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Original Article

In Vitro Comparison of the Cytotoxicity of Different Endodontic Sealers: AH Plus, AdSeal, Endoseal MTA, and GuttaFlow Bioseal

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Abstract

Introduction: Endodontic sealers are essential components of root canal treatment and contribute to the sealing of the root canal system. Their cytotoxicity is an important factor in evaluating their biocompatibility. This study aimed to assess the cytotoxicity of four commonly used endodontic sealers, namely, AH Plus, AdSeal, Endoseal MTA, and GuttaFlow Bioseal against human gingival fibroblasts (HGFs).

Methods: HGFs were exposed to the respective sealers in set form and in five different weights after sterilization. Then, the cytotoxicity of the sealers was evaluated after 1 day, 3 days, and 7 days using the methyl thiazolyl tetrazolium (MTT) assay. Ultimately, data were analyzed by repeated measures ANOVA followed by Tukey's post hoc test.

Results: After 24 hours, all sealers showed low cytotoxicity. However, sealers in 0.25 g and 0.5 g weights exhibited a marked reduction in cell viability (<30%) at 3 days and 7 days, indicating high cytotoxicity, while lighter weights (0.125 g, 0.1 g, and 0.08 g) demonstrated moderate-to-low cytotoxicity (30–70%). Moreover, AdSeal consistently displayed the highest cell viability, particularly at 0.08 g (around 70%), whereas AH Plus illustrated the lowest viability (approximately 5%) across all weights and time points.

Conclusion: Among the tested sealers, AdSeal had the lowest cytotoxicity and the highest biocompatibility. Therefore, its use may promote faster periapical healing while reducing the risk of inflammatory complications compared with AH Plus.

Keywords: Materials testing, Epoxy resin-based root canal sealer, Calcium silicate, Fibroblasts, Biocompatibility, MTT assay

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Introduction

The success rate of endodontic treatment has reached 92–98% due to recent advances in dental science and technology (1-3). Endodontic treatment is performed to hermetically seal the canal three-dimensionally and prevent coronal and apical leakage (4, 5). Such a hermetic seal is frequently achieved by the use of gutta-percha and sealer. Gutta-percha fills the root canal space and serves as a carrier for the sealer. In addition, sealer provides a hermetic seal against microleakage (6, 7). Ideally, endodontic sealers should exhibit no cytotoxic effects on periodontal cells that are likely to come into contact with the material during or after root canal treatment (8). Aside from the apical foramen, numerous microscopic and macroscopic communications exist between the root canal system and the periodontal ligament and the supporting bone, including dentinal tubules, accessory foramina, and accessory canals (9). Thus, the tissue fluid can easily penetrate into the root canal system and degrade and wash out the sealer. Further, the washed-out products may penetrate into the periodontal tissue and alveolar bone and trigger local inflammatory reactions and subsequent complications (10, 11). Long-term exposure of the periodontal tissue to sealer or its constituents can cause irritation and delay the healing of periapical lesions. Furthermore,

sealers can directly interact with the adjacent tissue when the canal is over-filled (sealer puff) (6), causing considerable irritation of the periradicular tissue (12). According to recent studies, biocompatibility is one of the most essential properties determining the clinical performance of endodontic sealers (13). A granular fibrovascular tissue is formed and the periapical lesion is healed following the debridement of the canal content and a correct non-surgical endodontic treatment (14). Biocompatible endodontic sealers can enhance the healing of apical periodontitis. According to Grossman (12), an ideal sealer should demonstrate optimal adhesion to root canal walls after mixing and provide a strong seal while not undergoing shrinkage after setting or causing tooth discoloration. Likewise, it should be radiopaque and visible on radiographs, easily mixable with liquid, bacteriostatic, insoluble in tissue fluids, and biocompatible. Moreover, it must have a slow setting, and no potential for the irritation of periradicular tissue (12).

Considering all the above-mentioned discussions, biocompatibility is an important property for endodontic sealers (15). Several methods are available for assessing the possible cytotoxicity of dental materials, such as cell culture, animal studies, and clinical investigations. The novel cell culture techniques aim to replace investigations on animal models (16). The methyl thiazolyl tetrazolium (MTT) assay is the most commonly used test for the evaluation of the biocompatibility of dental materials following exposure to gingival fibroblasts, dental pulp stem cells, stem cells of the apical papilla, and periodontal ligament stem cells (17-20). This test has been confirmed by the ISO standard for the assessment of cytotoxicity (17).

Depending on their chemical composition and structure, the currently available sealers can be divided into zinc oxide eugenol, resin, glass ionomer, silicone, and calcium hydroxide-based sealers, as well as bioactive sealers (21). AH Plus epoxy resin-based sealer has been frequently employed as a gold-standard reference sealer in endodontic studies. Considering the lack of studies comparing the cytotoxicity of different sealer types, this study aims to compare the cytotoxicity of AH Plus and AdSeal epoxy resin-based sealers, Endoseal MTA calcium silicate-based sealer, and GuttaFlow Bioseal silicone-based sealer against human gingival fibroblasts (HGFs). The null hypothesis is that no significant difference can be found in the cytotoxicity of the four sealer types.

Materials and Methods

This in vitro, experimental study evaluated the cytotoxicity of four typically used sealers against HGFs. The study protocol was approved by the Ethics Committee of AJA University of Medical Sciences (IR.AJAUMS.REC.1399.190). Table 1 presents the sealers and their composition.

Cell Culture

HGF (C10459; Iranian National Center for Genetic and Biologic Resources, Tehran, Iran) was cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Sigma-Aldrich, USA) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were daily observed, and the culture medium was replaced every two days until reaching approximately 90% confluence. After detachment with 0.25% trypsin–ethylenediaminetetraacetic acid, the cells were neutralized with complete medium, centrifuged, and resuspended for subsequent passages. In addition, the suspension included 45 µL pen-strep + 5 mL FBS per 50 mL DMEM. Cells at the fourth passage were then used for the experiments. The general procedure followed standard cell culture protocols as described in our previous study (8).

Moreover, 0.4% trypan blue was used to assess the cell viability (0.2 g added to 50 mL of saline). Next, the treated cells were detached with trypsin, collected in a Falcon tube, and centrifuged at 1,500 rpm for 6 minutes to obtain a homogenous cell suspension; more precisely, 20 µL of the cell suspension was collected and poured into a small microtube, followed by adding 20 µL of trypan blue. After 3–5 minutes, 20 µL of the suspension was transferred to a Neubauer chamber, and the number of cells was counted under a light microscope (Zeiss, Germany).

For cell counting, the fourth passage cells were exposed to trypsin for 4 minutes and detached, and 1 mL of the complete culture medium was used to neutralize the enzymatic activity. Subsequently, the collected cells were poured in a Falcon tube and centrifuged at 1,500 rpm for 5 minutes for the cell sediment to form. The supernatant was then discarded, and 1 mL of DMEM was added to the cell sediment in order to create a cell suspension; afterward, 10 µL of the cell suspension was mixed with 10 µL of trypan blue, and 10 µL of the mixture was injected between the slide and the cover slip. The number of cells

Table 1. Composition of Sealers Evaluated in This Study

Sealer	Composition	Manufacturer
AH Plus	*Paste A:* Epoxy resin, calcium tungstate, zirconium oxide, silica, and iron oxide *Paste B:* Amines, calcium tungstate, zirconium oxide, silica, and silicon oil	Dentsply Sirona, Tulsa, OK, USA
AdSeal	*Base:* Epoxy resin and calcium phosphate *Catalyst:* amines, and bismuth subcarbonate	Meta Biomed, Cheongju, Korea
Endoseal MTA	Calcium silicate, calcium aluminate, calcium aluminoferrite, calcium sulfate, radiopacifier, and thickening agent	Maruchi, Wonju, Korea
GuttaFlow Bioseal	Gutta-percha, zinc oxide, barium sulfate, poly dimethyl siloxane, bioactive glass ceramic, zirconia, platinum catalyst, pigments, and silver microparticles	Coltène/Whaledent AG, Altstätten, Switzerland

was counted under a light microscope as follows:

Number of cells per 1 mL = mean number of counted cells in four squares x 10^4

The cells were counted in 16 wells on a Neubauer chamber. Then, the mean value was calculated and multiplied by the dilution coefficient (cell count x distance between the slide and the cover slip x concentration of culture medium x dilution coefficient of trypan blue).

Moreover, the calculated number of cells and the culture medium were added to each well, and the well-plates were incubated for 24 hours for the cells to adhere to the wells.

Determination and Optimization of the Adequate Amount of Sealer for Addition to Human Gingival Fibroblasts

The sealers were mixed on sterile pads according to the manufacturers' instructions, and after setting, they were weighed under a class II biosafety cabinet by a scale (Scaltec) with 0.0001 accuracy to prepare sealer discs with 0.08 g, 0.1 g, 0.125 g, 0.25 g, and 0.5 g weight. Prior to use, the sealer discs were UV-sterilized for 30 minutes. Next, 5 x 10^4 cells were cultured in each well of a 24-well plate, and after 12 hours of culture, the cells were exposed to the set sealers and incubated at 37°C and 5% CO_2 for 24 hours. Next, cell viability was assessed at the respective time points using trypan blue. The final test was performed after optimizing the experiment. For this purpose, 5 x 10^4 cells were added to three 24-well plates (a total of 66 wells). Additionally, low-glucose DMEM containing 10% FBS was added to the wells, and the plates were incubated at 37°C and 5% CO_2 for 24 hours. To measure the cytotoxicity of five different weights of different sealers, the set sealers were added to 60 wells containing the cells. Three wells served as the positive control and were only exposed to dimethyl sulfoxide. The plates were incubated at 37°C and 5% CO_2. Then, cell viability was assessed after 3 days and 7 days of exposure of the cells to the sealers using the MTT assay. For this purpose, 50 µL of the MTT solution (5 mg/mL in the basic medium) was added to each well at 1 day, 3 days, and 7 days, and the plate was incubated at 37°C and 5% CO_2 for 4 hours. Next, 300 µL of the culture medium was removed by a pipette and replaced with 250 µL of dimethyl sulfoxide and placed at room temperature for 5 minutes. Finally, 100 µL of the solution was transferred into a 96-well plate, and the formed formazan crystals were quantified by measuring the optical density of the solution at 570 nm by an enzyme-linked immunosorbent assay reader (Anthos 2020.Austria). After determining the optical density, cell viability was calculated (in comparison with the negative control) and reported in percentage; cell viability of 0–30%, 30–60%, and 60–90% indicated high, moderate, and low cytotoxicity of the sealer, respectively. Viability of > 90% of the cells represented that the sealer was not cytotoxic.

Statistical Analysis

Data were analyzed using SPSS (version 11) at a significance level of $P < 0.05$. All experiments were repeated three times, and the results were reported as means ± standard deviations (SD). Moreover, data normality was checked using the Kolmogorov–Smirnov test. Additionally, paired t-test was used for within-group comparisons, while differences among the groups were analyzed using repeated measures analysis of variance, followed by Tukey's post-hoc test for pairwise comparisons.

Results

Cytotoxicity of 0.5-g Sealers

Comparison of the cytotoxicity of each sealer at different time points revealed that all sealers, except for AH Plus, were significantly more cytotoxic at 7 days than at 3 days. AdSeal, Endoseal MTA, and GuttaFlow Bioseal showed a significant reduction (approximately 30%) in cell viability at 7 days, while AH Plus exhibited no significant change.

Likewise, none of the sealers had a significant difference with the negative control group at 1 day. However, all sealers were significantly more toxic than the negative control group at 3 days and 7 days. At 7 days, all the sealers displayed cell viability < 30% (high cytotoxicity). Based on the comparison results of the sealers, AH Plus was substantially more cytotoxic than all others at 3 days; nonetheless, AH Plus was only significantly more cytotoxic than AdSeal at 7 days. Eventually, AdSeal, Endoseal MTA, and GuttaFlow Bioseal were not remarkably different regarding the percentage of cell viability at all three time points (Figure 1).

Cytotoxicity of 0.25-g Sealers

The results regarding the comparison of the cytotoxicity of each sealer at different time points demonstrated no sharp difference in cytotoxicity at 7 days compared with 3 days in any sealer. All sealers had considerably higher cytotoxicity at 3 days and 7 days compared with the negative control group. At 3 days, AdSeal was markedly more biocompatible than other sealers. Conversely, at 7 days, AH Plus showed significantly higher cytotoxicity

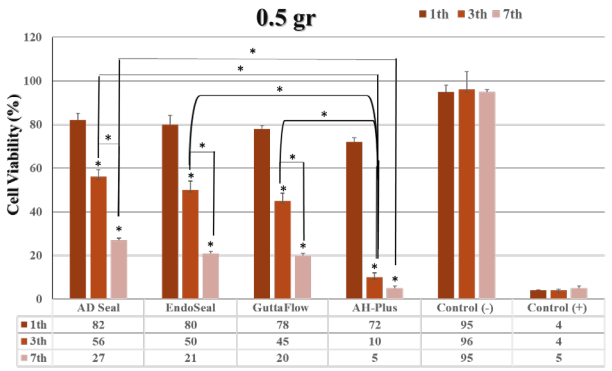

Figure 1. Viability of HGFs Following Exposure to 0.5 g of Different Sealers for 1, 3, and 7 Days
Note. HGF: Human gingival fibroblast. * indicates $P < 0.05$, and starred columns have a significant difference with the negative control group at the respective time point

than AdSeal. The results represented no other significant differences. Based on the results (Figure 2), Endoseal MTA and GuttaFlow Bioseal displayed similar cell viability with no significant difference. Except for day 1 at which, the percentage of the cell viability of sealer groups was close to the negative control group, AH Plus demonstrated high cytotoxicity (< 30% cell viability) at 3 days and 7 days, and other sealers illustrated moderate cytotoxicity (30–60%).

Cytotoxicity of 0.125-g Sealers

The comparison results of the cytotoxicity of each sealer at different time points (Figure 3) confirmed that AdSeal and Endoseal MTA were substantially more cytotoxic at 7 days compared with 3 days. At 3 days, only AH Plus showed a significant difference with the negative control group with 85% lower cell viability. However, at 7 days, all sealers were remarkably more cytotoxic than the negative control group. At both 3 and 7 days, AdSeal displayed significantly higher cell viability than AH Plus. The other three sealers were not considerably different. Except for AH Plus, all other sealers demonstrated low cytotoxicity (> 60% cell viability) at 3 days. Conversely, at 7 days, they represented moderate cytotoxicity (30–60% cell viability).

Cytotoxicity of 0.1-g Sealers

Comparison of the cytotoxicity of each sealer at different time points (Figure 4) revealed no significant difference in cytotoxicity at 7 days compared with 3 days in any sealer group. On day 3, only AH Plus was markedly more cytotoxic than the negative control group. Contrarily, at 7 days, all sealers showed significantly higher cytotoxicity than the negative control group. On day 3, AdSeal illustrated substantially lower cytotoxicity than GuttaFlow Bioseal and AH Plus. Endoseal MTA displayed lower cytotoxicity than AH Plus at 7 days, and cells demonstrated higher viability (by 25%) in the presence of Endoseal MTA. On day 3, all other sealers, except for AH Plus, exhibited cell viability > 60% and at 7 days, all sealers represented moderate cytotoxicity (30–60%).

Cytotoxicity of 0.08-g Sealers

Based on the comparison of the cytotoxicity of each sealer at different time points (Figure 5), no significant difference was observed in cytotoxicity at 7 days compared with 3 days in any sealer group. On day 3, only AH Plus was remarkably more cytotoxic than the negative control group. However, at 7 days, all sealers, except for

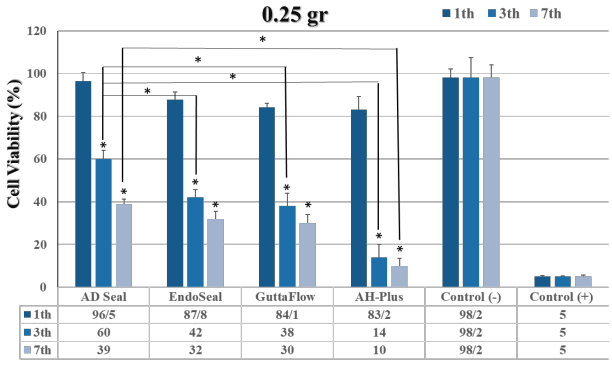

Figure 2. Viability of HGFs After Exposure to 0.25 g of Different Sealers for 1, 3, and 7 Days
Note. HGF: Human gingival fibroblast. * represents $P<0.05$, and starred columns have a sharp difference with the negative control group at the respective time point

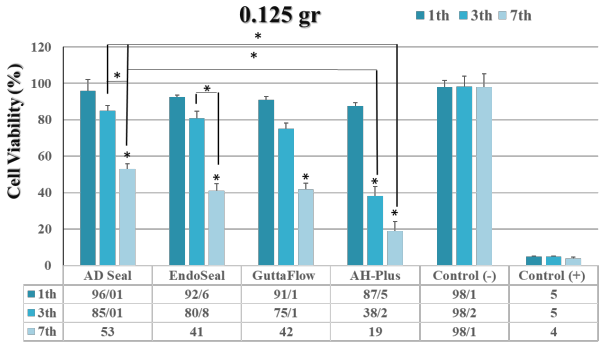

Figure 3. Viability of HGFs Following Exposure to 0.125 g of Different Sealers for 1, 3, and 7 Days
Note. HGF: Human gingival fibroblast. * denotes $P<0.05$, and starred columns display a marked difference with the negative control group at the respective time point

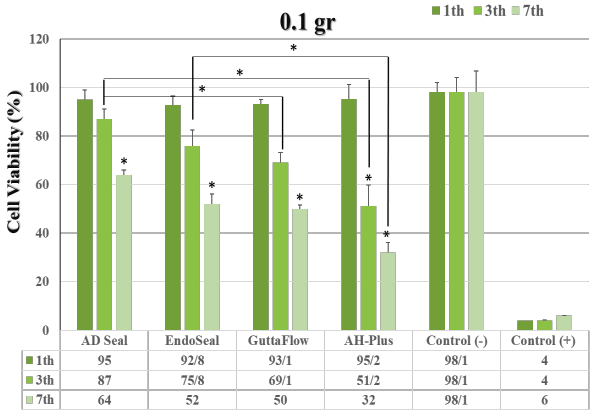

Figure 4. Viability of HGFs After Exposure to 0.1 g of Different Sealers for 1, 3, and 7 Days
Note. HGF: Human gingival fibroblast. * demonstrates $P<0.05$, and starred columns have a remarkable difference with the negative control group at the respective time point

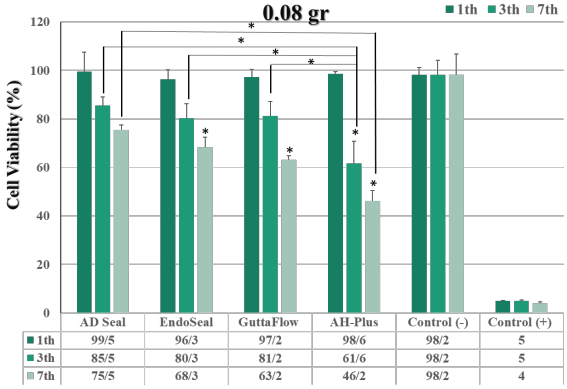

Figure 5. Viability of HGFs Following Exposure to 0.08 g of Different Sealers for 1, 3, and 7 Days
Note. HGF: Human gingival fibroblast. * implies $P<0.05$, and starred columns have a meaningful difference with the negative control group at the respective time point

AdSeal, showed considerably higher cytotoxicity than the negative control group. On day 3, AH Plus exhibited low cytotoxicity (> 60% cell viability) and other sealers became significantly more biocompatible. On day 7, only AH Plus displayed a significant difference with AdSeal, and the cells depicted around 30% higher viability in the presence of AdSeal. There were no other significant differences. Except for AH Plus that illustrated moderate cytotoxicity at 7 days, other sealers demonstrated > 60% viability (low cytotoxicity) at all time points.

Discussion

This study compared the cytotoxicity of AH Plus and AdSeal epoxy resin-based sealers, Endoseal MTA calcium silicate-based sealer, and GuttaFlow Bioseal silicone-based sealer. The null hypothesis was that no significant difference would be found in the cytotoxicity of the four sealer types. The results revealed that AdSeal had the highest biocompatibility, and AH Plus had the highest cytotoxicity among the tested sealers ($P < 0.05$). The null hypothesis of the study was rejected accordingly. HGFs were used in this study because they are directly exposed to sealers in the oral environment and play a fundamental role in the healing of wounds and periapical lesions. Injury of HGFs can substantially delay the healing course (22). In the present study, HGFs were directly exposed to sealers according to Seo et al (23), and the MTT assay was performed for the assessment of cell viability due to its high accuracy, simplicity, reliability, and cost-effectiveness (24). similar to previous studies, AH Plus was used as the gold-standard sealer for comparison with other sealers (25, 26). It should be noted that AH Plus has optimal physical and sealing properties (27). However, our findings showed the high cytotoxicity of AH Plus such that its cytotoxicity was higher than that of all other sealers. It further displayed markedly lower biocompatibility at 3 days and 7 days compared with the negative control group. The same results were reported by Collado-González et al (28), indicating no significant difference in cytotoxicity between AH Plus and the negative control group in the first 24 hours; however, AH Plus illustrated significantly higher cytotoxicity at 48 hours. Moreover, AH Plus was more cytotoxic than GuttaFlow Bioseal at week one. Likewise, Oh et al (29) evaluated the cytotoxicity of Endoseal MTA and AH Plus in freshly mixed and set forms using the CCK-8 test and found no significant difference between the two sealers in set form at 1 day, 3 days, and 7 days, which conforms to our results. However, in freshly set form, AH Plus was substantially more cytotoxic at all three time points. Similarly, some other studies have reported the high cytotoxicity of AH Plus (26, 30-33). AH Plus contains epoxy resin, which is cytotoxic even in low concentrations (34). Additionally, it is mutagenic and can break the DNA chain (35). According to the manufacturer, it releases formaldehyde (36), which has high penetration depth and is toxic. Thus, its release along with amine and epoxy resin can explain the high cytotoxicity of this sealer (33). Moreover, AdSeal showed the closest results to the negative control group in terms of cell viability. In 0.125 g and 0.1 g weights, it depicted insignificant cytotoxicity. In 0.08 g weight, it displayed no significant difference with the negative control group regarding cytotoxicity. Likewise, Kim et al (37) evaluated the biocompatibility of AdSeal compared with AH Plus and AH 26 using the agar diffusion test. Consistent with the present findings, they concluded that AdSeal was more biocompatible than the other two sealers; however, they reported no cytotoxicity for AdSeal. Some other studies demonstrated the higher biocompatibility of AdSeal than AH Plus (37, 38). Although both of them are epoxy resin sealers, the higher biocompatibility of AdSeal compared with AH Plus may be due to the presence of calcium phosphate in its composition since calcium phosphate has high biocompatibility and induces bone formation (39). In the comparison of AdSeal with Endoseal MTA, AdSeal only showed lower cytotoxicity in 0.25 g weight on day 3. No other significant differences were noted between these two sealers at other time points, which is in agreement with the results of Lee et al (40). They studied the cytotoxicity of a calcium-silicate-based sealer (i.e., Endoseal MTA) and two epoxy-resin-based sealers (i.e., AdSeal and AH Plus). They further assessed the cytotoxicity of sealers only at 1:5 concentration using the MTT assay. In line with our findings, they observed that AH Plus had the highest cytotoxicity. However, they found no significant difference between AdSeal and Endoseal MTA at any time point. In the present study, Bioseal GuttaFlow was more cytotoxic than AdSeal in 0.125 g and 0.1 g weights on day 3; no other significant differences were noted between them. Nonetheless, our results are not generalizable since, to the best of our knowledge, no study has so far compared these two sealers. Endoseal MTA is a calcium-silicate-based sealer, which is supplied as a paste in a syringe, and is injected into the root canal system (41). It is recommended for root perforation repair; thus, the periodontal ligament cells would be exposed to it (42). Additionally, given that it is directly injected into the root canal system, it has high risk of apical extrusion and formation of sealer puff and may directly contact the periapical tissue, causing acute inflammation in the periapical region (43). Kim et al (44) compared the cytotoxicity of Endoseal MTA with AH Plus and Sealapex using WST-1 kit and two different cell types. They used sealer extracts in 1:10 concentration. They found no significant difference between AH Plus and the two sealers regarding cytotoxicity against MG-63 cell line at 1 days, 3 days, and 7 days. However, in exposure to HGFs, Endoseal MTA showed significantly lower cytotoxicity at all three time points, which corroborates our findings. In the present study, AH Plus was more cytotoxic than Endoseal MTA in 0.5 g, 0.1 g, and 0.08 g weights on day 3. In the study by Seo et al (23), dental pulp stem cells were directly exposed to sealers for 24 hours, 48 hours, 72 hours, and 120 hours. The MTT assay displayed the higher biocompatibility of Endoseal

MTA than AH Plus at 3 days and 5 days. Nevertheless, our results cannot be generalized since no study has compared Endoseal MTA and GuttaFlow Bioseal. In the present study, these two sealers were highly similar and had no significant difference with each other in comparison with the negative control group regarding cytotoxicity. GuttaFlow Bioseal has two components, which are automatically mixed. It is easy to use and is based on silicone. Rodríguez-Lozano et al (45) concluded that this sealer can release apatite-forming ions and induce the formation of hydroxyapatite cores; this explains its higher biocompatibility even in comparison with GuttaFlow 2, which is from the same family of sealers. Likewise, Saygili et al (46) evaluated the cytotoxicity of sealers in 1:1 concentration using the MTT assay and the Tunnel test and reported the higher cytotoxicity of AH Plus compared with GuttaFlow Bioseal at 3 hours, 24 hours, 72 hours, and 168 hours, which is in conformity with our results obtained for 0.5 g and 0.08 g sealers at 3 days. In their study, GuttaFlow Bioseal depicted no significant difference with the negative control group, while such a result was only obtained on day 1 in the present study. Our findings conform to those of our previous investigation that evaluated the cytotoxicity and subcutaneous tissue response of Beta RCS, AdSeal, and AH Plus in both in vitro and in vivo conditions (8). In the mentioned study, AH Plus consistently exhibited the highest cytotoxicity, while AdSeal showed minimal cytotoxicity and tissue irritation, which corroborates the present results. Interestingly, Beta RCS and AdSeal demonstrated comparable levels of cell viability in vitro, and their in vivo tissue responses were substantially milder than those induced by AH Plus. Overall, these findings reinforce the superior biocompatibility of AdSeal across different biological models, highlighting the need for further evaluation of newly introduced calcium silicate-based and silicone-based sealers (e.g., Endoseal MTA and GuttaFlow Bioseal) in future in vivo studies.

The present study had certain limitations, including its in vitro design, use of a single cell type (HGFs), and lack of in vivo validation. Accordingly, it is suggested that future animal studies assess the biocompatibility of sealers in vivo. Moreover, the cytotoxicity of sealers should be investigated on other cell types present in the periapical tissue (e.g., the osteoblasts). Additionally, it is recommended that future research use other tests (e.g., WST-1) for assessing the cytotoxicity of sealers. Finally, cell migration and behavior (by studying cell morphology) following exposure to different sealers should be investigated in future studies.

Conclusion

AdSeal demonstrated the highest biocompatibility among all tested sealers, showing cell viability values closest to the negative control group. In contrast, AH Plus exhibited the highest cytotoxicity at all time points, which may potentially delay the healing of periapical tissues. Clinically, sealers with lower cytotoxicity, such as AdSeal, may promote faster periapical repair, thereby minimizing the risk of inflammatory complications following root canal therapy.

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Competing Interests

The authors declare that there is no conflict of interests.

Ethical Approval

The study protocol was approved by the Ethics Committee of AJA University of Medical Sciences (ethical approval No. IR.AJAUMS.REC.1399.190). In addition, all methods were performed in accordance with the relevant guidelines and regulations of AJA University of Medical Sciences.

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