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Cytotoxic and Apoptotic Effects of Simvastatin on Human Dental Pulp Cells

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Abstract: Statins, specifically hydroxymethylglutaryl-coenzyme-A reductase inhibitors (HMG-Co-A), are well-recognized for their capacity to reduce plasma cholesterol levels. Nevertheless, a comprehensive investigation into the biocompatibility of Simvastatin with human dental pulp cells has been somewhat lacking. The principal objective of this study was to conduct an in-depth examination of the effects of varying concentrations of Simvastatin on critical aspects such as cell attachment, proliferation, toxicity, cell cycle progression, and apoptosis in normal human dental pulp cells. To execute this, dental pulp cells derived from healthy human pulp tissue were subjected to Simvastatin treatments at concentrations ranging from 1 µmol/L to 100 µmol/L, with a control group at 0 µmol/L. The evaluation encompassed an assessment of cell attachment at a 16-hour interval. Subsequent investigations spanned proliferation rates and cytotoxicity assessments conducted at 7, 14, and 21 days. Additional analyses included cell cycle progression and apoptotic events at 1- and 3 days post-treatment. The statistical analysis relied on ANOVA, with a significance threshold set at p-values of ≤ 0.05. The results yielded several noteworthy findings. Notably, a concentration of 25 µmol/L demonstrated a substantial and statistically significant enhancement ($p < 0.001$) in cell attachment efficiency. However, it became evident that all tested concentrations of Simvastatin led to a marked reduction in the proliferation rate ($p < 0.001$) and a concurrent increase in cytotoxicity ($p < 0.001$). Furthermore, the analysis of cell cycle progression and apoptosis revealed a progressive and statistically significant increase with the passage of time ($p < 0.001$). In summary, the outcomes of this investigation underscore a prominent adverse effect of Simvastatin on normal human dental pulp cells. Specifically, this statin appears to detrimentally influence cell proliferation and overall cell viability through the induction of apoptosis, as indicated by the findings of this study.

Keywords: Simvastatin, Human Dental Pulp Cells, Toxicity, Apoptosis

1. Introduction

Hydroxymethylglutaryl-coenzyme A inhibitors (statins) are known to reduce plasma cholesterol levels and cardiovascular morbidity and mortality [1, 2]. Statins inhibit the rate-limiting step of cholesterol synthesis by preventing HMG-CoA from being reduced to mevalonate via HMG-CoA reductase [2]. By inhibiting the hepatic cholesterol biosynthesis at the level of HMG-CoA reductase, this drug increases hepatic low-density lipoprotein receptors, resulting in an increased uptake of low-density lipoprotein cholesterol from the blood and the subsequent lowering of circulating cholesterol levels.

Simvastatin has multiple functions, including anti-inflammation, the induction of angiogenesis, the improvement of vascular endothelial cell function [3], and advantageous effects on many diseases, such as multiple sclerosis [4] and osteoporosis [5], which have no direct correlation with cholesterol levels. It has also been reported that Simvastatin-induced odontoblastic odontogenic differentiation in HDPCs [6]. The addition of Simvastatin

with hydroxyapatite in scaffolds increased the expression of osteogenesis markers in dental pulp stem cells (DPSC), with a possible increase in cell differentiation and bone formation [7]. It was suggested that Simvastatin-treated DPSC could be used for dental coronal pulp tissue regeneration and dentin regeneration [8]. Statins along with α-Tricalcium Phosphate could increase the proliferation, differentiation, and mineralization of HDPCs and might be used as a pulp-capping material to accelerate reparative dentin formation [9] and as root canal treatment material [10]. It has also been reported that Simvastatin could improve cell growth and the differentiation of bismuth oxide-containing Portland cement in HDPCs [11] and suppress LPS-induced inflammatory cytokines in HDPCs [12]. However, the cytotoxic effects of Simvastatin on normal human dental pulp cells have not been studied prior to its potential applications in dental clinics. The primary aim of this study was to assess the possible detrimental impacts of varying concentrations of Simvastatin on normal human dental pulp cells.

2. Materials and Methods

2.1. Cell Culture

Healthy human dental pulp explants were procured from the freshly extracted third molars of individuals aged 18 to 26 years. Stringent screening procedures were employed to ensure that these individuals had no underlying systemic or metabolic bone disorders, acute infections, or recent steroid use within six months prior to the surgical extraction. Prior to the tooth extraction procedure, eligible participants provided informed consent. The dental pulp tissues were acquired from the teeth extracted during surgical procedures conducted at the Oral Surgery Clinic of Boston University School of Dental Medicine, operating under the approval of the Institutional Review Board (IRB). Exclusion criteria encompassed teeth with preexisting restorations, carious lesions, non-vital status, a history of trauma, endodontic pathologies, or any other conditions that could compromise tooth integrity and normal tooth formation. The isolation and culture of normal human dental pulp cells followed a previously established protocol, albeit with some procedural adjustments [13-15]. The pulp tissue was 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 Eagle's Basal Medium (BME)]. The pulp tissue was maintained at 37°C, in a standard CO_2 incubator with 5% carbon dioxide, and saturated humidity until the second passage. The growth medium was refreshed at three-day intervals until the cells achieved an approximate 80% confluence. Subsequently, the cells were dissociated from the culture flask using a 0.05% Trypsin-EDTA solution (Thermo Fisher Scientific, USA). Afterward, a centrifugation step was performed using a TJ-6 Beckman Centrifuge, spinning the cells at 1000 revolutions per minute (rpm) for a duration of 5 minutes. The resulting pellet of cells was quantified and employed in subsequent experimental procedures.

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 [7, 10, 16, 17].

2.3. Cell Attachment Efficiency and Proliferation Rates Assessment

Cell attachment efficiency was assessed at sixteen hours. The pulp cells were seeded in a 24-well plate (Fisher Scientific) at a density of 1×105 cells per well. The cells were seeded with BME 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. Following a 16-hour incubation period, the culture medium was aspirated from the wells, and a series of three washes with phosphate-buffered saline (PBS) (Thermo Fisher Scientific) were performed to eliminate any residual Simvastatin traces. Subsequently, the cells were subjected to fixation by introducing 500 µL of 10% neutral buffered formalin (Sigma) into each well, maintaining this condition for 1 hour at room temperature. After fixation, a staining step was initiated by adding 500 µL of 0.2% crystal violet stain (Sigma-Aldrich) to each well, with the staining process lasting for an additional hour. To conclude the staining procedure, all wells underwent another series of three PBS washes to remove any unbound dye. The optical density of the stained cells was quantified using a microplate reader at a wavelength of 590 nm.

The dental pulp 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 16 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 introducing Simvastatin, the attachment of dental pulp cells to each well was microscopically verified. Subsequently, the culture media containing Simvastatin were refreshed every three days. At various time intervals, the cells were subjected to fixation and staining, using the identical procedure employed in the attachment experiment. To assess proliferation rates, the optical densities at these specific time points were compared to the baseline, represented by the optical density of 5×10^3 cells at the 16-hour mark. It's important to note that the optical density derived from the crystal violet stain directly correlated with the number of attached dental pulp cells.

2.4. Cell Viability Assay

Measuring cell viability 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, dental pulp cells were

seeded at the concentration of 500 cells per well in 96-well plates and were incubated for 16 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 cellular metabolic activity as an indicator of cell viability.

The dental pulp cells were seeded at the concentration of 500 cells per well in 96-well plates and were incubated for 16 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, 14, 17, and 21 days of incubation, the MTT kit was used by the manufacturer's protocol. The media was aspirated. In each well, 50 uL of new serum-free media was added, followed by the addition of 50 uL of MTT Reagent. In the Background well, 50 uL of MTT Reagent and 50 uL of cell culture media were combined. The plate was incubated for three 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 fifteen 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. RNaseA is included in the kit to digest cellular RNA to minimize background RNA staining. 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, dental pulp 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%. Simvastatin was added at 16 hours. Flow Cytometry was used on days 1 and 3.

2.7. Flow Cytometry Annexin Apoptosis Assay

Apoptosis, or programmed cell death, is integral to normal cellular development and life cycle. 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 offers 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, dental pulp cells were seeded between 100-500,000 cells per well adding Simvastatin at different concentrations as designed at 16 hours and the Annexin kit was used as instructed on days 1 and 3 of cultures.

2.8. Statistics

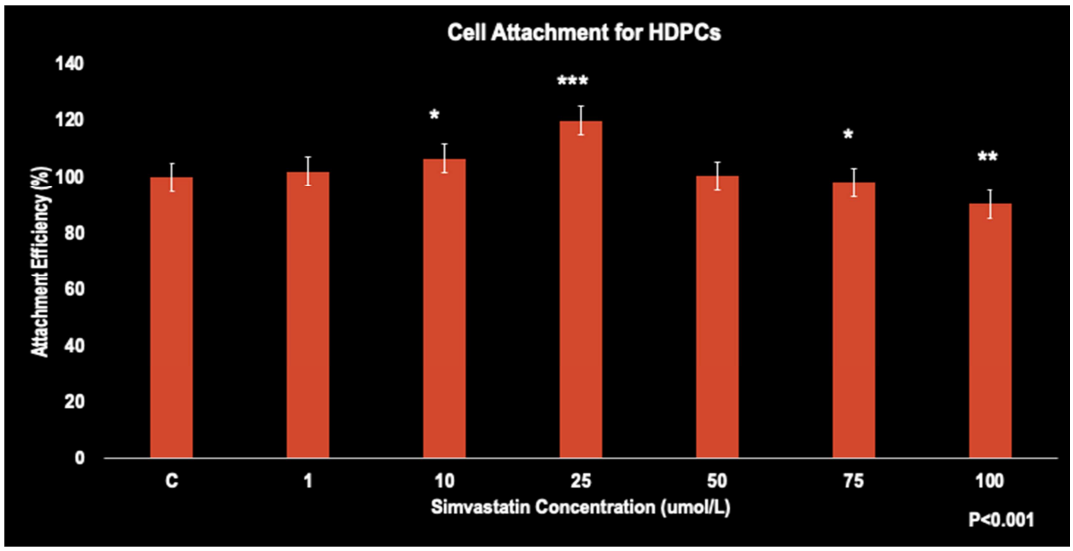
All experiments were conducted with six replicates, and the data are presented as means with corresponding standard deviations (SD). The means and SD values were determined for various parameters, including osteoblast cell attachment efficiency and proliferation rate at 9 hours, 7 days, 14 days, and 21 days. Additionally, cell viability and cytotoxicity were assessed at 7 days, 14 days, and 21 days, with an analysis of cell cycle and annexin. Statistical analysis was carried out using JMP Pro 12 software (version 12.1.0) with Student's t-test and ANOVA to identify any statistically significant differences between the groups. Statistical significance was considered for differences at p-values of ≤ 0.05.

3. Results

3.1. Cell Attachment Efficiency

An interesting observation of cell attachment efficiency was made at the 16-hour time point. Among the various concentrations of Simvastatin tested, the 25 µmol/L concentration was associated with a significant boost in

attachment efficiency ($P < 0.001$) (Figure 1).



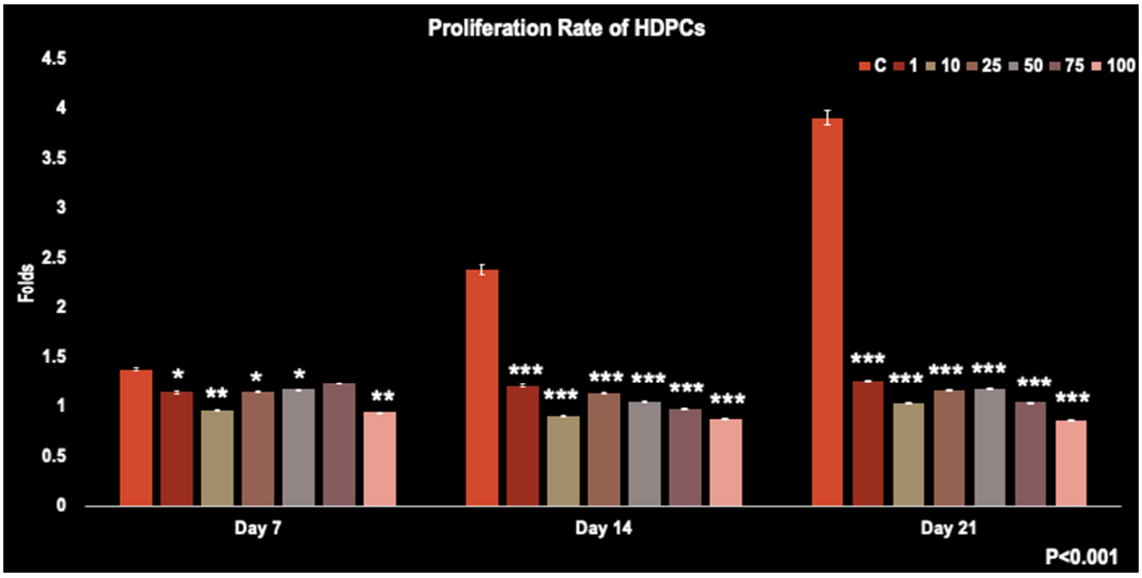
*, **, ***- represent the significant difference ($p<0.001$) of the concentrations when compared to the control.

Figure 1. *Cell attachment efficiency of HDPCs at 16 hours with all Simvastatin concentrations (N=6).*

The error bars represent the standard deviations of six replicates.

3.2. Proliferation Rates

From day 7 to 21, all groups of Simvastatin at different concentrations showed significant decreases in cell proliferation rates compared with the control group. A statistically significant difference was noted when the various concentrations were compared to the control at each time point ($p<0.001$) (Figure 2).



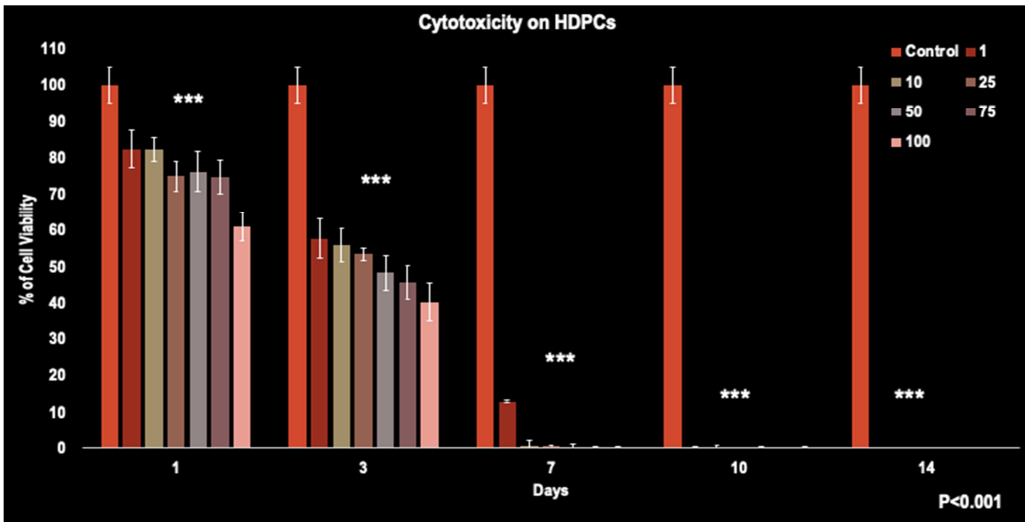
*, **, ***- represent the significant difference ($p<0.001$) of the concentrations when compared to the control.

Figure 2. *The proliferation rate of HDPCs affected by Simvastatin concentrations from days 7, 14, and 21 (N=6).*

The error bars represent the standard deviations of six replicates.

3.3. Cell Viability

All Simvastatin concentrations were found to severely impact the viability of HDPCs. While setting the control as 0 at all time points as the baselines, the viability of cells in all other concentrations had decreased so drastically by day 7 that only the 1 umol/L concentration remained at 17%. By day 10, all concentrations showed 0% cell viability, except for the control. All groups of Simvastatin concentrations showed significant decreases in cell viability compared to the control at each time point ($p<0.001$) (Figure 3).



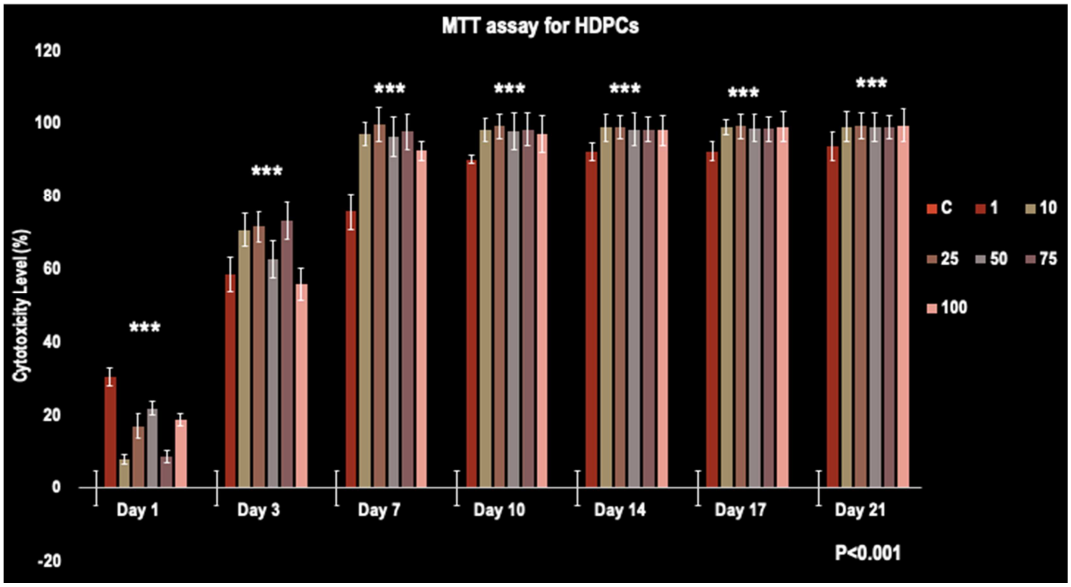
***- represents the statistical significance (p<0.001) of the Simvastatin concentrations when compared to the control.

Figure 3. *Cell Viability of HDPCs 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 Assay

All Simvastatin concentrations were found to severely impact HDPCs cell cytotoxicity. While the control was set at 0% as the baseline at all time points, all concentrations significantly increased HDPC's toxicity levels. By day 7, only the 1 umol/L concentration remained at 75% cytotoxicity. All concentrations showed significant increases in cytotoxicity levels compared to the control at each time point (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 assay of HDPCs 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.

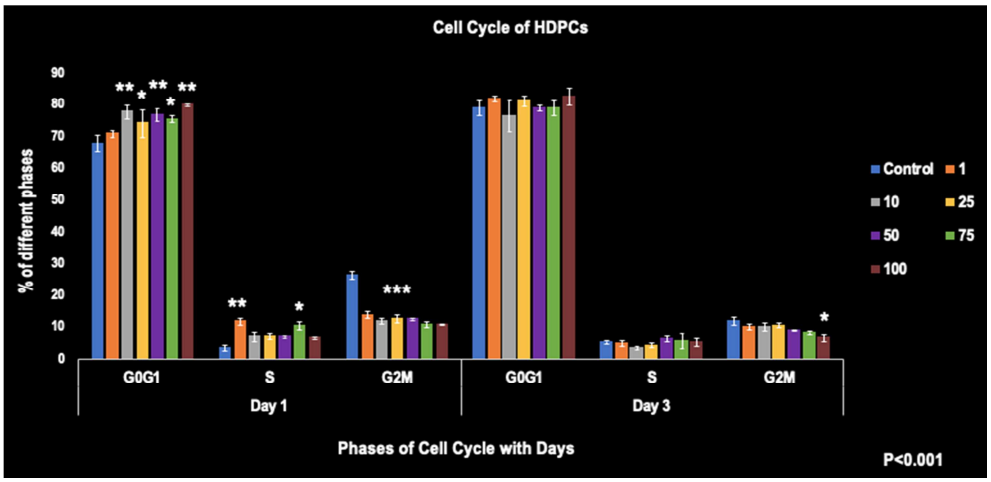
3.5. Cell Cycle

Day 1:

The figure indicates that there was a significant difference among the groups in the G0G1 phase, with Simvastatin-treated cells showing a dose-dependent response. An increase was observed in all concentrations during the S phase compared to the control, which showed increased numbers in the G2M phase (Figure 5).

Day 3:

No statistical significance was identified among the groups at the G0G1 and S phases, while the control presented an increase in the G2M phase compared to the rest of the groups (Figure 5).



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

Figure 5. *Cell cycle of HDPCs 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 the figure indicates, the control exhibited the lowest percentages of apoptosis on both days. On days 1 and 3, Simvastatin-treated HDPCs showed a dose- and time-dependent increase in early apoptotic cells. The concentrations of 25 and 50 umol/L showed a higher number of dead cells compared to the rest of the concentrations on day 3 (Figure 6).

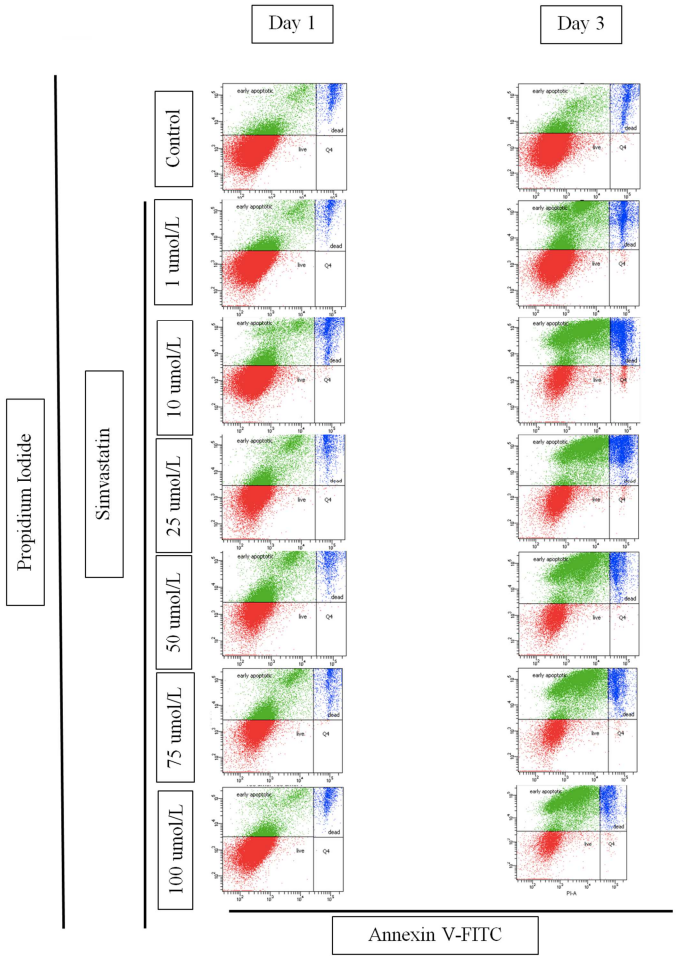


Figure 6. *Annexin levels of HDPCs were affected by different concentrations of Simvastatin at 1 and 3 days.*

4. Discussion

Statins are known to reduce plasma cholesterol levels and cardiovascular morbidity and mortality [1, 2]. It has been reported that Simvastatin induced odontoblastic differentiation and angiogenesis in HDPCs [18, 19] and in DPSCs [6]. The addition of Simvastatin with hydroxyapatite in scaffolds increased the expression of osteogenesis markers in DPSCs [7]. The present study observed first time a notable decline in the proliferation rates of normal HDPCs and significantly increased toxicity caused by Simvastatin at different concentrations. These data seem to contradict many previous reports on statin's stimulatory effect on HDPCs [6-8] and bone cells [10, 17, 20, 21]. 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. This study, which investigated the impact of Statins on normal human pulp cells, stands out for its ability to replicate a scenario that closely resembles clinical conditions. 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 rather than much later passages used in other studies [18]. It was experienced in the present study that the phenotypic behavior of normal human dental pulp cells would start to deteriorate significantly after the 2nd passage of 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 [6, 18, 20, 22]. The present study is unique because the long-term effects of Simvastatin on normal human dental pulp cells for up to 21 days were studied.

The current results showed that a concentration of 25 umol/L Simvastatin had the best effect on cell attachment for HDPCs. No previous study investigated the attachment of HDPCs with Simvastatin. However, it was observed a notable decline in the proliferation rates of pulp cells when treated with Simvastatin at all different concentrations. Agreeing with the present study, Okamoto et al, found that Simvastatin at concentrations of 1 and 10 umol/L was able to significantly suppress the proliferation of dental pulp stem cells at Day 3 and Day 5 cultures without inducing apoptosis [6]. The current study showed that Simvastatin at all concentrations ranging from 1 umol/L to 100 umol/L significantly suppressed the proliferation rate of normal human dental pulp cells at all time intervals of 7,14 and 21 days. This also contradicts many previous studies that suggested Simvastatin stimulates cell proliferation of HDPCs [9]. Simvastatin has been shown to suppress the cell proliferation rate of other cell types such as bone marrow stromal cells [22] and periodontal ligament fibroblasts (PDL) [20].

The toxicity of Simvastatin on normal human dental pulp cells was first assessed in this study, in which a dose and time-dependent increase in cytotoxicity was noticed. The previous study reported that Simvastatin decreased cell viability in a dose-dependent manner in bone marrow stromal cells [22]. The presence of Simvastatin led to a notable decrease in the viability of HDPCs and PDL cells, with this effect being dependent on both the dosage and duration of exposure [23]. The results of this study indicate a significant decrease in cell proliferation rate and cell viability of HDPCs by Simvastatin at all concentrations and time intervals tested, probably by inducing apoptosis. The induction of apoptosis by Statins is primarily attributed to the reduction of isoprenoid levels. This reduction, in turn, can inhibit the geranylgeranylation and/or farnesylation of proteins. These effects may result in increased cytosolic calcium levels, leading to the activation of calpain and the initiation of the mitochondrial-mediated apoptotic signaling pathway [24]. There was a significant increase in apoptosis observed in HDPCs and PDL, especially in cells treated with 10 mM simvastatin, as previously reported [23].

5. Conclusion

In conclusion, this research compels evidence that Simvastatin significantly influences the proliferation and viability of human dental pulp cells, presumably via the induction of apoptosis as confirmed by flow cytometry analysis. Although Simvastatin exhibits potential beneficial impacts within the realm of dental health, these investigations accentuate the intricate nature and potential obstacles related to its applications. The comprehension of Simvastatin's influences is crucial before its utilization in vivo experiments or clinical trials.

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