NEURAL REGENERATION RESEARCH
Volume 8, Issue 16, June 2013

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.16.004 [http://www.nrronline.org; http://www.sjzsyj.org]
Cui YC, Tian Y, Tang Y, Jia LJ, Wu AL, Peng P, Yang JZ, Du H, Wang XJ, Wu LK. Application of sodium alginate microspheres in ischemic stroke modeling of miniature pigs. Neural Regen Res. 2013;8(16):1473-1480.

Application of sodium alginate microspheres in ischemic stroke modeling in miniature pigs*☆

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Abstract

The miniature pig is an optimal animal model for studying nervous system disease because of its physiologic and pathologic features. However, the rete mirabile composed of arteries and veins at the skull base limits their application as a model of ischemic stroke by middle cerebral artery occlusion. The present study investigated the possibility of establishing an ischemic stroke model in the miniature pig by blocking the skull base retia with sodium alginate microspheres. Three Bama miniature pigs were used. Using the monitor of C-arm X-ray machine, sodium alginate microspheres (100–300 μm), a novel embolic material, were injected through the femoral artery, aortic arch, common carotid artery, ascending pharyngeal artery and the retia. Results were evaluated using carotid arteriography, MRI, behavior observation and histology. The unilateral rete mirabile was completely blocked, resulting in disturbance in blood supply to the basal ganglia, astasia of the right hind limb and salivation. MRI and hematoxylin-eosin staining showed an evident infarction focus in the basal ganglia. These findings indicate that sodium alginate microspheres are a suitable embolic material for blocking the skull base retia in miniature pigs to establish an ischemic stroke models.

Key Words

neural regeneration; brain injury; stroke; miniature pig; sodium alginate microsphere; basilar blood vessels; middle cerebral artery; grants-supported paper; neuroregeneration

Research Highlights

(1) Miniature pigs can survive for a long period of time, are cost-effective and controllable. Thus, they are regarded as excellent models for studying nervous system diseases.
(2) The rete mirabile composed of arteries and veins at the skull base limits their application as an ischemic stroke model by middle cerebral artery occlusion.
(3) Sodium alginate microspheres can occlude the skull base retia of miniature pigs to establish ischemic stroke models.
(4) This method challenges the concept that the swine cannot be used to establish models of ischemic cerebrovascular diseases.

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Received: 2013-03-06
Accepted: 2013-05-10
(N20120427006)

INTRODUCTION

Stroke has very high rates of disability and death worldwide. Thus, the pathogenesis and treatment of stroke remain a particular medical focus[1]. A large animal model of persistent focal brain ischemia with similar pathogenesis and progression is very important for these studies.

Approximately 85% of stroke is caused by thrombo-embolism of the middle cerebral artery[2]. Thrombus placed in the middle cerebral artery through an interventional catheter to occlude blood vessels and block blood flow is frequently used to establish brain ischemia models. The miniature pig is an optimal animal for studying nervous system disease because of physiologic and pathologic features[3]. However, the rete mirabile composed of thin arteries and veins at the skull base significantly limits the use of the miniature pig as an ischemic stroke model induced by middle cerebral artery occlusion[4].

To solve this problem, in the present study, we injected sodium alginate microspheres, a novel biodegradable material, to embolize the rete mirabile to establish an ischemic stroke model. This method would provide a stable and reliable model for studying pathogenesis, pathophysiologic changes, molecular biologic changes and development of safe and effective drugs for cerebral infarction-induced brain ischemia.

RESULTS

Quantitative analysis of experimental animals

Three Bama miniature pigs were used. Ischemic stroke model was successfully established by occluding the rete mirabile with sodium alginate microspheres. All three pigs survived and were included in the final analysis.

Alteration of neurological function in miniature pigs with ischemic stroke

All three pigs survived during the observation. Following model establishment, the pigs presented mild hemiplegia; they were able to stand and walk, but were not able to maintain balance of body or to evade barriers. Moreover, their activities were reduced, and they could not control their walking speed. The neurological function and motor function of the animal model was assessed prior to and 6, 12, 24, 48, 72 and 96 hours following model establishment. The scores of overall neurological function were decreased at 6 hours post surgery compared with that prior to surgery, although the difference was not significant ($P > 0.05$). The scores of overall neurological function and motor function were significantly reduced from 12 hours post surgery compared with that prior to surgery ($P < 0.05$). The scores of neurological function remained up to 96 hours (Figure 1).

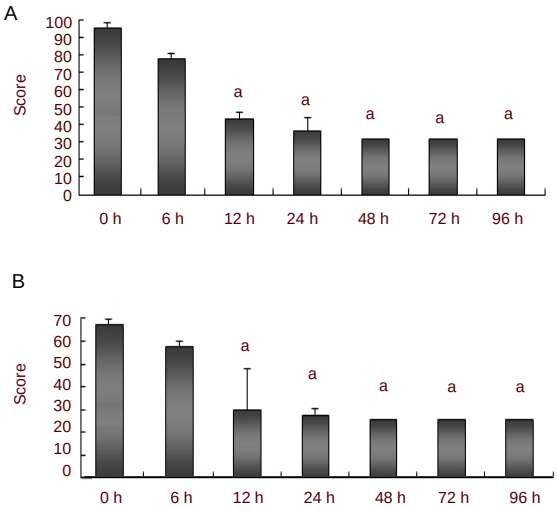

Figure 1 Changes of neurological function of ischemic stroke miniature pigs induced by sodium alginate microspheres.

(A) Scores of overall neurological function: total scores are 100; lower scores represent more severe neurological function disturbance.

(B) Scores of motor function: total scores are 70; lower scores represent more severe neurological function disturbance.

Data are expressed as mean ± SD of three miniature pigs at each time point. $^aP < 0.05$, *vs*. prior to model establishment (0 hour) using one-way analysis of variance followed by least significant difference test. The time in A, B mean hours following model establishment.

Alteration of cerebral blood flow in ischemic stroke miniature pigs

To elucidate changes of cerebral blood flow in the model miniature pigs prior to and following injection of sodium alginate microspheres, we conducted angiography of the right hemisphere involving the right femoral artery, right common carotid artery, right ascending pharyngeal artery and the retia prior to and 0.5 hour and 1 week following model establishment. Results showed that the brain volume and structure were similar between the miniature pig and human, but the cerebral vessel structure was significantly different. The ascending pharyngeal artery formed an abnormal artery-vein net, termed the rete mirabile, at the foramen lacerum of the

sella turcica (6 × 6 × 10 mm^3; Figure 2A). The rete mirabile converged to the internal carotid artery for the brain blood supply. The rete mirabile was completely occluded after the microspheres were injected into the right ascending pharyngeal artery (Figure 2B), resulting in significantly reduced blood flow and flow rate. Angiography at 1 week post surgery suggested that the occlusion of rete mirabile persisted (Figure 2C).

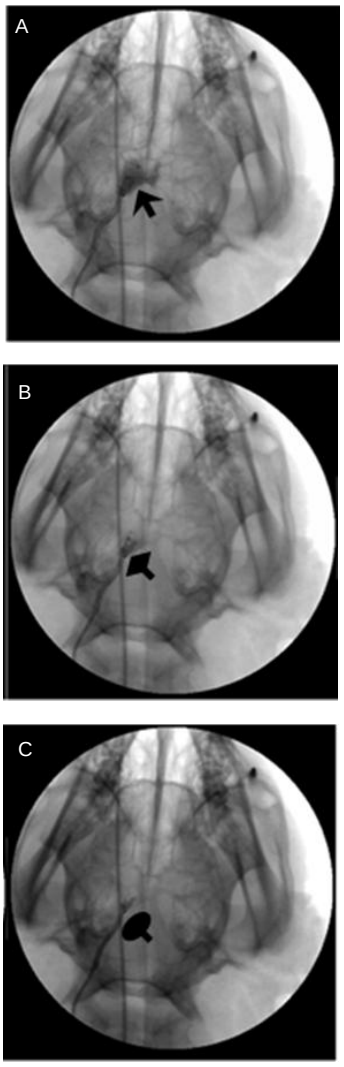

Figure 2 Changes of cerebral blood flow prior to and following occlusion of the rete mirabile with sodium alginate microspheres.

(A) Angiography shows normal blood flow in the rete mirabile prior to injection of sodium alginate microspheres (sharp arrow).

(B) Blood flow was reduced in the rete mirabile following injection of sodium alginate microspheres for 0.5 hour (square arrow).

(C) Blood flow disappeared in the rete mirabile following injection of sodium alginate microspheres for 1 week (circle arrow).

Ischemic condition in brain tissues of ischemic stroke miniature pigs

We also conducted brain MRI T2 weighted imaging and cerebral vessel diffusion enhanced imaging at 1 week post surgery to assess the ischemic condition following injection of sodium alginate microspheres. Coronal images of brain tissues were photographed, and the brain tissues and blood vessels were three-dimensionally reconstructed to observe the infarction and damaged areas. MRI showed hyperintensities of the ischemic focus, with a clear boundary (Figure 3).

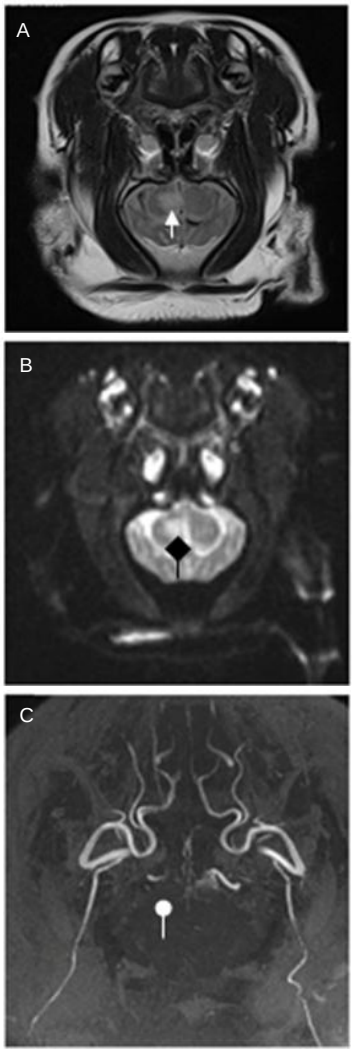

Figure 3 Ischemic changes of brain tissues of miniature pigs undergoing occlusion of the rete mirabile with sodium alginate microspheres.

Conventional MRI (A) and T2 weighted imaging (B) showed evidence of infarction foci in the basal ganglia of miniature pigs.

(C) Diffusion enhanced imaging of cerebral vessels showed that the rete mirabile of the right hemisphere was completely occluded, with no blood flow.

Arrows represent infarction foci.

The mean volume percent of the ischemic area *versus* the contralateral hemisphere was 30.9 ± 2.1% and 31.2 ± 4.3%, calculated by two independent observers. The diffusion enhanced imaging showed that the rete mirabile of the right hemisphere was completely occluded, and no branches were observed (Figure 3), consistent with results of angiography.

Pathologic changes of brain tissues of ischemic stroke miniature pigs

To further verify the model, we observed the brain tissues. The affected brain was swollen, with blood clots in brain surface blood vessels. White infarct areas of different sizes were found at the temporal lobe, parietal lobe and/or basal ganglia of the right hemisphere. The fixed entire brain was placed in anatomical molds and transected according to brain anatomical iconic structure (Figure 4A). At the cross section of the thalamus, an infarct focus was observed in the basal ganglia (~30% of the volume of the affected hemisphere), and edema was found surrounding the necrotic tissues, consistent with MRI results (Figure 4B). Hematoxylin-eosin staining of frozen brain tissue sections showed an infarct focus in the internal capsule, necrotic nerve cells in infarct areas and a large number of inflammatory cell infiltrates surrounding the infarct focus (Figure 4C).

DISCUSSION

Rodents are frequently used as animal models for ischemic stroke studies induced by middle cerebral artery occlusion[5-6]. However, their anatomic structure is significantly different from humans. Further, a number of large scale and multi-center clinical studies of Europe and USA showed that the pharmacodynamic results from rodents were not consistent with clinical study results[7-8]. Thus, recent studies have focused on developing stroke models in large animals with similar anatomic structure as the human brain. The present study used the swine as a model as they have several properties resembling the human brain, including brain volume and weight, quantity of cortical gyri and the percentage of white matter to gray matter. These properties allow evaluation of conventional cerebral imaging techniques and simulation of some surgical approaches. Moreover, the swine is more cost-effective, easy to feed and obtain compared with non-human rodents, and are thus an ideal model[9-10].

The abnormal capillary net (supplementary Figure 1 online) at the internal carotid artery (the branch of common carotid artery in swine)[11] makes it difficult to establish an ischemic stroke model by middle cerebral artery occlusion *via* the common carotid artery.

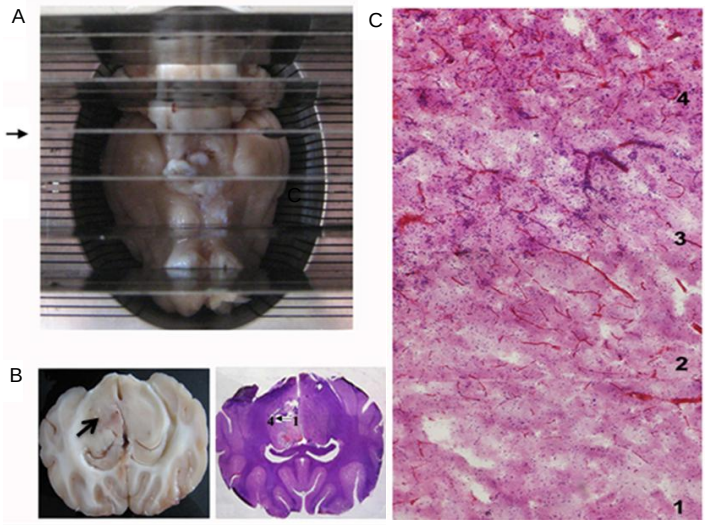

Figure 4 Pathologic analysis of brain tissues of miniature pig models of ischemic stroke.

(A) Selection of cross section position (arrow indicate infarct focus).

(B) Gross anatomic observation (left) and hematoxylin-eosin staining (right) showed clear ischemic focus at the cross section of the thalamus (arrow).

(C) Hematoxylin-eosin staining (× 200) showed that nuclei almost disappeared in the center of ischemic areas, the tissues were lysed, and evident tissue swelling and inflammatory cell infiltrate were observed around the ischemic areas.

1→4 in B and C represent infarct areas (1 and 2), the surroundings of infarct focus (3) and normal tissues (4).

Thus, the present study aimed to verify the possibility to inject sodium alginate microspheres, a novel embolic material, through the femoral artery, abdominal aorta, aortic arch, common carotid artery, ascending pharyngeal artery and the retia to induce ischemic stroke in miniature pigs.

The matrix material of sodium alginate microspheres was polysaccharide sodium salt extracted from natural plant brown algae. Giant molecule chain crosslinking and solidification occurs in response to Ca^{2+}. The Ca^{2+} in microspheres slowly migrates to the blood and gradually degrades by detaching from the chain. The degradation products are a kind of polysaccharide, mannose and glucose, which do not participate in metabolism and can be discharged with urine. They are atoxic and free of antigenicity. They can embolize the blood vessels while not producing stimulation *in vivo*, and thus do not influence the long-term results of study[12-13]. Thus, microspheres are regarded as an ideal material to establish ischemic stroke model in miniature pigs. The size of the sodium alginate microspheres (100–300 μm) was selected according previous studies reporting that the success rate was 100% in embolizing the rete mirabile of age- and body mass-matched miniature pigs[13-14].

Behavior evaluation in this study showed that all animals survived following embolism, but suffered mild hemiplegia and lateral falling. Neurological function scores were decreased after 6 hours of embolism compared with prior to embolism, although the difference was not statistically significant. However, the scores of overall neurological function and motor function were significantly reduced at 12 hours following embolism compared with prior to embolism. The neurological function deficits remained until up to 96 hours. These behavioral findings confirmed that the model was successfully established.

In addition, femoral artery-carotid arteriography was conducted at 0.5 hour and 1 week following injection of sodium alginate microspheres. Results showed that the rete mirabile was completely occluded, with no blood flow perfusion. Similarly, infarction and damaged areas were observed by coronal image of T2 weighted imaging and three-dimensional reconstruction of brain tissues and blood vessels at 1 week following microspheres injection. The mean volume percent of ischemic area versus the contralateral hemisphere was 30.9 ± 2.1% and 31.2 ± 4.3%, calculated by two independent observers. The diffusion enhanced imaging showed that the rete mirabile of the right hemisphere was completely occluded, and no branches were observed. These findings indicate that imaging results can confirm the stability and feasibility of an ischemic stroke model established by occlusion of rete mirabile with sodium alginate microspheres.

To confirm the results of imaging and behavior, we also analyzed gross observation and microstructure of brain tissues of the model animals. Results showed that the lesioned brains were swollen, with blood clots in the blood vessels, and white infarct areas of different sizes were found in the temporal lobe, parietal lobe and/or basal ganglia of the right hemisphere. The success rate of occlusion was 100%. The fixed brains were placed into anatomical molds and transected according to brain anatomical structure. At the cross section of the thalamus, an infarct focus was clearly observed in the basal ganglia. Hematoxylin-eosin staining of brain tissues showed an infarct focus in the internal capsule, necrotic nerve cells in the infarct areas and a large number of inflammatory cell infiltrates surrounding the infarct focus. The pathologic alteration of brain tissues was consistent with characteristics of imaging data and behavior results.

By using carotid angiography, MRI examination, behavior evaluation and histological analysis, we confirmed that injection of sodium alginate microspheres *via* the femoral artery-internal carotid artery-ascending pharyngeal artery and rete mirabile to occlude the rete mirabile can successfully establish a stable ischemic stroke model in miniature pigs.

The advantages and features of this model include: (1) the skull base rete mirabile consists of small and twining capillaries with slow blood flow. The afferent artery (ascending pharyngeal artery) was significantly thicker than the efferent artery (internal carotid artery), allowing stagnation of embolic agents. Thus, the problem that the embolic infarction is movable in the internal carotid artery of small animals and that the model was not stable were solved. (2) The normal rete mirabile diameter in the swine is 75–275 μm (154 μm in average). The present study found that microspheres of 100–300 μm could completely occlude the rete mirabile[15]. (3) As the main communicating branches of the brain go through the rete mirabile, occlusion of the rete mirabile can reduce blood supply of the collateral circulation, benefiting focal ischemia. (4) Use of a balloon catheter can completely prevent microsphere reflow. Moreover, this method is simple and practical.

Although we demonstrated that it was feasible to induce ischemic stroke in miniature pigs by rete mirabile occlusion with sodium alginate microspheres, there are some limitations in this study. For example, the size of samples was small, and homogenicity and stability of models required further observation. Future studies should increase the number of animals to provide more quantitative data.

MATERIALS AND METHODS

Design

A self-controlled animal study.

Time and setting

The experiment was conducted at the Animal Experimental Center of Fuwai Hospital & Cardiovascular Institute, Chinese Academy of Medical Sciences, China from June 2011 to March 2012.

Materials

Three male Bama miniature pigs, 6 months old, weighing 15–20 kg, were purchased from the Institute of Animal Husbandry of the Northeast (license No. 230502600072021). All procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[16].

Methods

Preparation of ischemic stroke models

The animals were deprived from food overnight and anesthetized by intramuscular injection of ketamine (35 mg/kg) and diazepam (1.5 mg/kg), followed by orotracheal intubation connected with breathing machine for positive pressure respiration. The animals were placed in supine position and their body temperature was maintained. Blood pressure, heart rate, respiratory frequency and other vital sign parameters were monitored by electrophysiolograph (BioPac, Goleta, CA, USA). The femoral artery was sterilely isolated, and a 0.85 inch nondetachable silicone balloon (Target Therapeutics, Fremont, CA, USA) and a 3-French microtubule (Cordis, Miami, Florida, USA) were advanced to the left ascending pharyngeal artery *via* the abdominal aorta-thoracic aorta-aortic arch-common carotid artery under the guidance of a C-arm OEC 9800 X-ray work station (GE, Bethesda, MD, USA). A 2 mL suspension (1 g/mL) of sodium alginate microspheres (100–300 μm in diameter; Beijing Starway Medical Technology, Beijing, China) and Iopromide contrast medium (Ultravist; Shenzhen Medicine Diffuse Technology, Guangdong Province, China) at a 1:10 volume ratio was injected through the guiding catheter (Cordis) until disappearance of blood flow in the unilateral rete mirabile. The balloon catheter was deflated and removed after a 15-minute occlusion. The wound was washed with benzylpenicillin sodium for injection and then sutured layer by layer. The animals were returned and recovered naturally.

Evaluation of neurological function

Total neurological function and motor function were assessed prior to and 6, 12, 24, 48, 72 and 96 hours following surgery with the Clinical Neurological Function Scale[17]: A, scores for severity of limb paralysis: 10 scores for severe degree, 25 for moderate degree, 55 for mild degree and 70 for normal; B, conscious state: 0 score for death, 1 for a state of unconsciousness, 5 for consciousness but motionless, 15 for consciousness and little motion and 20 for normal; C, facial paralysis: 1 score for facial paralysis and 5 for no facial paralysis; D, visual field defect: 1 score for visual field defect and 5 for no defect. The total scores are 100, and lower scores indicate more severe neurological impairment.

Angiography

Angiography involving the femoral artery-abdominal aorta-aortic arch-common carotid artery-ascending pharyngeal artery-rete mirabile was conducted at 1 day prior to and 0.5 hour and 1 week following surgery performed under general anesthesia. The microtubule and guide wire (0.035 inch loach guide wire; Cordis) were inserted using guidance *via* a short puncture needle, and were advanced to the internal carotid artery. The guide wire was removed and the tube was fixed to the internal carotid artery. Approximately 10 mL of contrast medium (ultravist) was injected. Dynamic scanning was conducted with a GE C-arm X-ray machine at 3 frames/second. The blood flow perfusion of the rete mirabile was observed.

MRI examination

Cranial multisequencing scanning was conducted using a 3.0 T MRI (Philips Healthcare, Amsterdam, Netherlands) at 1 week following model establishment. MRI T2 weighted imaging and diffusion-weighted imaging were conducted using a TSENSE gradient echo sequence with retrospective electrocardiogram gating. Following image enhancement, two independent observers calculated infarct area and the contralateral hemisphere area of coronal T2 weighted images (3 mm thickness) according to the formula (0.75 × slice height ×

sum of area of each scanning) using image processing software ImageJ (National Institutes of Health, Bethesda, MD, USA). The volume percentage of the injury region versus the contralateral hemisphere was quantitatively analyzed. A hemisphere midline shift due to cerebral edema was corrected according to a previously described method[18]. The repeatability was evaluated using a statistical method proposed by Bland-Altman.

Hematoxylin-eosin staining

The animals were anesthetized following imaging examination and sacrificed by intravenous injection of 10% potassium chloride. Paraformaldehyde (4%) was perfused *via* the common carotid artery. After fixation for 60 minutes, the entire brain was harvested and fixed for 48 hours. The brains were cut coronally according to pig brain molds (Figure 4A), washed with PBS, embedded with OCT compound (Sakura Finetek, Torrance, CA, USA) and stored at –80°C. The brain tissues were sectioned using a Leica CM3050 S cryostat (Leica Microsystems, Buffalo Grove, Illinois, USA), 10 μm thick, followed by hematoxylin-eosin staining. The distribution of neurons and blood vessels and inflammatory cell infiltrate in areas of necrosis, the surroundings of necrosis and normal tissues were observed by light microscopy (Leica DM12000 M; Wetzlar, Germany).

Statistical analysis

Data were expressed as mean ± SD. Comparisons at different time points were conducted using one-way analysis of variance, and comparisons between two time points were conducted using least significant difference test with SPSS 11.0 package (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Funding: This study was supported by the Science and Technology Support Program of Beijing Science and Technology Committee, No. Z101107052210004.

Author contributions: Yongchun Cui designed and conducted the study, analyzed experimental data and wrote the manuscript. Yi Tian established the animal models. Yue Tang was in charge of funds, designed the study, guided the conduction of experiments and revised the manuscript. Liujun Jia and Hong Du cared for the animals post surgery. Aili Wu assisted in the animal experiments. Peng Peng and Jianzhong Yang helped establish the animal models. Xiaojuan Wang assisted in study design. Like Wu designed the study, provided technical support and revised the manuscript. All authors approved the final version of the submitted manuscript.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Care and Ethics Committee of Fuwai Hospital & Cardiovascular Institute, Chinese Academy of Medical Sciences, 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.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

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(Reviewed by Dean J, Raye Z, Li AP, Ge PF, Zhao R)
(Edited by Wang LM, Su LL, Li CH, Song LP)