Dental Research Journal

Original Article

Cytotoxicity evaluation of a copaiba oil-based root canal sealer compared to three commonly used sealers in endodontics

Angela Delfina Bittencourt Garrido[1], Sueli Patricia Harumi Miyagi de Cara[2], Marcia Martins Marques[2], Emílio Carlos Sponchiado Jr.[1], Lucas da Fonseca Roberti Garcia[3], Manoel Damião de Sousa-Neto[4]

[1]Department of Endodontics, School of Dentistry, Federal University of Amazonas, Manaus, Amazonas, [2]Department of Dentistry, School of Dentistry, University of São Paulo, Departments of [3]Dental Materials and Prosthodontics and [4]Restorative Dentistry, Ribeirão Preto School of Dentistry, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

Received: November 2013
Accepted: January 2014

Address for correspondence:
Dr. Lucas da Fonseca Roberti Garcia,
Rua Siró Kaku, n° 72, apto. 73, Bairro Jardim Botânico, 14021-614 Ribeirão Preto, São Paulo, Brazil.
E-mail: drlucas.garcia@gmail.com

ABSTRACT

Background: The constant development of new root canal sealers has allowed the solution of a large number of clinical cases in endodontics, however, cytotoxicity of such sealers must be tested before their validation as filling materials. The aim of this study was to evaluate the cytotoxic effect of a new Copaiba oil-based root canal sealer (Biosealer [BS]) on osteoblast-like Osteo-1 cells.
Materials and Methods: The experimental groups were formed according to the culture medium conditioned with the tested sealers, as follows: Control group (CG) (culture medium without conditioning); Sealer 26 (S26) - culture medium + S26; Endofill (EF) - culture medium + EF; AH Plus (AHP) - culture medium + AHP; and BS - culture medium + BS (Copaiba oil-based sealer). The conditioned culture medium was placed in contact with 2×10^4 cells cultivated on 60 mm diameter Petri dishes for 24 h. Then, hemocytometer count was performed to evaluate cellular viability, using Trypan Blue assay. The normal distribution of data was tested by the Kolmogorov-Smirnov test and the values obtained for cellular viability were statistically analyzed (1-way ANOVA, Tukey's test - $P < 0.05$), with a significance level of 5%.
Results: S26, EF and AHP presented decreased cellular viability considerably, with statistical significance compared with CG ($P < 0.05$). BS maintained cellular viability similar to CG ($P > 0.05$).
Conclusion: The Copaiba oil-based root canal sealer presented promising results in terms of cytotoxicity which indicated its usefulness as a root canal sealer.

Key Words: Copaiba oil-resin, copaifera genus, cytotoxicity, natural products, root canal sealer

INTRODUCTION

Root canal sealers are generally irritating to the periapical tissues. Therefore, they may inhibit the healing processes and consequently influence the success of the endodontic treatments.[1]

In vitro cytotoxicity assays are extensively used in the preliminary testing of new sealers,[2,3] since such methods are considered simple, reproducible and reliable for biological evaluations.[4]

Several root canal sealers are currently available on the market and they are classified into five large groups according to their chemical composition: Zinc oxide-eugenol-based sealers, sealers containing calcium hydroxide, resin-based sealers, glass ionomers-based sealers and those based on silicone.[2,5-7] Despite the great variety of root canal sealers, there is still no material which fulfills the ideal requirements of the American National Standards Institute/American Dental Association (ANSI/ADA).[7] Thus, the development of new root canal sealers with adequate physico-chemical and biological properties is crucial.[6]

Access this article online

Website: http//:drj.mui.ac.ir

Copaiba is an oil-resin produced by the exudation of the trunks of trees from the *Copaifera* genus.[8] The material excreted is a transparent liquid, bright, with a coloration ranging from yellow to brown.[8] Moreover, pharmacological studies have demonstrate its anti-inflammatory, analgesic, reparative, anti-nociceptive, anti-tumoral and antimicrobial properties[9-14] and previous studies have demonstrated that Copaiba oil-resin is not cytotoxic toward Swiss mice (*Mus musculus*) liver cells.[14]

Given the properties and wide use of Copaiba oil-resin in folk medicine, studies related to the application of this phytotherapeutic substance in dentistry need to be carried out.

Therefore, a new endodontic root canal sealer (Biosealer [BS]) based on this phytotherapy substance was first developed in 2010,[15] comprised of powder and liquid. The powder is composed of zinc oxide, calcium hydroxide, bismuth subcarbonate and sodium tetraborate and the liquid is Copaiba oil-resin. Moreover, Garrido *et al.*[15] have reported that BS presented adequate physicochemical properties, such as setting-time, flow, film thickness, dimensional stability, radiopacity and solubility/disintegration, in accordance with the ANSI/ADA specification no. 57.[16]

The aim of the present study was to evaluate the potential cytotoxic effect of a Copaiba oil-based root canal sealer on cultured immortalized Osteo-1 cells line, in comparison with three different sealers: AH Plus (AHP), Endofill (EF) and Sealer 26 (S26). The null hypothesis tested was that there would be no difference in the cytotoxicity caused by the sealers.

MATERIALS AND METHODS

Culture of Osteo-1 cells

Immortalized osteoblast-like Osteo-1 cells were cultured in an incubator at 37°C in Dulbecco Modified Eagle Medium (DMEM, Sigma-Aldrich, Inc., St. Louis, MO, USA) supplemented with 10% - fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% - antibiotic-antimycotic solution (Sigma-Aldrich, Inc., MO, USA) in a humid atmosphere containing 5% CO_2 and 95% air. An uniform suspension containing single cells suspension was placed in a conical centrifuge tube. The cells suspension was pipetted up and down in the tube using a 10 ml pipette. The cells were daily checked for growth, using a light microscope under ×100 magnification, until an adequate number of cells were obtained to perform the study.

Conditioning or induction of cultivation media (indirect technique)

In order to carry out this study, five experimental groups were designed, as follows: Control group (CG) (culture medium without sealer); S26 - culture medium + S26 (Dentsply/Maillefer, Tulsa, OK, USA) (calcium hydroxide and resin-based sealer); EF - culture medium + EF (Dentsply/Maillefer, Tulsa, OK, USA) (zinc oxide and eugenol-based sealer); AH - culture medium + AHP (Dentsply/Maillefer, Tulsa, OK, USA) (resin-based sealer) and BS - culture medium + BS (Experimental sealer developed by the authors) (Copaiba oil-based sealer) [Table 1].

After preparation of each sealer according to manufacturer's recommendations, 5 mg of each root canal sealer was placed to the bottom of a test tube (50 ml) with the aid of a Pasteur pipette and 30 ml of the culture medium. The test tubes were properly identified according to the experimental group and maintained for 24 h in an incubator at 37°C in 5% CO_2. It is notable that in CG, the culture medium was not conditioned by any substance.

Cellular viability test (short-term)

Suspensions of Osteo-1 cells were seeded in 60 mm diameter Petri dishes (2×10^4 cells/dish). These cells

Table 1: Experimental groups and root canal sealers composition

Groups/ sealers	Composition	Manufacturer
CG	Culture medium alone	—
S26	Powder: Bismuth (III) oxide; hexamethylene tetramine; TiO_2; Ca $(OH)_2$	Dentsply/Maillefer, Tulsa, OK, USA
	Liquid: Bisphenol-A-diglycidylether	
EF	Powder: ZnO; resin (staybelite); bismuth subcarbonate; barium sulfate; borax	Dentsply/Maillefer, Tulsa, OK, USA
	Liquid: Eugenol; peanut oil	
AHP	Paste A: Epoxy resin; calcium tungstate; zirconium oxide; aerosil; iron oxide	Dentsply/Maillefer, Tulsa, OK, USA
	Paste B: Adamantane amine; N, N-dibenzoy l-5-oxanoname; diamine-1, 9-TCD-diamine; calcium tungstate; zirconium oxide; silicone oil; aerosil	
BS	Powder: ZnO; $Ca(OH)_2$; bismuth subcarbonate; nature resin; borax	Experimental root canal sealer developed by the authors
	Liquid: Copaiba oil-resin	

CG: Control group; S26: Sealer 26; EF: Endofill; AHP: AH Plus; BS: Biosealer; TCD: Tricyclodecane

grew adhering to the bottom of the Petri dish forming monolayers; and 3 days after the plaque formation, when the cultures became confluente, the culture media were substituted by fresh medium from the CG and by media induced with the tested sealers (S26, EF, AHP and BS). After 24 h of incubation at 5% CO_2 and 37°C, the cells were stained with 0.4% Trypan Blue in phosphate buffered saline. Hemocytometer counts of live and dead cells were performed blindly by a single examiner in a Neubauer Chamber coupled to an inverted phase microscope at ×100 magnification (TS100, Nikon, Tokio, Japan). Each large square of the hemocytometer represents a total volume of 0.1 mm^3 (1.0 mm × 1.0 mm × 0.1 mm). The cellular viability was obtained through the following mathematical formula: Number of viable cells (unstained)/total number of cells (viable and dead), multiplied by 100.

Statistical analysis

Five replicates of each group were performed in the test. The normal distribution of data was tested by the Kolmogorov-Smirnov test and the values obtained for cellular viability were statistically analyzed (1-way ANOVA, Tukey's test - $P < 0.05$), with a significance level of 5%.

RESULTS

Figures 1 and 2 present the cellular viability mean values and the comparison of data among the different groups. It could be observed that there was statistically significant difference among the groups ($P < 0.05$). The cell viability of CG (95.02 ± 1.95) was statistically different from S26 (2.77 ± 2.37), AHP (5.98 ± 5.13) and EF (76.70 ± 7.60). The results in GC were statistically similar - to BS (95.36 ± 1.53). Furthermore, S26 and AHP presented the lowest cellular viability mean values in comparison with the other groups.

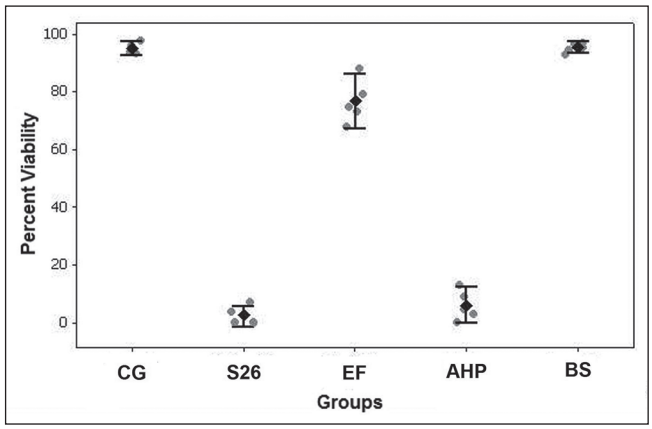

Figure 1: The viability of osteoblast-like Osteo-1 cells percentages according to the different root canal sealers (one-way ANOVA, Tukey's test - $P < 0.05$)

DISCUSSION

Cytotoxicity of new root canal sealers must be evaluated before their validation as a reliable option for endodontic therapy.[1-3] The aim of the present study was to evaluate the cytotoxic effect of a Copaiba oil-based root canal sealer (BS) on osteoblast-like Osteo-1 cells, comparing it with AHP, EF and S26. Based on the results, the tested null hypothesis was rejected, since the root canal sealers presented different behavior regarding cellular viability.

The biocompatibility study of a root canal sealer is a fundamental requirement before its clinical application.[7] Many studies have shown that the cytotoxicity of several endodontic materials can be investigated through cellular culture tests.[17,18]

Different cell cultures are used for cytotoxicity evaluation. In the present study, osteoblast-like

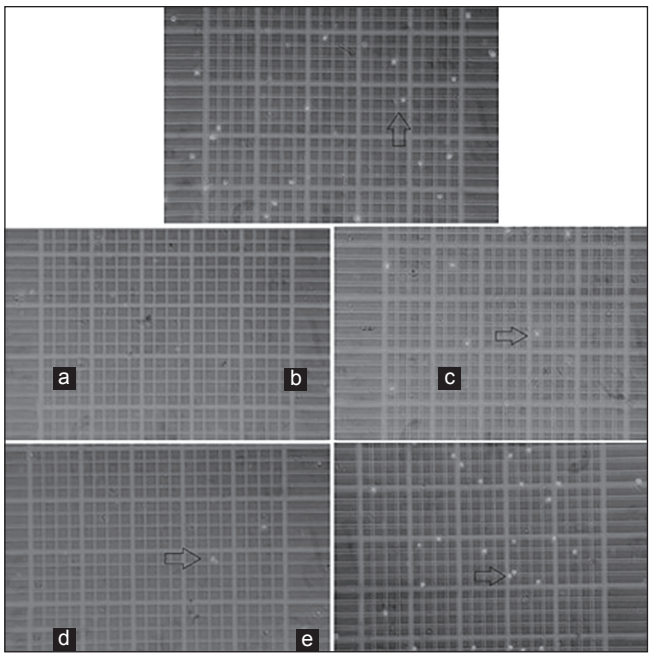

Figure 2: Cells counting procedure - photomicrographs of the Neubauer Chamber on inverted phase microscope. Live cells appear in suspension, colorless and bright (refractile) under phase contrast (arrow). Dead cells, non-viable, are stained with Trypan Blue and are non-refractile. (a) Control group, (b) sealer 26, (c) Endofill, (d) AH Plus and (e) Biosealer (×100)

Osteo-1 cells were selected because root canal sealers are constantly in contact with similar cells in periapical region.[6] Thus, sealers should not be cytotoxic, preventing the repair process of the apical region.[6] Regarding the method used to determine cellular viability, Trypan Blue dye exclusion assay is one of the most common methods for cell viability measurement tests.[17] The method consists of the alteration in the cells membrane integrity determined by the uptake of the dye by dead cells. However, despite this method allows a direct measure of cell viability, the absence of contrast in cells membrane makes it difficult to differentiate unstained and stained cells.[17] Based on the cellular viability test results, it was observed that the experimental sealer based on Copaiba oil-resin was not cytotoxic,. The authors believe that this fact may be related to the inherent properties of Copaiba oil-resin, such as biological compatibility,[13,14] reparative[19] and anti-inflammatory properties.[12] According to Kobayashi *et al*.,[12] Copaiba oil is composed of sesquiterpenes with the predominance of β-caryophyllene (36.0%), α-copaene (18.8%), β-bisabolene (8.5%) and α-trans-bergamotene (7.0%). Administration of 100 and 200 mg/kg doses at a concentration of 200 µl/ml Copaiba oil presented anti-inflammatory effects, decreasing leukocyte migration rates to the pleural cavity in rats and to the chemotactic agent lipopolysaccharide solution.

Garcia *et al*.,[13] in a recent study, evaluated the biological compatibility of the non-fractionated and volatile fraction of the Copaiba oil-resin in the connective tissue of rats. The authors reported a tissue reaction of chronic development, highly cellularized and vascularized at the initial time, with a predominance of mononuclear cells. The inflammatory reaction was moderate in the initial period, as a natural response in short periods of time; however, the histopathological events significantly decreased at the final period of observation.

Moreover, similar results were reported by Almeida *et al*.,[14] which evaluated the cytotoxic and genotoxic effect of the Copaiba-oil resin and its volatile and resinous fractions. Under the experimental conditions, the Copaiba-oil resin itself and volatile and resinous fractions from commercial Copaiba oil-resin presented no mutagenic or genotoxic effects, corroborating the results of the present study.

The reason that BS presented no cytotoxicity is probably due to its powder formulation, which did not allow the release of irritant substances. The choice of the powder components was based on their biological properties, with the aim of minimizing the toxic effect of the sealer. The zinc oxide, corresponding to 27.89% of the sealer formula, is an efficient antimicrobial agent and has been shown to have a cytoprotective effect on the living tissues.[20] The calcium hydroxide, corresponding to 27.89% of the sealer formula, is a substance which increases the biocompatibility due to the medium alkalinization, stimulating the periapical repair process through collagenization and mineralization.[21] The bismuth subcarbonate is selected as radiopacifying agent (21.91%), since bismuth is considered as one of the least toxic heavy metals.[22] Furthermore, according to Sousa Neto *et al*.,[23] bismuth subcarbonate gives a greater radiopacifying effect than barium sulfate and also allows achieving adequate physico-chemical properties.

Natural resin is another constituent of the cement powder, representing 21.91% of its formulation. Natural resin or pitch has a more acid pH than the hydrogenated resin,[23] and according to Söderberg,[24] increases the cellular membrane permeability and consequently, the cytotoxicity. However, Sunzel *et al*.[20] have demonstrated that although natural resins contain cytotoxic resinous acids, the addition of zinc effectively reduces the toxicity of the natural resin. Therefore, the low toxicity of the cement may also be related to the association of the zinc oxide powder with the natural resin in the cement powder formulation.[20] Finally, the sodium tetraborate (anhydrous) or borax was included to the formula in a very low quantity (0.40%) and did not interfere with the cement toxicity.

Furthermore, the lack of toxicity of the experimental sealer may be related to the setting mechanism of the material. BS results from an acid-base reaction of the acid components of the Copaiba oil-resin with the basic components of the powder, forming a low irritant salt.[15]

In the current study, AHP, S26 and EF presented high cytotoxicity. These results are in agreement with the reports of some published studies,[2,3,7] which showed that there is no ideal root canal sealer present in terms of biological compatibility.

S26 is a material which contains cytotoxic chemical substances such as hexamethylenetetramine, titanium dioxide and the resin bisphenol-A diglycidyl.[25,26] Huang *et al*.[21] in their study observed genotoxic effects of root canal sealers that release paraformaldehyde

or substances which have a mutagenic effect, such as bisphenol-A diglycidylether. Therefore, S26 has two factors which are responsible for its intense cytotoxicity: The presence of bisphenol-A diglycidylether in its composition; and the release of formaldehyde from the hexamethylenetetramine during its setting process.[21]

Many studies have confirmed the high cytotoxicity of AHP.[21,27,28] Cohen *et al*.,[27] conducted cytotoxicity tests with AHP, based on the methods described in the International Organization for Standardization. The biological reaction of L929 mouse fibroblast cells to the tested sealer demonstrated that AHP was cytotoxic and do not meet the requirement of the agar diffusion test. However, other studies have demonstrated the low cytotoxicity of this sealer.[3,7] The inconsistency of these results may be related to the experimental conditions, in which *in vitro* and *in vivo* tests were conducted.[29]

Endodontic sealers based on zinc oxide-eugenol release free eugenol molecules during the setting process of the material. Thus, the intensity of the inflammatory response is directly proportional to the quantity of eugenol released by the sealer.[30] According to Serene *et al*.,[30] eugenol can inhibit the adhesion of macrophages, inhibiting cellular activity. Furthermore, a sealer which hardens very slowly may irritate the periapical tissues. Thus, the setting-time must not be excessively long.[30]

Sealers with shorter setting-time, such as BS and EF,[15] may favor the biological compatibility when compared with sealers with longer setting-times, such as S26 and AHP, since the cytotoxic components in the plastic state, such as unreacted monomers (bisphenol-A diglycidylether), may be released into the medium for longer periods allowing greater cytotoxicity and tissue irritation.[25,26]

Although the results of the *in vitro* cytotoxicity experiments using cellular cultivation cannot be directly interpreted in terms of *in vivo* application, the fact that the experimental sealer based on Copaiba oil-resin did not cause damage to the osteoblasts-like cells line indicates the possible biocompatibility of this new root canal sealer.

CONCLUSION

Based on the results obtained, experimental sealer (BS) presented low toxicity *in vitro*, indicating that this is a promising material for endodontic application. However, it is valid to emphasize that new toxicity tests must be carried out *in vivo*, since different levels of biological tests should be performed prior to root canal sealer application in humans.

REFERENCES

1. Cardoso M, Marques RF, Lopes MF, Cabrita AS, Santos JM. *In vivo* biocompatibility of Resilon compared with gutta-percha in a pre-clinical model. Dent Res J (Isfahan) 2013;10:652-8.
2. Baraba A, Zelježić D, Kopjar N, Mladinić M, Anić I, Miletić I. Evaluation of cytotoxic and genotoxic effects of two resin-based root-canal sealers and their components on human leucocytes *in vitro*. Int Endod J 2011;44:652-61.
3. Lee BS, Wang CY, Fang YY, Hsieh KH, Lin CP. A novel urethane acrylate-based root canal sealer with improved degree of conversion, cytotoxicity, bond strengths, solubility, and dimensional stability. J Endod 2011;37:246-9.
4. Lima AF, Ribeiro AP, Soares DG, Sacono NT, Hebling J, de Souza Costa CA. Toxic effects of daily applications of 10% carbamide peroxide on odontoblast-like MDPC-23 cells. Acta Odontol Scand 2013;71:1319-25.
5. Yoshimine Y, Yamamoto M, Ogasawara T, Koishi Y, Tanabe K, Hashiguchi I, *et al*. *In vitro* evaluation of the cytocompatibility of a glass-lonomer cement sealer. J Endod 2003;29:453-5.
6. Gandolfi MG, Pagani S, Perut F, Ciapetti G, Baldini N, Mongiorgi R, *et al*. Innovative silicate-based cements for endodontics: A study of osteoblast-like cell response. J Biomed Mater Res A 2008;87:477-86.
7. Eldeniz AU, Mustafa K, Ørstavik D, Dahl JE. Cytotoxicity of new resin-, calcium hydroxide- and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines. Int Endod J 2007;40:329-37.
8. Veiga VF Jr, Zunino L, Calixto JB, Patitucci ML, Pinto AC. Phytochemical and antioedematogenic studies of commercial copaiba oils available in Brazil. Phytother Res 2001;15:476-80.
9. Paiva LA, de Alencar Cunha KM, Santos FA, Gramosa NV, Silveira ER, Rao VS. Investigation on the wound healing activity of oleo-resin from *Copaifera langsdorffi* in rats. Phytother Res 2002;16:737-9.
10. Lima SR, Junior VF, Christo HB, Pinto AC, Fernandes PD. *In vivo* and *in vitro* studies on the anticancer activity of *Copaifera multijuga* Hayne and its fractions. Phytother Res 2003;17:1048-53.
11. Carvalho JC, Cascon V, Possebon LS, Morimoto MS, Cardoso LG, Kaplan MA, *et al*. Topical antiinflammatory and analgesic activities of *Copaifera duckei* dwyer. Phytother Res 2005;19: 946-50.
12. Kobayashi C, Fontanive TO, Enzweiler BG, de Bona LR, Massoni T, Apel MA, *et al*. Pharmacological evaluation of *Copaifera multijuga* oil in rats. Pharm Biol 2011;49:306-13.
13. Garcia L, Cristiane S, Wilson M, Soraya M, Lopes RA, Mônica R, *et al*. Biocompatibility assessment of pastes containing Copaiba oilresin, propolis, and calcium hydroxide in the subcutaneous tissue of rats. J Conserv Dent 2011;14:108-12.

14. Almeida MR, Darin JD, Hernandes LC, de Souza Ramos MF, Antunes LM, de Freitas O. Genotoxicity assessment of Copaiba oil and its fractions in Swiss mice. Genet Mol Biol 2012;35: 664-72.
15. Garrido AD, Lia RC, França SC, da Silva JF, Astolfi-Filho S, Sousa-Neto MD. Laboratory evaluation of the physicochemical properties of a new root canal sealer based on *Copaifera multijuga* oil-resin. Int Endod J 2010;43:283-91.
16. ANSI/ADA Specification No. 57. Endodontic Sealing Material. Chicago, IL: ANSI/ADA; 2000.
17. Key JE, Rahemtulla FG, Eleazer PD. Cytotoxicity of a new root canal filling material on human gingival fibroblasts. J Endod 2006;32:756-8.
18. Heitman EP, Joyce AP, McPherson JC 3rd, Roberts S, Chuang A. An *in vitro* evaluation of the growth of human periodontal ligament fibroblasts after exposure to a methacrylate-based endodontic sealer. J Endod 2008;34:186-9.
19. Stupp T, de Freitas RA, Sierakowski MR, Deschamps FC, Wisniewski A Jr, Biavatti MW. Characterization and potential uses of *Copaifera langsdorfii* seeds and seed oil. Bioresour Technol 2008;99:2659-63.
20. Sunzel B, Söderberg TA, Johansson A, Hallmans G, Gref R. The protective effect of zinc on rosin and resin acid toxicity in human polymorphonuclear leukocytes and human gingival fibroblasts *in vitro*. J Biomed Mater Res 1997;37:20-8.
21. Huang TH, Yang JJ, Li H, Kao CT. The biocompatibility evaluation of epoxy resin-based root canal sealers *in vitro*. Biomaterials 2002;23:77-83.
22. Sano Y, Satoh H, Chiba M, Okamoto M, Serizawa K, Nakashima H, *et al*. Oral toxicity of bismuth in rat: Single and 28-day repeated administration studies. J Occup Health 2005;47:293-8.
23. Sousa Neto MD, Guimarães LF, Gariba Silva R, Saquy PC, Pécora JD. The influence of different grades of rosins and hydrogenated resins on the powder-liquid ratio of Grossman cements. Braz Dent J 1998;9:11-8.
24. Söderberg TA. Effects of zinc oxide, rosin and resin acids and their combinations on bacterial growth and inflammatory cells. Scand J Plast Reconstr Surg Hand Surg Suppl 1990;22:1-87.
25. Pulgar R, Segura-Egea JJ, Fernández MF, Serna A, Olea N. The effect of AH 26 and AH Plus on MCF-7 breast cancer cell proliferation *in vitro*. Int Endod J 2002;35:551-6.
26. Tai KW, Huang FM, Huang MS, Chang YC. Assessment of the genotoxicity of resin and zinc-oxide eugenol-based root canal sealers using an *in vitro* mammalian test system. J Biomed Mater Res 2002;59:73-7.
27. Cohen BI, Pagnillo MK, Musikant BL, Deutsch AS. An *in vitro* study of the cytotoxicity of two root canal sealers. J Endod 2000;26:228-9.
28. Jukić S, Miletić I, Anić I, Britvić S, Osmak M, Sistig S. The mutagenic potential of AH+ and AH26 by Salmonella/microsome assay. J Endod 2000;26:321-4.
29. Camps J, About I. Cytotoxicity testing of endodontic sealers: A new method. J Endod 2003;29:583-6.
30. Serene TP, Vesely J, Boackle RJ. Complement activation as a possible *in vitro* indication of the inflammatory potential of endodontic materials. Oral Surg Oral Med Oral Pathol 1988;65:354-7.

How to cite this article: Garrido AB, de Cara SH, Marques MM, Sponchiado Jr EC, Garcia LR, de Sousa-Neto MD. Cytotoxicity evaluation of a copaiba oil-based root canal sealer compared to three commonly used sealers in endodontics. Dent Res J 2015;12:121-6.

Source of Support: Nil. **Conflict of Interest:** None declared.