Research Article

Simvastatin and Lovastatin Induce Ectopic Bone Formation in Rat Subcutaneous Tissue

Behzad Houshmand[1] • Rahele Hassanizade[2] • Behnam Eslami[3] • Shohreh Amouei[2] • Ghazaleh Dashti[2] •

Golnaz Morad[2] • Arash Khojasteh[4]*

[1]Associate professor, Department of Periodontology, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]General Practitioner, Tehran, Iran
[3]Associate professor, Department of Oral and Maxillofacial Pathology, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]Assistant professor, Department of Oral and Maxillofacial Surgery, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran
*Corresponding Author; E-mail: arashkhojasteh@gmail.com

Abstract

Background and aims. Statins, the mevalonate pathway inhibitors, have effects on bone formation *in vitro* and *in vivo*. The aim of the present study was to investigate whether injection of statins could lead to ectopic bone formation in rats.

Materials and methods. A single dose (0.5 mg) of simvastatin, atrovastatin, lovastatin and polyethylene glycol (control material) were injected into four quadrants of dorsal subcutaneous tissue in twelve rats. Intramuscular injections of the same statins were also done in femur and arm muscles of eight other rats.

Results. Cartilage formation was observed in simvastatin-treated area in one rat after six weeks. Bone formation was also evident in lovastatin-treated area in one rat and simvastatin-treated area in another after this period. No hard tissue formation was detected in muscles.

Conclusion. Subcutaneous injection of simvastatin and lovastatin can induce ectopic bone formation.

Key words: Ectopic bone formation, lovastatin, simvastatin, subcutaneous injection.

Introduction

Commonly known as statins, 3-Hydroxy 3-methylglutaryl coenzyme A (HMG Co-A) reductase inhibitors are routinely prescribed as cholesterol lowering drugs. Over the past decade, special attention has been focused on the potential effects of these mevalonate pathway inhibitors on hard tissue metabolism. Mundy et al[1] first observed BMP-2 expression *in vivo* and new bone formation in calvaria following local administration and increased tibial bone volume following systemic administration of statins.

Studies on the effect of statins on bone formation *in vivo* have investigated the anabolic potential of these

mevalonate inhibitors. These animal studies have shown their anabolic effect in calvarial,[2-4] parietal,[5,6] tibial,[7-12] and femoral[13,14] bone defects. Animal studies on the effect of statin administration in oral and maxillofacial region have also confirmed their anabolic effects on bone.[15-21] Bradely et al[15] showed that simvastatin stimulates BMP-2 and nitric oxide formation and regional bone formation in rat mandible models. Lee et al[16] examined local application of statins onto rat mandibles and observed reduced soft tissue swelling while preserving bone growth, increased bone formation and higher maximum force to fracture, compared to control group. Ozec et al[17] examined the effect of local simvastatin application on 3 mm-bone defects in rat mandible. Radiologic assessment of newly-formed bone by peripheral quantitative computed tomography showed significantly increased density in the experimental group. Histologic examination also confirmed their radiological findings.[17] Wu et al[18] examined local application of simvastatin in extraction sockets of rat mandibular incisors. Relative height of the residual alveolar ridge and bone mineral density were significantly increased compared to control groups. Larger newly formed bone islands and higher bone formation rate and quality were also detected in simvastatin-treated groups.[18] In a similar study, Nishimura[19] examined local application of simvastatin in rat mandibular sockets. Bone mineral density measured by dual-energy X-ray absorptiometry was higher in simvastatin groups. Increased cortical thickness was also evident compared to control groups.[19] Vaziri et al[20] examined local application of statins in ligature-induced bone resorption in the mandibles of ovariectomized rats. They observed less periodontal breakdown in simvastatin group.[20] Morris et al[21] examined the effect of local simvastatin injection in healing of three-walled intrabony and furcation defects in beagle dogs. Improved ridge augmentation and new cementum formation was evident in simvastatin group compared to control groups.[21]

Effect of subcutaneous administration of statins on bone metabolism is also studied.[1,22-26] However, ectopic bone formation following single subcutaneous injection of statins has not been reported so far. We hypothesized that simvastatin would have the potential to induce ectopic bone formation in stem-cell rich areas without proximity to other active materials, as used in other studies. The aim of the present study was to investigate whether injection of statins could lead to ectopic bone formation in rats.

Materials and Methods

Twenty sixteen-week-old Sprague-Dawley rats (Pas-teur Institute, Tehran, Iran), including ten males each weighing 250 grams and ten females each weighing 300 grams, were used in this observational experimental study. All procedures were approved by ethics committee of Shahid Beheshti University of Medical Sciences. Animals were acclimatized for four weeks in polycarbonate cages. They were exposed to a 12-hour light-dark cycle and had free access to food and water. Male and female rats in one group were separately caged.

Statins were dissolved in polyethylene glycol 300 according to USP26 monographs in order to reach to the concentration of 1.0 mg/ml. The rats were randomly assigned to seven groups; in the first three groups (each containing 4 rats), three different statins (simvastatin, atorvastatin, lovastatin) and polyethylene glycol (as the vehicle and the control material) were injected subcutaneously in two sides of vertebral column. In group A, 0.5 ml of simvastatin, atrovastatin, lovastatin and polyethylene glycol were respectively injected in upper left, upper right, lower right and lower left quadrants. The same drugs were injected, with one clockwise shift, in upper right, lower right, lower left and upper left quadrants respectively in group B and the same scenario was repeated in group (c) with another clockwise shift. Groups D, E, F and G each containing two rats, received intramuscular (IM) injections of simvastatin, atorvastatin, lovastatin and polyethylene glycol respectively in both femur and arm muscles. All injections were done by 28-gauge needles under general anesthesia (inhalation of 2 ml of halothane for two minutes in anesthesia chambers).

Animals were sacrificed by an overdose of halothane inhalation after six weeks. Samples containing subcutaneous tissue and underlying muscular tissue in the first three groups and muscular tissue in the last four groups, were separately immersed in plastic containers of neutral (phosphate buffered) 10% formalin solution and sent to an oral pathology laboratory (Shahid Beheshti University of Medical Sciences, Tehran, Iran). Each sample was cut into 5 μm sections and 5 to 10 sections of each sample were observed by polarized microscope (Y-THM, Nikon, Japan).

Results

Three rats, belonging to groups B, E and G, died because of an overdose of anesthetic agent. Calcified tissue formation following statin administration was observed in subcutaneous dorsal areas in three rats, belonging to groups B and C (Table 1). Simvastatin injected area in one rat was filled with chondral tissue. No sign of inflammation or foreign body reaction was

Table 1. Description of microscopic views of specimens with hard-tissue formation areas

Injected drug	Location of injection	Calcified material	Bone type	Inflammation	Foreign-body granuloma	Giant cells	Active osteoblastic ream
Simvatatin	dorsal subcuataneous area	100% cartilage	---	does not exist	does not exist	does not exist	does not exist
Simvastatin	dorsal subcuataneous area	5% cartilage 95% bone	woven	exists	does not exist	exist	exists
Lovastation	dorsal subcuataneous area	5% cartilage 95% bone	lamellar woven	does not exist	does not exist	does not exist	exists

detected; while mesenchymal connective tissue undergoing cartilaginous differentiation was evident (Figure 1).

Bone formation was noticed in two other rats. Simvastatin-treated tissue in one rat developed two foci of bone (95%) and some chondral tissue (5%). Woven bone tissue with active osteoblastic ream close to newly formed bone was apparent in both foci. Single
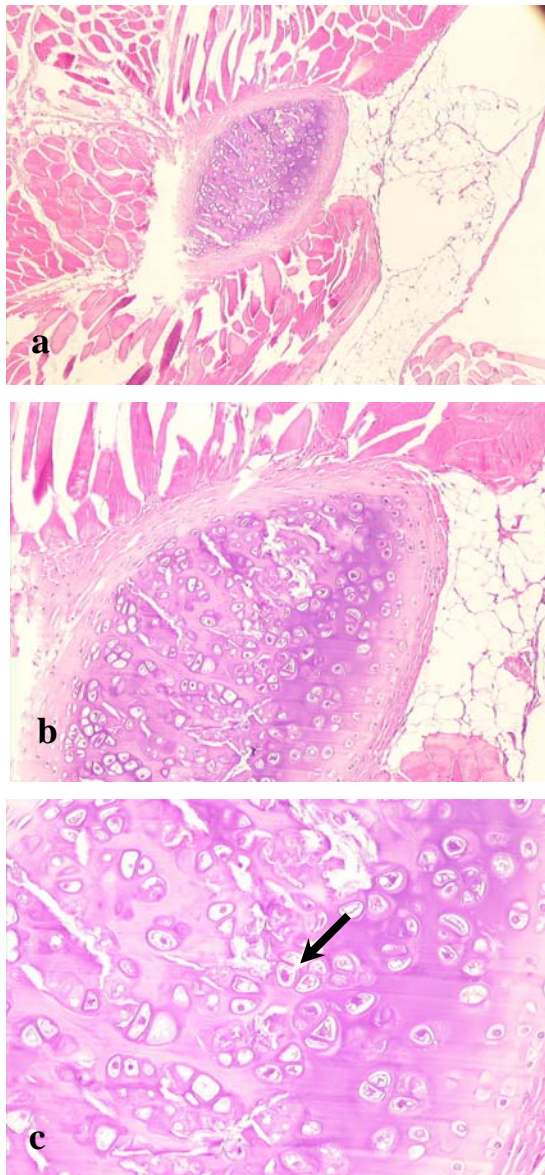

Figure 1. Mesenchymal tissue undergoing cartilaginous differentiation following injection of simvastatin in rat dorsal subcutaneous tissue after six weeks (a: 40X, b: 100X, c: 400X). Chondroblasts are evident in lacunae (*black arrow*).
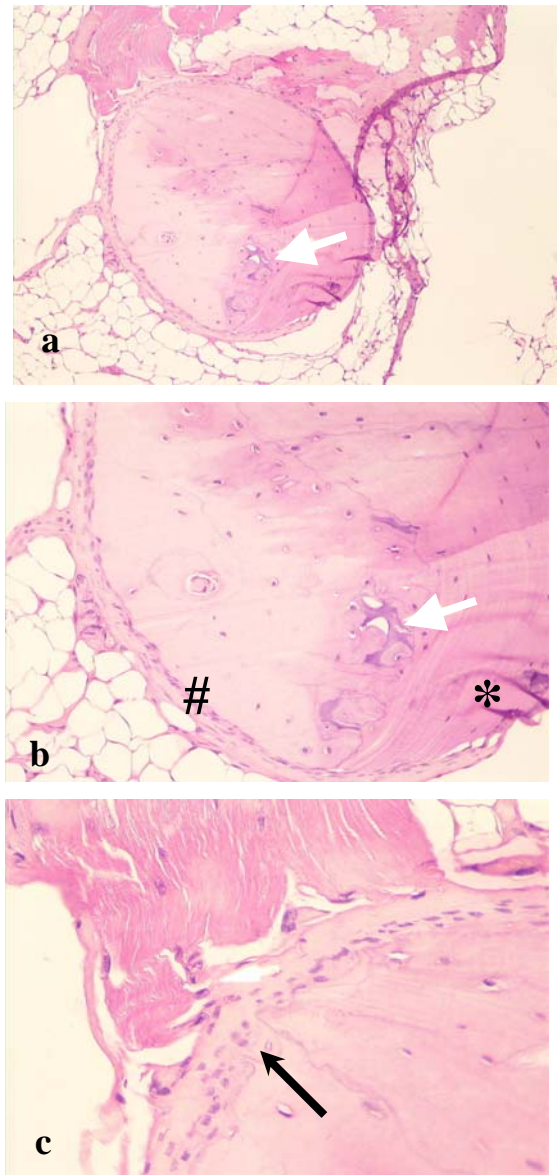

Figure 2. Bone formation following injection of lovastatin in rat dorsal subcutaneous tissue after six weeks. Lammellar bone (*), woven bone (#) and osteoblastic ream (*black arrow*) are evident (a: 100X, b: 200X, c: 400X). Bone marrow-like tissue is also detectable (*white arrow*).

giant cells were also found in some areas.

Two bone foci (95%) and some cartilage (5%) were also formed in lovastatin-treated dorsal subcutaneous tissue of one other rat. Lamellar and woven bone tissue and active osteoblastic ream were detected in both foci. The exclusive finding in this sample was formation of a bone marrow-like tissue in one bone focus. No sign of inflammation was detected in lovastatin-treated area (Figure 2).

Discussion

In the present study, the authors injected three types of statins in dorsal subcutaneous tissue and femur and arm muscles of rat. Ectopic hard tissue formation was detected in subcutaneous area in simvastatin- and lovastatin-treated areas in three rats. This is the first report of ectopic bone formation following a single injection of statins in subcutaneous tissue.

Adult subcutaneous tissue is an abundant source of mesenchymal stem cells which share with the bone marrow mesenchymal stem cells the capacity to differentiate into different mesenchymal lineages. Subcutaneous adipose cells can convert to osteoblast-like cells leading to ectopic bone formation in subcutaneous fat.[27]

Studies have shown the osseoinductive effect of statins on stem cells *in vitro*. Maeda et al[28] cultured MC3T3-E1 cells (a clonal pre-osteoblastic cell line derived from newborn mouse calvaria) in presence of simvastatin, atorvastatin or cerivastatin for 4-24 days. Simvastatin markedly increased mRNA expression for bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), alkaline phosphatase, type I collagen, bone sialoprotein, and osteocalcin (OCN) in MC3T3-E1 cells, while suppressing gene expression for collagenase-1, and collagenase-3. Statins were also shown to stimulate mineralization.[28] Baek et al[29] used simvastatin in rat bone marrow stromal cells culture and observed that simvastatin enhanced matrix calcification and increased alkaline phosphatase (ALP) activity. It was shown that simvastatin decreased the proliferation of cells, while osteocalcin mRNA expression level was enhanced.[29] A study on the effect of a fluvastatin-releasing hydrogel on human mesenchymal stem cells showed increased levels of CBFA1, alkaline phosphatase and type I collagen expression. BMP-2 formation and mineralization were also shown to enhance.[30] A recent *in vitro* study, investigating the effect of three doses of mevinolin (lovastatin) on bone differentiation in D1 cells cloned from bone marrow stromal cells, showed higher levels of alkaline phosphatase, type I collagen and osteocalcin after mevinolin administration. Os-

teoblast-specific surface molecules in mevinolin-treated groups were also detected.[31]

Subcutaneous bone formation following injection of statins over the calvarium has also been reported. In one study, an increase in new bone formation was observed following subcutaneous injection of simvastatin over the murine calvaria after 5 days.[1] More bone formation compared to control group was demonstrated 22 days following simvastatin injection over murine calvaria in another study.[32] Jinno et al[23] injected fluvastatin and atelocollagen-alpha-tricalcium phosphate (alpha-TCP) over the rat calvaria and observed more new bone formation compared to the control groups.

It can be hypothesized that subcutaneous injection of simvastatin and lovastatin can lead to differentiation of subcutaneous mesenchymal stem cells. Our findings demonstrate that this potential is not dependent on proximity of the subcutaneous tissue to bone (e.g. calvarium) or on statins use together with other active materials (e.g. alpha-TCP). It can be concluded that subcutaneous injection of simvastatin and lovastatin can induce ectopic bone formation in subcutaneous tissue. Further investigation is required to canvass the details of statins effect on bone formation mechanisms.

References

1. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, et al. Stimulation of bone formation in vitro and in rodents by statins. *Science* 1999;286:1946-9.
2. von Knoch F, Wedemeyer C, Heckelei A, Saxler G, Hilken G, Brankamp J, et al. Promotion of bone formation by simvastatin in polyethylene particle-induced osteolysis. *Biomaterials* 2005;26:5783-9.
3. Nyan M, Sato D, Kihara H, Machida T, Ohya K, Kasugai S. Effects of the combination with alpha-tricalcium phosphate and simvastatin on bone regeneration. *Clin Oral Implants Res* 2009;20:280-7.
4. Nyan M, Sato D, Oda M, Machida T, Kobayashi H, Nakamura T, et al. Bone formation with the combination of simvastatin and calcium sulfate in critical-sized rat calvarial defect. *J Pharmacol Sci* 2007;104:384-6.
5. Wong RW, Rabie AB. Early healing pattern of statin-induced osteogenesis. *Br J Oral Maxillofac Surg* 2005;43:46-50.
6. Wong RW, Rabie AB. Histologic and ultrastructural study on statin graft in rabbit skulls. *J Oral Maxillofac Surg* 2005;63:1515-21.
7. Ayukawa Y, Yasukawa E, Moriyama Y, Ogino Y, Wada H, Atsuta I, et al. Local application of statin promotes bone repair through the suppression of osteoclasts and the enhancement of osteoblasts at bone-healing sites in rats. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009;107:336-42.
8. 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.
9. Moriyama Y, Ayukawa Y, Ogino Y, Atsuta I, Koyano K. Topical application of statin affects bone healing around im-

plants. *Clin Oral Implants Res* 2008;19:600-5.

10. Pauly S, Luttosch F, Morawski M, Haas NP, Schmidmaier G, Wildemann B. Simvastatin locally applied from a biodegradable coating of osteosynthetic implants improves fracture healing comparable to BMP-2 application. *Bone* 2009;45:505-11.

11. Wang JW, Xu SW, Yang DS, Lv RK. Locally applied simvastatin promotes fracture healing in ovariectomized rat. *Osteoporos Int* 2007;18:1641-50.

12. Kolanczyk M, Kuhnisch J, Kossler N, Osswald M, Stumpp S, Thurisch B, et al. Modelling neurofibromatosis type 1 tibial dysplasia and its treatment with lovastatin. *BMC Med* 2008;6:21.

13. Adah F, Benghuzzi H, Tucci M, Russell G, England B. Cholesterol production inhibitor (statin) increased bone healing in surgically created femoral defect in an animal model. *Biomed Sci Instrum* 2007;43:95-103.

14. Garrett IR, Gutierrez GE, Rossini G, Nyman J, McCluskey B, Flores A, et al. Locally delivered lovastatin nanoparticles enhance fracture healing in rats. *J Orthop Res* 2007;25:1351-7.

15. Bradley JD, Cleverly DG, Burns AM, Helm NB, Schmid MJ, Marx DB, et al. Cyclooxygenase-2 inhibitor reduces simvastatin-induced bone morphogenetic protein-2 and bone formation *in vivo*. *J Periodontal Res* 2007;42:267-73.

16. Lee Y, Schmid MJ, Marx DB, Beatty MW, Cullen DM, Collins ME, et al. The effect of local simvastatin delivery strategies on mandibular bone formation *in vivo*. *Biomaterials* 2008;29:1940-9.

17. Ozec I, Kilic E, Gumus C, Goze F. Effect of local simvastatin application on mandibular defects. *J Craniofac Surg* 2007;18:546-50.

18. Wu Z, Liu C, Zang G, Sun H. The effect of simvastatin on remodelling of the alveolar bone following tooth extraction. *Int J Oral Maxillofac Surg* 2008;37:170-6.

19. Nishimura K. [Local application of simvastatin to rat incisor sockets augments bone]. *Kokubyo Gakkai Zasshi* 2008;75:49-54. [Japanese]

20. Vaziri H, Naserhojjati-Roodsari R, Tahsili-Fahadan N, Khojasteh A, Mashhadi-Abbas F, Eslami B, et al. Effect of simvastatin administration on periodontitis-associated bone loss in ovariectomized rats. *J Periodontol* 2007;78:1561-7.

21. Morris MS, Lee Y, Lavin MT, Giannini PJ, Schmid MJ, Marx DB, et al. Injectable simvastatin in periodontal defects and al-veolar ridges: pilot studies. *J Periodontol* 2008;79:1465-73.

22. Sugiyama T, Nakagawa T, Sato C, Fujii T, Mine K, Shimizu K, et al. Subcutaneous administration of lactone form of simvastatin stimulates ectopic osteoinduction by rhBMP-2. *Oral Dis* 2007;13:228-33.

23. Jinno Y, Ayukawa Y, Ogino Y, Atsuta I, Tsukiyama Y, Koyano K. Vertical bone augmentation with fluvastatin in an injectable delivery system: a rat study. *Clin Oral Implants Res* 2009;20:756-60.

24. Hughes A, Rogers MJ, Idris AI, Crockett JC. A comparison between the effects of hydrophobic and hydrophilic statins on osteoclast function *in vitro* and ovariectomy-induced bone loss in vivo. *Calcif Tissue Int* 2007;81:403-13.

25. Anbinder AL, Junqueira JC, Mancini MN, Balducci I, Rocha RF, Carvalho YR. Influence of simvastatin on bone regeneration of tibial defects and blood cholesterol level in rats. *Braz Dent J* 2006;17:267-73.

26. Galus R, Wlodarski P, Wlodarski K. Influence of fluvastatin on bone formation induced by demineralized bone matrix in mice. *Pharmacol Rep* 2006;58:443-7.

27. Baptista LS, Petrosa CGS, Silva KR, Otazu IB, Takiya CM, Dutra HS, et al. *Stem Cell Research Progress*, 1st ed. New York: Nova Science Publishers Co.; 2008.

28. Maeda T, Matsunuma A, Kurahashi I, Yanagawa T, Yoshida H, Horiuchi N. Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. *J Cell Biochem* 2004;92:458-71.

29. Baek KH, Lee WY, Oh KW, Tae HJ, Lee JM, Lee EJ, et al. The effect of simvastatin on the proliferation and differentiation of human bone marrow stromal cells. *J Korean Med Sci* 2005;20:438-44.

30. Benoit DS, Nuttelman CR, Collins SD, Anseth KS. Synthesis and characterization of a fluvastatin-releasing hydrogel delivery system to modulate hMSC differentiation and function for bone regeneration. *Biomaterials* 2006;27:6102-10.

31. Kim HK, Cho SG, Kim JH, Doan TK, Hu QS, Ulhaq R, et al. Mevinolin enhances osteogenic genes (ALP, type I collagen and osteocalcin), CD44, CD47 and CD51 expression during osteogenic differentiation. *Life Sci* 2009;84:290-5.

32. Thylin MR, McConnell JC, Schmid MJ, Reckling RR, Ojha J, Bhattacharyya I, et al. Effects of simvastatin gels on murine calvarial bone. *J Periodontol* 2002;73:1141-8.