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Basic fibroblast growth factor gene transfection in repair of internal carotid artery aneurysm wall☆

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Abstract

Surgery or interventional therapy has some risks in the treatment of cerebral aneurysm. We established an internal carotid artery aneurysm model by dripping elastase in the crotch of the right internal and external carotid arteries of New Zealand rabbits. Following model induction, lentivirus carrying basic fibroblast growth factor was injected through the ear vein. We found that the longer the action time of the lentivirus, the smaller the aneurysm volume. Moreover, platelet-derived growth factor expression in the aneurysm increased, but smooth muscle 22 alpha and hypertension-related gene 1 mRNA expression decreased. At 1, 2, 3, and 4 weeks following model establishment, following 1 week of injection of lentivirus carrying basic fibroblast growth factor, the later the intervention time, the more severe the blood vessel damage, and the bigger the aneurysm volume, the lower the smooth muscle 22 alpha and hypertension-related gene 1 mRNA expression. Simultaneously, platelet-derived growth factor expression decreased. These data suggest that recombinant lentivirus carrying basic fibroblast growth factor can repair damaged cells in the aneurysmal wall and inhibit aneurysm dynamic growth, and that the effect is dependent on therapeutic duration.

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Key Words

basic fibroblast growth factor; lentivirus; aneurysm; vascular smooth muscle cells; hypertension-related gene 1; smooth muscle 22 alpha; platelet-derived growth factor; gene therapy; brain injury; neural regeneration

Research Highlights

(1) Lentivirus carrying basic fibroblast growth factor can increase the number of proliferating vascular smooth muscle cells in the aneurysmal wall, and promote smooth muscle cell proliferation in the blood vessel wall.
(2) Lentivirus-mediated basic fibroblast growth factor can inhibit aneurysm growth.

Abbreviations

bFGF, basic fibroblast growth factor; SM22α, smooth muscle 22 alpha

INTRODUCTION

Intracranial aneurysm is the main reason for spontaneous subarachnoid hemorrhage, and its mortality and disability rates are high[1]. Surgical occlusion and intravascular interventional therapy are the main methods used for clinical treatment of aneurysms, although they carry some risks including perioperative hemorrhea, aneurysm rupture, relapse, and postoperative complications. Moreover, surgery for some complicated aneurysms is difficult, and interventional therapy cannot be performed[2]. Thus, alternative treatments such as gene

targeted therapy that could deliver drugs to the blood vessel wall with an aneurysm may be useful for delaying or preventing aneurysm rupture[3]. *In vivo* gene transplantation strategies initially used a scaffold coated with vascular cells or artificial graft. In animal experiments, cultured cells implanted in a gelatin sponge were introduced into aneurysms by a surgical method, or cells implanted in alginate were introduced into aneurysms by catheter intervention[4]. Gene therapy is mainly used to strengthen the aneurysm wall and to prevent aneurysm rupture, and growth factors are common genes used for gene therapy.

In vitro studies demonstrate that basic fibroblast growth factor (bFGF) can induce cell proliferation and regulate cell motility, cell differentiation, axonal extension, and survival[5-7]. Promoting aneurysm wall smooth muscle cell proliferation is important in aneurysm wall repair[8]. Platelet-derived growth factor is a marker for synthesis-type smooth muscle[9-10]. Hypertension-related gene 1 and smooth muscle 22 alpha (SM22α) are marker genes of smooth muscle cells[11-14]. In the present study, we targeted experimental aneurysms using a lentiviral vector carrying the bFGF gene, and provided theoretical evidence for repair of the aneurysm wall for prevention or delay of aneurysm rupture.

RESULTS

Quantitative analysis of experimental animals

A total of 48 healthy New Zealand rabbits were selected to establish an internal carotid artery aneurysm model. Immediately after model induction, 24 rabbits were injected daily with lentivirus carrying bFGF *via* the ear vein. Six rabbit models were separately analyzed at 1, 2, 3, and 4 weeks after injection. Of the remaining 24 rabbits, six were injected with lentivirus carrying bFGF *via* the ear vein at 1, 2, 3, and 4 weeks following model establishment, and these rabbits were observed following 1 week of lentivirus intervention. Normal internal carotid arteries of six randomly selected model rabbits were selected as normal controls.

Lentivirus carrying bFGF improves rabbit internal carotid artery aneurysm wall morphology

Hematoxylin-eosin staining results exhibited intact elastic fiber of the internal carotid artery of normal rabbits and regular smooth muscle cells. Compared with the control group, internal carotid artery aneurysm volume was smaller, elastic fiber breakage was lighter, and smooth muscle number was larger in the experimental group. At 3 and 4 weeks following lentivirus injection, the elastic layer of the aneurysmal wall was most lightly damaged in experimental rabbits with the highest number of smooth muscles and the thickest aneurysmal wall. The differences in aneurysmal wall morphology were not significant at 3 and 4 weeks after injection. These data suggest that bFGF promoted smooth muscle cell proliferation and suppressed aneurysmal growth. At 1 week following model establishment, smooth muscle cell proliferation was most significant. The later the intervention time, the more severe the blood vessel damage, and the lower the elastic fiber breakage, the fewer the number of proliferated smooth muscle cells (Figure 1). These data suggest that the repair effect of bFGF on the aneurysmal wall was obvious with early intervention.

Lentivirus carrying bFGF reduces rabbit internal carotid artery aneurysm volume

Internal carotid artery aneurysm volume was decreased with prolonged therapy times, and was smallest at 4 weeks following injection of lentivirus carrying bFGF (Table 1). The earlier the lentivirus intervention, the smaller the aneurysmal volume, while the later the lentivirus intervention, the larger the aneurysmal volume (Table 2). These data suggest that lentivirus-mediated bFGF contributed to smooth muscle cell proliferation in the vascular wall and suppressed aneurysmal growth. Thus, early intervention of lentivirus carrying bFGF has significant effects on aneurysm volume.

Lentivirus carrying bFGF upregulates platelet-derived growth factor expression in rabbit internal carotid artery aneurysm blood vessels

Platelet-derived growth factor expression could not be detected in the internal carotid artery vessel wall of normal rabbits (Figure 2A). However, platelet-derived growth factor expression was detected in rabbit aneurysms following injection of lentivirus carrying bFGF, and its expression was high at 3 and 4 weeks (Figures 2B–E, Table 3). Following model establishment, the later the lentivirus injection, the more severe the aneurysmal wall damage. In addition, platelet-derived growth factor was visible in each layer of the aneurysmal wall, and the number of smooth muscle cells was decreased at 4 weeks following model induction (Figures 2F–I, Table 4). Platelet-derived growth factor is a marker that can synthesize vascular smooth muscle cells. Thus, a lentivirus carrying bFGF can induce smooth muscle cells to transform from contraction type to synthesis type, providing greater ability to repair the aneurysmal wall. The transformation of vascular smooth muscle cells indicates that there were many vascular smooth muscle cells undergoing proliferation.

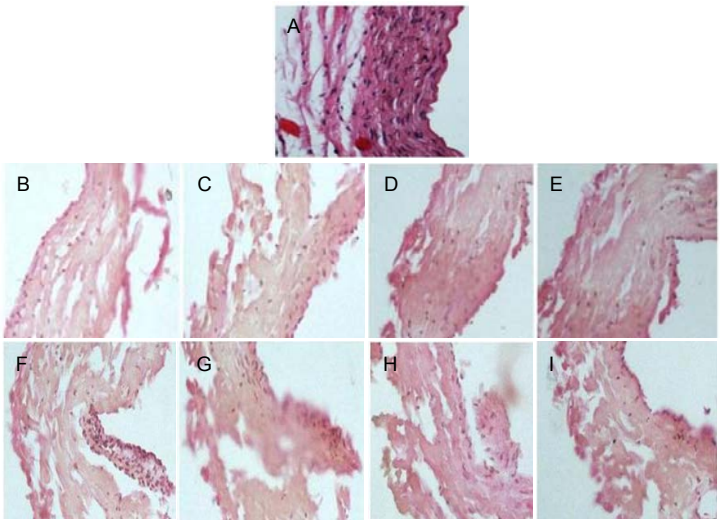

Figure 1 Effects of lentivirus carrying basic fibroblast growth factor (bFGF) on morphology of rabbit internal carotid artery aneurysmal wall (hematoxylin-eosin staining, × 200).

(A) Intact elastic fiber of the internal carotid artery and regular smooth muscle cells of normal control rabbits.

(B) Damaged whole elastic fiber of aneurysmal wall and reduced number of smooth muscle cells at 1 week following injection of lentivirus carrying bFGF immediately after model induction. (C) Highly damaged elastic fiber of the aneurysmal wall and reduced number of smooth muscle cells at 2 weeks. (D) Locally damaged elastic fiber of aneurysmal wall at 3 weeks. (E) Intact media layer of aneurysmal wall at 4 weeks.

(F) Increased number of smooth muscle cells of the aneurysmal wall following injection of lentivirus carrying bFGF at 1 week after internal carotid artery aneurysm model induction. (G) Highly damaged elastic fiber of the aneurysmal wall and increased number of smooth muscle cells in local regions following injection of lentivirus carrying bFGF at 2 weeks after internal carotid artery aneurysm model induction. (H) Damaged whole elastic fiber of aneurysmal wall following injection of lentivirus carrying bFGF at 3 weeks after internal carotid artery aneurysm model induction. (I) Damaged whole aneurysmal wall and reduced layer following injection of lentivirus carrying bFGF at 4 weeks after internal carotid artery aneurysm model induction.

Table 1 Effects of injection of lentivirus carrying basic fibroblast growth factor on internal carotid artery aneurysm volume at various time points following lentiviral injection immediately after aneurysmal model establishment

Index	Time after lentiviral injection (week)			
	1	2	3	4
Neck width (mm)	4.8±0.3	4.3±0.4[a]	4.0±0.3[b]	3.3±0.4[c]
Length (mm)	6.2±0.5	6.0±0.3[a]	5.7±0.4[b]	5.2±0.6[c]

Data are expressed as mean ± SD. There are six rabbits per time point. Least significant difference *t*-test was used for comparison. [a]*P* < 0.05, *vs.* 1 week; [b]*P* < 0.05, *vs.* 2 weeks; [c]*P* < 0.05, *vs.* 3 weeks.

Table 2 Effects of injection of lentivirus carrying basic fibroblast growth factor on internal carotid artery aneurysm volume following lentiviral injection at different time points after aneurysmal model establishment

Index	Time after model induction (week)			
	1	2	3	4
Neck width (mm)	6.0±0.4	6.4±0.4[a]	7.4±0.6[b]	7.8±0.7[c]
Length (mm)	9.3±0.6	10.8±0.6[a]	12.0±0.7[b]	13.6±0.8[c]

Data are expressed as mean ± SD. There are six rabbits per time point. Least significant difference *t*-test was used for comparison. [a]*P* < 0.05, *vs.* 1 week; [b]*P* < 0.05, *vs.* 2 weeks; [c]*P* < 0.05, *vs.* 3 weeks.

Lentivirus carrying bFGF diminishes SM22α and hypertension-related gene 1 mRNA expression

Hypertension-related gene 1 is a negative regulatory gene related to vascular smooth muscle cells, and is highly expressed in normal blood vessel wall smooth muscle[15]. Real-time PCR results demonstrated that the longer the duration of lentivirus injection, the lower the SM22α and hypertension-related gene 1 mRNA expression (Table 5). By contrast, the later the lentivirus injection, the lower the SM22α and hypertension-related gene 1 mRNA expression (Table 6).

DISCUSSION

In the present study, bFGF lentiviral intervention decreased the rabbit internal carotid artery aneurysm volume, reduced the elastic fiber breaks, and increased the number of smooth muscle cells. Moreover, aneurysm volume was smallest, the damage to the elastic layer was least, the number of smooth muscle cells was highest, and the aneurysmal wall was thickest at 3 and 4 weeks following lentiviral intervention.

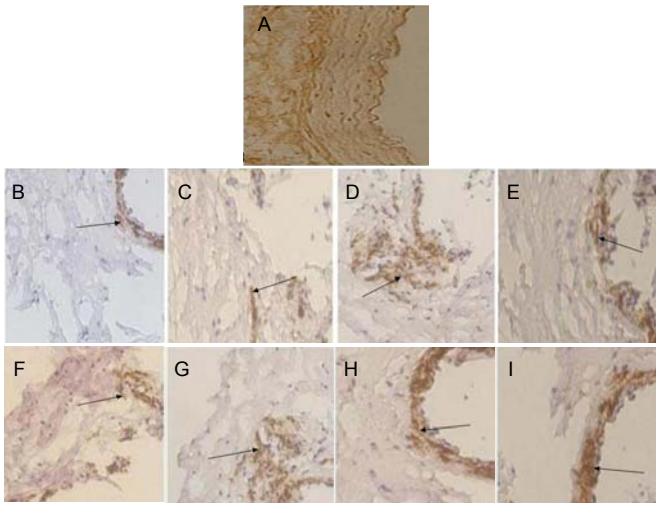

Figure 2 Effects of lentivirus carrying basic fibroblast growth factor (bFGF) on platelet-derived growth factor expression (arrows) in rabbit internal carotid artery aneurysm wall (immunohistochemistry, × 200).

(A) No platelet-derived growth factor expression in the internal carotid artery vessel wall of normal rabbits.

(B) Monolayer platelet-derived growth factor expression at 1 week following lentiviral injection immediately after aneurysmal model establishment. (C) Platelet-derived growth factor expression in local aneurysmal wall at 2 weeks following lentiviral injection. (D) Platelet-derived growth factor expression in a wide range of aneurysmal wall at 3 weeks following lentiviral injection. (E) High platelet-derived growth factor expression in the aneurysmal wall at 4 weeks after lentiviral injection.

(F) High platelet-derived growth factor expression following injection of lentivirus carrying bFGF at 1 week after aneurysmal model establishment. (G) High platelet-derived growth factor expression in a wide range of aneurysmal wall following injection of lentivirus carrying bFGF at 2 weeks after model establishment. (H) High platelet-derived growth factor expression in local aneurysmal wall following injection of lentivirus carrying bFGF at 3 weeks after model establishment. (I) Platelet-derived growth factor expression in whole aneurysmal wall following injection of lentivirus carrying bFGF at 4 weeks after model establishment.

Table 3 Platelet-derived growth factor expression in the aneurysmal wall at various time points following lentiviral injection immediately after aneurysmal model establishment

Index	Time after lentiviral injection (week)			
	1	2	3	4
Integrated absorbance	0.93±0.14	1.38±0.31[a]	2.10±0.54[b]	2.36±0.45[c]
Average absorbance	0.15±0.02	0.22±0.06[a]	0.38±0.27[b]	0.42±0.29[c]

High absorbance value represents high platelet-derived growth factor expression. Data are expressed as mean ± SD. There are six rabbits per time point. Least significant difference *t*-test was used for comparison. [a]*P* < 0.05, *vs*. 1 week; [b]*P* < 0.05, *vs*. 2 weeks; [c]*P* < 0.05, *vs*. 3 weeks.

Table 4 Platelet-derived growth factor expression in the aneurysmal wall following lentiviral injection at various time points after aneurysmal model establishment

Index	Time after model establishment (week)			
	1	2	3	4
Integrated absorbance	1.48±0.36	2.45±0.47[a]	2.53±0.69[b]	1.42±0.21[c]
Average absorbance	0.41±0.05	0.63±0.12[a]	0.67±0.09[b]	0.68±0.11[c]

High absorbance value represents high platelet-derived growth factor expression. Data are expressed as mean ± SD. There are six rabbits per time point. Least significant difference *t*-test was used for comparison. [a]*P* < 0.05, *vs*. 1 week; [b]*P* < 0.05, *vs*. 2 weeks; [c]*P* < 0.05, *vs*. 3 weeks.

Table 5 Smooth muscle 22 alpha (SM22α) and hypertension-related gene 1 mRNA expression (fluorescence signal intensity) in the aneurysmal wall at various time points following lentiviral injection immediately after aneurysmal model establishment

Index	Time after lentiviral injection (week)	
	1	2
SM22α	0.084±0.012	0.079±0.025[a]
Hypertension-related gene 1	0.174±0.146	0.134±0.084[a]

Index	Time after lentiviral injection (week)	
	3	4
SM22α	0.026±0.008[b]	0.022±0.012[c]
Hypertension-related gene 1	0.040±0.010[b]	0.039±0.016

High fluorescence signal intensity represents high SM22α and hypertension-related gene 1 mRNA expression. Data are expressed as mean ± SD. There are six rabbits per time point. Least significant difference *t*-test was used for comparison. [a]*P* < 0.05, *vs*. 1 week; [b]*P* < 0.05, *vs*. 2 weeks; [c]*P* < 0.05, *vs*. 3 weeks.

These data suggest that lentivirus-mediated bFGF contributed to the proliferation of smooth muscle cells in the blood vessel wall and inhibited aneurysmal growth. The proliferation of smooth muscle cells was most significant at 1 week following lentiviral injection. The later the intervention time, the more severe the blood vessel damage and the fewer the number of proliferating smooth muscle cells. These data also suggest that early

intervention of lentivirus carrying bFGF has significant effects on suppressing the injury to the blood vessel wall and aneurism growth.

Table 6 Smooth muscle 22 alpha (SM22α) and hypertension-related gene 1 mRNA expression (fluorescence signal intensity) in the aneurysmal wall following lentiviral injection at various time points after aneurysmal model establishment

Index	Time after model establishment (week)	
	1	2
SM22α	0.084±0.012	0.079±0.025[a]
Hypertension-related gene 1	0.174±0.146	0.134±0.084[a]

Index	Time after model establishment (week)	
	3	4
SM22α	0.026±0.008[b]	0.022±0.012[c]
Hypertension-related gene 1	0.040±0.010[b]	0.039±0.016

High fluorescence signal intensity represents high SM22α and hypertension-related gene 1 mRNA expression. Data are expressed as mean ± SD. There are six rabbits per time point. Least significant difference *t*-test was used for comparison. [a]$P < 0.05$, *vs.* 1 week; [b]$P < 0.05$, *vs.* 2 weeks; [c]$P < 0.05$, *vs.* 3 weeks.

Platelet-derived growth factor is a marker of synthesis-type vascular smooth muscle cells. As such, changes in platelet-derived growth factor expression are associated with smooth muscle phenotype transition[16]. Platelet-derived growth factor expression was high in rabbit aneurysms at 3 and 4 weeks following injection of lentivirus carrying bFGF. Platelet-derived growth factor was detectable in the whole aneurysmal wall at 4 weeks, but there was a trend for a decrease in numbers of smooth muscle cells. Lentivirus carrying bFGF induced vascular smooth muscle cells in the aneurysmal wall to transform from contraction type to synthesis type. Thus, early intervention of lentivirus carrying bFGF had significant effects on the repair of the aneurysmal wall.

Smooth muscle cells have either contraction or synthesis phenotypes[17-20], and the cellular phenotype determines its biological property and function. The phenotype transition from contraction type to synthesis type of vascular smooth muscle cells is a prerequisite for its proliferation. SM22α mRNA, a marker gene of vascular smooth muscle cell phenotype, is only expressed in contractive vascular smooth muscle cells[11]. Hypertension-related gene 1 is a negative regulatory gene related to vascular smooth muscle cells[12-13]. SM22α mRNA and hypertension-related gene 1 mRNA expression were high in the aneurysmal wall at 1 and 2 weeks following lentiviral injection immediately after aneurysmal model establishment. SM22α mRNA and hypertension-related gene 1 mRNA expression were high following lentiviral injection at 1 and 2 weeks after aneurysmal model establishment. These data suggest that early and prolonged lentivirus intervention can increase synthetic vascular smooth muscle cell numbers by inhibiting cell differentiation, and can repair the damaged aneurysmal wall.

In summary, lentivirus-mediated bFGF gene can be used for aneurysmal repair by inducing vascular smooth muscle cell phenotype change into synthesis type, promoting smooth muscle cell proliferation, and delaying early aneurysmal rupture. Blood vessel wall thickening after smooth muscle cell proliferation revealed that the opportunity for treatment of aneurismal rupture is small.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed at the Central Laboratory, Hospital Affiliated to Jiangsu University, China from December 2010 to August 2011.

Materials

Animals

A total of 48 clean New Zealand rabbits weighing 2.5–3.0 kg, of equal gender, were purchased from the Animal Center, Jiangsu University (license No. SCXK (Su) 2009-0002). Animals were housed in a dark room at 24°C with a relative humidity of 55 ± 5%. The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[21].

Virus

Lentivirus carrying bFGF was supplied by the First Affiliated Hospital of Soochow University, China[22]. PCR primer of bFGF gene was designed, and the pCI-bFGF plasmid was used as a template. Taq enzyme was employed for amplification. Restriction enzyme linearized the pGC-FU lentiviral vector. The bFGF gene fragment was amplified by PCR, followed by an exchange reaction. Lentivirus vector was packed and constructed using 293T cells.

Methods

Establishment of internal carotid artery aneurysm models

Rabbits were intraperitoneally anesthetized with 3.6%

chloral hydrate, and the crotch of the right internal and external carotid arteries was exposed and dripped with 0.2 mL of 100 U/mL elastase. After 20 minutes, heparin sodium 500 U was slowly injected *via* the ear vein[23]. The left internal carotid artery was considered as a normal control. Aneurysms were detected by CT angiography.

Lentivirus carrying bFGF injection via the ear vein

After sterilization with 75% ethanol, lentivirus carrying bFGF drip (2 μL; 1 × 10^8 TU/mL) was slowly injected *via* the ear vein, and the needle was maintained for 2 minutes[24-25], once per day.

Measurement of aneurysmal volume

The rabbits were intraperitoneally anesthetized with 3.6% chloral hydrate (3 mL/kg). The internal carotid artery aneurysm was obtained and the volume was measured using a CT angiography (Light Speed Pro 64-slice spiral CT; GE, Kansas, KS, USA).

Hematoxylin-eosin staining for morphology of rabbit internal carotid artery aneurysm

The rabbit internal carotid artery aneurysm was fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sliced into sections, stained with hematoxylin-eosin, and then observed under an inverted microscope (Chongqing Photoelectric Instrument Co., Ltd., Chongqing, China).

Immunohistochemistry for platelet-derived growth factor expression in the rabbit internal carotid artery aneurysm

Rabbit aneurysm paraffin sections were dewaxed, hydrated, blocked with 0.04% hydrogen peroxide in methanol for 15 minutes, and blocked in normal goat serum at room temperature for 20 minutes. The specimens were incubated in mouse anti-platelet-derived growth factor polyclonal antibody (1:200; Beijing Baoxin Technology Co., Ltd., Beijing, China) at 4°C overnight, goat anti-mouse IgG (1:200; Boster, Wuhan, China) at room temperature for 30 minutes, streptavidin-biotin complex at room temperature for 20 minutes, developed with 3,3'-diaminobenzidine for 3 minutes, and observed under an inverted light microscope (Olympus, Japan). A 0.01 M PBS wash was performed three times between each step. Sections were dehydrated, permeabilized in xylene, and mounted. Absorbance values were analyzed using Image-pro plus software (Media Cybernetics, Rockville, MD, USA).

Real-time PCR for SM22α and hypertension-related gene 1 mRNA expression in rabbit internal carotid artery aneurysm

Total RNA (2 μg in each group) was extracted from aneurysmal specimens. Reverse transcriptase was used to prepare cDNA from mRNA in a 20 μL reaction system, as follows: 42°C for 60 minutes and 70°C for 15 minutes. Primers were synthesized by the Shanghai Bioengineering Company, China, which are shown as follows:

Gene	Primer sequence	Length (bp)
SM22α	Upstream: 5'- TTC TGC CTC AAC ATG GCC AAC -3' Downstream: 5'- CAC CTT CAC TGG CTT GGA TC -3'	252
Hypertension-related gene 1	Upstream: 5'- TTG CTG GGC TAC AAT GAT-3' Downstream: 5'- CTT GCT GGC ACA GAT GAG -3'	309

A 2 μL of transcript was added to a fluorescence real-time quantitative PCR system. DNA was amplified using Taq polymerase in a 25 μL reaction system. Amplification conditions are: 35 cycles of 94°C for 2 minutes, 94°C for 15 seconds, and 60°C for 40 seconds. A 12 μL sample containing 10 μL product and 2 μL of 6 × reaction buffer was added into each well. Specimens were electrophoresed onto a 1.7% agarose gel at 100 V for 15 minutes, stained with ethidium bromide, and photographed using a GIS-2010 digital gel image analysis system (Bio-Rad, Hercules, CA, USA). The changes in fluorescence signal intensity were quantitatively analyzed. Each experiment was performed in triplicate.

Statistical analysis

Data are expressed as mean ± SD. Analysis of variance was conducted using SPSS 16.0 software (SPSS, Chicago, IL, USA). Multi-group data were analyzed using paired comparison and least significant difference *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Lei Jiao detected indexes and wrote the manuscript. Ming Jiang established animal models. Jinghai Fang performed immunohistochemistry. Yinsheng Deng raised the animals. Zejun Chen operated CT angiography. Min Wu participated in the study design. All authors approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Jiangsu University in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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