

Concentration-dependent effects of rapamycin on proliferation, migration and apoptosis of endothelial cells in human venous malformation

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Abstract. Rapamycin has been reported to be immunosuppressive and anti-proliferative towards vascular endothelial and smooth muscle cells. The purpose of the present study was to investigate the effects of rapamycin on the biological behaviors of endothelial cells that have been separated from the deformed vein in human venous malformation (VM). Cellular morphology was observed using inverted microscopy. An MTT assay was performed to measure the cell viability at different concentrations of rapamycin and different time points. Cell apoptosis and migration were detected using a terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay and a wound-healing assay, respectively. At 48 and 72 h, rapamycin inhibited proliferation of human VM endothelial cells, with the effects becoming more pronounced with increasing concentration. Only rapamycin at a concentration of 1,000 ng/ml had a significant effect at 24 h in repressing proliferation. At 48 h, compared with the blank group, the majority of cells maintained a clear nuclear boundary and a regular shape following treatment with 1 ng/ml rapamycin; 10 and 100 ng/ml rapamycin caused desquamation and rounded shape; and 1,000 ng/ml rapamycin caused even more marked desquamation, rounded shape and necrosis. Rapamycin at concentrations of 1, 10, 100 and 1,000 ng/ml reduced cell viability, increased the number of apoptotic cells and suppressed the migration capacity of human VM endothelial cells, and the effects became more pronounced with increasing concentration, when compared with the blank

group. These findings provide evidence that rapamycin induces apoptosis and inhibits proliferation and migration of human VM endothelial cells in a concentration-dependent manner.

Introduction

Venous malformations (VMs) consist of distorted and ectatic veins, which are enveloped sparsely by smooth muscle cells (SMCs) (1). VMs are low-flow vascular anomalies and will develop throughout life in the body without appropriate management (2,3). VMs comprise 50-75% of vascular malformations (4). Almost 40% of VMs are located in the head and neck region, particularly in the oral cavity, airway tract and related muscles (5). According to Legiehn and Heran, 98% of VMs are sporadic and non-inherited, and inherited VMs are typically multifocal (6). It has been estimated that VM occurs at an incidence of 1-2 individuals per 10,000 births (7). VMs in the temporal muscle of the face commonly cause cosmetic deformity and migraine, and those located in the extremities often lead to the development of hypotrophy or hypertrophy, as well as muscle weakness (8). Treatment regimens include sclerotherapy, laser therapy, embolization and surgical resection (9). The primary goals of treating VM include alleviating pain, mitigation of functional disability and amelioration of cosmetic deformity (10). Severe VMs often persist despite current treatment alternatives (11), and for this reason, a novel therapeutic strategy utilizing rapamycin to alleviate VM was explored in the present study.

Rapamycin, as a macrolide antibiotic, allosterically regulates the process of substrates entering the catalytic site of the mammalian target of rapamycin (mTOR) (12). Rapamycin not only binds to mTOR but also serves as the prototypical inhibitor of mTOR (13). Produced by *Streptomyces hygroscopicus*, rapamycin has multiple biological and pharmacological properties (14), including immunosuppressive (15), anti-neoplastic (16), neuro-protective (17) and anti-aging activities (18). Rapamycin serves a critical role in modulating endothelial cells by repressing mTOR, and it can not only suppress inflammation, but also prevent and mitigate post-angioplasty coronary artery

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restenosis (19). Cell contact in endothelial cells is mediated by the characteristics of endothelial monolayers, regulating the biological behaviors of endothelial cells and contributing to vascular homeostasis (20). Accordingly, aberrant disruptions of cell contact in endothelial cells are of substantial importance in cardiovascular diseases and are key features of pathologically altered vascular endothelium (21). Venous endothelial cells have differing responses to angiogenic signals, thus the biological behaviors of endothelial cells have been investigated previously in the pathogenesis and treatment of VM (4). The aim of the current study was to investigate the effects of rapamycin on the biological behaviors of endothelial cells, such as proliferation, migration and apoptosis in human VMs.

Materials and methods

Cell separation and culture. Between January 2016 and January 2017, 10 cases of single oral buccal and facial VM were reported at Department of Oral and Maxillofacial Surgery, The Affiliated Stomatological Hospital of Nanjing Medical University, including 4 male and 6 female patients, with a mean age of 29.4 ± 13.7 years. The diagnosis of buccal facial vein malformation was confirmed by preoperative pathological examination. The inclusion criteria were as follows: Patients who were diagnosed with single oral buccal and facial venous malformation by pathological examination. The exclusion criteria were as follows: Patients who were diagnosed with other types of venous malformations on the face, hypertension, serious cardiovascular and cerebrovascular diseases, and other patients who were not suitable for treatment (for example cardiopulmonary dysfunction or poor recovery). The present study was approved by the Ethics Committee of The Affiliated Stomatological Hospital of Nanjing Medical University (Nanjing, China) and written informed consent was obtained from each subject. Surgically resected VM tissues were separated under aseptic conditions, and the exposed malformed vascular mass was cut open. Sponge-like sinusoids were then visualized and were sliced into tissue blocks of approximately 3–5 mm². Following immersing in culture medium for minimal humidification, tissue blocks were inoculated in a culture flask that was subsequently plated on 1% gelatin, with the intima facing downward. Tissue blocks were separated by a distance of ~5 mm. The tissue blocks were then incubated in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with the culture flask inverted. Following 6 h, the culture flask was upended for incubation at 37°C. Tissue blocks and non-adherent cells were removed, and adherent cells were incubated in Endothelial Cell medium (cat. no. 1001; ScienCell Research Laboratories, Inc., San Diego, CA, USA) for 3 days at 37°C. Cellular morphology was observed under an inverted microscope at a magnification of x400, and cells outside the circle were removed using a cytopipette. Subsequently, tissue blocks were washed using PBS twice to remove other cells, and the culture medium was replaced. When cells had reached 70–90% confluency, 0.25% trypsin supplemented with ethylenediaminetetraacetic acid was used for cell subculture.

Identification of endovascular epithelial cells separated from deformed veins in human VM. Flow cytometry

analysis was used to detect endovascular epithelial markers in order to identify endovascular epithelial cells. Separated cells from deformed veins in human VM were detached using 2.5 g/l trypsin and centrifuged at 167 x g and 4°C for 5 min. The cells were washed with cold PBS at 4°C and centrifuged at 167 x g and 4°C for 5 min. The washing and centrifugation were repeated and then the cells were made into a single cell suspension. With 1×10^6 cells per tube, a 15-min incubation at 37°C was performed with the addition of 20 μ l phycoerythrin-conjugated anti-human cluster of differentiation (CD)31 antibody (cat. no. 566177; 1:500; BD Biosciences, San Jose, CA, USA), or a 30-min incubation at 4°C with the addition of 20 μ l mouse anti-human von Willebrand factor (vWF) monoclonal antibody (cat. no. 555849; 1:500; BD Biosciences). For determination of CD31/vWF, a simultaneous incubation was performed for 15 min at 37°C. Subsequently, a second incubation for 30 min at 4°C with the addition of 50 μ l fluorescein isothiocyanate-labeled rabbit anti-mouse IgG (cat. no. TA130002; 1:1,000; OriGene Technologies, Inc., Beijing, China) was performed. Cells were placed in tubes on the ice in the dark for 30 min, washed three times using 2 ml PBS and at 167 x g and 4°C and to remove the supernatant. Then 500 μ l PBS (containing 4% paraformaldehyde) was used to fix the cells at 4°C for 30 min prior to performing the flow cytometry. Cell identification was conducted using a FACSCan flow cytometer and the data was analyzed by Cell Quest Software (version 5.1; both BD Biosciences, Franklin Lakes, NJ, USA).

Cell grouping and treatment. The separated and determined endothelial cells of human VM were assigned into a blank group (cells cultured conventionally, without any treatment); a 1 ng/ml group (cells cultured in medium with rapamycin working solution at a concentration of 1 ng/ml); a 10 ng/ml group (cells cultured in medium with rapamycin working solution at a concentration of 10 ng/ml); a 100 ng/ml group (cells cultured in medium with rapamycin working solution at a concentration of 100 ng/ml), and a 1,000 ng/ml group (cells cultured in medium with rapamycin working solution at a concentration of 1,000 ng/ml). Rapamycin was purchased from Hunan TaiRen Pharmaceutical Co., Ltd. (Changsha, China).

MTT assay. Cell viability of the endothelial cells in each group was measured at 24, 48 and 72 h following incubation. The optimal reaction time point for rapamycin was determined for the observation of cell morphological changes under an inverted microscope and other experiments. A cell suspension for each group was inoculated in 96-well plates at a density of 5×10^4 cells/well following dilution. A total of 6 replicate wells were set up for each group. Upon reaching 80% confluency, endothelial cells were treated as described above. Following reoxygenation, 20 μ l MTT solution (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) was added and incubated for 4 h at 37°C, followed by the removal of MTT solution. Subsequently, each well was supplemented with 150 μ l dimethyl sulfoxide (Sigma Aldrich; Merck KGaA). Following shaking on a shaking table for 10 min, the optical density (OD) value of each well was measured using a microplate reader at a wavelength of 490 nm. The experiment was repeated 3 times, and the mean OD value

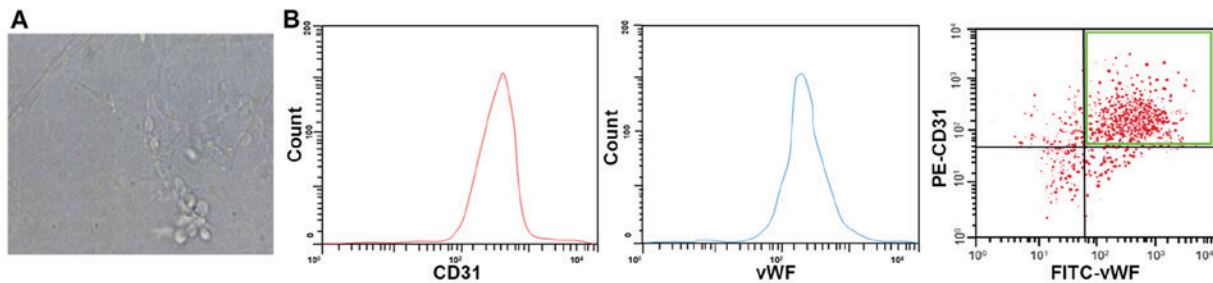


Figure 1. Cellular morphology of human venous malformation endothelial cells. (A) Observation of morphology of human venous malformation endothelial cells (original magnification, x200). (B) determination of the endovascular epithelial markers CD31, vWF, and CD31/vWF. CD, cluster of differentiation; vWF, von Willebrand factor; FITC, fluorescein isothiocyanate.

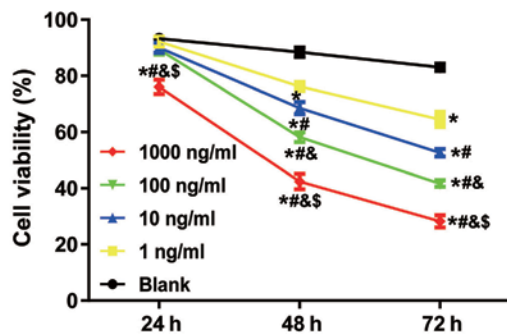


Figure 2. Comparison of the effects of rapamycin on the cell viability of human venous malformation endothelial cells at different concentrations and different time points. Comparisons among multiple groups were performed using two-way analysis of variance followed by a Tukey's post hoc test. * $P < 0.05$ vs. blank; $^{\#}P < 0.05$ vs. 1 ng/ml rapamycin; $^{\&}P < 0.05$ vs. 10 ng/ml rapamycin; $^{\$}P < 0.05$ vs. 100 ng/ml rapamycin.

was calculated. Cell viability was calculated as follows: $(\text{OD value}_{\text{experimental group}} - \text{OD value}_{\text{blank group}}) / \text{OD value}_{\text{blank group}}$.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) assay. Slides with attached cells were fixed in 4% (w/v) paraformaldehyde for 30 min at room temperature, rinsed in PBS, blocked with 0.3% H_2O_2 in methanol for 15 min at room temperature, and finally rinsed in PBS. Subsequently, slides were maintained in 0.1% Triton X-100 at 4°C for 20 min. The TUNEL assay (cat. no. C1098; Beyotime Institute of Biotechnology, Haimen, China) was performed according to the manufacturer's instructions. Slides were blocked with 3% bovine serum albumin (cat. no. C0225; Beyotime Institute of Biotechnology) at 37°C for 20 min, followed by 3 washes with PBS (5 min per wash). Subsequently, peroxidase solution was added, and the slides were incubated in a humidity chamber at 37°C for 30 min, followed by PBS washing 3 times (5 min/wash). Diaminobenzidine (0.3 ml) as a substrate was added as a chromogen, and the slides were incubated in a humidity chamber at 15–20°C for 10 min, followed by 3 washes with PBS (5 min per wash). Counterstaining was performed conventionally with hematoxylin at 37°C for 10 sec. Subsequently, ethanol-hydrochloric acid was used for differentiation, and ammonia was used to color the cells blue. The cells were mounted with 4% Paraformaldehyde Fix Solution (cat. no. P0099; Beyotime Institute of Biotechnology) and photographed. Normal endothelial cells were stained blue, and apoptotic cells were yellow and brown. Five fields of

vision were randomly selected. The apoptosis rate for a fixed area was calculated (apoptosis rate = apoptotic cells/total cells in a field).

Wound-healing assay. On the back of a 6-well plate, uniform horizontal lines at intervals of approximately 0.8 cm were drawn across the wells using a marker. Each well was crossed by at least 5 lines, and $\sim 5 \times 10^5$ cells were inserted into each well and grown to 100% confluency. The following day, a sterile 10- μl pipette tip, perpendicular to the horizontal lines on the back, was used to scratch wounds along a ruler. Importantly, the pipette tip was upright, rather than slanted. Following wound scratching, the cells were rinsed gently with PBS 3 times. Then, PBS was added along the wall, and the scratched cells were rinsed and removed. The endothelial cells were incubated with culture medium in a 5% CO_2 incubator at 37°C. Samples were taken at 48 h and then photographed. The rate of wound closure was calculated by measuring the distance between the two wound edges at 0 h and at 48 h. The experiment was repeated 3 times, and the mean value was calculated.

Statistical analysis. SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used to analyze data in the present study. Measurement data are presented as the mean \pm standard deviation. Comparisons among multiple groups were performed using one-way or two-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Observation and determination of human VM endothelial cells. The cellular morphology of separated human VM endothelial cells was observed under an inverted microscope. When the separated endothelial cells grew adherent to the wall, they were enlarged to different degrees and grew in clusters, resembling paving stones, with clear, homogeneous cytoplasm and a round or oval nucleus (Fig. 1A). For accurate identification of endovascular epithelial cells separated from the deformed vein of a human VM, flow cytometry was performed to evaluate a more specific marker for endovascular epithelial cells, CD31 and vWF, and it was demonstrated that there were 92.5% CD31-positive cells, 88.2% vWF-positive cells, and 76.3% CD31- and vWF-positive cells (Fig. 1B).

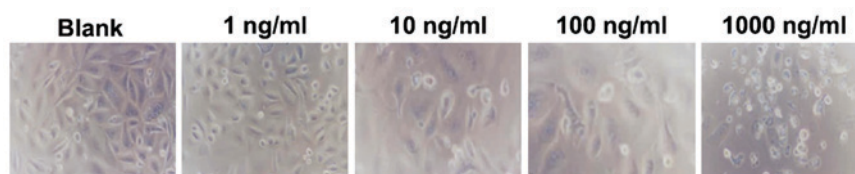


Figure 3. Comparison of the effects of rapamycin on cellular morphology of human venous malformation endothelial cells at different concentrations. Cellular morphology of human venous malformation endothelial cells treatment with increasing concentrations of rapamycin (original magnification, x400).

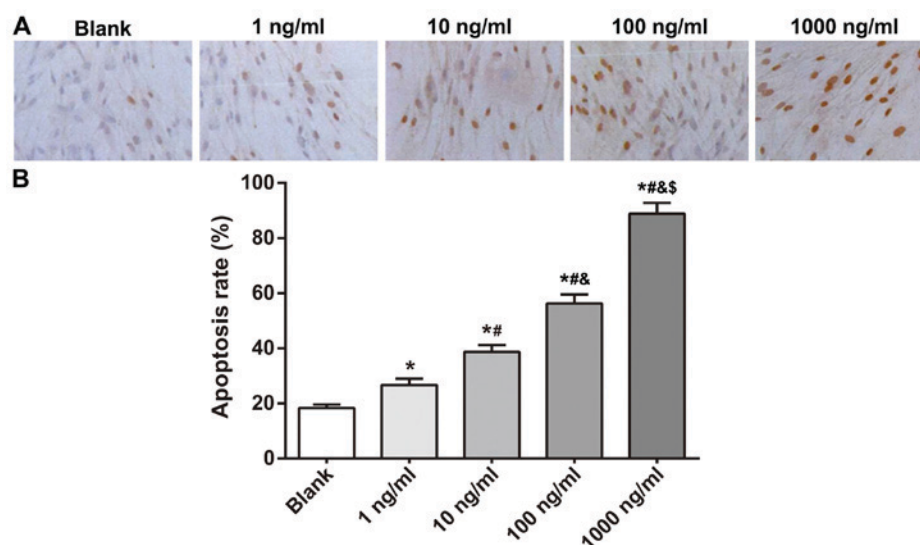


Figure 4. Comparison of the effects of rapamycin on cell apoptosis of human venous malformation endothelial cells at different concentrations. (A) Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling staining to evaluate cell apoptosis (original magnification, x400). (B) The apoptosis rate of cells among different groups. Comparisons among multiple groups were performed using one-way analysis of variance followed by a Tukey's post hoc test. * $P < 0.05$ vs. blank; # $P < 0.05$ vs. 1 ng/ml rapamycin; & $P < 0.05$ vs. 10 ng/ml rapamycin; \$ $P < 0.05$ vs. 100 ng/ml rapamycin.

Rapamycin inhibited the cell viability of human VM endothelial cells. An MTT assay was performed to detect the cell viability of human VM endothelial cells exposed to rapamycin (Fig. 2). Rapamycin significantly inhibited the proliferation of human VM endothelial cells at 48 and 72 h. Higher concentration of rapamycin induced greater inhibitory effects. However, only rapamycin at a concentration of 1,000 ng/ml significantly inhibited the viability of human VM endothelial cells at 24 h, suggesting that the inhibitory effects of rapamycin on human VM endothelial cells were concentration- and time-dependent. Therefore, 48 h following incubation was determined to be the optimal reaction time.

Effects of rapamycin on cellular morphology of human VM endothelial cells. An inverted microscope was used to observe the morphological changes of human VM endothelial cells exposed to rapamycin at different concentrations (Fig. 3). At 48 h, when compared with the blank group, treatment with rapamycin at a concentration of 1 ng/ml produced a few shrunken cells and a reduced cell number; however, the majority of cells exhibited a clear boundary and a regular shape. Treatment with rapamycin at 10 and 100 ng/ml reduced the number of cells even further and caused some cells to become round and desquamated; treatment with rapamycin at 1,000 ng/ml produced more obvious desquamation, round-shaped cells and necrosis.

Rapamycin enhanced apoptosis conditions in human VM endothelial cells. A TUNEL assay was conducted to determine the apoptosis conditions of human VM endothelial cells exposed to rapamycin at different concentrations (Fig. 4). In comparison with the blank group, apoptosis of human VM endothelial cells was significantly elevated when treated with rapamycin at 1, 10, 100 and 1,000 ng/ml. Higher concentrations yielded greater numbers of apoptotic endothelial cells, indicating that rapamycin treatment promoted the apoptosis of human VM endothelial cells.

Rapamycin inhibited the migration capacity of human VM endothelial cells. A wound-healing assay further verified the inhibitory effects of rapamycin on the migration capacity of human VM endothelial cells (Fig. 5). Compared with the blank group, rapamycin significantly suppressed the migration capacity of human VM endothelial cells at different concentrations. Higher concentrations yielded greater inhibitory effects.

Discussion

VMs damage the appearance and circulatory functioning of patients and even produce deadly bleeding and respiratory obstruction (3). Extensive VMs require lifelong treatment and may persist or become exacerbated (7,22). Rapamycin has already been reported in clinical trials to serve as an effective treatment for cancer, vascular restenosis and immune rejection

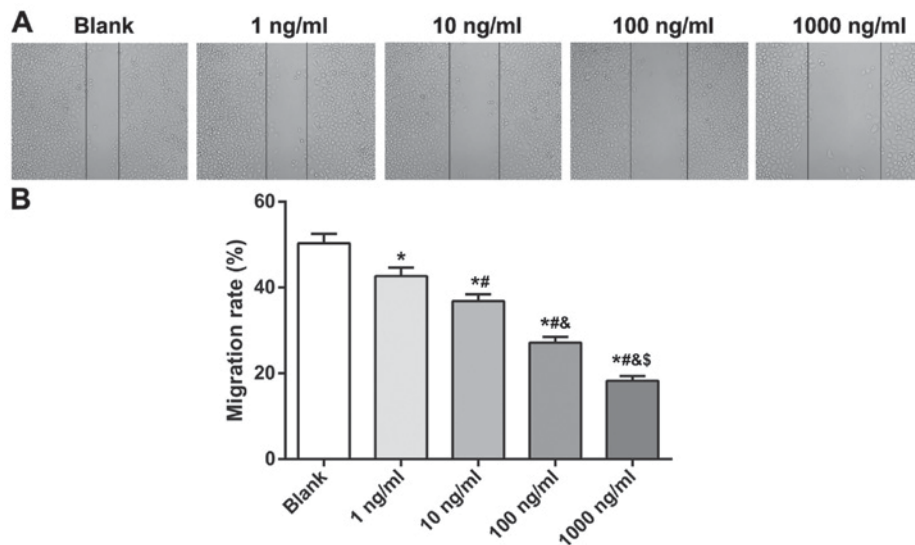


Figure 5. Comparison of the effects of rapamycin on cell migration of human venous malformation endothelial cells at different concentrations. (A) The migration ability of cells detected by wound-healing assay (original magnification, x200). (B) Migration rate of cells among different groups. Comparisons among multiple groups were performed using one-way analysis of variance followed by a Tukey's post hoc test. * $P<0.05$ vs. blank; # $P<0.05$ vs. 1 ng/ml rapamycin; & $P<0.05$ vs. 10 ng/ml rapamycin; \$ $P<0.05$ vs. 100 ng/ml rapamycin.

of transplanted organs (23,24). The aim of the present study was to explore the biological behaviors of human VM endothelial cells and their responses to rapamycin, which may be significant in the design of effective strategies for human VM treatment.

Initially, endothelial cells of human VM were separated and identified by detecting the positive expression of CD31 and vWF. CD31 and vWF are widely acknowledged as a specific marker for human endothelial cells (25); therefore, the strong positive expression of these markers confirmed the successful isolation of human VM endothelial cells. Endothelial cells secrete cytokines and adhesion molecules during the process of inflammation; additionally, accumulation of neutrophils within vascular endothelia is associated with the inflammatory process (2). Endothelial cells are also considered to be markers of endothelial dysfunction and endothelial injury (26). The number of proliferating endothelial cells and vascular SMCs is rather limited in normal vascular tissues, whereas a physiological or pathological stimulus can stimulate endothelial cells and SMCs to progress in the cell cycle (27). More specifically, it has been noted in a previous study that endothelial cells separated from VMs best represent the biological characteristics of VMs (4).

The cell viability of endothelial cells treated with rapamycin was determined by MTT assay at 24, 48 and 72 h following culture. The results indicated that rapamycin suppressed proliferation of human VM endothelial cells. As an mTOR inhibitor, rapamycin has been hypothesized to block the progression of cell cycle from G₁ phase to S phase by inhibiting p70 ribosomal protein S6 kinase (p70S6K) (13,28). mTOR, as a threonine kinase, phosphorylates S6K1 and 4E-BP1, and promotes transcription of key mRNAs associated with the progression of the cell cycle from G₁ phase to S phase (29,30). Through its interaction with p70S6K, it is associated with cell growth, proliferation and differentiation by regulating ribosome biogenesis, protein synthesis, cell cycle progression and metabolism (31). Consistent study demonstrates that rapamycin significantly represses the proliferation and

migration of hemangioma, and reduces vascular tumor growth by suppressing the mTOR complex, which regulates cell mass and cell number. In addition, it has been revealed that rapamycin delays the aging process of mice by attenuating the degeneration of the liver and the heart, reducing the proliferation of adrenal gland lesions and altering tendon elasticity (32). Via its anti-proliferative properties, rapamycin reduces the cell viability of lymphocytes, vascular endothelial cells and SMCs and decreases the fibro-proliferative responses in vascular injuries (33).

The migration capacity of human VM endothelial cells was demonstrated to be suppressed following treatment with rapamycin. At the same time, apoptosis conditions in human VM endothelial cells were enhanced, as detected using flow cytometry. These findings are corroborated by the results of Liu *et al* (34), which demonstrated that rapamycin suppressed reendothelialization by attenuating the migration and promoting the apoptosis of endothelial cells following percutaneous coronary intervention. Inhibition of mTOR by rapamycin, has been revealed to repress the activity of vascular endothelial cell growth factor (VEGF) by inhibiting VEGF signal transduction and synthesis (35). Notably, VEGF is a crucial modulator for endothelial cell migration and angiogenesis (36). Barilli *et al* (37) demonstrated that rapamycin could damage cell viability and reduce migration of human endothelial cells through inhibition of mTOR complex 2 (mTORC2). A previous study by Zhu *et al* (38) indicated that rapamycin inhibits proliferation and migration of human vascular SMCs, preventing arteriovenous grafts in hemodialysis patients. The present study demonstrated that the effects of rapamycin on the biological behaviors of human VM endothelial cells were concentration- and time-dependent. Similarly, rapamycin has been demonstrated in an *in vitro* study to suppress the migration and promote the apoptosis of aortic endothelial cells in rats in a time- and dose-dependent manner (39). Rapamycin has been reported to decrease further expansion of VMs in mouse models, and ameliorate such symptoms and signs as

pain, lesion size, bleeding and functional impairment in patients with VMs (40).

Altogether, evidence has been provided in this current study to suggest that rapamycin inhibits the proliferation and migration of human VM endothelial cells and accelerates their apoptosis. Furthermore, the effects of rapamycin exerted on human VM endothelial cells were concentration-dependent. However, the use of rapamycin may have adverse effects; therefore, further studies are required to investigate the safety and risk factors presented by the use of rapamycin in the treatment of VM.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

HJ and YS conceived of the study, designed the study, and wrote and reviewed the manuscript. YS designed the study. HC, RX and WZ acquired the data. TX, XS and YF analyzed and interpreted the data. HJ supervised the study.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Affiliated Stomatological Hospital of Nanjing Medical University and written informed consent was obtained from each subject.

Patient consent for publication

Written informed consent was obtained from each subject.

Competing interests

The authors declare that they have no competing interests.

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