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Single-Dose Local Simvastatin Injection Improves Implant Fixation via Increased Angiogenesis and Bone Formation in an Ovariectomized Rat Model

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Background: Statins have been reported to promote bone formation. However, taken orally, their bioavailability is low to the bones. Implant therapies require a local repair response, topical application of osteoinductive agents, or biomaterials that promote implant fixation.

Material/Methods: The present study evaluated the effect of a single local injection of simvastatin on screw fixation in an ovariectomized rat model of osteoporosis.

Results: Dual-energy X-ray absorptiometry, micro-computed tomography, histology, and biomechanical tests revealed that 5 and 10 mg simvastatin significantly improved bone mineral density by 18.2% and 22.4%, respectively ($P<0.05$); increased bone volume fraction by 51.0% and 57.9%, trabecular thickness by 16.4% and 18.9%, trabeculae number by 112.0% and 107.1%, and percentage of osseointegration by 115.7% and 126.3%; and decreased trabeculae separation by 34.1% and 36.6%, respectively (all $P<0.01$). Bone mineral apposition rate was significantly increased ($P<0.01$). Furthermore, implant fixation was significantly increased ($P<0.05$), and bone morphogenetic protein 2 (BMP2) expression was markedly increased. Local injection of a single dose of simvastatin also promoted angiogenesis. Vessel number, volume, thickness, surface area, and vascular volume per tissue volume were significantly increased (all $P<0.01$). Vascular endothelial growth factor (VEGF), VEGF receptor-2, von Willebrand factor, and platelet endothelial cell adhesion molecule-1 expression were enhanced.

Conclusions: A single local injection of simvastatin significantly increased bone formation, promoted osseointegration, and enhanced implant fixation in ovariectomized rats. The underlying mechanism appears to involve enhanced BMP2 expression and angiogenesis in the target bone.

MeSH Keywords: Angiogenesis Inducing Agents • Osseointegration • Osteoporosis • Simvastatin

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Background

Osteoporosis is characterized by low bone mass, deterioration of bone microarchitecture, extensive bone fragility, and increased risk of fracture, and is highly prevalent and often undertreated in the elderly. Osteoporosis leads to poor osseointegration and reduction of implant stability [1]. With an aging population, an associated increase in the proportion of patients with osteoporosis requiring implant treatment will likely occur [2,3]. Systemic administrations of anti-resorptive agents inhibit further bone loss rather than promote bone formation.

Statins are cholesterol-lowering drugs known to possess a number of pleiotropic effects [4,5], including promotion of bone formation [6]. Previous studies showed that simvastatin induces osteoblastic differentiation *in vitro* [7], promotes osteoblast viability and differentiation [8], stimulates bone formation *in vivo* [9], and stimulates tendon-bone healing *in vivo* via increased angiogenesis and osteogenesis [10]. However, the anabolic effect of statins on bone formation remains controversial [11,12]. Discrepancy likely exists because the primary target organ for statins is the liver, in which less than 5% of orally administered statin is present in the circulation, with even lower amounts being distributed into the bones [13]. Local application of statins might promote stimulation of bone formation. Multiple local injections of simvastatin with methylcellulose gel promote mandibular bone formation [14], and locally applied simvastatin to the site of fracture improves fracture healing [15,16]. Furthermore, simvastatin-coated implants have a positive impact on osseointegration and bone fracture healing [17,18]. Our previous studies showed that locally-applied simvastatin improved calvarial bone defect healing by the recruitment of autogenous osteogenic stem cells [19], and that a single local simvastatin injection increased target osteoporotic bone mass [20].

Osteogenesis and angiogenesis are closely related [10,21]. Postmenopausal osteoporosis is characterized by reduced number of sinusoidal and arterial capillaries in the bone marrow and reduced bone perfusion [22]. Increased bone vascularity and angiogenesis in the bone marrow might protect from bone loss in ovariectomized (OVX) rats [23]. Statins have been reported to amplify angiogenesis in stroke [24] and increase the density of capillaries in the chronic ischemic heart [25].

However, the effects of simvastatin on bones and implant stability have not been studied in an OVX rat model of osteoporosis. Therefore, we hypothesized that local injection of simvastatin enhances the local blood supply, which may be beneficial for bone formation and implant stability. The aim of the present study was to evaluate the effect of a single local injection of simvastatin on screw fixation in an OVX rat model of osteoporosis. Results from the present study might lead toward new strategies to improve implant stability in patients with osteoporosis.

Material and Methods

Preparation of injectable simvastatin and screw

Simvastatin (National Institute for Food and Drug Control, Beijing, China) was dissolved in phosphate-buffered saline (PBS) with 2% dimethylsulfoxide (DMSO, Sigma, St Louis, MO, USA) and 0.1% bovine serum albumin (BSA, Sigma, St Louis, MO, USA) [20]. Injectable simvastatin was examined by scanning transmission electron microscopy (STEM; JEM-200CX; JEOL, Tokyo, Japan) at 30 kV (Figure 1A). Briefly, samples (10-µl) were deposited onto a copper TEM grid for 5 s; after excess solution was absorbed, phosphotungstic acid was used to stain the sample [26].

Mini-titanium Ti-6Al-4V alloy screws (1.5 mm in outer diameter, 10 mm in length and 0.2 mm in pitch) were a gift from Weigao Orthopedic Device Co., Ltd. (Beijing, China).

Animals and implantation, double-fluorochrome labeling and Microfil® infusion

After 2 weeks of acclimation, 48 3-month-old Sprague-Dawley female rats (weight 260.68±16.85 g) underwent bilateral OVX to induce osteoporosis, as described previously [9]. Twelve weeks after OVX, the osteoporotic animals were randomly divided into 3 groups (n=16 rats /group): 1) implant with 5 mg simvastatin; 2) implant with 10 mg simvastatin; or 3) implant without simvastatin. All surgical procedures were carried out under general anesthesia with an intraperitoneal injection of 10% chloral hydrate (3.3 ml/kg). The Peking University Third Hospital Committee on Ethics in the Care and Use of Laboratory Animals approved all experimental protocols.

A hole penetrating the contralateral cortical bone was drilled perpendicular to the long axis of the left femur, 1 mm away from the distal growth plate, with a low-speed drill (diameter of 1.0 mm) and continuous cool saline irrigation. A mini-titanium Ti-6Al-4V alloy screw was then inserted into the hole and the skin was sutured. Simvastatin solution (100 µl containing 5 or 10 mg) or vehicle (100 µl) was injected into the femurs at the intercondylar notch using a 1-ml syringe with a 20-gauge needle. Bone wax was used to seal the hole and prevent leakage.

To monitor bone mineral apposition rates (MARs), double-fluorochrome labels were administered as follows. Briefly, under general anesthesia, calcine green (Sigma, St. Louis, MO, USA; 8m g/kg) was injected via the tail vein on day 7 after implantation.

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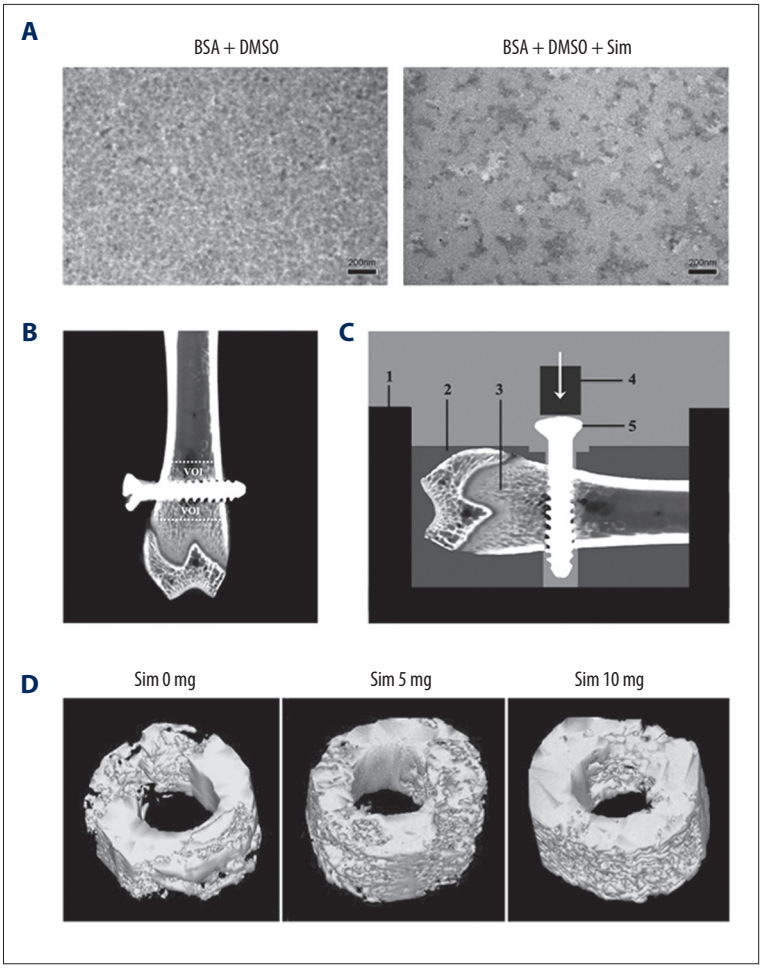

Figure 1. STEM, μCT, and schematic drawings of the push-in test. (**A**) STEM images of BSA-DMSO and BSA-DMSO-simvastatin injectable solutions. (**B**) Schematic 2D drawings of the VOI. (**C**) Schematic drawings of the push-in test: 1: custom-designed holder; 2: polymethyl methacrylate used to embed the femur; 3: cylindrical probe for testing; 4: implant; 5: distal femur. (**D**) 3D reconstruction of trabecular bone around the titanium implant 4 weeks after implantation and local simvastatin injection.

Alizarin red (Sigma, St. Louis, MO, USA; 20 mg/kg) was injected on day 21 after implantation [27,28].

Four weeks after implantation, 4 rats from each group were selected randomly for Microfil® perfusion (Flow Tech Inc., Carver, MA, USA). Microfil® is a silicone-based polymer that produces semi-rigid vascular casts to enable vessels to be visualized radiographically [29]. 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) at a constant rate of 0.75 ml/min [30]. Microfil compound was allowed to polymerize overnight at 4°C. Each sample was carefully dissected, leaving a large amount of muscles around the bone, prior to fixing in 10% neutral buffered formalin.

The remaining rats were euthanized and their left femurs excised. Four specimens from each group were randomly selected for undecalcified histology or scanning electron microscopy (SEM). After evaluation with dual-energy X-ray absorptiometry (DXA) and micro-computed tomography (μCT), 2 specimens from each group were randomly selected for immunohistochemistry to analyze the expression of bone morphogenetic protein 2 (BMP2), vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR-2), von Willebrand factor (vWF), and platelet endothelial cell adhesion molecule-1 (CD31). The remaining 6 specimens were evaluated using biomechanical push-in tests. To reduce bias, tests were conducted blinded.

DXA

Because peri-implant bone (PIB) mineral density (BMD) is difficult to detect in rats, we assessed BMD of the mid-diaphysis region of the femur. The region of interest (ROI) was set to 1.5 cm × 0.5 cm at the same position in the mid-shaft of each femur [31].

μCT

Specimens were placed in a sample holder with PBS and scanned using μCT (Inveon, Siemens, Erlangen, Germany) at a spatial resolution of 18 μm, 600 kV/300 μA, with 900 ms integration time and 360 projections per 360°.

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Bone microstructure

It is reported that anchorage failure occurs mainly in PIB, 0.5 to 1.0 mm away from the implant surface [32]. The volume of interest (VOI) was defined as 1.0 mm away from the implant surface (Figure 1B). The implant in the VOI was selected and extracted by means of binarization [33]. Three-dimensional (3D) images of the VOI were reconstructed (Figure 1B). Bone volume/tissue volume (BV/TV), trabeculae number (Tb.N), average trabecular thickness (Tb.Th), average trabeculae separation (Tb. Sp), and percentage osseointegration (%OI) were calculated.

Vascularity

Specimens with Microfil® perfusion were scanned twice, as previously described [29]. After μCT analysis, femurs were decalcified in a mixture of 4% formic acid and 10% formalin. Decalcified samples, with screws mechanically removed, were kept in 10% formalin until re-analysis to obtain the vascular system. Deep red was assigned to vessels external to the bone, to provide a clear-cut difference between external and internal bone vessels [30]. VOI was defined from the growth plate extending 10 mm below the diaphysis, and was measured in 3D to characterize vascular differences between the 3 groups. Vessel thickness was determined using the sphere algorithm, which is routinely used to measure mean bone trabecular thickness [29,30]. Parameters, including vessel volume (VV), VV per tissue volume (VV/TV), vessel number (V.N), vessel thickness (V.Th), and vessel surface area (V.SA), were also calculated [32].

Histology

Four specimens were selected randomly from each group and 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 screw (EXAKT Cutting & Grinding System, Norderstedt, Germany).

MARs were calculated under fluorescence microcopy (DM3000, Leica Microsystems, Wetzlar, Germany) by measuring the mean distance between the 2 fluorescent labels divided by 14 days (time interval between injections) [34]. MARs of different areas, peri-implant trabecular and cortical bone, were also calculated.

After observation of MARs, undecalcified sections were stained with toluidine blue or Goldner's trichrome and observed under light microscopy (E800, Nikon, Tokyo, Japan). Goldner's trichrome was used to identify maturity of new trabecular bone around the implant. Using this method, mature trabecular bone and osteoid were stained green and red, respectively [35].

SEM

SEM was performed as previously described [36,37]. In brief, 2 specimens, selected randomly from each group, were embedded in polymethyl methacrylate resin and cut into 100-μm-thick slices perpendicular to the long axis of the implant (EXAKT Cutting & Grinding System, Norderstedt, Germany). After grinding and polishing, the specimens were coated with gold-palladium (K550x, Quorum Technologies Ltd., Lewes, England) and processed for SEM (JSM-5600LV, JEOL, Tokyo, Japan) with accelerating voltages at 15 kV increments.

Push-in test

After carefully removing the surrounding soft tissues, specimens were frozen at -80°C for mechanical testing. Samples were slowly thawed at 7°C overnight and then allowed to reach room temperature prior to testing [38]. The strength of implant fixation was evaluated using a push-in test, via a mechanical testing system (MTS Landmark Servohydraulic Test System, MTS Systems Co., Eden Prairie, MN, USA). As previously described [1,39], specimens were embedded in polymethyl methacrylate and a round hole, a little larger than the screw, was made in the resin to expose the 2 ends of the implant. The compression indenter was centered over the long axis of the implant (Figure 1C) with a 2-N preload applied along the longitudinal axis of the screw. The biomechanical push-in test was accomplished at a rate of 2 mm/min. The maximum value of the ultimate load was recorded.

Immunohistochemistry

Two specimens, selected randomly from each group, were fixed in phosphate-buffered 4% paraformaldehyde and decalcified in 10% ethylene diamine tetraacetic acid (EDTA), prior to routine paraffin embedding. Sections (5-mm) were treated with 3% H_2O_2 for 10 min, then incubated in 10% goat serum diluted in PBS for 30 min, followed by incubation with either rat polyclonal anti-BMP2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-VEGF (1:300), rabbit anti-VEGFR-2 (1:300), rabbit anti-vWF (1:300), or rabbit anti-CD31 (1: 300) antibodies, overnight at 4°C. All primary antibodies, apart for BMP2, were purchased from Beijing Biosynthesis Biotechnology (Beijing, China). After incubation with biotinylated anti-rat secondary antibody for 30 min and peroxidase for 10 min, signals were detected using diaminobenzidine and observed under light microscopy (E800, Nikon, Tokyo, Japan).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was conducted to assess

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Table 1. BMD, MARs and maximum force.

	Simvastatin dose		
	0 mg	5 mg	10 mg
BMD[a]	170.8±13.8	201.0±23.3*	207.9±23.5*
MARs-Tb[b]	2.89±0.16	7.96±1.80**	6.61±0.71**
MARs-Ct[b]	3.64±0.57	9.57±0.39**	28.91±0.91**
Maximum Force[c]	145.7±9.6	161.5±9.4*	161.9±11.4*

BMD – bone mineral density; MARs – mineral apposition rates. [a] BMD in the mid-diaphysis of the femurs of osteoporotic rats 4 weeks after simvastatin injection. Values are mean ± SD, n=8 in each group. * $P<0.05$ *vs*. the control group. [b] MARs in the peri-implant trabecular (Tb) and cortical (Ct) bone. Values are mean ± SD, n=4 in each group. ** $P<0.01$ *vs*. the control group. [c] Effect of single simvastatin injection on push-in strength of screw inserted in the distal femoral metaphysis. Values are mean ±SD, n=6 in each group. * $P<0.05$ *vs*. the control group.

Table 2. Quantitative μCT analysis of PIB microstructural parameters.

PIB	Simvastatin dose		
	0 mg	5 mg	10 mg
BV/TV (%)	37.23±2.48	56.20±2.64**	58.77±4.20**
TbTh(mm)	140.92±14.11	157.41±10.47**	164.01±7.99**
Tb.Th (μm)	133.94±8.49	155.92±6.98**	159.31±7.83**
Tb.N (/mm)	2.66±0.44	5.64±0.26 **	5.51±0.78**
Tb.Sp (mm)	0.41±0.02	0.27±0.02**	0.26±0.01**
%OI (%)	22.18±3.04	47.85±2.55**	50.20±3.24**

Values are mean ± SD, n=8 in each group. ** $P < 0.01$ *vs*. the control group. BV/TV – bone volume/tissue volume; Tb.Th – average trabecular thickness; Tb.N – trabeculae number; Tb.Sp – average trabecular separation;%OI – percentage osseointegration.

differences among groups, followed by appropriate least significant difference (LSD) tests. *P*-values <0.05 were considered to be statistically significant.

Results

Establishment of the rat model of osteoporosis

Surgery was successful in all animals and recovery was uneventful. No signs of infection arose at implant sites. All rats survived the modeling.

Bone formation

Four weeks after implantation, BMD of groups treated with 5 or 10 mg simvastatin was increased by 18.2% and 22.4% (both $P<0.05$), respectively (Table 1).

μCT imaging confirmed that simvastatin increased bone formation around the screws compared with the control group (Figure 1D). Quantitative analysis revealed that a single local simvastatin injection (5 or 10 mg) significantly increased BV/TV by 51.0% and 57.9%, Tb.Th by 16.4% and 18.9%, Tb.N by 112.0% and 107.1% and%OI by 115.7% and 126.3%; and decreased Tb.Sp by 34.1% and 36.6% (all $P<0.01$), respectively (Table 2).

Histology results confirmed the μCT imaging findings. The PIB was significantly increased in the simvastatin-treated groups (Figure 2).

In Figure 3, areas labeled with green and red fluorescence represent regions of calcium precipitation labeled by double-fluorochrome at different moments of tissue mineralization. The peri-implant trabecular and cortical bone MARs of the simvastatin groups were markedly greater than those in the controls (Figure 3A, 3B). The peri-implant trabecular bone MARs of the 5 (7.96±1.80 μm/d) and 10 mg (6.61±0.71 μm/d) simvastatin groups were significantly greater than those in controls (2.89±0.16 μm/d) ($P<0.010$ (Table 2). The cortical bone MARs of the 5 (9.57±0.39 μm/d) and 10 mg (28.91±0.91 μm/d) simvastatin were significantly greater than those in controls (3.64±0.57 μm/d) ($P<0.01$) (Table 1).

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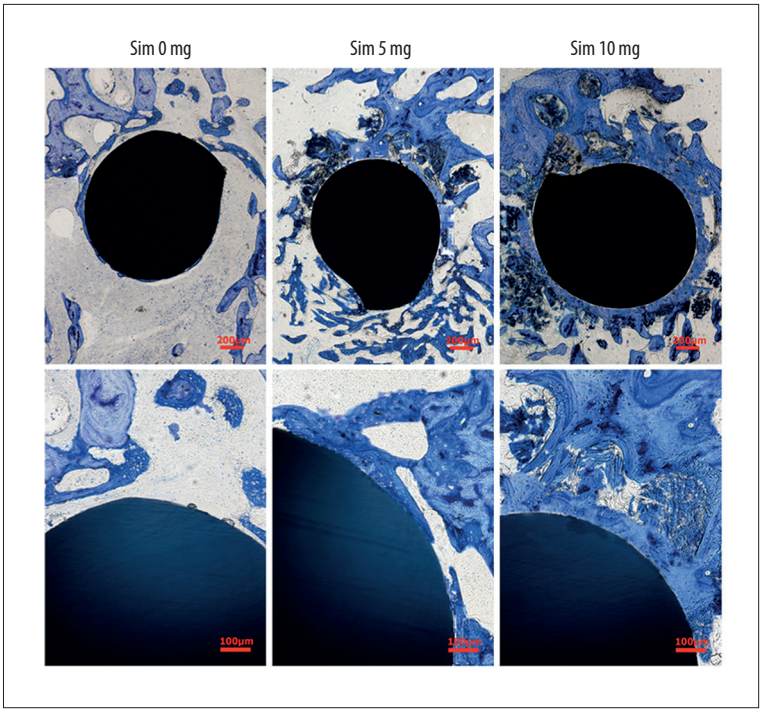

Figure 2. Representative photomicrographs of toluidine blue staining. Abundant bone was observed in the medullary canal region and around the titanium alloy screw. Bars represent 200 μm (upper panel) and 100 μm (lower panel).

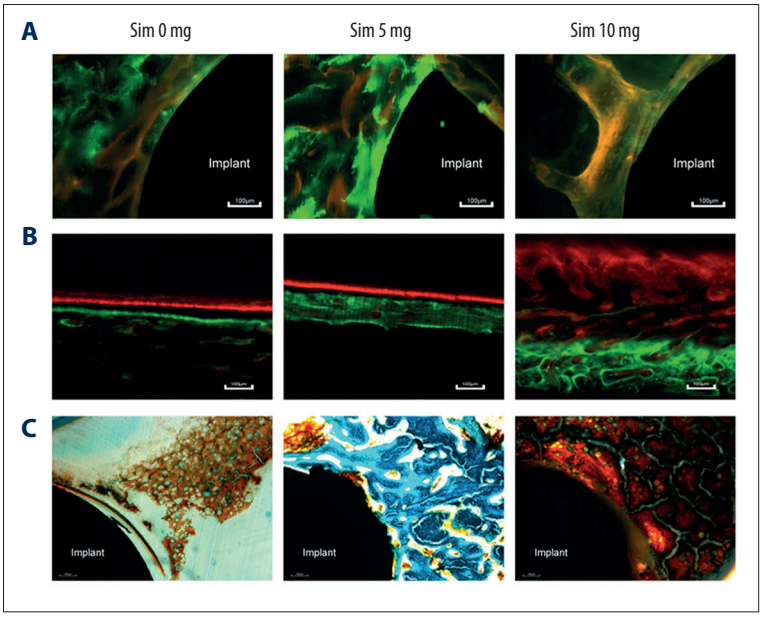

Figure 3. Double fluorochrome labeling and Goldner's trichrome staining. Fluorescence microcopy images of new trabecular bone (**A**) and cortical bone (**B**) formed surrounding the implant surface; green and red labels represents regions where calcium was precipitated on days 7 and 21 after implantation, respectively. (**C**) Photomicrographs of undecalcified sections stained with Goldner's trichrome four weeks after implantation and simvastatin injection. Mature trabecular bone and osteoid are stained green and red, respectively.

Goldner's trichrome staining showed that there was less peri-implant trabecular bone in the control group. However, a large amount of peri-implant trabecular bone could be observed in the simvastatin injection groups. Interestingly, the mature trabecular bone volume after injection of 5 mg simvastatin was markedly higher than that in the controls, and after injection of 10 mg simvastatin there was a large amount of osteoid around the implant (Figure 3C).

SEM images clearly illustrate that the PIB volume fraction and osseointegration was lower in the control group. In comparison, locally applied simvastatin markedly increased the peri-implant bone volume fraction and osseointegration in the osteoporotic bone of OVX rats (Figure 4).

The push-in test showed that 4 weeks after implantation, implant fixation in groups treated with simvastatin (5 and 10 mg) was significantly higher than that in controls (both $P<0.05$; Table 1).

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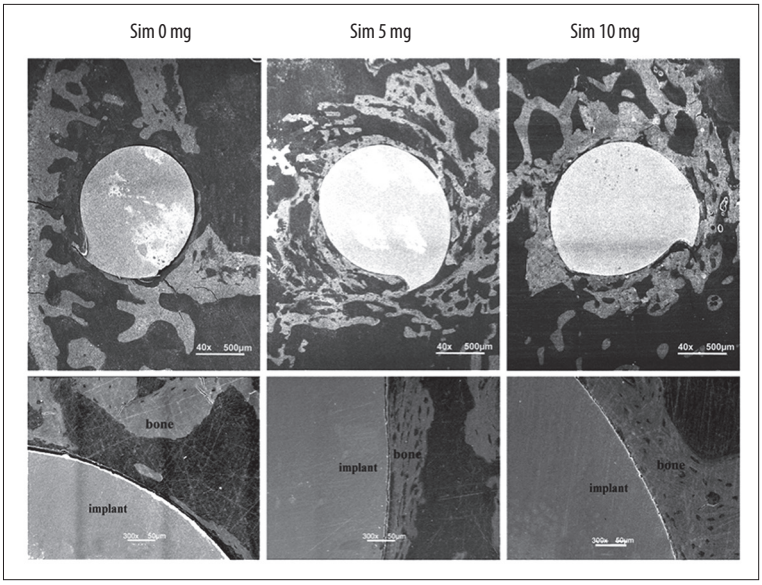

Figure 4. SEM. Osseointegration was poor in the control group and better in the simvastatin-injected groups.

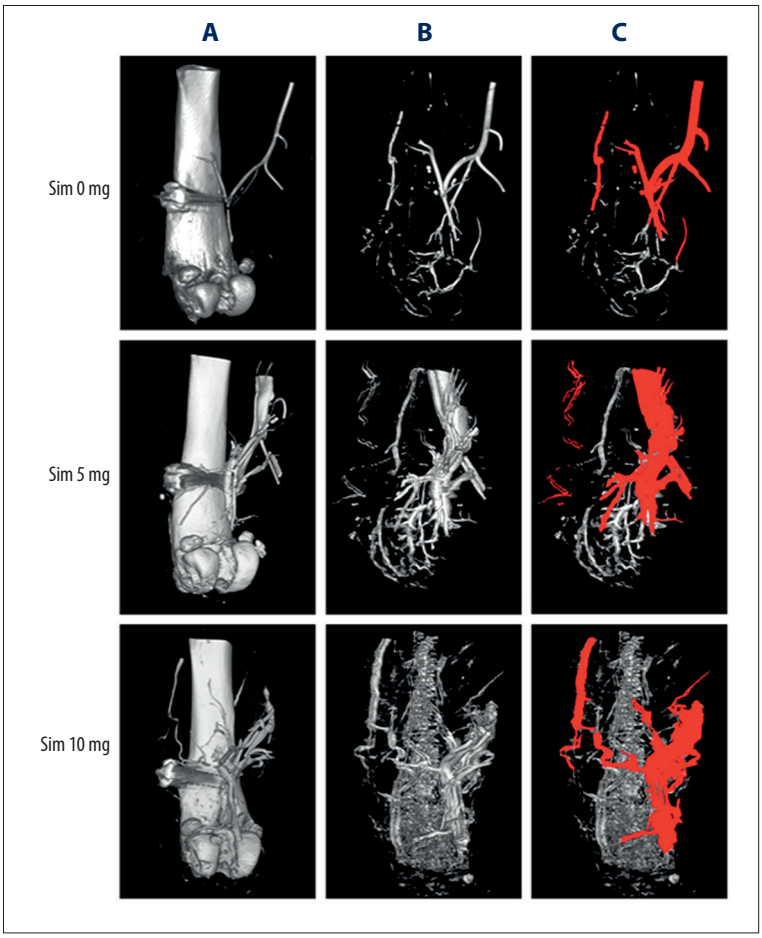

Figure 5. 3D images of vascularity after Microfil® perfusion. (**A**) Images obtained on undecalcified samples. (**B**) Images obtained after decalcification of identical femurs with screws removed. (**C**) Images of whole vessels, with deep red representative of external vessels.

Angiogenesis induction

Four weeks after a single local injection of simvastatin, the number of vessels was increased in the bone marrow compared with controls. In addition, more vessels were found externally to the bone in the simvastatin groups compared with controls (Figure 5).

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Table 3. Quantitative μCT analysis of vascularity in the bone marrow.

	Simvastatin		
	0 mg	5 mg	10 mg
VV (m^3)	3.36±0.52	17.74±3.93**	50.30±3.17**
VV/TV (%)	2.79±0.70	11.53±2.56**	25.15±1.59**
V.N (/mm)	0.27±0.03	0.91±0.07**	1.51±0.10**
V.Th (mm)	73.32±5.06	109.23±6.81**	154.28±9.89**
V.SA (mm^3)	62.10±2.54	294.82±7.86**	610.61±7.53**

Values are mean ± SD, n=4 in each group. ** *P*<0.01 *vs.* the control group. VV – vessel volume; VV/TV – vessel volume per tissue volume; V.N – vessel number; V.Th – vessel thickness; V.SA – vessel surface area.

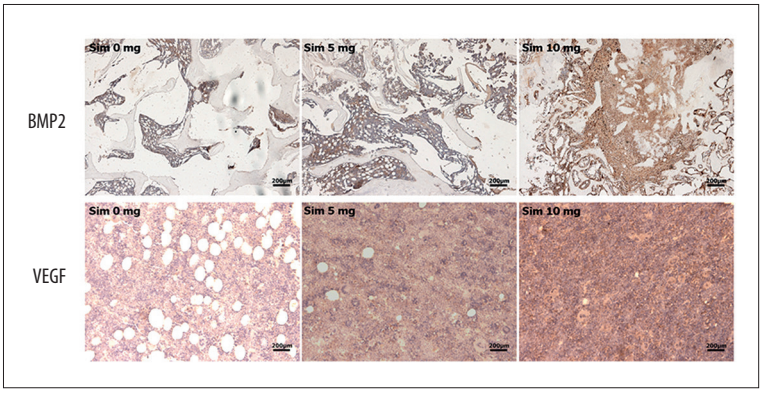

Figure 6. Immunohistochemistry for BMP-2 and VEGF. Increased BMP-2 and VEGF expression were observed in the simvastatin-treated groups compared with the control group.

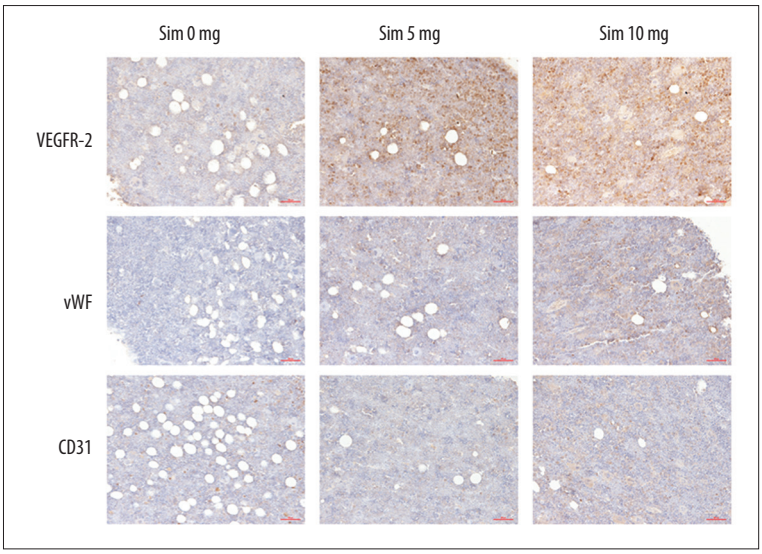

Figure 7. Immunohistochemical staining. Four weeks after implantation and simvastatin injection, increased expression of VEGFR-2, vWF, and CD31 were observed in the simvastatin-treated groups compared with the control group.

Quantitative analysis revealed that a single local simvastatin injection (5 or 10 mg) significantly increased VV by 427.98% and 1397.02%, VV/TV by 313.26% and 801.43%, V.N by 237.04% and 459.26%, V.Th by 48.98% and 110.42% and V.SA by 374.75% and 883.27% (all *P*<0.01) in the bone marrow, respectively (Table 3).

Immunohistochemistry showed that both BMP-2 and VEGF expression was markedly higher in the simvastatin-treated groups than that in the control group (Figure 6). Endothelial markers, including VEGFR-2, vWF, and CD31, were also higher in the simvastatin-treated groups than that in the control group (Figure 7). These results indicate that angiogenesis was induced by a single local simvastatin injection. Furthermore,

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femurs of OVX rats treated with a single dose of simvastatin had reduced numbers of adipocytes in the bone marrow cavity compared with the control group.

Discussion

Osteoporosis not only increases the risk of fracture, but also decreases the performance of implants [40, 41], and even increases the rate of implant failure associated with slow osseointegration in osteoporotic subjects [42]. Therefore, achieving sufficient implant fixation in osteoporotic bone might be a challenge. Current systemic therapies, including estrogen, bisphosphonates, calcitonin, and other anti-resorptive agents, are intended primarily to inhibit further bone loss rather than improving implant anchorage locally [43]. Therefore, the present study aimed to evaluate the effect of a single local injection of simvastatin on screw fixation in an ovariectomized rat model of osteoporosis. Results showed that simvastatin significantly improved BMD, increased BV/TV, Tb.Th, Tb.N., and %OI, and decreased trabecular separation. MAR was significantly increased. Furthermore, implant fixation significantly increased. Local injection of single-dose simvastatin also promoted angiogenesis. VN, VV, V.Th, V.Sa, and VV/TV were significantly increased, and were associated with increases in VEGF, VEGFR-2, vWF, and CD31.

Internal fixation requires improved PIB formation and stability of the bone-implant interface. Therefore, bioactive agents loaded directly onto the implant surface will be of great advantage for implantation in osteoporotic bone. Recently, various agents have been tried in attempts to locally enhance implant fixation, including bisphosphonates, bone cements, and BMPs [44–46]. Bisphosphonates inhibit excessive bone resorption, but because bone resorption and bone formation are related, this inhibitory effect likely affects bone formation [47]. Bone cements physically, but not physiologically, improve fixation strength and are poorly degraded, with a number of limitations [48]. BMPs are not used widely in the clinical setting because of their short shelf life and high cost [49].

Simvastatin is inexpensive and has been used safely for many years. Simvastatin administered orally at a daily dose of 5 mg/kg significantly improved osseointegration in osteoporotic rats [50]. Systemic administration of statins requires a relatively high daily dose to counter hepatic clearance, which likely elicits adverse effects. Evidence for an anabolic effect on bone following local application of simvastatin has been shown in the mandible [50] and critical-sized calvarial bone defects [51]. Furthermore, local application of simvastatin in PGA gel, as a slow-release carrier, has shown positive effects on bone around titanium implants in normal rats [38].

Low BMD affects initial stability of the implant and delays osseointegration [52]. In this study, we found that BMD at the mid-diaphysis region of the femur was significantly increased compared with controls. However, BMD is not the only factor that affects stability of the implant, and local bone microstructural quality also affects implant anchorage [40]. Gabet et al. found that changes in anchorage biomechanical properties, with respect to%OI, appeared substantially smaller than changes to the PIB structure [32]. Our μCT, histology and SEM results showed that a single local simvastatin injection significantly improved all of these parameters previously shown to be associated with better osseointegration. These results are in accordance with previous studies showing increased osteoblast differentiation and viability after simvastatin treatment [7,8]. A recent study showed that local simvastatin injections increased bone mass in rats [20]. A previous study also showed that simvastatin improved tendon-bone healing in a rabbit model [10], as well as bone fracture repair [15,16].

Both pull-out and push-in failures are caused by implant loosening and detachment. Pull-out has been more frequently assessed, as it is most often observed in the clinic, particularly when the screw penetrates just 1 side of the cortical bone. In the present study, screws penetrated both sides of the cortical bone wall with identical diameter to screws in the bone. Implant fixation relies strongly on early stability, which is coupled to the risk of later loosening [52,53]. PIB loss is significant during the first 3 months after implantation, mostly as a result of limited weight bearing and stress shielding during the repair process [43], and common anti-resorptives can only help in reducing bone loss after 3 months [36]. It is especially crucial to increase bone mass, improve the PIB microstructure, and enhance osseointegration at the early stages. In the present study, we selected a 4-week post-implantation time point for bone assessment because it is suggested to be an early and key period for mechanical stability and osseointegration in OVX rats [54]. Simvastatin increased BMD and peri-implant microstructure, and improved implant fixation within 4 weeks. In addition, the push-in strength was significantly higher in the simvastatin-treated groups than in the control group. However, the long-term effect of simvastatin on implant fixation warrants further study.

Undecalcified samples were stained with Goldner's trichrome to identify maturity of new trabecular bone around the implant. There was a large amount of mature bone around the implant in the 5 mg simvastatin group, while in the 10 mg simvastatin group there was a large amount of osteoid. From the MARs results obtained using a double-fluorochrome labeling of cortical bone, we observed that the red-labeled bands in the 10 mg group were thicker than in the 5 mg group. This implies that osteogenesis was still active in the 10 mg group on day 21 after administration of simvastatin. Taking the difference

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in performance of the 2 groups, we hypothesize that the higher simvastatin concentration tends to have a longer retention time after local injection into the bone marrow. To explore these phenomena, further research needs to be done.

Simvastatin has been shown to induce expression of BMP-2, a potent osteoinductive cytokine, in bone marrow stromal cells *in vitro* [7] and in rat models [18]. Here, we observed up-regulation of BMP-2 expression following a single local simvastatin injection *in vivo*. Previous mechanistic studies also suggest that an upregulation of the estrogen receptor by simvastatin [9], as well as an effect of the Ras/Smad/Erk/BMP-2 pathway [8]. However, more studies are necessary to assess the exact mechanisms involved in the effects of simvastatin on bone repair.

Angiogenesis is closely coupled with osteogenesis [55]. Angiogenesis plays a pivotal role in skeletal development and bone fracture repair [56]. Impairment of angiogenesis decreases trabecular bone formation [22]. One of the current limitations of bone tissue engineering is the inability to provide sufficient blood supply during the initial phase after implantation [57]. Factors that can couple the activity of angiogenesis with osteogenesis are likely to have a clear advantage [58]. In the present study, Microfil® perfusion revealed more blood vessels in the bone marrow and around the bone after a single local dose injection of simvastatin, which was supported by previous studies [8,16].

Vascularization is required for osteogenesis and is accomplished by a combination of factors, including adequate oxygen tension, compression forces, nutrients, growth factors, and differentiation factors [59]. Furthermore, the surrounding vasculature outside the bone provides an essential source of morphogens and a source from which regenerative cells may be recruited during injury-induced bone regeneration [59]. We hypothesize that local simvastatin application enhances angiogenesis, which is a novel explanation for the effect of simvastatin on bone formation. Results showed that the expression of angiogenesis markers, including VEGFR-2, vWF, and CD31, were markedly higher in the simvastatin-treated groups than in the control group. In addition, immunohistochemistry showed a marked increase in VEGF expression in simvastatin-treated groups, which acts as a major angiogenetic modulator involved in the process of blood vessel formation. Histology showed a reduced number of adipocytes in simvastatin-treated bone marrow, which was consistent with our earlier studies [7,20]

BSA is widely used as a carrier for drug delivery, and has been shown to be nontoxic, non-immunogenic, biocompatible, and biodegradable, and to have controlled release properties [60]. Furthermore, it provides a large number of reactive sites for multivalent coupling of bioactive molecules and improves the water-solubility of the drugs [61]. In this study, a single local injection of simvastatin affected bone metabolism for at least 4 weeks. BSA degradation rate and sustained release of simvastatin were not examined, both of which need to be elucidated in the future. A single local injection was easier to administer, removing the need for the tedious coating process.

In the present study, only a small amount of simvastatin was required to induce osseointegration. We determined this amount to be about 1–6% of the dose used previously for oral administration (5 or 10 mg/kg/day for 42 days) [9]. Other studies have reported favorable effects of systemic administration of simvastatin at higher concentration or for longer time (50 mg/kg/d for 42 days [62], or 5 mg or 10 mg/kg/day for 30 days [63]). This supports our hypothesis that local administration of statins may improve their bioavailability, increase bone repair, and reinforce fixation in osteoporotic bone. Results from the present study might lead to new, simple, and cost-effective strategies to improve implant stability in patients with osteoporosis. However, due to risks of rhabdomyolysis, statins are being used less for prevention of fall-induced hip fractures in the elderly [64]. Therefore, results of the present study probably could not be applied in the context of high statin use, but only from local application (e.g., slow-release beads), which would result in very low systemic levels. Further studies are required to assess this issue.

Conclusions

A single local injection of simvastatin significantly increased bone formation, promoted osseointegration, and enhanced implant fixation in OVX-induced osteoporotic rats. The underlying mechanism of action appears to involve up-regulation of BMP2 expression and enhanced angiogenesis in the target bone.

Conflict of interest

The authors declare that they have no conflict of interest.

References:

1. Li Y, Feng G, Gao Y et al: Strontium ranelate treatment enhances hydroxyapatite-coated titanium screws fixation in osteoporotic rats. J Orthop Res, 2010; 28: 578–82
2. Ponnusamy KE, Iyer S, Gupta G, Khanna AJ: Instrumentation of the osteoporotic spine: biomechanical and clinical considerations. Spine J, 2011; 11: 54–63
3. Cherubino P, Ratti C, Fagetti A, Binda T: Total hip arthroplasty and bone fragility. Aging Clin Exp Res, 2011; 23: 76–77
4. Davignon J: Pleiotropic effects of pitavastatin. Br J Clin Pharmacol, 2012; 73: 518–35
5. Davignon J: Emphasis on pleiotropic effects, a new paradigm shift? Coron Artery Dis, 2004; 15: 223–25

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS] [Index Copernicus]

6. Mundy G, Garrett R, Harris S et al: Stimulation of bone formation *in vitro* and in rodents by statins. Science, 1999; 286: 1946–49
7. Song C, Guo Z, Ma Q et al: Simvastatin induces osteoblastic differentiation and inhibits adipocytic differentiation in mouse bone marrow stromal cells. Biochem Biophys Res Commun, 2003; 308: 458–62
8. Chen PY, Sun JS, Tsuang YH et al: Simvastatin promotes osteoblast viability and differentiation via Ras/Smad/Erk/BMP-2 signaling pathway. Nutr Res, 2010; 30: 191–99
9. Li X, Song QS, Wang JY et al: Simvastatin induces estrogen receptor-alpha expression in bone, restores bone loss, and decreases ERalpha expression and uterine wet weight in ovariectomized rats. J Bone Miner Metab, 2011; 29: 396–403
10. Oka S, Matsumoto T, Kubo S et al: Local administration of low-dose simvastatin-conjugated gelatin hydrogel for tendon-bone healing in anterior cruciate ligament reconstruction. Tissue Eng Part A, 2013; 19: 1233–43
11. Tsartsalis AN, Dokos C, Kaiafa GD et al: Statins, bone formation and osteoporosis: hope or hype? Hormones (Athens), 2012; 11: 126–39
12. Patil S, Holt G, Raby N et al: Prospective, double blind, randomized, controlled trial of simvastatin in human fracture healing. J Orthop Res, 2009; 27: 281–85
13. Schachter M: Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. Fundam Clin Pharmacol, 2005; 19: 117–25
14. Lee Y, Schmid MJ, Marx DB et al: The effect of local simvastatin delivery strategies on mandibular bone formation *in vivo*. Biomaterials, 2008; 29: 1940–49
15. Wang JW, Xu SW, Yang DS, Lv RK: Locally applied simvastatin promotes fracture healing in ovariectomized rat. Osteoporos Int, 2007; 18: 1641–50
16. Fukui T, Ii M, Shoji T, Matsumoto T et al: Therapeutic effect of local administration of low-dose simvastatin-conjugated gelatin hydrogel for fracture healing. J Bone Miner Res, 2012; 27: 1118–31
17. Stadlinger B, Korn P, Todtmann N et al: Osseointegration of biochemically modified implants in an osteoporosis rodent model. Eur Cell Mater, 2013;25: 326–40; discussion 339–40
18. Pauly S, Luttosch F, Morawski M et al: Simvastatin locally applied from a biodegradable coating of osteosynthetic implants improves fracture healing comparable to BMP-2 application. Bone, 2009; 45: 505–11
19. Yueyi C, Xiaoguang H, Jingying W et al: Calvarial defect healing by recruitment of autogenous osteogenic stem cells using locally applied simvastatin. Biomaterials, 2013; 34: 9373–80
20. Yang N, Cui Y, Tan J et al: Local injection of a single dose of simvastatin augments osteoporotic bone mass in ovariectomized rats. J Bone Miner Metab, 2014; 32: 252–60
21. Gotz W, Reichert C, Canullo L et al: Coupling of osteogenesis and angiogenesis in bone substitute healing – a brief overview. Ann Anat, 2012; 194: 171–73
22. Zhao Q, Shen X, Zhang W et al: Mice with increased angiogenesis and osteogenesis due to conditional activation of HIF pathway in osteoblasts are protected from ovariectomy induced bone loss. Bone, 2012; 50: 763–70
23. Liu X, Tu Y, Zhang L et al: Prolyl hydroxylase inhibitors protect from the bone loss in ovariectomy rats by increasing bone vascularity. Cell Biochem Biophys, 2014; 69: 141–49
24. Cui X, Chopp M, Zacharek A et al: Chemokine, vascular and therapeutic effects of combination Simvastatin and BMSC treatment of stroke. Neurobiol Dis, 2009; 36: 35–41
25. Wang W, Lang JK, Suzuki G et al: Statins enhance clonal growth of late outgrowth endothelial progenitors and increase myocardial capillary density in the chronically ischemic heart. PLoS One, 2011; 6: e24868
26. Fang R, Jing H, Chai Z et al: Design and characterization of protein-quercetin bioactive nanoparticles. J Nanobiotechnology, 2011; 9: 19
27. Sun Z, Lee E, Herring SW: Cranial sutures and bones: growth and fusion in relation to masticatory strain. Anat Rec A Discov Mol Cell Evol Biol, 2004; 276: 150–61
28. Kajiwara H, Yamaza T, Yoshinari M et al: The bisphosphonate pamidronate on the surface of titanium stimulates bone formation around tibial implants in rats. Biomaterials, 2005; 26: 581–87
29. Nyangoga H, Mercier P, Libouban H et al: Three-dimensional characterization of the vascular bed in bone metastasis of the rat by microcomputed tomography (MicroCT). PLoS One, 2011; 6: e17336
30. Downey CM, Singla AK, Villemaire ML et al: Quantitative *ex-vivo* micro-computed tomographic imaging of blood vessels and necrotic regions within tumors. PLoS One, 2012; 7: e41685
31. Pastoureau P, Chomel A, Bonnet J: Specific evaluation of localized bone mass and bone loss in the rat using dual-energy X-ray absorptiometry subregional analysis. Osteoporos Int, 1995; 5: 143–49
32. Gabet Y, Kohavi D, Voide R et al: Endosseous implant anchorage is critically dependent on mechanostructural determinants of peri-implant bone trabeculae. J Bone Miner Res, 2010; 25: 575–83
33. Nyan M, Hao J, Miyahara T et al: Accelerated and Enhanced Bone Formation on Novel Simvastatin-Loaded Porous Titanium Oxide Surfaces. Clin Implant Dent Relat Res, 2014; 16(5): 675–83
34. Ruehe B, Heberer S, Bayreuther K, Nelson K: Effect of dehiscences to the bone response of implants with an Acid-etched surface: an experimental study in miniature pigs. J Oral Implantol, 2011; 37: 3–17
35. Ripamonti U, Klar RM, Renton LF, Ferretti C: Synergistic induction of bone formation by hOP-1, hTGF-beta3 and inhibition by zoledronate in macroporous coral-derived hydroxyapatites. Biomaterials, 2010; 31: 6400–10
36. Stadelmann VA, Gauthier O, Terrier A et al: Implants delivering bisphosphonate locally increase periprosthetic bone density in an osteoporotic sheep model. A pilot study. Eur Cell Mater, 2008; 16: 10–16
37. Peter B, Gauthier O, Laib S et al: Local delivery of bisphosphonate from coated orthopedic implants increases implants mechanical stability in osteoporotic rats. J Biomed Mater Res A, 2006; 76: 133–43
38. Moriyama Y, Ayukawa Y, Ogino Y et al: Local application of fluvastatin improves peri-implant bone quantity and mechanical properties: a rodent study. Acta Biomater, 2010; 6: 1610–18
39. Gao Y, Zou S, Liu X et al: The effect of surface immobilized bisphosphonates on the fixation of hydroxyapatite-coated titanium implants in ovariectomized rats. Biomaterials, 2009; 30: 1790–96
40. Wirth AJ, Goldhahn J, Flaig C et al: Implant stability is affected by local bone microstructural quality. Bone, 2011; 49: 473–78
41. Wirth AJ, Muller R, van Lenthe GH: Augmentation of peri-implant bone improves implant stability: quantification using simulated bone loss. J Orthop Res, 2012; 30: 178–84
42. Marco F, Milena F, Gianluca G, Vittoria O: Peri-implant osteogenesis in health and osteoporosis. Micron, 2005; 36: 630–44
43. Venesmaa PK, Kroger HP, Miettinen HJ et al: Monitoring of periprosthetic BMD after uncemented total hip arthroplasty with dual-energy X-ray absorptiometry – a 3-year follow-up study. J Bone Miner Res, 2001; 16: 1056–61
44. Wermelin K, Aspenberg P, Linderback P, Tengvall P: Bisphosphonate coating on titanium screws increases mechanical fixation in rat tibia after two weeks. J Biomed Mater Res A, 2008; 86: 220–27
45. Kim KH, Lee SH, Lee DY et al: Anterior bone cement augmentation in anterior lumbar interbody fusion and percutaneous pedicle screw fixation in patients with osteoporosis. J Neurosurg Spine, 2010; 12: 525–32
46. Sun P, Wang J, Zheng Y et al: BMP2/7 heterodimer is a stronger inducer of bone regeneration in peri-implant bone defects model than BMP2 or BMP7 homodimer. Dent Mater J, 2012; 31: 239–48
47. Feng X, McDonald JM: Disorders of bone remodeling. Annu Rev Pathol, 2011; 6: 121–45
48. Felix Lanao RP, Leeuwenburgh SC, Wolke JG, Jansen JA: *In vitro* degradation rate of apatitic calcium phosphate cement with incorporated PLGA microspheres. Acta Biomater, 2011; 7: 3459–68
49. Einhorn TA: Clinical applications of recombinant human BMPs: early experience and future development. J Bone Joint Surg Am, 2003; 85-A(Suppl.3): 82–88
50. Du Z, Chen J, Yan F, Xiao Y: Effects of Simvastatin on bone healing around titanium implants in osteoporotic rats. Clin Oral Implants Res, 2009; 20: 145–50
51. Nyan M, Sato D, Oda M et al: Bone formation with the combination of simvastatin and calcium sulfate in critical-sized rat calvarial defect. J Pharmacol Sci, 2007; 104: 384–86
52. Aro HT, Alm JJ, Moritz N et al: Low BMD affects initial stability and delays stem osseointegration in cementless total hip arthroplasty in women: a 2-year RSA study of 39 patients. Acta Orthop, 2012; 83: 107–14
53. Karrholm J, Borssen B, Lowenhielm G, Snorrason F: Does early micromotion of femoral stem prostheses matter? 4–7-year stereoradiographic follow-up of 84 cemented prostheses. J Bone Joint Surg Br, 1994; 76: 912–17

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS] [Index Copernicus]

54. Linderback P, Agholme F, Wermelin K et al: Weak effect of strontium on early implant fixation in rat tibia. Bone, 2012; 50: 350–56
55. Jeong BC, Kim HJ, Bae IH et al: COMP-Ang1, a chimeric form of Angiopoietin 1, enhances BMP2-induced osteoblast differentiation and bone formation. Bone, 2010; 46: 479–86
56. Kanczler JM, Oreffo RO: Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater, 2008; 15: 100–14
57. Bai Y, Yin G, Huang Z et al: Localized delivery of growth factors for angiogenesis and bone formation in tissue engineering. Int Immunopharmacol, 2013; 16: 214–23
58. Carano RA, Filvaroff EH: Angiogenesis and bone repair. Drug Discov Today, 2003; 8: 980–89
59. Brandi ML, Collin-Osdoby P: Vascular biology and the skeleton. J Bone Miner Res, 2006; 21: 183–92
60. Elzoghby AO, Samy WM, Elgindy NA: Albumin-based nanoparticles as potential controlled release drug delivery systems. J Control Release, 2012; 157: 168–82
61. Du C, Deng D, Shan L et al: A pH-sensitive doxorubicin prodrug based on folate-conjugated BSA for tumor-targeted drug delivery. Biomaterials, 2013; 34: 3087–97
62. Basarir K, Erdemli B, Can A et al: Osseointegration in arthroplasty: can simvastatin promote bone response to implants? Int Orthop, 2009; 33: 855–59
63. Ayukawa Y, Ogino Y, Moriyama Y et al: Simvastatin enhances bone formation around titanium implants in rat tibiae. J Oral Rehabil, 2010; 37: 123–30
64. Jansen JO, Lord JM, Thickett DR et al: Clinical review: Statins and trauma – a systematic review. Crit Care, 2013; 17: 227

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