Sarpogrelate and rosuvastatin synergistically ameliorate aortic damage induced by hyperlipidemia in apolipoprotein E-deficient mice

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Abstract. The current study aimed to investigate whether sarpogrelate and rosuvastatin possess anti-arterial injury, and attempted to elucidate the mechanism of action underlying this activity. Sarpogrelate, a 5-hydroxytryptamine type 2A antagonist, is extensively used to prevent arterial thrombosis; however, its effects on atherosclerosis remain unknown. In the present study, sarpogrelate combined with rosuvastatin or rosuvastatin alone were administered to male ApoE−/− mice fed a high-fat diet (HFD) for 8 weeks. Metabolic parameters in the blood samples were analyzed using an automatic analyzer. Aortic tissues were stained with hematoxylin and eosin for morphological analysis. The expression levels of oxidized-low density lipoprotein (LDL) specific scavenging receptors, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and cluster of differentiation 68 were detected via immunostaining. mRNA expression levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α were determined via reverse transcription-quantitative PCR analysis, while protein expression levels of LOX-1 and phosphor(p)-ERK were determined via western blot analysis. The results demonstrated that sarpogrelate combined with rosuvastatin treatment significantly decreased the expression levels of LOX-1 and p-ERK. Taken together, these results suggest that the positive effects of sarpogrelate combined with rosuvastatin treatment on aortic injury may be associated with the regulation of the LOX-1/p-ERK signaling pathway. Sarpogrelate and rosuvastatin synergistically decreased aortic damage in ApoE−/− HFD mice, and thus provide a basis for the treatment of aortic injury caused by hyperlipidemia with sarpogrelate.

Introduction

Hyperlipidemia is a high risk factor for cardiovascular disease (CVD), either by eroding large elastic arteries or causing damage to endotheliocytes (1,2). In ApoE−/− mice, hyperlipidemia induces lipid deposition and foam cell formation, which ultimately leads to atherosclerosis (3,4). Decreasing the blood lipid levels, particularly low-density lipoprotein cholesterol (LDL-C) levels, lowers the risk of CVD (5). Statins are a class of cholesterol-lowering agents that significantly decrease the severity of CVDs (6). For example, rosuvastatin is prescribed to lower cholesterol levels and thereby decrease the risk of CVD (7). Although statins are extensively used to prevent hyperlipidemia and CVD, concerns have been raised regarding their association with an increased risk of new-onset diabetes and other adverse effects, such as liver toxicity (8) and myopathy (9), leading to termination of treatment (10). In addition, previous studies have reported that a subset of patients who receive statins, even those with well-controlled LDL-C levels, still experience CVD events due to changes in the levels of other lipids/lipoproteins (11-13). Thus, novel strategies are currently being developed to improve the therapeutic effects and minimize the side effects.

Sarpogrelate is a selective 5-hydroxytryptamine type 2A serotonin receptor antagonist that possesses an extensive range of antiplatelet effects and can prevent arterial thrombosis (14). Acyl-coenzyme A cholesterol acyltransferase-1 is inhibited by sarpogrelate, which decreases the accumulation of lipid droplets in macrophages and blocks atherosclerosis (15). However,
the underlying molecular mechanism of sarpogrelate remains unclear, as only two previous studies on rabbits have been published (16,17). In rabbits, sarpogrelate delays the progression of atherosclerosis by upregulating endothelial nitric oxide synthase expression (17). Several clinical studies have demonstrated that sarpogrelate improves atherosclerosis (18-21). These studies focused on patients with peripheral artery disease (18-20) or cerebrovascular disease (21); however, the role of sarpogrelate on major arteries remains poorly understood. Thus, the present study aimed to investigate whether sarpogrelate synergistically protects against aortic damage in ApoE−/− HF mice, when combined with rosuvastatin treatment.

Materials and methods

Mice and diets. The present study was approved by the Ethics Committee of The Second Affiliated Hospital of Dalian Medical University (approval no. L20160153; Dalian, China), and all animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (17). A total of 22 male ApoE−/− mice (8 weeks old) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. The body weights of mice ranged from 22 to 26 g. All animals were housed at 24°C and 60% relative humidity with a 12 h light and dark cycle with free access to water and food. Mice were randomly divided into four treatment groups. The control group was fed a normal diet (NF; n=5; 20.3% protein, 66% carbohydrate, 5% fat; D10001; Research Diets, Inc.), whilst the other three groups were fed a high-fat diet (HFD), containing 1.5% cholesterol and 15% fat (HF group; n=5; Shanghai SLAC Laboratory Animal Co., Ltd.). Furthermore, two of these groups were additionally treated with rosuvastatin calcium (40 mg/kg/day) and sarpogrelate (50 mg/kg/day; both purchased from Mitsubishi Tanabe Pharma Corporation; HF+R group; n=6), or rosuvastatin calcium alone (40 mg/kg/day; HF+RS group; n=6), respectively. Following 8 weeks (22) of treatment, the mice were euthanized to analyze and characterize aortic injury.

Biochemical measurements. Following 8 weeks of treatment, all mice were sacrificed by a 1% sodium pentobarbital overdose. After fasting for 12 h, the heart was exposed and blood samples were taken by left ventricular puncture. The serum was subsequently separated, all samples were centrifuged at 1,200 x g for 5 min at 4 °C, and the expression levels of total cholesterol (TC), triglyceride (TG) and LDL-C were determined using an automatic analyzer (Hitachi, Ltd.). Sections were fixed on glass slides and sections were stained with hematoxylin for 6 min and eosin for another 1 min. Resinene were fixed on glass slides and observed using a light microscope (Olympus Corporation; magnification, x40).

Histological analysis. After the mice were euthanized, the complete aorta (from the aortic root to the abdominal aorta) was fixed with 4% formaldehyde at room temperature for 24 h and embedded in paraffin. The paraffin-embedded aortas were cut into 4 μm thick sections and dewaxed. Subsequently, the sections were stained with hematoxylin for 6 min and eosin for another 1 min. Resinene were fixed on glass slides and observed using a light microscope (Olympus Corporation; magnification, x40).

Immunohistochemistry (IHC) was performed using the Histofine Simple Stain kit (cat. no. 414142F; Nichirei) according to the manufacturer's protocol. Briefly, the sections were deparaffinized in xylene (3 times, 5 min each) and rehydrated (100, 90, 85, and 75% alcohol, 5 min each) following removal of the excess tissue outside the aorta at room temperature. Sections were incubated with 3% hydrogen peroxide at room temperature for 15 min to inhibit endogenous peroxidase activity. The tissue sections were incubated at 1% blocking solution (cat. no. P0220; Beyotime Institute of Biotechnology) at room temperature for 10 min. Subsequently, sections were incubated with primary antibodies against cluster of differentiation 68 (CD68; 1:500; cat. no. ab213363) and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1; 1:250; cat. no. ab60178; both purchased from Abcam) at room temperature for 10 min. Following the primary incubation, sections were incubated with goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. ab205718; Abcam) at 37°C for 30 min. The slides were observed under a light microscope (Olympus Corporation; magnification, x40).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the aorta using TRIzol® reagent (Nippon Gene, Co., Ltd.) and reverse transcribed into cDNA using the SuperScript VILO cDNA synthesis kit (cat. no. 11756050; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was subsequently performed using SYBR Green (Light Cycler; Roche Molecular Diagnostics) and in accordance with the manufacturer's instructions. The primer sequences used for qPCR primers are listed in Table I. The following thermocycling conditions were used for qPCR: 95°C for 30 sec, 38 cycles at 95°C for 10 sec, 60°C for 20 sec and 72°C for 15 sec. Relative mRNA levels were calculated using the 2^ΔΔCq method (23) and normalized to the internal reference gene β-actin.

Western blotting. The aorta was washed three times with PBS (cat. no. C0221A; Beyotime Institute of Biotechnology) and subsequently lysed using tissue lysate fluid (P0013G; Beyotime Institute of Biotechnology). The mixture was centrifuged at 12,000 x g for 7 min at 4°C, the suspension after centrifugation was absorbed and total protein was quantified using a bicinchoninic acid assay. Equal amounts of protein (35 μg) were subjected to electrophoresis using 10% SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes (EMD

Table I. Primer sequences used for quantitative PCR.

| Gene | Primer sequence |
|------|----------------|
| TNF-α | F:5'-TCTCATGCACCCATCAAGGACT-3' R:5'-ACCACCTTCTCTTGTGAGAATCTCA-3' |
| IL-1β | F:5'-TGCCACCTTTGACAGTGAT-3' R:5'-TGTGCTGTCGCGAGATTIGA-3' |
| IL-6 | F:5'-TACCAGTTGGCTTCTTGGAGCTGA-3' R:5'-TAAGGCTTCGGAGTGTGGAATGGT-3' |
| β-actin | F:5'-CGATGCCCTGAGGGTCTTT-3' R:5'-TGGATGCCACAGGATTCCCAT-3' |

TNF-α, tumor necrosis factor-α; IL, interleukin; F, forward; R, reverse.
Furthermore, there were no significant differences in the body following treatment with rosuvastatin alone, the differences were not as notable compared with the HF+RS group (P<0.05). Although the levels of TC and LDL-C also decreased (P<0.05), whereas the levels of TG only moderately decreased (P=0.51). Although the levels of TC and LDL-C also decreased (P<0.05), whereas the levels of TG only moderately decreased (P=0.51).

Conversely, sarpogrelate combined with rosuvastatin treatment significantly decreased the levels of TC and LDL-C. Following 8 weeks of dietary treatment, ApoE fed an HFD exhibited significantly increased lipid levels. Metabolic characterization. To determine the effects of the combined therapy on metabolism, the serum levels of lipids were assayed and presented in Table II, including TC, TG and LDL-C. Following 8 weeks of dietary treatment, ApoE+ mice fed an HFD exhibited significantly increased lipid levels. Metabolic characterization. To determine the effects of the combined therapy on metabolism, the serum levels of lipids were assayed and presented in Table II, including TC, TG and LDL-C. Following 8 weeks of dietary treatment, ApoE fed an HFD exhibited significantly increased lipid levels.

Table II. Metabolic data from the four groups following treatment for 8 weeks.

| Groups   | Body weight (g) | TC (mmol/l) | TG (mmol/l) | LDL-C (mmol/l) |
|----------|-----------------|-------------|-------------|---------------|
| NF       | 23.78±0.66      | 8.03±1.57   | 5.93±0.78   | 3.95±0.18     |
| HF       | 24.04±0.83      | 33.58±2.79  | 7.42±0.73   | 23.73±2.01    |
| HF+R     | 22.08±0.50      | 22.43±1.36  | 7.75±1.16   | 14.13±1.64    |
| HF+RS    | 22.83±1.16      | 14.45±0.77  | 5.45±1.30   | 6.83±1.07     |
| P-Value  | 0.351           | 0.000       | 0.000       | 0.357         |

Data are presented as the mean ± standard error of the mean (n=4 or 5 per group). TC, total cholesterol; TG, triglycerides; LDL-C, low density lipoprotein cholesterol; NF, normal diet group; HF, high-fat diet group; R, rosuvastatin treatment group; RS, sarpogrelate and rosuvastatin treatment group.

Millipore) and blocked with 5% skimmed milk at 37°C for 1 h. The membranes were incubated with primary antibodies against LOX-1 (rabbit anti-LOX-1; 1:250; cat. no. ab60178; Abcam), phospho (p)-ERK (rabbit anti-p-ERK; 1:1,000; cat. no. 9101; Cell Signaling Technology, Inc.), total-ERK (rabbit anti-ERK; 1:1,000; cat. no. 4695; Cell Signaling Technology, Inc.), β-tubulin (rabbit anti-β-tubulin; 1:1,000; cat. no. 2148; Cell Signaling Technology, Inc.) and β-actin (rabbit anti-β-actin; 1:1,000; cat. no. 4970S; Cell Signaling Technology, Inc.) overnight at 4°C. Following the primary incubation, membranes were incubated with anti-rabbit IgG secondary antibody (1:1,000; cat. no. 7074P2; Cell Signaling Technology, Inc.) at room temperature for 1 h. A and B chromogenic solutions (cat. no. P0013G; Beyotime Institute of Biotechnology) were mixed in 1:1 ratio, and 2 ml of the solution was added to the films. Protein signal intensity was determined using Image J 2.0 software (National Institutes of Health).

Statistical analysis. Each experiment was repeated three times. Statistical analysis was performed using SPSS 23.0 software (IBM Corp.) and all data are presented as the mean ± standard error of the mean. One-way analysis of variance followed by Tukey's post hoc test were used to compare differences between multiple groups. If the data did not show homogeneity of variance, a Tamhane's T2 test were used to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Metabolic characterization. Sarpogrelate combined with rosuvastatin inhibits macrophage infiltration and significantly decreases pro-inflammatory cytokine production. It is well known that the uptake of ox-LDL by macrophages is dependent on several scavenger receptors (SRs) (26). SR class A (SR-A), SR class B1 (SR-B1), LOX-1, cluster of differentiation 36 (CD36), and CD68 are relatively specific for ox-LDL (27) and CD68 is predominantly expressed in macrophages (28). It was hypothesized that, amongst others, CD68 plays a crucial role in the formation of fatty-streaks. Thus, the present study set out to determine whether the regulation of CD68 improved aortic injury in HFD ApoE+ mice. IHC analysis demonstrated that the expression of CD68+ cells was upregulated in the HF group, while the accumulation of CD68 decreased following combined treatment with sarpogrelate and rosuvastatin (P<0.05; Fig. 2B). The interaction between oxidized (ox)-LDL and LOX-1 results in ox-LDL uptake by macrophages and foam cell transformation (24,25). Collectively, these results indicate that sarpogrelate and rosuvastatin can synergistically prevent aortic damage induced by hyperlipidemia and reverse atherosclerosis, which may be associated with the regulation of LOX-1 expression.

Sarpogrelate combined with rosuvastatin suppresses aortic histopathological damage in ApoE−/− HFD mice. Aortic tissue damage was assessed via H&E staining. The results demonstrated that the increased intima thickness, lipid deposition and inflammatory cell infiltration induced by the HFD were reversed following combined treatment with sarpogrelate and rosuvastatin (Fig. 1). LOX-1 was analyzed via IHC analysis. The results demonstrated that LOX-1 staining significantly decreased in the HF+RS group compared with the HF+R group (P<0.05; Fig. 2B). The interaction between oxidized (ox)-LDL and LOX-1 results in ox-LDL uptake by macrophages and foam cell transformation (24,25). Collectively, these results indicate that sarpogrelate and rosuvastatin can synergistically prevent aortic damage induced by hyperlipidemia and reverse atherosclerosis, which may be associated with the regulation of LOX-1 expression.

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reversed following combined treatment with sarpogrelate and rosuvastatin (P<0.05; Fig. 3). Collectively, these results indicate that sarpogrelate and rosuvastatin synergistically inhibit macrophage infiltration into the aorta, and inflammatory cytokine release.

Sarpogrelate combined with rosuvastatin decreases LOX-1 protein expression in ApoE−/− HFD mice. IHC analysis demonstrated that combined treatment with sarpogrelate and rosuvastatin significantly decreased hyperlipemia-induced LOX-1-positive staining in the aorta. In order to verify these results, LOX-1

Figure 1. Aortic roots in ApoE−/− mice in different treatment groups. (A) Hematoxylin and eosin staining in (a) NF, (b) HF, (c) HF+RS (50 and 40 mg/kg/day, respectively), and (d) HF+R ApoE−/− mice fed a HFD and treated with rosuvastatin alone (40 mg/kg/day). The thickness of intima is indicated with a black line. (B) Quantitative analysis of aortic intimal thickness. Data are presented as the mean ± standard error of mean. *P<0.05 vs. NF; **P<0.01 vs. HF; ***P<0.001 vs. HF+R. NF, normal diet; HF, high-fat food; HF+R, HF treated with rosuvastatin; HF+RS, HF treated with sarpogrelate and rosuvastatin.

Figure 2. Immunohistochemistry analysis of aortic morphological abnormalities. Sections of aortic roots from the four groups were immunostained. Positive (A) CD68 and (B) LOX-1 signals appear dark brown. Data are presented as the mean ± standard error of the mean. *P<0.01 vs. NF and HF; *P<0.05 vs. HF+R. magnification, ×40 or ×100. NF, normal diet; HF, high-fat food; HF+R, HF treated with rosuvastatin; HF+RS, HF treated with sarpogrelate and rosuvastatin; CD68, cluster of differentiation 36; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1.
protein expression was determined via western blot analysis. The results demonstrated that LOX-1 protein expression significantly decreased in the HF+RS group compared with the HF+R group and the HF group, respectively (both P<0.001; Fig. 4).

Sarpogrelate combined with rosuvastatin decreases p-ERK expression in the aorta of ApoE-/- HFD mice. The ox-LDL-LOX-ERK signaling pathway is involved in atherosclerosis formation (29). To the best of our knowledge, the effects of sarpogrelate and statins on the hyperlipid-induced p-ERK pathway activation in aorta have not yet been investigated. Western blot analysis demonstrated that p-ERK levels were significantly higher in the HF group compared with the NF group (P<0.01; Fig. 5). Rosuvastatin monotherapy slightly decreased p-ERK levels compared with HF+RS group. Furthermore, combined treatment with sarpogrelate and rosuvastatin significantly decreased p-ERK levels compared with the HF and HF+R groups, respectively (both P<0.001; Fig. 5). Taken together, these results suggest that sarpogrelate and rosuvastatin may have a direct inhibitory effect on ox-LDL-induced p-ERK activation.
Discussion

The results of the present study demonstrated that combined treatment with sarpogrelate and rosuvastatin decreased hyperlipid-induced aortic injury by inhibiting p-ERK pathway activation and downregulating expression of the scavenger receptor protein, LOX-1. Furthermore, combined treatment with sarpogrelate and rosuvastatin prevented CD68+ macrophage recruitment and inflammatory cytokine release in ApoE−/− HFD mice.

A previous study reported a significant reduction in coronary stent restenosis in patients with stable angina administered sarpogrelate compared with the placebo group (30). Another study confirmed that sarpogrelate effectively decreases restenosis in patients with stable angina (31). Although these studies demonstrated the therapeutic effects of sarpogrelate in atherosclerotic heart disease, most studies focus on the thrombosis-inhibiting effects, whereas the effects of sarpogrelate on blood lipids have not yet been investigated. Statins are well recognized as lipid-lowering agents and are used to prevent atherosclerotic disease. Although statins have a certain therapeutic efficacy in patients with atherosclerosis, monotherapy is often insufficient to achieve the desired therapeutic outcomes (32). In the present study, combined treatment with sarpogrelate and rosuvastatin enhanced the protective effects of rosuvastatin alone. Combined treatment effectively decreased serum lipid levels, particularly TC and LDL-C, compared with rosuvastatin alone, and alleviated aortic injury in ApoE−/− HFD mice. In addition, combined treatment synergistically decreased intima thickness, lipid deposition and inflammatory cell infiltration induced by a HFD. Several possible reasons may underly these effects; a recent study demonstrated that sarpogrelate inhibits the accumulation of lipid droplets in macrophages and improves arteriosclerosis (15). Another study also indicated that 5-HT increased the uptake of LDL via LDL receptors, and that of ox-LDL via scavenger receptors in murine macrophages (33). Conversely, inhibition of 5-HT may decrease the uptake of LDL-C to prevent the formation of foam cells (33). To assess the potential involvement of such a mechanism, the morphological changes associated with aortic injury in hyperlipidemic ApoE−/− mice were assessed via immunohistochemical staining. LOX-1 and CD68 are relatively specific for ox-LDL (27), and the latter is expressed primarily in macrophages (28). LOX-1 and CD68−stained areas in cross-sectional aortic roots significantly decreased following combined treatment compared with either rosuvastatin alone or HFD. Thus, it is hypothesized that the significant synergistic lipid-lowering effects of sarpogrelate and rosuvastatin may be associated with the regulation of ox-LDL scavenging receptors.

In aortic diseases, hyperlipidemia is a key factor in the development of atherosclerosis as it increases the quantity of circulating inflammatory cells and induces inflammatory pathways (34,35). Reportedly, sarpogrelate also modulates inflammatory-macrophage accumulation and inflammatory responses (36). In a recent experimental study, treatment with sarpogrelate decreased inflammatory macrophage markers and inflammatory mediators in mice with type 2 diabetes and diabetic nephropathy (36). In the present study, combined treatment with sarpogrelate and rosuvastatin resulted in a significant decrease in the mRNA expression levels of inflammatory cytokines levels compared with rosuvastatin alone.

LOX-1 is a detrimental factor in hyperlipid-induced aortic injury (37). Physiological basal cellular expression of LOX-1 is low; however, LOX-1 expression is rapidly increased in response to proinflammatory cytokines (38,39). In turn, LOX-1 stimulates the release of inflammatory cytokines and activates inflammatory responses, aggravating disease pathogenesis (40,41). Thus, it was hypothesized that the key to blocking this positive feedback loop is to prevent LOX-1 expression. Administration of LOX-1 blockers or LOX-1 knockout can inhibit the binding of inflammatory factors to LOX-1 and prevent the progress of atherosclerosis (42). To verify the results of IHC, LOX-1 expression was measured via western blotting, which confirmed that sarpogrelate combined with rosuvastatin significantly suppressed hyperlipidemia-induced LOX-1 protein expression. This suggests that sarpogrelate and rosuvastatin can inhibit ox-LDL uptake in the arterial wall by interfering with LOX-1 activation. The 5-HT2a receptor is known to modulate both the MAPK/ERK and the PI3K/PDK/AKT pathways, which serve prominent roles in cell survival (41,43). Inhibitors of ERK, PKC and NF-κB attenuate LOX-1 expression, indicating that activation of the ERK/PKC/MAPK pathway is an initial signaling event in LOX-1 expression regulation (44). Other circumstantial evidence suggests that LDL induces inflammation via LOX-1 and increases phosphorylation of members of the ERK signaling pathway (45). Thus, it is hypothesized that p-ERK, which is downstream of LOX-1, is the target of aortic injury. Following 8 weeks of combined treatment with sarpogrelate and rosuvastatin, p-ERK levels significantly decreased in ApoE−/− HFD mice. A possible explanation for this may be that increased blood lipids level result in an increase in ox-LDL levels in artery walls. As a LOX-1 ligand, ox-LDL activates LOX-1 and its downstream signaling molecules, including p-ERK (30,29,46). Activated p-ERK results in LOX-1 upregulation and promotes arteriosclerosis (47), thereby resulting in aortic injury. This effect on ox-LDL/LOX-1/p-ERK signaling was more prominent in the HF+R group compared with the HF+R group. Thus, it is hypothesized that by blocking LOX-1 or downstream p-ERK signaling, sarpogrelate and rosuvastatin may improve hyperlipid-induced vascular remodeling and aortic injury.

Dyslipidaemia, characterized by increased plasma levels of LDL-C, VLDL-C, TG, and decreased plasma levels of HDL-C, is a key factor associated with atherosclerotic disease (48). The effects of sarpogrelate and rosuvastatin on VLDL-C, HDL-C and inflammatory factors in plasma of HFD ApoE−/− mice were not assessed in the present study. However, the results of the present study suggest that sarpogrelate may enhance the lipid-lowering effect of statins, improve the elevation of TG, TC and LDL-C caused by dyslipidaemia (Table II), and improve the formation of foam cells in aortic tissues and the infiltration of inflammatory cells (Fig. 1). Further studies are required to confirm the association between sarpogrelate enhanced statin therapy and cardiovascular outcomes to better understand the benefits of sarpogrelate in CVD.

In conclusion, the novel effects of sarpogrelate in synergistically acting with rosuvastatin to inhibit hyperlipid-induced aortic damage through the LOX-1/p-ERK pathway were
determined. These findings may provide novel insight into the roles of sarpogrelate and rosuvastatin in vascular protection and highlight the potential of a novel therapeutic intervention for the treatment of aortic lesions.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions
HL, YL and SX conceived and designed the present study. XF analyzed the data. GL, DL, QL, LH, and JZ have been involved in acquiring the data, analyzing and interpreting the data, and drafting the manuscript. HL, YL, JM and ZS performed the experiments. YL and HL revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of The Second Affiliated Hospital of Dalian Medical University (approval no. L20160153; Dalian, China), and all animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (21).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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