Original Research
Endodontic Therapy

Dose- and time-dependent effects of taxifolin on viability and mineralization markers of osteoblast-like cells

Loiane MASSUNARI[a]
Rafaela Laruzo RABELO[a]
Maria Luisa LEITE[b]
Diana Gabriela SOARES[c]
Giovana ANOVAZZI[b]
Carlos Alberto Souza COSTA[b]
Cristiane DUQUE[a]

[a]Universidade Estadual Paulista – Unesp, Araçatuba Dental School, Department of Preventive and Restorative Dentistry, Araçatuba, SP, Brazil.

[b]Universidade Estadual Paulista – Unesp, Araraquara School of Dentistry, Department of Physiology and Pathology, Araraquara, SP, Brazil.

[c]Universidade de São Paulo – USP, Bauru School of Dentistry, Department of Operative Dentistry, Endodontics and Dental Materials, Bauru, SP, Brazil.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

Corresponding Author:
Cristiane Duque
E-mail: cristianeduque@yahoo.com.br

https://doi.org/10.1590/1807-3107bor-2021.vol35.0140

Submitted: January 15, 2021
Accepted for publication: July 13, 2021
Last revision: August 18, 2021

Abstract: The current study evaluated the effects of taxifolin treatments on the viability of osteoblast-like cells, and on the expression of early mineralization markers, as part of the ongoing search for new endodontic materials able to induce periapical healing without causing cytotoxicity. Saos-2 osteoblast-like cells were exposed to different concentrations of taxifolin (5 and 10 μM), applied as pretreatments either for 24h and 72h, or continuously throughout the experimental protocol. Cell viability using the methylthiazole tetrazolium (MTT) assay, alkaline phosphatase activity using thymolphthalein monophosphate assays, deposition of mineralized nodules using alizarin red staining, and expression of ALP and COL-1 by qPCR were determined after 6 and 13 days of treatment. The data were analyzed statistically ($p<0.05$). Taxifolin was not cytotoxic in the concentrations tested. Pretreatments with taxifolin for 24h and 72h at 10 μM stimulated ALP activity, and increased mineralized nodule deposition by Saos-2 cells. Continuous treatment with taxifolin was not effective in stimulating ALP activity and mineralization. ALP and COL-1 gene expression increased with taxifolin pretreatments, since the highest mRNA levels were observed after 72h of pretreatment with taxifolin at 10 μM on day 13. In conclusion, taxifolin was cytocompatible, and induced mineralization markers when applied for short periods in osteoblast-like cell culture.

Keywords: Osteoblasts; Alkaline Phosphatase; Calcification, Physiologic.

Introduction

Bone formation is a complex process which involves different cell types, such as proliferating pre-osteoblasts, bone matrix-producing osteoblasts, osteocytes (or mature bone-lining cells), and osteoclasts, which are responsible for bone reabsorption and remodeling. The events of bone formation are modulated by several hormones and cell products secreted in response to tissue injury.[1,2] In vitro models of osteogenesis and bone nodule formation have been used to assess the effects of new compounds on the proliferation, differentiation, and mineralized matrix deposition of osteoblastic cells.[3] A substantial number of genes, including alkaline phosphatase (ALP), type I collagen (COL-1), bone morphogenetic protein-2 (BMP-2), and osteocalcin (OCN) are highly

expressed in the first periods of these processes. Following extracellular matrix deposition, osteoblasts begin mineral deposition, which extends along and within the collagen fibrils.[2,4]

A large variety of chronic inflammatory diseases are associated with bone loss. They occur when acute inflammation fails to eliminate infectious and non-infectious agents, such as microbial pathogens, damaged cells, or toxic products.[5] In dentistry, microorganisms that colonize necrotic root canal systems, or products released therefrom can diffuse through apical or lateral foramens, and cause damage to the periodontal ligament, cementum, and alveolar bone.[6] In response to microbial invasion, host defenses produce a variety of pro-inflammatory mediators, such as cytokines and prostaglandins, which are involved in the induction of inflammatory cell migration and osteoclastogenesis.[7,8] Chemical-mechanical treatment, and medicaments with multiple therapeutic effects are required to eliminate pulp and periapical infection, control the inflammatory response, and induce bone mineralization.[9]

Some authors have explored the osteogenic potential of natural compounds such as flavonoids for the treatment of bone diseases.[10,11,12] Taxifolin (or dihydroquercetin) is a pentahydroxyflavanone found in several plants, and included in food supplements by virtue of its broad therapeutic action, including antioxidant,[13] anti-inflammatory,[13] antibacterial,[14] and anticancer[15] activities. Taxifolin has been reported to induce osteoblast differentiation,[10,12] and increase bone sialoprotein and osteocalcin mRNA expression, as well as levels of secreted osteocalcin,[10] which are important markers of late mineralization produced by mature osteoblasts. Furthermore, taxifolin has also been found to inhibit in vitro osteoclastogenesis in RAW264.7 murine cells,[10,12] and in human bone marrow-derived macrophages (BMMs) induced by the receptor activator NF-κB ligand (RANKL).[11,12] The effect of taxifolin on ALP and COL-1 levels, on early markers of osteoblast differentiation, and on early mineralization has not yet been studied. In an effort to further the ongoing search for new endodontic materials able to induce periapical healing without causing cytotoxicity,[16,17] the present study aimed to evaluate the effects of taxifolin treatments on the viability of a human osteoblast-like cell line (Saos-2) and the expression of mineralization markers. The null hypothesis was that the taxifolin-based treatments do not stimulate the expression of mineralization markers (ALP activity, mineralized nodules, and ALP/COL-1 mRNA levels) in osteoblast-like cells.

Methods

Study design

Taxifolin (#78666, Sigma-Aldrich, Saint Louis, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), and the stock solution was stored at -20° C. Table 1 presents the experimental groups according to taxifolin concentrations and treatment time points, and Figure 1 shows the experimental design of the study. Saos-2 human osteoblast-like cells at passage 34 were cultured in Dulbecco's Modified Eagle's Medium – DMEM (GIBCO, Grand Island, USA), with 10% fetal bovine serum (FBS; GIBCO, Grand Island, USA), penicillin (100 IU/mL), streptomycin (100 µg/mL), and glutamine (2 mmol/L) (GIBCO). The cells were seeded ($2.5x10^3$ cells/well) in 96-well plates (P0), and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air (Thermo Plate, Fisher Scientific, Pittsburgh, USA) for 24 h. After incubation (P1), taxifolin treatments were performed as follows: 24 h pretreatment (P2) – cells were exposed once to the flavonoid treatment; 72 h pretreatment (P3) – cells were exposed three times daily until completing 72 h; continuous treatment (CT) – cells were exposed to taxifolin daily throughout the experimental protocol (Figure 1).[18] All the cells were cultivated up to two time points of analysis: 6 or 13 days (P4 and P5). Both pre- and continuous treatments were assayed with taxifolin at 5 and 10 µM (T5 and T10). Aspiration of P2 and P3 was followed by replacement with DMEM, which was refreshed every 24 h until completion of the experimental period (6 or 13 days). The control groups were supplemented with DMEM containing DMSO at 10 µM (Control DMSO), or not containing DMSO (Control DMEM). All the assays were performed in triplicate.[18]

Cell viability analysis

Cell viability was evaluated by using the methylthiazole tetrazolium (MTT) assay 1 day (P2), 3 days (P3), 6 days (P4), and 13 days (P5) after seeding the cells. The MTT assay is based on the succinate dehydrogenase enzyme produced by mitochondria, which reduces the MTT salt metabolically, converting it into formazan crystals. The treatments or DMEM were aspirated, after which 5 ug/mL MTT (Sigma-Aldrich) was applied to the wells, and the plate was incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 4 h. Thereafter, the MTT solution was aspirated, and replaced by an acidified isopropanol solution to dissolve the formazan crystals. Cell viability was calculated by absorbance at 570 nm in a spectrophotometer (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek Instruments, Winooski, USA). The data were standardized considering mean absorbance of the control group (Control DMEM) on day 1 as 100% cell viability.[18,19]

Table 1. Description of groups chosen for this study.

Group	Compound/ Concentration	Treatment
T5-24h	Taxifolin 5 μM	Pretreatment of 24h
T10-24h	Taxifolin 10 μM	Pretreatment of 24h
T5-72h	Taxifolin 5 μM	Pretreatment of 72h
T10-72h	Taxifolin 10 μM	Pretreatment of 72h
T5-CT	Taxifolin 5 μM	Continuous treatment
T10-CT	Taxifolin 10 μM	Continuous treatment
Control DMSO	DMSO 10 μM	Continuous treatment
Control DMEM	No compound	No treatment

Alkaline phosphatase assays

Total protein production

The quantification of total protein was performed according to Leite et al.,[18] with some modifications. On the sixth day, the treatments were aspirated, and 200μL of 0.1% sodium lauryl sulfate (Sigma-Aldrich) previously dissolved in deionized water was added to each well to lyse the cells. After the solution was left to rest 40 min at room temperature, it was homogenized and 100 μL was separated to perform the ALP activity assay. Next, Lowry reagent (Sigma-Aldrich) was added to the lysed cells, and incubated for 20 min at room temperature, followed by adding Folin-Ciocalteu's phenol reagent (Sigma-Aldrich), diluted in deionized water at a ratio of 1:3, for 30 min. After this period, all the samples were read in a spectrophotometer (Synergy H1) to determine absorbance at 655 nm. A standard curve of bovine serum albumin (BSA, Sigma-Aldrich) was determined to measure the total protein of each sample.[18]

Alkaline phosphatase activity

ALP activity was evaluated following a protocol based on dephosphorylation of thymolphthalein by

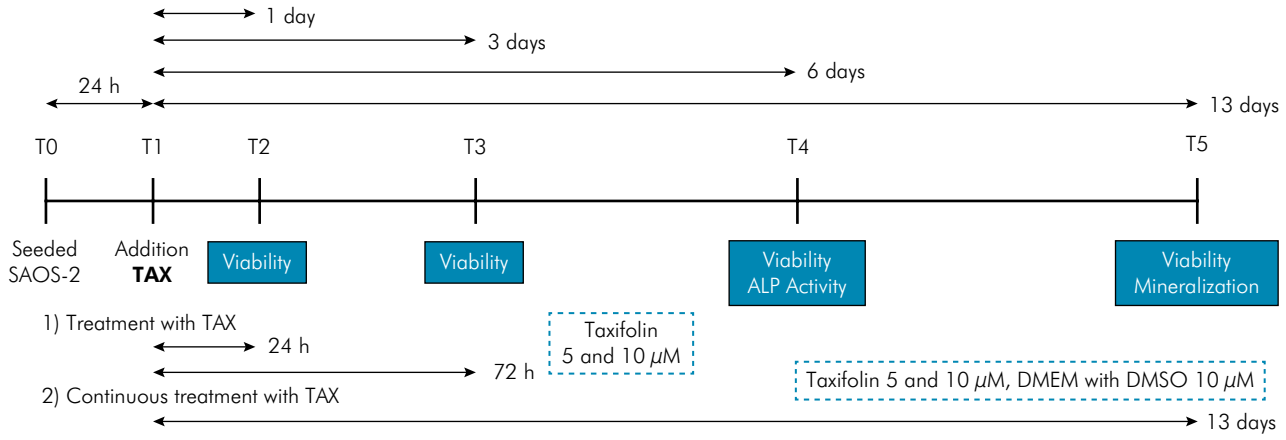


Figure 1. Experimental design of the study.

ALP - Alkaline Phosphatase Assay Kit-Test Endpoint (Labtest Diagnostics, Lagoa Santa, Brazil). After six days of experimentation, one tube containing 50 µL of thymolphthalein monophosphate 22 mmol/L, and 500 µL of buffer solution at 300 mmol/L (pH 10.1) was prepared for each well. Then, 50 µL of cell lysis solution (prepared in the previous assay) was added to the respective tubes and incubated for 20 min at 37°C. Afterwards, 2 mL of color reagent was placed in each tube and homogenized. The absorbance of the solution was determined at 590 nm using a spectrophotometer (Synergy H1). A standard ALP-based curve (Standard 45 U/L) measured the ALP activity, whose values were calculated by dividing the ALP dosage by those of total protein; the percentage for total protein was then calculated considering the control group (Control DMEM) as 100%.[18]

Mineralized nodule formation

Alizarin red staining was used to evaluate the mineralized nodule formation on the final days of treatment (days 6 and 13). This assay was based on quantifying the mineralized matrix produced by osteoblast-like cells. The treatments were aspirated, and the cells were fixed in 70% cold ethanol for 2 h. The ethanol was then aspirated, and each well received 100 µL of alizarin red (pH 4.2; Sigma-Aldrich), after which the samples were incubated under continuous shaking for 20 min. The wells were rinsed twice with deionized water, and incubated at room temperature to dry for 24 h. Next, mineral nodules of each group were identified and photographed by using a light microscope (Olympus BX51; Olympus, Miami, USA) equipped with a digital camera (Olympus C5060; Olympus, Miami, USA). Lastly, 150 µL of cetylpyridinium chloride solution (Sigma-Aldrich) was added to each well, and maintained under continuous shaking for 15 min to dissolve the nodules. The quantitative analysis was performed based on the mineralized nodule formation measured by absorbance at 570 nm in a spectrophotometer (Synergy H1). The mean absorbance value of the control group (Control DMEM) on day 6 was considered as 100% of mineralized matrix deposition.[18]

ALP and COL-1 gene expression

Based on the results of previous assays, real time quantitative PCR (qPCR) analysis was performed only with the highest concentrations (10 µM) of the pretreatments (24–72 h). On days 6 and 13, the treatments were aspirated, and the total RNA was extracted following the protocol of the RNAqueous Kit (Ambion, Austin, TX, USA). RNA quantification was performed by determining absorbance with 1 µL of total RNA in a spectrophotometer (Synergy H1). The cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), following the manufacturer's protocol. The qPCR was performed by SYBR® Green reagents (Applied Biosystems, Foster City, CA, USA), and specific primers and probe sets were designed for ALP, COL-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) analyses (Table 2). The data were analyzed using a Step One Plus software program (Applied Biosystems) with relative quantification of each mRNA according to GAPDH as the constitutive gene. Two independent experiments were performed in triplicate for each group. The data were calculated according to the $2^{\Delta\Delta CT}$ equation, using the control group for the purpose of normalization.[20,21]

Statistical analysis

Experiments were performed on three independent days using four wells per group. The results were submitted to the Kolmogorov-Smirnov test to ascertain normality. Normal data from cell viability, ALP activity, and mineralized nodule formation are presented as mean ± standard deviation, and were analyzed using ANOVA and Tukey's test. Data from gene expression did not present normality, and were

Table 2. Nucleotide sequence of primers used for each selected gene.

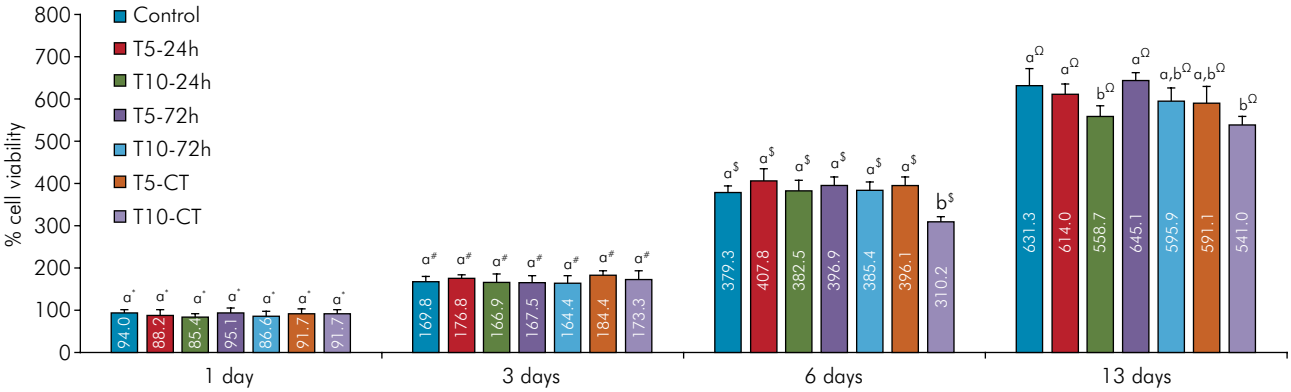
Gene	Sequence
ALP	Forward 5′-GACAAGAAGCCCTTCACTGC-3′
	Reverse 5′-AGACTGCGCCTGGTAGTTGT-3′
COL-1	Forward 5′-CAGCCGCTTCACCTACAGC-3′
	Reverse 5′-TTTTGTATTCAATCACTGTCTTGCC-3′
GAPDH	Forward 5′-CTCTGCTCCTCCTGTTCGAC-3′
	Reverse 5′-TTAAAAGCAGCCCTGGTGAC-3′

analyzed using the Kruskal-Wallis and Mann-Whitney tests. Boxplots were constructed to represent these results. The SPSS 17.0 software program (IBM Corp, Chicago, USA) was used to run the statistical analysis, considering $p< 0.05$.

Results

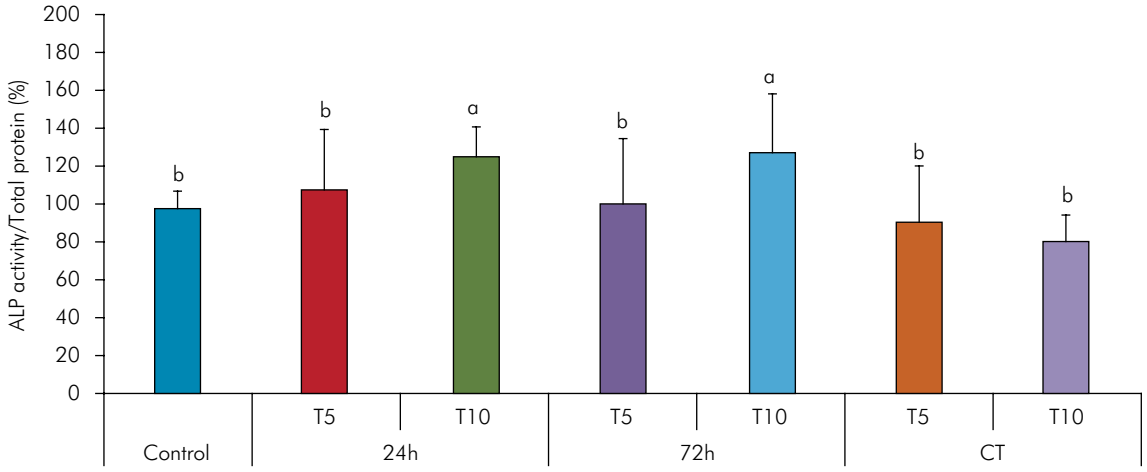
Figure 2 presents the percentage of Saos-2 cell viability immediately after exposure to different concentrations of taxifolin throughout the time points. There was a significant increase in cell viability, regardless of the treatment (p=0.001). No statistical differences were observed between the pretreatments and continuous treatments, except for T10-CT at 6 and 13 days. However, all these values were greater than 80%, as recommended by ISO 10993-12:2012,[19] thus showing that taxifolin was not cytotoxic to cells in the concentrations tested.

Taxifolin at 10 µM for 24-h or 72-h pretreatments stimulated ALP activity of Saos-2 cells, compared to the control group (Control DMSO). ALP activity from cells undergoing other types of treatments did not differ from the control (Figure 3).



[a]Different lowercase letters show statistical difference among the groups at each time point; *Different symbols show statistical difference among the time points considering each treatment condition separately. (ANOVA and Tukey test; $p < 0.05$).

Figure 2. Cell viability (mean/standard deviations) at the time points of 1, 3, 6, and 13 days of treatments with different concentrations of taxifolin (5 and 10 μM) for 24h, 72h and continuous treatment (CT), using MTT assays. Control: DMEM with 10 μM DMSO.



[a]Different lowercase letters show statistical difference among the groups at each time point. (ANOVA and Tukey test; $p < 0.05$).

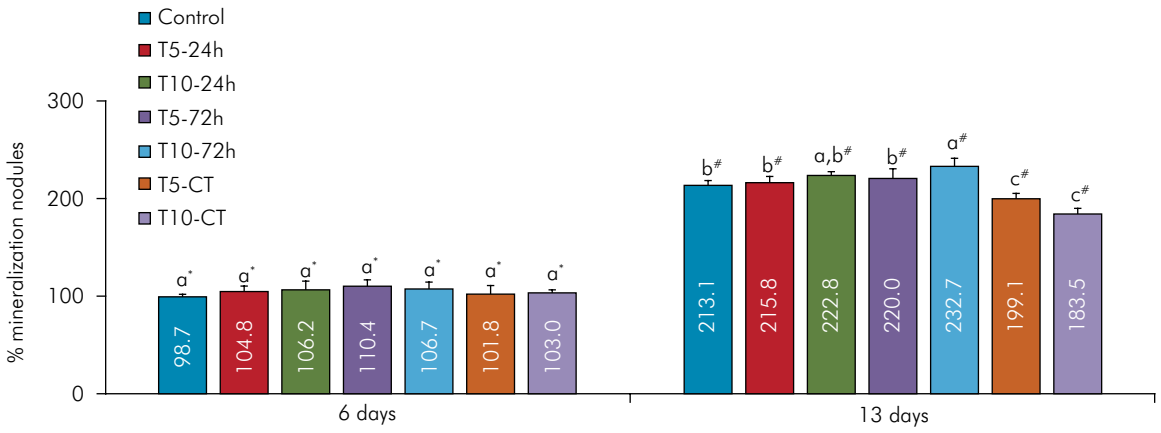
Figure 3. Alkaline phosphatase activity (means/standard deviations) after 6 days of treatment with taxifolin at 5 and 10 μM for 24h, 72h and continuous treatment (CT). Control: DMEM with 10 μM DMSO.

Figure 4 shows the mineralization ability of Saos-2 cells at 6 and 13 days in the three forms of treatment with taxifolin (24 h, 72 h pretreatments, and CT), determined by alizarin red staining. There was a significant increase in the percentage of mineralized nodule deposition over time ($p = 0.001$). At 6 days, no statistical difference was observed among the treatments. At 13 days, taxifolin at 10 µM for 72 h increased mineralized nodule deposition by Saos-2 cells. Continuous treatment with taxifolin was not effective in stimulating cell mineralization at any concentration tested. Representative images of alizarin red staining show an increase in mineralized nodule formation after 24-h and 72-h pretreatments with 10 µM taxifolin (Figures 5B and 5C), and lower mineral deposition after continuous treatment with 10 µM taxifolin (Figure 5D), compared with the control (Figure 5A – DMEM with DMSO at 10 µM).

ALP gene expression increased with taxifolin treatments, except for T10-72h pretreatment, which did not differ from the control group (Control DMSO) on day 6 (Figure 6A). There was also an increase in COL-1 gene expression on both days (6 and 13 days); however, no statistical difference was observed for T10-24h at 13 days (Figure 6B).

Discussion

The results of the present study showed that pretreatment with 10 µM taxifolin for 24 h or 72 h was not cytotoxic, and stimulated ALP activity, mineralized nodule deposition, and ALP/COL-1 gene expression, thus rejecting the null hypothesis. The MTT results demonstrated that taxifolin at 5 and 10 µM did not cause cytotoxic effects after 1, 3, 6, and 13 days of treatment, compared with the DMSO group. DMSO 10 µM was used as the control group, since all tested taxifolin concentrations were diluted in this solvent. Some treatments with the highest taxifolin concentration were able to cause a slight decrease in Saos-2 metabolism, mainly at the final time points (days 6 and 13); however, this reduction is not considered a cytotoxic effect according to ISO 10993-12:2012 recommendations.[19] Studies evaluating the stimulatory potential of taxifolin on osteoblastic differentiation in an osteogenic medium used higher concentrations[11,12] than those used in the present study. Since the aim was to biostimulate the immortalized osteoblast cell line, low concentrations had to be used to enhance the mineralization marker expression over time; these concentrations were tested at later time points, such as 6 and 13 days.[18]



[a]Different lowercase letters show statistical difference among the groups at each time point. Control: DMEM with 10 µM DMSO; *Different symbols show statistical difference among the time points, considering each treatment condition separately. (ANOVA and Tukey test; $p < 0.05$).

Figure 4. Mineralized nodule formation (means/standard deviations) at the time points of 6 and 13 days of the treatment with taxifolin at 5 and 10 µM for 24h, 72h and continuous treatment (CT), using alizarin red staining.

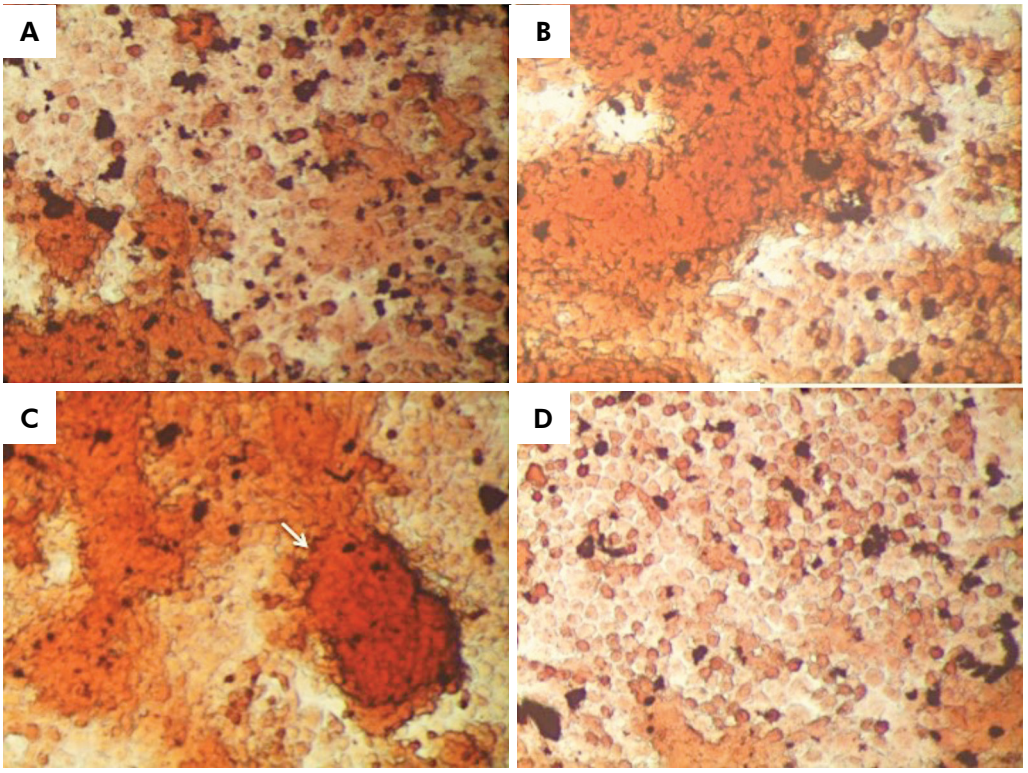
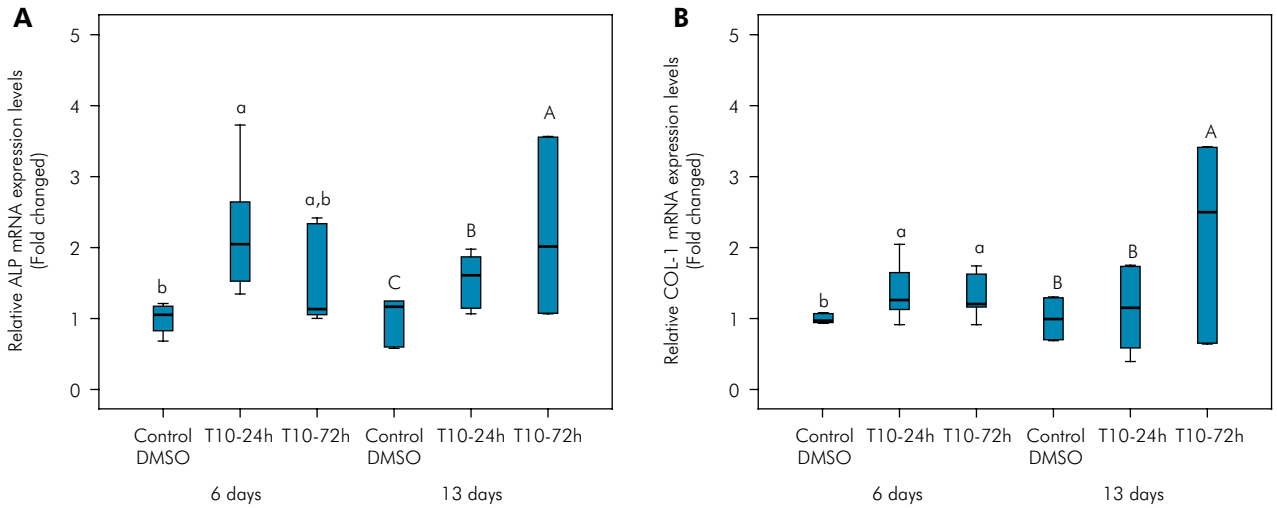


Figure 5. Representative images of alizarin red staining showing mineralization ability of Saos-2 cells after 13 days of taxifolin treatment. A: Control DMEM with μM DMSO, B: T10-24h, C: T10-72h, and D: T10-CT. White arrows show mineralized nodules. (Light microscopy, 4X).



[a]Different lowercase letters show statistical difference among the groups at 6 days; [A] Different uppercase letters show statistical difference among the groups at 13 days. (Kruskal-Wallis and Mann-Whitney tests; $p < 0.05$).

Figure 6. Expression of alkaline phosphatase - ALP (A) and collagen-1 – COL-1 (B) genes from Saos-2 cells after 6 and 13 days of treatment with taxifolin.

Saos-2 cells are considered to have a mature osteoblast phenotype;[22-25] hence, early osteogenic differentiation markers, such as ALP and COL-1, can be measured in short culture intervals, or even in a non-osteogenic medium. A study compared three osteosarcoma cell lines (MG-63, Saos-2, and

U-2 OS) with normal human osteoblasts using immunocytochemistry, cellular characteristics, and proliferation kinetics in a non-osteogenic medium. The authors observed that Saos-2 cells revealed the most mature osteoblastic labelling profile, with positive results for ALP, OC, bone sialoprotein, decorin, Col-I and collagen III[23]. In addition, natural compounds could have effects on cell differentiation and culture medium containing osteogenic supplements, such as ascorbic acid, and could mask or overlap the flavonoid effect. The effects of betulin, a naturally occurring pentacyclic triterpene, were evaluated in regard to the differentiation and mineralization of osteoblasts of two human cell lines (hFOB 1.19 and Saos-2). The cell lines were assessed with an osteogenic medium of ascorbic acid and β-glycerophosphate, and also without these supplements. Betulin increased the expression of RUNX2, COL-1A1, and OPN genes in both cell lines in the non-osteogenic medium. Moreover, the matrix mineralization in osteoblasts treated with betulin was also enhanced, showing bone nodule morphology in both cultures, even in the non-osteogenic medium.[25]

Both pretreatments (24 and 72 h) with 10 μM taxifolin were able to enhance ALP activity. Lee et al.[26] tested the same concentrations as the present study, and observed that 5 and 10 μM of the flavonoid baicalein increased ALP activity in human dental pulp cells (hDPCs) at the same time points. Some differences between their study and ours can be attributed to the characteristics of the flavonoids and cell types used in both studies, since primary cells are more sensitive than immortalized cell lines. Moreover, the hDPCs were incubated in osteogenic medium, which could stimulate the increase in ALP activity at lower concentrations of the flavonoid (5 μM). Similar to the ALP assay, the 24-h and 72-h pretreatments of 10 μM taxifolin showed better results for mineralized nodule formation. However only the T10-72h group at 13 days presented a significant increase in mineralized matrix deposition, compared with the control group. Wang et al.[12] tested higher concentrations of taxifolin and noted an increase in mineralized nodules in a dose-dependent manner. Our data corroborate these findings for both pretreatments.

The results of the ALP activity and mineralized nodule formation assays for pretreatments were better than those for continuous treatment, and indicated that low concentrations of taxifolin can stimulate Saos-2 osteoblast-like cell activity more effectively when applied for short periods of time rather than longer periods. There is a threshold of cytocompatibility for each cell type exposed to any agent, and excessive concentration of this agent can reduce the phenotype expression even when the cell remains viable.[18,27] Taxifolin stimulation seems to have reached this threshold at 72 h, and any increase in exposure time past this limit ceased to exert further positive biostimulatory effects on Saos-2 cells. This partially explains the reduction in cell viability, and in the mineralization markers of the Saos-2 cells treated with 5 to 10 μM taxifolin in continuous mode after 6 or 13 days of the experimental protocols.

ALP gene expression increased with the taxifolin treatments, except for T10-72h at 6 days. Corroborating our results, Lee et al.[26] showed an increase in ALP gene expression after 7 days of baicalein treatment at 10 μM. ALP is an enzyme attached to the cell surface of osteoblasts; it assists in the formation of an extracellular matrix, and renders the matrix competent for mineralization at the early stage of osteogenesis.[12] Taxifolin at 10 μM for 72 h increased COL-1 gene expression at the time points evaluated (6 and 13 days). Collagen is the main organic component of extracellular bone matrix, and promotes a binding site for mineral components also at the early stages of mineralization.[13] Effects similar to those presented in the current study were observed when human umbilical cord-derived mesenchymal stem cells (HUCMSCs) cultured on a bioactive surface coated with taxifolin increased COL-1 gene expression after 14 days.[13] It was speculated that collagenases would degrade the non-mineralized collagenous membrane – which covers mineralized bone – prior to osteoclastic resorption, at a later stage of mineralization.[28]

The properties of flavonoids are related to their structure. Their basic structure is a flavan nucleus consisting of two benzene rings combined with an oxygen-containing pyran ring.[29] Catechol-type flavonoids, such as taxifolin, present the o-dihydroxy

(3',4'-diOH, i.e., catechol) structure in the B ring, and have an important role in metal chelating and scavenging of free radicals, because of their high antioxidant properties.[29] According to Wang et al.,[12] the osteogenic effect of taxifolin is related to its role in suppressing the NF-Kβ signaling pathway. This pathway is activated when RANKL – a cytokine expressed in osteoblasts – is linked to a RANK receptor, expressed in osteoclast progenitor cells. NF-Kβ signaling pathway activation can promote the induction of osteoclast differentiation genes, prolonged survival of osteoclasts, increased bone resorption,[24] decreased osteogenic differentiation, and bone formation.[12] Binding between RANK and RANKL can be blocked by osteoprotegerin when it links to RANKL, thereby regulating osteoclast formation, and decreasing lesion progression. Hence, treatments able to increase the osteoprotegerin level or inhibit the NF-Kβ pathway are potential therapies to prevent bone degradation.[7]

Taxifolin significantly suppressed RANKL-induced gene expression, including tartrate-resistant acid phosphatase, matrix metalloproteinase-9 and other markers in the osteoclastogenesis model of human bone marrow-derived macrophage cultures induced by the receptor activator of RANKL.[30] A recent in vivo study showed that rats with periodontitis treated with taxifolin (1-10mg/kg/day) showed a decrease in alveolar bone loss. RANKL immunoexpression decreased with two doses of taxifolin. Although BMP-2, OCN, ALP and COL-1 expressions were also reduced, the effect was dose-dependent.[31]

This was a preliminary study that tested taxifolin in experimental protocols with Saos-2 cells. Further studies are needed to explore the effects of taxifolin in other bone mineralization markers and potential signaling pathways, and to assess possible vehicles for the application of taxifolin as a medicament for endodontic treatment, compared with gold standard medicaments.

Conclusion

Taxifolin pretreatments (24 h and 72 h) were cytocompatible and effective in inducing mineralization markers in osteoblast-like cells.

Acknowledgments

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) - grants #2015/00812-0, #2017/10940-1 and #2019/02129-7, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Funding Code 001, # 88881.068437/2014-01.

References

1. Eriksen EF. Cellular mechanisms of bone remodeling. Rev Endocr Metab Disord. 2010 Dec;11(4):219-27. https://doi.org/10.1007/s11154-010-9153-1
2. Blair HC, Larrouture QC, Li Y, Lin H, Beer-Stoltz D, Liu L, et al. Osteoblast differentiation and bone matrix formation *in vivo* and *in vitro*. Tissue Eng Part B Rev. 2017 Jun;23(3):268-80. https://doi.org/10.1089/ten.teb.2016.0454
3. Muthusami S, Senthilkumar K, Vignesh C, Ilangovan R, Stanley J, Selvamurugan N, et al. Effects of Cissus quadrangularis on the proliferation, differentiation and matrix mineralization of human osteoblast like SaOS-2 cells. J Cell Biochem. 2011 Apr;112(4):1035-45. https://doi.org/10.1002/jcb.23016
4. Beck GR Jr. Inorganic phosphate as a signaling molecule in osteoblast differentiation. J Cell Biochem 2003; 90:234-243. https://doi.org/10.1002/jcb.10622
5. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017 Dec;9(6):7204-18. https://doi.org/10.18632/oncotarget.23208
6. Siqueira Junior JF, Rôças IN. Bacterial pathogenesis and mediators in apical periodontitis. Braz Dent J. 2007;18(4):267-80. https://doi.org/10.1590/S0103-64402007000400001
7. Krum SA, Chang J, Miranda-Carboni G, Wang CY. Novel functions for NFκB: inhibition of bone formation. Nat Rev Rheumatol. 2010 Oct;6(10):607-11. https://doi.org/10.1038/nrrheum.2010.133
8. Bahuguna R, Jain A, Khan SA, Arvind MS. Role of odanacatib in reducing bone loss due to endodontic disease: an overview. J Int Soc Prev Community Dent. 2016 Dec;6(3 Suppl 3):S175-81. https://doi.org/10.4103/2231-0762.197183

9. Holland R, Gomes JE, Cintra LT, Queiroz ÍO, Estrela C. Factors affecting the periapical healing process of endodontically treated teeth. J Appl Oral Sci. 2017 Sep-Oct;25(5):465-76. https://doi.org/10.1590/1678-7757-2016-0464
10. Satué M, Arriero MM, Monjo M, Ramis JM. Quercitrin and taxifolin stimulate osteoblast differentiation in MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells. Biochem Pharmacol. 2013 Nov;86(10):1476-86. https://doi.org/10.1016/j.bcp.2013.09.009
11. Cai C, Liu C, Zhao L, Liu H, Li W, Guan H, et al. Effects of Taxifolin on osteoclastogenesis *in vitro* and *in vivo*. Front Pharmacol. 2018 Nov;9:1286. https://doi.org/10.3389/fphar.2018.01286
12. Wang YJ, Zhang HQ, Han HL, Zou YY, Gao QL, Yang GT. Taxifolin enhances osteogenic differentiation of human bone marrow mesenchymal stem cells partially via NF-κB pathway. Biochem Biophys Res Commun. 2017 Aug;490(1):36-43. https://doi.org/10.1016/j.bbrc.2017.06.002
13. Córdoba A, Satué M, Gómez-Florit M, Hierro-Oliva M, Petzold C, Lyngstadaas SP, et al. Flavonoid-modified surfaces: multifunctional bioactive biomaterials with osteopromotive, anti-inflammatory, and anti-fibrotic potential. Adv Healthcare Mat. 2015 Mar;11;4(4):540-9. https://doi.org/10.1002/adhm.201400587
14. Jeong KW, Lee JY, Kang DI, Lee JU, Shin SY, Kim Y. Screening of flavonoids as candidate antibiotics against Enterococcus faecalis. J Nat Prod. 2009 Apr;72(4):719-24. https://doi.org/10.1021/np800698d
15. Chen X, Gu N, Xue C, Li BR. Plant flavonoid taxifolin inhibits the growth, migration and invasion of human osteosarcoma cells. Mol Med Rep. 2018 Feb;17(2):3239-45. https://doi.org/10.3892/mmr.2017.8271
16. Labban N, Yassen GH, Windsor LJ, Platt JA. The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. Dent Traumatol. 2014 Dec;30(6):429-34. https://doi.org/10.1111/edt.12108
17. Yadlapati M, Souza LC, Dorn S, Garlet GP, Letra A, Silva RM. Deleterious effect of triple antibiotic paste on human periodontal ligament fibroblasts. Int Endod J. 2014 Aug;47(8):769-75. https://doi.org/10.1111/iej.12216
18. Leite ML, Soares DG, Basso FG, Hebling J, Costa CA. Biostimulatory effects of simvastatin on MDPC-23 odontoblast-like cells. Braz Oral Res. 2017;18:31:e104. https://doi.org/10.1590/1807-3107bor-2017.vol31.0104
19. International Standards Organization. ISO 10993-12:2012. Biological evaluation of medical devices - Part 5: Tests for *in vitro* cytotoxicity. 2ed. Geneva: ISO; 2012.
20. Soares DG, Bordini EA, Cassiano FB, Bronze-Uhle ES, Pacheco LE, Zabeo G, et al. Characterization of novel calcium hydroxide-mediated highly porous chitosan-calcium scaffolds for potential application in dentin tissue engineering. J Biomed Mater Res B Appl Biomater. 2020 Aug;108(6):2546-59. https://doi.org/10.1002/jbm.b.34586
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001 Dec;25(4):402-8. https://doi.org/10.1006/meth.2001.1262
22. Filova E, Vandrovcova M, Jelinek M, Zemek J, Houdkova J, Remsa J, et al. Adhesion and differentiation of Saos-2 osteoblast-like cells on chromium-doped diamond-like carbon coatings. J Mater Sci Mater Med. 2017 Jan;28(1):17. https://doi.org/10.1007/s10856-016-5830-2
23. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, et al. Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res. 2004 Nov-Dec;24(6):3743-8.
24. Prideaux M, Wijenayaka AR, Kumarasinghe DD, Ormsby RT, Evdokiou A, Findlay DM, et al. SaOS2 Osteosarcoma cells as an *in vitro* model for studying the transition of human osteoblasts to osteocytes. Calcif Tissue Int. 2014 Aug;95(2):183-93. https://doi.org/10.1007/s00223-014-9879-y
25. Mizerska-Kowalska M, Sławińska-Brych A, Kaławaj K, Żurek A, Pawińska B, Rzeski W, et al. Betulin promotes differentiation of human osteoblasts *in vitro* and exerts an osteoinductive effect on the hFOB 1.19 cell line through activation of JNK, ERK1/2, and mTOR kinases. Molecules. 2019 Jul;24(14):2637. https://doi.org/10.3390/molecules24142637
26. Lee SI, Kim SY, Park KR, Kim EC. Baicalein promotes angiogenesis and odontoblastic differentiation via the BMP and Wnt pathways in human dental pulp cells. Am J Chin Med. 2016;44(7):1457-72. https://doi.org/10.1142/S0192415X16500816
27. Meyers CD, Tannock LR, Wight TN, Chait A. Statin-exposed vascular smooth muscle cells secrete proteoglycans with decreased binding affinity for LDL. J Lipid Res. 2003 Nov;44(11):2152-60. https://doi.org/10.1194/jlr.M300252-JLR200
28. Chambers TJ, Fuller K. Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact. J Cell Sci. 1985 Jun;76(1):155-65. https://doi.org/10.1242/jcs.76.1.155
29. Amić D, Davidović-Amić D, Beslo D, Rastija V, Lucić B, Trinajstić N. SAR and QSAR of the antioxidant activity of flavonoids. Curr Med Chem. 2007;14(7):827-45. https://doi.org/10.2174/092986707780090954
30. Zhang HQ, Wang YJ, Yang GT, Gao QL, Tang MX. Taxifolin inhibits receptor activator of NF-κB ligand-induced osteoclastogenesis of human bone marrow-derived macrophages *in vitro* and prevents lipopolysaccharide-induced bone loss *in vivo*. Pharmacology. 2019;103(1-2):101-9. https://doi.org/10.1159/000495254
31. Lektemur Alpan A, Kızıldağ A, Özdede M, Karakan NC, Özmen Ö. The effects of taxifolin on alveolar bone in experimental periodontitis in rats. Arch Oral Biol. 2020 Sep;117:104823. https://doi.org/10.1016/j.archoralbio.2020.104823