Original Research
Endodontics Therapy

Cytotoxicity and biocompatibility of a new bioceramic endodontic sealer containing calcium hydroxide

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Abstract: This study evaluated the cytotoxicity and biocompatibility of a new bioceramic endodontic sealer (*i.e.*, Sealer Plus BC) in comparison with those of MTA Fillapex and AH Plus. L929 fibroblasts were cultured and Alamar Blue was used to evaluate cell viability of diluted extracts (1:50, 1:100, and 1:200) from each sealer at 24 h. Polyethylene tubes that were filled with material or empty (as a control) were implanted in the subcutaneous tissue of rats. The rats were killed after 7 and 30 d (n = 8), and the tubes were removed for histological analysis. Parametric data was analyzed using a one-way ANOVA test, and nonparametric data was analyzed via the Kruskal–Wallis test followed by the Dunn test ($p < 0.05$). A reduction in cell viability was observed in the extracts that were more diluted for Sealer Plus BC when compared to that of Control and AH Plus ($p < 0.05$). However, the 1:50 dilution of the Sealer Plus BC was similar to that of the Control ($p > 0.05$). Conversely, more diluted extracts of MTA Fillapex (1:200) and AH Plus (1:100 and 1:200) were similar to the Control ($p > 0.05$). Histological analysis performed at 7 d did not indicate any significant difference between tissue response for all materials, and the fibrous capsule was thick ($p > 0.05$). At 30 d, Sealer Plus BC was similar to the Control ($p > 0.05$) and MTA Fillapex and AH Plus exhibited greater inflammation than the Control ($p < 0.05$). The fibrous capsule was thin for the Control and for most specimens of Sealer Plus BC and AH Plus. Thus, Sealer Plus BC is biocompatible when compared to MTA Fillapex and AH Plus, and it is less cytotoxic when less-diluted extracts are used.

Keywords: Cell Culture Techniques; Endodontics; Inflammation; Materials Testing.

Introduction

Bioceramic materials are ceramics that are specially developed for biological applications[1] and are increasingly attractive in dentistry. Examples of the materials include alumina, zirconia, bioactive glass, ceramics glass, and those based on calcium silicates or phosphates.[1,2] In endodontics, silicate- and calcium phosphate-based materials are more prominent and are in constant development due to the ability of the materials to stimulate tissue repair via the deposition of mineralized tissue.[3,4]

Mineral trioxide aggregate (MTA) was the first bioceramic material introduced in endodontics and was highlighted by its high biocompatibility and bioactivity.[5] The material consists of hydrophilic particles of tricalcium silicate, dicalcium silicate, and tricalcium aluminum among other mineral oxides,[4,5] and its properties are preserved even when in contact with tissue fluids.[6] Its indications for clinical use include the repair of perforations and root resorption, filling cavities, pulp exposition, pulpotomies, and apexification.[4]

In addition to biocompatibility and bioactivity, bioceramic materials promote alkaline pH, which provides antibacterial activity, radiopacity, absence of volumetric contraction, and chemically stability in a biological environment.[7] The MTA is a root repair cement and does not exhibit physical handling properties for use as an endodontic sealer. Therefore, the development of new calcium-silicate-based endodontic sealers is possible due to the excellent biological properties of the MTA.[3,8]

The MTA Fillapex (Angelus Indústria de Produtos Odotontológicos S/A, Londrina, Brazil) is an endodontic sealer that contains MTA, radiopacifiers, and resins in its composition.[9] Previous studies exhibited that the sealer exhibited flowability, dimensional change, radiopacity, and solubility, and this is in agreement with International Organization for Standardization 6876 (ISO 6876/2001).[10,11] Additionally, it exhibited an adequate setting time that is suitable for an endodontic sealer.[12,13] However, a consensus with respect to the biocompatibility and cytotoxicity of the material is absent since extant studies indicate both favourable[9] and unfavorable results in terms of their use.[14,15]

Thus, new bioceramic materials appear in the market in order to obtain an ideal endodontic sealer. Thus, a new root canal sealer was developed, namely the Sealer Plus BC (MK Life Produtos Medical e Dental, Porto Alegre, Brazil). The sealer in addition to acting as a material with bioceramic compounds (zirconium oxide and di- and tri-calcium silicate) possesses an additional amount of calcium hydroxide in its composition and is resin free. Studies indicated that the addition of calcium hydroxide to the endodontic sealers improves the biological properties of the materials[15] and that resins present in the composition of the MTA Fillapex (salicylate resin, natural resin, and resin diluent) promotes adverse effects including cytotoxicity and genotoxicity.[16] Additionally, its compounds (such as the tri-calcium silicate) when in contact with the tissue's fluids provides calcium hydroxide itself as a product, thereby increasing the power of mineralization.[17] Although the new sealer exhibits superior results, its biological properties are not examined in detail.

For a new material to be used by clinicians, it is essential to evaluate its biocompatibility and cytotoxicity. Hence, the objective of the present study is to evaluate the *in vivo* biocompatibility and cytotoxicity in fibroblastic lineage cells of the new formulation for a bioceramic endodontic sealer, namely the Sealer Plus BC, when compared to the already examined MTA Fillapex. A resin-epoxy sealer, namely AH Plus (Dentsply, Konstanz, Germany), was also used as a comparison because it is considered as the gold standard in endodontic sealers.[18] The null hypothesis was adopted, and it is expected that the sealers do not exhibit significant differences in terms of biocompatibility and cytotoxicity.

Methodology

In vitro study

Sealers extract

The Sealer Plus BC is a premixed endodontic sealer. The sealers MTA Fillapex and AH Plus were mixed according to manufacturers' instructions. The discs of each sealer (diameter: 5mm; height: 3 mm) were prepared under aseptic conditions, and the discs of Sealer Plus BC were covered with sterile moist gauze, to ensure moisture required to set the same.[17] Subsequently, the discs were kept in an incubator at 37 °C for 6 h in a humid atmosphere with 5% CO_2. The discs were then removed from the mold and sterilized via ultraviolet irradiation for 1 h, and sealer extracts were performed based on Cintra *et al*.[4] The diluted extracts with a culture medium (1:50, 1:100, and 1:200) were used in the study.

Cell culture and viability assay

Specifically, L929 fibroblasts line cell were grown in Dulbecco Modified Eagle's Medium (DMEM)

supplemented with 10% fetal bovine serum (FBS, GIBCO Laboratories, Gaithersburg, MD, USA), streptomycin (50 g/mL), and 1% antibiotic/antimycotic cocktail (300 U/mL, 300 µg/mL streptomycin, 5 µg/mL amphotericin B) (GIBCO Laboratories, Gaithersburg, MD, USA) under standard cell culture conditions (37ºC, 100% humidity, 95% air, and 5% CO_2)[4].

The viability rate of L929 fibroblasts line cell grown in the presence of the different endodontic sealer extracts was evaluated via the Alamar Blue reduction assay (Alamar Blue® Cell Viability Reagent, Thermo Fisher Scientific, Waltham, USA) after 24 h based on the manufacturer's instructions. Additionally, L929 cultured in DMEM without extract was used as the Control. In summary, cells were seeded into the 96 well plates (10^4 cells/well) and incubated for 24 h under standard cell culture conditions. Subsequently, the sealer extracts (1:50, 1:100, and 1:200) and Alamar Blue reagent (1:100) were added to the cells. At each time point, 200 µL of the medium was transferred to another 96 well plate, and the OD (optimal density) was measured at wavelengths corresponding to 570 and 600 nm[4]. The reduction of Alamar Blue was calculated via a formula provided by the manufacturer. Each condition was analyzed in triplicate.

In vivo study

Subcutaneous implants

A total of 16 male Wistar albino rats (250–280 g) with an age of 2 months were used. The sample size was established based on previous studies involving the analysis of materials in subcutaneous tissues of rats.[19,20] The animals were housed in a temperature-controlled environment (22 ± 1°C, 70% humidity) with a 12-h light–dark cycle and received water and food *ad libitum*. The study was approved and performed based on the guidelines specified by the ethical committee (process nº 00937-2017).

Sixty-four polyethylene tubes (Abbott Laboratories of Brazil, São Paulo, Brazil) with an internal diameter of 1.0 mm, external diameter of 1.6 mm, and length of 10.0 mm were filled with endodontic sealers: Sealer Plus BC, MTA Fillapex, and AH Plus, which were prepared based on manufacturers' recommendations or were empty for control. The surgical procedure was performed by following those specified in extant studies:[4,20] the rats were anesthetized, their dorsum was shaved, and a 2.0-cm incision was made in a head-to-tail orientation with a #15 Bard–Parker blade (BD, Franklin Lakes, USA). The skin was reflected to create two pockets on the right side and a pocket on the left side of the incision. The tubes were then randomly implanted into the pockets and the skin was closed with 4-0 silk sutures.

Histologic analysis

After 7 and 30 d (n = 8/period), the rats were killed via an overdose of anesthetics solution. The implanted tubes and the surrounding tissues were removed and fixed in 10% formalin solution at a pH of 7.0. The fixed specimens were processed and embedded in paraffin and serially sectioned into 5-mm cuts for staining with hematoxylin-eosin.

Tissue reactions in contact with the material on the open end of the tubes were scored based on extant studies[4,21,22] as follows: 0, no or few inflammatory cells and no reaction; 1, fewer than 25 cells and mild reaction; 2, between 25 and 125 cells and moderate reaction; and 3, 125 or more cells and severe reaction. Fibrous capsules were considered thin when <150 µm and thick when ≥150 µm. All data were analyzed via a single calibrated operator in a blinded manner under light microscopy (DM 4000 B; Leica Microsystem, Wetzlar, Germany) to obtain a consensus between the results observed in the two sections analyzed. The assessed Cohen kappa coefficient value for the intra-investigator agreement exceeded 0.87 for all groups. The values indicate an almost perfect agreement in the analysis results.

Statistical analysis

Parametric data obtained from the cell viability tests was statistically analyzed via one-way ANOVA test using the Graph Pad Prism (version 5.0) software program. Nonparametric data obtained from histological analysis was statistically analyzed via the Kruskal–Wallis test, and this was followed by the Dunn test. The significance level corresponded to 5%.

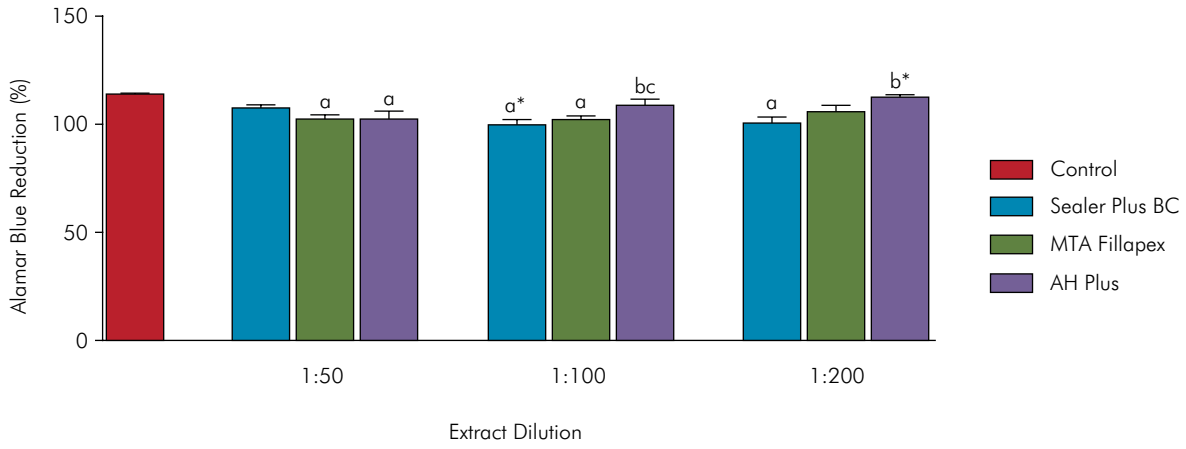

Figure 1. Cell viability of L929 fibroblasts after stimulation with serially diluted extracts of Sealer Plus BC, MTA Fillapex, and AH Plus at 24 h determined via Alamar Blue assay. The letters indicate differences observed while comparing different materials with the same dilution: a: $P < 0.05$ vs. Control; b: $P < 0.05$ vs. Sealer Plus BC; c: $P < 0.05$ vs. MTA Fillapex. The * symbol indicates the difference observed while comparing the 1:50 extract dilution with other dilutions of the same material ($P < 0.05$).

Results

Cell viability

The data of the L929 fibroblast viability are shown in Figure 1. Alamar Blue assay exhibited a significant reduction in cell viability stimulated by the Sealer Plus BC extract (1:100 and 1:200), MTA Fillapex (1:50 and 1:100), and AH Plus (1:50) relative to the Control group ($p < 0.05$). Conversely, a greater percentage of viability was identified on the L929 fibroblast that grew in the presence of the AH Plus extract when compared with that of the Sealer Plus BC (1:100 and 1:200) and MTA Fillapex (1:100) ($p < 0.05$). Additionally, the 1:200 dilution of AH Plus was less cytotoxic than the 1:50 dilution ($p < 0.05$).

It was observed that cell exposure to the 1:50 dilution of the Sealer Plus BC extract exhibited results similar to that of the Control group ($p > 0.05$) and significantly increased the cell proliferation when compared with the 1:100 dilution ($p < 0.05$).

Histologic analysis

Representative images of each group are shown in Figure 2 (A–P), and the histological analysis is shown in Table. After 7 d of the implantation, a severe inflammatory response was observed in the Sealer Plus BC group, moderate inflammatory response was observed in the MTA Fillapex and AH Plus groups, and mild inflammation was observed in the Control group. However, a significant difference was not observed among the groups ($p > 0.05$). The inflammatory cells observed corresponded to polymorphonuclear, macrophages, and multinucleated giant cells. The fibrous capsule was thick in all specimens of each group in this period.

At 30 d, the MTA Fillapex and AH Plus groups exhibited moderate inflammation while the Sealer Plus BC and Control groups exhibited mild and absent inflammation. However, a significant difference was absent between the bioceramic sealer and AH Plus or MTA Fillapex ($p > 0.05$), and a significant difference was observed among MTA Fillapex and AH Plus groups when compared to that of the Control group ($p < 0.05$). All specimens from the Control group exhibited a thin fibrous capsule at 30 d, and this was also applicable for most specimens from the Sealer Plus BC and AH Plus groups. However, several specimens from the MTA Fillapex group still exhibited a thick fibrous capsule in this period.

Discussion

Studies indicate the potential of bioceramic materials in endodontics due their ability to induce repair via the formation of mineralized tissue and excellent biocompatibility[7]. Thus, the study evaluated the biocompatibility and cytotoxicity of a

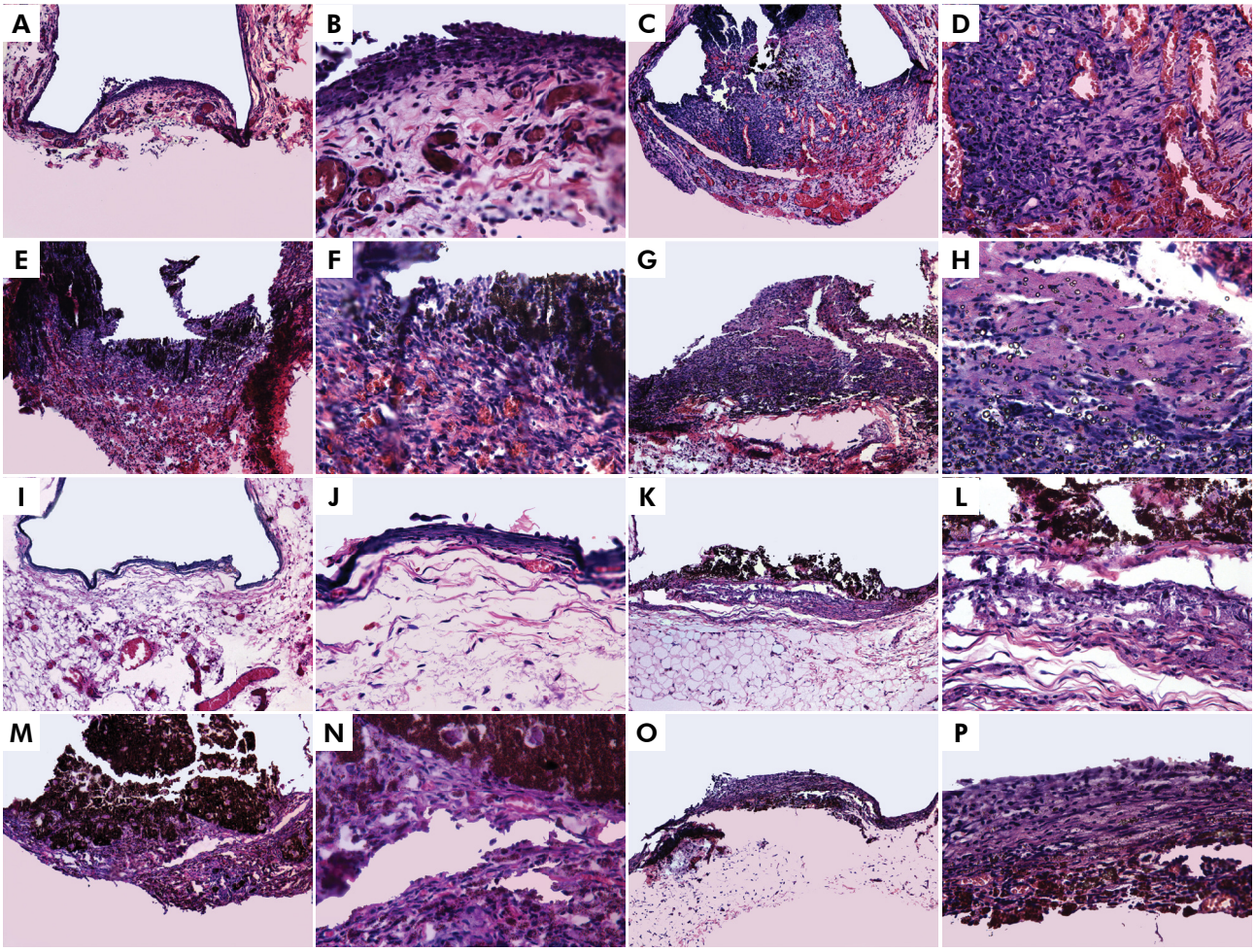

Figure 2. Representative images of the subcutaneous tissue reactions in the control and sealer groups. 7 d period: (A,B) control group with mild inflammatory cell infiltration; (C,D) the Sealer Plus BC group exhibits severe inflammation while the (E,F) MTA Fillapex and (G,H) AH Plus groups exhibits moderate inflammatory infiltrate. A thick fibrous capsule is observed in all groups in the period. 30 d period: (I,J) the control group did not exhibit inflammation in the periods and the thin fibrous capsule; (K,L) the Sealer Plus BC group exhibits mild inflammatory infiltrate; and (M,N) the MTA Fillapex and (O,P) AH Plus groups exhibit moderate inflammation. The thin fibrous capsule is observed in more specimens of the Sealer Plus BC and AH Plus groups in the period and in a few specimens of MTA Fillapex. Haematoxylin-eosin staining, (A,C,E,G,I,K,M,O) 100x, (B,D,F,H,J,L,N,P) 400x.

Table. Inflammatory score and thickness of the fibrous capsule for all groups.

Time (p-value)	Scores				Median	Capsule		n
	0	1	2	3		Thick	Thin	
7 days ($p < 0.001$)								
Control[a]	0	4	3	1	1	8	0	8
Sealer Plus BC[a]	0	0	3	5	3	8	0	
MTA Fillapex[a]	0	0	4	4	2	8	0	
AH Plus[a]	0	2	4	2	2	8	0	
30 days ($p < 0.001$)								
Control[a]	5	3	0	0	0	0	8	8
Sealer Plus BC[ab]	0	4	4	0	1	3	5	
MTA Fillapex[b]	0	3	5	0	2	4	4	
AH Plus[b]	0	2	6	0	2	2	6	

*Same letters indicate the absence of statistical difference among the groups in each analysis period ($p > 0.05$).

new bioceramic sealer, namely the Sealer Plus BC, which was developed for root canal filling. It was observed that the cytotoxicity of the material depends on the dilution of the extract used, and its cytotoxicity decreases when it is less diluted. An *in vivo* study demonstrated an increase in biocompatibility over time, and this was similar to the control group, thereby differing from the other materials tested. Therefore, we reject the null hypothesis presented.

Different dilutions of extracts from endodontic sealers are used in cytotoxicity assays.[4,16,22,23] An earlier study indicated that the cytotoxicity of a few endodontic sealers, such as MTA Fillapex and AH Plus, is already reduced with less diluted extracts.[16] However, we used different and higher dilutions of the extracts based on previous studies[4,24] and as recommended by ISO 10993-5.[25] The dilution use is also justified because when the material is in contact with the tissue, the leachable compounds are continuously eliminated by the extracellular fluids and their concentration progressively decreases.[23] The non-diluted extract of a material leads to cell death[23,26] and especially when the properties of the material are not known as in the case of Sealer Plus BC.

The MTA Fillapex is a material that was developed to combine the excellent biological properties of the MTA to resinous components to improve required physicochemical properties for endodontic sealers. The properties include flowability similar to that of AH Plus and an adequate setting time to facilitate its use by an operator.[14,27] However, studies indicated that the material affects cellular metabolism despite their interesting physiochemical properties.[8,14,28,29] The resins present in the MTA Fillapex include salicylate resin, natural resin, and resin diluent, which do not affect the bioactivity of the material[9] although it is suggested that the induction of cells death is attributed to the presence of the resins in its composition.[8]

The unfavorable results of MTA Fillapex in cell culture are reported even when compared to that of resin epoxy-based material such as AH Plus.[30] The results are observed in traditional two-dimensional (2D) and three-dimensional (3D) systems,[31] and are corroborated by results observed in our *in vitro* evaluation as follows: a greater reduction was observed in the cellular metabolism by MTA Fillapex when compared to that of the Control or AH Plus while using less-diluted extracts. The AH Plus exhibits less cytotoxicity with increases in dilution, and this is in agreement with the results of a previous study, which indicated that a greater dilution of AH Plus after 24 h caused minor cytotoxic effects toward L929 cells.[32]

The epoxy resin of AH Plus is considered as a mutagenic substance and mainly due to the addition of the amine component[16] although the material exhibits extremely low solubility since it is hydrophobic. It is hypothesized that the high solubility of MTA Fillapex (which is a hydrophilic material) also favors cytotoxicity because it indicates the increased release of toxic components.[33] The high solubility of MTA Fillapex is already considered in a few situations above the limits recommended by the ISO standard.[34] Furthermore, although the AH Plus exhibits an extremely long setting time, the possibility of the significant interference with the cytotoxicity of the material is not completely proven.[35] Thus, the unfavorable release of toxic components that potentially arises from the longer setting time of AH Plus is potentially compensated by the favorable results of the low solubility of the material, which does not occur with MTA Fillapex.

However, a study that evaluated long-term cytotoxicity indicated a favorable response of MTA Fillapex at 7 d of exposure to cell culture, which exhibited an evident recovery of its viability.[27] Our *in vitro* assay exhibits the limitation wherein only a 24-h period was used to analyze the cytotoxicity of the extracts. However, we evaluated inflammatory response *in vivo* following 7 d and 30 d of contact with materials based on previous studies.[4,22] In our histological analysis, a reduction in the inflammation caused by MTA Fillapex was observed over time despite increase in inflammation observed in relation to the Control group, thereby suggesting that it is more biocompatible with increases in the analysis times as reported in extant studies.[9] The same occurred with AH Plus given that the histological results indicate that the inflammation generated by the sealer decreases over time and can be better evaluated over longer periods of analysis. Other previous studies (both *in vitro* or *in vivo*) indicated that AH Plus is considered as

biocompatible by allowing reduction of inflammation and cytotoxicity over time.[14] When analyzed *in vivo* in bone tissue, both MTA Fillapex and AH Plus exhibited acute characteristics that were predominant in the initial period corresponding to 7 d. The response becomes chronic with respect to time (30 d), and it was almost absent subsequently (90 d).[36]

When we observed the fibrous capsule, the MTA Fillapex corresponded to the sealer that allowed a lower reduction in the fibrous capsule over time. However, the results should be analyzed in detail. It is known that the thickness of the fibrous capsule is a good marker of inflammation.[37] However, in the study, we observed several fibroblasts around the MTA Fillapex particles. Thus, we assign the largest number of specimens with a thick fibrous capsule at 30 d to the increased solubility of the MTA Fillapex, which can facilitate their extravasation.[38]

With respect to Sealer Plus BC, we observed a different profile of the cytotoxicity and biocompatibility. In contrast to the other tested sealers, the Sealer Plus BC was less cytotoxic when less diluted, and its biocompatibility was more rapid when compared to that of the other sealers, and this similar to the Control at 30 d. Bioceramics compounds, calcium hydroxide, and propylene glycol are part of the composition of the new endodontic sealer. The material is already available in Brazil although its composition is not yet analyzed in laboratory studies. Previous other studies did not analyze the properties of the Sealer Plus BC, and thus there are no previous results that are discussed in the present study. Furthermore, physicochemical studies are required to aid in explaining our results. For example, it is necessary to identify when the release of calcium hydroxide occurs, the point at which the pH becomes alkaline, and if other components are present in its formulation that can be used to interpret greater cytotoxicity while using a more diluted extract of the material.

However, our *in vivo* results indicate that the Sealer Plus BC allows a rapid reduction of the initial inflammation observed after implantation into the subcutaneous tissue of rats. Furthermore, its initial inflammation is justified by the addition of calcium hydroxide in its composition. As widely-known, the presence of calcium hydroxide can cause a superficial necrosis in the region of contact of the material with the tissue due to the increase in alkalinity. Subsequently, the region is repaired via the formation of hard tissue.[4,22] Calcium hydroxide also accelerates the tissue repair process[4,21] and is shown to improve the biological properties of endodontics sealers.[15]

There is another available bioceramic sealer on the market with a composition similar to that of the Sealer Plus BC, namely the Endosequence BC sealer (Brasseler, Savannah, USA), which in addition to the bioceramic components includes calcium hydroxide in its composition and is resin free.[39] Studies indicated more favorable cell viability and biocompatibility with the sealer when compared to that of the MTA Fillapex.[28,33]

In general, the results of this study indicate that the Sealer Plus BC is more cytotoxic than AH Plus and the Control with increase in dilution. However, the tissue response to the material exhibits biocompatibility superior to that of the other sealers because the reaction was similar to the Control at 30 d. However, an interesting result is observed *in vitro* while using its most concentrated extract. It is necessary to evaluate the properties of the Sealer Plus BC in detail given that it is a bioceramic sealer (which exhibits the capacity to induce biomineralization) and considering the initial results with respect to its biocompatibility because it is a bioceramic root canal sealer that can be used in the future by endodontists.

Conclusion

The Sealer Plus BC is less cytotoxic to L929 fibroblasts cells when a less-diluted extract is used. Additionally, it is more biocompatible than MTA Fillapex and AH Plus.

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