

**Serum bone formation marker correlation with improved osseointegration in osteoporotic rats treated with simvastatin**

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## **Abstract**

**Objective:** Simvastatin has been shown to enhance osseointegration of pure titanium implants in osteoporotic rats. This study aimed to evaluate the relationship between the serum level of bone formation markers and the osseointegration of pure titanium implants in osteoporotic rats treated with simvastatin.

**Materials and methods:** Fifty-four female Sprague Dawley rats, aged 3 months old, were randomly divided into three groups: Sham-operated group (SHAM; n=18), ovariectomized group (OVX; n=18), and ovariectomized with Simvastatin treatment group (OVX+SIM; n=18). Fifty-six days after ovariectomy, screw-shaped titanium implants were inserted into the tibiae. Simvastatin was administered orally at 5mg/kg each day after the placement of the implant in the OVX+SIM group. The animals were sacrificed at either 28 or 84 days after implantation and the undecalcified tissue sections were processed for histological analysis. Total alkaline phosphatase (ALP), bone specific alkaline phosphatase (BALP) and bone Gla protein (BGP) were measured in all animal sera collected at the time of euthanasia and correlated with the histological assessment of osseointegration.

**Results:** The level of ALP in the OVX group was higher than the SHAM group at day 28, with no differences between the three groups at day 84. The level of BALP in the OVX+SIM group was significantly higher than both OVX and SHAM groups at days 28 and 84. Compared with day 28, the BALP level of all three groups showed a significant decrease at day 84. There were no significant differences in BGP levels between the three groups at day 28, but at day 84 the OVX+SIM group showed significantly higher levels than both the OVX and SHAM groups. There was a significant increase in BGP levels between days 28 and 84 in the OVX+SIM group. The serum bone marker levels correlated with the histological assessment showing reduced osseointegration in the OVX compared to the SHAM group which is subsequently reversed in the OVX+SIM group.

**Conclusion:** The results from this study indicate that the serum level of bone formation markers, especially BALP and BGP, could be correlated with the degree of osseointegration around titanium implants in osteoporotic rats treated with simvastatin.

Medical conditions that affect bone quantity and quality may have a detrimental effect on osseointegration. Osteoporosis is a disease that has been reported to have negative effects on bone healing during dental implant osseointegration and is therefore considered a risk factor for implant failure (Motohashi et al. 1999, Yamazaki et al. 1999, Lugero et al. 2000, Pan et al. 2000, Duarte et al. 2005). Various therapeutic approaches have been proposed for the improvement of osseointegration of dental implants in patients with osteoporosis (Qi et al. 2004, Maimoun et al. 2010, Dayer et al. 2010, Du et al. 2009, Viera-Negron et al. 2008). Currently, most drugs used for the treatment of osteoporosis predominantly work by suppressing bone resorption by slowing down bone turnover and subsequently reducing bone loss. Theoretically, drugs that could enhance bone formation would be more likely to improve the osseointegration of dental implants in low quality bone such as that found in osteoporotic patients.

Simvastatin is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor. It is widely used as a cholesterol-lowering drug and inhibits hepatic cholesterol biosynthesis. Recent studies have shown a beneficial effect of statins on bone mineral density (BMD) (Serin-Kilicoglu & Erdemli 2007, Uzzan et al. 2007). It has been suggested that several statin drugs, including simvastatin, increase the mRNA expression of bone morphogenetic protein (BMP-2) in osteoblasts, with a subsequent increase in bone formation when injected subcutaneously adjacent to murine calvaria (Mundy et al. 1999). Several animal and human studies have been performed to elucidate the clinical importance of statins. Most experimental and epidemiological studies have shown statins to have beneficial effects on bone metabolism, as evaluated by BMD (Uzzan et al. 2007, Chuengsamarn et al. 2010, Montagnani et al. 2003, Rejnmark et al. 2004) and fracture risk (Skoglund & Aspenberg 2007, Pasco et al. 2002). Statins have therefore been proposed as potential agents in the

treatment of osteoporosis. Previous studies also suggest that simvastatin can promote osteogenesis around titanium implants (Ayukawa 2004, Ayukawa et al. 2010, Basarir et al. 2009).

Molecules associated with bone formation and turnover as well as their by-products, have been widely utilized as markers of bone metabolism and hence have an established role in osteoporotic status assessment and the evaluation of treatment outcomes. Alkaline phosphatase (ALP), and in particular bone specific alkaline phosphatase (BALP), and bone gamma-carboxyglutamic acid-containing (also known as osteocalcin) protein (BGP), are all associated with osteoblast activity and the serum levels of these proteins has been used to assess bone formation. BALP is associated with early stages of osteogenesis and BGP is associated with subsequent bone mineralization (Biver et al. 2011, Pagani et al. 2005).

In our previous report, simvastatin was shown to have a positive effect on the osseointegration of titanium in the tibias of osteoporetic rats (Du et al. 2009). However, the mechanism via which simvastatin influences osseointegration in ovariectomized (OVX) rats is unclear. This study aims to address this issue by evaluating the relationship between the systemic level of bone formation biochemical markers and the osseointegration of implants in OVX rats treated with simvastatin.

## **Materials and methods**

### **Experimental Design**

The study was conducted according to a protocol approved by the Animal Care and Use Committee of Fujian Medical University. The study protocol has been previously described (Du et al. 2009) and is summarized in Figure 1. Briefly, fifty-four 3-month-old female Sprague-Dawley rats (SLAC Laboratory Animal Co. Ltd, Shanghai, China) were divided into three groups. Each group contained 18 rats by randomized block design: i.e. sham-operated

(SHAM) ovariectomized (OVX) and ovariectomized with simvastatin treatment (OVX+SIM) groups. In the OVX and OVX+SIM groups, the ovaries were exposed and completely excised bilaterally using an abdominal approach. In the SHAM group, the ovaries were exposed and equal volume of fat tissue beside each ovary was excised. After surgery, the fascia and skin were closed and sutured. Commercial laboratory rat chow (Experimental Animal Centre of Zhejiang University, China) and water were available *ad libitum*. Osteoporotic changes due to ovariectomy were verified by sacrificing two rats from each group and recovering the proximal tibial metaphyses and uterine horns 56 days after ovariectomy as described previously (Du et al. 2009).

### **Implant placement**

Fifty-six days after ovariectomy surgery, screw-shaped machined surface titanium implants (length=5mm, diameter=2mm, thread pitch =1mm, Jiuzhou Co., Xian, China) were inserted in the left tibia of each rat as previously described (Du et al. 2009). Briefly, under general anesthesia attained by administration of 2.5% pentobarbital sodium (Chemical Agent Co., Shanghai, China) at 45mg/kg body weight, the surface of the proximal metaphyses of the tibia was exposed by an incision approximately 10mm in length. Under constant saline irrigation, bicortical implant beds were prepared with a dental bur (Zhongbang Co., Xian, China) at a rotary speed not exceeding 1500 rpm. The implants were then inserted until the screw threads were completely buried in bone (Fig. 1), after which the soft tissue was replaced and sutured. Simvastatin was administered orally at 5mg/kg per day after implant surgery to the OVX+SIM group. Saline was given as a placebo to the other two groups. Nine animals from each group were sacrificed either at 28 or 84 days after implantation (Fig 2).

### **Histological assessment**

After sacrificing the rats, the left tibiae containing the titanium implants were harvested and fixed in 4% neutral formalin for 48 hours. The specimens were dehydrated in a series of

graded alcohol and embedded in polyester resin without decalcification. Undecalcified sections approximately 30 µm thick and longitudinal to the implant, were cut with a Leitz 1600 saw microtome (LeitzWetzlar GmbH, Wetzlar, Germany) and prepared using the bone-grinding slice technique (Du et al. 2009). Three sections were obtained from each implant and stained with 0.1% toluidine blue (SigmaAldrich, St Louis, MO, USA) and methylene blue-basic fuchsin (Sigma-Aldrich, StLouis, MO, USA), and subsequently qualitatively analysed with light microscopy. The percentage of bone to-implant contact (BIC%) in the cancellous area was measured bilaterally as previously described (Du et al. 2009).

### **Serum biochemical markers**

To examine the relationship between serum markers of bone formation and implant osseointegration in the three groups, serum was collected at the time of sacrifice. According to the protocols provided by the manufacturers, the amount of BGP (Adlitteram Diagnostic Laboratories, USA) and BALP (Adlitteram Diagnostic Laboratories, USA) in rat serum were measured using an enzyme-linked immunosorbent assay (ELISA). Total serum ALP was also analyzed using an automated analyzer (Sysmex, Chemix 180, Japan).

### **Statistical methods**

Differences in serum bone marker levels between the three groups were assessed using a one-way analysis of variance (ANOVA) followed by Fisher's LSD *post hoc* test ( $\alpha=0.05$ ). Statistical analyses were performed using the Sigma Statt statistics package (SPSS Inc., Chicago, IL, USA).

## **Results**

### **Histological analysis**

A histomorphometric quantitative comparison of osseointegration in the three treatment groups has been previously presented (Du et al. 2009). In this study, the focus is on a descriptive analysis of the histological features that are relevant to osteogenesis and bone remodeling for the purpose of correlation with the serum level of markers that are indicative of bone catabolic activity. Figures 3-5 show the histological characteristics of the bone healing 28 and 84 days following implant placement in the three groups.

#### Day 28

In the OVX group, there was less newly formed bone around the implants compared with the OVX+SIM and SHAM groups at day 28 following implantation (Figs 3a-c, 4a-c). There were fewer osteoblasts in the newly formed bone matrix surrounding the implant and the bone matrix around the implants was thin and discontinuous. Evidence of osteoclastic activity was noted in the newly formed bone in the OVX group (Fig. 5a). Furthermore, in the cancellous bone away from the implant surface there were fewer mineralized trabeculae in the OVX group than in the SHAM and OVX+SIM groups. At day 28, the newly formed bone adjacent to the implants in both the SHAM and OVX±SIM groups showed similar morphology (Fig. 3a,c). Compared with the OVX group, both the OVX+SIM and SHAM groups exhibited more bone around the implants, both in terms of matrix thickness and continuity of mineralized tissue coverage of the implant surface (Figs 3a-c, 4a-c). In the OVX+SIM group, most of the newly formed bone matrix around the implants appeared to be less mature than the SHAM group at day 28 (Fig. 4c).



#### Day 84

At 84 days after implantation, the histological results showed more newly formed bone covering the implant surface than at 28 days in all three groups (Figs 3d-f, 4d-f). The changes in both the SHAM and OVX+SIM groups were similar, with the newly formed bone on the implant surface becoming thicker over time (Figs 3d,f, 4d,f). In the OVX group, the amount of new bone covering the implant surface was thinner compared with the new bone around implants seen in the OVX+SIM and SHAM groups (Figs 3 and 4). In the OVX group, there was greater evidence of both osteoblastic and osteoclastic activity in the bone matrix surrounding the implants (Fig. 5b) compared to the other groups. In the cancellous bone away from the implant surface, it was still evident that there were fewer mineralized trabeculae in the OVX group compared to the SHAM and OVX+SIM groups (Fig. 4). In the cortical area, the implant surfaces were covered with mature lamella bone, and no significant differences were noted between the three groups (data not shown) (Du et al. 2009).

#### **Analysis of biochemical markers**

The serum concentrations of ALP, BALP and BGP at both 28 and 84 days are shown in figure 6a-c. These measurements are related to the histomorphometric analysis of osseointegration in figure 6d.

#### ALP levels

The serum level of ALP at day 28 and day 84 are shown in figure 6a. At 28 days, the OVX group had significantly ( $p < 0.05$ ), higher levels of serum ALP ( $81.25 \pm 16.51$  U/L) than the SHAM group ( $63.25 \pm 13.42$  U/L), but there were no significant differences at 84 days

between the three groups. All three groups showed higher serum ALP levels at day 84 compared with day 28 (SHAM:  $92.63 \pm 10.89$  U/L VS  $63.25 \pm 13.42$  U/L,  $p < 0.05$ ; OVX:  $101.50 \pm 13.52$  U/L VS  $81.25 \pm 16.51$  U/L,  $p < 0.01$ ; OVX+SIM:  $93.88 \pm 16.21$  U/L VS  $69.25 \pm 13.99$  U/L,  $p < 0.01$ ).

#### BALP levels

The BALP levels at day 28 and day 84 are shown in Figure 6b. At 28 days, the serum level of BALP in the OVX+SIM group ( $3.73 \pm 0.46$  ng/ml) was significantly higher than both the OVX ( $2.97 \pm 0.55$  ng/ml  $p < 0.05$ ) and SHAM ( $2.84 \pm 0.75$  ng/ml  $p < 0.01$ ) groups. At day 84, the serum BALP level in the OVX+SIM group ( $2.22 \pm 0.28$  ng/ml) was significantly higher than the OVX ( $1.50 \pm 0.34$  ng/ml  $p > 0.01$ ), but not the SHAM group ( $2.33 \pm 0.77$  ng/ml). However, at day 84, the SHAM group serum BALP level was significantly higher than the OVX group ( $p < 0.01$ ). Compared to day 28, the BALP level in all three groups showed a statistically significant decrease at day 84 (SHAM,  $p < 0.05$ ; OVX,  $p < 0.01$ ; OVX+SIM,  $p < 0.01$ ).

#### BGP levels

The BGP levels at day 28 and day 84 are shown in Figure 6c. At day 28 there were no statistically significant differences in the level of BGP amongst all three groups (SHAM,  $4.43 \pm 0.52$   $\mu$ mol/l; OVX,  $4.99 \pm 0.64$   $\mu$ mol/l; OVX+SIM,  $4.80 \pm 0.96$   $\mu$ mol/l). However, by day 84, the OVX+SIM group had significantly ( $p < 0.01$ ) higher serum BGP levels ( $9.24 \pm 1.98$   $\mu$ mol/l) than both the OVX ( $4.02 \pm 0.72$   $\mu$ mol/l) and SHAM groups ( $5.05 \pm 0.76$   $\mu$ mol/l). No statistically significant differences were noted between the OVX and SHAM group. Compared with day 28, there was a statistically significant ( $p < 0.01$ ) increase in the serum BGP levels in the OVX+SIM group at day 84, but not the SHAM and OVX groups (Fig 6c).

### Summary of histomorphometric analysis

A summary of a previous quantitative analysis of bone to implant contact (BIC) around the implants at days 28 and 84 (Du et al. 2009) is shown in figure 6d for the purpose of comparison to the serum bone marker levels. The results showed that the BIC was higher in the SHAM and OVX+SIM groups compared with the OVX group at both 28 ( $p<0.05$ ) and 84 days ( $p<0.01$ ). There were no statistically significant differences in BIC between the SHAM and OVX+SIM groups at either 28 or 84 days after implantation. All three groups showed greater BIC at 84 days compared with 28 days ( $p<0.001$ ).

### **Discussion**

Simvastatin is a HMG-CoA reductase inhibitor which inhibits cholesterol biosynthesis and is widely used as a cholesterol-lowering drug. Recently, it has been reported that the liposoluble statin, Simvastatin, could increase the expression of BMP-2 mRNA in osteoblasts and, as a result, promote bone formation (Mundy et al. 1999). The anabolic effect of statins on bone metabolism has been further reported in a number of animal studies (Oxlund et al. 2001, Skoglund et al. 2002). This phenomenon has generated great interest amongst researchers to investigate potential applications of statins in the treatment of bone-related diseases, as well as in oral implantology. A limited number of studies have reported the effects of statins on the osseointegration of dental implants (Ayukawa 2004, Ayukawa et al. 2010), and we have previously shown that simvastatin can counter the compromised nature of osseointegration that is characteristic of osteoporosis (Du et al. 2009). However, the biological mechanisms for this effect are unknown. Given that serum levels of bone proteins (BALP, BGP) are utilized for osteoporotic status assessment and evaluation of osteoporosis treatment outcomes

(Biver et al. 2011, Pagani et al. 2005), we sought to correlate the serum levels of these markers with our histological assessment of osseointegration in simvastatin treated osteoporotic rats.

Total serum ALP, and more specifically, the serum activity of the bone-specific isoenzyme of BALP and serum bone gamma-carboxyglutamic acid-containing protein BGP or osteocalcin (OC) are widely accepted bone formation markers (Biver et al. 2011, Pagani et al. 2005). The total ALP level in serum is less sensitive and specific as a bone formation marker due to the contribution of alkaline phosphates from non-skeletal sources. Meanwhile, BALP, which is produced by immature osteoblasts, plays an essential role in the initiation of bone mineralization and is a more specific marker of bone formation (Whyte 1994). Evaluation of the serum concentration of these markers and correlation with a histological assessment of osseointegration would provide important information about the biological mechanisms associated with the influence of simvastatin treatment on bone healing in osteoporotic patients.

In this study, it was noted that after 28 days, the OVX group had higher ALP levels compared with SHAM group. This phenomenon might indicate that the OVX group had a higher proportion of bone turnover, in terms of bone resorption and bone formation. In the OVX+SIM group, the level of ALP was no different compared to the SHAM group. This implies that simvastatin might partly reverse the high turnover characteristic of osteoporosis through the enhancement of osteoblast activity and differentiation and reduced osteoclastic activity. The level of BALP in the OVX+SIM group was higher compared with the OVX and SHAM groups at day 28, which further confirmed that simvastatin could promote bone formation through enhanced osteoblast activity.

The BALP serum level results correlated with both qualitative and quantitative (BIC%) histological analysis. At day 28, the BIC of titanium implants in both the OVX+SIM and SHAM groups was significantly higher compared with the OVX group, suggesting that simvastatin promotes bone formation around titanium implant during the early stages of osseointegration. Simvastatin treatment resulted in more bone cells around the implants compared with the untreated OVX group, while displaying similar bone healing characteristics to the SHAM group. The results of the BALP serum levels analysis suggests that this may be through increased levels of osteoblastic activity in response to the application of simvastatin. BALP is a sensitive bone formation marker during early osteogenesis. BALP functions as an ectoenzyme and is attached to the osteoblast cell membrane (Magnusson et al. 1997). It has been suggested that BALP could act as a plasma membrane transporter for inorganic phosphate, or an extracellular calcium-binding protein that stimulates calcium phosphate precipitation and orients mineral deposition into osteoid (Debernard et al. 1986). BALP may also be involved in the mineralization process by hydrolyzing organic phosphates to release free inorganic phosphate at sites of mineralization.

At day 84, serum levels of both BGP and BALP in the OVX+SIM group were higher than OVX group. The serum levels of BGP in the OVX+SIM group was also higher than the SHAM group. These results indicate that simvastatin continued to promote osteoblastic activity as the newly formed bone around the implant matured, and is consistent with histological observations of increased bone formation and BIC in the OVX+SIM compared to the OVX group.

Interestingly, the serum levels of BALP and BGP showed divergent trends in expression between days 28 and 84, with BALP decreasing and BGP increasing over time. The relatively

weak correlation between these two markers may reflect the expression of these proteins at different stages of osteoblast differentiation (Mora et al. 1999). It is known that BGP and BALP are produced exclusively by osteoblasts but at different developmental stages. BALP is secreted by immature osteoblasts and BGP is produced by mature osteoblasts (Wang et al. 2006). BALP activity has been reported to be necessary for the initiation of mineralization but not for the continuation of the process (Hooper 1997). Indeed, reports indicating that statins promote osteocalcin expression by inhibiting the Rho and Rho-kinase pathway (Ohnaka et al. 2001) provide insights into the possible biological mechanism responsible for the increased serum levels of BGP in the OVX+SIM group. The levels of total ALP showed no significant differences between the three groups at 84 days, indicating that total ALP is not a reliable marker of enhanced bone formation in osteoporotic animals treated with simvastatin. The comparison of serum BALP and BGP levels with the extent of bone formation around dental implants showed that both of these proteins could be positively correlated with the enhanced osseointegration induced by simvastatin treatment of osteoporotic rats, with BALP associated with early bone formation and BGP with the later stages of osseointegration.

In conclusion, the present study demonstrated that serum level of biomarkers of bone formation, namely BALP and BGP, are correlated with osteointegration in osteoporotic and simvastatin treated rats.



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## Legends

**Figure 1.** X-ray image of the implant position within the rat tibia

**Figure 2.** Timeline of the experimental design.

**Figure 3.** Histological evaluation (methylene blue-basic fuchsin staining) of bone changes around implants at days 28 and 84. Compared with day 28, there was more new bone formed around the implants at 84 day in each group. At both timepoints, the OVX (b and e) group showed thinner and discontinuous osseointegration on the surface of implants compared with the SHAM (a and d) and OVX+SIM (c and f) groups. (bar=200 $\mu$ m)

**Figure 4.** Toluidine Blue staining of bone tissue in direct contact with implant surface. Bone matrix (yellow arrows) and bone cells (red arrows) are evident adjacent to the implant surface at both timepoints. Compared with day 28, the bone tissue around the implants became more mature in all three groups at day 84. At both days 28 and 84, the bone tissue in the OVX+SIM (c and f) group had more bone matrix and cells than the OVX (b and e) group, but similar bone volume to the SHAM (a and d) group. At day 28, a thick immature bone matrix was evident in the OVX+SIM group (c). (Bar=20 $\mu$ m).

**Figure 5.** Distribution of osteoclasts (yellow arrow) and osteoblasts (black arrow) during osseointegration in the ovariectomized rat (OVX) at day 28 (a) and day 84 (b) (Bar=50 $\mu$ m).

**Figure 6.** Concentration (mean $\pm$ 95% CI) of (a) serum total ALP, (b) serum BALP and (c) serum BGP in the three experimental groups at days 28 and 84. (d) Bone to implant contact (BIC, mean $\pm$ 95% CI) around the implants for the different experimental groups at days 28 and 84. Asterisks denote statistically significant differences between groups (\*  $p<0.05$ , \*\* $p<0.01$ ).

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