Journal of Dental Sciences 16 (2021) 1117–1124

Available online at www.sciencedirect.com

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journal homepage: www.e-jds.com

Original Article

Effects of treatment with local anesthetics on RANKL expression in MG63 and PDL cells

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Received 2 April 2021; Final revision received 23 April 2021
Available online 10 June 2021

KEYWORDS
Local anesthetics;
Inflammation markers;
RANKL;
Orthodontic tooth movement;
Bone cell

Abstract *Background/purpose:* Local anesthesia (LA) application is a routine dental work in clinic. The aim of present study was to evaluate the extent of biologic effects of LA on periodontal ligaments (PDL) or bone cells (MG63).
Materials and methods: Local anesthetics (LAs) at different concentrations were added to PDL and MG 63 cells. The viability of the cells was analyzed using an MTT assay. The inflammatory markers, COX-2, IL-1, IL-6 and TNF-A, of PDL and MG63 cells treated with LAs were analyzed with a Western blot assay. The extract medium of the LA-treated PDL cells was added to the MG63 cells for subsequent culture and to examine the RANKL, ALP, and OPG expression. The data were statistically analyzed with $p < 0.05$ set as an indication of significance.
Results: The viability of the PDL and MG63 cells was less 50% at LAs concentrations above the 10 mM. At high LA concentrations, the PDL and MG63 cells treated with LAs became spherical in shape, or vesicles developed in the cytoplasm. The IL-1, IL-6, and TNF-A expression in the PDL groups showed no statistical differences between Septanest and Scandonest ($p > 0.05$). The RANKL expression in the MG63 cells increased as the Septanest and Scandonest concentrations were increased in the PDL extract medium ($p < 0.05$) after 48 h of culturing.
Conclusion: The LAs with adrenaline increased inflammation in the PDL and MG63 cells. The LA-treated PDL extract medium increased the RANKL expression in the MG63 cells.
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https://doi.org/10.1016/j.jds.2021.04.020
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Introduction

Local anesthetics (LA) procedures are used to relieve pain when performing dental works. There are many types of infiltrative local anesthetics available, and those used in dentistry include lidocaine, articaine, mepivacaine, and prilocaine. They belong to one of two classes depending on the side chain present on the active molecule.[1] Local anesthetic administration complications include both local effects and systemic effects.[2] In inflamed tissues, a local anesthetic is not as effective as it would be otherwise. This is because the acidic tissue holds the LA in ionized form and reduces the LA cross the cell lipid membrane.[3] An overdose of a LA can affect both the central nervous system and the cardiovascular system.

A number of studies have shown that LAs may affect bone tissue and lead to apoptotic cell death. The findings include mitochondrial dysfunction by collapsing the mitochondrial membrane potential, uncoupling of oxygen consumption, and adenosine diphosphate phosphorylation, releasing cytochrome C.[4–8] Bovine chondrocyte cultures and osteochondral explants treated with bupivacaine indicated up to 90% cell death in culture experiments and 42% cell death in samples with intact cartilage.[9] *In vitro* studies have demonstrated that local anesthetics can induce cell death in both animal and human chondrocytes. Clinical evidence has revealed chondrotoxicity of local anesthetics in humans. Increasing evidence is being found for an association between intra-articular injections to manage pain and the development of chondrolysis, a catastrophic development of progressive pain and stiffness of the joints.[8,10,11]

In a cultured human chondrocytes study, ropivacaine was shown to potentially be safer than bupivacaine or levobupivacaine as an intra-articular analgesic. Their chondrotoxicity may be mediated via a reactive nitrogen species-dependent pathway.[12] The cytotoxic potency of local anesthetics on human mesenchymal stem cells (MSCs) before and after chondrogenic differentiation suggests that LAs are cytotoxic to MSCs in a concentration-, time-, and agent-dependent manner in monolayer cultures but not in whole-tissue probes.[13]

Orthodontic tooth movement (OTM) is accomplished through biologic bone modeling and remodeling with an aseptic inflammatory response. OTM causes physiologic changes in osteoclast cells accumulated on a pressure site and osteoblast cells on a tension site in alveolar bone.[14] The receptor activator of nuclear factor kappa-B ligand (RANKL) determines the activation of osteoclasts. The activity of RANKL is countered by osteoblast-secreted osteoprotegerin (OPG), which acts as a decoy receptor of RANKL.[15] The osteoclasts increase as the RANKL increases, and the osteoblasts increase as the OPG increases in the bone.

Time reductions during OTM are a goal for both patients and orthodontists. Each step in an orthodontic procedure is crucial to the OTM rate. If the orthodontic tooth extraction is performed using a periodontal ligament infiltrative injection or intrabony injection. And the TAD placement is by mucosa infiltrative injection, these LAs may affect the surrounding bone tissue, PDL, and bone cells, and thus lower the OTM rate. There is scant literature on this topic. The aim of present study was to evaluate the extent of the biologic effects of LAs on periodontal ligaments and bone cells. The cytologic effects, inflammatory effects, and osteoclastogenic effects of LAs are investigated.

Material and methods

Preparation of anesthetics

1.7 ml of Scandonest (Septodont Co., Saint-Maur-des-Fossés, France) without a vasoconstrictor was diluted to 0.5 mM, 1 mM, 2 mM, 2.5 mM, 5 mM, 10 mM, and 20 mM with a culture medium. 2.2 ml of Septanest (Septodont Co., Saint-Maur-des-Fossés, France) with adrenaline was diluted to 0.5 mM, 1 mM, 2 mM, 2.5 mM, 5 mM, 10 mM, and 20 mM using a culture medium. After the pilot test, the final concentrations were 1.25 μM, 2.5 μM, 5 μM, and 10 μM for the subsequent experiments (Table 1).

Cell culturing

The human periodontal ligament cell line (PDL) and human osteosarcoma cell line (MG63) was cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum and 1% antibiotic—antimycotic solution. The cells were cultured in 24 wells multiwell plates and incubated at 37 °C under a 5% CO2 atmosphere until use. The experimental groups were divided into three groups, for which the procedures are as follows: Group 1. The MG63 and PDL cells were treated with Scandonest (Septodont) and Septanest (Septodont) anesthetics at concentrations of 1.25 μM, 2.5 μM, 5 μM, and 10 μM separately to detect the cell viability and the inflammatory markers (COX-2, IL-1, IL-6, TNF-α) using a Western blot assay. Group 2. The MG63 cell cultures were treated with 2.5 μM, 5 μM, and 10 μM concentrations of Scandonest (Septodont) and Septanest (Septodont) for 24 h to detect the MG63 cells osteogenic-related proteins (RANKL,

Table 1 The composition of the local anesthetic drugs.

Products	Composition	Content	Concentration
Scandonest 3% without vasoconstrictor (Septodont Co., Saint-Maur-des-Fossés, France)	mepivacain hydrochloride	1.7 ml/tube	106.07 mM
Septanest with adrenaline (Septodont Co., Saint-Maur-des-Fossés, France)	articaine hydrochloride、adrenaline	2.2 ml/tube	125 mM

ALP, and OPG) using a Western blot assay. Group 3. The PDL cell culture were treated with 2.5 μM, 5 μM, and 10 μM concentrations of Scandonest (Septodont) and Septanest (Septodont) for 24 h, after which the cultured medium was extracted and added to the MG63 cell cultures for another 24 h of culturing in order to examine the MG63 culture medium for osteogenic-related proteins (RANKL, ALP and. OPG).

Microscopic observation of the cell morphology

The cells in multiwell plates were placed on the stage of a microscope (Zeiss Axiovert 200 M, Jena, Germany). Scandonest (Septodont) or Septanest (Septodont) was slowly added to the wells to be examined. The images were taken under the Zeiss microscope.

MTT-colorimetry assay

Cell viability was evaluated based on the method established by Mosmann[16] with some modifications. After seeding 3 or 4 days in multiwell plates, the cells were gently rinsed twice with Dulbecco's phosphate buffered saline with the addition of 10 mM glucose- (pH 7.4). Colorless DMEM medium 300 μL was then added to each well, and the cells were temperature-equilibrated for 5 min at 37 °C in a thermo-stated heat block.

Then, LAs were added to each well and incubated with the cells for 10 min at 37 °C. At the end of the LA treatment, the medium (containing detached cells) was collected in microcentrifuge tubes from each well and centrifuged for 1 min at 5000 rpm in an Eppendorf Benchtop centrifuge. Each pellet was resuspended in 300 μL of fresh colorless DMEM medium. In addition, 300 μL of fresh, colorless DMEM medium were added to each well containing the still-attached cells. Both the cells remaining in wells and those collected in microcentrifuge tubes were then mixed with 30 μL/well (or sample) of a yellow MTT-reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Promega, Madi-son, WI, USA) and incubated at 37 °C for 4 h in a tissue culture incubator.

The optic density (OD) of the homogenous purple solution was then measured using a spectrophotometer at 570 nm (OD570) and at 630 nm (the reference wavelength) (OD630). The difference between the OD570 and OD630 settings was used for the purpose of quantifying the specific MTT conversion rate. Total MTT conversion by cells in a given well was defined as the sum of the MTT conversion by both attached and detached cells. MTT conversion found in cells without LAs treatment (the control sample) was set as 100%, and the MTT conversion found in cells treated with LAs was normalized to the corresponding control values and used as an indicator for cell viability (in % versus the untreated control groups).

Western blot assay

The PDL and MG63 cells were harvested after treatment with different LA conditions. In the Group 3 experiment, LA-treated PDL cultured medium was extracted. Cells were lysed in an NP-40 lysis buffer at 4 °C for 30 min, and the lysates were then centrifuged at 13,000 g. Afterward, the culture medium and cell lysates (30 μg protein) were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After the cells had been blocked in 5% bovine serum albumin for 1 h, the membrane was immunoblotted with TNF-α, COX-2, IL-1, IL-6, ALP, OPG, RANKL, and GAPDH antibodies for 2 h, and then washed three times in tris-(hydoxymethyl) amonomethane-buffered saline containing 0.05% Tween 20. A horseradish peroxidase conjugated secondary antibody was subsequently added, and the proteins were viewed using enhanced chemiluminescence detection kits.

The stained bands were scanned and quantified with a densitometer and Image J (National Institutes of Health, Bethesda, MD, USA), after which the protein expression levels were normalized to the GAPDH band for each sample. The results were obtained in triplicate with the use of three separate samples for each test.

Statistical analysis

The data were collected and analyzed using a one-way variance statistical analysis. Scheffe's multiple comparison test was used to determine the significance of the deviations in the data for each specimen. In all cases, the results were considered statistically significant with a $p < 0.05$.

Results

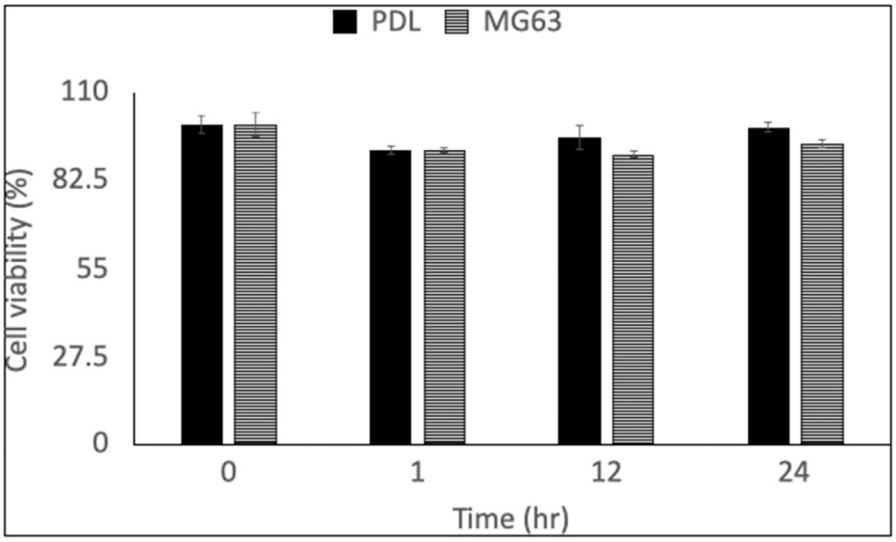
Cell viability

There were no statistically significant differences found in the cell viability of the PDS and MG63 cells within 24 h ($p > 0.05$, Fig. 1). The different concentrations of the LA anesthetics used on the PDL and MG63 cells and the cell viability are shown in Fig. 2. At concentrations higher than 10 mM, both Scandonest and Septanest groups had cell viability lower than 50%. The low cell viability of the PDL and MG63 groups after treatment with Septanest anesthetics was for the 2 mM, 2.5 mM, 5 mM, and 10 mM concentrations ($p < 0.05$, Fig. 2). The viability of the MG 63 groups was lower than that of the PDL groups ($p < 0.05$).

Changes in morphology

The morphology of the PDL and MG63 cells treated with the LAs is shown in Figs. 3—6. The morphology at the 2.5 mM and 5 mM concentrations for the Septanest-treated PDL cells was a spindle shape, but at 10 mM concentrations, the treated PDL cells developed increasing numbers of vesicles in the cytoplasm. At 20 mM concentrations, the morphology of the PDL cells became round, as shown at a 400x magnification (Fig. 3). At 2.5 mM and 5 mM concentrations of Scandonest the PDL cells formed a spindle shape but at 10 mM concentrations, the vesicles increased in the cytoplasm at a 400x magnification (Fig. 4). At 20 mM of LAs, the PDL cells appeared to float.

The morphology of 2.5mM and 5mM concentrations of Septanest treated MG63 cell showed triangular shape and



(hr)	0	1	12	24
PDL	100 ± 2.9	92.1 ± 1.6	96.0 ± 3.9	99.1 ± 1.8
MG63	100 ± 3.8	92.1 ± 1.1	90.7 ± 1.2	94.0 ± 1.5

Figure 1 The PDL and MG 63 cell viability assay. No statistical differences were found in cell viability.

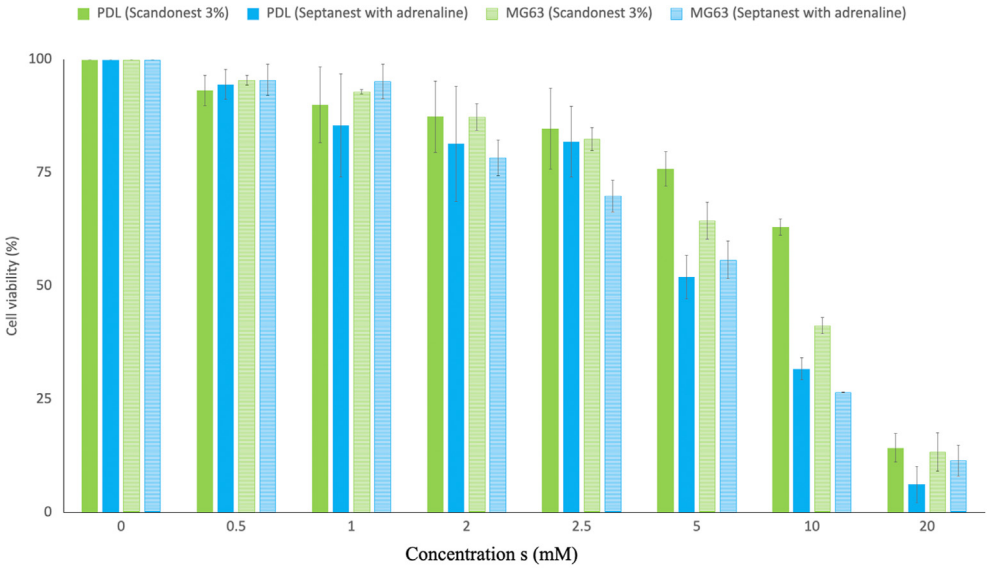


Figure 2 The effects of local anesthetic drugs on the PDL and MG63 cell survival rate. The MTT assay showed viability of less than 50% at concentrations of local anesthetic drugs higher than 10 mM.

decreased cell number. Above 10mM concentrations of Septanest, the MG63 cells became in round shape and appeared to float in the medium at a 400x magnification (Fig. 5). The morphology of 2.5 mM and 5 mM concentrations of Scandonest on MG63 cell showed triangular shape and decreased cell number. When above 10 mM concentrations of Scandonest, the MG63 cells number decreased and cells were floating in medium at 400x magnification (Fig. 6).

Inflammation markers expression

In the Septanest-treated MG63 treated group, increased expression of COX-2, IL-6, and TNF-α markers was found

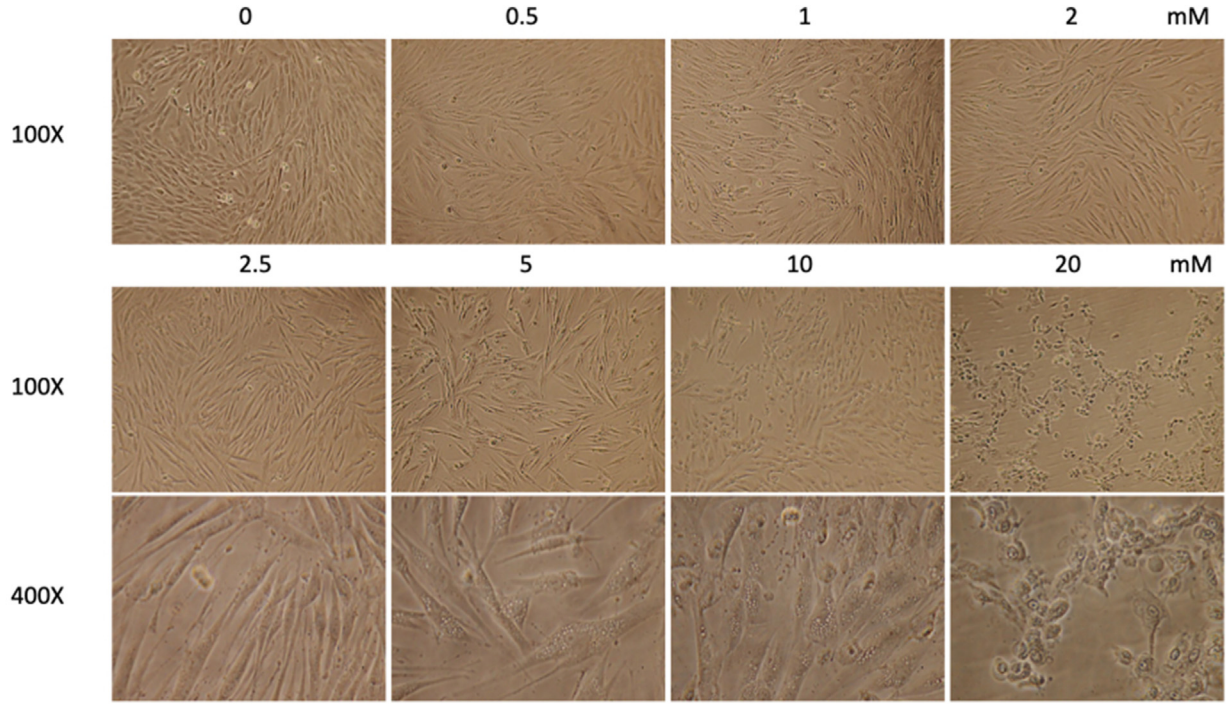


Figure 3 The PDL cell morphologies under different concentrations of Septanest anesthetic treatment at 100x and 400x magnifications. The cell numbers decreased as the treated concentrations increased. The 20 mM concentration group of PDL cells became spherical in shape.

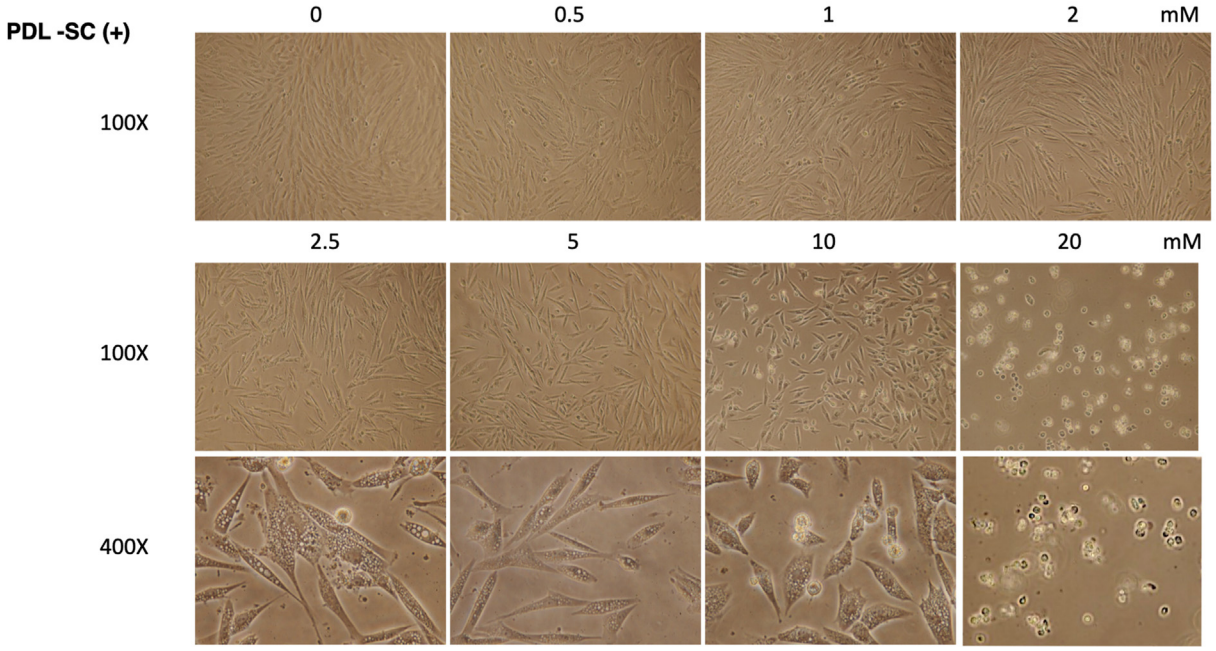


Figure 4 The PDL cell morphologies under different concentrations of Scandonest anesthetic treatment at 100x and 400x magnifications. The cell numbers decreased as the treatment concentrations increased. The 10 mM concentration group of PDL cells developed increases in the vesicles in the cytoplasm.

as the Septanest concentrations increased ($p < 0.05$ m, see Fig. 7A). The IL-1 expression in the MG63 significantly increased from as concentrations went from 0 to 5 μM. However, the band in the IL-1 expression on the 10uM Septanest-treated MG 63 cell group was unremarkable (Fig. 7A). The MG63 cells showed increased expression of COX-2, IL-1, IL-6, and TNF-A markers as the Scandonest concentrations increased ($p < 0.05$, Fig. 7A). The PDL

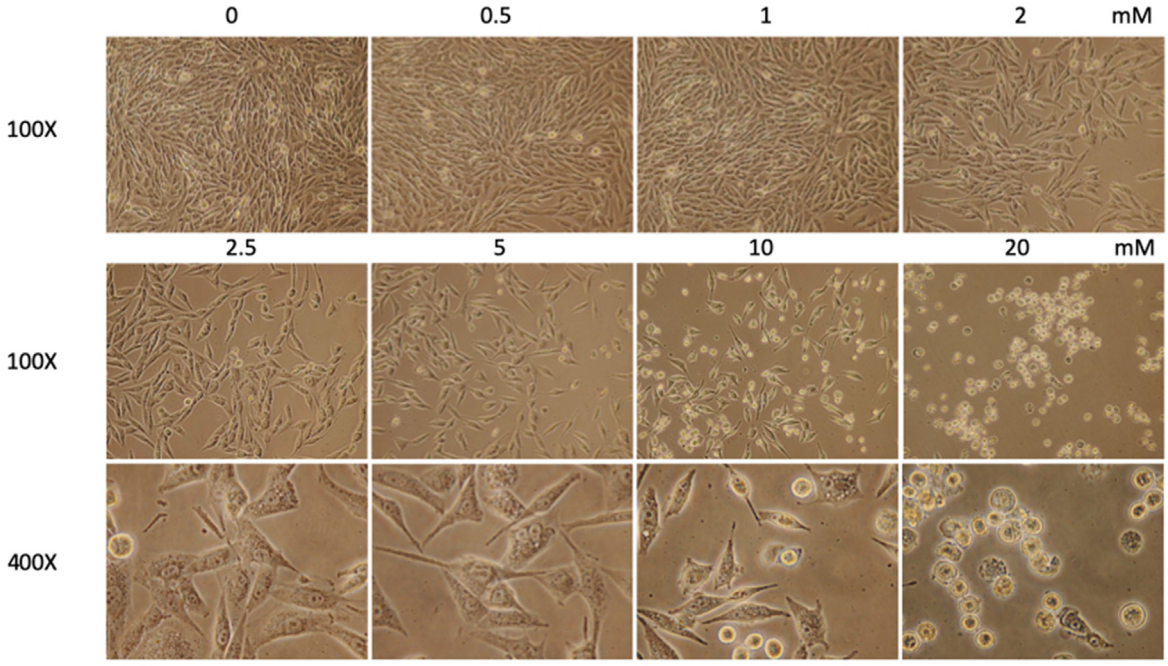


Figure 5 The MG63 cell morphologies under different concentrations of Septanest anesthetic treatment at 100x and 400x magnifications. The cell numbers decreased as the treatment concentrations increased. The 10 mM and 20 mM concentration groups of MG63 cells became spherical in shape.

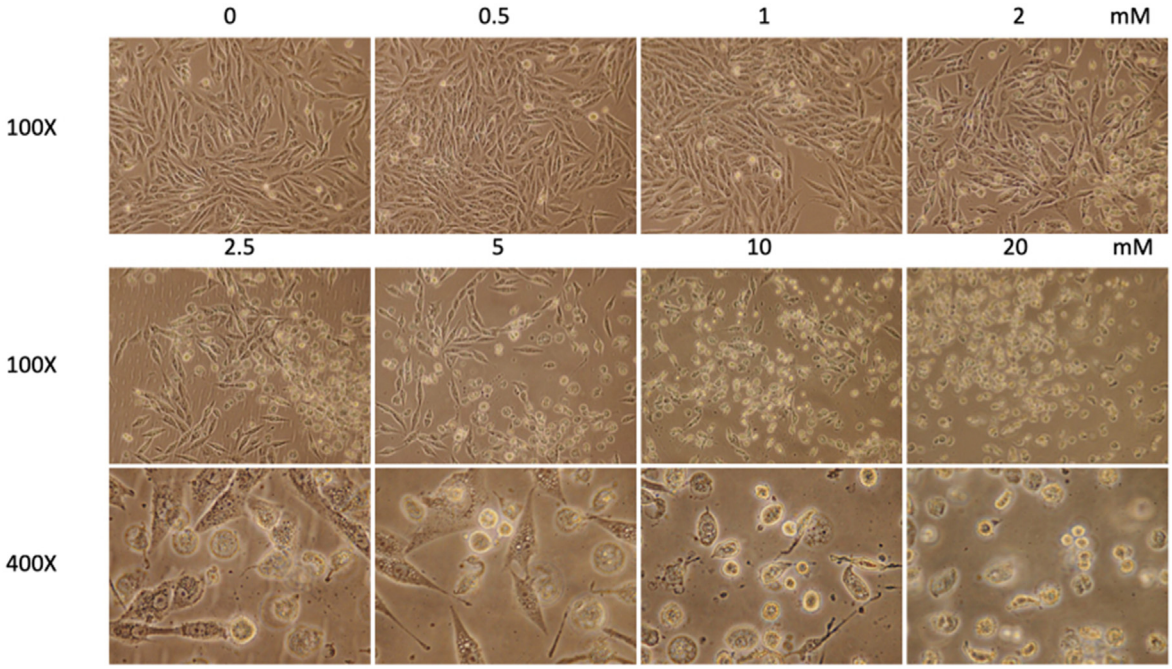


Figure 6 The MG63 cell morphologies under different concentrations of Scandonest anesthetic treatment at 100x and 400x magnifications. The cell numbers decreased at concentrations of 5 mM. Above concentrations of 5 mM, the cells floated in the medium.

cell showed a significant COX-2 expression on 5 μM and 10 μM concentrations of Septanest group and on 10 μM concentrations of Scandonest group ($p < 0.05$, Fig. 7B). The IL-1, IL-6 and TNF-α showed no statistical difference on Septanest group and Scandonest group ($p > 0.05$, Fig. 7B).

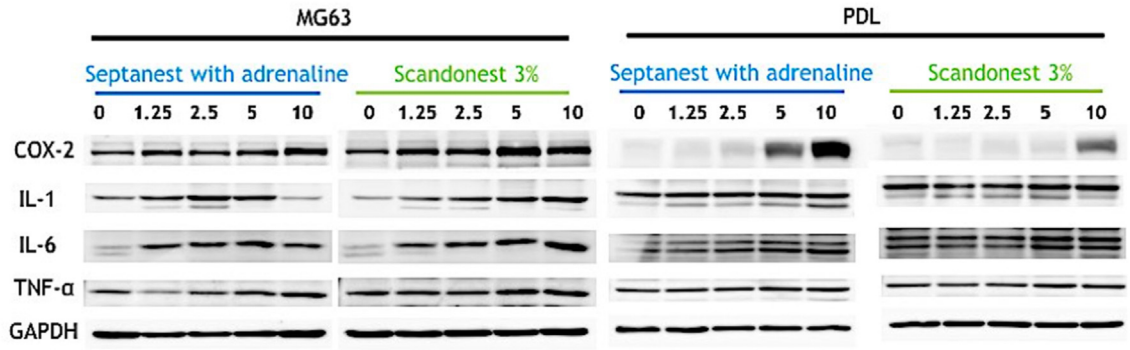


Figure 7 The expression of inflammatory markers (COX-2, IL-1, IL-6, and TNF -α) in the MG63 and PDL cells treated with Septanest and Scandonest.

Osteogenic markers expression

The osteogenic marker expression in MG63 cell treated with Septanest and Scandonest LAs is shown in Fig. 8. The RANKL expression of MG63 cell was higher at 5 and 10 μM of Septanest and at 2.5, 5, and 10 μM of Scandonest (Fig. 8). The ALP and OPG expression in the MG63 cells decreased as the Septanest and Scandonest concentrations increased (Fig. 8).

The osteogenic marker expression for the LA-treated PDL extract medium co-cultured with MG63 cells is shown in Fig. 9. In 24 h, the RANKL and ALP expression in the MG63 cells decreased with increases in the Septanest concentrations ($p < 0.05$, Fig. 9). The RANKL, ALP, and OC expression in the MG63 cells was not affected by the Scandonest concentrations ($p > 0.05$, see Fig. 9).

The RANKL expression in the MG63 cells increased as the Septanest and Scandonest concentrations of the PDL extract medium increased ($p < 0.05$) after 48 h of culturing. The ALP expression of the MG63 cells showed no statistical difference at different concentrations of Septanest and Scandonest ($p > 0.05$). The OPG expression of the MG63 cells increased as the concentration of Scandonest increased ($p < 0.05$).

Discussion

In the present study, LAs were used, which in Septanest is Articaine and in Scandonest is Mepivacaine. The difference between them is the lack or presence of adrenalin additives. The addition of adrenaline to an LA solution is done to reduce the plasma concentration of the drug and thus decrease its potential toxicity. But the toxicity is existed in high concentrations (20 mM) of present LAs. The PDL and MG63 cells morphologies became spherical in shape, or vesicles developed in the cytoplasm (Figs. 3—6).

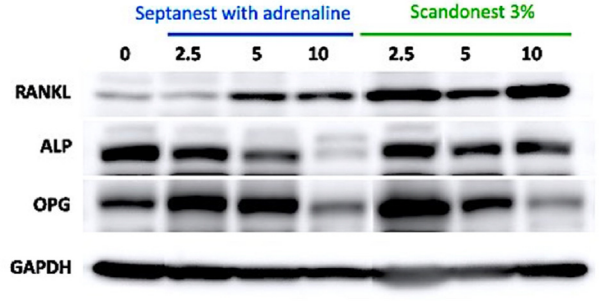


Figure 8 The expression of osteogenic markers (RANK, ALP, and OPG) in MG63 cells treated with Septanest and Scandonest.

Adrenaline may change bone metabolism and regulate osteogenic differentiation.[17] These adrenaline effects can suppress the osteogenic differentiation of hMSCs by down-regulation of miR-21.[18] The present study showed that the MG63 cells treated with Septanest containing adrenaline showed a high expression of inflammatory markers (COX-2, IL-6 and TNF-α Fig. 7) and RANKL (Fig. 8). COX-2 is an immediate-response gene that can be rapidly induced by inflammatory-related cytokines such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α).[19] The present study showed that high COX-2 expression occurred at high concentrations of LA treatment in both the MG63 and PDL cells (Fig. 7). The IL-1 and TNF-α expression was high in the MG63 cells treated with LAs with and without adrenaline (Fig. 7). Previous reports showed that COX-2 induced by IL-1 in bone marrow-derived cells promotes osteoclastogenesis, and another study with COX-2 knockout mice also showed the crucial role of this enzyme in osteoclastogenesis.[20,21]

Many orthodontists put a lot of effort into accelerating orthodontic tooth movement (OTM) using minimally invasive methods, such as low dose light energy, vibration, etc., or by invasive surgical interventions, such a corticision and piezocision, etc.[22,23] There are pros and cons to both approaches. OTM is achieved through compressive force on a tooth and causes the RANKL expression the increase in the gingival crevicular fluid in patients.[24,25] We posited that the products used for periodontal ligament stimulation will affect the subsequent bone changes. Thus, in the present study, the extracted mediums of PDL cells treated with LAs were used to subsequently culture with MG63 cells to evaluate osteogenic changes. The results showed that MG63 cells treated with PDL-LAs extracts exhibited increased RANKL expression as the LA concentrations were increased in the 48 h group (Fig. 9).

From this result, we suggest that PDL injections may induce positive influence in accelerating OTM after 48 h. It is concluded that higher LA concentrations were found to be toxic to PDL and MG63 cells. We suggest that LAs may

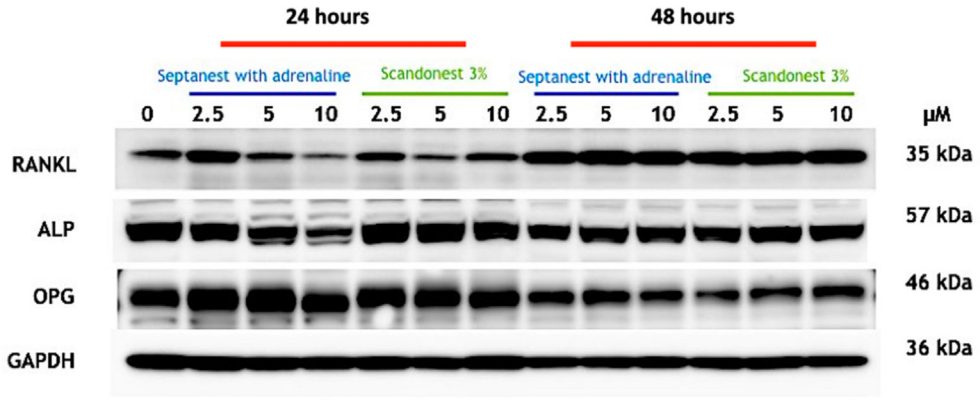


Figure 9 The extract medium of Septanest- and Scandonest-treated PDL cells added to the MG63 culture for 24 h and 48 h. The expression of osteogenic markers (RANK, ALP and OPG) in the MG63 cells.

increase PDL and MG63 cell inflammation and increase the MG63 RANKL expression affecting osteoclastogenesis.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This work was supported by the Ministry of Science and Technique, Taiwan (MOST 105-2314-B-040 -007 -MY3).

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