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Ginsenoside Rb1 promotes angiogenesis potentially by activating the JAK-STAT3 signalling pathway

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Conventional revascularization strategies for ischemic heart disease (IHD) are designed to prompt reperfusion of the coronary artery to the salvaged cardiomyocytes. However, these strategies may cause myocardial reperfusion injuries. Therefore, a safe and effective strategy needs to be developed to improve the conventional strategies. Here, we investigated the pro-angiogenic effect of Ginsenoside Rb1 (Rb1) to provide the experimental basis for angiogenesis-mediated drug therapy of IHD. Thus, Human umbilical vein endothelial cells (HUVECs) were treated with either a vehicle or Rb1 at 4, 8, 12 or 16 μM for 24 h. A model of hindlimb ischemia was established using C57BL/6J mice. In sham-operated mice, only the femoral artery was isolated without ligation whereas the other operations and supplementation control group were consistent. The mice in the supplementation group were injected with Rb1 (50 mg/kg body wt./day) for 7 days. The results indicated that Rb1 promotes cell proliferation, adhesion, migration and tube formation in the HUVECs in a dose-dependent manner. The ED50 of Rb1 to improve cell adhesion is 8 μM. In mice, Rb1 promoted angiogenesis after the ligation of the femoral artery and ameliorated the ischemic conditions. Intriguingly, more blood flow recovery was observed in the Rb1 supplemented mice than in the vehicle-treated mice (0.85 ± 0.05 *vs*. 0.71±0.10 on day 3; 0.94±0.10 *vs*. 0.75±0.08 on day 7). In HUVECs, Rb1 increased the phosphorylation of STAT3 and JAK, which may be the mechanism through which Rb1 mitigates IHD. Moreover, our results confirmed that Rb1mitigates IHD potentially by activating the JAK-STAT3 pathway. Further clinical trials are warranted to verify the clinical implications of Rb1.

Keywords: Hindlimb ischemia, Ischemic heart disease (IHD), Revascularization

According to the World Health Organization (WHO) report, ischemic heart disease (IHD) is the leading cause of death in developed and developing countries[1]. Traditional revascularization strategies, such as primary coronary intervention (PCI), mainly focus on promoting coronary reperfusion to replenish the cardiomyocytes[2,3]. However, these strategies may cause microcirculatory dysfunction[4,5]. Therefore, methods should be followed during the angiogenesis-mediated microvascular recovery to improve reperfusion and reduce adverse sequences.

Angiogenesis is a physiological or pathological process characterized by the sprouting of new blood vessels from existing vasculature[6,7]. In addition to IHD, angiogenesis plays a critical role in various physiological processes such as wound healing[8], where in the angiogenic capillary sprouts enter the fibrin/fibronectin-rich wound clot and generated a microvascular network in the granulation tissue. Signal transducer and activator of transcription (STAT) is a group of nuclear factors (NF) that modulate various cellular physiological and biological activities of cells[9]. Among the STAT family members, STAT3 is the most extensively studied NF due to its critical role in tumour formation and cardiac pathogenesis and angiogenesis[10-12]. Janus kinase (JAK) is a family of non-receptor tyrosine kinases[13]. In response to cytokine ligands, such as interleukin (IL)-6 and interferons (IFNs), cytokine receptors are activated, which may recruit JAK proteins by facilitating the binding of JAKs to the cytokine receptor subunit β (gp130) and the phosphorylation of STAT3 at tyrosine 705 residue[12,14]. Since the JAK-STAT3 signaling pathway plays a vital role in angiogenesis, it is receiving increasing attention in the cardiovascular field as a potential molecular target for angiogenesis-mediated therapy.

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Fufangtengyixinrecipe (FFTYX) isa well-defined herbal preparation which is widely and clinically used as an adjuvant therapy for IHD; FFTYX is composed of four traditional Chinese medicines: *Euonymus fortunei*, *Panax ginseng* C. A. Meyer, *Panax notoginseng*and*Acorus tatarinowii*[15]. Previously, we found that FFTYX synergistically enhances the activity of increasing coronary flow, improving hypoxia tolerance, lowering blood pressure and protecting myocardial ischemia in rats[16]. However,the underlying mechanisms and bioactive constituents remained unknown.FFTYXmainly contains a variety of pentacyclic triterpenoids, mainly including Ginsenosides—Rb1, Re, F2, Rb2[17]. In the past decades, many studies have shown the close relationship between ginsenosides and angiogenesis. Ginsenoside Rb1, the effective constituent of *ginseng* and *Panax notoginseng*, has been demonstrated to play a favorable role in improving the immunity system[18]. It also plays a role in osteogenic differentiation and angiogenesis factor expression of BMSCs[19]. However, the effects of Rb1 in treating IHD remain enigmatic. This study explores the potential efficacy of Rb1 in IHD treatment, using both in *vitro* and in *vivo* methods. We hypothesized that Rb1 can enhance blood flow recovery after IHD treatment by promoting angiogenesis. We also investigated the possible potential mechanism through which Rb1 maypromoted angiogenesis.

Materials and Methods

Animals

Six-week-old male C57BL/6J mice (N = 12) were obtained from the Department of Laboratory Animals, Chinese Academy of Sciences (Shanghai, China). All the mice received humane care, and all experimental procedures of the mice were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and approved by the Experimental Research Ethics Committee of Chinese Medical Science Guangxi University of Chinese medicine (GXUTCM0933162).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (Carlsbad, CA, USA) and cultured in MCDB131 (PAA, Pasching, Austria) containing 5% fetal bovine serum (Hyclone, Logan, UT), 1% endothelial cell growth supplement (ScienCell, Carlsbad, CA, USA), and 1% penicillin streptomycin (Gibco, Grand Island, NY, USA). The cells used for the experiments were in passages 4–6. Transformed HUVECs were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. Four biological replicates were used in the assays.

Cell viability assay

Rb1 was purchased from Push Bio-technology (Sichuan, China). Primary HUVECs were plated in 96-well plates and treated with 4, 8, 12 or 16 μMofRb1 (Push Biotechnology) for 24 h. Then, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay was conducted using a commercialized kit (ThermoFisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions. The absorbance at 570 nm in dimethyl sulfoxide (DMSO) was recorded as a reflection of cell viability.

Cell proliferation assay

Primary HUVECs were plated in 96-well plates. After Rb1 treatment for 24 h, the proliferating cell nuclei were stained with an EdU assay kit (RiboBio, Guangzhou, China) and detected by fluorescence microscopy. As a counterstain for cell nuclei, 4',6-diamidino-2-phenylindole) was used.

Cell adhesion assay

Primary HUVECs were plated in 48-well plates precoated with 50 μg/mL collagen I (BD, Franklin Lakes, NJ, USA) and treated with Rb1 at 4, 8, 12 or 16 μM of Rb1 for 10 min. Then, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde. Afterwards, the cells were stained with a crystal violet staining solution. The cells in three random fields from each well were counted, and the average was reported.

Wound healing assay

Primary HUVECs were plated in 48-well plates and treated with hydroxyurea (Sigma, St. Louis, MO, USA) to inhibit cell proliferation. Once a confluent cell monolayer was formed, it was scratched with a yellow pipette tip to create a scratch wound. Afterwards, the cells were treated with Rb1 at 16 μM of Rb1 for 24 h. Photographs were taken with a Zeiss digital camera at baseline (0 h) and 24 h. The wound area was calculated to determine the migration distance.

Transwell migration assay

Primary HUVECs were plated in the upper chamber pre-coated with 50 μg/mL collagen I, and Rb1 at 16 μMof Rb1 was added to the lower chamber. The cells were allowed to migrate for 8 h and then fixed with 4% formaldehyde. The non-migrating cells in the upper chamber were removed with a cotton swab, whereas migrating cells were stained with crystal violet. The cells in three random fields form each well were counted, and the average was reported.

Tube formation test

Primary HUVECs were plated in 48-well plates pre-coated with matrigel (BD Biosciences, CA, USA). The cells were treated with Rb1 for 6 h, and the tubule structure was subsequently photographed and quantified by measuring the length of each tube using the software Image-Pro Plus 6. The tube length in three random fields from each well was calculated, and the average was reported.

Hindlimb ischemia model

The mice were intraperitoneally administered with sodium pentobarbital at a dose of 20 mg/100 g body wt. A solution containing 16.7 mg/mL of sodium pentobarbital was prepared fresh daily. C57BL/6J mice were anesthetized with sodium pentobarbital and placed on a heating pad (37°C) under an Olympus stereotactic microscope. A longitudinal 5 mm incision was made along the left femoral vessel starting from the groin crease. The femoral artery was carefully isolated without injuring veins or nerves, and the ligation was performed using triple surgical knots 7.0 silk sutures. Successful ligation was verified by laser Doppler perfusion imaging, and color-coded perfusion images were recorded as a reflection of the perfusion defect in the left foot after hindlimb ischemia. In sham-operated mice, only the femoral artery was isolated without ligation whereas the other operations and supplementation control group were consistent. The mice in the supplementation group were intraperitoneally injected with Rb1 (50 mg/kg body wt./day)[20] for 7 days whereas the mice in the vehicle group were intraperitoneally injected with saline.

Laser Doppler perfusion imaging

Hindlimb perfusion was assessed using a PeriCam PSI system-hemoperfusion imager (Perimed, Stockholm, Sweden) by scanning the mice on a heating plate maintained at 37°C. Perfusion was evaluated at the indicated times (before, immediately after, and 7 days after femoral artery ligation), and the flow ratio was determined by dividing the perfusion value of the ischemic hindlimb by that of the non-ischemic hindlimb from the same mouse.

Immunohistochemistry (IHC)

On day 10 after the femoral artery ligation, mice were sacrificed, and their gastrocnemius muscles were excised for fixation and embedded in paraffin. The endothelial cells in paraffin sections were detected by using a polyclonal CD31 antibody (Santa Cruz, CA, USA) and then stained with HRP-conjugated secondary antibody and diaminobenzidine substrate (BlueGene Biotech, Shanghai, China). Three random fields per section were captured using a Zeiss digital camera.

Microangiography

For the hindlimb ischemia model, the mice were anesthetized with sodium pentobarbital on day 10 after femoral artery ligation, and catheters (PE-10, BD) were implanted into 0.5 cm of the abdominal aorta to reach the iliac bifurcation. The hindlimb vessels were then perfused with sequential manual injections of heparinized saline (10 U/mL), nitroglycerin (100 μg/mL), and barium sulfate (size, 1 μm; 0.1 g/mL). Subsequently, the mice were placed in an X-ray chamber and angiographic images were acquired using an in vivo FX PRO system (Carestream, Rochester, NY, USA). The vessel density was determined by pixel analysis using Image J software.

Protein extraction and Western blot

Whole cell lysates (WCLs) were extracted by using the methods described elsewhere[21,22]. Briefly, the cell medium was aspirated, followed by washing with chilled PBS and centrifugation at 2,500 rpm for 10 min. Afterwards, the WCL was extracted by using a radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitors. Western blot was performed as described previously[23,24]. In brief, the protein extracts were heated with 5× loading buffer (Fermentas, Vilnius, Lithuania) and then loaded onto sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels (Thermo Fisher Scientific, MA, USA). The separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA) and then blocked with 5% non-fat dry milk in TBS (Amresco) containing 0.1% Tween-20. Subsequently, the membranes were incubated with primary (p-STAT3, STAT3, p-JAK, JAK, and GAPDH) and secondary antibodies (Cell Signaling Technology, MA, USA). Horseradish peroxidase (HRP)-bound proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA).

Statistical analysis

The quantitative data were expressed as mean ± standard error of the mean (SEM). The statistical significance of differences was evaluated using the unpaired Student's t-test or one-way analysis of variance (ANOVA). A p-value <0.05 was considered statistically significant.

Results

Ginsenoside Rb1 promotes proliferation, adhesion and migration

As a result, Rb1 treatment at different dosages (4, 8, 12 or 16 μM) significantly increases the cell viability and cell proliferation in primary HUVECs (Fig. 1 A and B). Furthermore, we found that Rb1 treatment significantly promotes cell adhesion in a dose-dependent manner (Fig. 1C). The ED_{50} of Rb1 to improve cell adhesion is 8 μM. Interestingly, the cell adhesion assay showed that even a 10 min treatment with Rb1 can induce cell adhesion, suggesting that Rb1 may induce an acute response in the HUVECs cells.

Ginsenoside Rb1 promotes tube formation

Both wound healing assay and transwell migration assay showed an increased in the migration of HUVECs in response to Rb1 treatment (Fig. 2). In the 2D Matrigel experiments, the tube length and morphology have significantly improved with Rb1

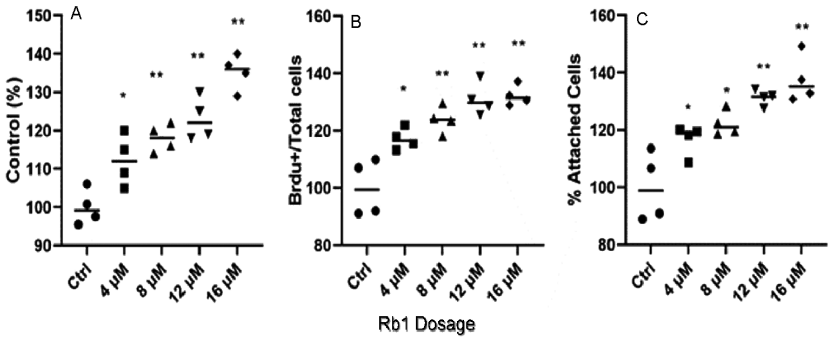

Fig. 1 — Ginsenoside Rb1 promotes cell viability, proliferation, adhesion and migration of primary HUVECs. (A) Cell viability assay. Primary HUVECs were treated with different concentrations (4, 8, 12 or 16 μM) of Rb1 for 24 h and then analysed with an MTT assay; (B) Cell proliferation assay. Primary HUVECs were treated with different concentrations of the drug for 24 h and then stained with EdU and DAPI. Red nuclei indicating proliferating cells were counted and statistically analysed; and (C) Cell adhesion and migration assays. [For adhesion assays, primary HUVECs were plated in collagen I pre-coated wells and treated with different concentrations of Rb1 for10 min. For wound healing assay, cell monolayers were scraped and treated with different concentrations of Rb1 for 24 h. HUVECs: human umbilical vein endothelial cells; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.* and** indicates significance @P<0.05 and$P$<0.01 compared to control group, respectively]

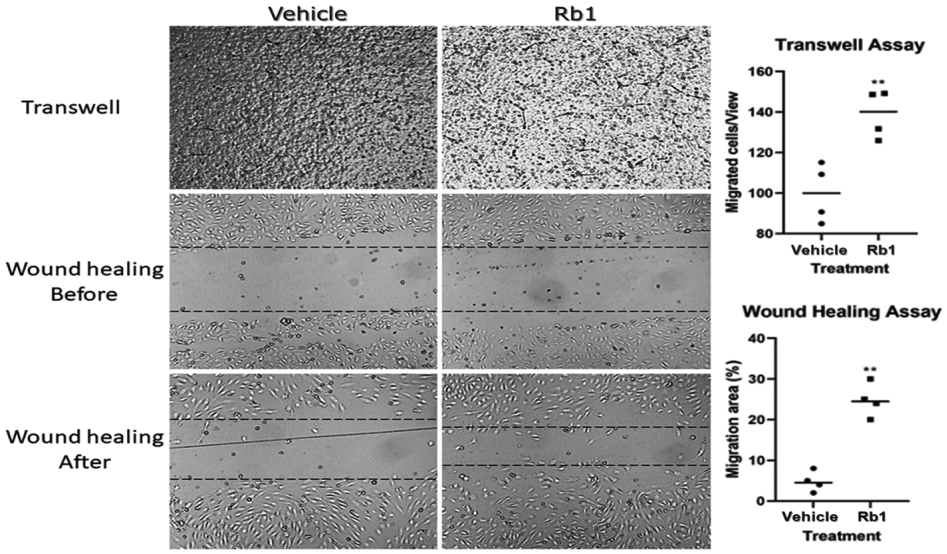

Fig. 2 — The pro-angiogenic effect of Rb1. For transwell migration assays, primary HUVECs were seeded in the upper layer of transwell inserts, whereas Rb1 (16 μmol/L) was added in the lower layer. Cells were treated for 8 h. **indicates P <0.01significance compared to control group.

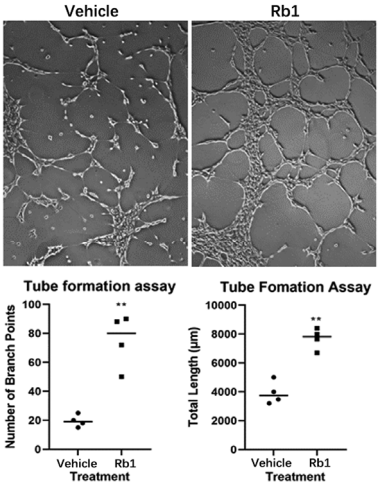

Fig. 3 — Regarding tube formation assay, primary HUVECs were seeded on gel matrigel and treated with Rb1 (16 μmol/L) for 6 h. ** indicates $P<0.01$ significance compared to control group.

treatment, suggesting that supplementation with Rb1 enhance the tube network formation in the endothelial cells (Fig. 3).

Ginsenoside Rb1 promotes angiogenesis *invivo* under both normal and ischemic conditions

To investigate the possible therapeutic potential of Rb1 in ischemic diseases, we used a mouse hindlimb ischemia model. The blood flow is significantly reduced in the ischemic hindlimbafter the femoral artery ligation (Fig. 4A). The serial analysis of hindlimb perfusion using the laser Doppler imaging revealed significantly more blood flow recovery in the Rb1 supplemented mice than in the vehicle-treated mice (0.85 ± 0.05 vs. 0.71 ± 0.10 on day 3; 0.94 ± 0.10 vs. 0.75 ± 0.08 on day 7). Our microangiography analysis showed that the ligation resulted in collateral artery development in the ischemic hindlimbs, which

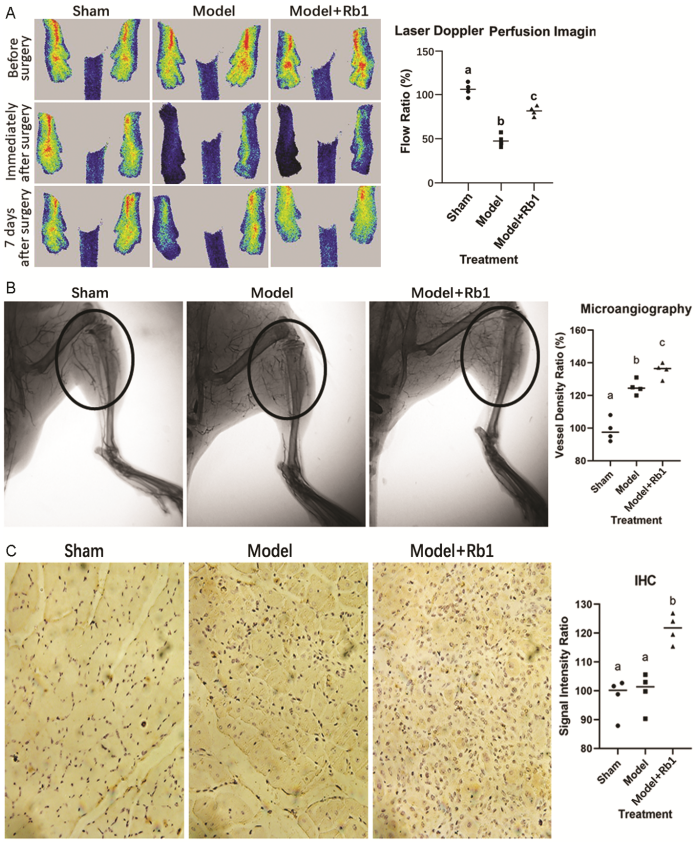

Fig. 4 — Ginsenoside Rb1 promotes angiogenesis *in vivo* under ischemic conditions. (A-B) Afterligation of left femoral artery, the treatment group mice were intraperitoneally injected with Rb1 (50 mg/kg/day) whereas the vehicle model group were intraperitoneally injected with saline. (A) The mice were scanned with laser Doppler perfusion imaging at the following times: mice before, immediately after, and 7 days after femoral artery ligation. The flow ratio was determined by dividing the perfusion value of the ischemic hindlimb by the perfusion value of non-ischemic hindlimb from the same mouse. $N = 6$ per group; (B) The mice were anesthetized by treatment with Rb1 or normal saline, and barium sulfate was injected from the abdominal aorta for microangiography, resulting in collateral vessels in the hind limbs. Solid arrows indicate the ligation site and arrows indicate "spiral" collateral vessels. $N = 6$ per group; and (C) Rb1 or saline treatment of seven, anesthetized mice. Gastrocnemius muscle was removed and stained with CD31 antibody. Solid arrows indicate vascular endothelial cells and arrows indicate capillaries. [Scale bar, 100 μm. $N = 8$ per group. [a]indicates$P<0.05$ singificance compared to control group,[b] indicates $P<0.01$ compared to control group, and[c]indicates $P<0.01$ compared to the model group]

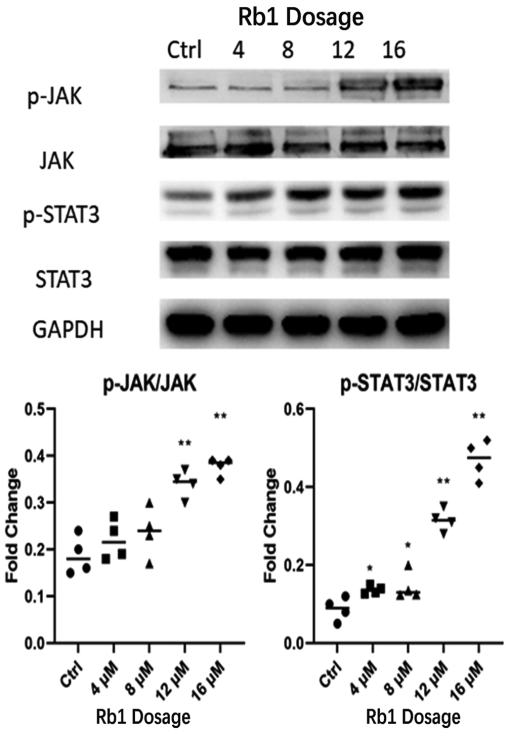

Fig. 5 — Ginsenoside Rb1 induces the phosphorylation of JAK and STAT3. Primary HUVECs were treated with GRb1 (4, 8, 12 or 16 μM) for 4 h. JAK: Janus kinase; STAT3: signal transducer and activator of transcription. * and ** indicates significance @P<0.05 and$P$<0.01 compared to control group, respectively]

is an adaptive pathway for tissue salvage during arterial occlusion. Intriguingly, Rb1 supplementation led to a significant increase in angiographically-visible collateral vessels with a typical "spiral" appearance (Fig. 4B).

We also found that CD31, a specific endothelial cell marker, has significantly increased in the gastrocnemius muscle of the Rb1 supplemented mice than in the muscle of the vehicle-treated mice **(Fig. 4C)**. These results suggest that Rb1 may enhance the blood supply by increasing the microvessel density of the hindlimb to mitigate ischemic conditions. Therefore, our in vivo results indicate that Rb1 may have therapeutic potential for ischemic diseases by promoting angiogenesis or arteriogenesis.

Rb1 enhances p-JAK and p-STAT3

We further explored the underlying molecular mechanism through which Rb1 enhances angiogenesis. The phosphorylation of JAK and STAT3 has significantly increased in the HUVECs treated with Rb1 of 4, 8, 12 or 16 μM (Fig. 5). This result suggests that Rb1 may improve angiogenesis by modulating the JAK-STAT3 signalling pathway.

Discussion

The current treatment strategies for IHD involve medications such as antiplatelet drugs (aspirin), antilipemic drugs (statins) or vasodilators as well as revascularization strategies, such as PCI or coronary artery bypass grafting (CABG), thereby prompting coronary reperfusion to save viable cardiomyocytes[25]. However, during the treatment, myocardial reperfusion injuries may occasionally occur, mainly due to microcirculatory dysfunction caused by oxidative stress, calcium overload, and inflammation[26,27]. In addition, considering the population such as elderly patients who are vulnerable to PCI or CABG procedures[28], novel strategies need to be explored to reduce the adverse effects and procedural risks. In a former study, fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) was injected as a plasmid into the ischemic myocardium of IHD patients, thereby reducing the symptoms and improving the myocardial perfusion by improving the angiogenesis[29]. Such results highlighted the potential efficacy of angiogenesis-mediated therapy in promoting the outcome of the IHD treatment.

Traditional Chinese Medicine has shown various beneficial effects against multiple diseases. In the past three decades, the cardiovascular protective effects of FFTYX have been investigated. The aqueous extract of FFTYX(200 mL/kg) can increase the coronary flow and significantly improves the hypoxia tolerance of the rats. It can improve endothelial function by improving the proliferation activity of endothelial cells after H/R, reducing the apoptosis rate of endothelial cells and increasing the concentrations of vascular endothelial growth factor receptor-2 and VEGF in the endothelial cells after H/R injury[15]. Interestingly, the extracted from *Euonymus fortunei*also displayed beneficial effects in enhancing blood circulation and antioxidative capacities in the rats with cerebral ischemia reperfusion injuries.

Since Rb1 is one of the main active ingredients of FFTYX, it could improve the outcomes of IHD treatment by reducing myocardial reperfusion injuries. However, one of the limitations of our study is that we were unable to determine whether the efficacy of Rb1 in preventing cerebral ischemia-reperfusion injuries is comparable with that of FFTYX. Since the multiple phytochemicals in a whole food may generate synergistic effects and display more potent beneficial effects than a single compound[30,31], it would be interesting to explore if the angiogenesis promoting

effects of FFTYXstemmed from itself or Rb1. Another limitation is that there is no rationale for selecting day-10 to observe microangiography. Therefore, multiple time-points will be adapted to observe microangiography in our future studies.

In this study, we demonstrated that Rb1 promotes proliferation, adhesion, migration, and tube formation of primary HUVECs under both normal and ischemic conditions. We have also shown that Rb1 promotes angiogenesis *invivo*. We revealed the therapeutic potential of Rb1 against IHD using a hindlimb ischemia model. As the JAK-STAT3 pathway plays an important role in cell survival, this pathway was first elucidated as a key transcription factor in tumour angiogenesis, and was subsequently shown to be required after ischemic injury[32,33]. Previous studies reported that STAT3 knockout in cardiomyocytes results in reduced left ventricular capillarization[34]. In contrast, cardiac specific activation of STAT3 was demonstrated to promote cardiac vascularization[35]. Given the important role of STAT3 in angiogenesis, we first examined whether Rb1 has any effect on STAT3 activity and found that Rb1 induces the phosphorylation of JAK and STAT3. Therefore, our results suggested that JAK-STAT3 might be a key signalling pathway through which Rb1 induces angiogenesis. However, this study showed only an association between JAK-STAT3 and angiogenesis, not causality. Therefore, in our future studies, we aim to investigate the causality of JAK-STAT3 activation in Rb1-induces angiogenesis using a STAT3-knockout mouse model. Moreover, JAK/STAT3 has been suggested to play a primary role in inflammation-associated pathogenic angiogenesis, such as retinopathy, and this might yield some side effects of Rb1. Nevertheless, we did not observe such side effects in the mice. Since the activation of JAK/STAT3 is a two-sided sword that could either rescue the ischemic tissue or induce pathogenic angiogenesis, it is critical to monitor the degree of JAK/STAT3 activation, which would be an interesting research topic that warrants further investigation.

Conclusion

Our study is the first one which shows that Rb1 promotes angiogenesis under both normal and ischemic conditions. In addition, this is the first instance a study has elucidated the molecular mechanism of how Rb1 improves the treatment outcomes of IHD by highlighting JAK-STAT3-mediated angiogenesis. Further clinical trials are warranted to verify its clinical implications.

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Conflict of Interest

Author declares no competing interests.

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