**In vivo** evaluation of a simvastatin-loaded nanostructured lipid carrier for bone tissue regeneration

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**Abstract**

Alveolar bone loss has long been a challenge in clinical dental implant therapy. Simvastatin (SV) has been demonstrated to exert excellent anabolic effects on bone. However, the successful use of SV to increase bone formation *in vivo* largely depends on the local concentration of SV at the site of action, and there have been continuing efforts to develop an appropriate delivery system. Specifically, nanostructured lipid carrier (NLC) systems have become a popular type of encapsulation carrier system. Therefore, SV-loaded NLCs (SNs) (179.4 nm in diameter) were fabricated in this study, and the osteogenic effect of the SNs was evaluated in a critical-sized rabbit calvarial defect. Our results revealed that the SNs significantly enhanced bone formation *in vivo*, as evaluated by hematoxylin and eosin (HE) staining, immunohistochemistry, and a fluorescence analysis. Thus, this novel nanostructured carrier system could be a potential encapsulation carrier system for SV in bone regeneration applications.

**Keywords:** nanostructured lipid carrier, simvastatin, critical-sized bone defect, osteogenesis

(Some figures may appear in colour only in the online journal)

**Introduction**

The resorption of alveolar bone, which reduces the bone height and width available for dental implant placement, results in restorative problems and esthetic challenges in clinical dental implant therapy [33]. Multiple treatments have been employed to augment bone regeneration, such as small molecule drugs, bone tissue engineering and the use of bone substitutes [11, 16, 30]. Since Mundy *et al* [22] found that statins, especially simvastatin (SV), a kind of small molecule drug and a well-known 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor that is mainly used to decrease serum cholesterol levels [8], had an excellent effect on new bone formation through increasing the expression of the bone morphogenetic protein-2 (BMP-2) gene in bone cells, significant research achievement obtained showed that SV has a distinct effect on the enhancement of bone formation and the reduction of bone resorption and fracture risk [4, 16, 27, 37]. Our previous study has also confirmed that optimal...
concentration of SV can improve the osteoblastic activity of human periodontal ligament cells [10]. However, it has been reported that less than 5% of the drug reaches the systemic circulation due to extensive first-pass metabolism in the liver and the clinical application of SV available in the bone is confined by this liposolubility [38, 39]. Therefore, drug delivery systems should be considered to increase the aqueous solubility and improve the bioavailability of SV.

In the development of nanomedicines, macromolecules (such as proteins and peptides) or small-molecule drugs could be loaded into nano-delivery systems, which show the ability of enhancing the entrance and accumulation of nanomedicines in specific cells or tissues, and in contrast to traditional small-molecule drugs, nanomedicines could minimize the side effects and toxicity [7]. Nano-delivery systems, on the whole, could be divided into two groups: polymer- and lipid-based systems [31]. Poly-(lactic acid) or poly(lactide-co-glycolide), owing to the excellent biocompatibility, biodegradability and incorporation capability for hydrophilic drugs, have been widely used to fabricate nanoparticles [28]. Wang et al fabricated the SIM-loaded nanoparticles and confirmed that the NPs exhibited a cumulative release of up to 80% within 72 h and had an excellent cellular uptake capacity and showed great biocompatibility with MC3T3-E1 cells [34]. Copolymer micellar nanoparticles of SV provided control drug release profiles and could control cholesterol level in hyperlipidemic rabbits [26]. However, the number of products based on the polymer system in the market is limited owing to their potential toxicity, the need to use organic solvents and the lack of suitable methods for large-scale production [3]. Lipid nanoparticles, due to their nonuse organic solvents, negligible toxicity, sustained drug release and suitability for large-scale production and sterilization [5, 12], have been considered as an alternative to traditional carriers such as polymeric liposomes and nanoparticles [13, 20], especially for drugs with poor water-solubility, peptides, and genes, as well as for other applications [18, 21]. Nanostructured lipid carriers (NLC), the second generation of lipid nanoparticles, have been demonstrated to load various small molecules [21, 31]. Tiwari et al successfully fabricated SV-loaded NLCs (SNs) (180.9 nm in diameter) with a high entrapment efficiency (>90%) and low recrystallization properties and, in mice, demonstrated a 4.8-fold increase in SV bioavailability compared with an orally administered SV suspension [32]. However, the osteogenic effect of SNs with a sustained-control release profile has not yet been investigated, and clinically, large alveolar defects or bone resorption caused by periodontal disease, tumor or trauma are challenging in dentistry. It is necessary to develop a simple and practical method to achieve bone augmentation. Therefore, the major objective of this study was to evaluate the early osteogenic effect of these novel lipid carriers loaded with SV by placing the SNs into a critical-sized defect in a rabbit calvaria in vivo, providing a theoretical basis for bone regeneration; critical-sized defects in bone will not heal without the use of bone augmentation procedures [19].

Materials and methods

Preparation and characterization of SNs

SNs were prepared using a microfluidization method [35] with optimized parameters, including the particle size, polydispersity index (PI), zeta potential, morphology, drug encapsulation efficiency (EE) and drug-loading capacity (DLC). In brief, SNs were constructed by dispersing 375 mg of stearic acid, 125 mg of oleic acid and 100 mg of SV melted at 80–85 °C into an aqueous phase containing 0.2% poloxamer 188. The mixture was then stirred at 10 000 rpm for 5 min to obtain a milky pre-emulsion. This pre-emulsion was processed using a microfluidizer (Nano De-BEE, South Easton, Mass, USA) and further dialyzed into a secondary water phase. The diameter, PI and zeta potential of the resultant particles were investigated via dynamic light scattering (Nano ZS, ZEN3600, Malvern, UK). The morphology of the nanoparticles was determined using transmission electron microscopy (TEM) (Hitachi (H-7500) 80 kV, Japan). The EE and DLC were analyzed via ultraviolet spectrophotometry (UV2100, Shimadzu, Japan) at 238 nm.

In vitro drug release

The release profile of SV from the SNs was evaluated using a dynamic dialysis method [15]. In brief, solutions of SNs containing 15 mg (20% theoretical DLC) of nanoparticles and 3 ml of human serum albumin (HSA) were placed into dialysis bags, which were then placed into an end-sealed vial. The total volume was 40 ml. The sealed vials were maintained at 37 °C and 75 rpm in a gas bath thermostatic oscillator (SHZ-82, Jintan Honghua Instruments Co. Ltd, Jintan, China). At predetermined time intervals, 2 ml of the release solution was replaced with the same volume of fresh HSA solution. The SV concentration was measured via high performance liquid chromatography (HPLC) at a UV absorbance of 238 nm. An equal amount of free SV was used for comparison.

Animal experiment

Experimental animals. Twenty New Zealand White rabbits (15–16 weeks, 2.8–3.3 kg) were used in this experiment. The experimental protocol was approved by the Institutional Committee for Animal Care at Southern Medical University.

Surgical procedure. All surgical procedures were performed under sterile conditions. First, the animals were induced with an intramuscular injection of sumianxin II (0.2 ml kg⁻¹) and anesthetized with an injection of sodium pentobarbital (30 mg kg⁻¹) into the lateral ear vein. The head of the rabbit was then shaved and sterilized with iodine solution. Next, 2 ml of 2% lidocaine was administered to the operating site as local anesthesia. A perpendicular incision was made, and a mucoperiosteal flap was raised. Four critical bone defects (8 mm in diameter) were then created and randomly divided into the following four groups: Group 1: No
augmentation (blank control, BC); Group 2: Bone substitute (BS); Group 3: Bone substitute with 0.5 mg of SV (SVBS); and Group 4: Bone substitute with 2.5 mg of nanoparticles (0.5 mg SV) (SNBS). The bone substitute used in this study was BioOss® (Geistlich Biomaterials, Wolhusen, Switzerland), with granules ranging from 0.25 mm to 1 mm in size. The surgical procedure is depicted in figure 1. The animals were sacrificed four weeks after surgery. To label the bone, the fluorochromes calcein was then subcutaneously injected at a concentration of 10 mg ml⁻¹ (10 mg kg⁻¹ body weight) three and four days before euthanizing the animals. The specimens were removed en bloc and soaked in 4% buffered formaldehyde.

Histomorphometric analysis. After the specimens were completely fixed, they were demineralized with 14% EDTA for four weeks. They were then dehydrated in a graded ethanol (70–100%) series, cleared with xylene and embedded in paraffin. All the samples were sectioned into 4 μm thick serial sections for hematoxylin and eosin (HE) or Masson’s trichrome staining; all samples were stained in triplicate. The ALP activity, an early marker of osteoblast differentiation, was also evaluated in these sections. The number of ALP-positive cells in the defect area was manually counted in a high-magnification photomicrograph (×400). The triplicate sections were separately evaluated by two blind observers (X Yue and T Zhang) with a digital microscope (BX 50, Olympus, Tokyo, Japan) and a digital camera (DP71, Olympus, Tokyo, Japan) at 40× magnification. The original critical bone defects were then outlined (figure 2) and evaluated using image-analysis software (Image-Pro Plus 6.0, Media Cybernetics, Silver Spring, MA, USA). The following histomorphometric parameter was determined: New bone area ratio (%) = (newly formed bone area, mm²)/(total critical bone defect area, mm²) × 100 (%).

Immunohistochemical examination. Subsequently, 4 μm-thick sections from independent samples were evaluated via an immunohistochemical analysis for osteocalcin (OC), a marker of late-stage osteoblast differentiation. The number of OC-positive cells in the defect area was manually counted in a high-magnification photomicrograph (×400). To confirm the reproducibility of the results, two previously calibrated blinded examiners (X Yue and Mao Niu) performed the quantitation, and the mean value was used in the results. All images of new bone formation in the defect area were obtained using the Image Pro Plus 6.0 software.

Fluorescence analysis and non-decalcified histomorphometry. The samples were dehydrated with a graded series of ethanol concentrations before being embedded in resin and sliced into 30 μm-thick sections. Fluorescence microscopy images of the calcified sections were then taken with a confocal microscope (Carl Zeiss LSM 510 Meta) using the appropriate filters. The newly formed mineralized bone in the defect area was observed using ZEN 2009 Light Edition software based on the grey values. Finally the non-decalcified 30 μm-thick cuts were stained with toluidine blue and the newly formed mineralized bone were calculated with the Image-Pro Plus 6.0 software. The
percentage of newly mineralized bone = newly formed mineralized bone area/total defect area.

**Statistical analysis**

All the data were expressed as the means ± standard deviation (SD). A one-way ANOVA was used for multiple comparisons among all the groups (Duncan’s multiple range test); the SPSS 18.0 software (SPSS Inc., Chicago, IL) was used. Differences were considered statistically significant when P < 0.05.

**Results**

**Characterization of SNs**

The particle size, PI and zeta potential of the SNs were determined by dynamic light scattering (Nano ZS, ZEN3600, Malvern, UK). From figure 3, we can see that the mean diameter of the nanoparticles was approximately 179.4 nm with a narrow size distribution. The mean PI of the SNs ranged from 0.14 to 0.2, and the zeta potential was approximately −35.8 mV, which indicated that the nanoparticles were physically stable. In addition, the SNs were approximately spherical in shape with a mean diameter of 170 nm, as determined by the TEM results (figure 3). These findings are consistent with the dynamic light scattering data. In this study, SNs produced under 20% theoretical DLC conditions presented relatively high EE and DLC values of 91.02 ± 1.4% and 18.1 ± 0.25%, respectively. These high values were considered to indicate a high SV loading capacity, and these nanoparticles were selected for a subsequent

**In vitro drug release**

In this study, the SV release profile of 75 mg of SNs, containing 15 mg of SV, was evaluated using a dynamic dialysis method. The in vitro cumulative release profiles of SV from the SNs are shown in figure 4 and clearly indicate controlled release. The data in figure 4 revealed that the free SV was nearly completely released at 12 h, whereas only approximately half of the total SV had been released from the nanoparticles. In addition, at the early stage of the release process (within 45 h), the SV was released from the nanoparticles in an approximately linear fashion, followed by a slow and sustained release until 96 h, at which point the level was eight times more than that observed with free SV.

**Animal experiment results**

The surgical procedures went well, and the course of postoperative healing was generally uneventful in all the animals;
no complications, such as redness, swelling, infection, dehiscence or exposure at the surgical wound site, were observed throughout the healing period.

**Gross appearance.** Based on the gross appearance, all the defects were covered with several soft fibrous tissues four weeks after surgery. On palpation, the tissue in the defects of the blank control group were soft to the touch, whereas some defects in the test groups were hard, especially in the SNBS group. These results suggested that new bone tissue had formed in the experimental groups.

**Histologic evaluation.** After four weeks, fibrous tissue, including osteoblasts, partly covered the defect area, and some irregular bone had formed around the defect edge (figure 5). Moreover, the bone substitute produced remarkably viable structures, and fibrous tissue and newly formed bone partially covered the defects. At high magnification, all of the newly regenerated bone had a woven composition, with irregular trabeculae around or far away from the bone substitute and osteoblasts surrounding the periphery of the trabeculae, and the presence of immature neovascularature was confirmed. These features were especially pronounced in the SNBS group, which exhibited abundant new bone and neovascularization in the defect area after four weeks. Moreover, some of the new bone had successfully transformed into the more mature lamellar phenotype, but cortical or diploic spaces remained absent.

A histomorphometric analysis revealed that the new bone area ratios were $4.05 \pm 0.56\%$, $4.80 \pm 0.64\%$, $10.78 \pm 0.25\%$ and $16.26 \pm 0.41\%$ in the BC, BS, SVBS and SNBS groups, respectively, as indicated in figure 6. The new bone area in the BC group was significantly smaller than those in the SVBS and SNBS groups ($P < 0.001$). Furthermore, the new bone area in the BS group was significantly smaller than those in the SVBS and SNBS groups ($P < 0.001$), indicating that the effect of SV was significant. However, the amount of new bone formed in the BC and BS groups did not significantly differ, but a large number of osteoblasts were readily found around the bone substitute. More importantly, the SVBS and SNBS groups significantly differed ($P < 0.001$), suggesting that the NLC system improved the osteogenic effect of SV.

The ALP activity is a marker of osteoblast differentiation. To determine the ability of SNs to induce early osteoblastic differentiation in vitro, ALP-positive cells located in or around the newly formed trabeculae were counted (figure 7). After four weeks, the SVBS and SNBS groups had significantly more ALP-positive cells than the BS group ($P < 0.001$) and the blank control group ($P < 0.001$), as indicated in figure 8. However, the number of these cells did not significantly differ between the SVBS and SNBS groups ($P > 0.05$) (figure 8).

Masson trichrome staining, which produces blue-stained tissue, is representative of collagen regeneration. As illustrated in figure 9, collagen, the most important component of the bone matrix, was copiously secreted; in both groups, the collagen fibers were connected to form the trabecular structure, which serves as the mineralization matrix. In addition, lines of active osteoblasts were observed around the
trabecular bone and a large number of neovascular channels were evident, especially in the SNBS group.

**Immunohistochemical analysis.** An immunohistochemical analysis revealed that the formation of new bone positively correlated with the number of OC-stained cells located in or around the newly formed bone trabeculae (figure 10). After four weeks, the SNBS group exhibited a significantly higher number of OC-positive cells than did the BS group (P < 0.001) and the BC group (P < 0.001), as shown in figure 11. Similarly, the SVBS group also had a significantly higher number of these cells than the BS group (P < 0.001) and the BC group (P < 0.001). Notably, in contrast to the new bone formation, the number of OC-stained cells in the SNBS group was significantly higher than that in the SVBS group after four weeks (P < 0.001).

**Fluorescence analysis and non-decalcified histomorphometry.** The fluorescence microscopy images were analyzed by segmenting the grey values with the imaging software. In figure 12, we can observe that a small amount of irregular and asymmetric bone had formed at the defect edge in all the groups at four weeks and that bone regeneration was obvious in the drug groups. The old host bone was melanocratic and unlabeled, whereas the newly formed bone was labeled by calcein with a diffuse green pattern and thin yellow lines. It was observed that new mineralized bone tissue grew at the edges of the bone defects and on the surface of the bone substitutes, but relatively less bone was found in the center. The shape of the bone substitutes was maintained; there was no indication of dissolution of the substitutes. Correspondent with a diffuse green pattern and thin yellow lines in the area of newly formed bone in the fluorescence analysis, the results of non-decalcified toluidine blue dye (figure 12) showed that the newly formed bone was hyacinthine at the defect edge and on the surface of the bone substitutes, but relatively less bone was found in the center. The shape of the bone substitutes was maintained; there was no indication of dissolution of the substitutes.
mineralized bone in the SNBS group was significantly higher than those in the BC, BS and SVBS groups (P < 0.001) (figure 13), which was in line with the decalcified histomorphometric analysis.

Discussion

SV has been reported to effectively promote osteogenesis and inhibit osteoclastic activity, but the successful use of SV to increase bone formation in vivo largely depends on the local concentration of the drug at the site of action [24]. To increase the aqueous solubility and improve the bioavailability of SV, a nanotechnology-based drug delivery system (NLC), which has been studied to a greater extent in pharmaceutical research [12], was successfully fabricated using a microfluidization method in the present study and loaded with SV. The SNs possess a high loading capacity for SV, and the in vitro release of SV from the nanoparticle formulations resulted in significantly prolonged release profiles, indicating that the nano-surface and sustained release properties of this nanoparticle formulation may improve the drug stability and osteogenic effects of SV under physiological conditions.

To investigate the in vivo stability, host reactions and the early bone regeneration capability of SNs, a critical-sized bone defect (CSD) model, which is commonly used to investigate the effectiveness of newly developed bone graft materials [1], was created in rabbit calvarias in our present study. However, the type of defect that can be considered a CSD remains unclear. The classical definition of a CSD is a ‘defect that has <10% bony regeneration during the lifetime of an animal’ or a defect that will not heal spontaneously when left untreated for a certain time period [2]. However, the healing capacity of defects of different sizes differs over time. In a systematic review, Rafael et al noted that the data on CSDs in the rabbit cranium lack homogeneity and suggested that smaller defects can be considered critical depending on the time of sacrifice [6]. Furthermore, Sohn et al concluded that four 8 mm defects created in a single rabbit cranium could be considered CSDs if several biomaterials must be evaluated for only the early phase of healing (two to four weeks), with the measurement of the spontaneous healing capacity of surgically produced cranial defects in rabbits at different healing periods [29]. Thus, four 8 mm-diameter CSDs were created in the rabbit cranium in this study.

The model in this animal study proved to be a proper animal model for evaluating the osteogenic effect of biomaterials. Furthermore, the SNs exhibited good stability and effectively induced osteogenesis to form mostly woven bone. The results of a histomorphometric analysis revealed that new bone covered only 4.05 ± 0.56% of the defect area in the BC group, indicating that the bone was far from healed and that the defect was of critical size (i.e. less than 10% healing during the lifetime of the animal) [2]. The new bone area ratio increased to 10.78 ± 0.25% with the addition of 0.5 mg SV and further increased to 16.26 ± 0.41% in the SN group, which contained 0.5 mg SV, indicating an abundance of newly formed bone in the defect area after four weeks without any inflammation in both test groups. Moreover, these results also suggest that SNs containing 0.5 mg SV constitute an appropriate dose for local application in an animal model, a finding that corroborates previous studies. Wong et al obtained a small but statistically significant increase in bone regeneration in parietal bone defects using collagen sponges containing 0.5 mg SV [36]. Lee et al confirmed that 0.5 mg of the statin represented the best compromise between bone growth and inflammation [14]. However, bone formation did not significantly differ between the SVBS and SNBS groups (P > 0.05), although new bone formation was slightly higher in the SNBS group. This finding may be due to confusion in the free SV and SV released from SNs during bone rebuilding. Alternatively, the bone substitute with granules ranging from 0.25 mm to 1 mm had a slow absorption rate in our study, which may result in a secondary environment for the released SV and perhaps lead to the incomplete release of SV from the nanoparticle formulations. However, the specific cause of the above findings should be studied further.

Moreover, the histology and fluorescence microscopy images indicated that some irregular and asymmetric bone had formed at the defect edge in all groups at four weeks. Some samples had small bony islands in the central area of the bone defect, away from the defect edge. Sohn et al [29] also found that bone regenerated in cranial defects starting from both the defect edges and from central islets of the defect within the dura mater and periosteum. The dura mater has been confirmed to be osteogenic [9], and the periosteum was shown to provide a blood supply to the cranial bone [2].
However, there were always uneven conditions because the dura mater can be easily damaged when creating defects, and the periosteum tears when sutured with a resorbable suture. Thus, the bone regeneration observed in our CSD model may originate from both the defect edges and from the central islets of the defect within the dura mater and periosteum. Furthermore, the Masson trichrome staining results demonstrated that the local application of SNs can significantly improve collagen regeneration and neovascularization in the bone defect at the early stages. The mechanism of angiogenesis may be due to the ability of statins to stimulate the expression of vascular endothelial growth factor, which stimulates the differentiation of endothelial progenitor cells to ultimately stimulate angiogenesis [40].

Furthermore, the expression of OC (a noncollagenous protein), a special indicator reflecting the calcification ability of the extracellular matrix by osteoblasts, was evaluated in the present study. Specifically, the expression of the OC protein was enhanced to a greater extent by the SNs, and the immunohistochemical analysis revealed that the formation of new bone positively correlated with the number of OC-stained cells, which was significantly higher in the SNBS group than in the SVBS group after four weeks (P < 0.05). This difference may explain the increased new bone formation in the SNBS group, suggesting that SNs recruited osteoblastic cells and accelerated osteoclast differentiation and mineralization. These results may be due to the controlled or slow release of SV from the SNs. Moreover, Marie noted that bone formation depends mainly on the number of osteoblastic cells rather than the activity of the osteoblasts, and the recruitment of osteoblastic cells plays a crucial role in osteogenesis [17]. Thus, the osteogenic effect of SNs was confirmed in vivo in our study.

However, our study was subject to several limitations. First, we evaluated the osteogenic effect of SNs in rabbit calvaria CSDs in vivo and observed that the osteogenic effect was more pronounced for drug-loaded nanoparticles than for an equal amount of free SV. However, the detailed mechanisms by which SNs enhance bone formation are not fully understood. Nevertheless, this regeneration may originate from both the defect edges and from the central islets of the defect within the dura mater and periosteum. The osteogenic mechanisms of SNs may be similar to those of free SV. That is, the SNs may promote osteogenesis by suppressing osteoblast apoptosis and inhibiting osteoclastogenesis [25]. Alternatively, these nanoparticles may promote osteogenesis via endocytosis and exocytosis mechanisms [23]. These mechanisms should be studied further. In addition to osteocalcin, the gene and protein expression of other osteoblast-related factors, such as BMP-2, osteopontin, type I collagen, and sialoprotein, should be quantitatively analyzed in future studies. Furthermore, this study evaluated the early phase of
Bars represent the corresponding standard error versus blank; the healing response and early osteogenic effect of SNs on calvarial CSDs, and future studies should examine the late phase of healing (eight weeks or more).

**Conclusion**

CSDs were successfully created in the rabbit cranium and proven to be a proper animal model for evaluating the osteogenic effect of biomaterials. The histologic evaluation, immunohistochemistry, and fluorescence analysis all suggested that the local application of SNs can significantly improve bone regeneration at the early stages of bone healing in this model. The mechanism of this bone regeneration may originate from both the defect edges and from the central islets of the defect within the dura mater and periosteum. Moreover, SNs improved the neovascularization and mineralization of newly formed bone. Thus, this novel nanostructured carrier system may serve as an encapsulation carrier system for SV in bone regeneration applications.

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