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Antimicrobial activity, viability, and physicochemical properties of an MTA-type cement with different concentrations of bismuth trioxide

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In medicine, bismuth is used as an antimicrobial agent. In dentistry, it is used primarily to increase radiopacity in some endodontic materials. The objective is to evaluate the antimicrobial activity, cell viability, pH, solubility, film thickness, and setting time of a mineral trioxide aggregated (MTA)-types of cement with different concentrations of bismuth trioxide. Three experimental MTA-types of cement with a bismuth trioxide (Bi_2O_3) concentration of 15 wt%, 20 wt%, and 25 wt% were used. The antimicrobial activity test was conducted on *Streptococcus mutans* and *Porphyromonas gingivalis* strains. Cell viability was measured by the quantitative colorimetric assay using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay on a mouse fibroblast cell line (L929). Solubility, film thickness, and setting time were performed according to ISO 6876. The lowest Bi_2O_3 concentrations showed the best antimicrobial activity and cell viability. pH, solubility, setting time, and film thickness did not show statistically significant differences between the different Bi_2O_3 concentrations tested.

Keywords: *MTA, bismuth trioxide, solubility, setting time, antimicrobial activity*

1. Introduction

As life expectancy increases, the need to maintain a healthy dentition is highlighted as an important factor in overall health [1], since tooth preservation is related to a person's quality of life [2]. In this regard, modern endodontic and restorative treatments offer alternatives to preserve severely decayed or damaged natural teeth. Although endodontic treatment is a highly predictable procedure, with reported success rates of ~96% [3], failures can occur due to recontamination or persistent infection of the root canal system [4]. Therefore, to help control contamination of the root canal system, appropriate endodontic materials should be used.

Endodontic materials should be biocompatible, insoluble, radiopaque, and ideally, have an antimicrobial effect. Calcium silicate (CS)-based types of cement, such as mineral trioxide aggregate (MTA), have demonstrated desirable properties in terms of biocompatibility, bioactivity, hydrophilicity, radiopacity, sealing capacity and low solubility, and

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therefore is used in different endodontic applications [5, 6] such as apexification, pulp capping, pulpotomy, and perforation sealing, among others, as they also stimulate mineralization and induce tissue repair [7], mainly due to the hydroxyapatite (HA) formation [8, 9].

Commercial MTA is a calcium silicate cement composed of 80% Portland cement and 20% bismuth trioxide (Bi_2O_3) added as a radiopacifying agent [10]. During the hydration process, MTA is converted into a colloidal gel, with calcium hydroxide being the main product of this reaction [11], which causes an increase in pH between 11 and 13, promotes bioactivity as apatite formation [8] and provides an antibacterial effect.

The literature reports that commercial MTA cement contains 20 wt% bismuth trioxide as a radiopacifying agent. Several studies report discrepant radiopacification values, however, and recently it was observed that bismuth trioxide concentrations in different batches of the same MTA product were recorded between 20% and 32% [12]. It has been observed that a high concentration of Bi_2O_3 affects the physical and chemical properties, reducing the compressive strength and increasing the solubility [13, 14], which could lead to a treatment failure. In this sense, bismuth has been studied as an antimicrobial agent, mainly in bismuth subsalicylate compound [15]. In this regard, the studies that evaluate its antimicrobial capacity offer contradictory results [16–18], but Bi_2O_3 displays remarkable antibacterial activity and low cytotoxicity [19, 20]. Nevertheless, the effect of Bi_2O_3 as part of MTA cement on the cell viability and as an antimicrobial agent has not been evaluated. The objective of the present work was to evaluate the influence of different concentrations of bismuth trioxide in an MTA cement on antimicrobial activity, cell viability, pH, solubility, setting time, and film thickness.

2. Materials and methods

Three study groups were developed using white Portland cement (WPC) (CPO40B, Cruz Azul, Mexico) previously characterized [21] and adding 15 wt%, 20 wt%, and 25 wt% bismuth trioxide (Aldrich Chemical Company Inc. Batch 1304-76-3, USA) identified as Bi15, Bi20, and Bi25, respectively. The particle sizes of the experimental cement and Bi_2O_3 were standardized (<0.62 μm). To achieve homogeneity, the cement and trioxide of bismuth were ground together with acetone for five minutes using an agate mortar. The cement was hand mixed with a powder-to-liquid ratio of 1 g to 0.33 mL [22]. Tang's method was used to estimate the sample size required to detect the effects of a given magnitude with analysis of variance tests, using a method based on the F statistic, considering the variance, the level of significance, and the mean square error, obtained from an ANOVA test of a previous pilot test [23, 24], the number of samples for each test was calculated with an 80% power analysis and a 95% confidence interval.

2.1. Antibacterial activity

In order to evaluate the antimicrobial effect of the Bi15, Bi20, and Bi25 cements, broth diffusion tests were performed, for which discs of each experimental group were made using a stainless-steel mold with an internal diameter of 10 ± 1 mm and a height of 1.5 ± 0.1 mm ($n = 6$). In brief, each cement was mixed in the ratio previously indicated. The molds were filled with the mixture of each cement to a slight excess, a polyethylene sheet and a glass plate were placed, and pressure was exerted with a "C" press (C-shaped press, Toolcraft, Mexico). The samples were conditioned at $37\pm1^{\circ}$C and 90% relative humidity for 24 h in a conditioning chamber (Water Bath, Polyscience, USA). After that time, the samples were removed from the chamber, and the removal of surface irregularities was performed with water sandpaper (SiC 1500 grit). Before antimicrobial evaluation tests, the samples were sterilized by radiation, following ISO 11137-1:2015 [25] and according to previously established standards [26].

The bacterial species used for broth diffusion tests were obtained from lyophilized pure cultures from the American Type Culture Collection (ATCC, Rockville, MD, USA) and are listed in Table 1. *P. gingivalis* and *S. mutans* strains were grown on enriched *Mycoplasma* agar plates

Table 1. Reference strains used to determine the antimicrobial activity

Bacterial strain	ATCC	Gram/Respiration	Associated with
Porphyromonas gingivalis	33277	−/anaerobic	Periodontitis
Streptococcus mutans	25175	+/facultative anaerobic	Dental caries

(BBL, Becton-Dickinson) supplemented with 5% defibrinated sheep blood, 5 μg/mL hemin (Sigma-Aldrich, St. Louis, MO) and 0.3 μg/mL menadione (Sigma-Aldrich) at 35°C in an anaerobic chamber under atmospheric conditions of 80% N_2, 10% CO_2, and 10% H_2, for 7 days.

Once pure cultures of both microorganisms were obtained, bacterial growth was collected from the agar surface and placed in tubes with *Mycoplasma* broth. The optical density (OD) of each tube was adjusted to 1 on a 600-nm wavelength spectrometer to obtain the same number of bacterial cells (1×10^9 cells/mL).

Next, experimental disks corresponding to the different concentrations of bismuth trioxide added to the MTA-type cement (Bi15, Bi20, and Bi25) were individually placed in 24-well plates where they were added with a suspension of 10 μL of each bacterial species, either *P. gingivalis* or *S. mutans*. Chlorhexidine (CHX) (0.20%) was used as a positive control since it is considered the gold standard for the control of oral bacterial growth [27]. The culture medium added to the bacterial suspension was used as a negative control. Each of the 24-well plates, with the experimental cements supplemented with the bacterial suspension, was incubated for 48 h at 35°C under anaerobic conditions.

After incubation for 48 hours, each sample was washed twice 1 mL with *Mycoplasma* broth medium (BBL, Becton-Dickinson, Sparks, MD). After the washes, 1 mL of the broth was placed in each well, then the experimental discs were sonicated using an ultrasonic processor for 5 periods of 10 sec each, to detach the bacteria adhering to each of the experimental blocks of cement. The ultrasonic processor was operated at a frequency of 20 kHz and output power of 60 W. Subsequently, four serial dilutions (×1, ×3, ×4 and ×6) of the supernatant of each of the sonicated samples were prepared, and then 100 μL of the −4 and −6 dilutions were seeded on enriched *Mycoplasma* agar plates. After 7 days of anaerobic incubation of *P. gingivalis* and *S. mutans*, visual counts of CFUs were performed to determine the percentage of growth on each of the experimental cements evaluated. All the experiments were performed in triplicate (n = 3).

2.2. Cell viability

Cell viability was measured by the quantitative colorimetric assay using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay [28]. The evaluations were performed on mouse fibroblast cell line (L929). Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies Corporation, NY, USA) containing antibiotics (penicillin 100 U/mL, streptomycin 125 l g/mL, and amphotericin 5 lg/mL) (Sigma Chemical Co, Saint Louis, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. In all experiments, 5–15 passages of cells were used. L929 were grown in 96-well microplates (Corning 96-well, NY, USA) (2.5×10^3 cells per well) until confluence reached 75%. Cement disc samples (10 mm diameter × 1 mm height) with the three bismuth concentrations were allowed to be set for 24 h at 37°C and 95% relative humidity in a sterile environment, after which one cement sample of each group was placed in 100 μL of DMEM for 24 h. This DMEM was identified as the conditioned medium (the conditioned media were labeled Bi15, Bi20, and Bi25).

Cells were then washed with DMEM and exposed to each conditioned medium for 24, 48, and 72 h. After this treatment, the cells were washed with PBS and supplemented with 10 μL of MTT solution (0.5 mg/mL in PBS) in each well, and incubated for 3 h at 37°C. The medium was removed, and the formazan crystals formed from the viable cells were solubilized with 100 μL of dimethyl sulfoxide (DMSO, MP Biomedicals, LLC, France).

Each well was evaluated in a dual-beam microtiter plate reader at 570-nm absorbance. Data were expressed as a percentage of the control (cells without any bismuth content). All experiments were conducted three times in a 6-fold sequence ($n = 3$).

2.3. pH measurements

Cement disk-shape samples ($n = 5$) were made (10 mm diameter × 1.5 mm height) in a stainless-steel mold between two glass blocks and pressed with a C-clamp, then placed at 37°C and 95% relative humidity (Water Bath, Polyscience, USA) for 1 h to allow the cement to set. After this period, the cement disks were removed from the molds and placed in sterile plastic jars with 10 mL of deionized water (Hycell Chemical Reactives, Mexico) at 37°C (Felisa Stove, Mexico). The pH was measured after 1 h, 3 h, 24 h, 48 h, 72 h, and 7 days with a pH electrode and potentiometer (Orion 4 Star Plus, Thermo Scientific, Waltham, MA, USA), the calibration of the potentiometer was performed with buffer solutions of pH = 7.0, 4.0, and 10.0 (J.T. Barker, Mexico) before each measurement.

2.4. Solubility and setting time

Solubility and setting time tests were performed in accordance with the ISO 6876 Standard [29]. Experimental cement disks (Bi15, Bi20, and Bi25) ($n = 10$), fabricated according to the process previously described in this paper, were weighted to an accuracy of 0.001 g and recorded as "$m1$" (Analytical balance Mod 203797, Adventurer Ohaus Corp, China) and immersed in 5 mL of deionized water (Hycell Chemical Reactives, Mexico) for 7 days at 37°C (Felisa Stove, Mexico). The disks were hung in a metal holder, in order to avoid the disk touching the wall or the bottom surface of the glass flask. After the incubation time, the specimens were placed in a desiccator chamber with freshly dried silica gel and weighed daily until no significant change in the weight of each specimen was observed, recorded as "$m2$". The weight difference between the first and final measurement, expressed as a percentage from original mass, was taken as indicative of the solubility of the material, according to the following formula:

$$\frac{m2 - m1}{m1} \times 100$$

For setting time, a type IV gypsum mold with a cavity of 10 mm internal diameter and 1 mm height was made. The cements were hand mixed as previously described and placed into the gypsum mold that was previously preconditioned at 37°C and 95% relative humidity for 24 h. The setting time test was performed inside a chamber at 37°C and 95% of relative humidity (Polyscience, Mod. 106B 00351, USA) using Gillmore equipment with a 2.0 ± 0.01 mm diameter flat-ended indenter needle and 100 ± 0.05 g load, during 15-s intervals. Setting time was recorded (Chronometer, Sper Scientific, Hong Kong) when any indention could be achieved after the end of the mixing.

2.5. Film thickness

Two square glass plates with a thickness of 5 mm and a contact surface area of 200 ± 10 mm^2 were brought into contact, and the combined thickness of the two pieces of glass was measured to the nearest 0.001 mm with a micrometer (Mitutoyo Micrometer, Digimatic, Tokyo, Japan). The cements were hand mixed; 0.5 mL of each cement was placed in the center of one of the glass plates; the second glass was placed centered on top. After 180 ± 10 s from the start of mixing, a force of 150 N was carefully applied vertically on the top of the plate with a loading device, making sure that the cement was completely covering the area between the glass plates. After 10 min from the start of mixing, the thickness of the two plates and the cement film was measured with the micrometer. The difference between the measurements with and without cement was recorded as the film thickness in μm.

2.6. Scanning electron microscopy

After a solubility test of each cement group, the bismuth trioxide powder, the unhydrated powder, and cement disk samples were fixed on a carbon tape placed on an aluminum sample holder. The SEM images were obtained in low-vacuum mode with backscattered electrons at 20–25 kV and 20–25 Pa pressure in the sample chamber (JEOL

JSM5600-LV, Tokyo, Japan) for all the samples, while secondary electron images were also taken for disk samples after the solubility test.

2.7. Statistical analysis

Means and standard deviations were calculated, and data were analyzed using the multivariable Shapiro-Wilks test to probe normality; cytotoxicity ($p = 0.21$), cell viability (24 h $p = 0.99$, 48 h $p = 0.99$, 72 h; $p = 0.80$, pH ($p<0.001$), solubility ($p = 0.10$), setting time ($p = 0.60$) and film thickness ($p = 0.15$). Analysis of variance (ANOVA), *post hoc* Dunnett test for antibacterial activity versus control, and multiple comparisons including all of the experiments with *post hoc* Tukey test were applied in IBM SPSS. The Kruskall Wallis test was applied in the pH test because the data did not show normal distribution. Statistics software version 23 (Statistical Package for the Social Science, Chicago, Ill), $\alpha = 0.05$ was used as the cutoff for statistical significance in all tests.

3. Results

3.1. Antibacterial activity

Figure 1A shows the results obtained from the evaluation of the antibacterial effect of the experimental groups: Bi15, Bi20, and Bi25, on bacterial strain *S. mutans*. As can be seen, Bi15 showed the highest antibacterial effect on *S. mutans*, compared with Bi20 and Bi25. While the experimental groups that showed the least antibacterial effect on the bacterial strain were Bi20 followed by Bi25, the difference in bacterial inhibition between the latter two groups was not significant. Finally, the highest inhibitory effect on the *S. mutans* strain was achieved through the positive control (0.20% CHX), which reached 100% inhibition of bacterial growth; such inhibition was statistically significant when compared to each of the experimental groups evaluated (F = 33.67, ANOVA $p < 0.01$; *post hoc* Tukey test $p < 0.05$). When the groups were compared to the positive control of 0.20% CHX, statistically significant differences were found in all three experimental groups, (ANOVA $p < 0.01$; *post hoc* Dunnett test $p < 0.05$).

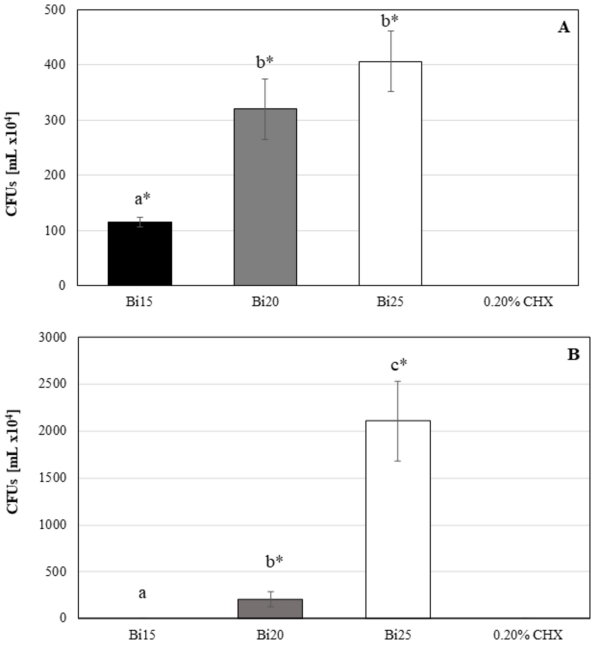

Fig. 1. (A) Evaluation of the antibacterial effect of the experimental cements: Bi15, Bi20, and Bi25, on *S. mutans*. Positive control: 0.20% chlorhexidine (ANOVA $p < 0.05$; *post hoc* Tukey test $p < 0.01$). 0.20% CHX was significant against all experimental groups (ANOVA $p < 0.01$; *post hoc* Dunnett test $p < 0.05$). Lowercase letters are used to compare means; means sharing a superscript letter are not significantly different, $n = 3$. (B) Evaluation of the antimicrobial effect of the experimental cements: Bi15, Bi20, and Bi25 on *P. gingivalis*. Positive control: 0.20% chlorhexidine solution. (ANOVA $p < 0.01$; *post hoc* Tukey test $p < 0.05$). 0.20% CHX was significant against the experimental groups Bi20 and Bi25 (ANOVA $p < 0.05$; *post hoc* Dunnett test $p < 0.05$). Lowercase letters are used to compare means; means sharing a superscript letter are not significantly different, $n = 3$

The results obtained from the evaluation of the antibacterial effect of the experimental cements are as follows: Bi15, Bi20, and Bi25 on *P. gingivalis* are depicted in Figure 1B. As can be seen, the experimental cements Bi15 and Bi20 showed the highest inhibitory effect on the *P. gingivalis* strain. Importantly, the antibacterial effect observed in the experimental group Bi15 was comparable to the gold standard (0.20% CHX). In addition, like what was observed with the *S. mutans* strain, the experi-

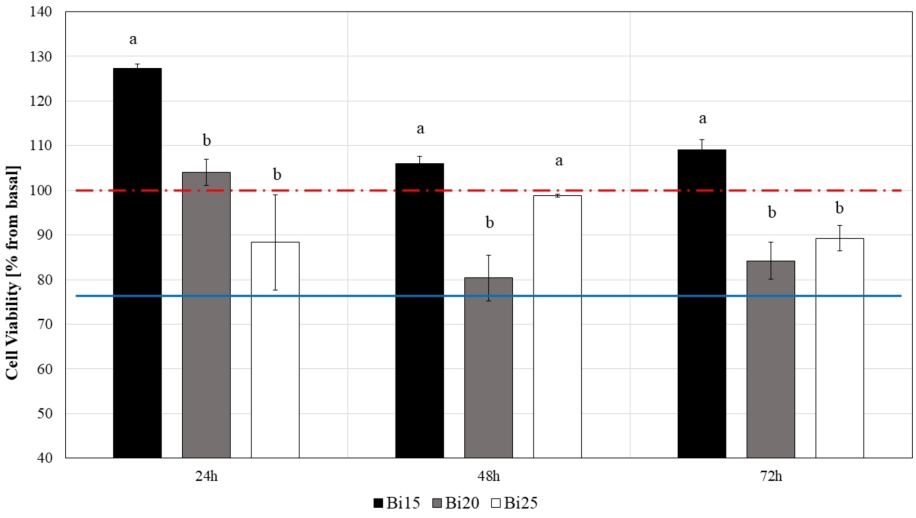

Fig. 2. Effect of bismuth on cell viability of Bi15, Bi20, and Bi25 cements. L929 cells were seeded in 96-well plates (2.5×10^3 cells/well) and treated with different percentages of bismuth followed by MTT assay for 24, 48, and 72 h. The red horizontal dotted line represents the basal group as 100%, and a blue continuous line represents the threshold for a cytotoxicity level of 75% of cell viability compared to the basal group (negative control). Each bar represents mean ± SD calculated from three independent experiments. ANOVA $p < 0.01$; *post hoc* Tukey test $p < 0.05$, lowercase letters are used to compare means; means sharing a superscript letter are not significantly different, $n = 3$

Table 2. pH means of the Bi15, Bi20, and Bi25 cements

	1 h	**3 h**	**24 h**	**48 h**	**72 h**	**7 d**
Bi15	10.28 (0.25)^a	10.68 (0.15)^a	11.16 (0.21)^a	10.94 (0.48)^a	10.78 (0.70)^a	9.98 (1.08) ^{a}
Bi20	10.49 (0.12)^a	10.87 (0.10)^a	11.32 (0.16)^a	11.33 (0.22)^{a,b}	11.07 (0.53)^a	10.11 (0.92) ^{a}
Bi25	10.49 (0.13)^a	10.88 (0.06)^{a,}	11.37 (0.12)^a	11.47 (0.18)^b	11.35 (0.35)^a	10.96 (0.88) ^{a}
p	0.309	0.061	0.119	*0.043*	0.237	0.181

^{a}Mean and standard deviation (in parentheses). Lowercase letters are used to compare means in the same column; means sharing a superscript letter are not significantly different. Any statistically significant difference is observed in pH over all time periods (Kruskall Wallis test $p > 0.05$), $n = 5$ for each group

mental group that had the lowest effect on inhibiting the growth of *P. gingivalis* was the experimental cement Bi25 (F = 201.02, ANOVA $p < 0.01$; *post hoc* Tukey test $p < 0.05$). In comparison with the positive control 0.20% CHX, the Bi20 and Bi25 groups showed statistically significant differences (ANOVA $p < 0.01$; *post hoc* Dunnett test $p < 0.05$).

The viability of L929 cells exposed to bismuth was assessed using the MTT assay. Cells were pretreated with various percentages (15, 20, and 25 wt%) of bismuth in MTA cement for different periods (24, 48, and 72 h). Treatment of L929 cells with 15% and 30% bismuth showed a significant increase in cell viability at all three time periods (24, 48, and 72 h). It was observed, however, that the Bi20 and Bi25 experimental types of cement did not exert an increase in cell viability and presented a decrease compared to the basal condition at all evaluation times (see Figure 2). Nevertheless, the different percentages of bismuth did not show a cytotoxic effect on L929 cells (a viability value of cells in contact with bismuth should be <70% of the basal condition to be recognized as a cytotoxic effect). (24 h F = 28.07, 48 h F = 54.89, 72 h F = 50.33, ANOVA $p < 0.01$; *post hoc* Tukey test $p < 0.05$).

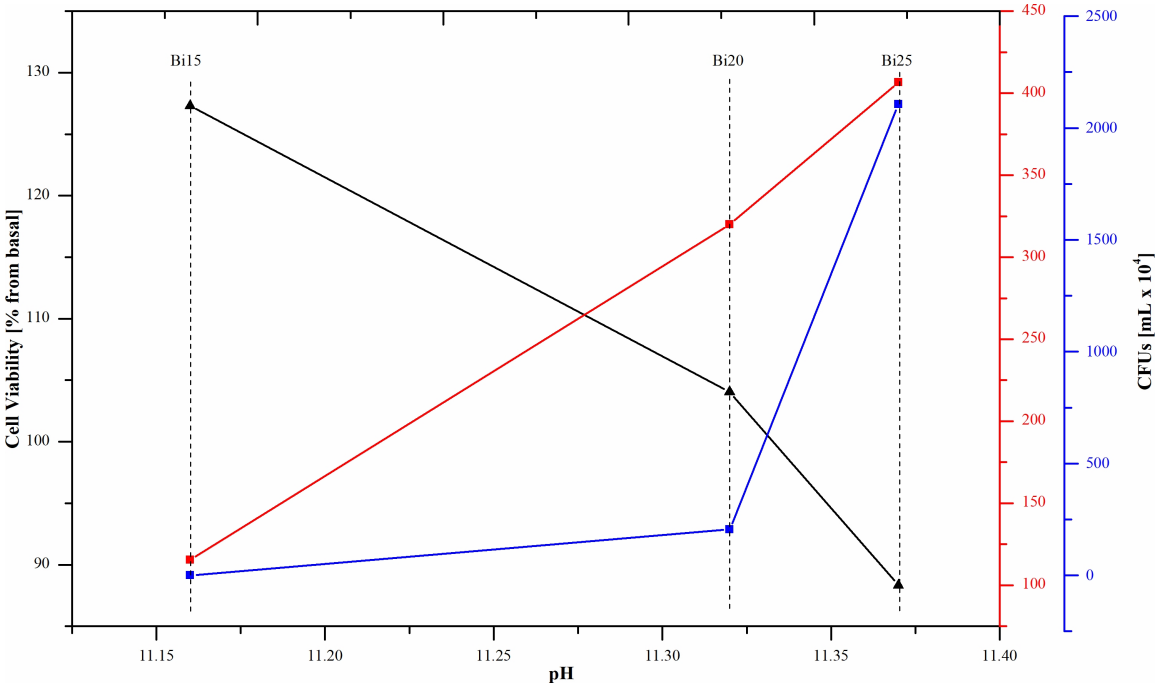

Fig. 3. Effect of pH on the antibacterial activity and cell viability at 24 h. Black line: Cell viability, L929 cells. Red line: Antibacterial activity on *S. mutans*. Blue line: Antibacterial activity on *P. gingivalis*

3.2. pH measurements

The basal pH of the solution was 7.01, at 1 h the three groups display a rapid rise in pH, from 10.28 to 10.50, with similar results at 3 h between 10.68 and 10.92. After 24 h, the pH of all the groups reached pH values above 11. These values were constant until the 72-h period; after 7 days, the values decreased from 9.98 to 10.96. The Bi15 group shows the highest pH value with 11.16 at 24 h, in contrast with the Bi20 and Bi25, which reached the highest pH at 48 h (Table 2). Any statistically significant differences were found at all time periods, with at 1 h (H = 2.34), 3 h (H = 5.59), 24 h (H = 4.24), 72 h (H = 2.88), and 7 days (H = 4.42), except for 48 h Bi15 and Bi25 (H = 6.26, Kruskal-Wallis Test $p < 0.05$).

The effect of the pH on the antibacterial activity and cell viability at 24 h is shown in Figure 3. It has been observed that the less alkaline pH values are related to major antibacterial activity on both bacteria strains, *S. mutans* and *P. gingivalis*, in contrast with the cell viability where the highest viability was found with the less alkaline pH values.

3.3. Solubility, Setting time, and Film Thickness

The solubility results showed that Bi15 presented the highest solubility, with −2.7%, followed by Bi25 with −0.27% (a negative result indicates solubility, whereas a positive result indicates weight gain). However, the Bi20 group presented an increase in weight or water intake (F = 49.47, ANOVA $p < 0.001$, Tukey test *post hoc*, $p < 0.05$, Table 3), and statistically significant differences were found between Bi15–Bi20 and Bi15–Bi25. The setting time was 5:19, 4:59, and 4:41 min for Bi15, Bi20, and Bi25, respectively. The results on film thickness in the three groups show a range from 0.01217 to 0.01238 mm; there were no statistically significant differences in setting time (F = 0.945, ANOVA $p = 0.401$) and film thickness (F = 0.235, ANOVA $p = 0.792$).

3.4. Scanning electron microscopy

The particles of the bismuth trioxide powder and the unhydrated powder of the cements are shown in Figure 4. The bismuth trioxide is pre-

Table 3. Means and standard deviation (in parenthesis) of solubility, setting time, and film thickness of Bi15, Bi20, and Bi25

	Solubility (%)	Setting time (min)	Film thickness (mm)
Bi15	−2.70 (0.41)^a	5:19 (1:41)^a	0.01238 (0.00027)^a
Bi20	0.24 (0.81)^b	4:59 (1:26)^a	0.01229 (0.00023)^a
Bi25	−0.27 (0.82)^b	4:41 (1:31)^a	0.01230 (0.00043)^a
ISO 6876 requirements	$< 3\%$	No more than 110% of the manufacturer's specifications.	< 0.05 mm

Lowercase letters are used to compare means in the same column; means sharing a superscript letter are not significantly different. No significant differences were found in setting time (ANOVA $p = 0.401$) or film thickness (ANOVA $p = 0.972$); $n = 10$ for each group and test.
Solubility at 24 h show statistically significant differences between Bi15 and Bi20 and Bi15 and Bi25 (ANOVA $p < 0.05$, *post hoc* Tukey test $p < 0.05$), negative signs indicate solubility

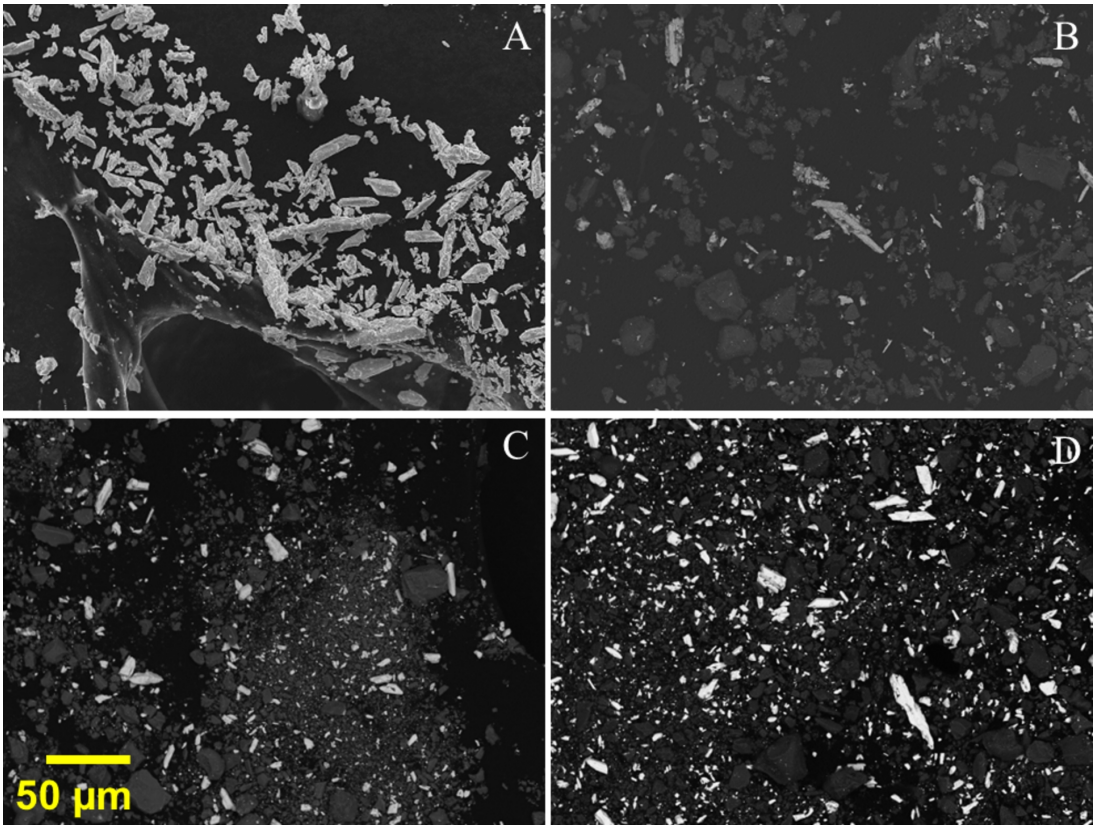

Fig. 4. Micrographs of bismuth trioxide and the unhydrated cement sample. A. Bismuth trioxide; B, Bi15; C, Bi20; and D-Bi25. Yellow bar = 50µm, 350 × magnification

sented as white, long, acicular particles. The unhydrated cements do not show differences in surface morphology.

The solubility cements disk micrographs show an irregular surface with some bismuth agglomerates on the surface. The images with backscattered electrons allow us to see the bright white bismuth particles; these particles are not integrated into the cement. The most representative images of the hydrated cements after the solubility test are shown in Figure 5.

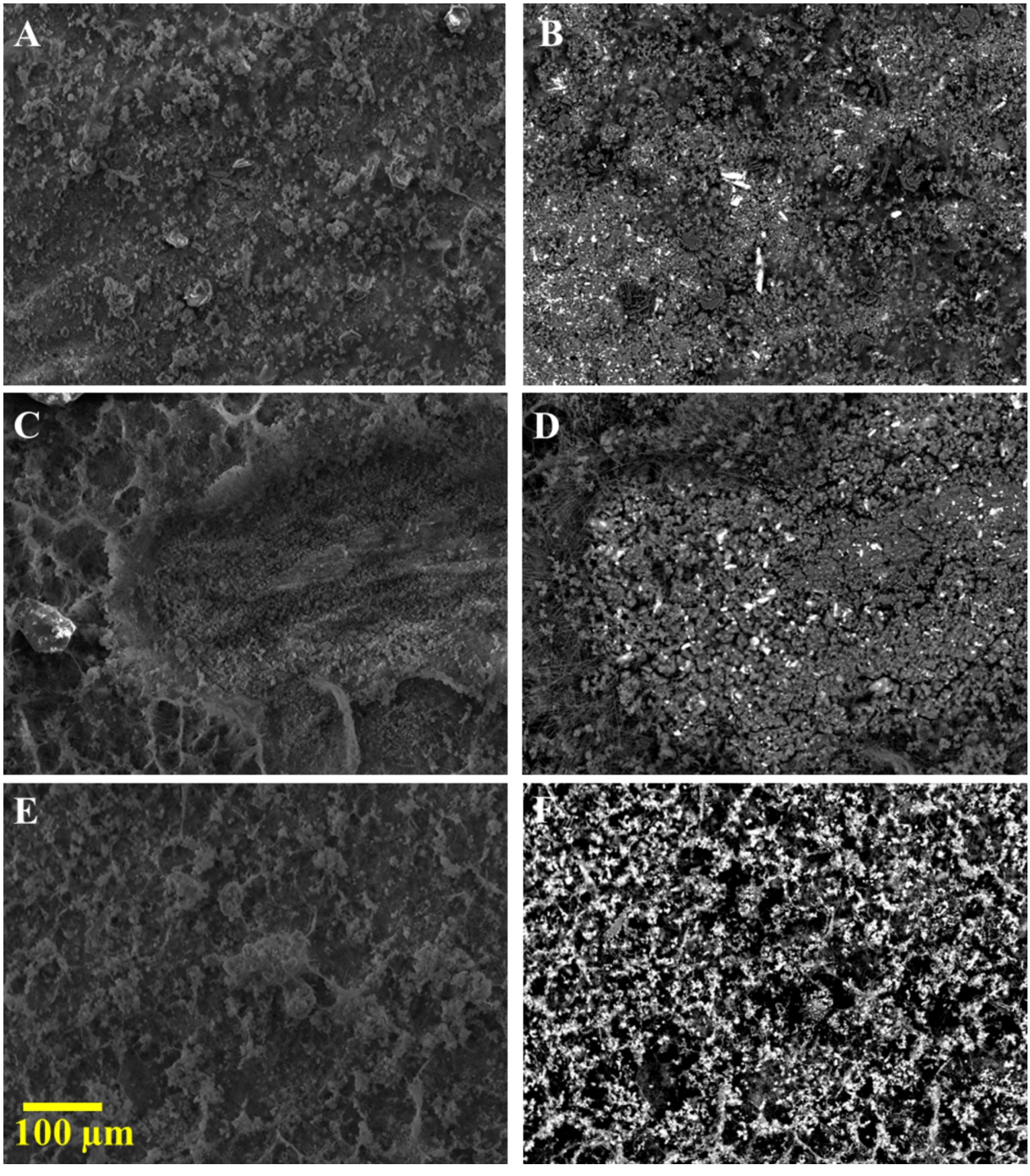

Fig. 5. Surface micrographs of the hydrated cement sample. The long white particles corresponding to bismuth trioxide are not well incorporated into the cement. Left columns (A, C, E) are micrographs with secondary electrons. The right columns (B, D, F) are backscattering electron images. A–B: Bi15, C–D: Bi20, and E–F: Bi25. Yellow scale bar = 100 µm, 200 × magnification

4. Discussion

This study evaluated the different concentration of bismuth trioxide as a radiopacifier for antimicrobial activity, cell viability, and some physicochemical properties on an MTA-like cement. Similar to Portland cement, MTA is composed of dicalcium silicate, tricalcium silicate, dicalcium aluminate, and calcium sulfate [21]. When this cement is mixed with water, it initiates a chemical process in which these mineral compounds begin to release calcium hydroxides in the mineral form Portlandite [8, 30]. These ions produce an increase in pH of around 9 to 13, which produces an antibacterial effect [31] and favors bioactivity [8]. The addition of 20% Bi_2O_3 to Portland cement in the formulation of MTA has the sole function of providing radiopacity to the cement, favoring its radiographic identification, warranting that the material has been placed properly, and ensuring that the material is well compacted and easily differentiated from the dental tissues. It has been demonstrated that bismuth trioxide does not react within the MTA hydration process [8], leading to the bismuth particles remaining unreacted, weakening the structure of the material, and compromising the mechanical properties. Recent studies have shown that some brands of commercial MTA contain bismuth trioxide in concentrations other than the 20% recommended, ranging from 20% to more than 30% [12]. Some authors suggest that concentrations of 10% to 15% are sufficient to provide the necessary radiopacity (3 mm Al) for radiographic identification [32, 33].

On the other hand, it is important to consider that a root canal sealing system with restorative materials possessing antibacterial activity could directly contribute to the success of endodontic treatment. In this regard, bismuth subsalicylate, a component of different endodontic biomaterials, has been studied as an antimicrobial agent [15]. Due to the above, in the present study, the antimicrobial effect of the MTA-type cements with different concentrations of bismuth trioxide was assessed on two bacteria of dental relevance. The bacterial strain *S. mutans* was evaluated due to its direct relationship with the occurrence of carious lesions [34], while *P. gingivalis* was tested because it is considered one of the main bacteria associated with periodontal diseases and is commonly called periodontopathogenic, in addition to being a microorganism frequently isolated from endo-periodontal lesions and sites of pulp necrosis [35].

When the antimicrobial activity of the experimental cements on *S. mutans* and *P. gingivalis* was evaluated, we found that the highest inhibitory effect on both strains was Bi15, the cement with the lowest concentration of bismuth trioxide. This finding is consistent with a previous report showing that *S. mutans* and *P. gingivalis* are sensitive to bismuth trioxide-containing materials at concentrations close to 14% [18]. In general, *S. mutans* showed higher sensitivity to all concentrations of bismuth trioxide present in the experimental cements, while *P. gingivalis* was also sensitive to the Bi20 group. It has been previously shown that bismuth can alter the iron metabolism of *P. gingivalis*, thus compromising their virulence and survival [36]. Both bacteria showed higher sensitivity to Bi15 cement, according to the measurements performed. This could be because those cements showed the greatest increase in pH, which could correlate with its higher antimicrobial capacity, as well as higher solubility; therefore, more bismuth ions could be released into the culture media. Bismuth ions are known to reduce bacterial growth through the inhibition of ATP synthesis [37], besides inducing structural damage to the bacterial cells [38].

Yet even the antibacterial effect of the different MTA-type cement tested here was not always correlated with the bismuth concentration. It was observed that the inhibition of bacterial growth was sustained and could be attributed to the release of bismuth ions from the experimental cements as a result of the degradation process of the experimental cements under the incubation conditions.

On the other hand, concerning the results of cell viability in the L929 line, it was shown that the different percentages of bismuth did not exert a cytotoxic effect on the L929 cells, which is consistent with previous studies [39, 40]. The ability of bismuth to promote viability in cell culture was significant compared to the control [41]. However, the increase in the percentage of bismuth could decrease

the viability in periods longer than 48 to 72 hours [42]; it is possible that the decrease could be causing by the association of the bismuth with dicalcium silicate cement.

One of the most important properties of this type of cements is the alkaline pH, which allows them to provide antimicrobial properties and favors the formation and deposition of apatite [8, 43]. In the first few hours after mixing, MTA cement's pH rises rapidly, increasing at least by 3 points from its initial value [8, 44] as a result of the calcium release. This allows the activation of some proteins associated with the remineralization process [45, 46]. Bismuth is considered a basic oxide insoluble in water; in this study, it was observed that the concentrations showed a directly proportional increase in pH with time. At 1 h, the three groups display a rapid increase of the pH, from 10.28 to 10.50, that reaches values above 11 after 24 h. However, the Bi15 group reached the highest pH at 24 h, while the Bi20 and Bi25 groups presented the highest values at 48 h. Although the pH increased over time, it never exceeded 12 in any group, as has been reported by Padrón-Alvarado [14]. The pH values were similar to those reported by Flores-Ledesma [8], where the highest pH was 11.5, but this was reached after 7 days of immersion; in the present study, it was observed that after 7 days the pH started to decrease. Human cells grow in a neutral pH environment, but it has been observed that a different pH environment decelerated cell migration in comparison to neutral environments [47], but especially an alkaline environment around soft tissues enhances cell proliferation, favoring wound healing [47]. In dental furcal or pulpal areas, alkaline pH activates alkaline phosphatase, which is related to the mineralization process and hydroxyapatite deposition [8, 48]. In contrast, a high pH has an adverse effect on the cell membrane of bacteria, generating antimicrobial activity [49]. *P. gingivalis* has optimum pH for its growth between 6.5 and 7.0 [50] and the *S. mutans* optimum is around 5.2 [51]: an increase in pH has a significant antimicrobial effect.

It has been observed in other studies that the higher the amount of radiopacifier, the higher the solubility will be [52]. The high solubility of Portland cements has been associated with their long setting time [53]. In accord with the present results, it was observed that, in terms of solubility, the Bi15 cements presented a higher solubility, although none of them exceeds the 3% established by ISO Standard 6876. But as these cements are known for their bioactivity; the hydroxyapatite growing on the surface of the probes could mask the weight loss due to solubility. This phenomenon could also occur with the transformation of Portlandite (calcium hydroxides) to calcite (calcium carbonate), which are common phases observed as hydration products in MTA cements that lead to apatite formation. It is important to consider that the synthesis or formation method could influence in the surface and adsorption properties [54]. Bismuth trioxide particles could be observed as nanoflowers [55] or as oblique prism-like structures [56]. In this study, the oblique prism-like particles could be identified in the non-hydrated powder SEM images, similarly to those reported by Padrón-Alvarado [14] and Wang [56]. The bismuth trioxide added to the cements does not undergo any chemical reaction, but instead occupies the interstitial spaces within the network, as can be seen in SEM images where the bismuth particles are not incorporated into the cement composite, as has been observed previously [14]. It is important to notice that in the images, the characteristic apatite structure (clusters with acicular growth) was not observed; this is because the cements were immersed in deionized water and not in simulated body fluid.

The setting time is an important characteristic, because if the setting time is too long, liquids present in the placement area such as saliva, crevicular fluid, blood, etc. could cause the material to disintegrate. It should be remembered that these materials harden in the presence of moisture. Again, the Bi15 group was the one that presented a setting time greater than 5 min; however, no statistical differences were found with the control group Bi20, which had a set time of 4:59 min, very close to the rest of the groups. Some studies have shown that as the amount of radiopacifier becomes greater, the setting time increases almost three times, going from 134 min without radiopacifier to 397 min with zirconium oxide [52]. It is worth mentioning

that the setting time on the experimental groups were very short, and this can be attributed to the absence of gypsum in the composition of the MTA (WPC with 15, 20, and 25 wt% bismuth trioxide) as opposed to other commercial MTA cements that have gypsum added by the manufacturers to delay its setting time [53].

To accelerate the hydration process and thus its setting time, various substances, such as citric acid, lactic acid, and calcium chloride, among others, are added, improving its setting time; however, its compressive strength decreases and with calcium chloride cell viability is also reduced [57]. Sharifi et Al., reported that increasing the temperature of the water used for the MTA contributes to a quicker setting time, and this benefits retrograde fillings and root canal sealing [58].

Finally, with respect to film thickness measurements, all groups ranged between 12.17 and 12.38 μm, with no statistically significant differences. ISO 6876 stipulates that these cements must have a thickness of less than 50 μm, so in this case, all groups comply. This property is not affected by changes in bismuth trioxide concentration. According to a recent study, the addition of 1% and 2% methylcellulose and calcium chloride to MTA did not alter its pH, which is close to 13, or the biological or physical properties of the composite, but it did reduce its flowability to make it suitable as a root canal sealer [59].

It has been observed that the placement of MTA causes dental dyschromia (color change). Initially, it was believed that the color change was due to the presence of iron in the gray MTA cement, but the the presence of white MTA, in which the iron-containing phase is eliminated, dyschromia is still observed. Studies have shown that the cause of these color changes is the sedimentation of the bismuth oxide to metallic bismuth in contact with saliva or dentin [60]. Despite the limitations, in the present study it was found that the bismuth concentration affected the antimicrobial activity, viability, pH, and solubility of the experimental cements evaluated. Therefore, to prevent adverse effects from the physicochemical and biological properties of MTA-type cements, the bismuth trioxide concentration may need to be regulated. Finally, further studies exploring the release of bismuth ions under different experimental conditions are recommended to better understand how the lixiviates modify the physico-biological properties of the material.

5. Conclusions

Within the limitations of this study, it was concluded that the different percentages of trioxide bismuth studied do not affect the physicochemical properties such as pH, solubility, setting time, or film thickness. It was observed, however, that lower concentrations of bismuth trioxide (15%) showed greater antibacterial effect on both bacteria strains, *S. mutans* and *P. gingivalis*, and showed the highest cell viability. This can improve the cost-effectiveness of MTA cement without sacrificing its physicochemical properties, high antibacterial activity, and cell viability.

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