and after tracheal intubation, hearts were exposed via left thoracotomy. After pericardiotomy, the left coronary artery was ligated by suturing with 4–0 prolene at the location 2 mm below the left atrial appendix. Then, the heart was returned to its nomal position, and the thorax was closed. For the sham operation, suturing was performed without ligation. In the present study, the operation-related mortality was approximately 30%, 24 h after operation.

4.4. Experimental protocol

Animals were divided randomly into 3 groups: sham-operated group (n = 15); MI group (n = 10); MI + Tan IIA (30 mg/kg, i.g.) (n = 10). Thirty min before the operation, Tan IIA or vehicle was given. After the surgery, animals received Tan IIA or vehicle daily for another 7 days.

4.5. Hemodynamic studies

Hemodynamic studies were performed 2 weeks after coronary ligation. Firstly, rats were anaesthetized with urethane (1.2 g/kg, i.p.). The right carotid artery was exteriorized and A P50 catheter connecting a pressure transducer (MP150, Biopac system, USA) was inserted into the left atria and left ventricle for measurement of hemodynamic parameters [LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), maximal rate of LV systolic pressure (LV + dp/dtmax) and minimum rate of LV systolic pressure (LV − dp/dtmax)]. After completion of hemodynamic measurements, the hearts were removed, washed with physiological saline, photographed and weighed.

4.6. Enzymic antioxidants and thiobarbituric acid-reactive substances (TBARs) in the serum

Oxidative stress was assessed by the concentrations of superoxide dismutase (SOD) and glutathione perioxidase (GSH-Px) in serum. The TBARs content of lipoproteins was employed as a measure of lipid peroxidation. The serum SOD, GSH-Px and malondialdehyde (MDA) levels were detected by the use of diagnostic kits (Jiancheng Bio-engineering Company, Nanjing, China) with a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) according to the supplier's instructions.

4.7. Histological examination

The hearts were cut into 3 transverse slices from apex to base. The middle slice was embedded in paraffin and processed for histology. Sections (5 μm thickness) of the paraffin-embedded tissue were stained with hematoxylin and eosin, as well as Masson's trichrome. The infarct size was measured on Masson's trichrome slides and expressed as previously study (Dai et al. 2005). Newly formed blood vessels were differentiated by HE staining of sections (Ji et al. 2003). The number of vessels in ten random and non-overlapping high-power fields (HPF) was counted (Nagaya et al. 2004) within the peri-infarct area using a light microscope at 400 × magnification, then, averaged and expressed as number of vessel per HPF. These morphometric studies were performed by two examiners who were blinded to treatment.

4.8. Immunohistochemistry for VEGF

Hearts were fixed in 10% neutral-buffered formaldehyde for 12 h, embedded in paraffin and cut into 5 μm-thick, transmural and consecutive serial sections for immunostaining of the VEGF expression. The sections were pretreated in 10 mM sodium citrate buffer (pH 6.0) at 120 °C for 2 min to preserve the antigenicity, and then allowed to cool to room temperature for approximately 30 min. The slides were then placed in 100% methanol with 0.3% (vol/vol) hydrogen peroxidase activity and then treated with blocking buffer (PBS containing 10% goat serum) for 15 min at room temperature in a moisture chamber. Polyclonal antibodies against VEGF (1 : 200, Boster, China) were applied onto the tissue sections for 18 h at 4 °C. After washing in 0.01 M PBS, the sections were incubated with biotin labeled goat anti-rabbit immunoglobulin (Ig)G (Santa Cruz Biotechnology) for 30 min at room temperature. The sections were then incubated with the SABC complex (Santa Cruz Biotechnology) and the antigen-antibody complexes were visualized by immersion in 3.3′-diaminobenzidine solution (0.01 M 3.3′-diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6; and 0.006% hydrogen peroxide). They were counterstained with hematoxylin, then dehydrated, cleared and covered with coverslips. The experiment was repeated on three different sections at least for each group. Ten random fields of each stained section were pictured and analyzed by a reader who was blinded to the animals' treatment status using morphometric software (Chansan, Shanghai, China).

4.9. Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) reagents were obtained from Promega (Madison, WI). Oligonucleotides for the primers were all synthesized by Invitrogen (Shanghai, China). Total RNA was extracted using Trizol according to the manufacturer's instructions; 5 μg RNA was used to synthesize the first strand of cDNA using II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and was applied as a template in the following PCR reactions (Eppendorf Mastercycler, Germany): mRNA expression of HIF-1α in border LV areas. The products were resolved on a 1.7% agarose gel followed by ethidium bromide staining, and the bands were analyzed by LABWORKS imaging acquisition and analysis software (Ultra-Violet products, Cambridge, UK).

4.10. Statistical analysis

All data were expressed as means ± SD. Comparisons of treatment group and vehicle group were made using one-way analysis of variance (ANOVA) by Dunnet's test. $P < 0.05$ was considered statistically significant.

Acknowledgements: This study was supported by a common project from National Natural Science Foundation of China (No: 30500661) and a grant from National Important Basic Development Plan of China (also called 973 project, No: 2005CB523405).

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