Evaluation of the Cytotoxicity and Biocompatibility of New Resin Epoxy–based Endodontic Sealer Containing Calcium Hydroxide

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Abstract

Introduction: Many endodontic sealers are available, but the search for the ideal sealer continues. This study evaluated the cytotoxicity and biocompatibility of Sealer Plus, a new resin epoxy–based endodontic sealer containing calcium hydroxide. AH Plus, Endofill, and SimpliSeal endodontics sealers were used for comparison. **Methods:** L929 fibroblasts were cultured, and an MTT assay was used to determine the cytotoxicity of the sealer extracts at 6, 24, 48, and 72 hours. Tubes containing materials or empty tubes for control were inserted into the subcutaneous tissues of 20 rats. After 7 and 30 days, the rats were killed, and the tubes were removed with the surrounding tissues for histologic analysis. The data were submitted to statistical tests ($P < .05$). **Results:** Undiluted Sealer Plus exhibited less cytotoxicity compared with other undiluted extracts at 6 hours ($P < .05$), and cell viability was higher for all Sealer Plus extracts after 24 hours ($P < .05$). At 48 hours, the undiluted and ½ Sealer Plus dilution were the extracts with less cytotoxicity ($P < .05$). At 72 hours, cell viability was higher for the undiluted and ½ Sealer Plus dilution compared with the other sealers ($P < .05$). At 7 days, Endofill and SimpliSeal had higher inflammation compared with the control and Sealer Plus ($P < .05$); AH Plus had moderate inflammation ($P > .05$). At 30 days, control, Sealer Plus, and AH Plus had less inflammation ($P < .05$). The fibrous capsule was thick at 7 days and thin at 30 days, except for SimpliSeal. **Conclusions:** In general, Sealer Plus promoted greater cell viability and was more biocompatible compared with the other sealers. *(J Endod 2017;43:2088–2092)*

Key Words

Biocompatibility, calcium hydroxide, cytotoxicity, endodontic sealers, resin epoxy

Significance

Our results indicate that Sealer Plus promoted greater cell viability and was more biocompatible compared with AH Plus, Endofill, and SimpliSeal sealers.

Biocompatibility is one of the most important properties that endodontic sealers must present, because they will be in close contact with periapical tissues (1, 2). This property is directly related to the composition of the material. For example, zinc oxide–eugenol–based materials release toxic substances during a prolonged period and may maintain constant inflammation (3); materials that are based on methacrylate resin have components that may not polymerize and are toxic to periapical tissues (4); and materials containing epoxy resin show a degree of cytotoxicity (5, 6) and severe inflammation (7, 8), and some were considered mutagenic for releasing bisphenol A diglycidyl ether and formaldehyde, potential carcinogens (9).

Despite the poor biocompatibility of these materials, some are still widely used; zinc oxide–eugenol–based materials such as Endofill have long and satisfactory usage history (10). AH Plus, a resin epoxy–based sealer, is considered the gold standard of endodontic sealers because of its excellent physicochemical properties (11, 12). SimpliSeal, another sealer containing epoxy resin, is also widely used (13), with calcium oxide and calcium phosphate in its composition, which may contribute to improve biocompatibility (13). However, the search for more biocompatible sealers with good physicochemical properties is ongoing.

A new resin epoxy–based endodontic sealer is commercially available and has not yet had its properties studied, Sealer Plus (MK Life, Porto Alegre, RS, Brazil). This sealer has a composition similar to AH Plus (Dentsply, Konstanz, Germany). In its composition AH Plus contains radiopaque fillers, calcium tungstate and zirconium oxide, which are also present in Sealer Plus, but the biggest difference is the presence of calcium hydroxide in the new sealer; calcium hydroxide is present in the base and catalyst pastes. Previous studies observed that the addition of calcium hydroxide to AH Plus significantly decreased the inflammation in rat subcutaneous tissue (14) without altering the sealer's physical properties (15). Thus, this new formulation seems to be promising for the use of endodontists if it combines the good physical properties of AH Plus with enhanced biological properties, which still needs to be investigated.

Thus, our aim was to evaluate the cell viability and reaction in the subcutaneous tissue of rats against Sealer Plus compared with previously known cements: AH Plus,

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Endofill (Dentsply-Latin America, Rio de Janeiro, Brazil), and SimpliSeal (Kerr, Orange, CA).

Materials and Methods

In Vitro Study

Cell Line. Fibroblast line cells L929 were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD), streptomycin (50 g/mL), and 1% antibiotic/antimycotic cocktail (300 U/mL, 300 μg/mL streptomycin, 5 μg/mL amphotericin B) (Gibco BRL) under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% CO_2) (16).

Cell Viability Assay. The endodontic sealers Sealer Plus, AH Plus, Endofill, and SimpliSeal were mixed according to the manufacturers' instructions, and sealer extracts were prepared following previous investigations (16). Briefly, disks containing these materials were prepared under aseptic conditions by using a sterile cylindrical polyethylene tube (diameter, 5 mm; height, 3 mm). The disks were kept in a 5% CO_2 incubator at 37°C for 6 hours for setting. After 6 hours, the disks were removed from the mold and sterilized by ultraviolet light for 1 hour (16). Each disk was immersed into 1 mL DMEM with 10% FBS and incubated in a humidified atmosphere containing 5% CO_2 for 3 days. Then the disks were discarded, and the supernatants (eluate extract) were collected and filtered through a sterile 0.22-μm filter (Sigma-Aldrich, St Louis, MO). The supernatant collected was referred to as sealer extract (16). The undiluted extracts (1/1) and 2 dilutions with culture medium (½ and ¼) were used.

L929 fibroblasts were seeded into the 96-well plates (104 cells/well) and incubated for 24 hours in a humidified air atmosphere of 5% CO_2 at 37°C to allow cell attachment. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cell viability (17). Briefly, after cell attachment, the sealer extracts were added to the cells. The controls were cultured in medium without any sealer extracts. At 6, 24, 48, and 72 hours after addition, the MTT solution (Sigma-Aldrich) was added to the cells, and the fibroblasts were incubated at 37°C for 4 hours protected from light. Then MTT solution was discarded, and 200 μL isopropyl alcohol was added to each well. The plate was kept under continuous agitation for 30 minutes to dissolve the dark blue crystals. The blue solution was transferred to a 96-well plate to measure the optical density at 570 nm in a spectrophotometer. The experiments were performed in triplicate.

In Vivo Study

Subcutaneous Implants. Twenty 3-month-old male Wistar rats weighing between 250 and 280 g were used. The sample size was established on the basis of previous studies (18, 19). The animals were housed in a temperature-controlled environment (22°C ± 1°C) with a 12-hour light-dark cycle and received water and food *ad libitum*. This study was approved by the institutional ethics committee at UNESP-Universidade Estadual Paulista (São Paulo, Brazil) and conducted in accordance with relevant guidelines (CEUA 2014-01052).

Polyethylene tubes ("80"; Abbott Labs of Brazil, São Paulo, SP, Brazil) (1.0 mm internal diameter, 1.6 mm external diameter, and 10.0 mm length) were filled with Sealer Plus, AH Plus, Endofill, or SimpliSeal prepared according to the manufacturers' recommendations or were empty for control. For the surgical procedure (16), the rats were anesthetized, their dorsa were shaved, and a 2.0-cm incision was made in a head-to-tail orientation with a #15 Bard-Parker blade (BD, Franklin Lakes, NJ). The skin was reflected to create 2 pockets on the right side and 2 pockets on the left side of the incision. After the tubes were randomly implanted into the pockets, subsequently identified according to the material received, the skin was closed with 4.0 silk sutures.

Histologic Analysis. At 7 and 30 days, the rats were killed with an overdose of the anesthetic solution, and the polyethylene tubes, together with the surrounding tissues, were removed and fixed in 10% buffered formalin at neutral pH. The specimens were processed and embedded in paraffin. The paraffin blocks were oriented parallel to the long axis of the tubes, and longitudinal serial sections of 5 μm were obtained from the central areas of the implants for staining with hematoxylin-eosin. For histologic analysis, 5 sections of each specimen were analyzed by a single calibrated operator in a blinded manner under light microscopy (DM 4000 B; Leica Microsystem, Wetzlar, Germany). The inflammation in the tissues close to the material was scored as follows: 1, no or few inflammatory cells and no reaction; 2, less than 25 cells and mild reaction; 3, between 25 and 125 cells and moderate reaction; and 4, 125 or more cells and severe reaction (16). Fibrous capsules were considered thin when thickness was <150 μm and thick at >150 μm (16).

Statistical Analysis

The GraphPad Prism (version 5.0; GraphPad Software, Inc, La Jolla, CA) statistical software program was used. Analysis of variance followed by Bonferroni correction was performed for parametric data. The Kruskal-Wallis test was followed by the Dunn test for nonparametric data. A P value <.05 was considered significant.

Results

Cell Viability

The data of cell viability in the presence of the different sealer extracts are shown in Figure 1. At 6 hours, undiluted Sealer Plus exhibited higher cell viability when compared with other undiluted extracts ($P < .05$). In addition, cell viability was higher for all Sealer Plus extracts (1/1, ½, and ¼) after 24 hours of exposure, compared with control and the other sealers ($P < .05$). At 48 hours, the undiluted and ½ Sealer Plus dilution were the less cytotoxic extract to the L929 fibroblasts, compared with other sealer extracts ($P < .05$); only at 48 hours, ½ and ¼ Sealer Plus dilutions were less toxic than undiluted Sealer Plus ($P < .05$). At 72 hours, undiluted and ½ Sealer Plus dilution and ¼ Endofill dilution were less cytotoxic compared with the other sealer extracts ($P < .05$).

At 48 and 72 hours, the cytotoxicity of AH Plus and Endofill extract was lower in presence of ½ and ¼ dilutions than the undiluted extract ($P < .05$). Moreover, at 24, 48, and 72 hours, cell exposure to undiluted SimpliSeal significantly increased cell viability compared with undiluted AH Plus and Endofill ($P < .05$).

The presence of sealer extracts (undiluted, ½, and ¼) significantly decreased cell viability compared with control at 72 hours ($P < .05$).

Tissue Response

Representative images of the tissue response of each group can be observed in Figure 2 (*A, a-J, j*), and the histologic analysis is shown in Table 1. At 7 days, large numbers of polymorphonuclear cells were observed at the opening of the tubes in addition to macrophages and multinucleated giant cells. Inflammatory cells were observed mainly in the Endofill and SimpliSeal groups, which had severe inflammation compared with the control and Sealer Plus groups, with mild inflammatory infiltrate ($P < .05$). The AH Plus group had moderate inflammation, without significant differences from the other groups ($P > .05$).

At 30 days, most of the specimens of the control, Sealer Plus, and AH Plus groups presented mild inflammation, which was different from the Endofill and SimpliSeal groups, which had moderate to severe

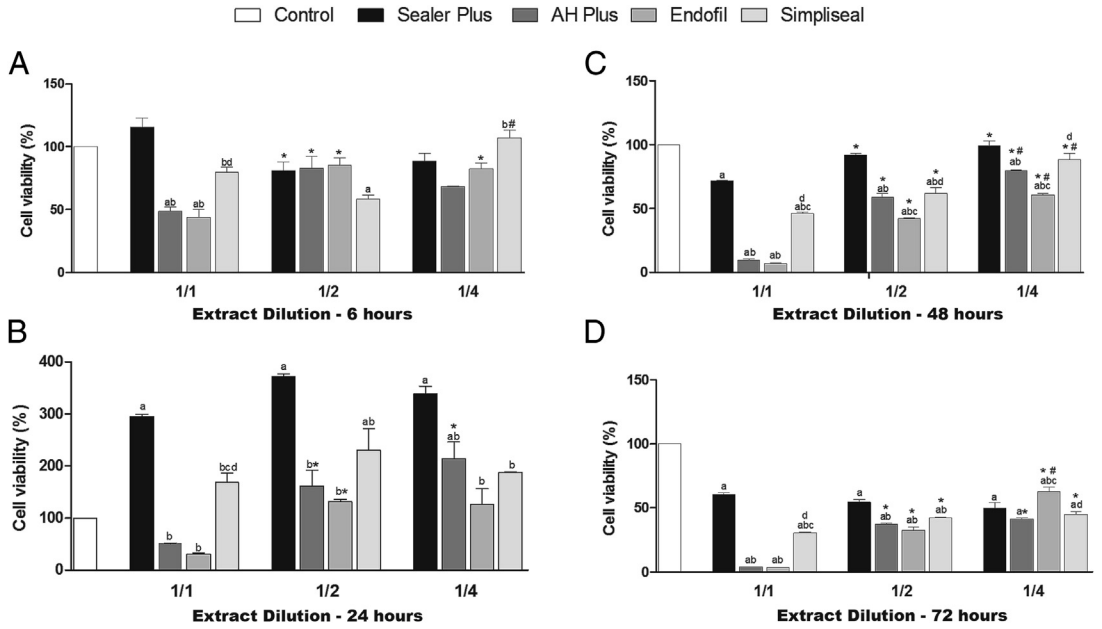

Figure 1. Cell viability of L929 fibroblasts after exposure with serially diluted extracts of Sealer Plus, AH Plus, Endofill, and SimpliSeal for 6 (*A*), 24 (*B*), 48 (*C*), and 72 hours (*D*) determined by MTT assay. Note that Sealer Plus extract (undiluted, ½, and ¼) significantly enhanced viability above the other sealer extracts ($P < .05$). Differences observed when comparing different materials at the same dilution are indicated by letters: a, $P < .05$ vs control; b, $P < .05$ vs Sealer Plus; c, $P < .05$ vs AH Plus, and d, $P < .05$ vs Endofill. Differences observed in comparison among extract dilution of the same material are indicated by symbols: *$P < .05$ vs undiluted extract; #$P < .05$ vs ½ dilution.

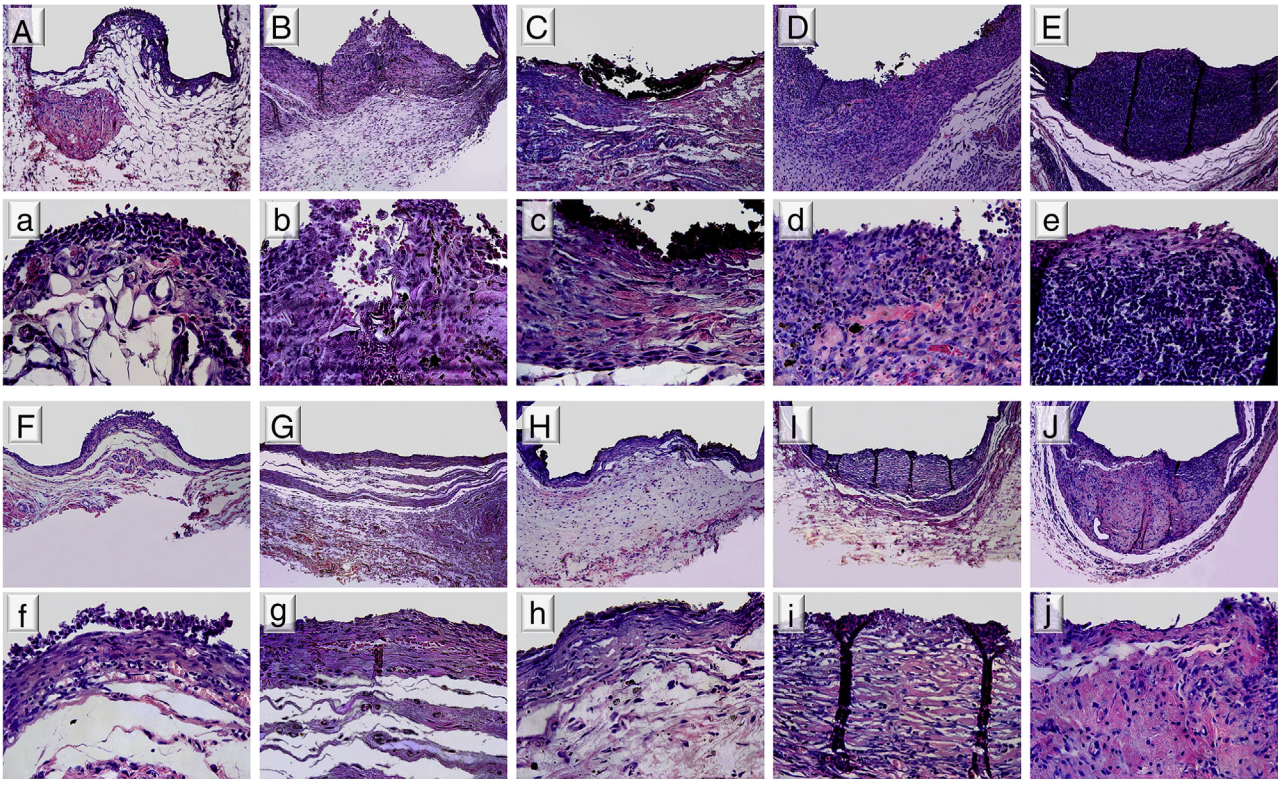

Figure 2. Representative images of subcutaneous tissue reactions in control and sealer groups. (*A, a–E, e*) At 7 days: (*A, a*) control group and (*B, b*) Sealer Plus group with the presence of mild inflammatory cell infiltration and a thick fibrous capsule; (*C, c*) AH Plus group showed moderate inflammatory cell infiltration in the thick fibrous capsule; and (*D, d*) Endofill and (*E, e*) SimpliSeal groups had severe inflammatory infiltrate and a thick fibrous capsule in the tube opening. (*F, f–J, j*) At 30 days: (*F, f*) control group, (*G, g*) Sealer Plus group, and (*H, h*) AH Plus group with mild inflammatory infiltrate and a thin fibrous capsule; (*I, i*) Endofill group with moderate inflammatory cell infiltration and a thin fibrous capsule; and (*J, j*) SimpliSeal group with moderate inflammatory cell infiltration and a thick fibrous capsule. Hematoxylin-eosin staining. Original magnification: (*A–J*) ×100, (*a–j*) ×400.

TABLE 1. Inflammatory Score and Thickness of Fibrous Capsule of All Groups

Time/*P* value	Groups*	Scores 1	2	3	4	Median	Capsule Thick	Thin	*n*
7 days/*P* < .001	Control[a]	1	4	3	0	2	8	0	8
	Sealer Plus[a]	0	5	3	0	2	8	0	
	AH Plus[ab]	0	0	5	3	3	8	0	
	Endofill[b]	0	0	2	6	4	8	0	
	SimpliSeal[b]	0	0	1	7	4	8	0	
30 days/*P* < .001	Control[a]	2	6	0	0	2	0	8	8
	Sealer Plus[a]	1	5	2	0	2	0	8	
	AH Plus[ab]	1	5	2	0	2	2	6	
	Endofill[b]	0	0	4	4	3	3	5	
	SimpliSeal[b]	0	0	4	4	3	4	4	

*Same superscript letters indicate no statistical difference among the groups ($P > .05$).

inflammation with polymorphonuclear cells and some lymphocytes ($P < .05$).

The fibrous capsule was thick in all specimens at 7 days. At 30 days, all specimens from the control and Sealer Plus groups had a thin fibrous capsule as well as most specimens from the AH Plus and Endofill groups. However, several specimens from the SimpliSeal group still had a thick fibrous capsule in this period.

Discussion

An ideal endodontic sealer must have biocompatibility, adequate physicochemical properties, bioactivity, and antimicrobial activity (1, 20). Although many endodontic sealers are available on the market, no sealer meets all these requirements. This study analyzed the cytotoxicity and biocompatibility of a new formulation of a commercially available endodontic sealer, Sealer Plus, and observed that this sealer had a superior response compared with previous sealers.

Our *in vitro* results showed that Sealer Plus could increase cell viability at almost all analyzed experimental periods and dilutions used in comparison with the other sealers. The composition of Sealer Plus resembles AH Plus, with the addition of calcium hydroxide. However, AH Plus was one of the most cytotoxic among the tested materials. Other studies have also shown that sealers containing calcium hydroxide induce less cytotoxicity than zinc oxide–eugenol–based or resin-based sealers without calcium hydroxide (21) or even no cytotoxic effect (22).

Sealer Plus also had the best histologic results, with mild inflammation at 7 days, similar to control, and a thin fibrous capsule in all specimens at 30 days. Although AH Plus had similar results for biocompatibility, Sealer Plus was the only sealer to differ significantly from Endofill and SimpliSeal, which produced more severe inflammation.

Calcium hydroxide has important effects on tissue; it promotes alkaline pH, has an antibacterial effect, and accelerates the process of tissue repair (14, 23). These properties may explain the Sealer Plus results. In a previous study, the addition of calcium hydroxide to AH Plus improved the biological properties of this cement, with reduction of the inflammatory response in rat subcutaneous tissue (14) without affecting most of the physical properties of AH Plus (15). Calcium hydroxide had already been added to AH 26, resulting in improvements in the apical sealing and biocompatibility of this sealer (24, 25). These results, together with those of the present study, help demonstrate the ability of calcium hydroxide to improve the properties of endodontic materials.

The cytotoxicity of AH Plus had been previously observed, similar to this study, but mainly in the initial periods, reducing over time (26–28). It should be noted that *in vitro* cytotoxicity assays comprise the first level of biocompatibility analysis of a material and that it may undergo influences, depending, for example, on the cell type used (26, 29). The model used for cell testing may also be an influence; cytotoxicity tests usually use traditional two-dimensional culture (30, 31), as in our study. However, three-dimensional models of cells best represent the cell in *in vivo* conditions because they allow the cells to grow and perform their functions (31). AH Plus showed reduced cytotoxicity in this three-dimensional model (31).

Compensating for the deficiencies of an *in vitro* model, we present the results *in vivo* in subcutaneous tissue, which comprises the second level of biocompatibility analysis of a material (29). We saw that at 7 days, AH Plus promoted a moderate inflammatory reaction that reduced over time. This result corroborates previous *in vitro* or *in vivo* studies; after 1 week, AH Plus can be considered biocompatible (27, 28).

In general, Endofill had the least favorable characteristics regarding cell viability, according to previous studies (32). Furthermore, in the histologic analysis, severe inflammation was observed at 7 days with the presence of multinucleated giant cells, as previously noted (33). In addition, most specimens presented severe inflammation even after 30 days. These results can be explained by the presence of eugenol, which is also responsible for its strong antimicrobial action (34).

Regarding the presence of a fibrous capsule, Endofill and SimpliSeal had specimens with a thick fibrous capsule even after 30 days. This result is similar to a previous study that showed no organized fibrous capsule in Endofill, which could favor the extent of the inflammatory process (1). However, histologically, more severe results were observed in the SimpliSeal group, unlike the results of cytotoxicity. At 7 days, severe inflammation was observed for SimpliSeal, which remained in part of the specimens at 30 days. It is possible that this inflammation will decrease over time, but further studies are necessary to determine this sealer's biocompatibility.

Regarding cellular viability, an earlier study showed that SimpliSeal was similar to the control group at 30 hours of cell contact with the dilutions of the extract, decreasing at 90 hours (13). These results are similar to ours when we observed that the cytotoxicity of this cement increased over time. However, its cytotoxicity was lower than AH Plus and Endofill, which can be explained by the presence of calcium in its composition.

It should be noted that in this study, *in vitro* analysis assessed the cytotoxicity of the material after setting, whereas in the *in vivo* study, the sealers used were still fresh. It is known that fresh or set sealers can cause different reactions in cells and/or tissues (16), which may explain the variation of *in vitro* and *in vivo* results observed in this study. However, we showed that when fresh or even after setting, Sealer Plus showed promising results.

Regarding endodontic therapy, the presence of obturation materials such as gutta-percha should be considered; they may influence the result of root canal filling, which was not evaluated in this study. Although traditional zinc oxide–eugenol–based sealers do not exhibit good biocompatibility (32, 33), previous studies concerning these sealers presented the lowest shrinkage and dimensional changes of the obturation mass, when compared with other sealers (35). This is explained by the chelation reaction of eugenol with zinc from the sealer and zinc oxide present in gutta-percha, which is not observed with other eugenol-free materials (35). Furthermore, the reaction of eugenol with gutta-percha may lead to less release of free eugenol into the periapical tissues, reducing the cytotoxicity (35). Perhaps these properties help to explain the longtime use of zinc oxide–eugenol–based sealers.

This first study analyzed only the cytotoxicity and biocompatibility of this new material, and other important properties such as physicochemical and bioactivity should be analyzed.

Conclusions

Sealer Plus promoted greater cell viability for almost all analyzed periods and dilutions, compared with the other sealers. In addition, this endodontic sealer was more histologically biocompatible.

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