International Journal of Materials Science and Applications
2023; 12(2): 26-35
http://www.sciencepublishinggroup.com/j/ijmsa
doi: 10.11648/j.ijmsa.20231202.12
ISSN: 2327-2635 (Print); ISSN: 2327-2643 (Online)

Cytotoxic Effects of Simvastatin on Normal Human Osteoblasts

Kanwal Maheshwari[1, 2, *], Laisheng Chou[1]

[1]Department of Restorative Sciences & Biomaterials, Henry M. Goldman School of Dental Medicine, Boston University, Boston, United States
[2]Department of Endodontics, Henry M. Goldman School of Dental Medicine, Boston University, Boston, United States

Email address:

kanwalrm@bu.edu (Kanwal Maheshwari)

[*]Corresponding author

To cite this article:

Kanwal Maheshwari, Laisheng Chou. Cytotoxic Effects of Simvastatin on Normal Human Osteoblasts. *International Journal of Materials Science and Applications*. Vol. 12, No. 2, 2023, pp. 26-35. doi: 10.11648/j.ijmsa.20231202.12

Received: June 8, 2023; **Accepted**: July 10, 2023; **Published**: July 21, 2023

Abstract: Statins, hydroxymethylglutaryl-coenzyme-A reductase inhibitors (HMG-Co-A), are known to reduce plasma cholesterol levels. However, the biocompatibility of Simvastatin with human bone tissue has not been studied thoroughly. The purpose of this study was to further investigate the effectiveness of different concentrations of simvastatin on the attachment, proliferation, toxicity, cell cycle, and apoptosis of normal human osteoblasts. Osteoblasts derived from normal human alveolar bone chips were cultured with simvastatin at concentrations of 1, 10, 25, 50, 75, 100 µmol/L, and 0 µmol/L as a control. The cell attachment was evaluated at 9 hours. The proliferation rate and cytotoxicity were investigated at 7, 14, and 21 days. Cell cycle and apoptosis were assessed at 1 and 3 days. Statistical analysis was performed using ANOVA. P-values ≤0.05 were considered statistically significant. The results showed that there was no statistical significance (P>0.05) amongst the groups in the cell attachment efficiency. All tested concentrations of Simvastatin showed a significant decrease in the proliferation rate (P<0.001) and an increase in cytotoxicity (P<0.001). Cell cycle and apoptosis significantly increased as time increased (P<0.001). In conclusion, the present findings showed that Simvastatin adversely affects human osteoblasts' proliferation and cell viability by inducing apoptosis.

Keywords: Simvastatin, Osteoblasts, Toxicity

1. Introduction

Simvastatin has multiple functions, including anti-inflammation, the induction of angiogenesis, and the improvement of vascular endothelial cell function [1]. Simvastatin has been shown to have advantageous effects on many diseases, such as multiple sclerosis [2] and osteoporosis [3], which have no direct correlation with cholesterol levels.

In rats, simvastatin given periorally increased both tibial and vertebral trabecular bone volume as well as vertebral compressive strength [4]. It also reduced ovariectomy-induced trabecular bone loss in vertebrae [5, 6]. In addition, there was evidence that simvastatin delivered in the diet could improve fracture repair [7] and increase bone formation and cancellous bone volume [6, 8]. The local application of simvastatin in the tooth extraction socket was reported to enhance the alveolar bone remodeling [9, 10] and the proliferation and osteoblastic differentiation of human PDL cells [11].

In-vitro studies suggested that simvastatin promoted osteoblast differentiation and mineralization demonstrated by an increased number of osteoblasts at all stages of differentiation [6]. It is further suggested that these effects led to the up-regulation of BMP-2 in osteoblast-like cells, MC3T3-E1 [12], and osteosarcoma cells [13]. The osseointegration of titanium implants was improved by Simvastatin [14] and fracture healing was enhanced [15]. It was noted that Simvastatin enhanced the repressive action of FoxO3 on the synthesis of Cyr61 in primary human osteoblasts and U2OS cell lines and subsequently decreased CCL_2 production and macrophage recruitment [16].

It was also reported that Simvastatin might inhibit periapical bone resorption by diminishing macrophage

chemotaxis to the inflammation site [17]. The anti-inflammatory action of simvastatin might be associated with its effects to induce autophagy and inhibit apoptosis in osteoblasts [18]. Anti-apoptotic effect of simvastatin has been shown in osteoblasts [18]. Inflammatory arthritis disease progression and osteoblastic expression of Cyr61 were decreased by simvastatin, which also inhibited cytokine-stimulated Cyr61 expression in osteoblastic cells. [19]. Simvastatin may slow the course of apical periodontitis in rats [17]. However, it is interestingly noted that there has not been an article about the toxicity of Simvastatin. The present study was designed to study the cytotoxic effects of simvastatin on normal human osteoblasts.

2. Materials and Methods

2.1. Cell Culture

Human intra-oral alveolar bone fragments were taken from healthy individuals between the ages of 18 and 50 who did not use steroids in the six months prior to surgery and did not have any systemic or metabolic bone disorders, acute infections, or fractures. Under IRB approval, bone samples were taken from wasted bone tissue during ostectomy procedures and the excision of dental teeth. Human osteoblast cells were obtained using a modified version of a previously reported approach [20-23]. First, a sterile surgical blade (Henry Schein) was used to cut away the soft tissue that was connected to the bone. Then, using a clean #11 surgical blade and sterile micro dissecting scissors, bone fragments were divided into 2-4 mm pieces. After the enzymatic digestion of the soft tissue and fibroblasts, bone fragments were cultured in a 12.5 cm^2 flask containing 6 mL growth media [10% fetal bovine serum (FBS), 1X Penicillin/Streptomycin antibiotic (100 U/mL), Amphotericin B anti-fungal (2.5 mg/ml) in Dulbecco's Modified Eagle Medium (DMEM)]. Up until the second passage, the bone pieces were kept at 37°C in a typical CO_2 incubator with 5% carbon dioxide and saturated humidity. Once the cells had attained 80% confluence, the culture media was changed every three days. After using 0.05% Trypsin-EDTA (Thermo Fisher Scientific, USA) to separate the cells from the flask, they were centrifuged (TJ-6 Beckman Centrifuge) for 5 minutes at 1000 rpm. To conduct the studies, the cells gathered in the pellet were counted.

2.2. Simvastatin Preparation

Simvastatin was activated by dissolving 25 mg of Simvastatin in 100 uL of Ethanol. 150 uL of 0.1 N NaOH was added to the solution. The mix was incubated at 50°C for 2 hours. The pH was tested, and it was brought down to 7.2 by HCL. The final concentration of the stock solution is 25 mg/ml. The stock solution was kept at -20°C for up to a month [16-18, 24].

2.3. Cell Attachment Efficiency and Proliferation Rates Assessment

Cell attachment efficiency was assessed at nine hours. The osteoblasts were seeded in a 24-well plate (Fisher Scientific) at a density of 1×105 cells per well. The cells were seeded with DMEM culture medium supplemented with concentrations of 1 umol/L, 10 umol/L, 25 umol/L, 50 umol/L, 75 umol/L, 100 umol/L, and 0 umol/L as a control. After 9 hours, the medium was discarded, and to get rid of any traces of Simvastatin, the wells were rinsed three times with phosphate-buffered saline (PBS) from Thermo Fisher Scientific. Following that, 500 uL of 10% neutral buffered formalin (Sigma) was added to the cells to fix them for an additional hour at room temperature. After that, 500 uL of 0.2% crystal violet stain (Sigma-Aldrich) was added and left on the fixed cells for an additional hour to stain them. After that, PBS was used to wash each well three times to get rid of any unbound stains. The microplate reader used a 590 nm wavelength to calculate the density of the labeled cells.

The cell proliferation rates were monitored on days 7, 14, and 21. The cells were seeded in 24-well plates at a density of 1.5×103 cells per well. After an optimal attachment was achieved in each well and before the first round of proliferation, i.e., after 9 hours, 1 umol/L, 10 umol/L, 25 umol/L, 50 umol/L, 75 umol/L, 100 umol/L of Simvastatin were added except for the control. Before adding Simvastatin, the microscopically validated cell attachment in each well. Every three days, new culture media were added to the Simvastatin-supplemented media. The same procedure used in the attachment experiment was followed to fix and dye the cells at each time point. To calculate the rates of proliferation, the optical densities at each point in time were contrasted with the baseline optical density of 5×10^3 cells at 9 hours. The associated cell counts were directly inversely correlated with the optical density of the crystal violet stain.

2.4. Cytotoxicity Assay

Measuring cytotoxicity is one of the most essential tasks for studying cell functions. The Abcam AB112118 kit utilizes a water-soluble dye that changes its absorption spectrum upon cellular reduction. The absorption ratio is directly proportional to the number of living cells. Briefly, osteoblasts were also seeded at the concentration of 500 cells per well in 96-well plates and were incubated for 9 hours until full attachment. Then, the media was replaced with 200 µL of fresh media containing the designated test groups. After 1, 3, 7, 10, and 14 days of incubation, the Abcam cytotoxicity kit was used by the manufacturer's protocol. 40 µL of the assay solution was added to each well, and the samples were incubated for one hour. Finally, a spectrophotometer was used to measure the absorbance at 570 nm (OD570) and 605 nm (OD605).

2.5. MTT Assay

MTT (3- (4, 5- dimethylthiazon-2-yl)-2, 5-dephenyl-2H-tetrazolium bromide) Assay Kit ab211091 is an easy-to-use, non-radioactive, and high-throughput assay for measuring cell proliferation, cell viability, and cytotoxicity.

Briefly, osteoblasts were seeded at the concentration of 500

cells per well in 96-well plates and were incubated for 9 hours until full attachment. Then, the media was replaced with 200µl of fresh media containing the designated test groups. After 1, 3, 7, 10, 14, 17, and 21 days of incubation, the MTT kit was used by the manufacturer's protocol. The media was aspirated. New serum-free media of 50 uL and 50 uL of MTT Reagent was added to each well. For the Background well, 50 uL MTT Reagent and 50 uL of cell culture media were added. The plate was incubated for 3 hours at 37°C. After incubation, remove the MTT Reagent-supplemented media. Add 150 µL of MTT Solvent into each well. Wrap the plate in foil and shake on an orbital shaker for 15 minutes. Read absorbance at OD = 590 nm.

2.6. Flow Cytometry Cell Cycle Assay

The Propidium Iodide Flow Cytometry Kit ab139418 has been developed for carrying out DNA content analysis in tissue culture cells. This is accomplished by staining the cells with propidium iodide and subsequently performing flow cytometry analysis. Propidium iodide, a classic agent for cell cycle analysis, binds to DNA and fluoresces, allowing for quantification. The staining process is time-efficient, taking less than an hour, and the cells, once fixed in ethanol, maintain stability for a few weeks at 4°C. The kit provides enough content for 200 assays.

Propidium iodide is a fluorescent compound with a broad binding affinity for nucleic acids, including both DNA and RNA. To minimize background RNA staining, RNaseA is included in the kit to digest cellular RNA. Ethanol is used to fix and permeabilize cells, as propidium iodide cannot permeate cell membranes. Compatibility extends to cells from any species, given they can be prepared as a single-cell suspension. Quantitative analysis requires a flow cytometer.

Briefly, cells were seeded between 100-500,000 cells per well in 6-well plates. 24 hours after seeding cells, serum deprives the cells, about 0.05%. Add Simvastatin at 9 hours for osteoblasts. Use the Flow Cytometry kit on days 1 and 3.

2.7. Flow Cytometry Annexin Apoptosis Assay

Apoptosis, or programmed cell death, is an integral part of normal cellular development and life cycle. Its deregulation, however, is associated with various disease conditions including Alzheimer's disease and cancer. It's differentiated from necrosis, an accidental form of cell death, by distinctive morphological and biochemical alterations, such as nuclear chromatin condensation and fragmentation, cytoplasmic shrinkage, and alteration in membrane asymmetry. In healthy cells, phosphatidyl serine (PS) is positioned on the inner side of the cell membrane, but during apoptosis, it shifts to the outer side, thus becoming exposed to the external environment. This PS exposure acts as a signal for macrophages to recognize and engulf apoptotic cells during leukocyte apoptosis. Annexin V, a human anticoagulant, and a phospholipid-binding protein, binds to PS with high affinity. This binding can be visualized using fluorophore or biotin-labeled Annexin V, thereby allowing the identification of apoptotic cells.

The Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI offer a fast and user-friendly assay for apoptosis. The kit uses recombinant annexin V tagged with Alexa Fluor® 488 dye, providing optimum sensitivity due to its brightness and photostability, making it an excellent choice over fluorescein (FITC). The kit also includes propidium iodide (PI), a red fluorescent dye that binds to the nucleic acids in the cells but is impermeant to live and apoptotic cells. The staining results in apoptotic cells fluorescing green, dead cells fluorescing both red and green, and live cells showing negligible fluorescence. These cell populations can be distinguished using a flow cytometer. In the case of cells stained with Annexin V and propidium iodide, green fluorescence in the FITC channel corresponds to apoptotic cells, while necrotic or dead cells exhibit bright red fluorescence and no green fluorescence. Live cells, on the other hand, exhibit neither green nor red fluorescence.

Concisely, cells were seeded between 100-500,000 cells per well. Add Simvastatin at 9 hours for osteoblasts. Use the Annexin kit on days 1 and 3.

2.8. Statistics

All experiments were performed in six replicates. Means and standard deviations are used to present data. Cell cycle and annexin were determined, as well as the means and standard deviations (SD) of osteoblast cell attachment efficiency, proliferation rate at 9 hours, 7, 14, and 21 days, and cytotoxicity were examined at 7, 14, and 21 days. ANOVA and the Student's t-test were used in statistical analysis using the program JMP Pro 12 (ver. 12.1.0) to find statistical differences between the groups. Differences were deemed statistically significant at $p \leq 0.05$.

3. Results

3.1. Cell Attachment Efficiency

It was noted that at 9 hours when comparing different concentrations of Simvastatin, there was no statistical significance amongst the groups ($p > 0.05$) (Figure 1).

There was no statistically significant difference among the concentrations when compared to the control ($p>0.05$).

The error bars represent the standard deviations of six replicates.

3.2. Proliferation Rates

It was noted that from days 7 to 21, the control group had significantly higher proliferation rates by multiple folds compared to all study groups with various concentrations of Simvastatin at each time point ($p<0.001$) (Figure 2).

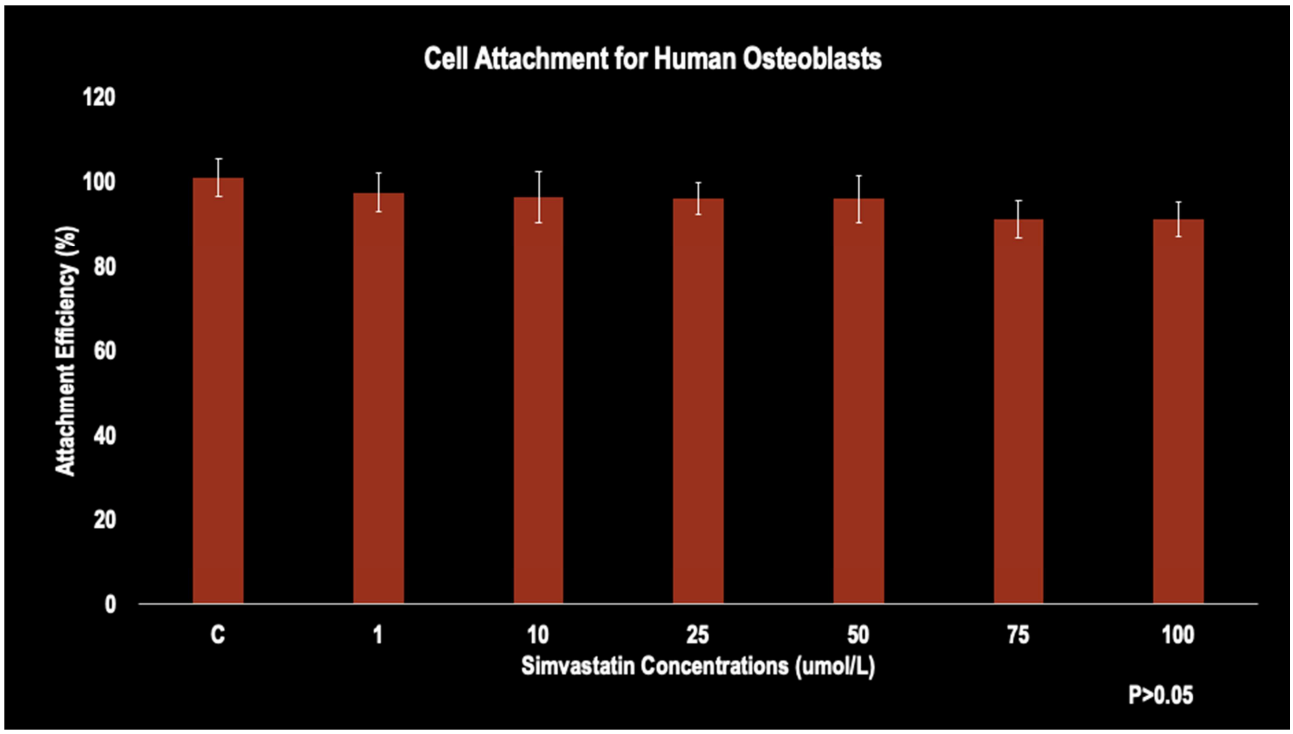


Figure 1. *Cell attachment efficiency of Osteoblasts at 9 hours with all Simvastatin concentrations (N=6).*

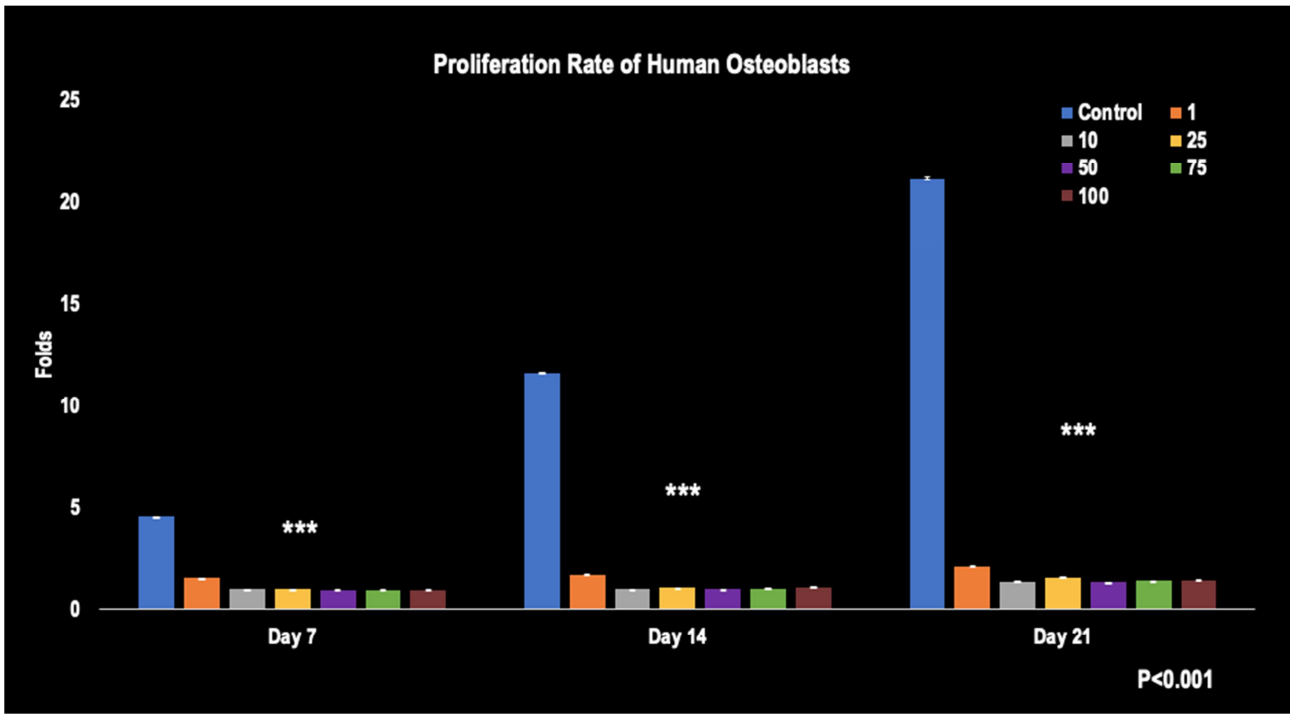


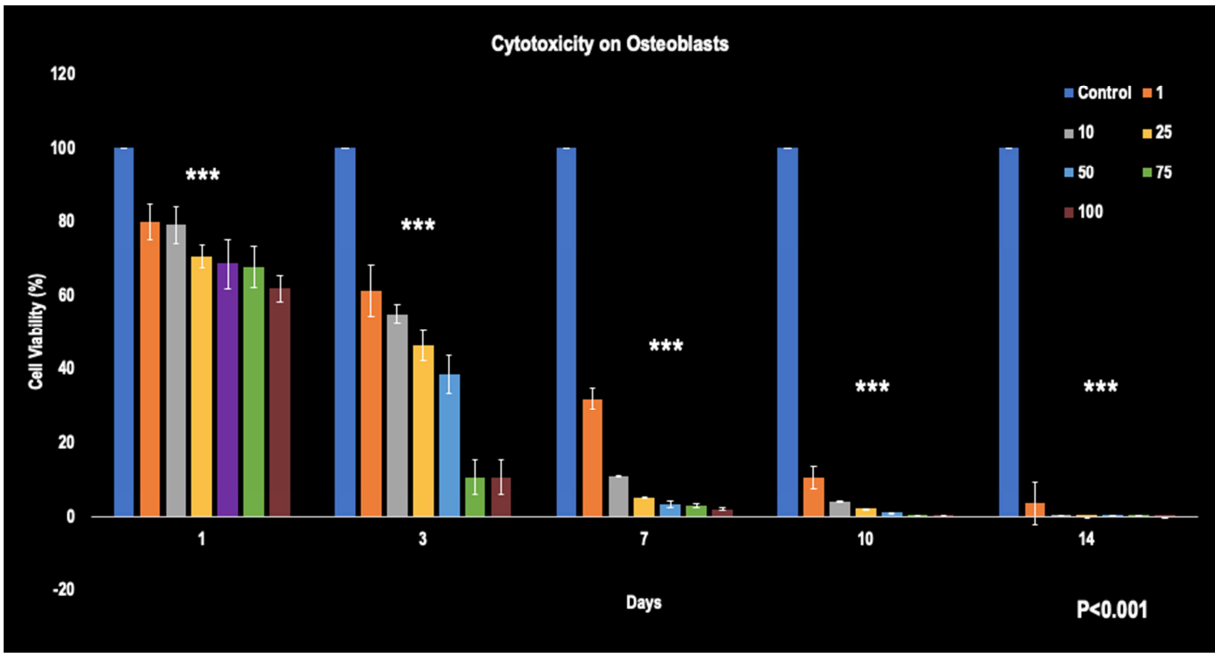
Figure 2. *The proliferation rates of Human Osteoblasts when affected by Simvastatin concentrations from days 7 to 21 (N=6).*

*, **, ***- represent the significant difference ($p<0.001$) in cell proliferation rates between the control group and all study groups with various concentrations of Simvastatin

The error bars represent the standard deviations of six replicates.

3.3. Cytotoxicity

It was noted that the osteoblasts' cell viability was drastically downregulated by all Simvastatin concentrations. Having the control at 100% cell viability at all time points, the Simvastatin of various concentrations decreased the HDPC's viability significantly. There was statistical significance amongst the concentrations when compared to the control at all time points ($p<0.001$) (Figure 3).



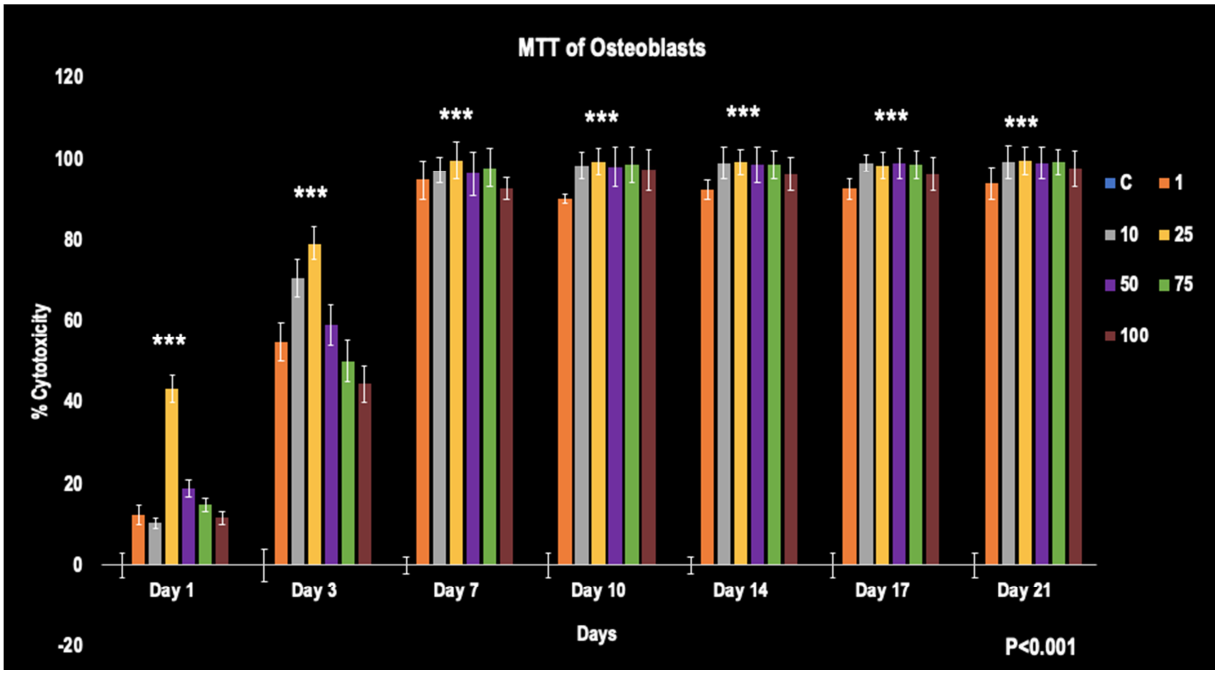
***- represents the statistical significance (p<0.001) of the Simvastatin concentrations when compared to the control at all time points.

Figure 3. *Cytotoxicity of osteoblasts affected by different concentrations of Simvastatin at 1, 3, 7, 10, and 14 days. (N=6).*

The error bars represent the standard deviations of six replicates.

3.4. MTT

It was noted that the osteoblasts cell cytotoxicity was drastically affected by all Simvastatin concentrations. The control was at 0% cell toxicity at all time points. On day 1, 25 umol/L showed the highest cytotoxicity at 43% when comes to the rest of the concentrations. By day 7, almost all concentrations showed 100% toxicity except 1 umol/L which was at 95%. At day 21, 1 umol/L was still showing consistent results of being at 95% whereas the rest of the concentrations were at 100% toxicity. There was statistical significance amongst the concentrations when compared to the control at all time points (p<0.001). (Figure 4)



***- represents the statistical significance (p<0.001) of the Simvastatin concentrations when compared to the control at all time points.

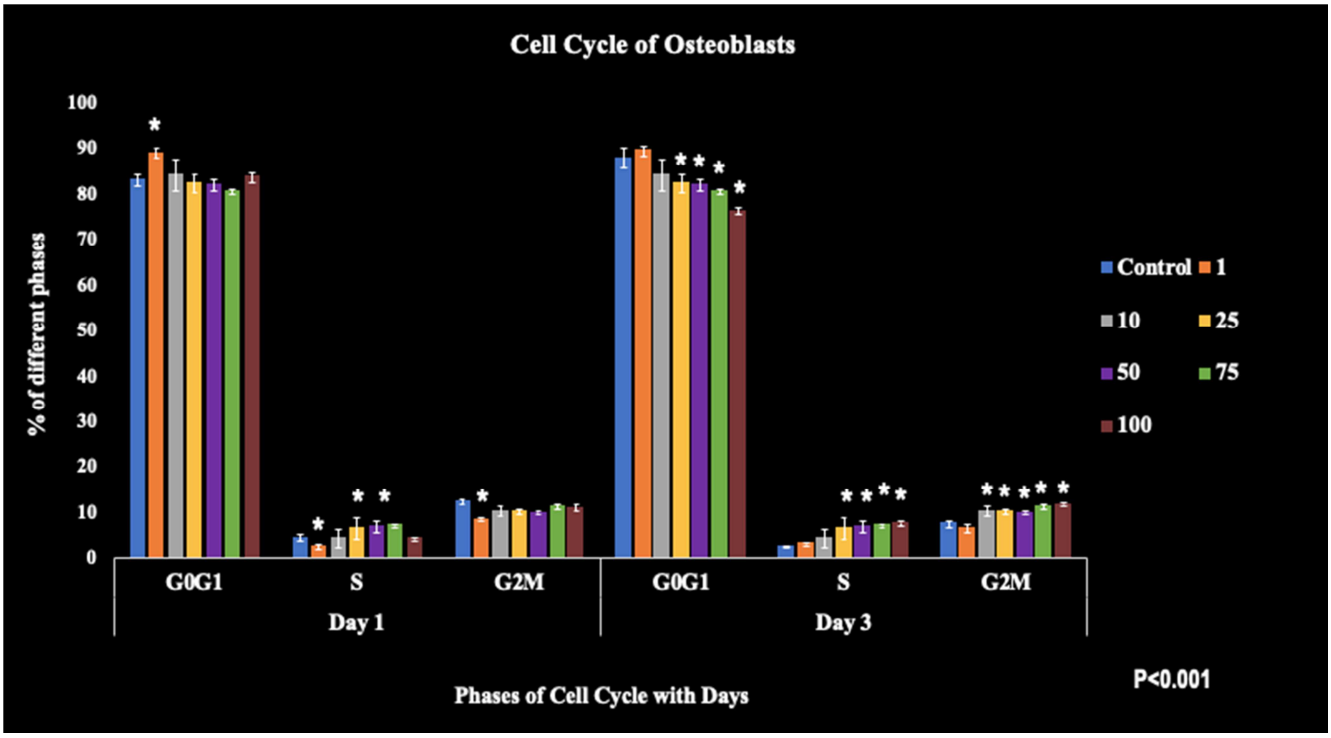
Figure 4. *MTT of osteoblasts affected by different concentrations of Simvastatin at 1, 3, 7, 10, 14, 17, and 21 days. (N=6).*

The error bars represent the standard deviations of six replicates.

3.5. Cell Cycle

On day 1, 1 umol/L showed an increase in the G0G1 phase when compared to the rest of the concentrations. In the S phase, 25, 50, and 75 umol/L were rising than the other groups. The control grew in the G2M phase (Figure 5).

On day 3, the control and 1 umol/L had increased cells in the G0G1 phase. 25, 50, 75, and 100 umol/L had a significant number of cells in the S phase when compared to the control (Figure 5).



*, **- represents the statistical significance ($p<0.001$) of the Simvastatin concentrations when compared to the control at all time points.

Figure 5. *The cell cycle of Osteoblasts is affected by different concentrations of Simvastatin at 1 and 3 days. (N=4).*

The error bars represent the standard deviations of four replicates.

3.6. Annexin Apoptosis

As shown in Figure 6, the control produced the lowest percentages of apoptosis on days 1 and 3. On day 1, cells treated with Simvastatin expressed fewer early apoptotic cells when compared to day 3. Surprisingly, 1 umol/L and 100 umol/L showed similar percentages in early apoptotic cells. At day 3, all concentrations except for the control increased in early apoptotic levels and 10 umol/L expressed more dead cells when compared to the rest of the concentrations (Figure 6).

4. Discussion

The effects of statins on bone formation have long been a topic of interest in medical research. One of the first pieces of evidence for this connection came from Mundy et al., who reported that statins could stimulate bone formation in rodents [6]. The mechanism appeared to be through the upregulation of bone morphogenic protein-2 (BMP-2) in osteoblasts, which promoted their differentiation into bone-forming cells [25]. Furthermore, statins may inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a process that also has a dampening effect on osteoclasts, the cells responsible for bone resorption [26]. More recent studies have also suggested that statins could modulate inflammation, promote osteogenesis and angiogenesis, and inhibit osteoblast apoptosis and osteoclastogenesis [4, 6, 7, 27-37]. However, this positive picture [38-40] has been complicated by other studies showing that statins might not always have beneficial effects on bone health [41-43]. In a randomized controlled trial, Simvastatin had no significant effect on bone mineral density and bone turnover in postmenopausal osteopenic women [44]. Some studies have also suggested that statins could be harmful to bone healing [45-54].

The present study observed a notable decline in the proliferation rates of osteoblasts and increased toxicity, which was confirmed by Annexin levels observed in this study with Simvastatin. These data seem to contradict to many previous reports on statin's stimulatory effect on bone cells. The possible reasons for this difference could partially be the cell types and experimental design for the study. Many previous studies investigating statins' effects on osteoblasts were

performed either on animal cell lines, immortalized cell lines, malignant osteosarcoma cell lines, or stem cells. The present study that examined the influence of Statins on normal human osteoblasts makes the study unique as it mimics a situation more relevant to the clinical scenario. In addition, different passages of cells were used in this present study and other studies. The present experiments were performed on cell cultures at 2nd passage. Min used cell cultures between the 5th and 7th passages [55]. In this experience, the phenotypic behavior of normal osteoblasts would start to deter significantly after the 2nd passage of the culture. The phenotype of the cells can drastically affect how Simvastatin reacts to it. Further, time intervals of experiment design also played a big role in this study. Most studies were short-term [11, 55-57]. The present study is unique because the long-term effects of Simvastatin on normal human osteoblasts for up to 21 days were studied.

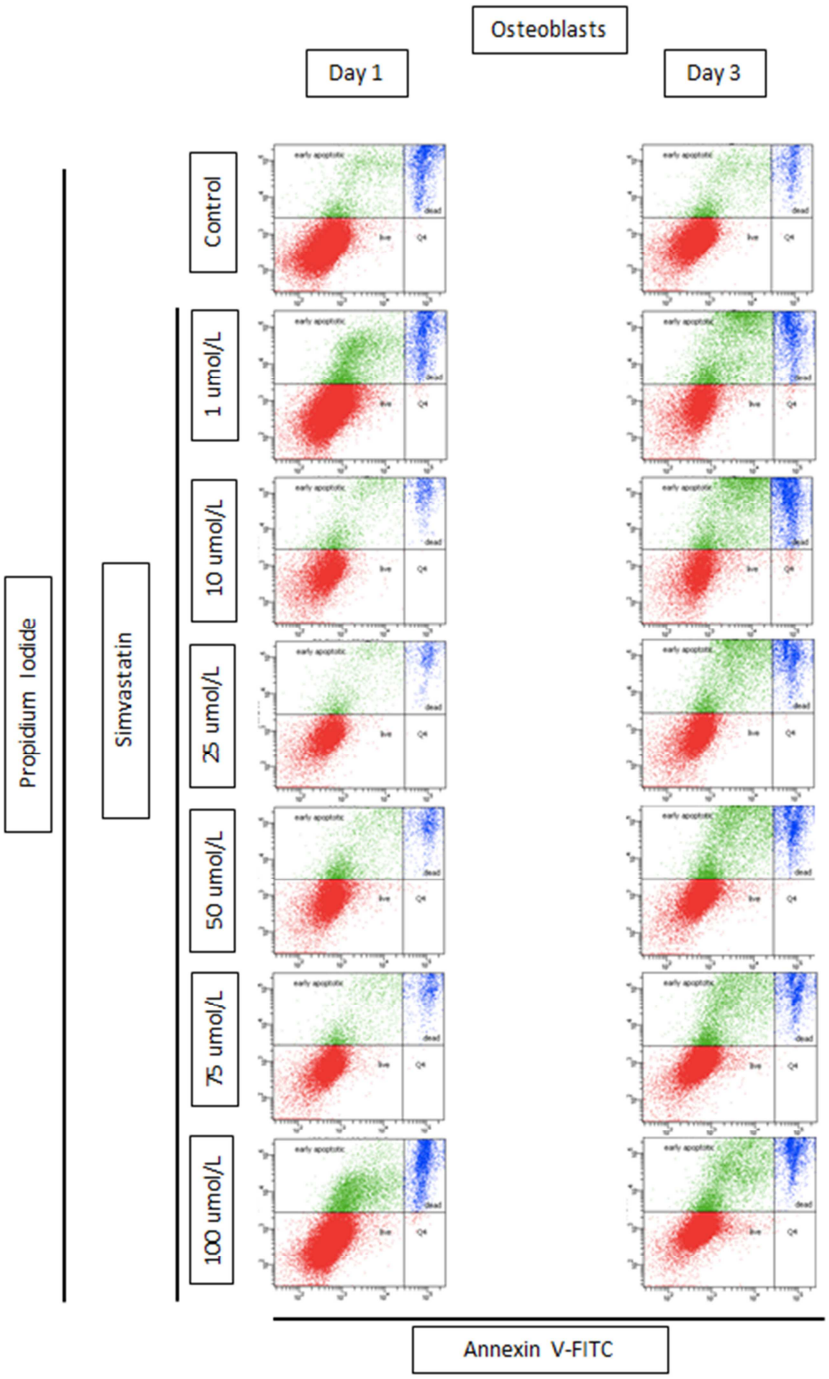


Figure 6. *Annexin levels of Osteoblasts were affected by different concentrations of Simvastatin at 1 and 3 days.*

Cell attachment efficiency of osteoblasts in the present study showed no statistical significance amongst the concentrations of Simvastatin. No previous study has investigated the attachment of osteoblasts with Simvastatin.

However, one previous article looked at cell attachment and proliferation on the SIM-loaded scaffold with pre-osteoblast MC3T3-E1 cells, which significantly improved the efficiency and the rates [58].

Proliferation rates decreased in all Simvastatin concentrations and at all time intervals of the present study (Figure 2). Baek et al, had similar results that the addition of Simvastatin decreased cell proliferation in a dose-dependent manner [56]. The results of the present study partially disagreed with many of the other studies, in which they had found an initial increase in proliferation rates dose-dependently at 24 hours, but after 72 hours, it had decreased [11, 59]. It has also been noticed that Simvastatin inhibited the cell proliferation of smooth muscle cells and endothelial cells [60, 61].

The toxicity of Simvastatin on normal human osteoblasts was first assessed in this study showing a dose and time-dependent increase in the cytotoxic effect. This finding confirmed the previous study that Simvastatin decreased cell viability in a dose-dependent manner in bone marrow stromal cells [56]. Multiple studies reported that higher concentrations of Simvastatin resulted in a decrease in cell viability [62, 63]. Contradicting the results of the present study, the MTT assay in Yazawa et al [11], noticed an increase in cell metabolism in human PDL cells in the initial 24 hours but decreased in the 72 hours.

The results in this study indicate that there was a significant difference among the groups in the distribution of the G0G1 phase, with Simvastatin-treated cells showing a dose-dependent increased response in osteoblasts. An increase was observed in all concentrations during the S phase compared to the control, which showed increased numbers in the G2M phase. The treatment of Simvastatin at 1 umol/L led to a decrease in the peak of the cells in the G2/M phase [57], like the present results. Another study used MG63 malignant human osteosarcoma cells showing an increase in the G0G1 phase but a decrease in the S phase [64]. Statins delay cell cycling in the G1 and G2/M phases and led to apoptosis of smooth muscle cells [65].

There is a lack of consistency in the published data detailing the anabolic effects of statins on bone [66]. Von Stechow evaluated the effect of statins on the elimination of bony defects in the mandible of rabbits and reported that the amount of regenerated bone was not significantly different with or without exposure to Statins [46]. However, the inhibitory function of Simvastatin on bone cell proliferation demonstrated in this study also demonstrated that it might interfere with new bone formation.

5. Conclusion

In conclusion, the findings of the present study compel evidence that Simvastatin exerts a discernible impact on the proliferation and cell viability of osteoblasts, ostensibly through the induction of apoptosis as corroborated by flow cytometric analysis.

Despite the apparent potential of Simvastatin in the context of dental and bone health, these studies underscore the complexities and potential pitfalls in its application. It is of paramount importance that Simvastatin's effects are fully comprehended prior to in vivo or clinical studies.

References

[1] Kwak B, Mulhaupt F, Veillard N, Pelli G, Mach F. The HMG-CoA reductase inhibitor simvastatin inhibits IFN-gamma induced MHC class II expression in human vascular endothelial cells. Swiss Med Wkly 2001; 31 (3-4): 41-46.

[2] Vollmer T, Key L, Durkalski V, Tyor W, Corboy J, Markovic-Plese S, et al. Oral simvastatin treatment in relapsing-remitting multiple sclerosis. Lancet 2004; 63 (9421): 1607-1608.

[3] Hatzigeorgiou C, Jackson JL. Hydroxymethylglutaryl-coenzyme A reductase inhibitors and osteoporosis: a meta-analysis. Osteoporos Int 2005; 6 (8): 990-998.

[4] Oxlund H, Dalstra M, Andreassen TT. Statin given perorally to adult rats increases cancellous bone mass and compressive strength. Calcif Tissue Int 2001; 9 (5): 299-304.

[5] Oxlund H, Andreassen TT. Simvastatin treatment partially prevents ovariectomy-induced bone loss while increasing cortical bone formation. Bone 2004; 4 (4): 609-618.

[6] 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 (5446): 1946-1949.

[7] Skoglund B, Forslund C, Aspenberg P. Simvastatin improves fracture healing in mice. J Bone Miner Res 2002; 17 (11): 2004-2008.

[8] Gutierrez G, Lalka D, Garrett I, Rossini G, Mundy G. Transdermal application of lovastatin to rats causes profound increases in bone formation and plasma concentrations. Osteoporosis international 2006; 7: 1033-1042.

[9] Liu C, Wu Z, Sun HC. The effect of simvastatin on mRNA expression of transforming growth factor-beta1, bone morphogenetic protein-2 and vascular endothelial growth factor in tooth extraction socket. Int J Oral Sci 2009; 1 (2): 90-98.

[10] Liu M, Wang K, Tang T, Dai K, Zhu Z. The effect of simvastatin on the differentiation of marrow stromal cells from aging rats. Die Pharmazie-An International Journal of Pharmaceutical Sciences 2009; 64 (1): 43-48.

[11] Yazawa H, Zimmermann B, Asami Y, Bernimoulin JP. Simvastatin promotes cell metabolism, proliferation, and osteoblastic differentiation in human periodontal ligament cells. J Periodontol 2005; 76 (2): 295-302.

[12] Maeda T, Matsunuma A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochemical and biophysical research communications 2001; 280 (3): 874-877.

[13] Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. Biochem Biophys Res Commun 2000; 271 (3): 688-692.

[14] 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 (2): 145-150.

[15] Sallam MM. The influence of oral administration of simvastatin on delayed non-union facial fractures—clinical study. J Am Sci 2011; 7 (5): 812-818.

[16] Lin LD, Lin SK, Chao YL, Kok SH, Hong CY, Hou KL, et al. Simvastatin suppresses osteoblastic expression of Cyr61 and progression of apical periodontitis through enhancement of the transcription factor Forkhead/winged helix box protein O3a. J Endod 2013; 39 (5): 619-625.

[17] Lin SK, Kok SH, Lee YL, Hou KL, Lin YT, Chen MH, et al. Simvastatin as a novel strategy to alleviate periapical lesions. J Endod 2009; 35 (5): 657-662.

[18] Lai EH, Hong CY, Kok SH, Hou KL, Chao LH, Lin LD, et al. Simvastatin alleviates the progression of periapical lesions by modulating autophagy and apoptosis in osteoblasts. J Endod 2012; 38 (6): 757-763.

[19] Kok SH, Hou KL, Hong CY, Wang JS, Liang PC, Chang CC, et al. Simvastatin inhibits cytokine-stimulated Cyr61 expression in osteoblastic cells: a therapeutic benefit for arthritis. Arthritis Rheum 2011; 63 (4): 1010-1020.

[20] Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences 2000; 97 (25): 13625-13630.

[21] Suchanek J, Soukup T, Ivancakova R, Karbanova J, Hubkova V, Pytlik R, et al. Human dental pulp stem cells--isolation and long term cultivation. Acta Medica (Hradec Kralove) 2007; 50 (3): 195-201.

[22] Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, et al. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell and tissue research 2013; 53 (1): 65-78.

[23] Raoof M, Yaghoobi MM, Derakhshani A, Kamal-Abadi AM, Ebrahimi B, Abbasnejad M, et al. A modified efficient method for dental pulp stem cell isolation. Dental Research Journal 2014; 1 (2): 244.

[24] Samiei M, Aghazadeh M, Alizadeh E, Aslaminabadi N, Davaran S, Shirazi S, et al. Osteogenic/Odontogenic Bioengineering with co-Administration of Simvastatin and Hydroxyapatite on Poly Caprolactone Based Nanofibrous Scaffold. Adv Pharm Bull 2016; (3): 353-365.

[25] Garrett IR, Mundy GR. The role of statins as potential targets for bone formation. Arthritis research & therapy 2002; (4): 1-4.

[26] Edwards C, Russell R, Spector T. Statins and bone: myth or reality? Calcified tissue international 2001; 9: 63-66.

[27] Moshiri A, Shahrezaee M, Shekarchi B, Oryan A, Azma K. Three-dimensional porous gelapin–simvastatin scaffolds promoted bone defect healing in rabbits. Calcified tissue international 2015; 6: 552-564.

[28] Maritz FJ, Conradie MM, Hulley PA, Gopal R, Hough S. Effect of statins on bone mineral density and bone histomorphometry in rodents. Arteriosclerosis, thrombosis, and vascular biology 2001; 1 (10): 1636-1641.

[29] Junqueira JC, Mancini MN, Carvalho YR, Anbinder AL, Balducci I, Rocha RF. Effects of simvastatin on bone regeneration in the mandibles of ovariectomized rats and on blood cholesterol levels. Journal of oral science 2002; 4 (3-4): 117-124.

[30] 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; 3 (10): 1141-1148.

[31] Wong R, Rabie A. Statin collagen grafts used to repair defects in the parietal bone of rabbits. British Journal of Oral and Maxillofacial Surgery 2003; 1 (4): 244-248.

[32] Saraf S, Singh A, Garbyal R, Singh V. Effect of simvastatin on fracture healing—an experimental study. 2007.

[33] Moriyama Y, Ayukawa Y, Ogino Y, Atsuta I, Koyano K. Topical application of statin affects bone healing around implants. Clinical oral implants research 2008; 9 (6): 600-605.

[34] Pauly S, Back DA, Kaeppler K, Haas NP, Schmidmaier G, Wildemann B. Influence of statins locally applied from orthopedic implants on osseous integration. BMC musculoskeletal disorders 2012; 3 (1): 1-8.

[35] Ayukawa Y, Ogino Y, Moriyama Y, Atsuta I, Jinno Y, Kihara M, et al. Simvastatin enhances bone formation around titanium implants in rat tibiae. Journal of oral rehabilitation 2010; 7 (2): 123-130.

[36] Goes P, Lima APS, Melo IM, Rêgo ROCC, Lima V. Effect of Atorvastatin in radiographic density on alveolar bone loss in wistar rats. Brazilian Dental Journal 2010; 1: 193-198.

[37] Yoshii T, Hafeman AE, Nyman JS, Esparza JM, Shinomiya K, Spengler DM, et al. A sustained release of lovastatin from biodegradable, elastomeric polyurethane scaffolds for enhanced bone regeneration. Tissue Engineering Part A 2010; 6 (7): 2369-2379.

[38] Edwards C, Hart D, Spector T. Oral statins and increased bone-mineral density in postmenopausal women. The Lancet 2000; 55 (9222): 2218-2219.

[39] Meier CR, Schlienger RG, Kraenzlin ME, Schlegel B, Jick H. Statin Drugs and the Risk of Fracture—Reply. Jama 2000; 284 (15): 1921-1922.

[40] Chan KA, Andrade SE, Boles M, Buist DS, Chase GA, Donahue JG, et al. Inhibitors of hydroxymethylglutaryl-coenzyme A reductase and risk of fracture among older women. The Lancet 2000; 55 (9222): 2185-2188.

[41] van Staa T-P, Wegman S, de Vries F, Leufkens B, Cooper C. Use of statins and risk of fractures. Jama 2001; 85 (14): 1850-1855.

[42] Bjarnason N, Riis B, Christiansen C. The effect of fluvastatin on parameters of bone remodeling. Osteoporosis International 2001; 2: 380-384.

[43] Sirola J, Sirola J, Honkanen R, Kröger H, Jurvelin J, Mäenpää P, et al. Relation of statin use and bone loss: a prospective population-based cohort study in early postmenopausal women. Osteoporosis International 2002; 3: 537-541.

[44] Rejnmark L, Buus HN, Vestergaard P, Heickendorff L, Andreasen F, Larsen LM, et al. Effects of simvastatin on bone turnover and BMD: a 1-year randomized controlled trial in postmenopausal osteopenic women. Journal of Bone and Mineral Research 2004; 9 (5): 737-744.

[45] Staal A, Frith JC, French MH, Swartz J, Güngör T, Harrity TW, et al. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. Journal of Bone and Mineral Research 2003; 8 (1): 88-96.

[46] Von Stechow D, Fish S, Yahalom D, Bab I, Chorev M, Müller R, et al. Does simvastatin stimulate bone formation in vivo? BMC musculoskeletal disorders 2003; : 1-10.

[47] Murray MM, Spindler KP, Devin C, Snyder BS, Muller J, Takahashi M, et al. Use of a collagen-platelet rich plasma scaffold to stimulate healing of a central defect in the canine ACL. Journal of Orthopaedic Research 2006; 4 (4): 820-830.

[48] Golomb BA, Dimsdale JE, White HL, Ritchie JB, Criqui MH. Reduction in blood pressure with statins: results from the UCSD Statin Study, a randomized trial. Archives of internal medicine 2008; 68 (7): 721-727.

[49] Mammen AL, Amato AA. Statin myopathy: a review of recent progress. Current opinion in rheumatology 2010; 2 (6): 644-650.

[50] Godlee F. Adverse effects of statins. In.: British Medical Journal Publishing Group; 2014.

[51] Oryan A, Kamali A, Moshiri A. Potential mechanisms and applications of statins on osteogenesis: Current modalities, conflicts and future directions. Journal of controlled release 2015; 15: 12-24.

[52] Tomlinson SS, Mangione KK. Potential adverse effects of statins on muscle. Physical therapy 2005; 5 (5): 459-465.

[53] Mansi I, Frei CR, Pugh MJ, Makris U, Mortensen EM. Statins and musculoskeletal conditions, arthropathies, and injuries. JAMA internal medicine 2013; 73 (14): 1318-1326.

[54] Peeters G, Tett SE, Conaghan PG, Mishra GD, Dobson AJ. Is statin use associated with new joint-related symptoms, physical function, and quality of life? Results from two population-based cohorts of women. Arthritis care & research 2015; 7 (1): 13-20.

[55] Min KS, Lee YM, Hong SO, Kim EC. Simvastatin promotes odontoblastic differentiation and expression of angiogenic factors via heme oxygenase-1 in primary cultured human dental pulp cells. J Endod 2010; 6 (3): 447-452.

[56] 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. Journal of Korean medical science 2005; 0 (3): 438-444.

[57] Okamoto Y, Sonoyama W, Ono M, Akiyama K, Fujisawa T, Oshima M, et al. Simvastatin induces the odontogenic differentiation of human dental pulp stem cells in vitro and in vivo. J Endod 2009; 5 (3): 367-372.

[58] Nath SD, Linh NT, Sadiasa A, Lee BT. Encapsulation of simvastatin in PLGA microspheres loaded into hydrogel loaded BCP porous spongy scaffold as a controlled drug delivery system for bone tissue regeneration. Journal of Biomaterials Applications 2014; 8 (8): 1151-1163.

[59] Liu S, Bertl K, Sun H, Liu Z-H, Andrukhov O, Rausch-Fan X. Effect of simvastatin on the osteogenetic behavior of alveolar osteoblasts and periodontal ligament cells. Human Cell 2012; 5: 29-35.

[60] van Vliet AK, Nègre-Aminou P, van Thiel GCF, Bolhuis PA, Cohen LH. Action of lovastatin, simvastatin, and pravastatin on sterol synthesis and their antiproliferative effect in cultured myoblasts from human striated muscle. Biochemical pharmacology 1996; 2 (9): 1387-1392.

[61] Nègre-Aminou P, van Vliet AK, van Erck M, van Thiel GCF, van Leeuwen RE, Cohen LH. Inhibition of proliferation of human smooth muscle cells by various HMG-CoA reductase inhibitors; comparison with other human cell types. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 1997; 345 (3): 259-268.

[62] Chen P-Y, Sun J-S, Tsuang Y-H, Chen M-H, Weng P-W, Lin F-H. Simvastatin promotes osteoblast viability and differentiation via Ras/Smad/Erk/BMP-2 signaling pathway. Nutrition research 2010; 0 (3): 191-199.

[63] Yang Y-m, Huang W-d, Xie Q-m, Xu Z-r, Zhao Q-j, Wu X-m, et al. Simvastatin attenuates TNF-α-induced growth inhibition and apoptosis in murine osteoblastic MC3T3-E1 cells. Inflammation research 2010; 9: 151-157.

[64] Feng X, Yue X, Niu M. Simvastatin-Loaded Nanomicelles Enhance the Osteogenic Effect of Simvastatin. Journal of Nanomaterials 2020; 020: 1-14.

[65] Guijarro C, Blanco-Colio LM, Ortego M, Alonso C, Ortiz A, Plaza JJ, et al. 3-Hydroxy-3-methylglutaryl coenzyme a reductase and isoprenylation inhibitors induce apoptosis of vascular smooth muscle cells in culture. Circulation research 1998; 3 (5): 490-500.

[66] Yao W, Farmer R, Cooper R, Chmielewski P, Tian X, Setterberg R, et al. Simvastatin did not prevent nor restore ovariectomy-induced bone loss in adult rats. JOURNAL OF MUSCULOSKELETAL AND NEURONAL INTERACTIONS 2006; (3): 277.