Influence of ultrasonic agitation on bond strength, marginal adaptation, and tooth discoloration provided by three coronary barrier endodontic materials

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Title Page

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Title:

Influence of Ultrasonic Agitation on Bond Strength, Marginal Adaptation and Tooth Discoloration Provided by Three Coronary Barrier Endodontic Materials.

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ABSTRACT

Objectives: The effect of ultrasonic agitation (UA) on bond strength and adaptation of cervical plugs prepared with MTA Angelus (MTA), MTA Repair HP (MTAHP) and Biodentine (BIO) was evaluated. Dentin discoloration caused by the materials/treatment was also assessed. Materials and Methods: Seventy-two single rooted teeth were divided into six groups depending on the materials/treatment. After cervical plug preparation, dentin discs were excised for the push-out test; additional discs were analysed under the confocal microscope to determine adaptation (gaps occurrence). For dentin discoloration analysis (ΔE), blocks of bovine incisors had cavities prepared and filled with the materials/treatment (from 7 to 180 days). Results: Both bond strength and adaptation were positively influenced by UA ($P<0.05$). Comparison between materials showed an advantage for BIO when compared to MTAHP ($P<0.05$). The best and worst results were provided by BIO/UA (12.66 MPa and 1.87%) and MTAHP (2.54 MPa and 28.58%), respectively. For ΔE, significant differences were observed throughout the periods. Just the MTA without UA exhibited noticeable discoloration at 180 days ($P<0.05$). Conclusions: UA favoured a better adaptation of the materials to the dentin root, resulting in higher bond strength and adaptation of the materials to the root canal walls. Moreover, UA reduced MTA discoloration, keeping it imperceptible over the period evaluated. Clinical Relevance: The better adaptation and higher bond strength provided by UA can be considered clinically relevant due to the importance of maintaining blood clot integrity and the possible aesthetic compromise provided by reparative materials when used as coronary barrier in regenerative procedures

Key words: Endodontics, hydraulic calcium silicate cement, marginal adaptation, tooth discoloration, ultrasonic agitation.

INTRODUCTION

The technique of pulpal revascularization has been considered a promising alternative for endodontic treatment of teeth with incomplete rhizogenesis and pulp necrosis, since it allows the continuity of root formation and thickening of the dentin walls [1, 2]. In this technique, a preparation of a coronal barrier with repairing material is necessary to preserve the integrity of the blood clot and to stimulate the production of a new tissue capable of re-establishing root formation [3-7].

The mineral trioxide aggregate (MTA) was initially developed to be used as root perforation repair material [8, 9], however, it has been commonly used as a coronal barrier due to its physico-chemical and biocompatibility characteristics [6, 9]. However, it presents limitations such as difficulty of insertion and induction of tooth discoloration [10].

In order to overcome these limitations new materials were developed. Biodentine (Septodont, Saint Maur des Fosse, Cedex, France) was introduced as a bioactive material. It was based on tricalcium and dicalcium silicate, main components of Portland cement; commercial MTA materials presents 70-90% of

Portland cement. Biodentine also presents calcium carbonate and oxides of iron and zirconia, the latter added as a radiopacifier [10]. This material liquid was composed of water, calcium chloride, added as a settling accelerator and a water-soluble polymer to improve its plasticity. This cement has adequate radiopacity, resistance to displacement, short curing time [11, 12], color stability [10], high dimensional stability and good sealing capacity [13, 14]. Another material developed to overcome the limitations of conventional MTA is the MTA Repair HP (Angelus, Londrina, PR, Brazil). It is a repair cement based on tricalcium silicate which also contains additives. According to the manufacturer, these changes provide better handling, insertion and color stability. The liquid includes an unidentified plasticizer [12, 15]. So far, the information on the MTA Repair HP is limited; until now its biocompatibility, biomineralization and resistance to displacement were evaluated showing promising results [12, 15, 16].

In addition to changes in materials composition aiming to improve clinical performance, the protocols of material insertion were also investigated. The use of ultrasonic indirect agitation was suggested to improve the sealing ability provided by MTA when used as root-end filling material [17, 18]. The ultrasonic agitation was also tested directly on filling materials and provided better sealer the penetration into dentinal tubules [19, 20]. The changes brought by the manufacturers caused a lack of information on its physical and chemical properties since there is no study under confocal microscopy for the proper evaluation of the marginal adaptation.

Moreover, considering the use of these materials in the aesthetic area at the cervical level and considering that the MTA presents notably the capacity to induce the dentin discoloration, it would be relevant to evaluate the influence of this event by the radiopacifiers of the new cements. As well as, the use of the ultrasonic agitation in the evaluation of the adaptation would be interesting to evaluate its effect in the darkening.

Considering the novel materials disposable for coronal barrier preparation, the possible influence of ultrasonic agitation on its adaptation and the parallel risk of tooth discoloration, the current study evaluated the influence of ultrasonic agitation on push-out bond strength and marginal adaptation to root canal walls in the cervical region of MTA Angelus (Angelus), MTA Repair HP and Biodentine; also evaluated the tooth discoloration promoted by these materials. It will be considered as null hypotheses the absence of differences in bond strength, marginal adaptation, and tooth discoloration provided by the materials in function of the use or not of the ultrasonic agitation.

MATERIALS AND METHODS

Bond strength tests and marginal adaptation

The sample calculation was performed using the G*Power v3.1 for Mac (Heinrich Heine, Universität Düsseldorf) by selecting the Anova One Way fixed test. Data from an earlier study were considered and the effect of sample size in the present study was established [20]. The effect size in the present study was established (=1.36) and the alpha type error of 0.05 in the beta power of 0.80. A total of 9 samples were indicated as the ideal size to observe significant differences; the sample was increased by 30% for safety if specimens were to be lost (*n* = 12).

Selection and preparation of the sample

After approval by the Local Ethics Committee (#1.900.129/2017), 72 maxillary uni-radicular human teeth extracted for unknown reasons were collected for the studies of bond resistance and marginal adaptation. Coronary access with diamond tips (#1014 and #3081; KG Sorensen, Cotia, SP, Brazil) was carried out, followed by preparation of the cervical and middle thirds with Largo Reamer #5 (Dentsply-Sirona, Ballaigues, Switzerland). After the preparation specimens were evaluated for circularity and standardization of the cavity diameter. Where the Largo Reamer #5 did not enter just the same has been replaced. The teeth were then cross-sectioned 5.0 mm below the cemento-enamel junction by means of a precision cut double-face diamond disk (Isomet 1000; Buhler Ltda., Lake Bluff, NY, USA). After being sectioned, the teeth were submitted to ultrasonic baths with 2.5% sodium hypochlorite solution (Asfer Indústria Química Ltda, São Caetano do Sul, SP, Brazil) for 15 minutes, with distilled water (Asfer Indústria Química Ltda.) for 1 minute, with 17% ethylenediaminetetraacetic acid (EDTA) (Biodinâmica Química e Farmacêutica Ltda., Ibiporã, PR, Brazil) for 3 minutes and finally with distilled water for 3 minutes.

After specimen preparation, collagen sponge plugs (Technew, Rio de Janeiro, RJ, Brazil) were inserted into the canal with the aid of a Schilder condenser (Odous de Deus, Belo Horizonte, MG, Brazil) obliterating the apical 2 mm. Regardless of the group in which they were to be allocated, the specimens were irrigated with 1 mL of distilled water. Afterwards, gentle aspiration was performed with the aid of the endodontic aspirator for 4 seconds and additional drying with absorbent paper cones (Dentsply-Sirona).

Manipulation and insertion of materials

The manipulation of the repair cements was carried out by a single operator previously calibrated, following the manufacturer's instructions; Fluo-3 fluorophore (Sigma-Aldrich, Warrington, PA, USA) at a concentration of approximately 0.1% (w/w) was incorporated into the materials to determine marginal adaptation [21].

The specimens were initially randomly assigned to three experimental groups, depending on the material (*n* = 24). For insertion of the materials a 1.2 mm diameter MTA microapplicator (Angelus) was used followed by condensation with the Schilder condenser. The procedure was repeated until cervical plugs reached 3.0 mm thickness. Then, the specimens were randomly divided again between the subgroups with and without ultrasonic agitation (UA) (*n* = 12). In the groups which received ultrasonic agitation (MTA/UA, MTAHP/UA and BIO/UA), piezoelectric ultrasound equipment (Piezon Master 200; EMS, Nyon, Geneva, Switzerland) was set to 10% of power. A specific ultrasonic tip (E5; Helse Ultrassonic, Santa Rosa do Viterbo, SP, Brazil) was inserted into the material at a depth of 2.0 mm. Two 20-second activation cycles were performed, one in the mesio-distal direction and one in the vestibular-palatine direction [19]. Then, a final vertical condensation was performed to accommodate the materials; similarly, the specimens allocated in groups without UA also received an additional vertical condensation.

After concluding the cervical plugs, temporary restorations with glass ionomer cement (GIC; FGM Produtos Odontológicos Ltda, Joinville, SC, Brazil) were performed in order to simulate clinical procedures. The specimens were then stored with the apical portions immersed in phosphate-buffered saline solution (PBS; Quimlab, Jacareí, SP, Brazil) in an oven at 37 °C for 7 days.

After the storage period, two 1.0 mm thickness dentin discs were extracted from each plug on its central portion using a cutting machine (Isomet 1000; Buhler Ltda., Lake Bluff, NY, USA) totalling 144 discs; each disk obtained were allocated to one of the tests proposed - the cervical one for the bond strength test and the apical one for the marginal adaptation test - the portion directly in contact with GIC was discharged in order to allow any interference.

Evaluation of the bond strength (push-out)

The 72 dentin disks allocated for the bond strength test were tested using a Universal Testing Machine (#443; Instron, Norwood, MA, USA) handled by a blind operator; for performing the push-out test using a constant velocity of 0.5 mm/min. For this, punches with 1.2 mm diameter were positioned in the center of the upper face of the plugs; on the underside, a steel plate with a 3.0 mm diameter central bore was adapted to serve as a support for the specimen and enable material to escape from the plugs. The maximum force required for cement displacement was measured in Newtons (N) and converted to MPa by the division of force (N) by the area in mm^2 ($1MPa = 1N/mm^2$).

For analysis of fracture types after the push-out test, all slices were examined in a scanning electron microscope (Aspex Express; FEI Europe, Eindhoven, Netherlands) at a magnification of 30x. The failures were classified as: adhesive - rupture of the union at the interface dentin/material, cohesive - rupture on the material and, mixed – rupture cohesive of the material and adhesive in the dentin.

Assessment of marginal adaptation

The remaining 72 discs were adapted to glass slides and fixed with wax with the coronary face facing upwards. The sets were taken to the politriz (Arotec, Cotia, SP, Brazil) where, using 600, 900 and 1200 granulometry water sands, were polished. Then the samples were taken to Confocal Laser Scanning Microscope (LSM 710; Carl Zeiss Microscopy GmbH, Baden-Württemberg, Germany) handled by a blind operator and examined 10 μm below the surface under 20x magnification; 10 section scans of 1 μm were recorded at a resolution of 1024 x 1024 pixels using the ZEN 2012 program (Carl Zeiss Microscopy GmbH) and saved in TIFF format.

To measure the adaptation (*i.e.* presence of gaps), the images were evaluated in the Image J software (National Institute of Health, Bethesda, MD, USA), using as a calibration tool the scale offered by confocal microscopy images. The total perimeter of the canals and the length of gaps were determined in millimetres using the software polygonal and linear tools, respectively; once these measurements were obtained, the percentages of gaps were calculated according to the total perimeter of the root canals.

Study of tooth discoloration

Before performing this evaluation, a sample size calculation was performed following the same parameters used in the previous test; based on the preliminary study results the test pointed to a sample of 10 specimens as a sufficient sample to determine possible statistical differences [22]. An experimental design of this study methods was show in Figure 1.

Selection and preparation of the sample

For the analysis of dentinal discoloration, bovine incisor blocks prepared according to the model proposed by Marciano et al. [22]. Sixty-five blocks (10 mm x 10 mm) were prepared with the aid of a diamond blade driven by a cutting machine. After being prepared, they had their lingual faces sectioned in manner to expose their pulp chambers. Cavities 5 mm in diameter and approximately 1.5 mm deep were prepared in the center of the lingual face of each specimen using diamond tips #4054 (KG Sorensen); specimens where a remainder of 2 ± 0.2 mm of dentin and enamel thickness were not obtained at the bottom of the cavity were replaced.

Subsequently, the specimens were submitted to ultrasonic baths following the previously used protocol. Afterwards, after being dried with filter paper, the wells had their external boundary conditioned with 37% phosphoric acid for 15 seconds, washed with distilled water for 1 minute and gently air-dried for 15 seconds. An adhesive layer (Adper Single Bond 2; 3M ESPE, Sumaré, SP, Brazil) was applied in the conditioned and polymerized area (Optilight LD Max; Gnatus, Ribeirão Preto, SP, Brazil) for 20 seconds to allow interface resin.

Materials manipulation and insertion

After specimen preparation, they were randomly divided among the six experimental groups (n = 10). The cements, after being manipulated, were inserted into the cavities. For those who underwent ultrasonic agitation (MTA/UA, MTAHP/UA and BIO/UA), it was accomplished by means of the use of piezoelectric ultrasound programmed for power 3 (approximately 30%), with a smooth tapered tip (E5; Helse Ultrasonic). After gentle condensation of the cements in the cavities, the tip was inserted at a depth of 1 mm. Then, two cycles of agitation of 20 seconds, one in the mesio-distal and one in the buccal-palatal direction were performed [19]; independent of the group to which they took part, the materials were once again condensed to fill the entire cavity.

After completion, restorations were performed with flow resin (New DFL, Rio de Janeiro, RJ, Brazil), which was polymerized with LED light (Optilight LD Max, Gnatus) for 60 seconds. The remaining 5 blocks comprised the negative control group in which the cavities were completely filled with restorative material. Regardless of the group, the specimens were immersed in individual containers containing 2 mL of distilled water, where they remained throughout the experimental period at 37ºC and 100% humidity.

Evaluation of tooth discoloration

Color determination was performed using a digital spectrophotometer (VITA Easyshade compact; VITA Zahnfabrik AG, Bad Sachington, Germany). The equipment was calibrated prior to the measurement of each specimen. Measurements were obtained immediately after placement of the materials (reference color; 0) after 7, 14, 30 and 180 days. Color parameters were recorded as determined by the *International Commission On Illumination* (CIE, 1978), considering "L", "a" and "b", where "L" represents the values of color luminosity, "a" corresponds measurement along the red-green axis and "b" is the measurement along the yellow-blue axis. The change in color (ΔE), in relation to time intervals, was calculated based on the initial values using the following formula:

$$\Delta E = [(L_1-L_0)^2 + (a_1-a_0)^2 + (b_1-b_0)^2]^{1/2}$$

Statistical analysis

The data of the evaluations were tabulated and tested for their normality by the Shapiro-Wilk test, which indicated a parametric nature for the bond strength and dentin discoloration and non-parametric data for the analysis of the adaptation to the canal walls. Thus, ANOVA and Tukey tests were used for the bond strength and discoloration tests, and Kruskal-Wallis and Dunn tests were used for analysis of adaptation. In addition, the influence of ultrasonic agitation was statistically tested in all evaluations, regardless of the material used, and Student's t-test and Mann-Whitney tests, respectively. For all comparisons, the significance levels were established at 5%.

Results

Bond strength

Table 1 presents the results of the push-out test. Premature failures were not observed in any groups/subgroups. The BIO/UA group exhibited the best bond strengths while the MTAHP group without agitation had the worst. Considering each material with and without treatment, statistically significant differences were observed between the BIO groups; the UA positively influenced their bond strength ($P<0.05$). Comparing the materials submitted or not to UA, significant differences were observed in both conditions, with MTAHP offering significantly lower results than the others ($P<0.05$); no differences were observed between BIO and MTA. Considering only the use or not of UA, significant differences were observed, where the UA proportioned superior results ($P<0.05$). The MTA/UA and BIO groups, with and without UA, presented a predominance of the cohesive failures, and the MTAHP group presented predominance of the cohesive type as could be observed in Table 1; Figure 2 presents the failure types illustration.

Analysis of the interface of adaptation to the dentin wall

Percentage of gaps observed in the sections of the cervical plugs prepared with the repair materials with or without UA are shown in Table 2. Once more the BIO/UA exhibited the optimal interface with adequate marginal adaptation, giving only 1,87% of gaps. The worst results were exhibited by MTAHP without UA which presented 28.58% of maladaptation. The comparisons between groups on both materials and treatment did not show significant differences ($P>0.05$). However, isolating the variables, significant difference was observed between BIO and MTAHP ($P<0.05$). A significant difference was also observed when only the treatment was considered, with UA increasing the adaptation of the materials to the root canal walls ($P<0.05$). Figure 3 shows representative images of specimens from the experimental groups obtained by confocal microscopy.

Analysis of tooth discoloration

The specimens of the control group exhibited no color change (ΔE). The data relating to ΔE for the experimental groups are shown in Table 3. The MTA specimens were the only ones to show discoloration above the clinically perceptible ($\Delta E \geq 3.7$). This occurred after 7 days of observation. Between 15 and 180 days it presented stability, offering the maximum of ΔE at 30 days (7,16). Significant differences between the MTA and the other groups were observed in all evaluated periods ($P<0.05$); the BIO and MTAHP/UA groups took turns as the best results. Representative images of the specimens from each group can be seen in Figure 4.

Discussion

The materials used were quite similar in composition and were all based on tricalcium silicate with similar hydration reaction. The MTA Angelus is a first-generation MTA with slight modifications. The Biodentine and MTA Repair HP are no longer based on Portland cement but on laboratory synthesized calcium silicates, both still include additives such as calcium carbonate, calcium oxide and polymer that enhance the material performance. The radiopacifiers are also different for the materials tested. The MTA Angelus has retained the bismuth oxide of the original formulation while Biodentine and MTA Repair HP include alternative radiopacifiers namely zirconium oxide and calcium tungstate, respectively. Although these materials have already been tested as retro plugs. This simple fact does not respond to possible differences when used as cervical plugs by the aspect of cavity size in relation to agitation / volume of material and aesthetic by use at the cervical level. Considering the results obtained, the null hypothesis suggested was rejected since the ultrasonic agitation favoured better bond strength and marginal adaptation of the materials to the canal walls and altered the tooth discoloration exhibited by the MTA Angelus.

The methods used closely simulated the clinical conditions and the protocols employed were based on the suggestions of the American Association of Endodontists, especially regarding the coronary barrier preparation for regenerative procedures [4]. Still, simulating teeth with incomplete rhizogenesis, the circularity and the cavity diameter were standardized with a Largo Reamer #5 (Dentsply-Sirona). When a restorative material is used, one of its most desirable property is sealing, therefore, it must withstand the forces that may cause its displacement, such as functional pressures, or the application of other restorative materials [12]. In this way, the push-out test has been used as an efficient and feasible method to evaluate the strength of bond strength between dentin/material and material/material [11, 12]. The protocol used for this type of evaluation has been widely described in the literature [11, 12, 20].

The marginal adaptation is a characteristic which is closely related to the bond strength. For this evaluation, a confocal laser scanning microscope was used as employed in large number of previous studies [19, 23, 24]. Previously, a pilot study was carried out not finding differences in the characteristics of the cements when using the flurophore, in agreement with Wiesse et al. [20]. The fluorophore Fluo3 was the chosen fluorescence indicator. Fluo-3 take the place of the commonly employed Rhodamine B once it binds to the calcium ions, which is an element present in the great quantity in the materials based on tricalcium silicate, as pointed out by Jeong et al. [24].

The tooth discoloration was investigated using methods previously described in the literature [25-27]. Even recognizing that the dental substrate employed is not exactly the same used in bond strength and adaptation analyzes, it should be considered that it presents advantages in function of, as opposed to human teeth that have a smaller surface and usually have restorations or caries that can interfere in the color analysis, the bovine teeth provide a larger flat surface area, allowing adequate and standardized color evaluation [26]. In addition, bovine teeth are similar to human teeth in the composition of type I collagen of the organic matrix, which accredits the method for microstructural issues considering that the interaction between radiopacifiers and this protein are blamed for the darkening process [22, 26-28].

Ultrasonic agitation is based on the transmission of micrometric acoustic energy. Such mechanical vibration energy is expended from the insert and transmitted to the material, favouring a greater penetration into the dentinal tubules and a better adaptation of cement/dentin interface [19, 20, 29]. In the present study, bond strength and improvement in dentin adaptation values were observed when ultrasonic agitation was used. It is postulated that ultrasonic agitation produced an increase of the cement pressure against the canal walls favouring a more effective filling of the irregularities, improving adaptation to root canal walls and, consequently, their bonding resistance [19]. Considering this observation, it could be suggested that this better adaptation increases the materials adhesive strength making it superior than its cohesive strength.

Additionally, evaluating the role of the materials independently of the treatment, Biodentine presented results of bond strength and adaptation to the dentine significantly superior to those provided by the MTA Repair HP. It is suggested that the results obtained by Biodentine can be attributed to the more plastic consistency of the material [20, 30].

Regarding dentinal discoloration, the results found in the present study confirms the color stability of the teeth treated with Biodentine and MTA Repair HP, corroborating with findings from previous studies that pointed the color stability of the first material [27, 31]. To date there was no information on the discoloration if any caused by MTA Repair HP. Knowledge of the discoloration mechanisms and it being attributed to the presence of bismuth oxide in materials, the findings in this study were as anticipated. The materials that were bismuth oxide free did not discolour. The novelty lies with the effect of ultrasonic agitation on the discoloration where use of ultrasonic agitation mitigated the discoloration caused by MTA Angelus. Thus, ultrasonic agitation can be suggested as another way of reducing the bismuth oxide induced discoloration. The dentin discoloration caused by the bismuth oxide present in MTA Angelus and its interaction with the dental structures had already been reported [22, 27, 28]. This finding was also observed in the present study, being this the only material to produce a color change higher than 3.7, an index that was perceived as being perceptible to the human eye [32]. However, results of the present study indicate that ultrasonic agitation is capable of positively influencing the color stability of bismuth oxide, consequently of MTA Angelus, an aspect highlighted by the statistically significant difference observed at 15 and 180 days between the MTA groups with and without agitation. Yet, in the observed periods the ultrasonically agitated MTA Angelus did not show color alteration higher than the perceptible values. It is known that discoloration caused by bismuth oxide results during the hydration process in its interaction with strong oxidants such as the amino acids present in the dentin collagen [22]. It is believed that this finding may be related to the reduction of water available for hydration - the result of the warming generated by the ultrasound action, reducing the number of particles reacted with the collagen matrix. However, other investigations are necessary to prove these hypotheses.

Considering the importance of maintaining blood clot integrity and the possible aesthetic compromise provided by reparative materials when used as coronary barrier in regenerative procedures, the findings of the present study suggest that ultrasonic agitation can improve the resistance to displacement of materials and their marginal adaptation; yet, positively influence the color stability of the MTA Angelus. Thus, although more studies are necessary to confirm the hypotheses raised and to evaluate other possible interactions, it can be indicated that the use of ultrasonic agitation during the revascularization procedures presents itself as an accessible and feasible tool for clinical use, capable of increasing the quality of the procedures performed.

Conclusion

It was concluded that the repair materials evaluated were unable to provide total adaptation to the root canal walls, however, the use of ultrasonic agitation provided better bond strength and marginal adaptation of these. Furthermore, the Biodentine and MTA Repair HP cements did not cause dentine discoloration, however the white MTA Angelus promoted it, having its effect reduced by the use of ultrasonic agitation.

Compliance with Ethical Standards

Conflict of Interest: The authors deny any conflicts of interest related to this study.

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Ethical approval: This study was previously approved by Local Ethics Committee (#1.900.129/2017) and were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figures and legends

Figure 1. Experimental design of discoloration analysis methods.

Figure 2. Representative images of failure types observed; A. Cohesive failure (Biodentine with ultrasonic agitation), B. Adhesive failure (MTA Repair HP without ultrasonic agitation), and C. Mixed failure (MTA Angelus withou ultrasonic agitation).

Figure 3. Confocal microscopy of the perimeter of gaps at the interface of the cervical dentin with the repairing material using Fluo-3 with wavelength of 506 nm of excitation and 526 nm of emission. Images made at 10 µm below the surface of the sample with a magnification of 20x. 10 sections of 1µm were obtained. Images recorded in CZI with resolution of 1024 x 1024 pixels using the program ZEN 2012 (Carl Zeiss Microscopy GmbH, Baden-Württemberg, Germany) and saved in TIFF format. Measurement of adaptation defects at the cement/dentin interface (gaps) in the Image J program (National Institute of Health, Bethesda, MD, USA) with 100 µm scale.

Figure 4. Representation of bovine teeth samples filled with MTA Angelus, MTA Repair HP and Biodentine as a function of ultrasonic agitation. The discoloration is evident in the MTA Angelus group without using the ultrasound with color change of the peripheral dentine to the material. The other groups do not show any color changes.

Figure 1

Click here to access/download;Figure;Figure_1.jpeg

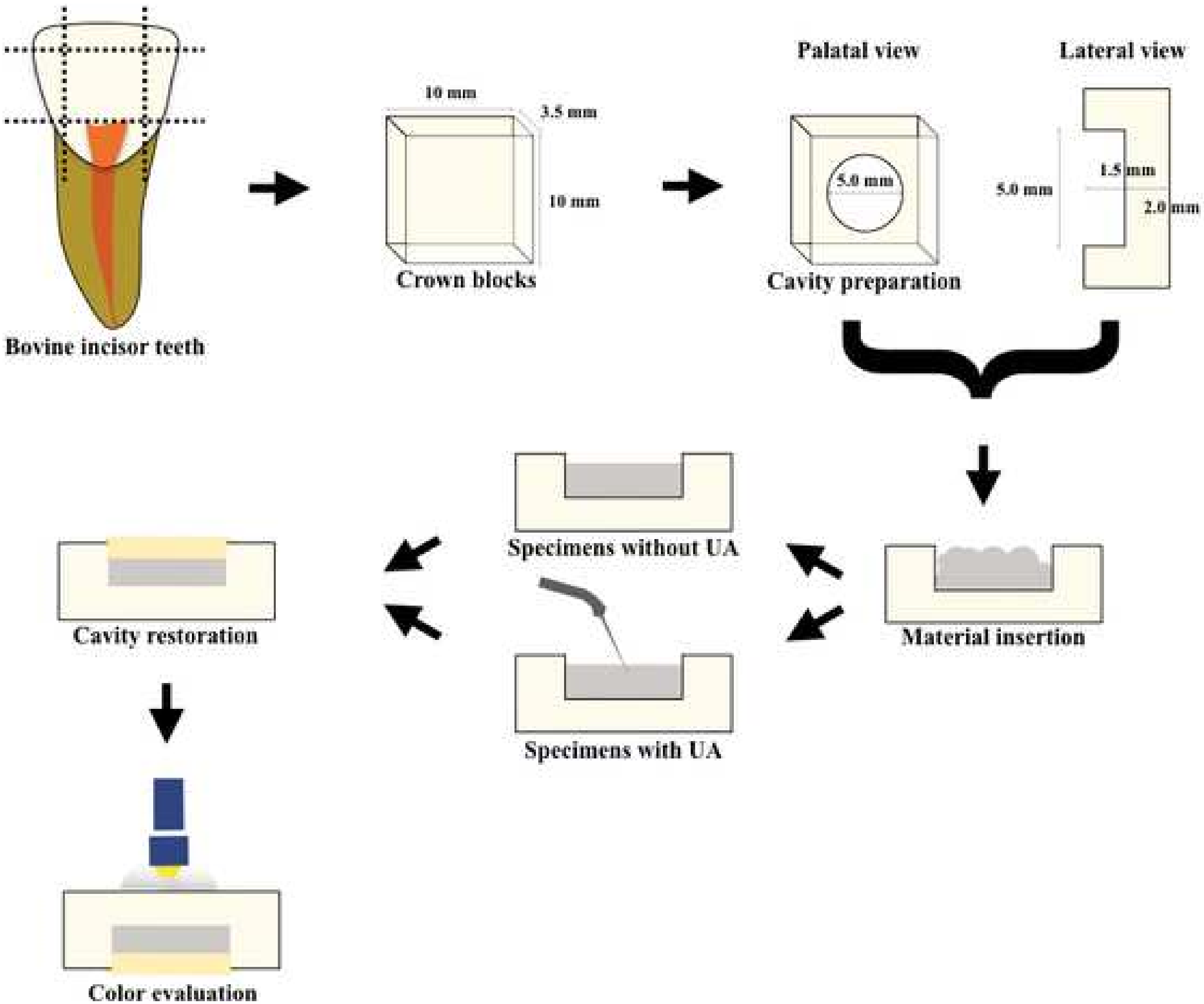


Table 1

Table 1. Bonding strength (MPa) of restorative materials to root dentin as a function of ultrasonic agitation and distribution of failure types (%).

Material	Agitation	Bond strength	Failure type		
		Mean ± sd	Adhesive	Coesive	Mixed
MTA-Angelus	No	6.83 ± 1.99[b,c]	31.25	12.5	56.25
	Yes	9.54 ± 3.35[a,b]	12.5	68.75	18.75
MTA Repair HP	No	2.54 ± 1.26[d]	56.25	37.5	6.25
	Yes	4.13 ± 2.43[c,d]	25	25	50
Biodentine	No	5.86 ± 2.55[c]	6.25	68.75	25
	Yes	12.66 ± 5.92[a]	6.25	68.75	25

[a,b]Different lowercase letters overlapped represent significant differences between the groups of material according to the ANOVA and Tukey tests ($P < 0.05$).

Figure 2

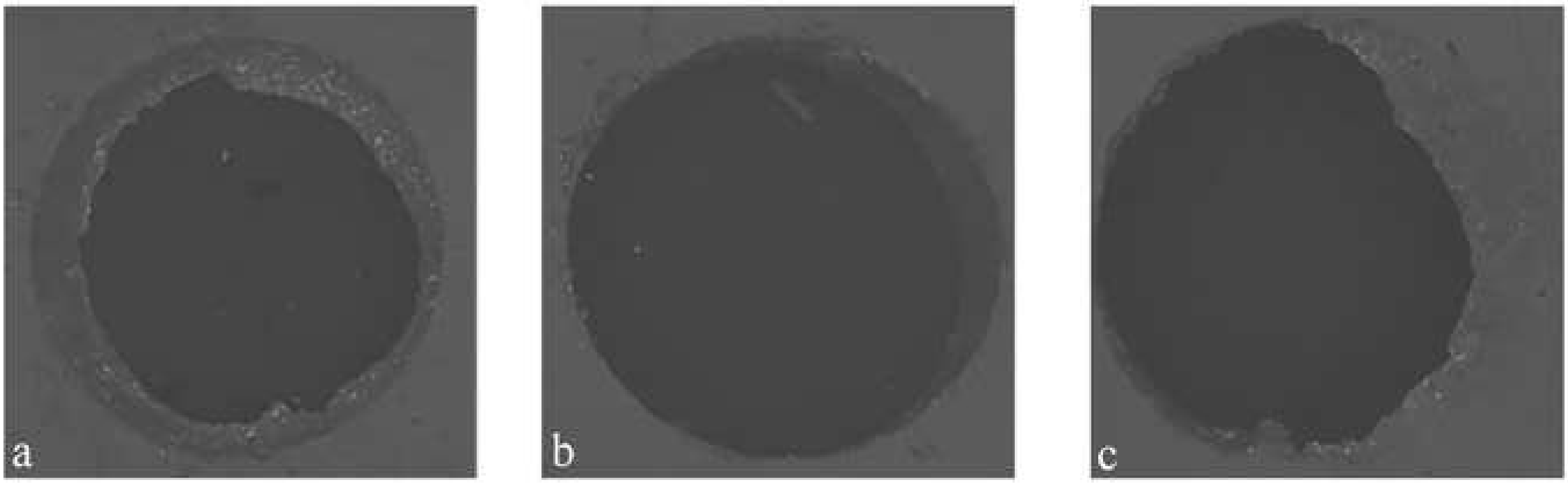
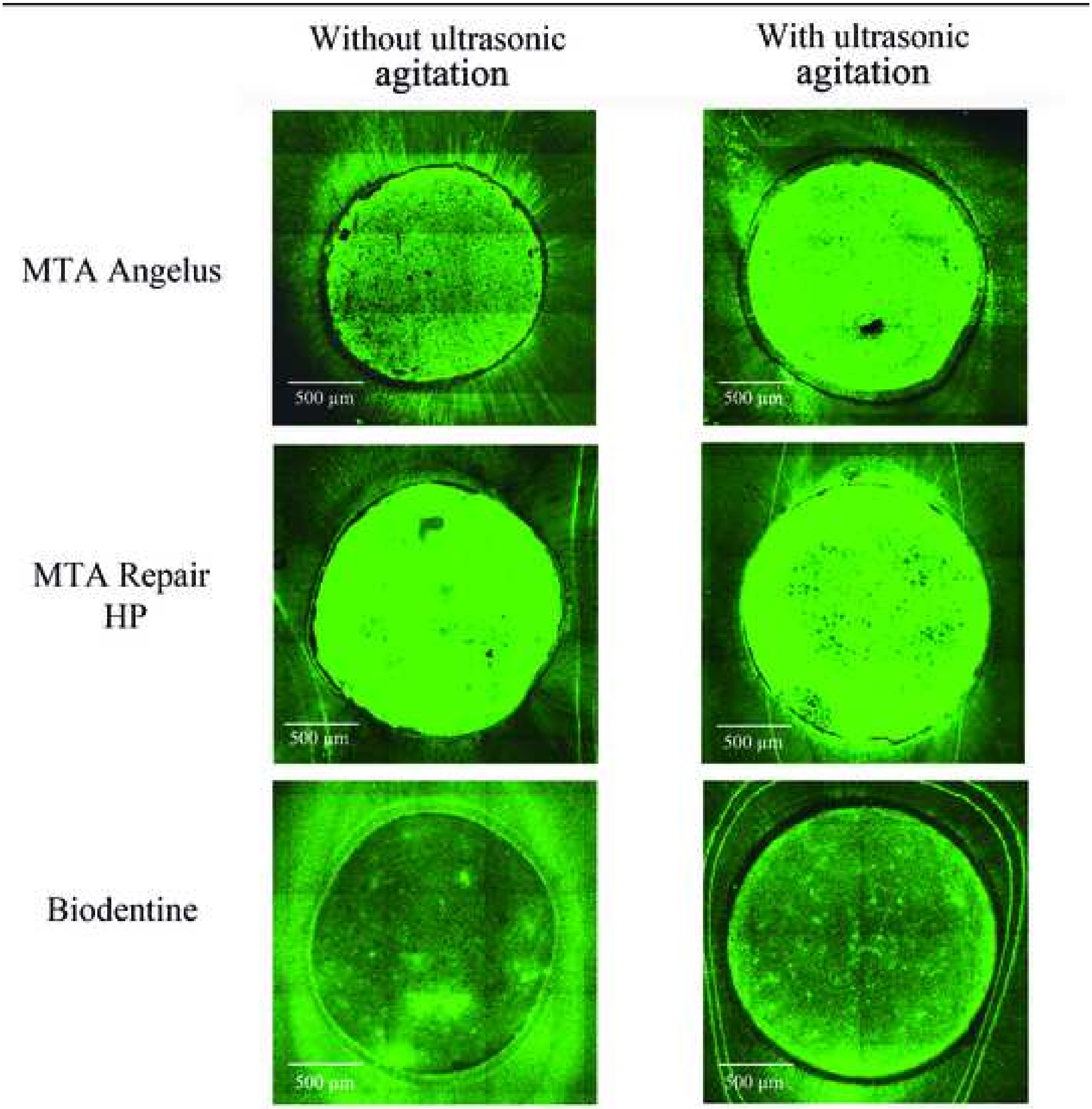


Figure 3

Click here to access/download;Figure;Figure_3.jpg



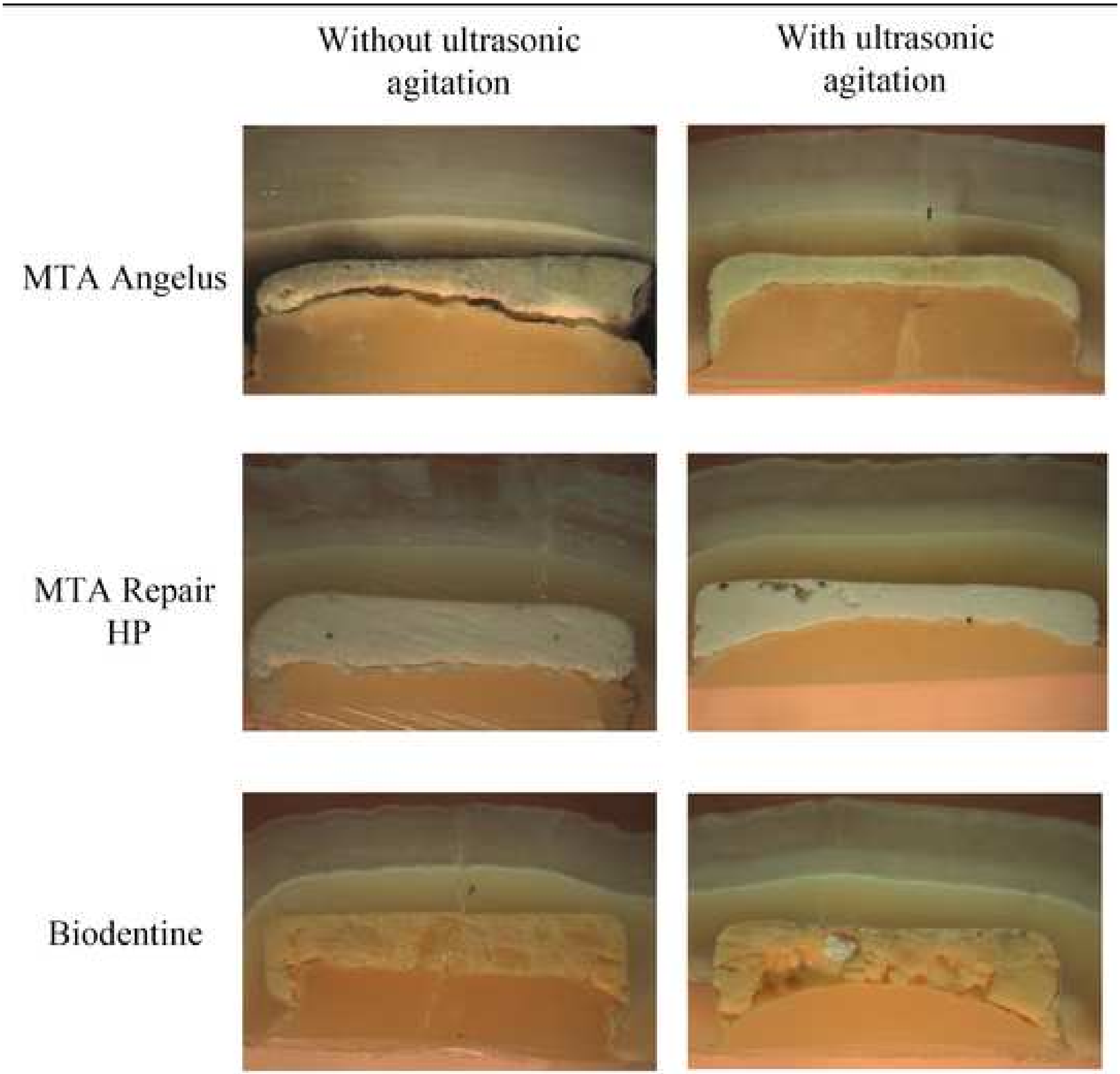

Without ultrasonic agitation
With ultrasonic agitation
MTA Angelus
MTA Repair HP
Biodentine

Table 2

Table 2. Percentage of gaps in relation to the perimeter provided by the repair materials as a function of the ultrasonic agitation.

Material	Agitation	Percentage of gaps Median (min. - max.)
MTA-Angelus	No	11.92 (0.0 – 48.65)[a,A]
	Yes	5.34 (0.0 – 65.47)[a,A]
MTA Repair HP	No	28.58 (8.01 – 63.73)[a,A]
	Yes	17.87 (0.0 – 43.26)[a,A]
Biodentine	No	10.14 (0.0 – 27.56)[a,A]
	Yes	1.87 (0.0 – 16.33)[a,A]

[a,b]Different lowercase letters overlapped represent significant differences between the groups according to the materials according to the Kruskal-Wallis and Dunn tests (*P* <0.05).

Table 3

Table 3. Mean and standard deviation of the discoloration analysis provided by the repair materials as a function of ultrasonic agitation.

Material	Agitation	7 days	15 days	30 days	180 days
		Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
MTA-Angelus	No	4.91 ± 2.85[b]	6.05 ± 4.05[b]	7.16 ± 3.81[b]	6.10 ± 1.79[b]
	Yes	2.11 ± 0.97[a]	4.09 ± 1.64[ab]	2.59 ± 1.36[ab]	2.68 ± 1.18[a]
MTA Repair HP	No	2.77 ± 1.12[ab]	2.70 ± 0.74[ab]	3.26 ± 1.47[ab]	2.60 ± 0.79[a]
	Yes	1.95 ± 1.38[a]	1.75 ± 1.46[a]	1.44 ± 1.33[a]	1.68 ± 0.67[a]
Biodentine	No	1.86 ± 0.87[a]	2.04 ± 0.68[a]	2.72 ± 0.86[ab]	2.33 ± 0.71[a]
	Yes	2.35 ± 0.36[ab]	2.61 ± 0.99[ab]	3.39 ± 0.67[ab]	2.25 ± 1.36[a]

[a,b]Different lowercase letters overlapped represent significant differences between groups, considering each evaluation period, according to the ANOVA and Tukey tests (*P* <0.05).

AUTHOR'S RESPONSE TO EDITOR/REVIEWERS

Dear Prof. Imad About, Ph.D.
Associate Editor of the Clinical Oral Investigations.

Thank you for allowing us to make the changes suggested by the Reviewers in order to qualify this paper for possible publication in the *Clinical Oral Investigations*.

Below are some answers to the Reviewers comments:

Reply to Reviewers # 1

Initially thanks for your considerations and your commendation. Our response for your concerns are listed below:

1. Concern of the Reviewer: Methods: What did the authors use for root canal preparation? diamond bur, gates glidden or lentulo?? Please see page 8-line 46 and page 4-line 8. Is not it difficult to insert the diamond bur up to the middle third of the root? I think, this part is a bit confusing.

Our response: For cervical and middle thirds preparation a Largo Peeso Reamer #5 (Dentsply-Sirona, Ballaigues, Switzerland) was used in long axis of the teeth. Please, apologies for this typing error.

Revised text:
Page 4, Lines 8 and 11: …was carried out, followed by preparation of the cervical and middle thirds with Largo Reamer #5 (Dentsply-Sirona, Ballaigues, Switzerland). After the preparation specimens were evaluated for circularity and standardization of the cavity diameter. Where the Largo Reamer #5 did not enter…

Page 8, Line 52: … and the cavity diameter were standardized with a Largo Reamer #5 (Dentsply-Sirona).

2. Concern of the Reviewer: Table 1-Table 3: I suggest giving mean and standard deviation values in one column in order to make table simple. Please see following examples;
mean ± sd
6.83± 1.99bc
For Table 2:
median (min - max)
11.92 (0 - 48.65) a,A

Our response: Following the guidance of the Reviewers the alterations was performed in Tables 1-3.

Revised text:
Revisions were highlighted in Tables 1-3.

3. Concern of the Reviewer: Could you please insert a scale bar on confocal images? Additionally, the way to be outlined the region of interest (ROI) using the polygonal tool in one of the sections could be stated if possible.

Our response: Regards of the scale bar, it was inserted in Confocal images at Figure 3 (Figure 1 in original submission). With respect to the inclusion of measurement procedures performed in Image J we do not understand that an illustration of it in Figure 3 was indicated; we think that it could turn difficult images comprehension and group comparisons. Whereas, a text better explaining which were Image J tools used was included in the text.

Revised text:

Page 5, Line 53: The total perimeter of the canals and the length of gaps were determined in millimetres using the software polygonal and linear tools, respectively.

4. Concern of the Reviewer: Did you observe premature failure during push-out bond strength test?

Our response: No premature failures were observed during sample preparation for the push-out bond strength test or during it. Considering this an important information a sentence stating it was inserted in Results chapter.

Revised text:
Page 7, Line 36 and 37: Table 1 presents the results of the push-out test. Premature failures were not observed in any groups/subgroups. The BIO/UA…

5. Concern of the Reviewer: I suggest adding a photograph of representative dentin discs showing cohesive failure or other failure types, if it is available. Furthermore, authors could state the percentage of failure types for each group (page 7-line 45).

Our response: Considering the asked revision, the percentages of failures types were inserted in Table 1 for better comprehension. Related to the failure types illustration, images were included (Figure 2).

Revised text:
Following the guidance of the Reviewers Table 1 was improved.
Page 7, Line 49 to 51: The MTA/UA and BIO groups, with and without UA, presented a predominance of the cohesive failures, and the MTAHP group presented predominance of the cohesive type as could be observed in Table 1. Figure 2 presents the failure types illustration.

6. Concern of the Reviewer: Discussion: Please improve discussion with the explanation of why most of the groups exhibited cohesive type of failure.

Our response: Related to this point, observed in UA and Biodentine groups, we understand that the cohesive failures observed predominantly in the groups under ultrasonic agitation could be provided by material better adaptation to the root canal walls. This adaptation increases the adhesive strength making it superior than the cohesive strength of the materials.

Revised text:
Page 9, Line 29 to 35: It is postulated that ultrasonic agitation produced an increase of the cement pressure against the canal walls favouring a more effective filling of the irregularities, improving adaptation to root canal walls and, consequently, their bonding resistance [19]. Considering this observation, it could be suggested that this better adaptation increases the materials adhesive strength making it superior than its cohesive strength.

7. Concern of the Reviewer: Are doi addresses necessary? Please check the journal reference style.
Following is an example of the reference of journal article;
Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325-329.

Our response: Taking account the Instruction for Authors accessed in December 26^{th} the most complete example available is:

Journal article:
Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Thus, considering the Instructions we think that was indicated to state the DOI number for all listed References.

Revised text:

Not applicable.

Reply to Reviewer # 2

Thanks for considering this study clinically relevant and to permit us to explain our typing and methodological options.

1. Concern of the Reviewer: My main major comment is: why didn't researchers consider this study in two separate parts (adhesive/interface analysis and discoloration). Too many parameters in one paper made the text confusing.

Our response: Honestly, at first, we thought of writing two papers considering the differences between the methodologies employed. However, when we started writing the first of them, we understood that, in clinical terms, one analysis could reinforce or compromise the other. We believe that it would be ambiguous to conclude that the ultrasonic agitation would be favorable in terms of adaptation and bond strength if it would compromise the color stability of the materials. Based on the above, we chose to write a single paper, even more complex, but more complete and with greater clinical impact and citation potential. We hope that this explanation could make sense, in any case, we are open to suggestions.

Revised text:
Not applicable.

2. Concern of the Reviewer: Furthermore, one part used extracted teeth, the second part used bovine teeth. The first part: the method is understandable; the second part (discoloration part) needs a schematic drawing to be able to make the method understandable.

Our response: We agree the comprehension improvement that an Experimental Design could produce in method description of Discoloration Evaluation. Thus, an image presenting it sequence was included (Figure 1).

Revised text:
Not applicable.

3. Concern of the Reviewer: If the authors could divide the paper into two separate parts, they could add SEM pictures from the failed samples.

Our response: This point was also commented by the Reviewer #1; representative images of the failure types were now present in paper as Figure 2.

Revised text:
Not applicable.

4. Concern of the Reviewers: …could make a more specific discussion and could give more knowledge.

Our response: We understand the point of view highlighted by the Reviewer and tried to better separate the Discussion related to each studied subject. Some information was really mixed in the original submission. We think that after corrections and inclusion asked during this review that confusion was mitigated.

Revised text:
Not applicable, spread throughout the discussion chapter.

5. Concern of the Reviewers: Abstract: needs revision. Method does not include the details of the study groups;

Our response: During this paper preparation, the most difficult part to write was the Abstract, mainly considering the recommendation in Authors Instructions to respect the number of 250 words. Thus, considering the word limitation we opted to describe the groups in the Objective section, it was stated as: *"Objectives: The effect of ultrasonic agitation (UA) on bond strength and adaptation of cervical plugs prepared with MTA-Angelus (MTA), MTA Repair HP (MTAHP) and Biodentine (BIO) was evaluated."* – it intends to presents MTA, MTA/UA, MTAHP, MTAHP/UA, BIO, and BIO/UA. We wish to better describe this group division; however, the word limitation makes such inclusion almost impossible.

Revised text:
Not applicable.

6. Concern of the Reviewer: Discussion: needs discussion of the differences between the test samples (bovine and human teeth).

Our response: As requested by the Reviewer an extended Discussion about the method option for bovine blocks and its advantages was inserted in the text.

Revised text:
Page 9, Line 12 to 22: The tooth discoloration was investigated using methods previously described in the literature [25-27]. Even recognizing that the dental substrate employed is not exactly the same used in bond strength and adaptation analyzes, it should be considered that it presents advantages in function of, as opposed to human teeth that have a smaller surface and usually have restorations or caries that can interfere in the color analysis, the bovine teeth provide a larger flat surface area, allowing adequate and standardized color evaluation [26]. In addition, bovine teeth are similar to human teeth in the composition of type I collagen of the organic matrix, which accredits the method for microstructural issues considering that the interaction between radiopacifiers and this protein are blamed for the darkening process [22, 26-28].