Original Article

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Dentistry Section

Endoflas FS Decreases the Viability and Mineralisation Process in Human Alveolar Osteoblastic Cells

SAUL ERNESTO CIFUENTES-MENDIOLA[1], MARLEN BARRERA-FRANCISCO[2], MANUEL ALAN GARCÍA-NAVARRO[3], EDUARDO FULGENSIO LLAMOSAS-HERNÁNDEZ[4], ISAAC OBED PÉREZ-MARTÍNEZ[5], ANA LILIA GARCÍA-HERNÁNDEZ[6]

ABSTRACT

Introduction: Endoflas FS, a root canal filling material, is indicated for the endodontic management of deciduous teeth. Several studies have shown undesirable effects of Endoflas FS on periodontal tissues. It is important to determine the possible toxic effects of Endoflas FS on osteoblast cells, as a small amount of microfiltration may exist following obturation of the root canal resulting in a direct contact with bone cells.

Aim: To evaluate the effect of different amounts of Endoflas FS on viability, mineralisation and production of TNF-α and IL-6 in a human alveolar osteoblastic cell line.

Materials and Methods: The present in vitro study was done using a human alveolar osteoblastic cell line which was exposed to different amounts of Endoflas FS. The MTT assay was performed to determine the cell viability (2-24 hours). Mineralisation was evaluated by red alizarin at 7-14 days. The production of TNF-α and IL-6 was determined by Enzyme-Linked Immunosorbent Assay (ELISA) at 12 and 24 hours.

Results: At high amounts Endoflas FS decreased the viability of osteoblasts and reduced the formation of mineralisation nodules and the production of IL-6 and TNF-α.

Conclusion: At low amounts Endoflas FS may not generate changes in osteoblastic cells; however, at high amounts Endolfas FS may be toxic, decreasing cell viability and mineralisation.

Keywords: Bone cells, Cytotoxicity, Mineral matrix, Root canal sealer

INTRODUCTION

Endoflas FS is a resorbable root canal filling for deciduous teeth, comprised of zinc oxide eugenol, barium sulfate, triiodomethane, calcium hydroxide, iodine dibutylorthocresol and a liquid consisting of eugenol and paramonoclorophenol [1]. It has the desirable properties of hydrophilicity, firm adherence to the surface of the root canal walls and high bacteriostatic and bactericidal activity in the dentinal tubules [2]; moreover, one of its constituents is iodoform, which adds resorbability, biocompatibility and antibacterial properties [3].

There is controversy about the use of Endoflas FS; some studies suggest that Endoflas FS causes the development of root cysts [4]. However, other studies have reported an effectiveness of up to 93.3% and no periapical lesions after nine months of follow-up; compared with other root canal fillings with a calcium hydroxide base of zinc oxide eugenol, Endoflas FS showed a higher success rate [5,6].

There are currently few studies on the cytotoxic effect of Endoflas FS in periodontal cells. In gingival fibroblasts it was observed that Endoflas FS dramatically decreased protein synthesis [7]. The effect of Endoflas FS on macrophage and epithelial cell cultures was also evaluated. At high concentrations Endoflas FS has a toxic effect and decreases the production of TNF-α, whereas at low concentrations it induces the proliferation of these cells [8]. Moskovitz M et al., reported that the components of Endoflas FS can be resorbed by phagocytosis [9].

Currently, as far as we know, no study has evaluated the effect of Endoflas FS on human alveolar osteoblasts or on the mineralisation process. Given the importance of bone tissue for the success of a root canal treatment, as well as the frequency with which the root canal filling has direct or indirect contact with bone, it is imperative to know the cytotoxic effects of Endoflas FS on alveolar osteoblasts and mineralisation.

The aim of the present study was to evaluate the effect of Endoflas FS on the viability, mineralisation and inflammation of human alveolar bone cells.

MATERIALS AND METHODS

The present in vitro study was performed using a human osteoblastic cell line. We have the endorsement of ethics for the culture of human osteoblasts trade: CE/FESI/082017/1159.

Cell Culture

Dr. Arzate from the Laboratory of Mineralised Tissues of the Dentistry Faculty of UNAM kindly provided the human alveolar osteoblastic cell line [10]. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen), supplemented with 10% Foetal Bovineserum (FBS) (Microlab) and antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin) (Sigma-Aldrich).

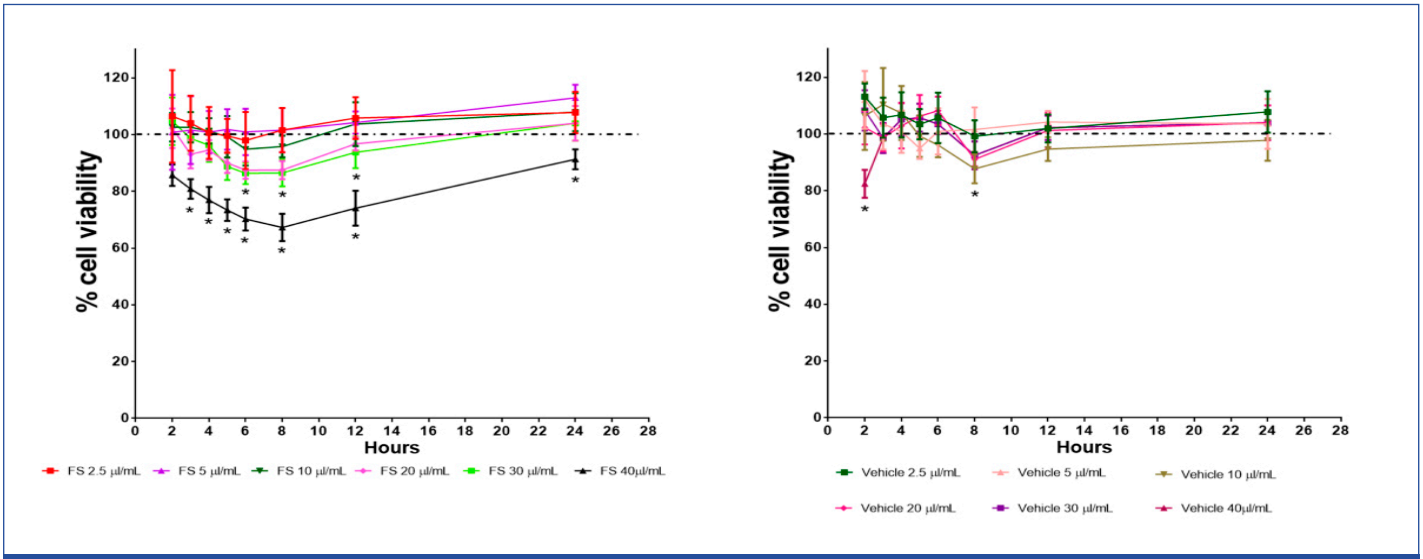
Preparation of Endoflas FS

The Endoflas FS (Sanlor Laboratories, Cali, Colombia) was prepared to obtain the recommended consistency necessary for a root canal filling according to the manufacturer's instructions. The proportions used in this study to achieve the ideal consistency of the material were: 0.1 gm of powder (zinc oxide, barium sulfate, triiodomethane and calcium hydroxide), 0.05 gm of zinc acetate and 100 µL or liquid (eugenol, paramonoclorophenol). It was dissolved in 10 mL of 96% ethanol and passed through a 0.22 µm filter; this solution served as stock solution for evaluation of the cellular effect of 2.5 µL (25 µg zinc oxide, barium sulfate, triiodomethane and calcium hydroxide; 12.5 µg zinc acetate and 25 nL of eugenol, paramonoclorophenol); 5 µL (50 µg zinc oxide, barium sulfate, triiodomethane and calcium hydroxide; 25 µg zinc acetate and 50 nL of eugenol, paramonoclorophenol); 10 µL (100 µg zinc oxide, barium sulfate, triiodomethane and calcium hydroxide; 50 µg zinc acetate and 100 nL of eugenol, paramonoclorophenol); 20 µL (200 µg zinc oxide,

barium sulfate, triiodomethane and calcium hydroxide; 100 µg zinc acetate and 200 nL of eugenol, paramonoclorophenol); 30 µL (300 µg zinc oxide, barium sulfate, triiodomethane and calcium hydroxide; 150 µg zinc acetate and 300 nL of eugenol, paramonoclorophenol); 40 µL (400 µg zinc oxide, barium sulfate, triiodomethane and calcium hydroxide; 200 µg zinc acetate and 400 nL of eugenol, paramonoclorophenol); in 1 mL of culture medium (DMEM). Human alveolar osteoblastic cells were treated with this concentration and amounts of endoflas FS or vehicle from one hour to 14 days of culture according to the determinations made.

Quantification of IL-6 and TNF-α (ELISA)

In these assays, 3×10^5 cells were seeded in 24-well cell culture plates and allowed to adhere overnight. After that, the cells were co-cultured with 1×10^9 *P. gingivalis* and treated with different concentrations of either Endoflas FS or vehicle or 40 mM of H_2O_2 as positive control for 12 and 24 hours in separate experiments. The supernatant was recovered and kept at -70°C until analysis. Using the recovered supernatant, ELISAs were performed following the technical specifications of the manufacturer for each cytokine, IL-6, and TNF-α (PeproTech) (Rocky Hill, NJ, USA).



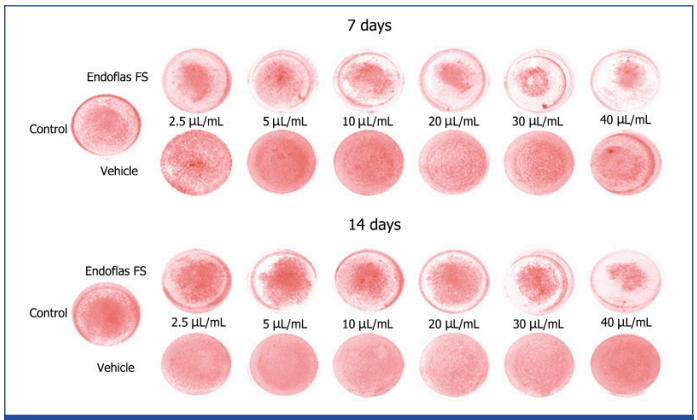
[Table/Fig-1]: Effect of the Endoflas FS or vehicle on the viability of alveolar osteoblastic cells. High amount of Endoflas FS decreases cell viability. Graphs represent the mean±SE (n=4), *p <0.001. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test.

Cytotoxicity Assay by MTT Reduction

We evaluate the reaction for determination of the ability of cells to reduce MTT to formazan after exposure to Endoflas FS to assess both the toxicity of the compound and cell viability [11]. Cells were seeded at density of 3×10^4 cell/well in 96-well cell culture plates. The cells were allowed to adhere overnight. The next day, the cells were treated with different concentrations of Endoflas FS or vehicle and maintained for 2,3,4,5,6,8,12 and 24 hours. Then, 10 µL of 0.5% MTT was added and incubated at 37°C for five hours. At the end of the incubation period, the formation of formazan crystals was observed under a microscope. To dissolve the crystals, 100 µL 75% v/v isopropanol, 15% v/v 0.1 M HCl and 15% v/v triton were added. The amount of formazan formed was evaluated by colorimetric reading, using absorbance (λ 540 nm, Biotek multi scan plate reader). Cytotoxicity analyses were determined taking the OD value of control cells (untreated cells) was taken as 100% viability (0% cytotoxicity). Data were reported as mean±SD of three independent experiments performed in quadruplicate.

Mineralisation Test

For this test, 1.5×10^5 cells were seeded in 12-well cell culture plates and treated with different concentrations of either Endoflas FS or vehicle 24 hours later. The cells were cultured in osteogenic medium to induce the formation of mineralisation nodules, (DMEM supplemented with 10% FBS plus 50 mg/mL ascorbic acid and 10 mM of β-glycerophosphate) for seven and 14 days. To evaluate biomineralisation, the cells and the minerals formed were fixed with 70% cold methanol, and the mineral matrix was identified using a saturated solution of 2% red Alizarin, pH 4.1 (Sigma Chemical Co., St. Louis, MO) which was added to the cell cultures for 60 minutes. Excess dye was repeatedly removed with phosphate buffer (PBS 1x), and the wells were inverted to dry. Wells with fixed and stained mineral were documented with a microscope stereoscope Zeiss (Germany).



[Table/Fig-2]: Represents photomicrographs of mineralisation nodules formed after different treatments of Endoflas FS or vehicle and stained with red alizarin. Amounts of 10, 20, 30 and 40 µL of Endoflas FS decreased the formation of mineralisation nodules after A) seven and B) 14 days of treatment.

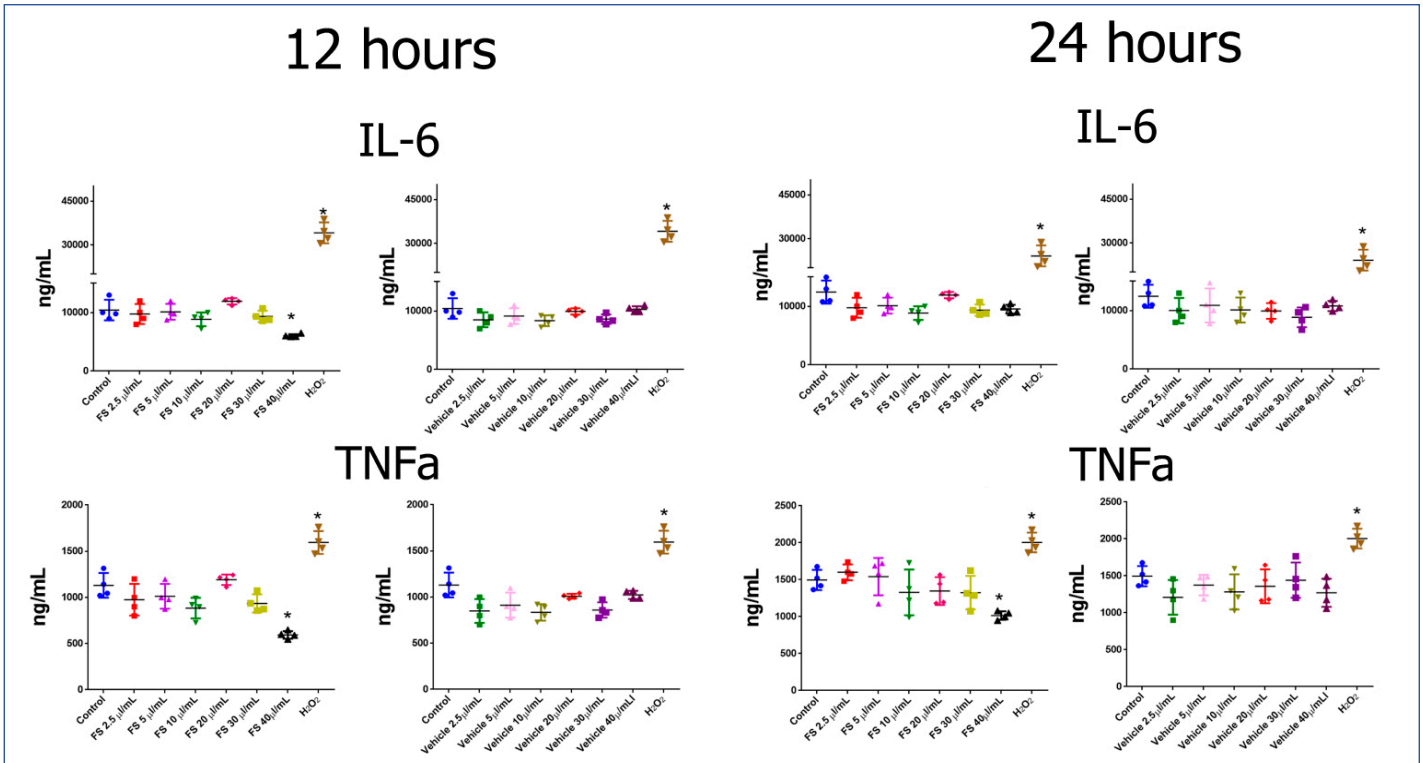
STATISTICAL ANALYSIS

All experiments were performed at least in triplicate. All data were analysed by analysis of variance (ANOVA), followed by Tukey's post-hoc test, using GraphPad Prism 6. The differences were considered statistically significant if * p-value ≤0.001.

RESULTS

a) High amounts of Endoflas FS decrease cell viability in a human alveolar osteoblastic cell line.

Alveolar osteoblastic cells were treated with different amounts of Endoflas FS or vehicle [Table/Fig-1] shows the percentage of cellular viability at various times and amounts of both Endoflas FS and vehicle. The higher amount of Endoflas FS (40 µL) decreased cell viability to 80% after three hours of treatment. The extent of reduction increased with increasing incubation time: Endoflas FS decreased viability to 75% at



[Table/Fig-3]: Effect of Endoflas FS or vehicle on the production of IL-6 and TNF-α at 12 and 24 hours in alveolar osteoblastic cells. The higher amount of Endoflas FS (40 µL) decreased the production of TNF-α at 12 and 24 hours and that of IL-6 at 12 hours. Graphs represent the mean±SE (n=4), *p <0.001. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test.

4 hours, 70% at 5 hours, 68% at 12 hours and 89% at 24 hours. Amounts of 20 and 30 µL decreased viability at 5, 6 and 8 hours (ANOVA, p<0.001), but viability at 12 hours was not significantly different from control.

b) Endoflas FS decreases the formation of mineralisation nodules evidenced by alizarin red staining

To determine the effect of Endoflas FS on mineralisation, alveolar osteoblastic cells were cultured with different amounts of this root-filling material in mineralisation medium for seven and 14 days [Table/Fig-2]. Results showed fewer nodules of mineralisation in wells treated with high amount of Endoflas FS compared with the control and vehicle at 10 µL, 20 µL and 30 µL amounts and at both time points.

c) A higher amount of Endoflas FS decreases the expression of IL-6 and TNF-α

We wanted to evaluate the capacity of Endoflas FS to induce the production of IL-6 and TNF-α [Table/Fig-3]. Compared with the control, the expression of TNF-α decreased at both 12 and 24 hours at a higher amount of Endoflas FS (40 µL). The expression of IL-6 also decreased with 40 µL of Endoflas FS at 12 hours. The positive control (40 mM of H_2O_2) showed an increase in the production of IL-6 and TNF-α.

DISCUSSION

The success of a root canal treatment is highly dependent on the material used and its biofunctional properties. One of the essential proprieties of root-filling materials is their biocompatibility, which makes them well tolerated by peri-radicular tissues; otherwise, inflammatory reactions could compromise the success of treatment. Although, most endodontic sealers are confined within the root canal, the material may extrude through the apex during sealing or there may be microfiltration that causes partial degradation of the root-filling material. The products of this degradation can irritate surrounding tissues such as alveolar bone; therefore, in this work we evaluated the cytotoxic effect and mineralisation of Endoflas FS in a cell line of alveolar osteoblasts.

Our results indicate that Endoflas FS is not cytotoxic to alveolar osteoblasts at low amounts. It does not induce the production of proinflammatory cytokines IL-6 or TNF-α and does not affect mineralisation; however, at the high amount tested in this study (40 µM), Endoflas FS is cytotoxic, decreases mineralisation and decreases the production of IL-6 and TNF-α. Our results coincide with the results of Petel R et al., in which they observed that at high concentrations Endoflas FS decreases the viability of macrophages and epithelial cells, as well as the production of TNF-α [8]. Also, Scelza MZ et al., observed a reduction of viability on human osteoblast cells with different endodontic sealers including one material with a zinc oxide eugenol sealer [12]. The decrease in the production of IL-6 and TNF-α we observed could have been due to the same toxic effect of the paste on the osteoblastic cells, which reduces their number and therefore, the concentration of these cytokines.

This article is the first to report the effect of Endoflas FS on mineralisation in the human alveolar osteoblastic cell line. Other studies have found that calcium phosphate-based root canal scalers promoted the formation of mineralisation nodules in mouse osteoblasts; thus, they have a beneficial effect on periapical tissue repair [13]. We observed a decrease in mineralisation caused by Endoflas FS. The present study's results differed from those of Rodríguez LC et al., they reported that cement with zinc oxide-eugenol did not decrease cell viability in a mouse osteoblastic cell line compared with the control [14]. This difference may have occurred because the sealing material was encapsulated in a solid state when placed in contact with cells, whereas we tested the effect of diluting the material. We consider the chronic exposure to the cells and the effects of the products formed as a result of its dilution to be caused by microfiltration post-obturation of the root canal. Even though the effect of Endoflas FS on mineralisation has not previously been evaluated, a few studies have assessed zinc oxide-eugenol-based endodontic sealers in rat osteoblasts and observed decreased cell viability, decreased ALP activity and the formation of mineralised nodules [15,16].

The cytotoxic effects of Endoflas FS on osteoblast cells could be due to its eugenol content. Ho YC et al., associated the toxic effects of Endoflas FS with eugenol content and glutathione levels; therefore, eugenol could cause cytotoxicity by inhibiting the antioxidant response [17]. Endoflas FS is used because of its antimicrobial proprieties, but its toxic effects are related to its eugenol or paramonoclorophenol component, which causes cytotoxicity, and inflammatory reactions [8,12,16,18] and activation of NF-κβ, a transcription factor responsible for the synthesis of proinflammatory cytokines [8,19].

The liquid component of the Endoflas FS cement contains paramonoclorophenol, which is an antibacterial drug known for its toxic activity. However, when used in combination with calcium hydroxide, the genotoxic effect of paramonoclorophenol is decreased significantly due to the denaturing effect of calcium hydroxide, because paramonoclorophenol is released slowly from this paste [20]. In the present study the combination of Endoflas FS elements, (zinc oxide, eugenol, barium sulfate, triiodomethane and calcium hydroxide) and observed a decrease in the toxic effects of paramonoclorophenol and eugenol at low concentrations, similar to that described by Siqueira JF Jr. [20]. The results of the present study indicate that in vivo, contact between high concentrations of Endoflas FS and bone could likely cause bone damage.

CONCLUSION

Endoflas FS at a low amount does not cause alterations in human alveolar osteoblasts, but at high amounts it is cytotoxic and diminishes mineralisation.

The results of the present study indicated that it is important to take care in the filling process to avoid direct contact of Endoflas FS with bone tissue to maintain the good qualities of the root canal sealer. It is important to do effect-causality, controlled and chronic clinical studies about the toxicity of Endoflas FS in the periradicular tissues in order to integrally evaluate the effect of Endoflas FS in the interaction of all cellular types near apical zone.

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Conflict of interest: The authors do not have a financial affiliation or involvement with any commercial organisation with a direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements that existed in the past three years.

REFERENCES

[1] Fuks AB, Eidelman E, Pauker N. Root fillings with Endoflas in primary teeth: a retrospective study. The Journal of Clinical Pediatric Dentistry. 2002;27(1):41-45.
[2] Navit S, Jaiswal N, Khan SA, Malhotra S, Sharma A, Mukesh, et al. Antimicrobial efficacy of contemporary obturating materials used in primary teeth-an in-vitro study. J Clin Diagn Res. 2016;10(9):ZC09-12.
[3] Mortazavi M, Mesbahi M. Comparison of zinc oxide and eugenol, and Vitapex for root canal treatment of necrotic primary teeth. International Journal of Paediatric Dentistry. 2004;14(6):417-24.
[4] Moskovitz M, Yahav D, Tickotsky N, Holan G. Long-term follow up of root canal treated primary molars. International Journal of Paediatric Dentistry. 2010;20(3):207-13.
[5] Rewal N, Thakur AS, Sachdev V, Mahajan N. Comparison of endoflas and zinc oxide eugenol as root canal filling materials in primary dentition. Journal of the Indian Society of Pedodontics and Preventive Dentistry. 2014;32(4):317-21.
[6] Subramaniam P, Gilhotra K. Endoflas, zinc oxide eugenol and metapex as root canal filling materials in primary molars--a comparative clinical study. The Journal of Clinical Pediatric Dentistry. 2011;35(4):365-69.
[7] Briseno BM, Willershausen B. Root canal sealer cytotoxicity with human gingival Fibroblasts. III. Calcium hydroxide-based sealers. Journal of Endodontics. 1992;18(3):110-13.
[8] Petel R, Moskovitz M, Tickotsky N, Halabi A, Goldstein J, Houri-Haddad Y. Cytotoxicity and proliferative effects of Iodoform-containing root canal-filling material on RAW 264.7 macrophage and RKO epithelial cell lines. Archives of Oral Biology. 2013;58(1):75-81.
[9] Moskovitz M, Sammara E, Holan G. Success rate of root canal treatment in primary molars. Journal of Dentistry. 2005;33(1):41-47.
[10] Arzate H, Alvarez-Perez MA, Aguilar-Mendoza ME, Alvarez-Fregoso O. Human cementum tumor cells have different features from human osteoblastic cells in vitro. Journal of Periodontal Research. 1998;33(5):249-58.
[11] Gerlier D, Thomasset N. Use of MTT colorimetric assay to measure cell activation. Journal of Immunological Methods. 1986;94(1-2):57-63.
[12] Scelza MZ, Linhares AB, da Silva LE, Granjeiro JM, Alves GG. A multiparametric assay to compare the cytotoxicity of endodontic sealers with primary human osteoblasts. International Endodontic Journal. 2012;45(1):12-18.
[13] Shon WJ, Bae KS, Baek SH, Kum KY, Han AR, Lee WC. Effects of calcium phosphate endodontic sealers on the behavior of human periodontal ligament fibroblasts and MG63 osteoblast-like cells. Journal of biomedical materials research Part B, Applied biomaterials. 2012;100(8):2141-47.
[14] Rodriguez LC, Saba JN, Chung KH, Wadhwani C, Rodrigues DC. In vitro effects of dental cements on hard and soft tissues associated with dental implants. The Journal of Prosthetic Dentistry. 2017;118(1):31-35.
[15] Ogata H, Hayashi M, Tsuda H, Suzuki N, Maeno M, Sugawara A, et al. Effects of a calcium phosphate cement on mineralized nodule formation compared with endodontic cements. Dental Materials Journal. 2012;31(1):92-97.
[16] Huang FM, Yang SF, Chang YC. Effects of root canal sealers on alkaline phosphatase in human osteoblastic cells. Journal of Endodontics. 2010;36(7):1230-33.
[17] Ho YC, Huang FM, Chang YC. Mechanisms of cytotoxicity of eugenol in human osteoblastic cells in vitro. International Endodontic Journal. 2006;39(5):389-93.
[18] Chang YC, Tai KW, Huang FM, Huang MF. Cytotoxic and nongenotoxic effects of phenolic compounds in human pulp cell cultures. Journal of Endodontics. 2000;26(8):440-43.
[19] Lee YY, Yang SF, Ho WH, Lee YH, Hung SL. Eugenol modulates cyclooxygenase-2 expression through the activation of nuclear factor kappa B in human osteoblasts. Journal of Endodontics. 2007;33(10):1177-82.
[20] Siqueira JF Jr. Strategies to treat infected root canals. Journal of the California Dental Association. 2001;29(12):825-37.

PARTICULARS OF CONTRIBUTORS:
1. Student, Department of Laboratorio de Investigación Odontológica, Inmunidad Oral y regulación ósea. (Laboratory of Dental R), Facultad de Estudios Superiores Iztacala UNAM, México.
2. Student, Laboratorio de Investigación Odontológica, Inmunidad Oral y regulación ósea. (Laboratory of Dental R), Facultad de Estudios Superiores Iztacala UNAM, México.
3. Student, Laboratorio de Investigación Odontológica, Inmunidad Oral y regulación ósea. (Laboratory of Dental R), Facultad de Estudios Superiores Iztacala UNAM, México.
4. Professor, Especialidad de Endoperiodontología, Facultad de Estudios Superiores Iztacala UNAM, México.
5. Professor, Laboratorio de Investigación Odontológica. Sección Neurobiología de las Sensaciones Orales (Laborato), Facultad de Estudios Superiores Iztacala UNAM, Mexico.
6. Professor, Laboratorio de Investigación Odontológica, Inmunidad Oral y regulación ósea. (Laboratory of Dental R), Facultad de Estudios Superiores Iztacala UNAM, Mexico.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Ana Lilia García-Hernández,
Professor, Department of Laboratorio de Investigación Odontológica, Av. Jiménez Gallardo S/N, San Sebastián Xhala,
54714 Cuautitlán Izcalli, Méx, México.
E-mail: draalgh@icloud.com

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