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In Vitro Cytotoxicity Analysis of Bioceramic Root Canal Sealers on Human Gingival Fibroblast Cells

Siti Aisyah Nadirah Ja'apar[a], Solachuddin Jauhari Arief Ichwan[b], Musliana Mustaffa[c*]

[a]*Department of Biotechnology, Kulliyyah of Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia*

[b]*Dentistry Programme, Pengiran Anak Puteri Rashidah Sa'adatul Bolkiah Institute of Health Sciences, Universiti Brunei Darussalam, Jalan Tungku Link, BE1410 Brunei Darussalam*

[c]*Department of Restorative Dentistry, Kulliyyah of Dentistry, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia*

[*]Corresponding author: muslianamustaffa@iium.edu.my

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ABSTRACT

This study evaluated the cytotoxicity of four bioceramic root canal sealers (bioceramic sealers): GuttaFlow Bioseal (GB), MTA Fillapex, CeraSeal Bioceramic root canal sealer (CS), and iRoot SP root canal sealer (iRSP). The viability of human gingival fibroblast (HGF) cells was used to evaluate the cytotoxicity of these bioceramic sealers. HGF cells were cultured and exposed to bioceramic sealer extracts for 24 hours, 48 hours and 72 hours at 37°C in an incubator humidified with 5% CO_2. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide or MTT assay was conducted to determine cell viability at each incubation period and compared among all bioceramic sealers. The Kruskal-Wallis test revealed statistically significant differences between the positive control group and MTA Fillapex, MTA Fillapex and GB, and between GB and iRSP with $p < 0.05$. However, no statistical differences were found in cell viability for each material across all the incubation periods. GB was the least cytotoxic bioceramic sealer with cell viability exceeding 90% throughout the 72-hour incubation followed by CS, iRSP, and MTA Fillapex with non-cytotoxicity after 72-hour incubation, mild cytotoxicity after 72-hour incubation, and mild cytotoxicity after 72-hour incubation, respectively. However, iRSP showed moderate cytotoxicity, and MTA Fillapex was severely cytotoxic (< 30% cell viability) after 24-hour incubation.

Keywords: *Bioceramic root canal sealers; cell viability; cytotoxicity; endodontics; human gingival fibroblast cells*

INTRODUCTION

Ceramic materials known as bioceramics are specially manufactured for medical and dental applications, such as relief of tissues or joints in orthopaedics and coating of metallic implants to enhance biocompatibility (Al-Haddad & Che Ab Aziz, 2016). Biological interaction is described as an evaluation of the cytotoxicity, biocompatibility, bioactive characteristics, differentiation potential, and cell plasticity (Sanz *et al.*, 2021) when exposing a foreign material to a specific cells.

The cytotoxicity is a hazardous effect of material on living tissues (Maru *et al.*, 2021) while biocompatibility is a capability of a material to perform in a particular condition and generate the proper host reaction (Williams, 2008). In dental applications, the cytotoxicity of conventional root canal sealers, such as AH-Plus (Dentsply, DeTrey, Konstanz, Germany), zinc oxide eugenol (Tubli-seal, SybronEndo), and AH26 (Dentsply Maillefer, Baillagues, Switzerland), induces severe degeneration while delaying healing when they come into contact with the periapical tissues, thus interfering with periapical repair (Almeida *et al.*, 2020). In contrast, bioceramic root canal sealers have been reported to have effectively repaired the periapical tissues while providing several benefits, such as improved biocompatibility, antibacterial properties, non-toxicity, bioinert, bioactive or biodegradable, ease of application, and great sealing properties (Nair *et al.*, 2018; Almeida *et al.*, 2020).

In the past decade or so, several bioceramic root canal sealers were developed. At present, there are at least four commonly used bioceramic root canal sealers: GuttaFlow Bioseal (GB), MTA Fillapex, iRoot SP root canal sealer (iRSP), and CeraSeal Bioceramic root canal sealer (CS). However, their cytotoxicity remains poorly understood. Previous studies focused mainly on comparing bioceramic and conventional root canal sealers such as GB (Collado-González *et al.*, 2017), GuttaFlow 2 (Saygili *et al.*, 2017), MTA Fillapex versus AH Plus (Rodríguez-Lozano *et al.*, 2019), GB versus AH26 (Ferreira *et al.*, 2020), MTA Fillapex, TotalFill BC Sealer versus AH Plus (Almeida *et al.*, 2020), iRSP, MTA Fillapex, Sankin Apatite Root Sealer versus Sealapex (Chang *et al.*, 2014b), CS, EndoSeal TCS versus AH Plus (Oh *et al.*, 2020), CS, Well-Root ST versus AH Plus (Elgendy & Hassan, 2021). However, the comparison between GB and other bioceramic root canal sealers remain unavailable.

On the other hand, the cytotoxicity results of the endodontic MTA Fillapex sealer are inconsistent (Saygili *et al.*, 2017). Such inconsistency is probably due to the use of different cell lines in assessing cell viability and cytotoxicity (Soares *et al.*, 2018) and experimental conditions (e.g., incubation time and concentration of sealer extracts). For example, Chang *et al.* (2014a) concluded that MTA Fillapex was less cytotoxic via the viability assessment of human periodontal ligament cells (hPDLCs). However, other authors (Yoshino *et al.*, 2013; Baraba *et al.*, 2016; Silva *et al.*, 2016a; Collado-González *et al.*, 2017; Victoria-Escandell *et al.*, 2017; Colombo *et al.*, 2018; Rodríguez-Lozano *et al.*, 2019; Almeida *et al.*, 2020; Benetti *et al.*, 2021) found it to be cytotoxic. Similarly, iRSP showed a higher proportion of cell viability (less toxic) in hPDLSCs (Chang *et al.*, 2014b; López-García *et al.*, 2019) and murine lung fibroblasts L929 (Nair *et al.*, 2018), but Mukhtar-Fayyad (2011) reported a lower proportion of cell viability of iRSP in human fibroblast cells (MRC-5). Besides, CS showed a relatively high fraction of cell viability when exposed to the hPDLSCs (López-García *et al.*, 2019; Oh *et al.*, 2020). Nevertheless, the current state of studies about the cytotoxicity of CS remains scarce, and therefore it warrants further evaluation.

This study aimed to evaluate the cytotoxicity of four bioceramic root canal sealers: GB, MTA Fillapex, iRSP, and CS. Human gingival fibroblast (HGF) cells were used to assess cytotoxicity in this study because of their close contact with cement and endodontic sealers, and they could be cultured in few passages to ensure the cells could prolong life and expand the number of cells in the culture with minimal cell alterations (Poggio *et al.*, 2017). Also, this approach was corroborated in other studies (Key *et al.*, 2006; Candeiro *et al.*, 2016). Above all, HGF cells are one of the most abundant cells (Soares *et al.*, 2018), i.e., readily available, and they show good potential for wound-healing with no scars than skin fibroblast cells.

MATERIALS AND METHODS

Cell Culture and Preparation of Bioceramic Root Canal Sealer Samples and Extraction Solution

HGF cell lines (American Type Culture Collection [ATCC], catalogue number PCS-201-018) were grown in the T-flasks (25 cm^2 and 75 cm^2), tissue culture dishes (10 cm^2 and 6 cm^2) and 96-well plates containing growth medium such as Dulbecco's Modified Essential Medium (DMEM) (F0445, Biochrom, Germany) at pH 7.4 and 37°C with the incorporation of 10% fetal bovine serum (FBS) (S0113, Biochrom, Germany) and 1% mixture of penicillin-streptomycin (PenStrep). Cells were subcultured under sterile conditions using biosafety cabinet for seven days (168 hours). The cells were maintained in a 25 cm^2 T-flasks at 37°C in an incubator humidified with 5% CO_2. Cells were examined every 24 hours using Evos XL Core Imaging System (ThermoFisher) to allow confluency of 70% to 80% only for evading overcrowding. Table 1 shows the compositions of the bioceramic root canal sealers.

Following the manufacturer's instructions, each of these bioceramic root canal sealers was mixed in a cylindrical disc (5 mm in diameter, 3 mm in thickness) and incubated at 37°C in an incubator humidified with 5% CO_2 for 24 hours. Each disc was placed into a 1.5-mL Eppendorf tube containing 1,000 µL of the growth medium and incubated for another 24 hours to obtain the extract solution for the subsequent 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Fourier Transform Infrared (FTIR) Analysis

The FTIR spectroscopy was performed to identify key functional groups of the DMEM standard and constituents of each bioceramic root canal sealer before and after they were immersed in DMEM. The FTIR analysis was conducted using a spectrometer (Perkin Elmer Spectrum Two, USA) with adsorption spectra read between 600 cm^{-1} and 4,000 cm^{-1} at a resolution of 2 cm^{-1}. Selection of spectral range was based on the chemical compositions of these bioceramic root canal sealers. With sufficient pressure applied on the samples, a smooth graph could be generated to display their transmittance and bonds. All measurements were conducted in triplicates in a dry environment at room temperature (25 ± 0.5°C).

Cell Viability Assay

Each of the 18 separate 96-well plates was seeded with 5,000 HGF cells at a density of

Table 1 Bioceramic root canal sealers

Bioceramic root canal sealers	Compositions	Manufacturer	Reference
GuttaFlow Bioseal	Polydimethylsiloxane, gutta-percha particles, and bioactive glass-ceramics	(Coltene/Whaledent AG, Switzerland)	Saygili *et al.*, 2017
MTA Fillapex	Consists of two types of paste, i.e., salicylate resin, bismuth trioxide, and silica in Paste A and titanium dioxide, MTA (40%), and resin in Paste B	(Angelus, Brazil)	Saygili *et al.*, 2017
CeraSeal Bioceramic root canal sealer	Calcium aluminates, dicalcium silicates, tricalcium silicates, thickening agents, and zirconium oxides	(Meta Biomed Co. Ltd, Korea)	Oh *et al.*, 2020
iRoot SP root canal sealer	Zirconium oxide, calcium silicates, calcium phosphate, calcium hydroxide, filler, and thickening agents	(Innovative BioCreamix Inc, Canada)	Chang *et al.*, 2014b

3×10^4 cells/mL with 100 µL medium from a 25 cm^2 T-flasks and incubated at 37°C in an incubator humidified with 5% CO_2. Experiments were performed in triplicates for each of the four bioceramic root canal sealers on three 96-well flat-bottom microplates. The HGF cells in each well were observed using Evos XL Core Imaging System (ThermoFisher) to ensure the cells were mature enough for exposing the materials. The growth medium, containing DMEM with the incorporation of FBS and PenStrep, was discarded from each well that contained the HGF cells. Each well was then added with 100 µL of the corresponding extract solution, except for the positive control, which was added with 100 µL of growth medium. The growth medium (with no HGF cells) served as a blank in a separate well. The microplate that contained HGF cells together with the extract solutions was then incubated at 37°C in an incubator humidified with 5% CO_2 for 24 hours, 48 hours and 72 hours, one microplate per specified duration.

Examining the cytotoxicity of the bioceramic root canal sealers over different incubation periods was crucial because the viability of HGF cells might change due to the diffusion of hazardous substances following the breakdown of the sealers' constituents. In this study, the colourimetric assay of MTT was used to evaluate the cytotoxicity. MTT would be converted into a purple-coloured formazan product in viable cells with an active metabolism, and non-dividing dormant cents and dead cells would not be able to metabolise MTT (Maioli *et al.*, 2009). The darker the solution the higher the number of metabolically active cells.

Meanwhile, a baseline unit of 24-hour incubation was selected for exposing HGF cells to the extract solutions because the constituents of bioceramic root canal sealers might have yet to exert their full effect on the cells, whereas after 48-hour incubation, the diffusion of sealer materials into the cells was likely more substantial than that of 24-hour incubation and the time required to see the actual effect of cell proliferation considering it as 72-hour incubation. Moreover, several other authors used similar incubation periods (Yoshino *et al.*, 2013; Poggio *et al.*, 2017; Colombo *et al.*, 2018; López-García *et al.*, 2019).

Upon incubation, the extract medium was drained from each sample. Then, each sample was washed with 10 µL of 0.1 M phosphate-buffered saline (pH 7.4) and added with 20 µL of MTT solution. These microplates were incubated further at 37°C for 4 hours. The MTT solution was drained, and each sample was added with 120 µL dimethyl sulfoxide (Sigma-Aldrich, USA) to solubilise the purple formazan crystals. The absorbance of each sample was measured at 570 nm (Abs_{570}) using a microtiter plate reader (Infinite 200 PRO, Tecan Austria) with absorbance at 630 nm (Abs_{630}) as the reference wavelength. The results were expressed as a percentage of absorbance, with the absorbance of control cells indicating 100% viability. Cell viability was calculated using the following formula (Mukhtar-Fayyad, 2011):

$$\text{Cell viability} = (A/B) \times 100\%$$

Where A is the absorbance of viable cells in the experimental well and B is the viable cells in the control group.

The cell viability was qualitatively graded as severely cytotoxic (< 30% viability), moderately cytotoxic (30%–60% viability), mildly cytotoxic (60%–90% viability), and non-cytotoxic (> 90% viability) (Loushine *et al.*, 2011; Mukhtar-Fayyad, 2011; Poggio *et al.*, 2017; Colombo *et al.*, 2018).

Statistical Analyses

The software Statistical Package for Social Sciences (SPSS) for Windows, version 25.0 was used for statistical analysis. The Kruskal-Wallis and post-hoc Tukey tests were used to assess the percentage of cell viability across bioceramic root canal sealers and incubation period.

RESULTS

FTIR Analysis of Bioceramic Root Canal Sealers

Fig. 1 shows the FTIR spectra of GB; the signal-to-noise ratio enhanced by each spectrum was scanned multiple times. The GB spectrum in DMEM (graph C) was nearly identical to the principal absorption peaks of the GB before the immersion of DMEM (graph A), notably at 1,081 cm^{-1} and 791 cm^{-1}, suggesting congruent vibrations for the same functional groups. The broad absorption peak at 3,350 cm^{-1} in GB after the immersion of DMEM (graph B) in graphs C and D indicated the presence of O-H bonds. No O-H bonds occurred in the GB spectrum before the immersion of DMEM (graph A) due to the dry (solid) state of the material. For all the four spectra, the peak between 1,666 cm^{-1} and 1,550 cm^{-1} generally corresponded to the absorption caused by the C=O double bonds. The sharp peak at 2960 cm^{-1} in A, B, and weak peak in C was associated with the C-H functional group, indicating the presence of methylene group ($-CH_2$).

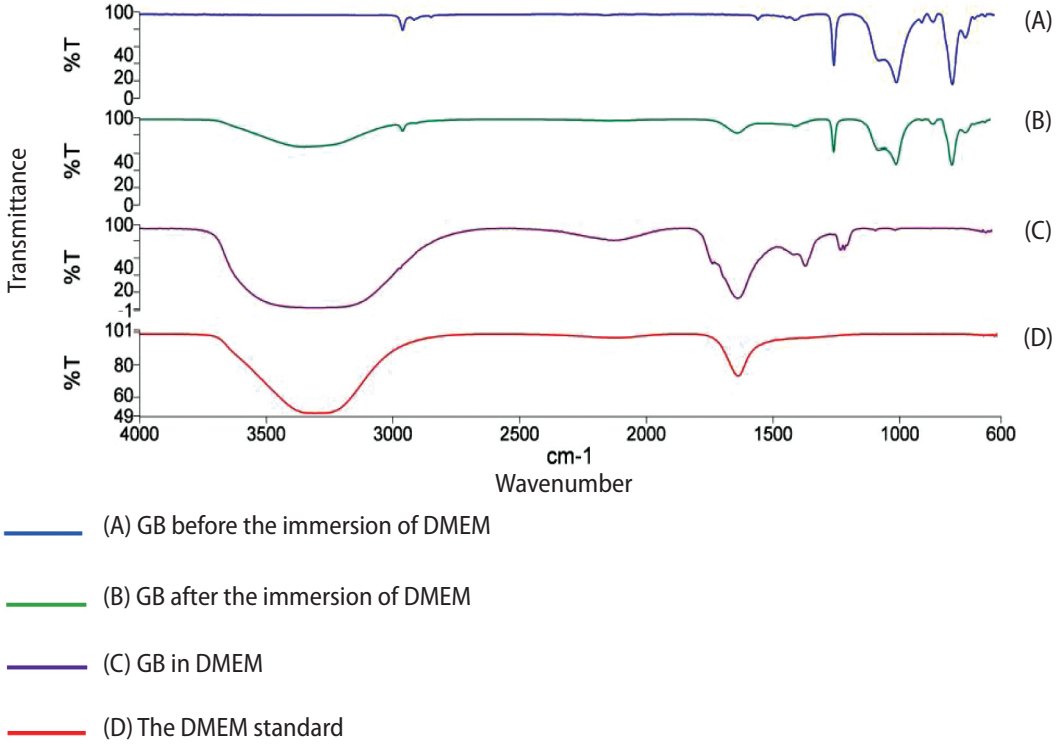


Fig. 1 The FTIR spectrum between 600 cm^{-1} and 4,000 cm^{-1}: (A) GB before the immersion of DMEM; (B) GB after the immersion of DMEM; (C) GB in the DMEM; and (D) the DMEM standard.

Meanwhile, various fingerprint regions contained the peak of a functional group with a single bond. Specifically, adsorption peaks between 1,412 cm^{-1} and 1,259 cm^{-1} in graph C were assigned to the N-H bond. Sharp peaks at 1,081 cm^{-1}, 1,012 cm^{-1}, and 867 cm^{-1} in graphs A, B and weak peaks in C corresponded to and barium sulphate ($BaSO_4$), which were absent in the spectrum of the DMEM standard (D). Peaks between 1,081 cm^{-1} and 867 cm^{-1} in graphs A, B, and C were attributable to the sulphur-oxygen (S-O) bond of inorganic sulphates. Meanwhile, Zn-O bonds were identified between 791 cm^{-1} and 660 cm^{-1} in graphs A, B, and C, but not in D. The fingerprint region in C differed from D, but they were nearly identical to A. Thus, the functional groups in the FTIR spectrum of C indicated that GB had been released into DMEM (Table 2).

Fig. 2 shows the FTIR spectra of MTA Fillapex. When compared with the DMEM standard (graph D), MTA Fillapex peaks occurred before the immersion of DMEM (graph A), after the immersion of DMEM (graph B), and in DMEM (graph C) were due to the presence of functional groups. Broad peaks between 3,287 cm^{-1} and 2,978 cm^{-1} in graph C were attributable to O-H bonds, whereas those between 1.666 cm^{-1} and 1,550 cm^{-1} of all the spectra corresponded to absorption caused by the C=O double bonds. Peaks between 1,248 cm^{-1} and 1,156 cm^{-1} were attributable to the stretching vibration of the C-H bond, indicating the presence of a methylene group ($-CH_2$) in graphs A, B, and C, but absent in graph D. Within the fingerprint region of MTA Fillapex from 1.248 cm^{-1} to 658 cm^{-1}, sharp peaks at 797 cm^{-1}, 756 cm^{-1}, 675 cm^{-1}, and 658 cm^{-1} in graphs A and B, and weak peaks in graph C were due to the Bi-O bond. This bond was contributed by bismuth oxide, one of the constituents of MTA Fillapex. However, the DMEM standard did not contain this chemical, and hence no Bi-O bond was detected in its FTIR spectrum (graph D). Thus, the presence of the Bi-O functional group in the fingerprint region of graph C (also in Table 3) indicated that MTA Fillapex was released into DMEM.

Table 2 Wavenumbers and selected functional groups in DMEM after the immersion of GB

Wavenumber (cm^{-1})	Functional group
3,350	O-H (water absorption band)
2,960	H-C-H stretching (CH_2)
1,638	C=O stretching
1,412	N-H bending
1,259	N-H bending
1,081	S-O bending
1,012	S-O bending
867	S-O bending
791	Zn-O stretching
738	Zn-O stretching
660	Zn-O stretching

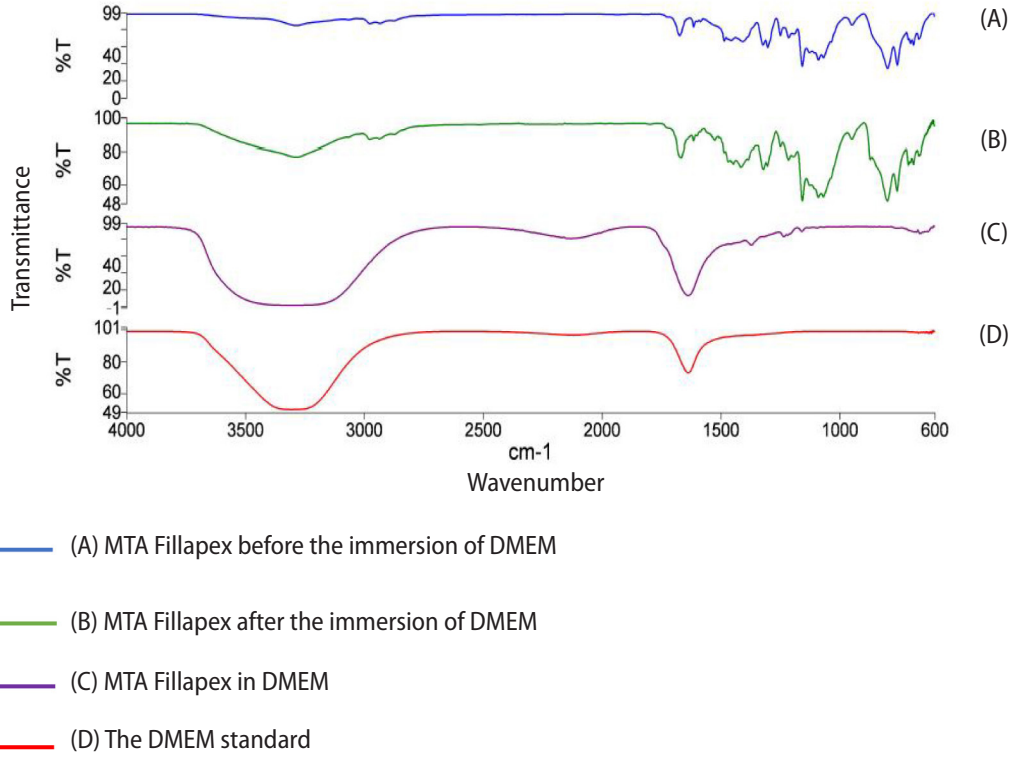


Fig. 2 The FTIR spectrum between 600 cm^{-1} and 4,000 cm^{-1}: (A) MTA Fillapex before the immersion of DMEM; (B) MTA Fillapex after the immersion of DMEM; (C) MTA Fillapex in the DMEM; and (D) the DMEM standard.

Table 3 Wavenumbers and the selected functional groups in DMEM after the immersion of MTA Fillapex

Wavenumber (cm^{-1})	Functional group
3287	O-H stretching
2978	O-H water-adsorbed stretching
1666	C=O stretching
1625	C=O stretching
1550	C=O bending
1248	C-H stretching
1234	C-H stretching
1156	C-H stretching
1067	C-O stretching in C-O-H group
947	C-C stretching
797	Bi-O bending
756	Bi-O bending
675	Bi-O bending
658	Bi-O bending

Fig. 3 shows the FTIR spectra of CS. The CS spectrum in DMEM (graph C) showed a strong but broad peak in the region between 3,000 cm^{-1} and 3,700 cm^{-1}, indicating the stretching vibration of the O-H group. The peak at 1,650 cm^{-1} of all the spectra generally corresponded to the adsorption due to the C=O double bonds, whereas the weak peak at 1,062 cm^{-1} in graphs A, B, and C was attributable to the C=H functional group. Meanwhile, peaks in the fingerprint region 938 cm^{-1}, 853 cm^{-1}, 871 cm^{-1}, 711 cm^{-1}, 676 cm^{-1} and 655 cm^{-1} corresponded to the adsorption of the characteristic molecules of CS, notably silicate. Specifically, the Si=O bonds and SiO_4 in the fingerprint regions of graphs (A, B, and C correspond to calcium silicate. These functional groups were absent in the DMEM standard (graph D). Thus, the presence of Si=O bonds and SiO_4 in graph C (also in Table 4) indicated the release of CS into DMEM.

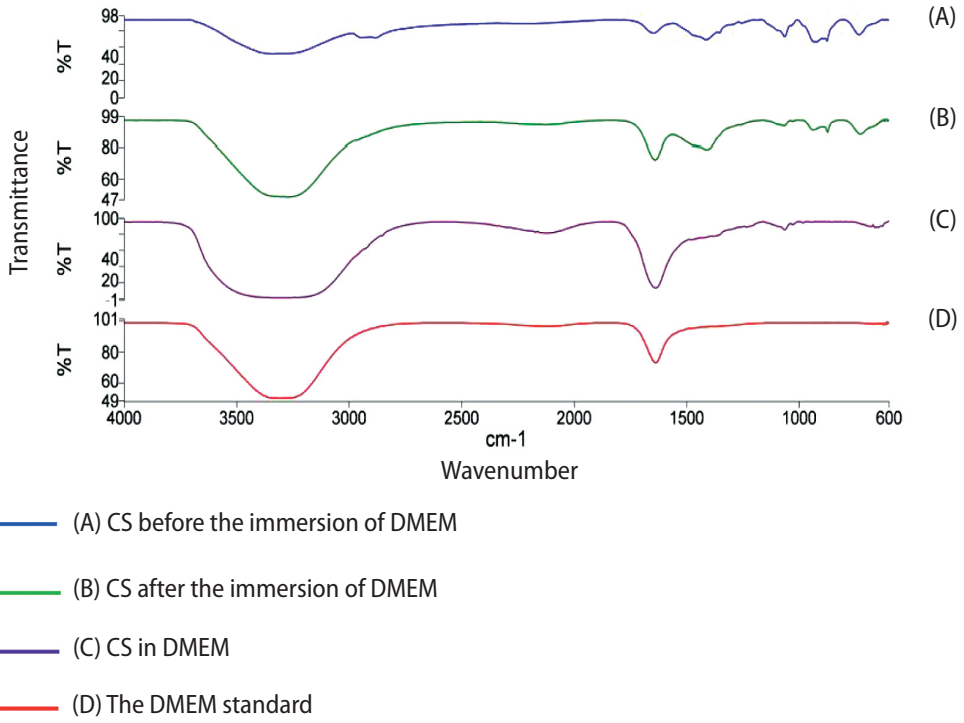


Fig. 3 The FTIR spectrum between 600 cm^{-1} and 4,000 cm^{-1}: (A) CS before the immersion of DMEM; (B) CS after the immersion of DMEM; (C) CS in the DMEM; and (D) the DMEM standard.

Table 4 Wavenumbers and selected functional groups in DMEM after the immersion of CS

Wavenumber (cm^{-1})	Functional group
3,300	O-H stretching
1,650	C=O stretching
1,062	C=H bending
938	Si=O stretching
853	Si=O stretching
871	Si=O stretching
711	SiO_4 bending
676	SiO_4 bending
655	SiO_4 bending

Fig. 4 shows the FTIR spectra of iRSP. Compared to the standard DMEM (graph D), the iRSP spectra at different experimental conditions showed major reflection peaks that corresponded to the presence of various functional groups. The broad peak at 3,326 cm^{-1} was attributable to the O-H bonds. The weak peak of iRSP before the immersion of DMEM (graph A), the medium peak in iRSP after the immersion of DMEM (graph B), the sharp peak of iRSP in DMEM (graph C), and the medium peak in graph D at 1,650 cm^{-1} was assigned to the general C=O bonds. The fingerprint region between 1,408 cm^{-1} and 660 cm^{-1} contained specific functional groups. In particular, the functional groups of phosphate and zirconium occurred in graphs A, B, and C. Meanwhile, peaks at 1,408 cm^{-1} and 1,065 cm^{-1} in graphs A, B, and C represented general C-H bonds, whereas peaks at 944 cm^{-1}, 871 cm^{-1}, 734 cm^{-1}, 691 cm^{-1}, and 660 cm^{-1} corresponded to Zr-O bonds and PO_4. These two functional groups came from zirconium oxide and calcium phosphate, which are characteristics of iRSP; they were absent in the DMEM standard, and hence graph D. Thus, the presence of these two specific functional groups in the fingerprint region of graph C (also in Table 5) indicated that iRoot SP was released into DMEM.

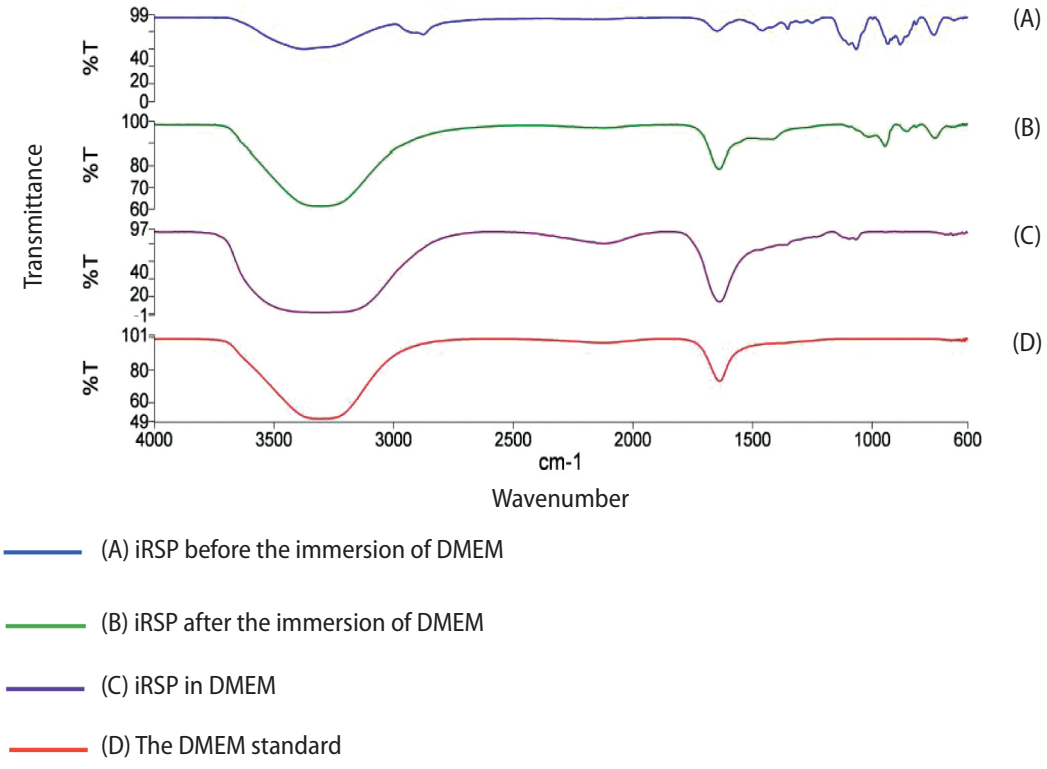


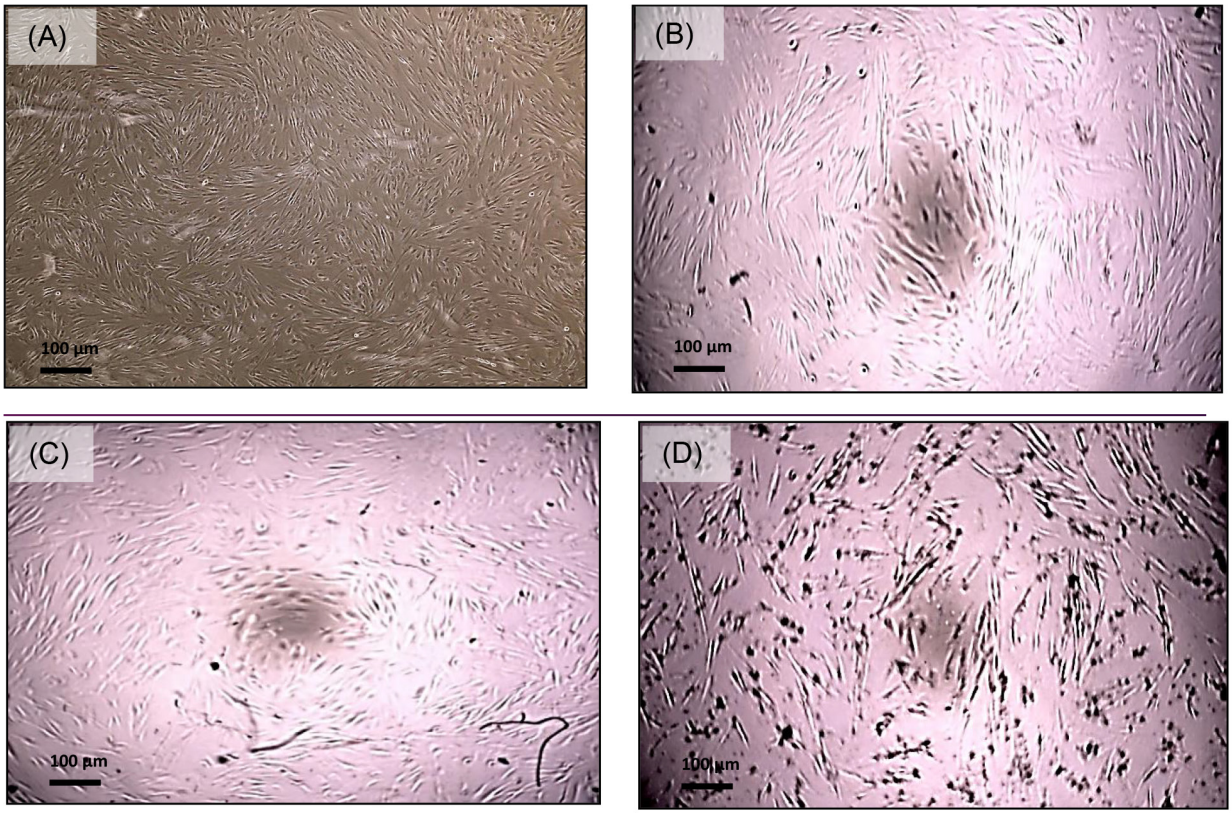
Fig. 4 The FTIR spectrum between 600 cm^{-1} and 4,000 cm^{-1}: (A) iRSP before the immersion of DMEM; (B) iRSP after the immersion of DMEM; (C) iRSP in the DMEM; and (D) the DMEM standard.

Table 5 Wavenumbers and selected functional groups in DMEM after the immersion of iRSP

Wavenumber (cm^{-1})	Functional group
3,326	O-H stretching
1,650	C=O stretching
1,408	C-H bending
1,065	C-H bending
944	Zr-O bending
871	Zr-O bending
734	Asymmetric stretching PO_4
691	Asymmetric bending PO_4
660	Asymmetric bending PO_4

The Morphology of HGF Cells

Fig. 5 shows the morphology of HGF cells at the 80% confluence in the extract of various bioceramic root canal sealers and the control group. The elongated cells were visible when the confluency reached 80% in the positive control group (Fig. 5B) and the tested group GB (Fig. 5C), i.e., nearly all cells (> 90%) were viable in these two groups. In the CS extract, about 60%–90% of cells were viable (Fig. 5D), whereas about 30%–60% of the cells were alive in iRSP (Fig. 5E). In contrast, only a small fraction (< 30%; Fig. 5F) of cells was viable in the MTA Fillapex extract.



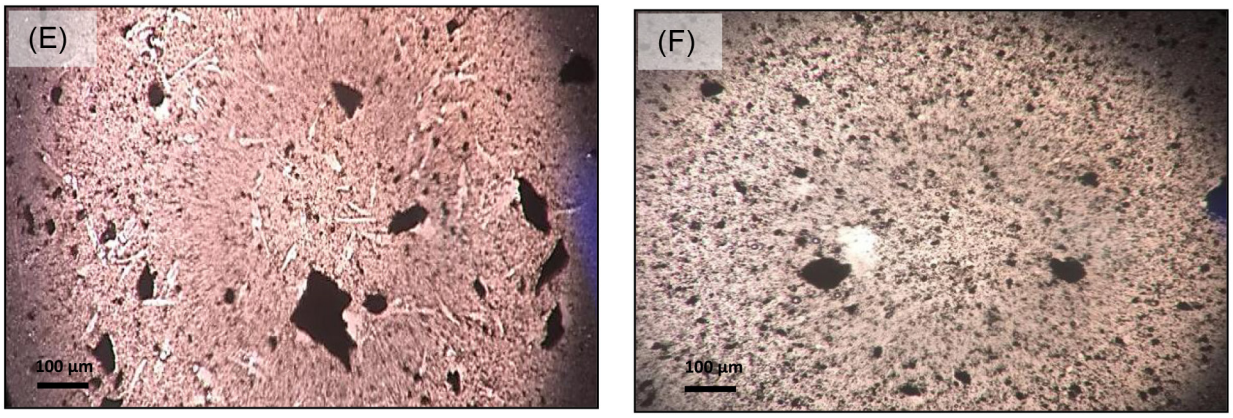


Fig. 5 The morphology of HGF cells in the subculture flask at 10x magnification: (A) at 80% confluency after seven days of subculture; (B) the positive control; (C) GB with most cells (> 90%) were viable; (D) CS with 60%–90% living cells; (E) iRSP with 30%–60% viable cells; and F) MTA Fillapex with < 30% living cells.

Cytotoxicity Analysis

Fig. 6 shows the viability of HGF cells in contact with the extract solution of bioceramic root canal sealers after incubating for 24 hours, 48 hours and 72 hours. MTA Fillapex showed a cell viability of 28.8%, 32.5%, and 36.1% after incubating for 24 hours, 48 hours and 72 hours, respectively, indicating a reduction in cytotoxicity through time. Similarly, the proportion of cell viability for iRoot SP was 49.7% and 52.9% at 24 hours and 48 hours respectively; it then increased rather substantially to 63.5% at 72 hours. Meanwhile, CS showed a cell viability of 84.5%, 86.8%, and 91.7% at 24 hours, 48 hours and 72 hours, respectively. The percentage of cell viability for GB was 98.0%, 98.1%, and 99.6% at 24 hours, 48 hours and 72 hours, respectively; it was nearly similar to the positive control group.

The Kruskal-Wallis test showed differences in cell viability across the positive control and four tested bioceramic root canal sealers (χ^2 (4) = 11.7, p = 0.019) with mean rank viability of 11.8 for the control, 12.2 for GB, 9.0 for CS, 5.0 for iRSP, and 2.0 for MTA Fillapex. Post-hoc Tukey's test also revealed significant differences on cell viability between MTA Fillapex and the control (p = 0.007), between MTA Fillapex and GB (p = 0.005), between MTA Fillapex and iRSP (p = 0.049), and between MTA Fillapex and GB (p = 0.049). However, the Kruskal-Wallis test showed no significant differences in cell viability for each material across 24 hours, 48 hours and 72 hours (χ^2 (2) = 0.035, p = 0.983) with a mean rank cell viability of 11.8 for the control, 12.2 for GB, 9.0 for CS, 5.0 for iRS, and 2.0 for MTA Fillapex.

DISCUSSION

Cytotoxicity of material is commonly evaluated via the viability of cells exposed to such material. In the present study, at the first observation period of 24-hour incubation, severe cytotoxicity occurred in MTA Fillapex (< 30% cell viability), moderate cytotoxicity in iRSP (30%–60% cell viability), mild cytotoxicity in CS (60%–90% cell viability, and non-cytotoxicity in GB (> 90% cell viability). The proportion of cell viability increased over time. After 72-hour incubation, MTA Fillapex and iRSP became moderately cytotoxic, and CS was mildly toxic; GB appeared to be non-cytotoxic, i.e., nearly similar to the control group. An increase in the cell viability over time indicated an enhancement of growth associated with the regeneration of cell

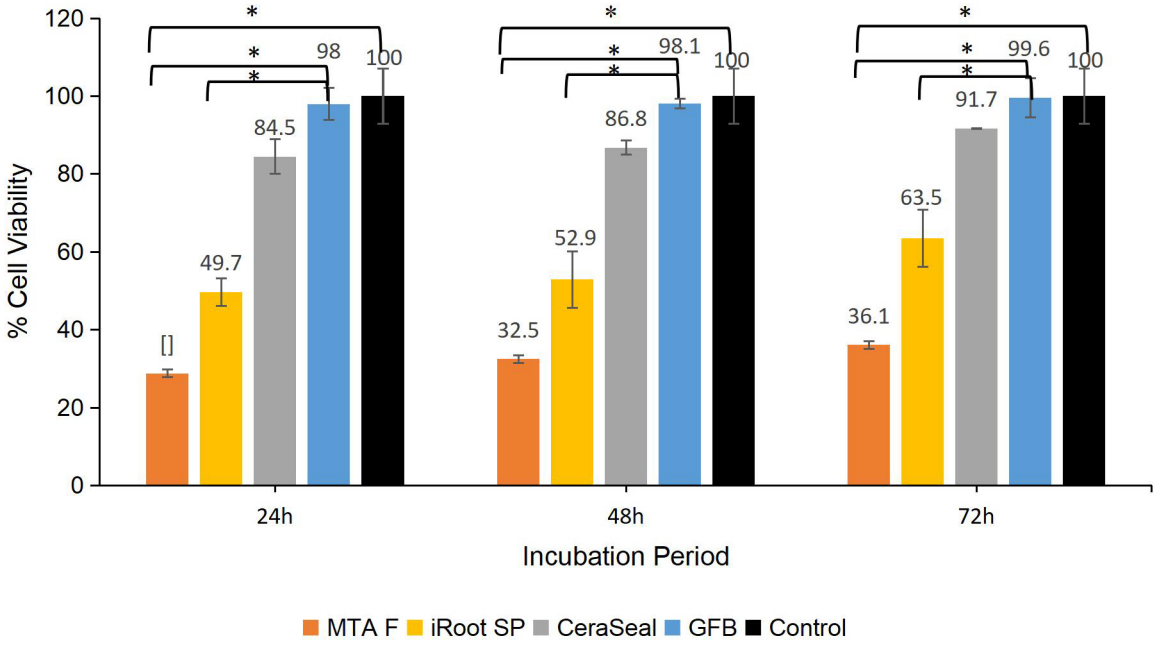


Fig. 6 Cytotoxic effects of MTA Fillapex, iRSP, CS, and GB on HGF cells after incubating for 24 hours, 48 hours and 72 hours.

Note: * indicates statistically significant difference when $p < 0.05$.

proliferation activities, thus increasing the number of dividing cells (Mukhtar-Fayyad, 2011). Different levels of cytotoxicity were observed at each incubation period between the present study and other studies (Saygili *et al.*, 2017; Nair *et al.*, 2018; Oh *et al.*, 2020) owing to the use of different cell lines and bioceramic root canal sealer concentrations exposed to the cell lines. In this respect, cytotoxic response was dependent on the material concentration (Mukhtar-Fayyad, 2011; Collado-González *et al.*, 2017; López-García *et al.*, 2019; Almeida *et al.*, 2020; Ferreira *et al.*, 2020; Benetti *et al.*, 2021). However, the present study did not evaluate the effect of different cell lines and bioceramic root canal sealer concentrations.

High cytotoxicity of MTA Fillapex was evident after the 24-hour incubation compared to the control group and GB. Such toxicity might be due to the presence of several components, such as salicylate resin, diluted resin, silica, and bismuth oxide in MTA Fillapex (Yoshino *et al.*, 2013; Silva *et al.*, 2016b; Almeida *et al.*, 2020). Besides, a higher level of salicylate resin content than MTA had been attributed to one of the possible causes for such toxicity (Colombo *et al.*, 2018). The finding of this study was consistent with that of other studies (Yoshino *et al.*, 2013; Baraba *et al.*, 2016; Silva *et al.*, 2016b; Collado-González *et al.*, 2017; Victoria-Escandell *et al.*, 2017; Colombo *et al.*, 2018; Rodríguez-Lozano *et al.*, 2019; Almeida *et al.*, 2020; Benetti *et al.*, 2021) despite differences in incubation periods, types of cell lines, and bioceramic root canal sealer concentrations.

The toxicity of iRSP changed from moderate upon incubation for 24 hours and 48 hours to mild cytotoxic after 72-hour incubation, suggested that iRSP was more cytotoxic than GB, probably due to the presence of calcium silicate, zirconium oxide, and thickening agents in iRSP (Nair *et al.*, 2018). Meanwhile, the increased proportion of cell viability in iRSP over time could be attributable to the key component calcium phosphate in this bioceramic root canal sealer. It is postulated that calcium phosphate facilitates the reproduction of

cells, effecting cell adhesion and growth via the release of calcium and phosphorus ions (Jeong *et al.*, 2019). In addition, calcium phosphate is the primary inorganic constituent of hard tissue, and the free calcium and phosphate ions generated can be employed in metabolism (Mukhtar-Fayyad, 2011). The present results at 24-hour and 48-hour incubation periods were consistent with the finding from Mukhtar-Fayyad (2011) who reported that iRSP showed moderate cytotoxicity throughout incubation periods of 24 hours, 72 hours and 168 hours (seven days). This consistency in results was probably due to the high pH (up to 11) of the bioceramic root canal sealer which might induce adjacent cells and proteins to denature (Zhang *et al.*, 2010). However, another study (López-García *et al.*, 2019) reported that iRSP showed no cytotoxicity on hPDLSC cells throughout the incubation periods of 24 hours, 48 hours and 72 hours. This finding (López-García *et al.*, 2019) is contradictory with the result of the present study. Even though the incubation periods were the same, different cell lines clearly showed different sensitivity towards the eluted toxic compounds (López-García *et al.*, 2019).

Meanwhile, CS showed mild cytotoxic at incubation periods 24 hours and 48 hours and became non-cytotoxic after 72-hour incubation. Such a change in toxicity was probably due to the presence of calcium silicate and zirconium oxide in CS. Dubbed as ceramic steel, zirconium oxide has excellent durability, resilience, fatigue resistance, wear properties, and biocompatibility, making it ideal for dental use (Bona *et al.*, 2015). No local or systemic cytotoxic effects or adverse reactions were observed in an extensive evaluation of zirconia's biocompatibility (Nistor *et al.*, 2019). Besides, CS showed a nearly similar proportion of cell viability to the control group (López-García *et al.*, 2019; Oh *et al.*, 2020), and this finding agreed with that of the present study after the 72-hour incubation. In general, that materials containing zirconium oxide would stimulate fibroblast proliferation (López-García *et al.*, 2019). However, studies evaluating CS are scarce, thus limiting the comparison of results.

On the other hand, GB showed no cytotoxicity throughout the entire 72-hour incubation, with results nearly similar to the control group. These results suggest that bioactive constituents such as bioactive glass, which consists of silica, calcium oxide, sodium oxide, and phosphorus oxide were the causes of low cytotoxicity of GB (Saygili *et al.*, 2017). Silicates are now employed in numerous biocompatible materials for perforation repair, retrograde filling, and regeneration treatment (Saygili *et al.*, 2017). The finding of the present study was consistent with the result of other studies (Collado-González *et al.*, 2017; Saygili *et al.*, 2017; Rodríguez-Lozano *et al.*, 2019; Ferreira *et al.*, 2020), i.e. a general lack of cytotoxicity in GB might be due to its bioactive components such as calcium silicate and the absence of resin in its formulation, and GB extracts exhibited substantially higher cell viability than MTA Fillapex (Collado-González *et al.*, 2017). Indeed, GB was a more biocompatible endodontic bioceramic root canal sealer compared to other conventional root canal sealers (e.g. AH-Plus, AH 26) currently used in clinical practice (Collado-González *et al.*, 2017; Saygili *et al.*, 2017; Ferreira *et al.*, 2020).

The limitations of the present study included: (1) a two-dimensional cell culture model instead of a more powerful three-dimensional model; (2) only one cell line (HGF) was used instead of comparing several other cell lines in the same study; (3) bioceramic root canal sealers were assessed after being set by putting all the materials into a specified state for 24 hours, rather than comparing freshly mixed materials immediately, and (4) only three incubation periods were evaluated. Further *in vitro* and *in vivo* experiments would be beneficial for better understanding on cytotoxicity of bioceramic root canal sealers for clinical application. In order to

comprehend their cytotoxicity, evaluating the available bioceramic root canal sealers using various cell lines at longer incubation periods, or three-dimensional cell culture models and *in vivo* studies involving various animal models are required. The outcomes of basic research protocols may also lead to the effective sustainability of *in vivo* and clinical applications.

CONCLUSION

This study showed that GB was the lowest cytotoxicity followed by CS, iRSP, and MTA Fillapex. Clinicians should select bioceramic root canal sealers that have low cytotoxic effects during root canal treatment procedure.

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