

## Original Article

# Effect of fasudil on restenosis after balloon injury in rats

Nana Pan, Min Leng, Lijuan Tan, Renchao Yu, Shanglang Cai

Department of Cardiology, The Affiliated Hospital of Qingdao University, Qingdao 266555, Shandong, China

Received October 10, 2016; Accepted February 16, 2017; Epub April 15, 2017; Published April 30, 2017

**Abstract:** Aims: The aim of this study was to observe the effect of the Rho kinase (ROCK) inhibitor fasudil on apoptosis of vascular smooth muscle cells (VSMCs) in rat aortas after balloon injury. Methods: Forty rats subjected to balloon injury were randomly divided into untreated (A) and fasudil treated (B) groups. Twelve rats were randomly selected for use as the sham-operated group (C). Group B were given 5 mg/kg fasudil by intraperitoneal injection, while Groups A and C received vehicle. After 4 weeks, aorta intima and media thickness were measured and VSMC apoptosis detected by TUNEL staining. Levels of ROCK mRNA were determined by RT-PCR and expression of the apoptosis-related proteins Bcl-2 and Bax in VSMCs was determined by immunohistochemistry. Results: In the balloon injury model, proliferation in the intima and media of the aorta varied, and the expression of ROCK mRNA and Bcl-2 increased, while the expression of Bax decreased ( $P < 0.01$ ). Additionally, apoptotic VSMCs were detected. After the administration of fasudil, the thickness of the intima and media decreased, the expression of ROCK mRNA and Bcl-2 decreased in VSMCs, whereas the expression of Bax ( $P < 0.01$ ) and the number of apoptotic VSMCs ( $P < 0.01$ ) increased. Conclusion: By inhibiting ROCK expression, fasudil downregulated anti-apoptotic proteins and upregulated proapoptotic proteins to increase VSMC apoptosis and reduce the degree of hyperplasia of the intima and media in the aorta after endothelial injury, thus reducing vascular stenosis. Therefore, fasudil could have therapeutic value in preventing restenosis after intravascular interventional therapy.

**Keywords:** Rho kinase, apoptosis, restenosis, fasudil, vascular smooth muscle cells

## Introduction

Percutaneous coronary intervention is the main treatment for coronary artery stenosis. However, postoperative restenosis remains a difficult clinical problem. Numerous studies have shown that Rho kinase (ROCK) and restenosis are closely related, with ROCK participating in the regulation of restenosis in various ways [1, 2]. With regard to the relationship between ROCK and VSMC apoptosis, there are few reports, and from these controversial results have been obtained [3, 4]. Preliminary animal experiments have shown that, after myocardial infarction, the expression level of ROCK and apoptosis of surviving myocardial cells were increased. Application of the ROCK-specific inhibitor fasudil could protect the ischemic myocardium, reduce myocardial infarct size and improve cardiac function. Moreover, its protective role is related to its anti-apoptotic role [5, 6]. In contrast, it has also been shown that fasudil can promote

the apoptosis of proliferating VSMCs [7], which may indicate a possible tissue-specific role for ROCK [8]. However, at present, most VSMC studies are carried out in vitro. Therefore, in this study, we established a rat model of aortic balloon injury, and observed the effect of fasudil on VSMCs after endothelial injury. This provides an experimental basis for finding potential preventive and therapeutic treatments for restenosis after coronary intervention.

## Materials and methods

### Materials

Fasudil (2 ml:30 mg) was purchased from Tianjin Red Sun Pharmaceutical Co., Ltd. (Tianjin, China). RNA extraction and RT-PCR kits were purchased from the Bo Biological Engineering Co., Ltd. (Hangzhou, China). The TUNEL kit was purchased from the Beijing BIOTEKE Biological Engineering Co., Ltd. (Beijing, China). Bcl-2 and Bax antibodies were

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**Table 1.** Changes of aortic intima and media thickness in rats with balloon-injury ( $\bar{x} \pm s$ )

Group	Intima ( $\mu\text{m}$ )	Media ( $\mu\text{m}$ )
A (n = 18)	99.6 $\pm$ 5.42 <sup>a</sup>	311.6 $\pm$ 17.00 <sup>a</sup>
B (n = 19)	91.3 $\pm$ 9.11 <sup>a,b</sup>	291.6 $\pm$ 19.74 <sup>a,b</sup>
C (n = 12)	21.8 $\pm$ 2.93	194.3 $\pm$ 9.09

<sup>a</sup>P<0.01 compared with the sham-operated group (C);

<sup>b</sup>P<0.01 compared with untreated group (A).

purchased from the Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China).

### *Animal treatment groups*

3-month-old male Wistar rats (body weight 350-400 g) were purchased from Qingdao Institute for Drug Control (Qingdao, China). Fifty-two male rats were randomly divided into three groups: untreated control group (group A, n = 20), Fasudil treatment group (group B, n = 20), and sham-operated group (group C, n = 12). A rat model of balloon injury was established as follows: the rats in group A and B were anesthetized with 10% chloralhydrate, and placed on the operating table. A self-made 2F Fogarty catheter was inserted into the rat's left common carotid artery and delivered to the lower end of the abdominal aorta. Then 0.1 ml normal saline was injected to fill the balloon and the catheter was pulled in different directions to completely strip the artery endothelium. Rats in group C underwent the same procedures, except that the balloon catheter was not inserted. The rats of group B were given 5 mg/kg fasudil by intraperitoneal injection twice a day for 4 weeks. The rats of group A and C received intraperitoneal injection of the same volume of saline. All procedures were approved by the Institutional Animal Care and Use Committee of Qingdao University and were conducted in conformity with institutional guidelines. All efforts were made to minimize the number of animals and their suffering.

### *Specimen collection and processing*

After 4 weeks, all rats were anesthetized with 10% chloral hydrate, soaked in 75% alcohol for 5 s, and then under sterile conditions an aortic vascular segment of approximately 4-5 cm was isolated. The proximal aorta was cut to 5-10 mm and fixed in formalin, embedded in paraffin, and then stained with hematoxylin and

eosin (HE) for histopathological examination. The remainder of the aorta was divided into two parts: one part was stored at -70°C for RNA extraction; the other part was put into 10% formaldehyde fixation solution for immunohistochemical staining.

### *Determination of the thickness of intima and media by HE staining*

HE stained paraffin sections were used for histopathological observations by light microscopy. The thickness of intima and media were measured using the Simple PCI pattern analysis system [9].

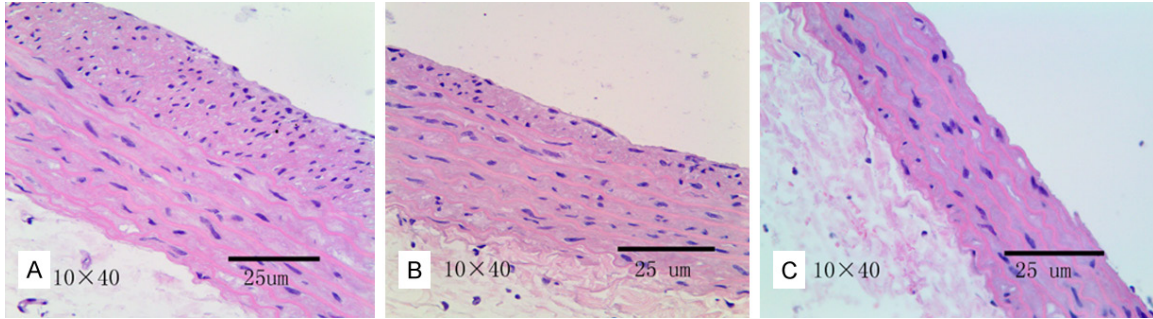
### *Determination of Rho-kinase mRNA expression*

Total RNA was isolated from frozen vascular tissue (50 mg) and was reverse transcribed to cDNA, according to the manufacturer's instructions. PCR primers were designed and synthesized by the Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China). The primers for GAPDH (433 bp) were as follows: forward, 5-CCGCATCTTCTTGTGCACTG-3, reverse, 5-TCACAAACATGGGGGCATCA-3. Primers for ROCK (194 bp) were as follows: forward primer, 5-GACATGCAAGCGCAATTGGTA-3, reverse primer, 5-TAAGGAATGCAGGCAGAACCA-3. The PCR amplification consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 30 s denaturation at 95°C, annealing for 30 s at 50°C for GAPDH and 55°C for ROCK, and extension for 45 s at 72°C. The final extension step was carried out at 72°C for 5 min. 10  $\mu\text{l}$  of the PCR product was run on a 1% agarose gel. The relative expression level of ROCK mRNA was normalized to that of GAPDH as an internal reference.

### *Detection of apoptosis of vascular smooth muscle cells*

TUNEL staining of 5- $\mu\text{m}$ -thick paraffin sections was performed according to the manufacturer's instructions to observe the apoptosis of VSMCs. Phosphate buffered saline (PBS) was used as a negative control. Sections were divided into four quadrants and observed at 40 $\times$  magnification. Five high-magnification visual fields in each quadrant were randomly selected and the TUNEL-positive and total cells counted, then the percentage of apoptotic cells

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**Figure 1.** HE staining. Balloon injury causes hyperplasia of the aortic intima and media, which is attenuated by fasudil. By HE staining the intima and media have different degrees of thickening in groups A (untreated) and B (5 mg/kg fasudil) compared with group C (sham-operated). Notably, after fasudil treatment, the thickening of intima and media was decreased.

**Table 2.** TUNEL-positive rate and integral optical density of apoptotic cells in the aorta of rats with balloon-injury ( $\bar{x} \pm s$ )

Group	Intima		Media	
	p-rate (%)	IOD	p-rate (%)	IOD
A (n = 18)	6.28±2.69	0.10±0.03	7.32±2.41	0.11±0.04
B (n = 19)	9.64±2.76 <sup>c</sup>	0.13±0.04 <sup>c</sup>	10.51±2.71 <sup>c</sup>	0.14±0.04 <sup>c</sup>

<sup>c</sup>P<0.01 compared with group A (untreated group).

calculated (apoptosis rate = positive cells in the same field of view/total cells ×100). The average integral optical density (IOD) of positive cells in each field of view was determined and recorded with a commercial image analysis system (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

### Measurement of the expression of apoptosis-related proteins Bcl-2 and Bax

All specimens were fixed in 10% formalin, paraffin embedded, and serial 2- $\mu$ m paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. After blocking the sections with 20% (v/v) goat serum in PBS, sections were incubated with anti-Bcl-2 and anti-Bax antibodies overnight at 4°C. Sections were then incubated with the appropriate secondary antibodies (Goat anti-rabbit, from the Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China). Positive areas were determined and the results were expressed as the percentage of positive area compared with the total area. In the positive areas, Bcl-2 and Bax proteins were identified as yellow or brown particles, diffused within a

localized area in the membrane or the cytoplasm. Image acquisition was obtained by digital photomicrography, where five high-power fields were randomly selected per slide. Blinded analysis of positive immunostained sections was performed with Image Pro Plus (Media Cybernetics). This was used to calculate the sum of the positive points of the tissue slices of each field and IOD values. The mean optical density value represents a semi-quantitative analysis of Bcl-2 and Bax protein expression.

### Statistical analysis

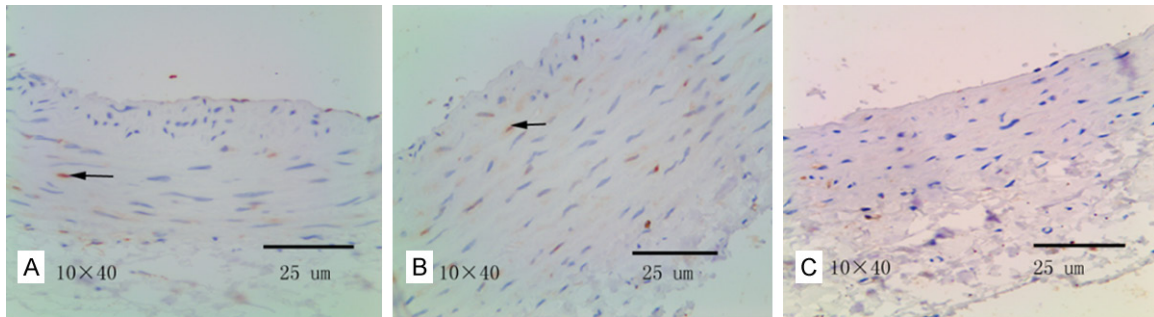
The data were analyzed using the statistical analysis software SPSS, Version 22.0 (IBM SPSS, Armonk, NY, USA). All the data are expressed as means  $\pm$  standard deviation (S.D.). Independent t-test was used for comparison between two groups, while one-way ANOVA analysis q-test, followed by post-hoc tests (using Least Significant Difference test, LSD-t), was used for comparison of more than two groups.  $\chi^2$  test was used for count data. A P-value of P<0.05 was considered statistically significant.

## Results

### Establishment of the aortic balloon-injury model

Forty rats survived 24 h after the operation but 3 died in the next four weeks during the intervention period; 2 in group A and 1 in group B. Statistically, there was no difference in mortal-

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**Figure 2.** TUNEL staining. Balloon injury increases apoptosis of VSMCs in areas of hyperplasia in the rat aorta, which is further increased by fasudil. The number of apoptotic cells by TUNEL staining: compared with group C (sham operated), the number of TUNEL-positive cells in groups A and B is increased. Notably, after fasudil treatment, the number of TUNEL-positive cells further increased.

**Table 3.** Comparison of ROCK mRNA and the expression of Bcl-2 and Bax protein in VSMCs of rats in the 3 groups ( $\bar{x} \pm s$ )

Group	ROCK	Bcl-2	Bax
A (n = 19)	0.51±0.036 <sup>d</sup>	2.12±0.12 <sup>d</sup>	1.64±0.13 <sup>d</sup>
B (n = 18)	0.31±0.041 <sup>d,e</sup>	1.15±0.09 <sup>d,e</sup>	2.39±0.16 <sup>d,e</sup>
C (n = 12)	0.43±0.041	0.93±0.08	0.82±0.54

<sup>d</sup>P<0.01 compared with the sham-operated group (C);

<sup>e</sup>P<0.01 compared with the untreated group (A).

ity between the groups. There was no death in the sham group, and therefore the number of rats included in the final data analysis consisted of 18 in group A, 19 in group B, and 12 in group C.

### *Histopathological changes in the balloon-injury model*

HE staining demonstrated that the aorta intima and media have different degrees of thickening in the balloon-injured rats, with the difference mainly being due to the proliferation of VSMCs. After fasudil treatment, the thickness of intima and mediastissue was significantly decreased in rats with balloon injury (P<0.01; **Table 1**, **Figure 1**).

### *Detection of apoptosis of VSMCs*

TUNEL-positive cells were found in the aortas of the balloon-injured rats (groups A and B), where as no TUNEL-positive cells were detected in the sham operation group. After fasudil treatment, the number of TUNEL-positive cells was increased compared with the untreated group (**Table 2**, **Figure 2**).

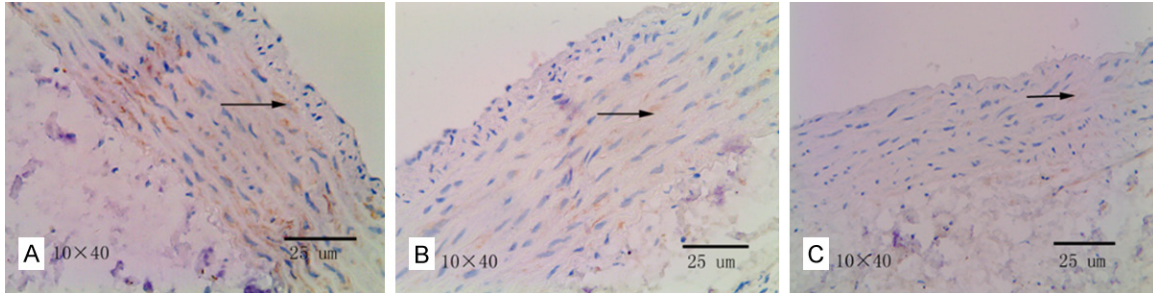
### *Expression changes of ROCK mRNA, Bcl-2 and Bax in VSMCs*

ROCK mRNA and the expression of Bcl-2 were significantly increased, while the expression of Bax decreased, in rats with balloon injury, compared with the sham group (P<0.01). Furthermore, compared with the untreated group, the administration of fasudil reduced ROCK mRNA and Bcl-2 expression, while the expression of Bax was increased (**Table 3**, **Figures 3, 4**).

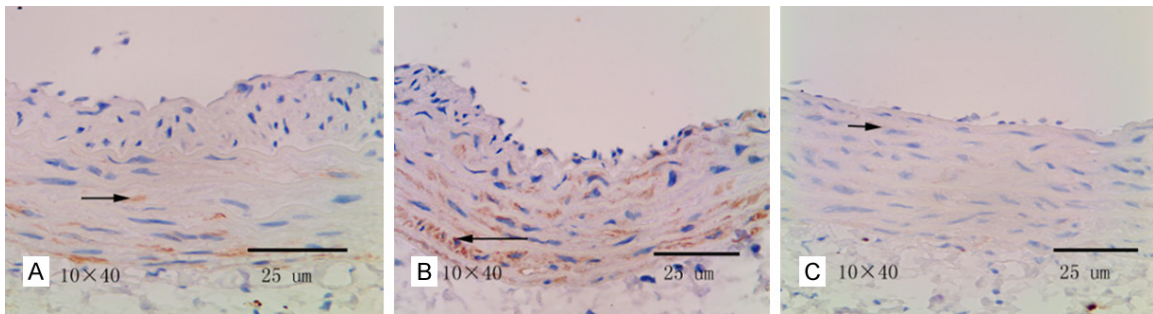
### **Discussion**

Interventional therapy is an effective method for the treatment of coronary heart disease and other vascular diseases. However, the high incidence of restenosis has a serious impact on the surgical outcome of interventional therapy. Thus, restenosis after interventional therapy remains a difficult problem for clinicians [10]. VSMCs are considered to be one of the major contributors to the development of restenosis [11]. In this study, we found that VSMCs proliferation and aortic intima and media tissue thickness were significantly increased after balloon injury in rats. Therefore, it may be of great benefit to inhibit excessive proliferation of VSMCs and promote VSMCs apoptosis in the prevention and treatment of atherosclerosis and restenosis after vascular injury. VSMCs apoptosis, which is associated with vascular formation and remodeling after injury, is an important pathological change caused by vascular atherosclerosis, restenosis and hypertension, and is essential for vascular structure and function.

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**Figure 3.** Expression of Bcl-2 in VSMCs by immunohistochemistry. Compared with Group C (sham-operated), the expression of Bcl-2 increased in the balloon-injury model groups, both Group A (untreated) and Group B (fasudil treated). After fasudil treatment, the expression of Bcl-2 decreased compared with the untreated control.



**Figure 4.** Expression of Bax in VSMCs by immunohistochemistry. Compared with Group C (sham operated), the expression of Bax in the balloon-injury model groups, Group A (untreated) and Group B (fasudil treated), is decreased. After fasudil treatment, the expression of Bax increased.

The balance between pro- and anti-apoptotic pathways has an important role in the new intima forming. Therefore, a relative deficiency of VSMCs apoptosis could be an important cause of restenosis [12]. Apoptosis can reduce the overall number of cells, thereby reducing intimal hyperplasia. Therefore, promoting VSMCs apoptosis represents a promising therapeutic target for the treatment of restenosis after vascular intimal injury [13]. In this study, we also found that the proliferation of VSMCs in balloon-injured rats was accompanied by VSMCs apoptosis, and the intimal and medial hyperplasia of the artery was significantly increased. However, the promotion of VSMCs apoptosis can reduce the degree of restenosis.

Apoptosis is an active death process controlled by multiple genes. Many factors can regulate the expression of apoptosis-related genes and start the process of apoptosis. Bcl-2 and Bax are recognized as major apoptosis regulatory proteins. Bcl-2 is mainly distributed in the mitochondrial membrane and can regulate the

permeability of the membrane. Its main function is to promote cell survival and inhibit apoptosis. Bax is located in the cytoplasm. When Bax is highly expressed, it can translocate from the cytoplasm to the mitochondrial membrane and combine with Bcl-2 to form a dimer, changing the permeability of the mitochondrial membrane, thus causing damage to mitochondria and promoting apoptosis of the cell [14, 15].

Rho is involved in the regulation of various biological activities of cells. ROCK is a downstream effector of the small G protein Rho, and it can interact with a variety of vasoactive substances. Notably, by regulating myosin light chain phosphorylation and dephosphorylation, it can affect the structure and function of smooth muscle cells. ROCK is thus closely associated with the development of cardiovascular disease [16, 17]. ROCK is also important for apoptosis. Specifically, it is involved in the regulation of apoptosis, of both the endogenous and exogenous pathways, and it is widely linked with other signaling pathways in the cell [18]. However, ROCK may have different bio-

logical effects in each tissue, and even in different states of the same tissue [19]. Moreover, ROCK inhibitor was applied to treat cardiovascular disease as a therapy for animals in experimental clinical trials and achieved impressive results. Fasudil as the first ROCK inhibitor which was discovered and applied in clinical, is becoming more extensively used in the cardiovascular field, where it has been applied successfully in a significant number of cases. In recent years, with research into its pharmacological mode of action and novel clinical applications, fasudil has increasingly been applied in the field of cardiovascular disease. From the initial results demonstrated, fasudil is expected to become the new drug of choice for prevention and treatment of cardiovascular diseases [20, 21].

In this study, we established a rat aortic balloon-injury model, and observed the proliferation and apoptosis of VSMCs after artery injury. The results demonstrated that the aortic intima and media were obviously thickened after balloon injury and VSMCs underwent marked hyperplasia accompanied by apoptosis. Furthermore, ROCK mRNA expression was increased. Following the administration of fasudil for 4 weeks, ROCK mRNA expression was decreased, the expression of pro-apoptotic proteins was increased, VSMCs apoptosis was more obvious, and vascular intima and media hyperplasia was reduced. Importantly, fasudil promotes apoptosis of VSMCs undergoing abnormal proliferation, but not steady state VSMCs in areas unaffected by hyperplasia. This indicates that fasudil could have therapeutic value in the treatment of restenosis after interventional therapy. However, further studies are needed to discover the mechanism responsible for the induction of apoptosis by fasudil.

In summary, VSMCs play an important role in the development of restenosis after interventional therapy. The dysregulated reaction of VSMCs to vascular endothelial injury leads to excessive proliferation, which is considered to be the main contributor to the occurrence and development of restenosis. ROCK also plays an important role in restenosis, by increasing the hyperplasia of VSMCs, inhibiting apoptosis of proliferating cells and aggravating vascular stenosis. Fasudil, by inhibiting the expression of ROCK, up regulates pro-apoptotic proteins and down regulates anti-apoptotic protein,

increasing VSMC apoptosis and reducing the degree of VSMCs hyperplasia after aortic injury, thereby reducing the degree of vascular stenosis. Therefore, fasudil may be valuable in preventing the occurrence of restenosis after interventional therapy.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Shanglang Cai, Department of Cardiology, The Affiliated Hospital of Qingdao University, Jiangsu Road No.16, Qingdao, Shandong, China. Tel: 18661805505; Fax: 0532-86983286; E-mail: caishanglang@126.com

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