Increase of Angiogenesis and Osteogenesis by Local Application of Simvastatin Depends on VEGF

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Abstract

Aims

Simvastatin stimulates both BMP-2 and VEGF expression, but it is unknown which is more important for bone formation. This study was undertaken to determine whether these effects could be blocked by the anti-VEGF antibody bevacizumab.

Methods

60 Sprague–Dawley male rats were randomly divided into five groups (n = 12 per group): normal control; simvastatin 0 mg or 0.5 mg; and bevacizumab with simvastatin 0 mg or 0.5 mg. Simvastatin groups were administered intraosseous injections of simvastatin delivered by thermosensitive poloxamer. Bevacizumab groups were given bevacizumab intraperitoneally or the same volume of saline. Serum samples were collected before the treatment and every two weeks thereafter. Four weeks after the treatment, four rats randomly selected from each group were subjected to Microfil® perfusion. The remaining eight rats was evaluated with dual energy X-ray absorptiometry (DXA) and micro-computed tomography (μCT). Four specimens (left tibias) were randomly selected from each group for undecalcified histology, the other four specimens selected for Western blot to analyse the changes of expression of BMP-2.

Results

local injection of simvastatin significantly increased bone formation. Microfil® perfusion showed that there were more vessels both in the bone marrow and around the bone after a single-dose simvastatin injection. Western blot analysis also confirmed that the expression levels of BMP2 were significantly higher in the simvastatin-treated groups compared with the control group. Compared with the simvastatin group, bevacizumab blunted the simvastatin-induced increase in bone mass and angiogenesis.

Conclusion

The anabolic effect of simvastatin on bone formation is through VEGF-related mechanisms.

Introduction

Statins play an important role in hyperlipidemia treatment as the first-line drug for the prevention of cardio-cerebrovascular events. Mundy first reported that statins induce bone morphogenetic protein 2 (BMP-2) expression in bone cells and promote bone formation(1). We have found that simvastatin also induces high BMP-2 expression in bone marrow stromal cells, stimulating osteoblastic differentiation and

enhancing bone formation(2). Furthermore, we found that the osteogenic effect of simvastatin could not be blocked by a neutralizing BMP-2 antibody(3). The anabolic effect of statins on bone formation cannot be completely explained by the induction of BMP-2.

Bone is a highly vascularized tissue, and as angiogenesis is crucial for bone regeneration, osteogenesis and angiogenesis are closely coupled(4). A key candidate regulator of osteoblasts and endothelial cell communication is vascular endothelial cell growth factor (VEGF), a known pro-angiogenic factor with well-established actions on endothelial cells that is required for bone development(5, 6). Simvastatin enhances VEGF production, promotes angiogenesis and ameliorates impaired wound healing(7). The local application of simvastatin at fracture sites successfully induces fracture union via promoting angiogenesis and osteogenesis(8). We also found that the local application of simvastatin improves bone defect healing, inducing a high expression of VEGF and BMP-2 and promoting osteogenesis and angiogenesis(9).

Bone formation is a coordinated process involving BMPs and VEGFs in angiogenesis and osteogenesis(10). BMPs and VEGFs combined, used as osteogenic and angiogenic factors, act synergistically toward bone regeneration(11). Simvastatin stimulates both BMP-2 and VEGF expression, but it is unknown which is more important for bone formation. We hypothesized that VEGF-mediated angiogenesis is required for osteogenesis stimulated by simvastatin. This study was undertaken to determine whether these effects could be blocked by the anti VEGF-antibody bevacizumab.

Materials And Methods

Preparation of the injectable simvastatin/poloxamer 407 hydrogel

Poloxamer 407 (22.5% w/w) and poloxamer 188 (2.5% w/w) (all from BASF, Ludwigshafen, Germany) were added to isotonic phosphate-buffered saline (PBS, pH 7.4, 4 °C) with gentle mixing until complete dissolution(12). Simvastatin (National Institute for Food and Drug Control, Beijing, China) was added to the prepared poloxamer solutions, and the final concentrations of simvastatin were 0, 0.5 mg/mL.

Animals and treatments

In total, 60 Sprague–Dawley male rats, aged 12 weeks (380–400 g) were randomly divided into five groups (n = 12 per group): normal control; simvastatin 0 mg or 0.5 mg; and bevacizumab with simvastatin 0 mg or 0.5 mg. The rats were anesthetized with 10% chloral hydrate (3.3 mL/kg, i.p.), 100 μL of the poloxamer gel with 0 or 0.5 mg of simvastatin was injected into the left tibia via the tibial plateau. Rats were administered intraperitoneal injections of bevacizumab (Bev, Avastin®, Roche, France. 0.70 mg/kg/d) or the same volume of saline according the experimental design(13).

Serum samples were collected through the orbital sinus before the treatment and every two weeks thereafter, centrifuged at 3000 rpm for 15 min and stored at -80 °C for the determination of serum

osteocalcin (OCN), VEGF and tartrate-resistant acid phosphatase (TRAP) by enzyme-linked immunosorbent assay (ELISA).

Four weeks after the treatment, four rats randomly selected from each group were subjected to Microfil® perfusion. Prior to sacrifice, the abdominal aorta was cannulated and infused with heparinized saline, followed immediately by perfusion with 10 mL of Microfil® (42% of MV-122 yellow, 53% of diluent solution and 5% of curing agent; Flow-Tech, Carver, MA, USA) as described previously(14) and stored overnight at 4 °C to allow the Microfil® compound to polymerize. Each sample was carefully dissected prior to fixing in 10% neutral buffered formalin.

The remaining eight rats in each group were euthanized by overdose of anesthesia, and the left tibias were excised. After evaluation with dual energy X-ray absorptiometry (DXA) and micro-computed tomography (μCT), four specimens were randomly selected from each group for undecalcified histology, the other four specimens selected for Western blot to analyze the changes of expression of BMP-2.

Dual energy X-ray absorption (DXA)

Bone mineral density (BMD) of the tibias was measured by DXA using a small-animal high-resolution collimator (Discovery™, Hologic Inc., Boston, MA, USA). In order to measure the same area of different femurs, each specimen was aligned and positioned identically during the scan, and the ROIs were set 0.5 cm away from growth plate(9).

Bone microstructures assessment by micro-CT

After the DXA scanning, the specimens were placed in a sample holder with PBS and scanned using μCT (Inveon, Siemens Medical Solutions USA, Inc., IL, USA) at a 36-μm spatial resolution, 80-kV voltage, 500-μA current, 900-ms integration time, and 360 projections per 360°. The volumes of interest (VOI) were determined as the trabecular compartment 5 mm x 5 mm starting adjacent to the proximal tibia epiphyseal plate. Bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were calculated.

Mineral apposition rates (MARs)

To monitor MARs, double fluorochrome labels were conducted by calcein green (Sigma, St. Louis, MO, USA; 8 mg/kg) and alizarin red (Sigma, St. Louis, MO, USA; 20 mg/kg) injected via the tail vein on days 7 and 21(14, 15).

Four specimens (left tibias) randomly selected from each group were fixed in 10% neutral buffered formalin, dehydrated in increasing gradients of alcohol and embedded in methylmethacrylate resin. Undecalcified sections of 30 μm were cut and ground perpendicular to the long axis of the bone (EXAKT Cutting & Grinding System, Germany). MARs were calculated under fluorescence microcopy (Leica DM3000, Germany) by measuring the mean distance between the two fluorescent labels divided by 14 days (the time interval between injections)(16).

Vascularity

Specimens with Microfil® perfusion were scanned and analyzed by μCT as described previously(14). After μCT analysis of bone microstructures, tibias were decalcified in a mixture of 4% formic acid in 10% formalin. The decalcified samples were re-analyzed to obtain the vascular system. VOI was defined from the growth plate extending 10 mm below the diaphysis and was measured in 3D to characterize vascular differences between the five groups. Vessel parameters, including vessel volume (VV), vessel volume per tissue volume (VV/TV), vessel number (V.N), vessel thickness (V.Th) and vessel surface area (V.SA), were calculated.

Enzyme-linked immunosorbent assay (ELISA)

A 96-well microplate was precoated with polyclonal antibody for rat OCN or VEGF according to the protocol; then, 50 mL of assay diluent and 50 mL of samples were added. After incubation for 2 h at room temperature, the wells were washed, and anti-OCN and anti-VEGF conjugated to horseradish peroxidase were added. Incubation was continued for 2 h, and the plates were washed; substrate solution was added to each well and incubated for 30 min. The value of O.D. at 450 nm was measured using a micro-Quant microplate spectrophotometer (Varioskan Flash, Thermo Electron, Finland).

As a marker for bone resorption, the activity of TRAP, which is normally produced only by osteoclasts, was measured using a colorimetric assay(17).

Western blot

The lyophilized samples were crushed by a Mixer Mill MM400 (Retsch technology, Germany). The powdered samples were added to RIPA lysis buffer. After centrifugation at 14,000 g for 15 min, the supernatant was collected, and protein concentrations were determined with the BCA assay (Biyuntian Biological Co., Ltd., Shanghai, China). Equal amounts of proteins were separated by 10% SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes that were blocked and incubated with rabbit polyclonal anti-BMP2 and β-actin (Santa Cruz biotechnology) according to standard protocols. Then, the membranes were re-probed with secondary antibodies conjugated with horseradish peroxidase for 1 h. The blots were processed using an ECL kit (Santa Cruz Biotechnology) and exposed to film. Images were obtained using the Odyssey Infrared Imaging System from LI-COR Biosciences.

Statistical analyses

All data are expressed as the mean ± SD. Statistical analyses were performed using SPSS16.0 (Chicago, IL, USA). One-way ANOVA was conducted to assess differences among groups, followed by least significant difference (LSD) tests. $P < 0.05$ was considered significant.

Results

The minimally invasive injections were successful in all animals. No signs of infection or significant side effects or morbidity associated with treatment were observed in any of the animals, and all the rats

survived.

BMD

The BMD of the simvastatin 0.5 mg group (0.266 ± 0.01 g/cm^2) was significantly higher than that of the normal control (NC; 0.233 ± 0.011 g/cm^2) and vehicle (0.234 ± 0.013 g/cm^2) groups (Table 1, P < 0.01). While the BMD value in the bevacizumab with simvastatin (0.5 mg) group (0.229 ± 0.008 g/cm^2) was substantially lower than that of the simvastatin 0.5 mg group (Fig. 1, P < 0.01), it was similar to the BMD values in the NC and vehicle groups (Table 1, P > 0.05).

Table 1
BMD and quantitative μCT analysis of microstructure parameters.

	NC	Bevacizumab		No- Bevacizumab	
		Sim 0 mg	Sim 0.5 mg	Sim 0 mg	Sim 0.5 mg
BMD (g/cm^2)	0.23 ± 0.01	0.23 ± 0.01$^{\$\$}$	0.23 ± 0.01$^{\#\#}$	0.23 ± 0.01	0.27 ± 0.01*
BV/TV (%) (%)	0.24 ± 0.02	0.28 ± 0.02$^{\$\$}$	0.22 ± 0.10$^{\#\#}$	0.24 ± 0.04	0.38 ± 0.06*
TbTh (mm)			140.92 ± 14.11	157.41 ± 10.47**	164.01 ± 7.99**
Tb.Th (μm) (μm)	0.07 ± 0.01	0.07 ± 0.01$^{\$\$}$	0.08 ± 0.01$^{\#\#}$	0.06 ± 0.01	0.09 ± 0.01*
Tb.N (/mm) (/mm)	3.18 ± 0.21	3.79 ± 0.45	3.23 ± 1.28$^{\#\#}$	3.09 ± 0.21	3.98 ± 0.41*
Tb.Sp (mm) (mm)	0.24 ± 0.02	0.18 ± 0.01	0.25 ± 0.09$^{\#\#}$	0.25 ± 0.02	0.16 ± 0.03*

The values are the mean ± SD, n = 8 in each group. $^{*}P$< 0.05, vs. the NC and Sim 0-mg groups. $^{**}P$< 0.01, vs. the NC and Sim 0-mg group; $^{\$}P$< 0.05, vs. the Sim 0.5 mg group. $^{\$\$}P$< 0.01, vs. the Sim 0 group. $^{\#}P$< 0.05, vs. the Sim 0.5 mg group. $^{\#\#}P$< 0.01, vs. the Sim 0.5 mg group.

Bone microstructures

The μCT imaging also confirmed that simvastatin augmented bone formation and increased bone microstructure (Fig. 1). Quantitative analysis revealed that simvastatin significantly increased BV/TV, Tb.Th, Tb.N and decreased Tb.Sp (Table 1, P < 0.01).

Anti-VEGF bevacizumab treatment completely blunted the osteogenic effect induced by simvastatin (Table 1, P < 0.01). Interestingly, bevacizumab (i.p., 0.70 mg/kg/d) for 4 weeks did not influence BV/TV, but decreased Tb.Th and Tb.Sp (Table 1).

Mineral apposition rates (MARs)

Images with areas labeled in green and red fluorescence represent regions of mineralization labeled by double fluorochrome at different times (Fig. 2). The MARs of the simvastatin 0.5 mg (3.34 ± 0.42 μm/d) group were significantly higher than those of the NC group (0.62 ± 0.06 μm/d) and simvastatin 0 mg group (0.44 ± 0.08 μm/d) ($P < 0.01$; Table 2). Bevacizumab completely blunted the rise in MARs induced by simvastatin.

Table 2
Effect of bevacizumab and simvastatin on MARs

	NC	Bevacizumab		No- Bevacizumab	
		Sim 0 mg	Sim 0.5 mg	Sim 0 mg	Sim 0.5 mg
MARs (μm/day)	0.62 ± 0.06	$0.56 \pm 0.19^{\$\$}$	$0.82 \pm 0.15^{\#\#}$	0.44 ± 0.08	$3.34 \pm 0.42^{**}$

The values are the mean ± SD, n = 4 in each group. $^{**}P < 0.01$, vs. the NC and Sim 0-mg group; $^{\$\$}P < 0.01$, vs. the Sim 0 group. $^{\#\#}P < 0.01$, vs. the Sim 0.5 mg group.

Vascularity

Four weeks after single injection, more external bone vessels were found in the simvastatin groups than in the control (Fig. 3a). Quantitative analysis revealed that single, local simvastatin injection significantly increased VV, VV/TV, V.N, V.Th and V.SA ($P < 0.01$, Table 3) for internal bone vessels (Fig. 3b). In addition, bevacizumab completely blunted the rise in internal and external bone vessels induced by simvastatin.

Table 3
Quantitative μCT analysis of vascularity.

	NC	Bevacizumab		No- Bevacizumab	
		Sim 0 mg	Sim 0.5 mg	Sim 0 mg	Sim 0.5 mg
VV (m^3)	4.78 ± 0.35	$2.50 \pm 0.44^{\$\$}$	$3.20 \pm 0.37^{\#\#}$	4.36 ± 0.70	$17.25 \pm 0.79^{**}$
VV/TV (%)	0.02 ± 0.02	$0.01 \pm 0.01^{\$\$}$	$0.01 \pm 0.01^{\#\#}$	0.25 ± 0.01	$0.11 \pm 0.02^{**}$
V.N (/mm)	0.35 ± 0.05	$0.12 \pm 0.07^{\$\$}$	$0.12 \pm 0.04^{\#\#}$	0.19 ± 0.03	$2.69 \pm 0.50^{**}$
V.Th (mm)	0.12 ± 0.01	$0.11 \pm 0.03^{\$\$}$	$0.12 \pm 0.02^{\#\#}$	0.12 ± 0.01	$0.25 \pm 0.01^{**}$
V.Sp (mm)	7.42 ± 2.22	$14.39 \pm 1.84^{\$\$}$	$15.36 \pm 0.46^{\#\#}$	6.64 ± 1.25	$0.39 \pm 0.13^{**}$

The values are the mean ± SD, n = 4 in each group. $^{**}P < 0.01$, vs. the NC and Sim 0-mg group; $^{\$\$}P < 0.01$, vs. the Sim 0 group. $^{\#\#}P < 0.01$, vs. the Sim 0.5 mg group.

Bone turnover biomarkers and VEGF in the serum

Serum OCN levels were significantly higher in the simvastatin groups than in the control groups at 2 and 4 weeks ($P < 0.01$), and bevacizumab blocked the increase of OCN secretion induced by simvastatin (Fig. 4).

Simvastatin significantly increased the level of VEGF in the serum at 4 weeks compared with that in the other groups ($P < 0.01$, Fig. 4), while bevacizumab almost abolished the increase of VEGF induced by simvastatin ($P < 0.01$, Fig. 4).

At 2 weeks, the serum TRAP activity in the simvastatin 0.5 mg group were significantly higher than that of all the other groups ($P < 0.01$, Fig. 4). However, there were no significant differences among all groups at 4 weeks, as TRAP activity in the simvastatin 0.5 mg group decreased.

Expression of BMP2

Western blot analysis of BMP2 demonstrated that these proteins were expressed at significantly higher levels in the simvastatin-treated groups than in the control group. In contrast, BMP-2 expression induced by simvastatin was inhibited in the presence of bevacizumab (Fig. 5).

Discussion

Bone is a highly vascularized tissue, and angiogenesis is crucial for bone formation(18). Bone formation and angiogenesis are closely associated during bone development(5, 19) and fracture healing(20). Insufficient bone vascularity results in decreased bone formation and mass. During bone formation and fracture healing, there is crosstalk between endothelial cells and osteoblasts, which are tightly coupled(21). VEGF and BMPs play important role during angiogenesis and osteogenesis(22).

BMP signaling is required for both bone development and angiogenesis(23). BMPs induce angiogenesis via osteoblast-derived VEGF(24) and play a central role in bone regeneration and neovascularization(22, 25). VEGF-A, generally called VEGF, is a key regulator of angiogenesis and differentiation of endothelial cells. Blocking VEGF activity inhibits VEGF-stimulated angiogenesis but has no effect on osteoblast differentiation(24). VEGF expression is induced by most osteoinductive factors(26), such as prostaglandins(27), exercise(13) and intermittent PTH(28). An anti-VEGF antibody fully prevented bone vascular events and bone formation induced with treadmill training(13), PTH(28) or BMP-2(29). These factors stimulating bone formation might be mediated, at least partly, by VEGF. Furthermore, VEGF can promote osteogenesis by directing the function of osteo-related cells(30, 31).

Statins have been reported to amplify angiogenesis in stroke(31), increase capillary density in chronic ischemic heart(32), and accelerate wound recovery by promoting angiogenesis(7). Many reports have shown the therapeutic effect of statins on bone formation and fracture healing(1, 8). However, the primary target organ for statins is the liver; less than 5% of orally administered statin reach the circulation, and the concentration is even lower in bone(33). Local use might increase its bioavailability for bone formation. It has been found that local application of statins increases bone defect(34) and fracture healing(8), and promotes angiogenesis and osteogenesis(14). Our team has developed a method

to locally administrate simvastatin by thermosensitive hydrogel, and found that osteogenesis remained active at least 3 months after local injection(12). Also we found that the local application of simvastatin induced both BMP-2 and VEGF expression, which are important cytokines for bone formation(9, 14, 15). However, in our previous study, we found that the ALP activity induced by simvastatin could not be blocked by a neutralizing BMP-2 antibody(3), which indicates that the bone augmentation induced by simvastatin could not be completely explained by the up-regulation of BMP-2. So we designed this research to determine whether these effects could be blocked by bevacizumab (anti-VEGF).

In this study, we found that local injection of simvastatin significantly increased BMD, improved bone microstructures, accelerated mineral apposition rates and enhanced serum levels of OCN, TRAP and VEGF. Furthermore, Microfil® perfusion and μCT scanning showed that there were more vessels both in the bone marrow and around the bone after a single-dose simvastatin injection. Western blot analysis also confirmed that the expression levels of BMP2 were significantly higher in the simvastatin-treated groups compared with the control group. Bevacizumab is a monoclonal antibody against VEGF-A; binding to VEGF-A results in the prevention of VEGFR activation and the subsequent signaling cascades. Based on our results, we found bevacizumab significantly decreased the BMP-2 expression induced by simvastatin in the bone tissue. Along with the μCT, mineral apposition rates and Microfil® perfusion tests, we could conclude that bevacizumab blunted the angiogenesis and osteogenesis effects induced by simvastatin completely.

In summary, simvastatin promotes bone formation via inducing angiogenesis with VEGF involved in this process.

Abbreviations

BMP-2
Bone Morphogenetic Protein-2
VEGF
Vascular Endothelial Growth Factor
DXA
Dual Energy X-Ray Absorptiometry
μCT
Micro-Computed Tomography
OCN
Osteocalcin
TRAP
Tartrate-Resistant Acid Phosphatase
ELISA
Enzyme-Linked Immunosorbent Assay
BMD
Bone Mineral Density

VOI
Volumes of Interest
BV/TV
Bone volume/tissue volume
Tb. N
Trabecular Number
Tb. Th
Trabecular Thickness
Tb. Sp
Trabecular Separation
MARs
Mineral Apposition Rates
VV
Vessel Volume
VV/TV
Vessel Volume Per Tissue Volume
V. N
Vessel Number
V. Th
Vessel Thickness
V. SA
Vessel Surface Area
LSD
Least Significant Difference
PTH
Parathyroid Hormone

Declarations

Ethics approval and consent to participate:

The Ethics Committee of Peking University Third Hospital approved all animal experimental protocols.

Consent for publication:

Not applicable

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests

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Authors' Contributions

JT: Designed the research, completed the animal study, analyzed the statistics, wrote and reviewed the manuscript.

HW: Completed the animal study, reviewed the manuscript

GHD: Completed the animal study, reviewed the manuscript

HJL: reviewed the manuscript

CLS: Designed the research, analyzed the statistics, wrote and reviewed the manuscript.

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Figures

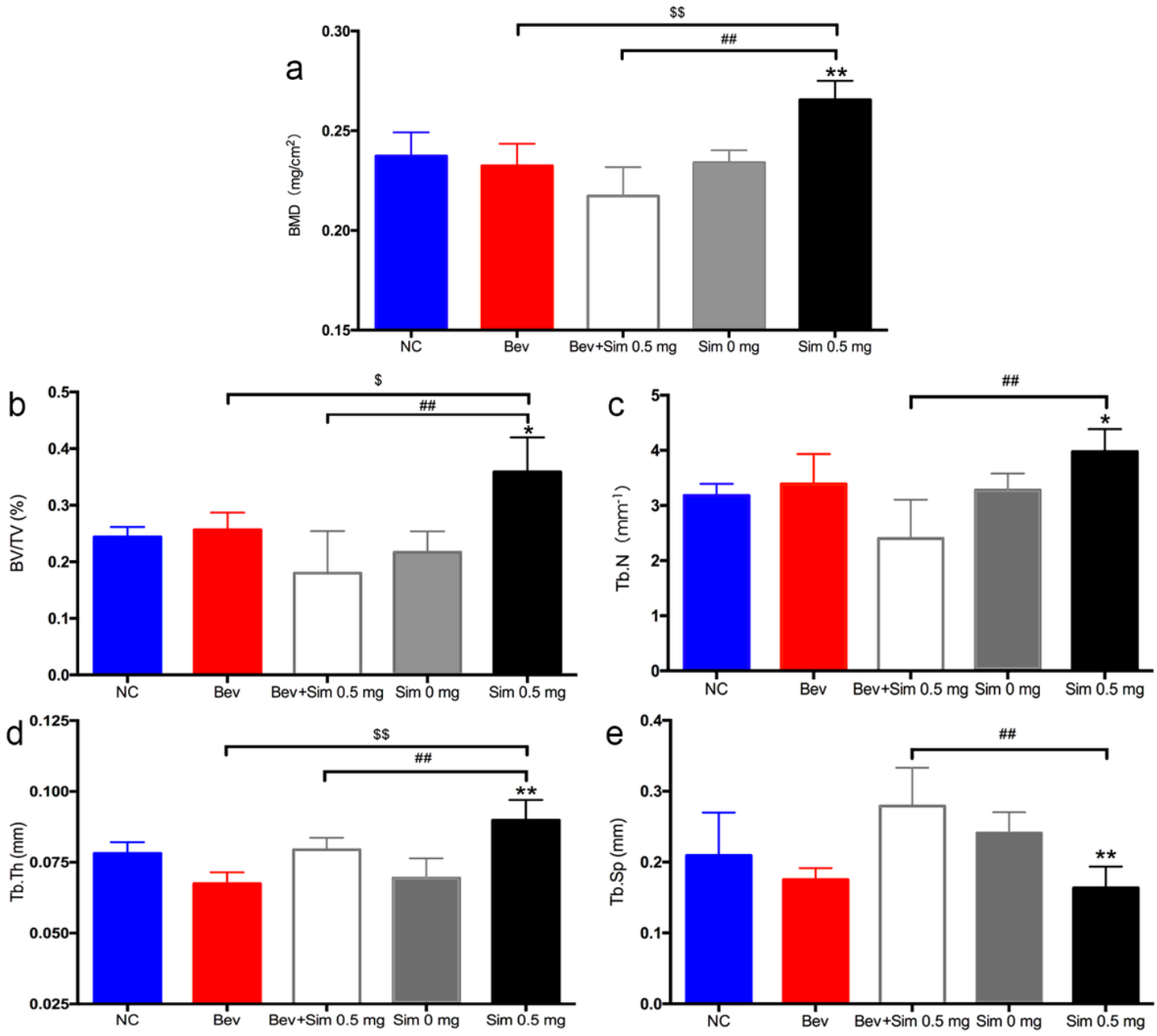

Figure 1

Figure 1

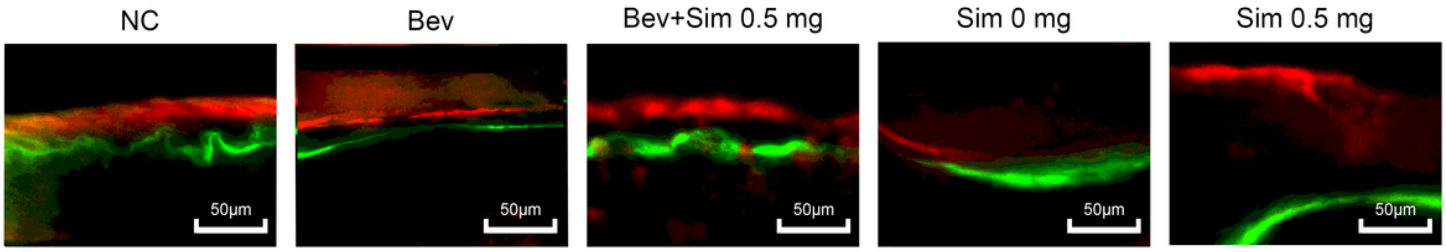

Figure 2

Figure 2

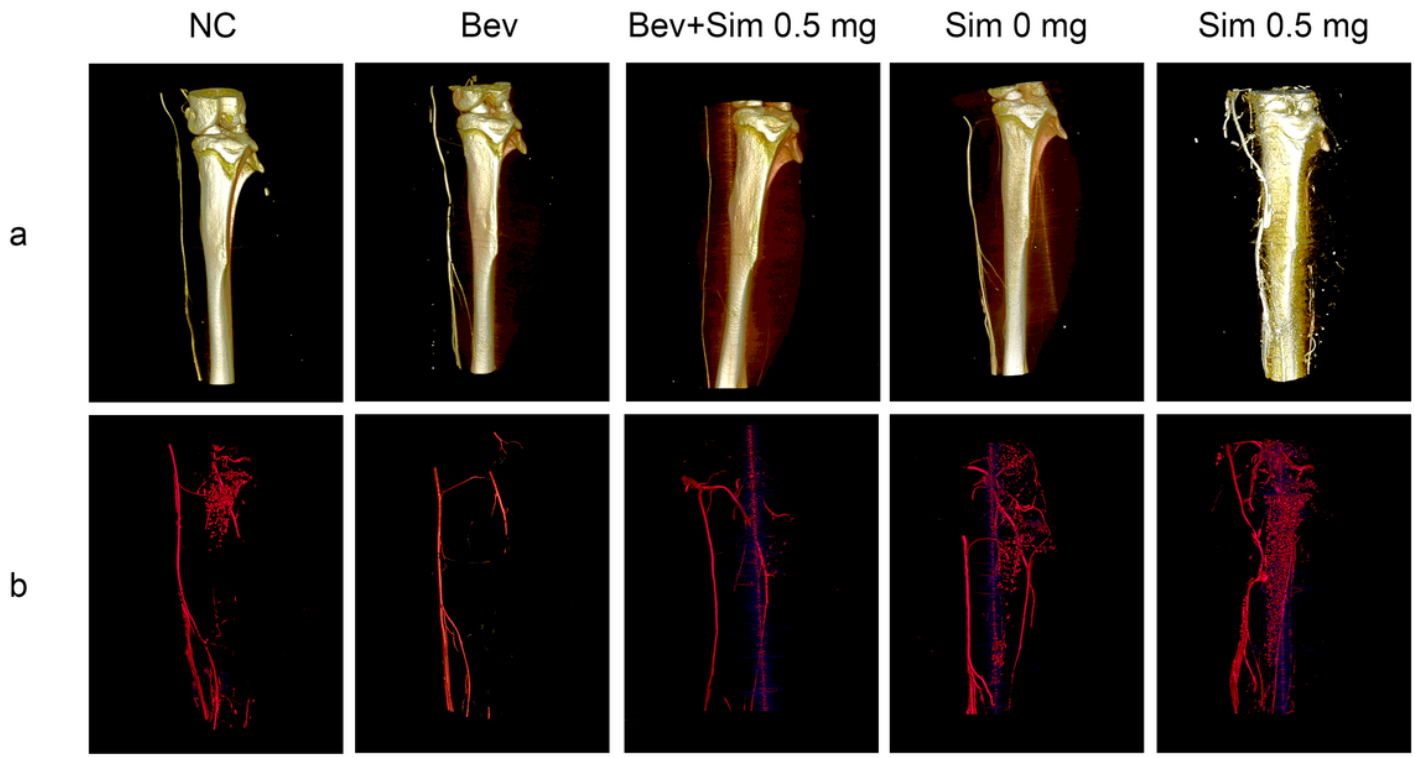

Figure 3

Figure 3

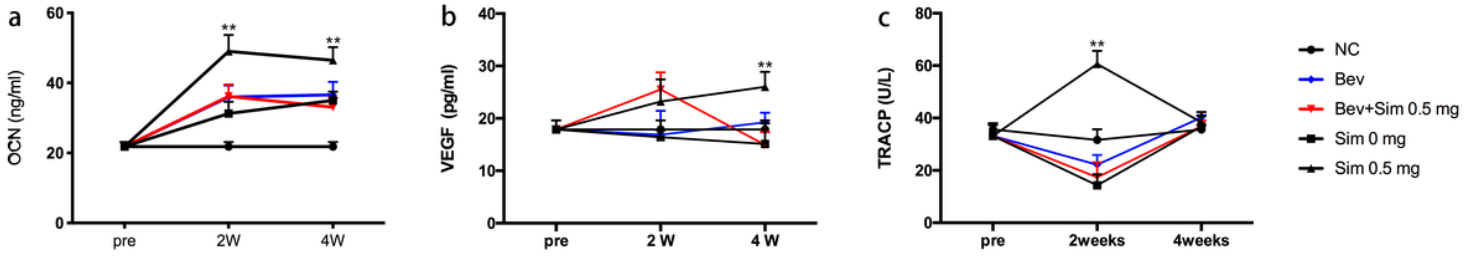

Figure 4

Figure 4

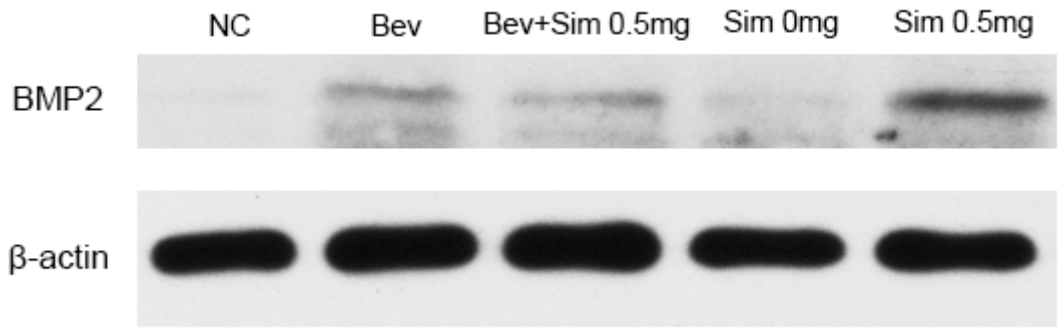

Figure 5

Figure 5