UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

LAUTER ESTON PELEPENKO TEIXEIRA

AVALIAÇÃO DAS PROPRIEDADES FÍSICO-QUÍMICAS E ANTIMICROBIANAS DE BIOMATERIAIS À BASE DE SILICATO TRICÁLCIO

PHYSICOCHEMICAL PROPERTIES AND ANTIMICROBIAL EVALUATION OF TRICALCIUM SILICATE-BASED BIOMATERIALS

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Clínica Odontológica, na Área de Endodontia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Dental Clinic, in Endodontics area.

Orientadora: Prof.ª Dr.ª Marina Angélica Marciano da Silva

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PROF. DR. MARCO ANTÔNIO HUNGARO DUARTE

PROFª. DRª. BRENDA PAULA FIGUEIREDO DE ALMEIDA GOMES

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RESUMO

Novos cimentos reparadores de alta plasticidade foram introduzidos melhorando a sua manipulação e inserção, sendo a estabilidade de cor, à longo prazo, outro importante fator a ser levado em consideração. O objetivo do estudo foi avaliar tais materiais quanto às propriedades físico-químicas e atividade antimicrobiana. Cinco composições foram testadas: MTA Repair HP (Angelus), MTA Flow (Ultradent), Biodentine (Septodont), MTA Flow contendo 5% de óxido de zinco (ZnO) e um cimento experimental composto por silicato tricálcico, óxido de bismuto, 5% de ZnO e em seu líquido, água destilada e éter policarboxílico modificado. A hipótese testada é que o ZnO usado como aditivo de cimento teria efeito duplo, primeiro, como inibidor da alteração de cor e, segundo, aumentaria as propriedades antimicrobianas dos cimentos. A alteração de cor (ΔE) e a luminosidade (L) dos materiais foram avaliadas em dentes bovinos (n = 5), antes, 24 horas, 28 dias e 90 dias após o contato com a dentina. Foram testadas as propriedades de escoamento ISO 6876 e volumétrico, tempo de presa, radiopacidade, alteração volumétrica, liberação de cálcio e pH. A atividade antimicrobiana dos cimentos testados foi avaliada através do teste de difusão em ágar (halos) e método de contato direto (turbidez) contra *Enterococcus faecalis* e *Porphyromonas gingivalis*. A análise estatística dos dados foi realizada utilizando Kruskal-Wallis, ANOVA e teste T ($p<0,05$). Todos os cimentos apresentaram alteração de cor, com maiores valores de L sendo encontrados para o MTA Repair HP, Biodentine e o cimento experimental em comparação ao MTA Flow ($p<0,05$). O ZnO reduziu o escurecimento dental causado pelo MTA Flow, porém sem diferença estatística significante. Foi detectada a migração dos íons bismuto e silício na dentina. Todos os cimentos testados apresentaram radiopacidade adequada e pH alcalino. O Biodentine e o cimento experimental apresentaram a maior liberação de cálcio e alteração volumétrica ($p<0,05$). MTA Repair HP apresentou o menor tempo de presa. O MTA Repair HP e Biodentine apresentaram os maiores valores volumétricos de preenchimento da cavidade central no teste de escoamento volumétrico ($p<0,05$). Todas as formulações testadas apresentaram propriedades favoráveis e a adição de ZnO não alterou as propriedades do MTA Flow. Não foram observados halos de inibição para qualquer material testado. O controle utilizando clorexidina apresentou halos médios de 19,1 mm contra *Enterococcus faecalis* e 42,2 mm para *Porphyromonas gingivalis* ($p<0,05$). Todos os materiais testados apresentaram níveis significativamente mais altos de turbidez quando comparados aos controles ($p <0,05$). A clorexidina apresentou os menores valores de turbidez em todos os períodos testados ($p <0,05$). Concluiu-se que todos os cimentos exibiram alteração de cor, sendo que o MTA Flow apresentou os menores valores de L. Todos os materiais testados apresentaram características físico-químicas adequadas em diferentes níveis nas metodologias testadas. Nenhum dos materiais apresentou efeito antimicrobiano satisfatório, sugerindo que a desinfecção adicional em locais onde esses materiais são utilizados clinicamente é necessária.

Palavras-chave: Endodontia. Biomateriais. Silicato tricálcio. Óxido de Zinco.

ABSTRACT

Novel high plasticity repair cements were introduced, improving its handling and insertion, being color stability, in long term, another important factor to be considered. The aim of the study was to evaluate such materials regarding the physicochemical properties and antimicrobial activity. Five compositions were tested: MTA Repair HP (Angelus), MTA Flow (Ultradent), Biodentine (Septodont), MTA Flow containing 5% zinc oxide (ZnO) and an experimental cement composed of tricalcium silicate, bismuth oxide, 5% ZnO and in its liquid, distilled water and a modified polycarboxylic ether. The hypothesis tested is that the ZnO used as a cement additive would have a double effect, first, as a color change inhibitor and secondly, it would increase the antimicrobial properties of the cements. The color change (ΔE) and the luminosity (L) of the materials were evaluated in bovine teeth (n = 5), before, 24 hours, 28 days and 90 days after contact with dentin. The properties of ISO 6876 and volumetric flow, setting time, radiopacity, volumetric change, calcium release and pH were tested. The antimicrobial activity was evaluated using agar diffusion test (halos) and direct contact method (turbidity). For the antimicrobial essays, storage was at 37°C, carried out in triplicates and had the bacterial viability verified. As a control, 2% gel chlorhexidine filter paper discs were used. Statistical analysis of the data was performed using Kruskal-Wallis, ANOVA and T-test ($p<0.05$). All the cements presented color changes, with higher values of L being found for MTA Repair HP, Biodentine and experimental cement when compared to MTA Flow ($p<0.05$). ZnO reduced dental darkening caused by MTA Flow, but without significant difference. The migration of the bismuth and silicon ions into the dentin was detected. All tested cements presented proper radiopacity and an alkaline pH. Biodentine and experimental cement showed the highest calcium ion release and volumetric change ($p<0.05$). MTA Repair HP showed the shortest setting time. MTA Repair HP and Biodentine presented the highest volumetric flow values of the central cavity in the volumetric flow test ($p<0.05$). All tested formulations presented favorable properties and the addition of ZnO did not alter the properties of the MTA Flow. No inhibition halos were observed for any material tested. The control using chlorhexidine showed average halos of 19.1 mm against *Enterococcus faecalis* and 42.2 mm for *Porphyromonas gingivalis* ($p <0.05$). All materials tested showed significantly higher levels of turbidity when compared to controls ($p <0.05$). Chlorhexidine showed the lowest values of turbidity in all the periods tested ($p <0.05$). It was concluded that all cements exhibited a color change, and the MTA Flow presented the lowest values of L. All tested materials presented adequate physicochemical characteristics at different levels in the methodologies tested. None of the materials showed a satisfactory antimicrobial effect, suggesting that additional disinfection in places where these materials are used clinically is necessary.

Key-words: Endodontics. Biomaterials. Tricalcium Silicate. Zinc oxide.

SUMÁRIO

1 INTRODUÇÃO

O agregado trióxido mineral (MTA), cimento à base de silicato tricálcio, tem sido amplamente utilizado na Endodontia em procedimentos reparadores como o selamento de perfurações (Hashem e Hassanien 2008; Estrela et al., 2018), o tratamento de fraturas radiculares (Roig et al.2011), capeamentos pulpares (Farsi et al., 2007; Linu et al. 2017), apicificação (Simon et al., 2007), retrobturações em cirurgias apicais (Baek et al., 2005) e como barreira cervical em procedimentos de revascularização (Banchs e Trope 2004; Kahler et al., 2016). Desde o seu desenvolvimento na década de 90, o MTA tem apresentado resultados clínicos satisfatórios no reparo tecidual (Torabinejad et al., 1995). A aplicação clínica do MTA envolve o contato direto com os tecidos e fluidos biológicos (Nekoofar et al., 2010). Assim, alterações físicas, químicas e biológicas são previstas (Camilleri et al., 2013; Guimarães et al., 2015).

Os cimentos endodônticos reparadores idealmente devem apresentar características como: estabilidade de cor e dimensional (Camilleri e Mallia 2011; Marciano et al., 2014, 2017); radiopacidade, para acompanhamentos futuros por exames de imagem (Islam et al., 2006); tempo de presa adequado para sua manipulação e não solubilizar quanto em contato com fluídos biológicos (Fridland e Rosado 2003; Cavenago et al., 2014); possuir escoamento favorável para um adequado selamento (Duarte et al., 2012; Duque et al., 2018); apresentar ações químicas e biológicas que resultem em alcalinidade e liberação de íons cálcio (Duarte et al., 2003); bioatividade (Gandolfi et al., 2010); permitir adesão celular (Balto 2004); biocompatibilidade (Holland et al., 2002; Camilleri et al., 2004); e, por fim, propriedades antimicrobianas satisfatórias (Tanomaru-Filho et al., 2007). Diversas das propriedades ideais para um cimento reparador foram contempladas pelo MTA, porém outras ainda necessitam de aprimoramento como a sua estabilidade de cor, dificuldade de manipulação e escoamento (Duarte et al., 2012; Marciano et al., 2016; 2017).

O MTA apresenta potencial para causar alteração de cor promovendo o escurecimento dentário, quando em contato com a dentina, o que restringe a sua utilização em regiões estéticas (Belobrov e Parashos 2011; Camilleri 2014; Marciano et al. 2014). O óxido de bismuto, agente radiopacificador presente em sua composição, é apontado como o elemento responsável pelo escurecimento (Marciano et al., 2017). O óxido de bismuto quando em contato com um agente oxidante, como o sangue, colágeno da dentina ou remanescentes de hipoclorito de sódio, se desestabiliza formando um precipitado enegrecido (Camilleri 2014; Marciano et al. 2014). Radiopacificadores alternativos como o tungstato de cálcio e o óxido de zircônio têm sido estudados para substituir o óxido de bismuto (Duarte et al., 2009; Camilleri et al., 2011; Cutajar et al., 2011). Tais agentes não causam manchamento dentário (Marciano et al., 2015), porém, há estudos mostrando que para obter uma radiopacidade similar ao óxido de bismuto seria necessário utilizar altas proporções destes componentes (Duarte et al., 2009). Por outro lado, o óxido de bismuto apresenta alto peso molecular, permitindo assim a sua utilização em menor volume (Duarte et al., 2009). Foi demonstrado que a adição de radiopacificadores em quantidades maiores à matriz do cimento de silicato tricálcio resulta em alterações nas propriedades do material, afetando tanto a hidratação quanto a presa do cimento (Camilleri 2008; Camilleri et al., 2011; Tanomaru-Filho et al., 2012). Com o objetivo de inibir o escurecimento dental do MTA causado pelo óxido de bismuto, sem a substituição deste, foi proposta a incorporação de substâncias que impeçam a sua desestabilização (Marciano et al., 2017).

O óxido de zinco (ZnO) apresentou resultados satisfatórios em estudo prévio (Marciano et al., 2017), quando as proporções de 5%, 15% e 45% foram acrescentadas ao MTA. A concentração de 5% de ZnO foi a mínima necessária para que ocorresse a inibição de alteração de cor, sem afetar a radiopacidade, solubilidade, pH, tempo de presa e liberação de íons cálcio e a resposta tecidual do MTA (Marciano et al., 2017).

A consistência do MTA é uma propriedade também discutida. A textura arenosa e a baixa viscosidade dificultam a sua inserção clínica. Tanto o MTA quanto o silicato de cálcio, na sua forma pura, apresentam escoamento baixo, interferindo no correto preenchimento em determinados casos. A adição de diferentes componentes vem sendo estudada e cimentos de alta plasticidade desenvolvidos com o objetivo de melhorar tais características (Holland et al., 2007; Duarte et al., 2012).

Um cimento reparador de alta plasticidade recentemente introduzido no mercado foi o MTA Repair HP (MTA High Plasticity, Angelus, Londrina, Paraná, Brasil). A formulação apresenta biocompatibilidade, biomineralização, tempo de presa, radiopacidade e solubilidade semelhantes ao MTA Angelus (Cintra et al., 2017; Ferreira et al., 2019), com uma resistência de união cimento/dentina e escoamento maiores em relação a formulação original (Silva et al., 2016, Ferreira et al., 2019). O MTA Repair HP é composto por silicato tricálcio, silicato dicálcio, aluminato tricálcio, óxido de cálcio e tungstato de cálcio e, em seu líquido, água destilada e polyvinylpyrrolidone. Como principais alterações nesta formulação em relação ao MTA Angelus original estão a incorporação de agente plastificante ao líquido e a substituição do radiopacificador anteriormente utilizado, óxido de bismuto, por tungstato de cálcio.

Outra formulação que propõe uma substituição do óxido de bismuto é o Biodentine (Septodont, Saint Maur des Fossés, França). Este cimento de alta plasticidade, em apresentação pó/líquido contém silicato tricálcio, silicato dicálcio, óxido de cálcio, carbonato de cálcio e óxido de zircônio como agente radiopacificador. Já o líquido contém cloreto de cálcio para reduzir o tempo de presa e polímeros hidrossolúveis à base de policarboxilato com intuito de aumentar a plasticidade do material (Camilleri 2013). O desenvolvimento deste cimento buscou agregar a biocompatibilidade e bioatividade dos cimentos à base de silicato de cálcio com propriedades de tempo de presa curto e alta resistência, resultado da baixa proporção água/cimento devido a adição de polímero à base de água. Estudos mostram que o Biodentine é biocompatível (Nowicka et al., 2013) e apresenta bioatividade (Camilleri et al., 2013), bem como resultados clínicos semelhantes ao MTA (Nowicka et al., 2013; Katge e Patil 2017).

Também em apresentação pó/líquido, o MTA Flow (Ultradent, Utah, Estados Unidos) manteve o óxido de bismuto como radiopacificador, em uma formulação de alta plasticidade. Além dessa substância, este cimento é composto por silicato tricálcio e silicato dicálcio. Seu líquido é composto por gel à base de silicone e água destilada. Como um diferencial na apresentação deste material, a sua consistência permite ser levada ao local de aplicação por meio de seringa e agulha. Essa forma de inserção é inovadora em relação aos demais cimentos disponíveis, o que pode facilitar a sua utilização em diversos procedimentos reparadores. Este material apresenta adequada biocompatibilidade e indução de biomineralização (Bueno et al., 2019). Em relação às propriedades físico-químicas, esse material apresentou alcalinidade, radiopacidade, solubilidade e bioatividade adequadas (Guimarães et al., 2017). Devido a presença de óxido de bismuto em sua composição, postula-se que pode ocorrer alteração de cor quando em contato com a dentina. Embora este cimento apresente uma tonalidade acinzentada, seu escurecimento pode ser intensificado pela reação do bismuto com agentes oxidantes, resultando no manchamento dentário. Neste sentido, a adição de ZnO poderá inibir tal reação, como previamente demonstrado (Marciano et al., 2017).

Neste trabalho é proposta uma formulação de cimento reparador com éter policarboxílico modificado, adicionado no intuito de se obter uma melhor plasticidade, e o óxido de bismuto associado ao ZnO para promover uma alta radiopacidade sem alteração de cor, foi um dos objetivos desse trabalho. Esse cimento foi composto por silicato tricálcio (80%), óxido de bismuto (20%), óxido de zinco (5% em peso, como aditivo) e, em seu líquido adicionados em volume, água destilada (95%) e éter policarboxílico modificado (5%). O ZnO adicionado na composição do cimento experimental buscou, além de impedir a alteração de cor causada pelo óxido de bismuto, investigar um possível efeito benéfico em relação às propriedades antimicrobianas dessa formulação. O ZnO é reportado na literatura como tendo atividade antimicrobiana, propriedade altamente desejável em materiais endodônticos reparadores (Sevinç e Hanley 2010). Além disso, a associação de um agente plastificante polimérico à formulação do líquido parece ser benéfica em relação a facilidade de inserção desse material no sítio a ser tratado. Neste sentido, a adição de ZnO a materiais contendo bismuto poderia ser uma alternativa viável para prevenir a alteração de cor, sem retirar o bismuto da composição devido à sua excelente radiopacidade proporcionada. Além disso, polímeros solúveis em água foram utilizados com sucesso em estudos anteriores com o objetivo de melhorar as características de manipulação do MTA e estão presentes na composição dos materiais testados, fornecendo alta plasticidade a estes cimentos (Camilleri 2009; Camilleri e Mallia 2011; Camilleri et al., 2013). Supostamente, a adição de polímeros solúveis em água não altera as características de hidratação do cimento, uma vez que tal processo é dependente da reação das partículas de pó com a água constante no líquido da formulação (Camilleri 2009).

O objetivo deste estudo foi avaliar as propriedades físicas (alteração de cor, alteração de volume, radiopacidade, tempo de presa, escoamento padrão e volumétrico, liberação de cálcio e pH) e antimicrobianas em cimentos à base de silicato tricálcio presentes no mercado (MTA Repair HP, Biodentine e MTA Flow). Além disso, foi avaliada a incorporação de ZnO à composição original do MTA Flow para inibição da descoloração dentária e avaliar possíveis diferenças em relação às suas propriedades físicas e antimicrobianas após essa adição. Foi também proposto o desenvolvimento de uma formulação experimental de um cimento de alta plasticidade, agregando o óxido de bismuto ao ZnO em uma apresentação de elevada fluidez. A divisão da apresentação dos resultados seguiu as normas do método alternativo de apresentação (Elaboração e Normalização de Teses e Dissertações da FOP/Unicamp, 2015), em formato de três (3) artigos, divididos de acordo com cada característica avaliada.

2 ARTIGOS

2.1 Colour stability and elemental migration of tricalcium silicate-based dental biomaterials in dentine

Artigo submetido ao periódico: *Clinical Oral Investigations* (Anexo 01)

Lauter Eston Pelepenko, Fernanda Romeiro De Gennaro, Marco Antonio Hungaro Duarte, Josette Camilleri, Adriana de Jesus Soares, Marina Angélica Marciano

Colour Stability and Elemental Migration of Tricalcium Silicate-Based Dental Biomaterials in Dentine

Lauter Eston Pelepenko[1], Fernanda Romeiro De Gennaro[1], Marco Antonio Hungaro Duarte[2], Josette Camilleri[3], Adriana de-Jesus-Soares[1], Marina Angélica Marciano[1]

[1] Department of Restorative Dentistry, School of Dentistry of Piracicaba, State University of Campinas, Piracicaba, São Paulo, Brazil.

[2] Department of Dentistry, Endodontics and Dental Materials, School of Dentistry of Bauru, University of São Paulo, Bauru, São Paulo, Brazil.

[3] College of Medical Sciences, Dental School, University of Birmingham, Birmingham, United Kingdom.

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Correspondence author:

Marina Angélica Marciano

Department of Restorative Dentistry, School of Dentistry of Piracicaba, State University of Campinas, Piracicaba, São Paulo, Brazil.

Av. Limeira, 901, Areião, CEP 13414-903, Piracicaba-SP

Telephone: +55 19 2106 5343

marinama@unicamp.br

Colour Stability and Elemental Migration of Tricalcium Silicate-Based Dental Biomaterials in Dentine

Abstract

Objective Evaluate colour stability, chemical element migration in contact with dentine of calcium silicate-based cements and evaluate whether the addition of zinc oxide (ZnO) to formulations containing bismuth oxide intending to prevent dental discolouration.

Materials and methods Thirty-five bovine teeth were prepared and filled with MTA Repair HP, Biodentine, MTA Flow, MTA Flow+5% of ZnO and an experimental biomaterial. Teeth were sealed with composite and stored in separate flasks immersed in tap water at 37ºC. Colour assessment was performed with a spectrophotometer before filling, 24 hours, 28 days and 90 days after filling. Colour change (ΔE) and luminosity (L) were calculated. Representative samples were sectioned for stereomicroscopy. Elemental mapping in scanning electron microscope (SEM) and energy dispersive spectroscopy (EDS) was evaluated. Statistical analysis was performed using nonparametric Kruskal–Wallis and Dunn test ($p<0.05$).

Results Higher L values were found for MTA Repair HP, Biodentine and experimental biomaterial in comparison to MTA Flow at 90d ($p<0.05$). The addition of ZnO inhibited dental staining of MTA Flow after 90d of contact with dentine. The mapping of elements revealed migration of bismuth and silicon in dentine.

Conclusions MTA Flow exhibited potential for dental discoloration, which was visually inhibited by the ZnO addition, but without significant difference. Experimental biomaterial containing bismuth oxide and ZnO, had similar luminosity values to that of MTA Repair HP containing calcium tungstate and Biodentine containing zirconium dioxide as radiopacifiers.

Clinical relevance: The bismuth oxide containing material MTA Flow, has potential for dental discoloration, which is inhibited by addition of ZnO. MTA Repair HP and Biodentine were tested with satisfactory L values.

Key words Discolouration · Dental material · Bismuth oxide · Zinc oxide (ZnO)

Introduction

Biomaterials based on tricalcium silicate have been widely used for endodontic therapy since the 1990s, when mineral trioxide aggregate (MTA) was introduced [1]. The tricalcium silicate-based materials are an important addition to the range of endodontic materials due to their interaction with the host environment and their optimal biological properties. The main shortcoming with the use of MTA is its poor handling properties and the susceptibility to tooth discoloration which is caused by the interaction of bismuth oxide in MTA with the dental hard structures [2] and reagents used during root canal therapy [3, 4].

The radiopacifier bismuth oxide contained in MTA was shown to be implicated in the interactions with dentine and other components, resulting in migration of ions into the dentine and dental staining [2, 3, 5]. Novel endodontic biomaterials were introduced with alternative radiopacifiers to prevent dental discolouration. MTA Repair HP (MTA High Plasticity, Angelus, Londrina, Paraná, Brazil) contains calcium tungstate while Biodentine (Septodont, Saint-Maur-des-Fossés, France) contains zirconium dioxide as radiopacifiers [6]. Studies have demonstrated that MTA Repair HP presents biocompatibility, cytotoxicity and bio-mineralization similar to those of MTA Angelus [6, 7]. Despite the promising biological results of MTA Repair HP, there are no studies demonstrating its colour stability. On the other hand, the colour stability of Biodentine has been widely demonstrated [8–10].

Another biomaterial recently introduced was MTA Flow (Ultradent Products Inc., South Jordan, UT). This material is composed of tricalcium and dicalcium silicate and bismuth oxide, being available in both powder and water-based gel forms. This formulation can be prepared at variable consistencies, including a thin consistency, allowing it to be delivered with syringe and needle, which facilitates its insertion during certain procedures. MTA Flow showed alkalinizing capability, low solubility, adequate radiopacity, biocompatibility and induction of biomineralization [11, 12]. No studies have yet evaluated its colour stability. This material itself is grey in colour, but the presence of bismuth oxide in its composition is a factor which may cause discolouration after contact with the dentine [2]. The addition of zinc oxide (ZnO) to MTA at 5%, 15% and 45% prevented dental discolouration caused by bismuth oxide, with 5% being the minimal required for inhibition without significant materials' properties alterations [13]. It is possible that the addition of ZnO to MTA Flow cement can prevent an expected darkening of the tooth. A similar addition is being tested in a prototype biomaterial based on tricalcium silicate.

The aim of the study is to evaluate colour stability and elemental migration in dental structures filled with the reparative cements, namely, MTA Repair HP, Biodentine and MTA Flow and to test the addition of ZnO to MTA Flow and an experimental formulation. The hypothesis tested is that, first, the MTA Flow, containing bismuth oxide, presents dental discolouration which is inhibited by addition of 5% ZnO, and, secondly, the cements containing alternative radiopacifiers does not present dental staining.

Materials and methods

Specimen Preparation

A total of thirty-five bovine teeth were selected for this study, all without stains or irregularities in the crown [2]. They were cleaned and sectioned with a 0.3-mm diamond disc (Isomet, Buehler, Lake Bluff, Illinois, USA) to obtain enamel-dentine blocks measuring 10 × 10 mm. The thickness of each block was standardized at 3.5 ± 0.1 mm and checked with a thickness gauge. A cavity with a diameter corresponding to 5.0 mm in diameter and 1.5 mm in depth was prepared at the centre of the dentine surface with a high-speed diamond bur (Model 4054, Medical Burs Sorensen, São Paulo, SP, Brazil). The specimens were then immersed in 1% sodium hypochlorite for 30 minutes, washed with distilled water and placed in 20% EDTA (pH 7.7) for 2 minutes. Next, the specimens were irrigated with a final flush of distilled water before being dried with gauze and then they were randomly divided into 5 groups (n = 5). Complete materials composition is provided in Table 1, as follows:

- MTA Repair HP (Angelus, Londrina, PR, Brazil);
- Biodentine (Septodont, Saint-Maur-des-Fossés, France);
- MTA Flow (Ultradent, Ultradent Products Inc., South Jordan, UT);
- MTA Flow (Ultradent, Ultradent Products Inc., South Jordan, UT) with 5% ZnO (ZnO, Sigma-Aldrich, St. Louis, Missouri, USA);
- Experimental biomaterial composed of 80% tricalcium silicate (SARL Mineral Research Processing, Garnier, France), 20% bismuth oxide (BiO, Sigma-Aldrich), 5% ZnO (ZnO, Sigma-Aldrich), distilled water and modified polycarboxylic ether.

The cavities were filled with the biomaterials, whereas a positive control was filled with triple antibiotic paste (n = 5) and a negative control samples were evaluated without biomaterial (n = 5). MTA Repair HP and Biodentine were mixed according to their manufacturer's instructions. In MTA Flow groups, the material was mixed until having a thick consistency (0.26 g of powder to 2 drops of liquid). In MTA Flow + ZnO group, a total of 5% ZnO was added to the powder by weight. As for the experimental biomaterial, each component of the powder was proportioned by weight and prepared by mixing 1 g of powder to 0.25 mL of liquid.

The triple antibiotic paste was prepared with metronidazole, minocycline and ciprofloxacin [14]. The edge of the cavities was conditioned with 37% phosphoric acid for 30 seconds, washed with distilled water for 1 minute and dried with an air syringe. A layer of adhesive (Adper Single Bond 2, 3M ESPE, Sumaré, SP, Brazil) was applied to the conditioned edge of the cavity and then light cured (Optilight LD Max, Gnatus, Ribeirão Preto, SP, Brazil) for 20 seconds to allow the sealing of the interface with resin. The biomaterials were inserted into the prepared cavities at a depth of 1.5 mm. After the material setting, the cavities were sealed with a natural flow resin (B2, Nova DFL, Rio de Janeiro, RJ, Brazil). The polymerization was performed with LED curing light (Optilight LD Max) for 60 seconds and the specimens were stored in separated flasks containing tap water at 37ºC throughout the test period.

Colour Assessment

The colour assessments were performed as follows: before filling (B) and 24 hours (24 h), 28 days (28 d) and 90 days after filling (90 d) [2]. A spectrophotometer (Vita Easyshade V, VITA Zahnfabrik, Bad Sackingen, Germany) was used for assessments, which was performed in a light controlled room. The measurement was performed once for each specimen in each period. The excess water in the specimen was removed with gauze and then the colour assessed. The values of CIE L*, a* and b* were recorded and the colour change (ΔE) corresponding to the intervals was calculated by using the following formula: $\Delta E = [(L_1-L_0)^2 + (a_1-a_0)^2 + (b_1-b_0)^2]^{1/2}$. The values of colour change (ΔE) and lightness (L) were considered to evaluate the tooth darkening in the groups.

Characterization of Specimens

Stereomicroscopy

After 90 days, representative specimens of each group were selected and photographed with a camera (T5i Camera, Canon Inc., Tokyo, Japan) operating at 1/200, f 20 and ISO 100 and with a micro ring flash lite MR-14 EX II, 1/8. The specimens were then impregnated with epoxy resin (EpoxyFix, Struers, Ballerup, Denmark), sectioned at the centre by using a 0.3-mm diamond disc (Buehler) and polished with progressively finer grits of carbide discs. The sections were viewed under a stereomicroscope (SZX9, Olympus, Tokyo, Japan) at 2x magnification. The images were acquired with software IM 50 (Leica, Wetzlar, Germany).

Scanning Electron Microscopy and Energy Dispersive Mapping

The specimens were mounted on an aluminium stub, carbon coated and viewed under scanning electron microscope (SEM; JSM 5600 Lv JEOL, Akishima, Tokyo, Japan). Scanning electron micrographs of the material adhered to tooth interface were captured at different magnifications and elemental maps were plotted for calcium, silicon, phosphorus, zinc and radiopacifier (i.e. bismuth, zirconium or tungsten).

Statistical Analysis

Statistical analysis was performed by using non-parametric Kruskal–Wallis and Dunn's tests ($p<0.05$) as a result of the absence of normal distribution, as confirmed in D'Agostino and Pearson's analysis.

Results

Colour Assessment

Mean, standard deviation and statistical difference for colour change (ΔE) and luminosity (L) are shown in Table 2 and Figure 1. Analysis of ΔE values showed that all the materials exhibited colour alteration after the test periods, with statistical differences in relation to negative and positive controls ($p < 0.05$). With regard to luminosity, at 24h and 28d, experimental and Biodentine cements showed higher values, respectively ($p > 0.05$). At 90d, MTA Flow presented the lower values among all biomaterials, with statistical difference in comparison to MTA Repair HP and experimental cements ($p > 0.05$). The addition of ZnO to MTA Flow resulted in increase in the luminosity after 90 days of contact with dentine.

Representative specimens of each group are shown in Figure 2. Tooth discoloration was evident in the buccal surface of teeth with MTA Flow cement. Stains in the dentine were marked, indicating the penetration inside dentinal tubules. The addition of ZnO to MTA Flow cement prevented discoloration and altered the original colour of the material from grey to white. As for the other biomaterials, no evidence of tooth discolouration was verified.

Elemental Migration

Scanning electron micrographs and elemental maps of the bovine teeth filled with the biomaterials are shown in Figure 3. The radiopacifiers bismuth, zirconium and tungsten migrated into the tooth structure, with bismuth showing more intense migration into dentine/material interface in the MTA Flow specimens. This was not verified in the MTA Flow + ZnO group, in which bismuth ions were concentrated in the material matrix. The migration of Si into dentine was also evident in the maps for all biomaterials.

Discussion

Tooth discolouration caused by MTA was widely demonstrated *in vitro* and clinically. This drawback occurs due to interaction between the radiopacifier bismuth oxide and dental structures [15, 16]. In clinical conditions, the bismuth oxide interacts not only with dentine, but also with blood and sodium hypochlorite [2, 3, 17] showing different discolouration levels under these conditions. Some alternatives were proposed to inhibit discolouration caused by the presence of bismuth in MTA. These strategies include replacement of bismuth oxide by another radiopacifier or retention of bismuth and addition of ZnO or aluminum fluoride [13, 18, 19]. The first approach, bismuth oxide replacement, was undertaken in MTA Repair HP and Biodentine formulations, with bismuth oxide being substituted by calcium tungstate and zirconium oxide, respectively [20, 21].

Luminosity (L) is a parameter that indicates whether the surface absorbed light, and thus presented darkening, or reflected it. On the other hand, Delta E values represent colour alteration, which can be darker or clearer. Thus, analysis of the two parameters together allows for identification of tooth discolouration [2]. In this study, high Delta E values were found for the biomaterials, with statistical differences compared to negative and positive controls ($p < 0.05$). A reduction in L values was observed in all biomaterials. MTA Repair HP and Biodentine cements, both containing alternative radiopacifiers to bismuth oxide, did not cause tooth discolouration, accepting the second hypothesis [4, 9]. The present study was the first to demonstrate the colour stability of MTA Repair HP cement, corroborating other studies evaluating tricalcium silicate associated with calcium tungstate [4].

The presence of bismuth oxide in MTA Flow cement formulation could promote discolouration along the period of contact with dental structures [2]. This was confirmed in the first tested hypothesis in this study, with L values decreasing constantly since the insertion of this material. This material presents a grey powder color which may be partially responsible for the results. Tooth discolouration was evident after 90 days and the staining was intense in dentine where the migration of bismuth ions was detected. This is in accordance with other study [4], which found migration of bismuth and silicon into dentine. The sealing of dentine with a bonding agent could avoid migration of components from the material into dentinal tubules [22, 23]. In the study, the bonding agent was applied only to the edges of the cavities for adhesion of the composite resin. Thus, the inner surface of the cavities was not sealed, allowing for interaction between material and collagen of dentine and penetration of ions into dentinal tubules to be observed.

The addition of ZnO to MTA Flow cement inhibited the discolouration of this material, as reported for MTA Angelus, which contains bismuth oxide, validating the hypothesis [13]. Bismuth ions were detected in dentine for this group, although discolouration was not verified. For MTA Flow + ZnO, Delta E value was statistically higher than the other biomaterials tested ($p < 0.05$). After 28 days, analysis showed that L values were similar in the specimens filled with MTA Flow cement, with or without ZnO. However, after 90 days, the L values for MTA Flow + ZnO were higher. Overall, this difference was not statistically significant, except in the buccal surface of the teeth and in sectioned specimens. These results, in accordance with previous findings, suggest that ZnO is a plausible alternative to keep bismuth oxide as radiopacifier without leading to tooth discolouration [13]. Furthermore, the literature showed that ZnO addition does not interfere with physical, chemical and biological properties of MTA Angelus cement in a previous study [13] – a finding which further investigation can demonstrate also for MTA Flow. The use of bismuth oxide as a radiopacifier is preferred over its replacement due to its high molecular weight, demanding lower volumes of this compound to reach high radiopacity levels, in comparison with other components (i.e. zirconium or tungsten) [24, 25].

A prototype biomaterial was tested with the aim to test bismuth oxide in combination with ZnO in an alternative formula. The powder composition was comprised of tricalcium silicate, bismuth oxide and ZnO, including distilled water and a modified polycarboxylic ether [26]. The ZnO was added to inhibit destabilization of bismuth oxide. High values of luminosity were observed for this cement, similar to that verified for MTA Repair HP and Biodentine, suggesting that colour was stable for this test formulation. In the sectioned specimens, there was an evident area with different colour at the dentine/material interface. The elemental mapping did not reveal different composition for this layer. The cement probably precipitated during setting, which was longer than in other cements. Addition of calcium chloride might reduce the setting time and thus prevent the precipitation of the components [27].

Conclusions

Colour stability was verified for MTA Repair HP, Biodentine and prototype cement. MTA Flow presented potential for tooth discolouration, which was visually inhibited by the ZnO addition, but without significant difference. In all biomaterials tested, the radiopacifiers ions from the material penetrated into the dentine as there was evident interaction and colour alteration in the MTA Flow specimens.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval

The research did not involve Humans or Animals.

Informed consent

The research did not involve Humans.

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FIGURE LEGENDS

Figure 1 - Luminosity (L) values of the cements in the test periods (24 hours, 28 days and 90 days). L values decreased in all cements, from the initial (24h) to intermediate (28d) assessments. At 90d, there was an increase in L for MTA Repair HP, MTA Flow + 5% ZnO and experimental cements. MTA Flow showed a constant decrease in L from the initial to final assessments, which indicates darkening of the tooth. Addition of 5% ZnO to MTA Flow cement increased the L values between 28d and 90d

Figure 2 - Representative specimens of bovine teeth filled with Biodentine (A, B), MTA Repair HP (C, D), MTA Flow (E, F), MTA Flow + ZnO (G, H), experimental cements (I, J) and negative control (K, L). Darkening is evident in MTA Flow specimens. The dentine is stained, with grey colour visible in the buccal surface. Stereomicroscopic images at x2 magnification

Figure 3 - Scanning electron micrographs and energy dispersive analysis with elemental maps of sectioned teeth filled with the tested materials. MTA Repair HP (A), Biodentine (B), MTA Flow (C), MTA Flow + 5% ZnO (D) and experimental cements (E). Calcium (Ca), silicon (Si) and phosphorous (P) were found in all specimens. Bismuth (Bi) was found in MTA Flow, MTA Flow + 5% ZnO and experimental cements. Tungsten (W) was found in MTA Repair HP and zirconium (Zr) in Biodentine cements, corresponding to the radiopacifiers. Zinc (Zn) was verified in MTA Flow + 5% ZnO and in experimental cement. The migration of radiopacifiers and Si into dentine was evident in the maps. High molecular weight of Bi was detected in MTA Flow specimens, with a high incidence of these ions at the cement/dentine interface. This was not verified in MTA Flow + 5% ZnO, whose molecular weight of Bi was lower and ions evenly distributed in the material matrix

Table 1 - Material compositions used in analysis.

Tested material	Manufacturer	Composition
MTA HP	Angelus, Brazil	**Powder** Tricalcium silicate Dicalcium silicate Tricalcium aluminate Calcium oxide Calcium Tungstate (radio-opacifier) **Liquid** Water Polyvinylpyrrolidone
Biodentine	Septodont, France	**Powder** Tricalcium silicate Dicalcium silicate Calcium carbonate and oxide Iron oxide Zirconium oxide (radio-opacifier) **Liquid** Water Calcium chloride Polycarboxylate
MTA Flow	Ultradent, USA	**Powder** Tricalcium Silicate Dicalcium silicate Calcium sulfate Silica Bismuth trioxide (radio-opacifier) **Liquid** Water Water soluble silicone-based gel
Experimental		**Powder** Tricalcium silicate Zinc Oxide Bismuth oxide (radio-opacifier) **Liquid** Water Modified polycarboxylic ether

Table 2 – Mean and standard deviation of colour change (ΔE) after 90 days and the lightness (L) at 24h, 28d and 90d of analysis. Different lowercase letters in each column indicate statistically significant differences ($p < 0.05$).

Group	ΔE	L 24h	L 28d	L 90d
MTA HP	$90.8^{ab} \pm 0.4$	$87.2^{ab} \pm 1.3$	$76.1^{abd} \pm 0.1$	$90.7^{a} \pm 0.4$
Biodentine	$57.1^{a} \pm 10.3$	$88.1^{ab} \pm 1.3$	$80.8^{ab} \pm 4.9$	$82.2^{ab} \pm 3.1$
MTA Flow	$67.4^{ab} \pm 1.7$	$85.1^{ab} \pm 1.7$	$76.5^{abd} \pm 0.5$	$67.8^{b} \pm 1.7$
MTA Flow + 5% ZnO	$152.3^{b} \pm 6.2$	$84.0^{bc} \pm 0.4$	$73.1^{ad} \pm 0.7$	$74.2^{ab} \pm 0.5$
Experimental	$48.5^{a} \pm 12.9$	$94.0^{a} \pm 1.8$	$78.3^{ab} \pm 0.2$	$85.1^{a} \pm 4.0$
Negative control	$6.3^{c} \pm 0.8$	$92.4^{a} \pm 0.7$	$90.0^{b} \pm 1.7$	$90.7^{a} \pm 1.3$
Positive control	$1520.0^{d} \pm 28.4$	$63.2^{bc} \pm 13.0$	$44.5^{d} \pm 2.3$	$30.1^{c} \pm 14.6$

Figure 1

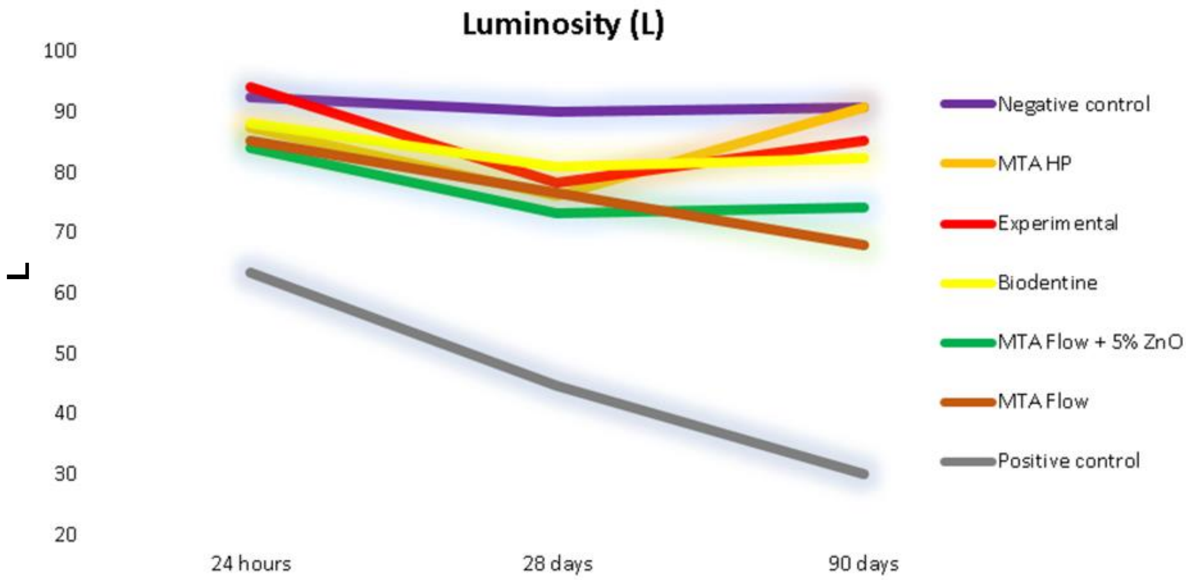

Luminosity (L)
L
100
90
80
70
60
50
40
30
20
24 hours
28 days
90 days
Negative control
MTA HP
Experimental
Biodentine
MTA Flow + 5% ZnO
MTA Flow
Positive control

Figure 2

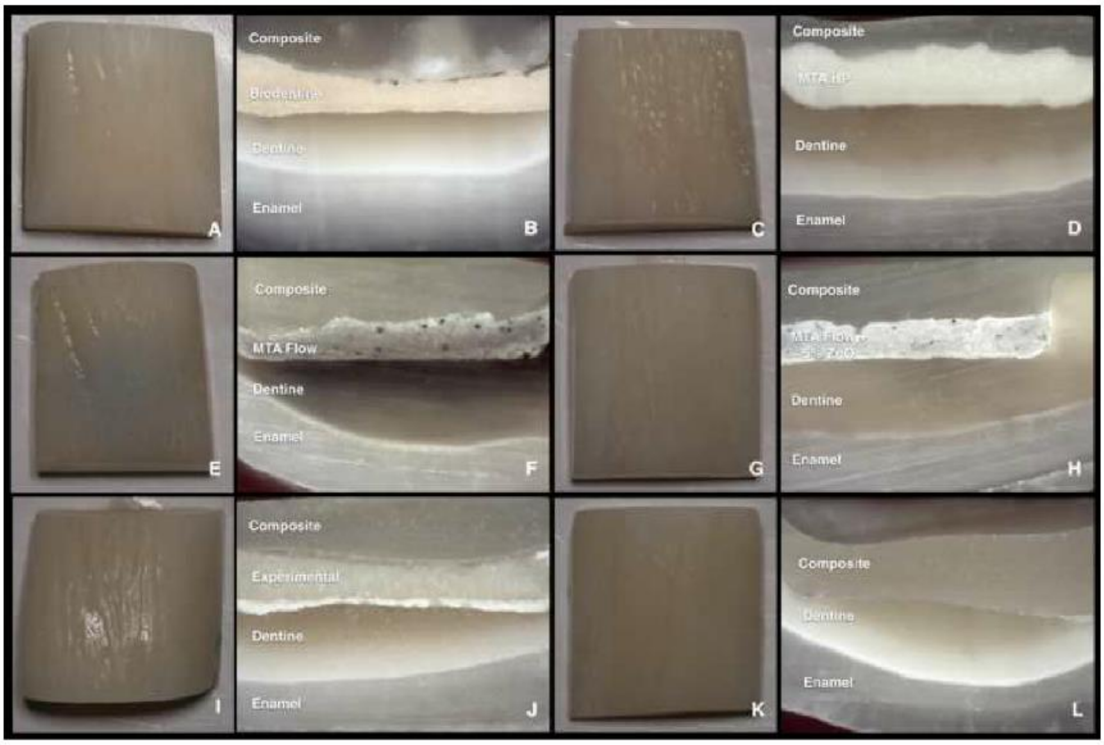
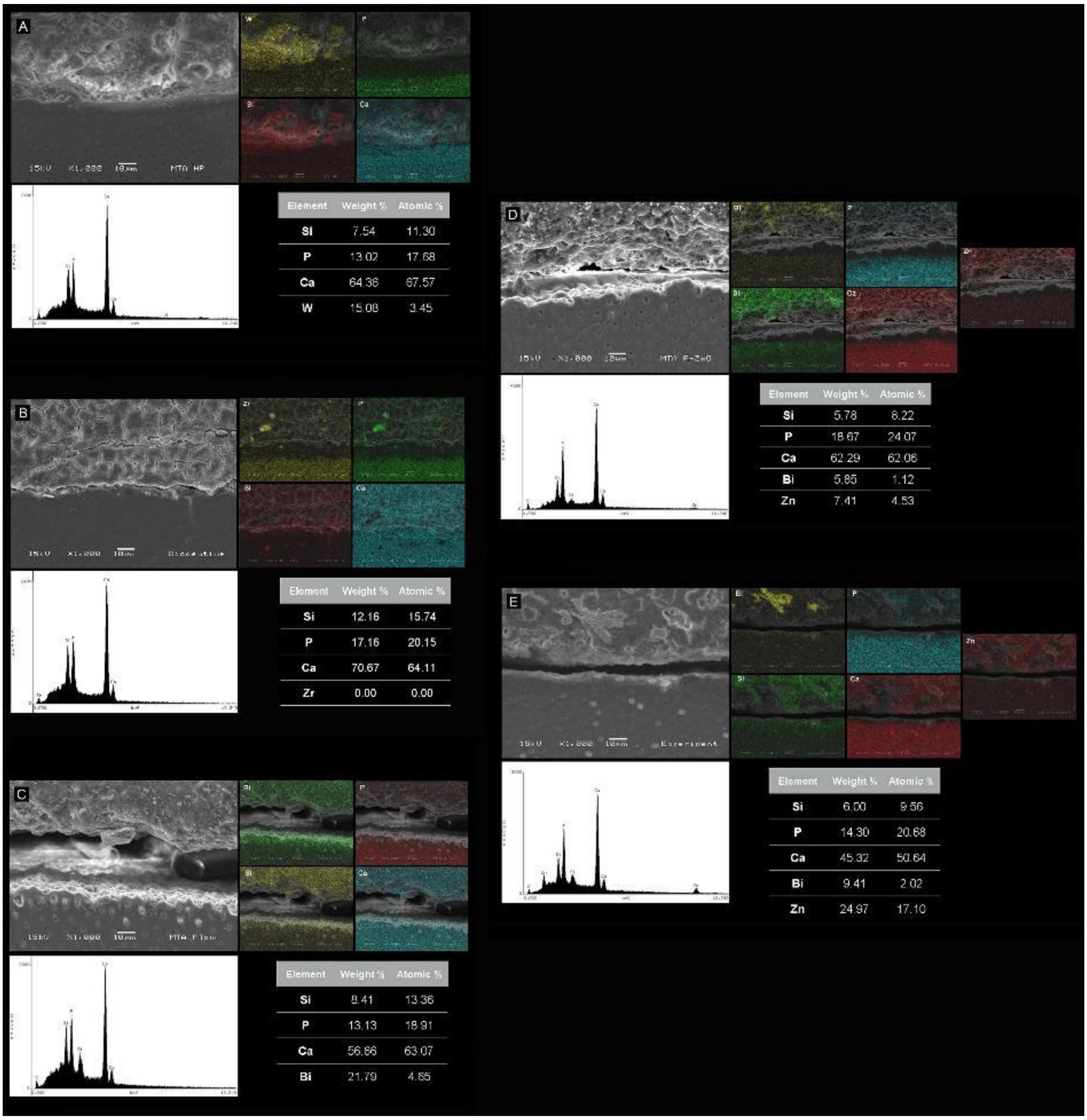

Composite
Biodentine
Dentine
Enamel
A
B
Composite
MTA HP
Dentine
Enamel
C
D
Composite
MTA Flow
Dentine
Enamel
E
F
Composite
Dentine
Enamel
G
H
Composite
Experimental
Dentine
Enamel
I
J
Composite
Dentine
Enamel
K
L

Figure 3


A
15kV X1,000 10µm MTA HP
Element Weight % Atomic %
Si 7.54 11.30
P 13.02 17.68
Ca 64.36 67.57
W 15.08 3.45
B
Element Weight % Atomic %
Si 12.16 15.74
P 17.16 20.15
Ca 70.67 64.11
Zr 0.00 0.00
C
Element Weight % Atomic %
Si 8.41 13.36
P 13.13 18.91
Ca 56.66 63.07
Bi 21.79 4.65
D
15kV X1,000 10µm MTA F-ZnO
Element Weight % Atomic %
Si 5.78 8.22
P 18.67 24.07
Ca 62.29 62.06
Bi 5.85 1.12
Zn 7.41 4.53
E
Element Weight % Atomic %
Si 6.00 9.56
P 14.30 20.68
Ca 45.32 50.64
Bi 9.41 2.02
Zn 24.97 17.10

2.2 Physicochemical properties of high-plasticity reparative biomaterials

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Lauter Eston Pelepenko, Brenda PFA Gomes, Adriana de-Jesus-Soares, Mario Tanomaru-Filho, Bruno Martini Guimarães, Alexandre Augusto Zaia, José Flávio Affonso Almeida, Marina Angélica Marciano

Physicochemical properties of high-plasticity reparative biomaterials

Abstract

Novel endodontic reparative biomaterials were introduced with improvements in handling and flow. The ideal consistency and clinical handling of a cement allows its proper insertion, improves its quality of sealing and interferes directly with treatment success. Objective: Evaluate biomaterials regarding its physicochemical properties and, whether, 5% zinc oxide (ZnO) added to bismuth-containing materials alters its physicochemical properties. Material and Methods: MTA Repair HP, MTA Flow, Biodentine, MTA Flow + 5% ZnO and an experimental cement (powder: tricalcium silicate, bismuth oxide and ZnO; liquid: distilled water and modified polycarboxylic ether), were tested regarding pH, radiopacity, calcium release, volume change, setting time and, both, standard and three-dimensional flows. Results: All cements presented alkaline pH after 28 days in solution. Radiopacity higher than 3 mm Al was observed in all materials tested. Biodentine and experimental cement presented the highest calcium ion release and volume change. MTA Repair HP presented the shortest setting time (4.6 min) and the experimental cement the longest (20.0 min). MTA Repair HP and Biodentine presented the highest results regarding volumetric cavity filling. As for the lateral cavity filling, MTA Flow, MTA Flow + 5% ZnO and the experimental cement presented the highest results. Conclusion: The high-plasticity materials being tested showed proper physicochemical properties at different levels, especially regarding three-dimensional flow. The cements presented calcium ion release and elevated pH. The addition of 5% ZnO to the MTA Flow original formulation for color issues significantly enhanced radiopacity and ISO 6876 flow values. The other properties were not significantly altered by this additive. The experimental cement showed properties comparable to the other materials tested.

Key words: Endodontics. Dental Cements. X-Ray Microtomography. Root repair materials.

Introduction

Reparative biomaterials are used in several endodontic treatments. Pulp capping, sealing of perforations, cervical barrier in regenerative procedures and orthograde/retrograde apical plug are some examples of indication for these materials.[1–3] Proper interaction between biomaterial and tissue is crucial for repair.[2] Alkaline pH and calcium release are properties expected for healing due to calcium hydroxide release. Clinically, the material consistency is of high significance once appropriate insertion and filling improve the quality of sealing and directly affect the treatment success.

Endodontic biomaterials have been recently introduced with improvements in handling and flow.[4,5] Tricalcium silicate cements are hydrophilic and, thus, require water before setting begins.[6,7] The high-plasticity formulations are based on tricalcium silicate, but they have different plasticizers added to the liquid, such as polyvinylpyrrolidone and silicon-based gels. Tanomaru-Filho *et al*. (2017)[5] have recently proposed a three-dimensional methodology to test the flow properties of reparative biomaterials indicated for root canal sealing. Micro-tomography (μCT) analysis increases the accuracy in the assessment of flow and appears to be adequate for novel high-plasticity biomaterials especially due to the volumetric filling expected from this material category. In opposition to endodontic sealers, reparative endodontic materials seem to be better evaluated in a three-dimensional method.

The radiopacifier content vary in the novel formulations. This alteration in comparison with traditional formulas intended to avoid discoloration, which was widely demonstrated when bismuth oxide is used.[2] MTA Repair HP (MTA High Plasticity, Angelus, Londrina, Paraná, Brazil) contains calcium tungstate and Biodentine (Septodont, Saint-Maur-des-Fossés, Île-de-France, France) zirconium dioxide as radiopacifiers.[7,8] MTA Flow (Ultradent Products Inc., South Jordan, UT, USA) contains bismuth oxide, which is associated with tooth staining.[2,4] An experimental formulation was proposed with the addition of ZnO in order to inhibit tooth discoloration caused by bismuth while maintaining its radiopacity properties.

The aim of the present study is to evaluate the high-plasticity biomaterials, namely, MTA Repair HP, MTA Flow, Biodentine, MTA Flow with an addition of 5% ZnO; and an experimental cement containing bismuth oxide and ZnO regarding three-dimensional volumetric change, radiopacity, setting time, standard and three-dimensional flows, calcium release and pH. The tested hypothesis is that the addition of ZnO as an additive to bismuth-containing formulations does not alter its properties in comparison to the other tested materials.

Material and methods

The biomaterials included were the following:

- MTA Repair HP (Angelus);
- MTA Flow (Ultradent);
- Biodentine (Septodont);
- MTA Flow + 5% ZnO (Sigma-Aldrich, St Louis, USA);
- Experimental cement.

MTA Repair HP and Biodentine were prepared according to their manufacturers' instructions. MTA Flow was mixed in thin consistency following the recommended powder/liquid ratio in order to obtain the most flowable characteristic of this material. ZnO was added to the MTA Flow original formula at a weight percentage of 5% by using an electronic analytical balance scale (Gehaka AND-GR-202, Tokyo, Japan) with 10^{-3} precision.

The experimental cement was prepared with 80% of tricalcium silicate, 20% of bismuth oxide and 5% of ZnO (added in weight). The liquid consisted of 95% of distilled water and 5% of modified polycarboxylic ether. For an ideal handling consistency, the experimental cement was mixed at ratio of 1 g of powder and 0.25 mL of liquid.

Volume Change

By simulating a retrograde filling, µCT scanning was used to evaluate the volume change.[9] Twenty-five acrylic models of single-rooted teeth had their root end resected at 3 mm before retro-cavity preparation (3 x 1 ± 0.1 mm; depth versus width) (Figure 1). The cavities were filled with the cements (n = 5) and initial scanning was performed by using a µCT (SkyScan 1174, Bruker, Kontich, Belgium) with a 0.5 mm aluminum filter, 31.03 µm pixel size, 360º rotation with 1.0º step, reconstructed with NRecon software (Bruker, Kontich, Belgium) and analysed with CTan software (Bruker, Kontich, Belgium). After the initial scanning and cement set final time (according to tested material), each tooth was individually immersed into flasks containing 15 mL of deionized water and stored at 37ºC for 28 days. After this period, the samples were removed from the flasks, dried with filter paper and rescanned with the same initial parameters. The rescanned volume values, compared to the initial ones, represented the percentage volume change.

Radiopacity

The radiopacity was evaluated following the ISO 6876:2012 standard. Three metal rings *per* group were used to mould the cement samples (10 mm x 1 mm), which were kept at 37°C ± 1°C and relative humidity until complete setting. After this period, the samples were radiographed with digital sensor (Micro Imagem, Indaiatuba, Sao Paulo, Brazil) instead of a radiographic film in order to exclude film revelations biases. An aluminum scale at 60 kV, 10 mA, 0.3 seconds of exposure and a focus-film distance of 30 cm. The radiographic density was evaluated in grayscale values and converted into aluminum equivalent thickness (mm Al) by using the ImageJ software (National Institutes of Health, Bethesda, MD).

Setting Time

The ISO 6876:2012 standard was followed for setting time. Freshly mixed cements were placed into metal rings of 10 mm internal diameter and 2 mm thick (n = 3). The samples were kept at a temperature of 37°C ± 1°C and humidity of 95% ± 5% during the test and periodically subjected to vertical pressure by using Gilmore needles (ASTM- 266/2008). A room temperature 113.4 g needle was used for initial setting time and a 453.6 g one for final setting. The initial and final setting times (in minutes) were determined from the mixing start to the moment that it was no longer possible to observe the marking of each needle on the surface of the samples.

Standard and Three-Dimensional Flow

For flow analysis, two different methodologies were used. First, according to the ISO 6876:2012 standard, a total volume of 0.05 mL of each cement (n = 3) was placed in the center of a glass plate by using a plastic syringe. After 3 minutes from the mixing start, another glass plate weighting 20 ± 0.5 g was positioned above the cement along with a 100 g weight (total weight of 120 g). After 10 minutes from the start of the mixing, the 100 g weight was removed and the largest and smallest diameters of the cement interposed between the plates were measured by using a digital calliper (Mitutoyo, MTI Corporation, Tokyo, Japan). The mean between both diameters was considered as the cement flow.

The second flow test was performed as previously reported (Tanomaru-Filho *et al*. 2017). This methodology was based on the three-dimensional cement flow by using μCT scans. Twenty-five glass plates (n = 5) were fabricated with a central cavity of 2

mm^3 and four lateral grooves of equal size measuring 12 mm (Figure 2). An amount of 0.05 ± 0.005 mL of each freshly mixed material was placed into the central cavity. Next, another glass plate (20 ± 0.5 g) and an extra weight (100 g) were immediately superposed for 10 minutes. For assessment of linear lateral flow (in mm), the 100 g weight was removed and photographed with a ruler alongside for calibration purposes. ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used for this measurement. Linear lateral flow was represented by the amount of material present in the lateral grooves, expressed by the average of the four measurements of the lateral grooves from the central cavity. For volumetric flow (mm^3), two measurements were evaluated: central and lateral volumetric flows. The samples were scanned by using μCT and reconstructed with the same previous parameters. CTan software was used for analysis of central and lateral volumes using the region of interest measurement. Volume assessments were performed individually for the central cavity with 2 mm^3 (Figure 2 f) and for each one of the four lateral grooves up to 2 mm from the central cavity with a total of 4 mm^3 (Figure 2 g). Lateral volumetric flow was represented by four lateral grooves as average volumes.

Calcium Ion Release and pH Analysis

Twenty-five acrylic teeth (n = 5) with a standardized root-end cavity with 3 x 1 ± 0.1 mm; depth versus width were used.[9] The cavities were filled with the test cements and immersed into individual flasks containing 10 mL of deionized water. For assessment of calcium ion release (mg/L), periods of 3 hours, 24 hours and 28 days were evaluated using atomic absorption spectroscopy. Standard calcium solutions were used as reference for calcium ion release of each cement sample. For analysis of pH in the same time periods, a pH-meter (371 model; Micronal, Sao Paulo, Brazil) was used with previous calibration.[9]

Statistical Analysis

For statistical analysis, the software BioEstat 5.3 (Mamiraua, Tefe, Brazil) was used. One-way ANOVA and Tukey's tests were used in parametric data distribution (radiopacity, volumetric flow and calcium ion release), then confirmed by Kolmogorov-Smirnov and Lilliefors tests ($p < 0.05$). For nonparametric data, Kruskal-Wallis and Dunn (volume change, setting time, ISO flow and pH) tests were used for comparisons.

Results

The results for volume change, radiopacity, initial and final setting times are listed in Table 1. Initial and final setting times varied between the biomaterials, with lower values being verified for MTA HP ($p < 0.05$). However, Biodentine also showed the fast setting times similar to MTA HP. MTA Flow showed an increase in volume (expressed as positive value), whereas Biodentine had a higher percentage of volume reduction ($p < 0.05$). As for radiopacity, all test cements achieved the minimum required by the ISO standard of 3 mm Al. A significant increase in radiopacity was observed for MTA Flow + 5% ZnO in comparison to MTA Flow ($p < 0.05$). Initial and final setting times varied between the biomaterials, with lower values being verified for MTA Repair HP ($p < 0.05$). The addition of ZnO did not significantly reduce the setting time of MTA Flow ($p > 0.05$).

The results for standard and three-dimensional flows are listed in Table 2. Analysis of standard flow shows that the experimental cement presented significantly higher flow, followed by MTA Flow + 5% ZnO, with statistical difference compared to the other biomaterials ($p < 0.05$). As for linear lateral flow, the experimental cement also showed significantly higher values compared to MTA Repair HP and Biodentine ($p < 0.05$). In opposition to linear evaluations, volumetric analysis revealed that the experimental cement had the lowest filling values ($p < 0.05$), regarding central volumetric flow. In opposition, Biodentine showed the highest volumetric filling in the central cavity.

The mean values of pH and calcium release obtained in the study periods are shown in Figures 3 and 4, respectively. All the test cements presented alkaline pH during analysis without significant differences regardless of the study period ($p > 0.05$). As for calcium ion release, no significant difference ($p > 0.05$) was found between the two initial periods (i.e. 3 and 24 hours). In the 28-day period, significantly higher values ($p < 0.05$) were observed for all the materials tested.

Discussion

Endodontic biomaterials with high-plasticity characteristic has been recently introduced in the market. The modifications in their formula had the main objective to improve handling, clinical insertion and filling. The high-plasticity cements MTA Repair HP, MTA Flow, Biodentine and experimental cement were tested regarding physical and chemical properties. Additionally, the MTA Flow containing 5% zinc oxide (ZnO) was tested. The association of a bismuth-containing cement and ZnO was previously shown to inhibit color change.[4]

As the main characteristic of these materials is the plasticity, the flow test is fundamental to determinate their fluidity. ISO standard test, originally designed for sealers, considers linear measurements only, but the clinical conditions in which the material is inserted are three-dimensional. Thus, Tanomaru-Filho *et al*. (2017)[5] proposed a microtomographic evaluation of the flow regarding reparative biomaterials considering its volumetric alterations in opposition to ISO tests that consider a bi-dimensional approach. With this methodology, one can assess the linear, lateral and volumetric filling and flow. In the present study, MTA Repair HP and Biodentine presented similar results regarding volumetric flow, which agrees with previous results.[5] Although these cements showed lower values of linear lateral flow, their lateral and central volumetric flows were high, which suggest that these cements would provide an adequate cavity sealing when used in clinical conditions. MTA Flow and experimental cement, probably due to their consistency, presented higher results for lateral volumetric and linear lateral flows in the ISO tests.

Considering the clinical procedures in which these reparative biomaterials are used, a short initial setting time is an important characteristic.[10,11] Contact with organic tissues, blood and fluids should not alter the material's surface and its biological properties, thus being necessary an initial setting when this contact occurs. MTA Repair HP presented the shortest setting time between the cements tested, whereas the experimental cement the longest. This clinically relevant prolonged final setting time was probably related to powder/liquid ratio and particle size used for this cement in our study. Initial setting time is directly related to volumetric filling because once the material starts its initial setting, it no longer continues to flow inside the cavity or onto the surface. These different conditions of application to cavities, grooves and plain surfaces were tested in high-plasticity cements according to previous studies.[5,9,10,12–14]

Adequate radiopacity is mandatory in order to obtain an adequate follow-up, once it is used in regions not visible clinically.[9,14] All the high-plasticity cements tested showed adequate radiopacity, that is, at least 3 mm Al according to ISO standard recommendations. MTA Flow presented similar radiopacity values of a previous study.[10] In the present study, ZnO addition to the MTA Flow original composition significantly increased its radiopacity values. These results could be explained by the radiopacity presented by ZnO.[15] MTA Repair HP and Biodentine contain calcium tungstate and zirconium oxide, respectively, as radiopacifier.[16–18] These radiopacifiers have lower molecular weight compared to bismuth, requiring higher volumes to achieve similar radiopacity as demonstrated by Duarte et al. (2009)[15].

When using reparative cements, an interaction with the surroundings tissues and fluids occurs and change in the material's volume is expected. The methodology used in this study[9] is based on clinically compatible materials by using retro-cavities in contact with water and analysis by using µCT scanning before and after their immersion. All test cements presented volume change, mainly on the surface in contact with fluid. Biodentine presented the highest volume loss in this study, which is in agreement with previous studies.[19] This high solubility has been associated with its plasticizer.[19] Another possibility may be related to the radiopacifier volume added to its composition. [20] Due to its high molecular weight, the use of bismuth demands lower volumes for adequate radiopacity. On the other hand, alternative radiopacifiers require higher volume to obtain similar radiopacity values, which may compromise the material's properties. MTA Repair HP and MTA Flow + 5% ZnO showed similar volume losses. MTA Flow presented a slight volume increase after immersion, probably due to absorption during the immersion period. This increase in volume was previously reported in conventional MTA formulations. [11] Volume loss was found for MTA Flow when tested with similar methodology in a previous study in 'putty' consistency recommended by the manufacturer.[10] In the present study, the 'thin' consistency was used which might be the reason for this variability in the results. Another important aspect is the silicon-based gel used in the MTA Flow liquid that is practically water insoluble and might be the responsible for this stability.

Previous studies reported higher values of alkaline pH as a result of cement hydration and calcium hydroxide formation on its surface (e.g. portlandite).[22] In the present study, a progressive increase of pH values for Biodentine and experimental cement was observed during the study period. This fact can be explained due to higher solubility presented by these two materials. All test materials presented pH values between 8 and 9 after 28-day immersion in water. Guimarães et al. (2017)[10] found higher pH values for MTA Flow, in 'putty' consistency possibly associated to increased solubility values, in comparison to this study which used 'thin' consistency for this material. Calcium ion release in water presented significant higher values after 28 days, with this property being important to provide healing conditions. The calcium hydroxide formed on the material's surface in contact with water was present in the tissues.[17] Biodentine and the experimental cement presented the highest calcium ion release. Balance between component release and stability is important to provide sealing and biocompatibility after long periods, since these materials are not expected to be replaced. Considering these new high-plasticity materials, biocompatibility should be expected due to calcium ion release and alkaline pH.

Conclusions

All the materials tested have presented adequate physicochemical properties at different levels, especially regarding the three-dimensional flow. The addition of 5% ZnO to the MTA Flow original formulation for color issues significantly enhanced radiopacity and ISO 6876 flow values. The experimental cement (containing bismuth oxide and ZnO) presented physicochemical properties comparable to the other materials tested.

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Figure legends

Figure 1 Schematic diagram for microtomography (μCT) in the volume change test. (a) Retrograde cavities filled with test cements prepared for μCT scanning. (b) Scanned cavity (3 x 1 mm). (c) Reconstructed cement sample viewed in different aspects before and after assessment of the volume change. (d) MTA Repair HP sample before immersion with stereolithography (STL) image in blue. In red, STL image after immersion followed by superimpositions showing differences in the material's top surface where the volumetric reduction occurred. (e) STL images of MTA Flow material showing a slight volume increase on the top surface evaluated on μCT scans before and after immersion. (f) STL images of MTA Flow + 5% ZnO showing the material surface where a slight volumetric reduction occurred. (g) STL images of the experimental cement showing the material surface with significant volumetric reduction. (h) STL images of Biodentine presenting material on the top and lateral (arrow) surfaces with significant volumetric reduction.

Figure 2 Schematic diagram for microtomography (μCT) in the evaluation of volumetric flow. (a) Glass plate with 0.05 mL of fresh cement placed in central cavity immediately after manipulation. (b) From bottom to top: volumetric glass plate containing the material (1), 20 g flat glass plate placed onto it (2), 100 g extra weight placed for 10 minutes (3). (c) Scanned volumetric glass plate containing the material tested. (d) Reconstructed image showing the flow pattern for volumetric evaluation. (e) Distance of 2 mm where lateral volumetric flow was considered. (f) Stereolithography (STL) representation of cement flow in the central cavity (rectangle). (g) STL representation of cement flow in the lateral cavity (rectangle).

Figure 3 Graphical representation of pH values for the test materials at 3 hours, 24 hours and 28 days. All the test cements showed alkaline pH during the study periods without significant difference between groups and evaluated periods.

Figure 4 Calcium ion release (mg/L) for the test materials at 3 hours, 24 hours and 28 days. Significantly higher values ($p < 0.05$) were observed in the 28-day period for all materials tested. Different letters represent significant differences between evaluated periods.

Tables legends

Table 1 - Mean and standard deviation of volume change, radiopacity, initial and final setting time. Different letters in each column indicate statistically significant differences between tested materials ($p < 0.05$).

Table 2 - Mean and standard deviation of tested material volumetric and linear flow. Different letters in each column indicate statistically significant differences between tested materials ($p < 0.05$).

Figure 1

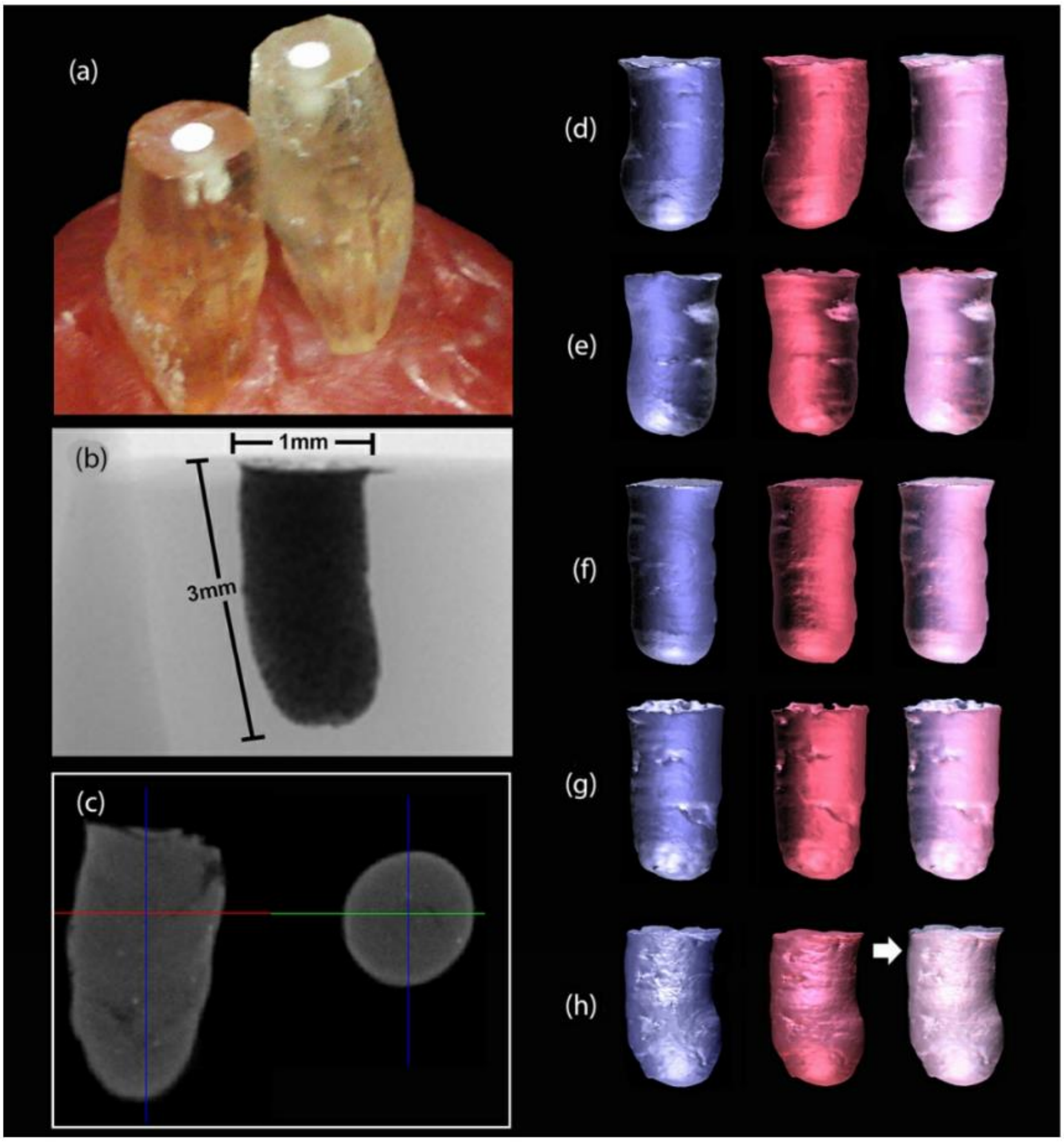

Figure 2

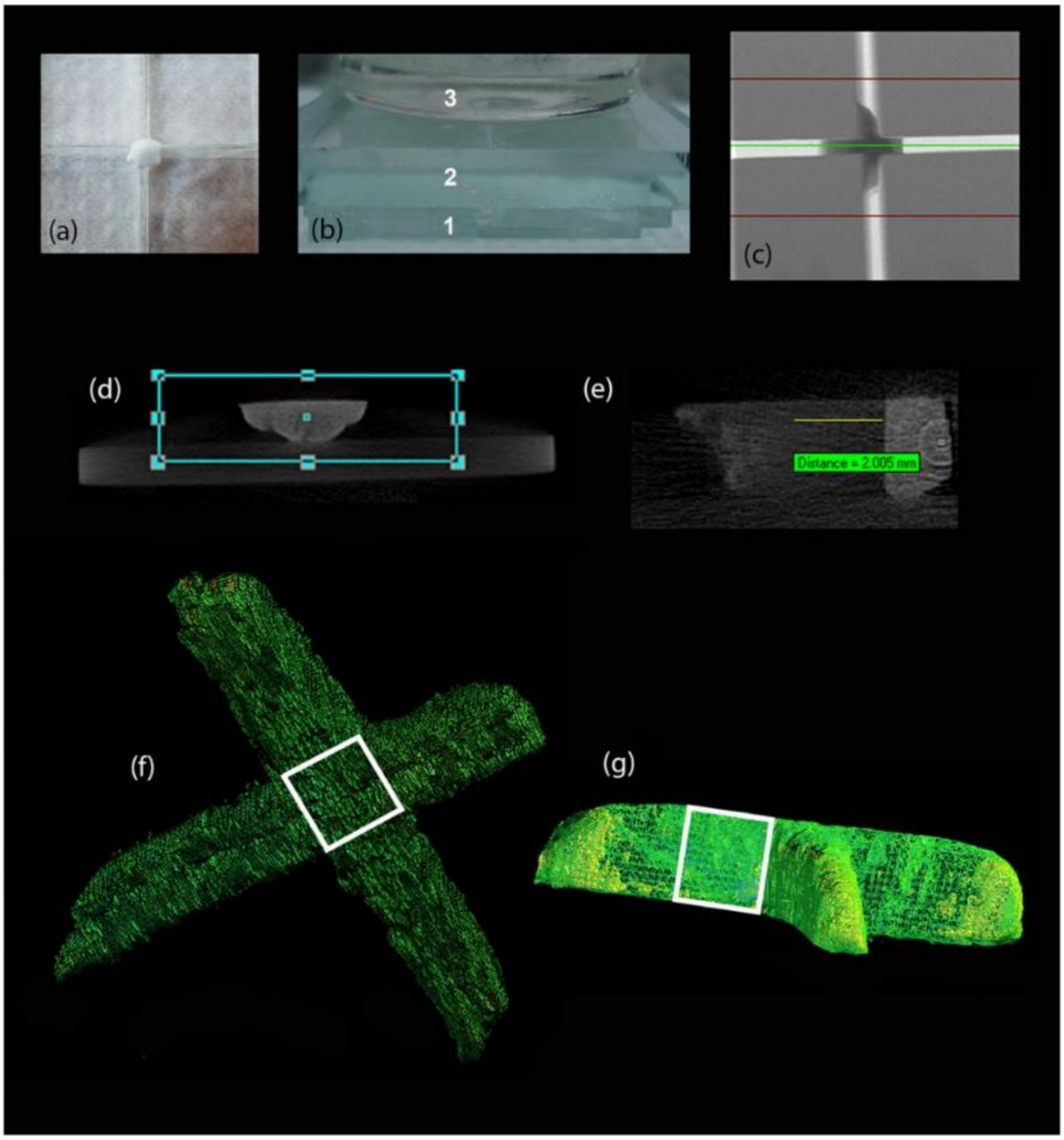

Figure 3

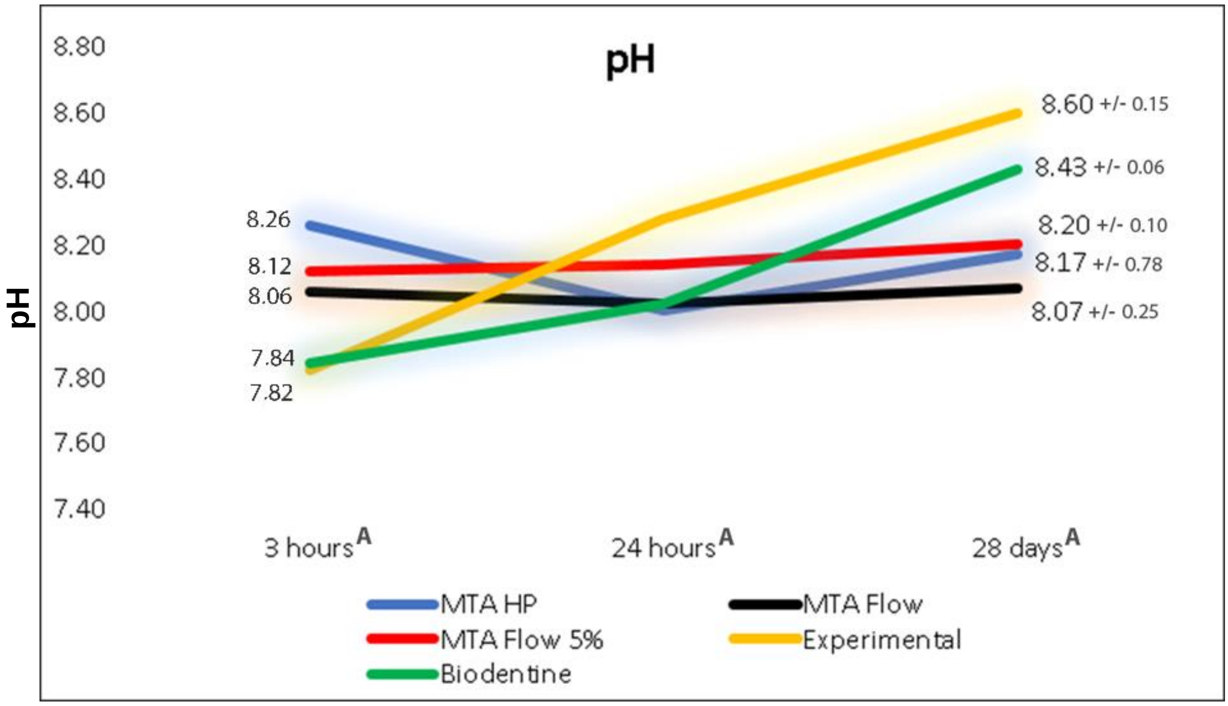
pH
pH
8.80
8.60
8.40
8.20
8.00
7.80
7.60
7.40
8.26
8.12
8.06
7.84
7.82
8.60 +/- 0.15
8.43 +/- 0.06
8.20 +/- 0.10
8.17 +/- 0.78
8.07 +/- 0.25
3 hours A
24 hours A
28 days A
MTA HP
MTA Flow
MTA Flow 5%
Experimental
Biodentine

Figure 4

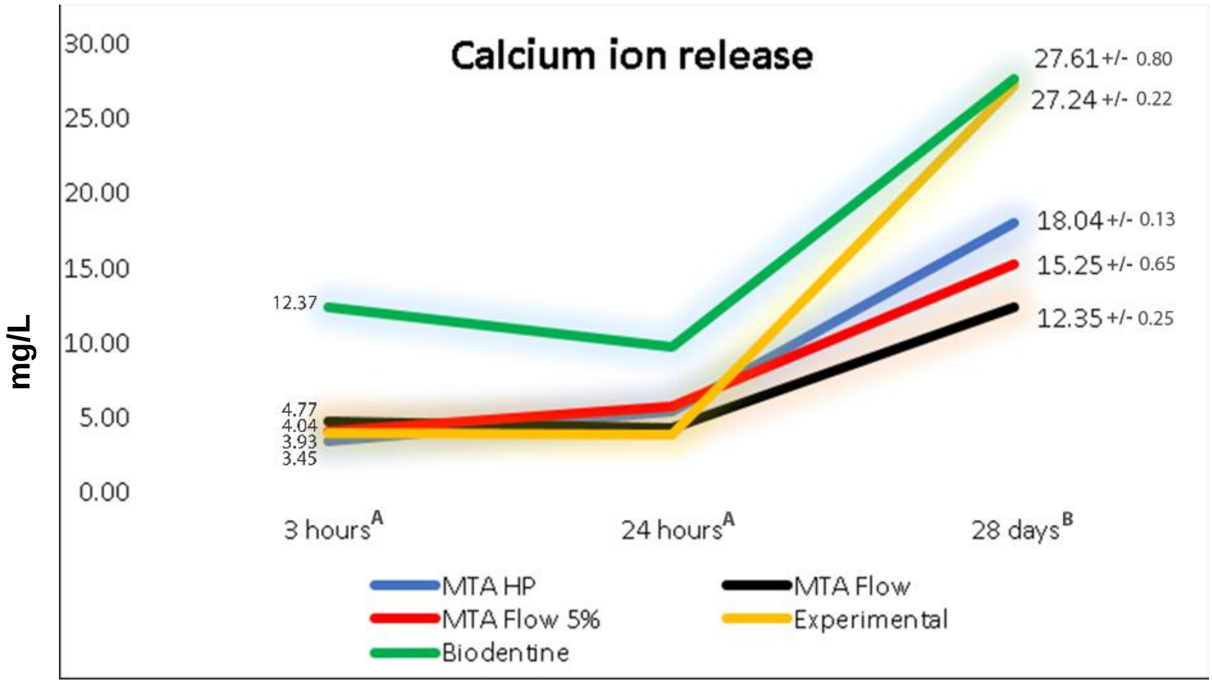
Calcium ion release
mg/L
30.00
25.00
20.00
15.00
10.00
5.00
0.00
12.37
4.77
4.04
3.93
3.45
27.61 +/- 0.80
27.24 +/- 0.22
18.04 +/- 0.13
15.25 +/- 0.65
12.35 +/- 0.25
3 hours A
24 hours A
28 days B
MTA HP
MTA Flow
MTA Flow 5%
Experimental
Biodentine

Table 1

Group	Volume Change (%)	Radiopacity (mmAl)	Initial setting time (min)	Final setting time (min)
MTA HP	-2.0 ± 3.3[ab]	5.1 ± 0.3[ab]	4.6 ± 0.3[a]	25.0 ± 0.4[a]
MTA Flow	0.4 ± 1.8[a]	4.9 ± 0.2[a]	8.7 ± 0.2[b]	52.2 ± 0.2[b]
MTA Flow 5%	-0.5 ± 2.1[a]	6.8 ± 0.1[b]	7.9 ± 0.4[ab]	49.0 ± 1.0[b]
Experimental	-4.6 ± 1.3[bc]	6.0 ± 1.3[ab]	20.0 ± 2.0[c]	165.0 ± 5.0[c]
Biodentine	-7.9 ± 1.0[c]	5.4 ± 0.4[ab]	11.6 ± 2.1[b]	29.0 ± 2.4[a]

*Volume change negative values represent volume loss and positive values represent expansion.

Table 2

Group	Flow ISO 6876 (mm)	Lateral linear flow (mm)	Lateral 4 mm^3 volumetric flow (mm^3)	Central 2 mm^3 volumetric flow (mm3)
MTA HP	9.1 ± 0.1[a]	3.4 ± 1.4[a]	1.9 ± 0.5[a]	1.3 ± 0.2[ab]
MTA Flow	10.9 ± 0.2[a]	6.7 ± 1.4[b]	2.4 ± 0.4[a]	1.1 ± 0.3[b]
MTA Flow 5%	11.4 ± 0.2[c]	6.3 ± 0.6[b]	2.4 ± 0.2[a]	0.9 ± 0.2[b]
Experimental	13.6 ± 0.6[c]	6.8 ± 0.9[b]	1.7 ± 0.4[a]	0.3 ± 0.2[c]
Biodentine	8.2 ± 0.2[b]	3.7 ± 0.6[a]	2.5 ± 0.2[a]	1.6 ± 0.2[a]

2.3 Antimicrobial evaluation of tricalcium silicate-based cements

Artigo em revisão para ser submetido (Periódico pretendido: *Journal of Endodontics*)

Lauter Eston Pelepenko, Brenda P. F. A. Gomes, Adriana de-Jesus-Soares, Alexandre Augusto Zaia, José Flávio A Almeida, Marina Angélica Marciano

Antimicrobial evaluation of tricalcium silicate-based cements

Lauter Eston Pelepenko, Brenda P. F. A. Gomes, Adriana de-Jesus-Soares, Alexandre Augusto Zaia, José Flávio A Almeida, Marina Angélica Marciano

Short title: Antimicrobial properties of cements

Abstract

Introduction: Novel reparative endodontic cements (RECs) have been introduced claiming for improved handling and flowability. Antimicrobial activity is a critical aspect of investigation once these materials are frequently used in contact with infected sites. The aim of this study was to evaluate the antimicrobial effect of this new formulations and test whether 5% ZnO added to bismuth-containing materials influence its antimicrobial property. **Methods:** MTA Repair HP, MTA Flow, Biodentine and an experimental cement were evaluated. The addition of 5% zinc oxide (in weight) to the original MTA Flow to inhibit the potential color change of this material was also tested. Agar diffusion test and direct contact methods were used against *Enterococcus faecalis* and *Porphyromonas gingivalis.* Chlorhexidine gel was used as positive control for the agar diffusion test. Data were statistically evaluated using Anova and Tukey ($P < .05$). **Results:** No inhibition halos were observed from any RECs regardless the bacteria tested. Control presented average halos of 19.1 mm against *Enterococcus faecalis* and 42.2 mm for *Porphyromonas gingivalis* with significant difference ($P < .01$). Regarding direct contact method, all RECs presented significant higher levels of turbidity when compared to controls ($P < .01$). Viable bacteria were recollected from all tested RECs tubes at all tested periods. Chlorhexidine presented the lowest values of turbidity from 3 hours to 7 days ($P < .01$). **Conclusions:** None of the RECs formulations presented satisfactory antimicrobial effect for tested methodologies.

Introduction

Reparative endodontic procedures must provide healing conditions for affected sites after its execution (1,2). Reparative endodontic cements (RECs) are used for sealing (3), regenerative procedures (4) and pulp vitality maintenance (5,6). These materials must present several characteristics including biological properties (2), marginal adaptation (7) and antimicrobial properties (1). Physical, chemical and biological properties of the RECs have been studied for decades and new substances introduced into the market. However, advances are still necessary to obtain a composition that provides all the ideal characteristics for a repairing material (8).

Enterococcus faecalis and *Porphyromonas gingivalis* are bacterial species frequently isolated from endodontic infected sites (9,10) and different levels of prevalence and resistance are attributed to these microorganisms (11). The presence of infection in affected endodontic sites delays substantially their repair (12).

Recently developed RECs' compositions supposedly provides better marginal adaptation due to its improved handling characteristics and reduced particle size (13). MTA Repair HP (Angelus, Paraná, Brazil) (14), MTA Flow (Ultradent, Utah, USA) (15) and Biodentine (Septodont, Besançon, France) (16) are material with this characteristics. However, previous studies showed that when in contact with blood and organic fluids RECs significantly reduces its antimicrobial activity in vitro and material properties were altered (1,17). In clinical situations, this contact occurs in several procedures and these alterations are also expected (18,19).

Another clinical aspect is aesthetics, when using RECs. Tooth color alterations caused by dental materials are undesired (17,20). RECs are reactive materials and consequently promotes interactions with adjacent tissues. Bismuth-containing materials for radiopacity purposes induces ions migration to adjacent tissues resulting in the formation of a black precipitate (Bismite) (21) with potential color alteration. Alternative radiopacifiers (22–24) or additives intending to inhibit bismuth pigmentation (17,18) were previously proposed. Zinc oxide (ZnO) used as an additive with a concentration of 5% did not altered materials' properties and inhibited dental color alteration (17).

Zinc ions potentially exhibit antimicrobial activity against bacterial and fungal strains (25). The presence of ZnO in RECs composition could hypothetically provide two roles, first, as color alteration inhibitor (17) and, second, as an antimicrobial substance. The aim of this study was to evaluate the antimicrobial effect of commercially available RECs, comparatively to an experimental cement formulation, using agar diffusion test for freshly mixed materials and direct contact method with inoculated broth in contact with RECs' discs, regarding its turbidity. Also, to test whether the addition of ZnO to bismuth-containing RECs alters its antimicrobial potential.

Materials and Methods

The materials included in this study were:

- MTA Repair HP (Angelus, Paraná, Brazil);
- MTA Flow (Ultradent, Utah, USA);

- Biodentine (Septodont, Besançon, France);
- MTA Flow + 5% ZnO (Sigma-Aldrich, St. Louis, USA);
- Experimental cement.

MTA Repair HP and Biodentine were prepared according to manufacturer's instructions. MTA Flow was mixed in thin consistency, following the recommended powder/liquid ratio. ZnO was added to the original formula of MTA Flow at a percentage of 5% in weight using an electronic analytical balance (Gehaka AND-GR-202, Tokyo, Japan) with 10^{-3} precision. The experimental cement was prepared using 80% of tricalcium silicate, 20% of bismuth oxide and 5% of ZnO, proportioned in weight. The liquid was composed, by volume, of 95% distilled water and 5% modified polycarboxylic ether. The experimental cement was mixed using 1 g of powder to 0.25 mL of liquid ratio.

Antimicrobial tests

Enterococcus faecalis (ATCC 29212) and *Porphyromonas gingivalis* (ATCC 49417) bacterial strains were previously subcultured on appropriate culture media plates and under gaseous conditions to confirm their purity. *Enterococcus faecalis* was subcultured on Brain Heart Infusion (BHI) agar plates + 5% defibrinated sheep blood (Ebefarma, Araras, SP) and incubated for 18 h at 37°C under aerobic conditions. *Porphyromonas gingivalis* was subcultured on Fastidious Anaerobe Agar (FAA) + 5% defibrinated sheep blood and incubated for 48 h in an anaerobic chamber (Don Whitley Scientific, Bradford, UK) with controlled atmosphere containing 80% N_2, 10% CO_2 and 10% H_2.

Agar diffusion test

The methodology used was previously described (28).

After growth and purity tests, *Enterococcus faecalis* strain was individually suspended into tubes containing 5 mL of 0.85% NaCl solution. The suspension was adjusted spectrophotometrically at 800nm (OD_{800}) to match the transmittance of 90 T (equivalent to 0.5 of the McFarland scale = $1.5x10^8$ bacteria/mL). For *Porphyromonas gingivalis*, the suspension was adjusted to match the transmittance of 2.0 in McFarland scale (Transmittance of 60 T = $6x10^8$ bacteria/mL).

Next, 150 µL of *Enterococcus faecalis* inoculum and 150 µL of *P. gingivalis* was evenly plated directly onto BHI and FAA plates respectively.

Inside a laminar flow chamber, 6 sterile stainless-steel cylinders (4.0 x 1.0 x 10 mm; inner diameter, 6 mm), were added to the surfaces of the inoculated media and filled with 40 µL of freshly spatulated RECs. The plates were kept for 2 h at room temperature (*P. gingivalis* plates were kept inside the anaerobic jar) to allow the diffusion of the agents through the agar. After, they were incubated at 37^{o}C under appropriate gaseous conditions and for an appropriate period of time: facultative: 24-48 h; strict anaerobe:5-7 days). As controls, a metal cylinder containing a sterile paper disc impregnated with 2% chlorhexidine gel was used. Isolated RECs' liquids were also tested.

Zones of inhibition of microbial growth around the cylinder containing the tested substances were measured and recorded after the incubation period. The inhibitory zone was considered to be the shortest distance (mm) from the outer margin of the cylinder to the initial point of microbial growth.

Plates were photographed next to a ruler for software calibration measurement references (ImageJ Version 1.46, National Institutes of Health, USA).

Complementary tests after agar diffusion test

Tested plates and RECs discs in contact with agar surface were submitted to complementary procedures to clarify inhibition halos interpretation (26).

Culture purity

BHI and FAA plates were submitted to culture purity confirmations. One colony-forming unit (CFU), collected laterally to the RECs metallic disc was diluted in 50 µL of distilled water and Gram stained for immediate microscope optic observation.

Bacterial growth viability

Enterococcus faecalis viability after final analysis period (7 days), with a re-assay of one CFU diluted in 50 µL of distilled water and seeded on BHI to confirm growth after 24 and 72 hours. This species exhibits known resistance in cultivation conditions (27).

RECs discs scanning electron microscopy (SEM)

Tested RECs discs in contact with the inoculated agar surfaces were prepared for SEM. SEM images were captured starting at 1000x magnification.

Agar surface analysis without bacterial inoculation

It was assumed that the water of BHI interfered with the RECs powders and its surface also suffered alterations regardless bacterial inoculation (26). Metallic discs containing 1g of powder, without hydration of MTA Repair HP, MTA Flow and Biodentine were put in contact with BHI agar plates without the bacterial inoculum. In addition, calcium chloride (Sigma-Aldrich, Darmstadt, Germany), present in the composition of Biodentine, and, known to be a powerful deliquescent compound, was placed over BHI in two different amounts: 0.3 g inside the metal disc and 4 g directly over BHI, to observe its effect in BHI surface.

Direct contact method

Direct contact method used a methodology previously described (28).

Pure cultures of *E. faecalis* were grown on 5% sheep blood plus BHI agar plates (Oxoid, Basingstoke, UK). After 24 h, the colonies were suspended in tubes containing 5 mL of brain heart infusion (BHI) broth (Oxoid). The cell suspension in each tube was adjusted spectrophotometrically at 800 nm (O.D.800) to match the transmittance of 90 T (equivalent to 0.5 McFarland scale =1.5 x 108 cfu).

Hydrated discs prepared weighing 0.10 g of the RECs tested were used with 24 hours set at 37ºC.

The following systems were tested

• Tube 1 to 5 - Bacterial inoculum + disc 0.10 g of MTA Repair HP; MTA Flow; MTA Flow + 5% ZnO; Biodentine; and Experimental cement.
• Tube 6 - Bacterial inoculum + felt paper disc + 0.10 g 2% chlorhexidine gel (antimicrobial control);
• Tube 7 - Bacterial inoculum only (bacterial growth control);
• Tube 8 - 'Blank solution' BHI without the bacterial inoculum nor cement for calibration purposes.

Turbidity measurements were performed using a calibrated spectrophotometer (Spectronic 20d+, Milton Roy, Houston, USA), discounting the broth turbidity, using ‘blank solution’ tube calibration before the readings (tubes were vortexed for 5 seconds) in initial, 3 hours, 24 hours, 48 hours and 7 days.

Parallelly to the spectrophotometer readings, bacterial growth viability was confirmed by dripping 10 µL broth aliquots, at each period, in BHI plates stored at 37°C for analysis after 24 hours.

Considering the RECs dissolution, further 6 tubes (A to F) without the bacterial inoculum were kept in parallel under the same storage and readings conditions:

- Tube A to E - BHI broth + disc 0.10 g of MTA Repair HP; MTA Flow; MTA Flow + 5% ZnO; Biodentine; and Experimental cement;
- Tube F - BHI broth + disc 0.10 g Chlorhexidine.

Dissolution turbidity values were discounted from the expressed results.

Statistical Analysis

Data were submitted to D'Agostino and Pearson's tests ($P < 0.05$) and one-way Anova and Tukey tests used, with a significance level of 5%.

Results

Agar diffusion test with freshly mixed materials did not show any inhibition halos for any tested RECs regardless the analysis period. Controls with 2% chlorhexidine gel showed inhibition halos with an average diameter of 19.1 mm against *Enterococcus faecalis* and 42.2 mm against *Porphyromonas gingivalis* with a significant difference ($P < .01$). None of the isolate RECs liquids showed any inhibition halos.

Purity of the tested cultures stained with Gram were confirmed after final test periods (Fig. 1). Bacterial growth viability was observed after 7 days of contact of RECs with *Enterococcus faecalis*.

SEM analysis of the cements surface in contact with bacteria showed biofilm structures in all RECs. Detailed images in higher magnifications showed the presence of structured biofilm developed on RECs surface (Fig. 2).

Complementary agar surface analysis without bacterial inoculation showed important alterations in its surface caused by the application of the RECs. Calcium Chloride in a small amount (0.3 g) showed changes in the plate surface in 24 hours showing a halo-like image. Calcium chloride (4 g) suffered complete liquefaction in approximately 2 minutes and showed a significant change in the BHI surface in 24h and 7 days. The discs containing the powders of MTA Repair HP, MTA Flow and

Biodentine were removed from the agar surface without spreading the powder after 7 days due to its partial hydration.

In direct contact method RECs showed the highest values of turbidity after the dissolution values were discounted from the results ($P < .05$), without significant difference between them (Fig. 3). Bacterial growth control showed significant lower turbidity values when compared to RECs. Chlorhexidine used as an antimicrobial control showed significantly lower values of turbidity in all periods after 3 hours. Control test tubes presented bacterial growth viability for all periods ($P < .05$). Chlorhexidine effectively inhibited bacterial viability in all periods after 3 hours ($P < .05$). *Enterococcus faecalis* presented viability for up to 7 days.

Discussion

The aim of this work was to evaluate the antimicrobial properties of MTA Repair HP, Biodentine and MTA Flow using two methodologies: the agar diffusion method and the direct contact. In addition, it was also aimed propose the development of an experimental cement and evaluate the incorporation of ZnO to the original MTA Flow composition. The antimicrobial activity of the experimental cement and the MTA Flow + 5% ZnO was compared against other tested RECs. This property is an important aspect that must be considered in new materials development to obtain materials that can be clinically used with predictability. Our results showed that no material formulation had satisfactory bacterial growth inhibition capacity, regardless the methodology tested or the bacterial species. Similar results were reported in previous studies (1,29).

In the agar diffusion test, *Enterococcus faecalis* presented viability even after 7 days in contact with the RECs. Even considering that cement hydration occurred in contact with the inoculated surface, no inhibition halos were observed in the agar diffusion test. SEM analysis of the surface of the RECs that remained in contact with the inoculated surfaces showed that all tested materials had biofilm directly developed in its surface. None of the previous studies performed SEM analysis of the cement discs after contact with bacteria.

Complementary analysis of BHI without bacterial inoculation with the RECs powders showed partial powder hydration, probably due to the absorption of water from the medium. This effect is an indication that the use of inhibition halos tests for RECs that sets mixed with water seems to be inadequate due to possible misleading interpretation of the halos. A double halo can occur, being either the inhibition of bacterial growth and/or an alteration of the medium by the interaction with the RECs absorbing water from BHI. A previous study reported similar observation regarding Biodentine in contact with agar (26). This effect was clearly demonstrated by the damage in BHI surface caused when a large amount of calcium chloride was placed in BHI surface, justifying the false inhibition halos observed around the metal discs containing Biodentine. Nonetheless, the bacterial viability was maintained. Several studies (31–33) performed wells on the agar surface in order to contain the cement samples tested. In our understanding, the use of metallic discs containing the tested materials guarantees the BHI surface integrity and the uniformity bacterial inoculation.

Used as control for the antimicrobial activity, 2% chlorhexidine gel is a large spectrum antimicrobial agent (30). Its antimicrobial mechanism is related to its cationic molecular structure of bis-biguanide which is absorbed by the inner cell membrane and causes leakage of intracellular components. In this study, chlorhexidine showed significantly larger halos in the FAA medium inoculated with *Porphyromonas gingivalis* when compared to those observed in the BHI medium inoculated with *Enterococcus faecalis*. These results can be attributed to the greater susceptibility of *Porphyromonas gingivalis* to this substance (29). The absence of antimicrobial activity presented by tested cements suggests that a previous disinfection at infected sites is crucial prior to insertion of these materials (34).

Direct contact test used a previously described methodology (28), which is based on the reading of the transmittance values in the spectrophotometer, which provides turbidimetrically the determination of microbial growth. The higher the transmittance value the higher was the antimicrobial activity (i.e. less microbial growth). In our work, the RECs showed significant higher values of turbidity and an apparent upregulation of the bacterial growth. Bacterial growth control showed significant lower turbidity values when compared to RECs. Control test tubes presented bacterial growth viability for all tested periods. Further studies with longer analysis periods are necessary to clarify these findings.

In conclusion, none of the RECs formulations presented satisfactory antimicrobial effect for the tested methodologies. ZnO used as additive of 5% did not influence the antimicrobial activity.

Acknowledgments

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Figure legends

Fig. 1 – (A) BHI, *Enterococcus faecalis* - Arrow representing CFU collection area for the smear. (B) FAA, *Porphyromonas gingivalis* - Arrow representing CFU collection area for the smear. (C) Smear of *Enterococcus faecalis* after 7 days of contact with fresh cement. (D) *Porphyromonas gingivalis* smear after 5 days contact with fresh cement.

Fig. 2 – Representative images in higher SEM augmentation of the surface of the cements in contact with the different bacteria. Above: *Enterococcus faecalis* at 1,000x, 2,000x and 5,000x. Below: *Porphyromonas gingivalis* at 1,000x, 2,500x and 10,000x.

Fig. 3 – Spectrophotometry turbidity representation from tested RECs showing significantly the highest values of turbidity after RECs dissolution discounted values significant difference between them (different lower-case letters represents statistical difference between test tubes and controls). Positive control with *Enterococcus faecalis* inoculation showed lower average turbidity values when compared to tested RECs. Chlorhexidine presented the lowest values of turbidity in all periods after 3 hours (different upper-case letters represents statistical difference between test periods).

Figure 01

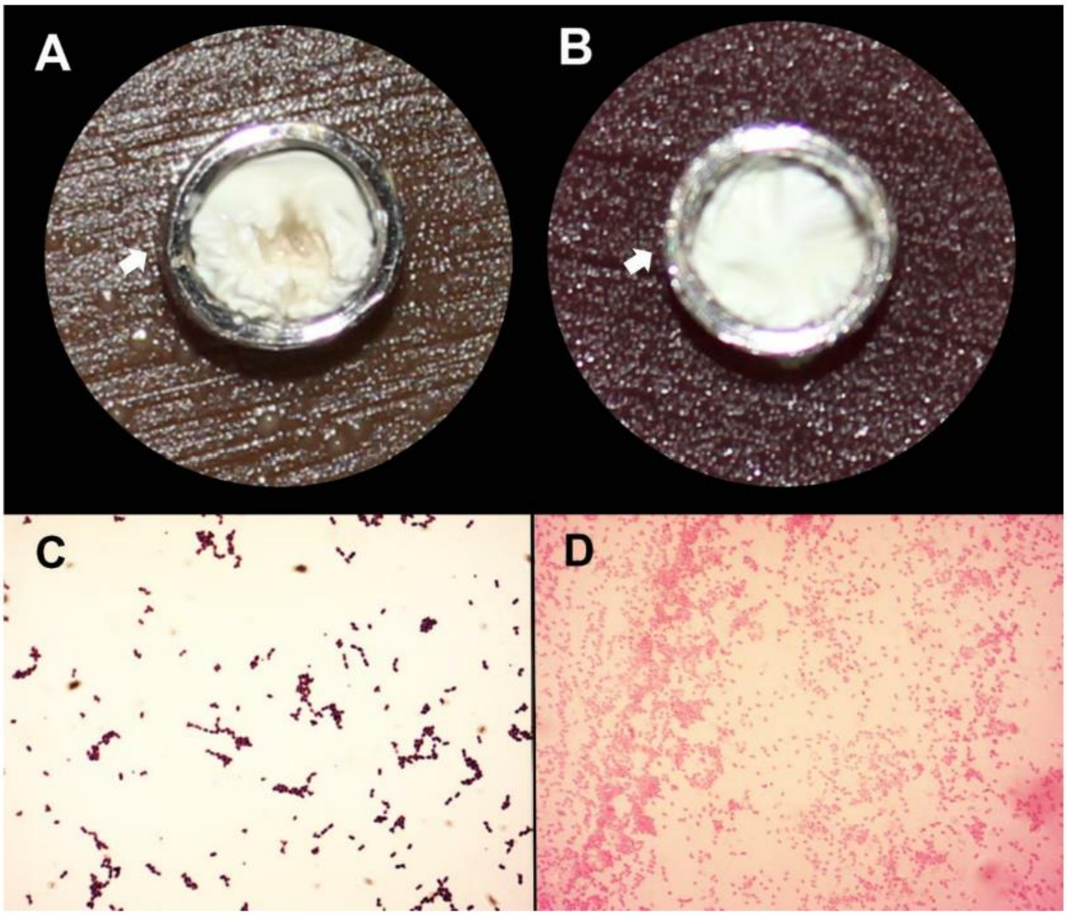


Figure 2

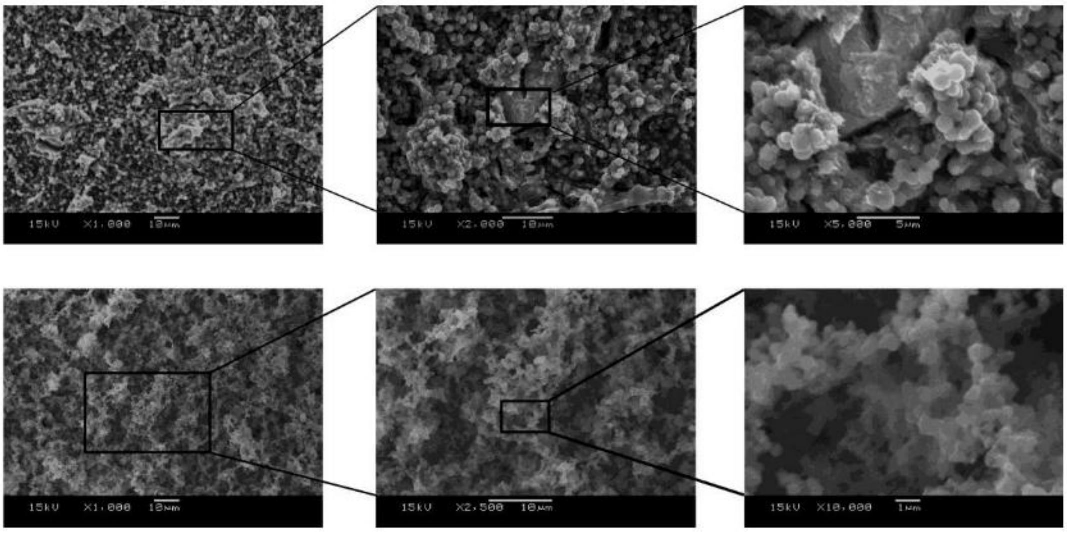
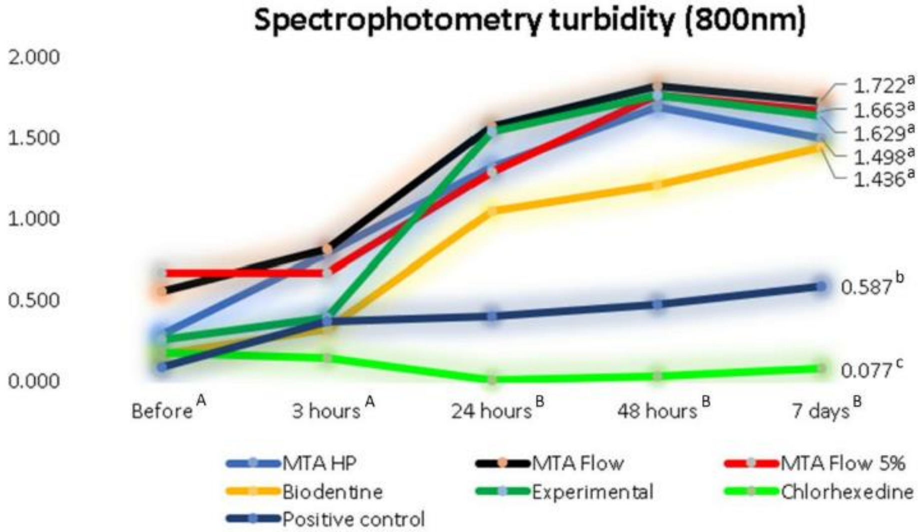


Figure 3



Spectrophotometry turbidity (800nm)
2.000
1.500
1.000
0.500
0.000
1.722a
1.663a
1.629a
1.498a
1.436a
0.587b
0.077c
BeforeA
3 hoursA
24 hoursB
48 hoursB
7 daysB
MTA HP
MTA Flow
MTA Flow 5%
Biodentine
Experimental
Chlorhexedine
Positive control

3 DISCUSSÃO

O presente estudo avaliou as propriedades físicas, químicas e antimicrobianas dos cimentos à base de silicato tricálcio de alta plasticidade presentes no mercado (MTA Repair HP, Biodentine e MTA Flow) e propôs o desenvolvimento de uma nova formulação de cimento reparador experimental. Além disso, avaliou a incorporação de ZnO ao MTA Flow, material que contém bismuto como radiopacificador, buscando a inibição da descoloração dentária e uma possível influência do ZnO sobre as propriedades antimicrobianas.

A descoloração dentária causada pelo MTA foi amplamente demonstrada *in vitro* e clinicamente. Essa desvantagem ocorre devido à interação entre o óxido de bismuto (radiopacificador) e as estruturas dentárias (Belobrov e Parashos 2011; Ioannidis et al., 2013). Em condições clínicas, o óxido de bismuto interage não apenas com a dentina, mas também com o sangue e o hipoclorito de sódio (Camilleri 2014; Marciano et al., 2014), apresentando diferentes níveis de descoloração nessas condições. Algumas alternativas foram propostas para inibir a descoloração causada pela presença de bismuto no MTA. Essas estratégias incluem a substituição do óxido de bismuto por outro radiopacificador; ou a manutenção do bismuto e a adição de ZnO ou de fluoreto de alumínio (Duarte et al., 2009; Marciano et al., 2017, 2019). Neste estudo foi testada a manutenção do óxido do bismuto como agente radiopacificador na formulação experimental e a adição de ZnO com o intuito de inibir a alteração de cor. A adição de ZnO também foi realizada à formulação original do MTA Flow. Em ambas as formulações testadas, com adição de ZnO, a inibição da alteração de cor foi observada, o que corrobora com os resultados de estudos anteriores (Marciano et al., 2017, 2019).

Quando os cimentos reparadores são utilizados, ocorre a interação entre íons do material aplicado e os tecidos adjacentes (Gandolfi et al., 2012; Josette et al., 2014). Quando considerado o colágeno presente na dentina, a reação com o óxido de bismuto resulta em uma alteração de cor acinzentada neste tecido dental (Marciano et al., 2014). A análise de mapeamento de elementos foi realizada neste estudo na interface entre os cimentos reparadores e a dentina. Esta análise mostrou que íons de bismuto foram detectados na dentina para os cimentos MTA Flow, MTA Flow + 5% ZnO e, também, para o cimento experimental. No entanto, a descoloração não foi verificada quando da adição de ZnO como aditivo (MTA Flow + 5% ZnO e cimento experimental). A alteração de cor é mensurada quantitativamente por meio do Delta E, permitindo a identificação da mudança na cor dos materiais, no entanto, quando se associa esses dados com a luminosidade (L), que indica a absorção de luz (baixos valores) ou a reflexão de luz (altos valores), é possível identificar se houve o escurecimento das amostras. Para o MTA Flow + 5% ZnO, o valor do Delta E foi estatisticamente maior quando comparado ao MTA Flow ($p < 0{,}05$). Após 28 dias, a análise mostrou que os valores de L foram semelhantes nos espécimes preenchidos com cimento MTA Flow, com ou sem ZnO. No entanto, após 90 dias, os valores de L para MTA Flow + 5% ZnO foram maiores, evidente a alteração de cor nas amostras do MTA Flow em comparação com o MTA Flow + 5% ZnO. Os resultados obtidos neste estudo estão de acordo com estudos anteriores, que sugerem que o ZnO, como aditivo, é uma alternativa plausível para se manter o óxido de bismuto como radiopacificador sem que ocorra a descoloração dentária (Marciano et al., 2017).

Como a principal característica desses materiais é a plasticidade, o teste de escoamento é fundamental para se determinar a fluidez dos materiais testados. O teste padrão ISO, originalmente projetado para cimentos obturadores endodônticos, considera apenas medidas lineares bidimensionais. Porém, as condições clínicas nas quais os cimentos reparadores são inseridos parecem ser melhor consideradas de forma tridimensional. Assim, foi proposta em 2017 por Tanomaru-Filho et al., uma avaliação com microtomografia do escoamento aplicável à cimentos reparadores considerando suas alterações volumétricas. Com esta metodologia, pode-se avaliar de forma mais esclarecedora os escoamentos linear, lateral e volumétrico. No presente estudo, o MTA Repair HP e o Biodentine apresentaram resultados semelhantes em relação ao escoamento volumétrico. Tanomaru-Filho et al., (2017) verificaram valores semelhantes quando avaliaram o Biodentine. Embora esses dois cimentos tenham apresentado menores valores de escoamentos linear e lateral, seus valores volumétricos elevados sugerem que esses cimentos proporcionariam um selamento adequado de cavidade quando utilizados em condições clínicas. O MTA Flow e o cimento experimental, provavelmente devido à sua consistência, apresentaram maiores resultados quando considerados os outros critérios de escoamento.

O tempo de presa reduzido também influencia no comportamento dos cimentos (Duque et al., 2018; Guimarães et al., 2018) quando estes são aplicados clinicamente.

O dueto plasticidade/tempo de presa inicial deve ter um equilíbrio que contemple o tempo de inserção (ainda plástico), que o material fique na posição em que foi inserido sem excessivo escoamento lateral e, por fim, que seu tempo de presa inicial seja relativamente baixo. O MTA Repair HP apresentou o menor tempo de presa inicial neste trabalho e o cimento experimental o maior. Estudos anteriores descreveram que a estabilidade dimensional, após a sua aplicação e presa final, é importante para a longevidade do material no sítio de aplicação (Camilleri 2011) e, também, para a sua capacidade de preenchimento volumétrico no selamento de cavidades (Tanomaru et al, 2017).

A radiopacidade é importante em cimentos reparadores para se obter um acompanhamento adequado (Cavenago et al., 2014; Guimarães et al., 2015). Todos os cimentos de alta plasticidade testados neste estudo apresentaram radiopacidade adequada, ou seja, pelo menos 3 mm Al de acordo com as recomendações da norma ISO. O MTA Flow + 5% ZnO e o cimento experimental, ambos contendo óxido de bismuto como radiopacificador, apresentaram os valores mais altos dessa propriedade. Neste estudo, o Biodentine e o MTA Repair HP que possuem em suas formulações radiopacificadores alternativos (óxido de zircônio e tungstato de cálcio, respectivamente) apresentaram índices de radiopacidade similares aos demais cimentos testados. A adição de ZnO à formulação original do MTA Flow aumentou significativamente a radiopacidade desse material. Estudos anteriores também relataram valores similares de radiopacidade (acima de 3 mm Al) para cimentos reparadores (Guimarães et al., 2017; Marciano et al., 2017). O óxido de zircônio e o tungstato de cálcio possuem menor peso molecular quando comparados ao bismuto, necessitando a adição de maiores volumes para alcançar uma radiopacidade similar, como demonstrado por Duarte et al. (2009).

Ao se usar cimentos reparadores, uma interação com os tecidos e fluidos adjacentes ocorre e a mudança no volume do material é esperada. A metodologia utilizada neste estudo (Cavenago et al., 2014) baseou-se na utilização de retro-cavidades preenchidas com os cimentos testados em contato com água e posterior análise utilizando-se microtomografia antes e depois da imersão. Todos os cimentos testados apresentaram alteração de volume, principalmente na superfície em contato com o fluido. O Biodentine apresentou a maior perda de volume neste estudo, o que está de acordo com estudos anteriores (Torres et al., 2017). O MTA Repair HP e MTA Flow + 5% ZnO apresentaram perdas de volume semelhantes. Por outro lado, o MTA

Flow apresentou um ligeiro aumento de volume após a imersão, provavelmente devido à absorção de água durante o período de imersão. Esse aumento no volume foi relatado anteriormente em formulações convencionais de MTA (Duque et al., 2018).

O equilíbrio entre a liberação dos componentes e a estabilidade dos materiais é importante para fornecer selamento e prover biocompatibilidade, uma vez que esses materiais permanecem durante longo período em contato com os tecidos. No presente estudo, um aumento progressivo dos valores de pH para o Biodentine e o cimento experimental foi observado durante o período do estudo e a maior liberação de íons cálcio também foi associada a esses dois materiais. Este fato pode ser explicado devido à maior solubilidade apresentada pelo Biodentine e pelo cimento experimental. Guimarães et al. (2017) encontraram valores de pH mais altos para o MTA Flow, em consistência "putty" associado a maiores valores de solubilidade, em comparação com este estudo que usou consistência "fina" para este material. Neste estudo, todos os materiais testados apresentaram valores de pH entre 8 e 9 após 28 dias de imersão em água e a liberação de íons cálcio apresentou valores significativamente maiores após 28 dias. Resultados semelhantes foram observados em estudos anteriores (Camilleri 2011; Guimarães et al., 2018). O Biodentine apresentou a maior perda de volume neste estudo, o que está de acordo com estudos anteriores, sendo essa alta solubilidade associada ao seu plastificante (Torres et al., 2017). Outra possibilidade pode estar relacionada ao volume do radiopacificador adicionado à sua composição (Cutajar et al., 2011). Devido ao seu alto peso molecular, o uso do bismuto exige volumes menores para uma adequada radiopacidade. Por outro lado, os radiopacificadores alternativos requerem maior volume para obter valores de radiopacidade semelhantes, o que pode comprometer as propriedades do material. O MTA Flow apresentou um discreto aumento de volume após a imersão, provavelmente devido à absorção de líquido durante o período de imersão. Esse aumento no volume foi relatado anteriormente em formulações convencionais de MTA (Duque et al., 2018). Perda volumétrica foi encontrada em um estudo anterior para o MTA Flow quando avaliado com metodologia similar a deste estudo, porém na consistência *putty*, a qual também é recomendada pelo fabricante (Guimarães et al. 2017). No presente estudo, foi utilizada a consistência '*thin*', o que pode explicar a razão dessa variabilidade nesses resultados.

Em relação às propriedades antimicrobianas dos cimentos, nossos resultados mostraram que nenhuma formulação de material apresentou capacidade de inibição do crescimento bacteriano, independentemente da metodologia testada ou da espécie bacteriana. Resultados semelhantes foram relatados em estudos anteriores (Arias-Moliz e Camilleri 2016; Farrugia et al., 2017). A capacidade antimicrobiana de cimentos é um importante aspecto do material, uma vez que este deveria evitar a recontaminação bacteriana em locais onde estes cimentos são utilizados. A viabilidade bacteriana do *Enterococcus faecalis* após 7 dias mostrou que, mesmo após um tempo relativamente longo em contato com os cimentos reparadores, não houve evidências de inibição. Mesmo considerando que a hidratação do cimento ocorreu em contato com a superfície inoculada, não foi observado qualquer tipo de halo de inibição no teste de difusão em ágar. No intuito de avaliar a superfície do cimento reparador, após o contato entre o cimento reparador com o inóculo bacteriano, durante a presa do material, realizou-se a análise com microscopia eletrônica de varredura dos discos de cimento após contato com bactérias. Essa análise não havia sido anteriormente relatada, e, neste estudo, mostrou a presença tanto de *Enterococcus faecalis* quanto *Porphyromonas gingivalis* agregados em forma de biofilme na superfície de todos os cimentos testados.

O teste de contato direto inóculo/cimento utilizou uma metodologia descrita anteriormente (Gomes et al., 2004), contra *Enterococcus faecalis*, no qual discos de cimentos reparadores foram testados e sua dissolução considerada em uma análise paralela. Em nossos achados, os cimentos mostraram valores significativamente maiores de turbidez em relação aos controles do crescimento bacteriano e ao controle antimicrobiano (clorexidina gel 2%). As confirmações de crescimento bacteriano mostraram, para todos os tubos testados, viabilidade bacteriana mesmo após 7 dias em contato direto para todos os cimentos, o que corrobora com os resultados do teste de difusão em ágar deste estudo. Resultados diversos foram relatados em estudos anteriores utilizando o contato direto entre cimentos reparadores e meios bacterianos inoculados (Al-Hezaimi et al., 2006; Holt et al., 2007; Odabaş et al., 2011; Arias-Moliz et al., 2017) .

O desenvolvimento de novos materiais endodônticos reparadores com propriedades similares às do cimento MTA objetiva aprimorar as características de manipulação e estabilidade de cor dentária. Os cimentos testados neste estudo apresentaram um avanço nas características de manipulação, evidenciado pelos valores de escoamento encontrados. A alteração de cor visualizada no MTA Flow foi inibida pela adição de ZnO à sua formulação contendo bismuto, o que parece ser uma alternativa para que se mantenha o óxido de bismuto como agente radiopacificador, com alto peso molecular exigindo adição menor em volume, porém tendo a sua potencial descoloração inibida por essa adição. Todas as propriedades físicas e químicas dos cimentos testados, contendo o aditivo ZnO, apresentaram resultados comparáveis aos demais materiais, do que se pode inferir que essa adição não altera significativamente tais propriedades dos cimentos contendo óxido de bismuto. A ausência de um efeito antimicrobiano significativo desses materiais sugere que uma desinfecção prévia de locais infectados onde esses materiais serão usados clinicamente é necessária, uma vez que se espera obter resultados bem-sucedidos a longo prazo, sem que ocorra recolonização bacteriana. A caracterização química dos cimentos, o comportamento frente ao contato com diferentes irrigantes utilizados em Endodontia, o contato com sangue, a citotoxicidade, a biocompatibilidade em contato com tecidos *in vivo* e avaliações clínicas à longo prazo ainda são aspectos a serem analisados para se assegurar previsibilidade no uso desses materiais em suas diferentes indicações clínicas.

4 CONCLUSÕES

O estudo permitiu concluir que em todos os materiais avaliados houve interação entre o cimento e a dentina verificado por meio da migração de elementos. A alteração de cor (ΔE) ocorreu para todos os cimentos testados, porém diferentes níveis de luminosidade (L) foram detectados indicando o escurecimento especialmente dos espécimes do MTA Flow. O MTA Flow apresentou potencial para descoloração dentária que foi reduzida pela adição de ZnO.

Todos os materiais testados apresentaram propriedades físico-químicas em diferentes níveis em relação a alteração de volume, radiopacidade, tempo de presa, liberação de íons cálcio e pH. A adição de 5% de ZnO à formulação original do MTA Flow melhorou significativamente os valores obtidos de radiopacidade e escoamento ISO e não alterou significativamente as demais propriedades. O cimento experimental apresentou propriedades físico-químicas comparáveis aos demais materiais testados.

Nenhuma das formulações testadas apresentou efeito antimicrobiano satisfatório para as metodologias avaliadas. O ZnO utilizado como aditivo a 5% não influenciou a atividade antimicrobiana.

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ANEXOS

Anexo 1 – Carta submissão ao periódico *Clinical Oral Investigations*.

Clinical Oral Investigations

Colour Stability and Elemental Migration of Tricalcium Silicate-Based Dental Biomaterials in Dentine

--Manuscript Draft--

Manuscript Number:	CLOI-D-19-00064	
Full Title:	Colour Stability and Elemental Migration of Tricalcium Silicate-Based Dental Biomaterials in Dentine	
Article Type:	Original Article	
Corresponding Author:	Marina Angélica Marciano, PhD State University of Campinas Piracicaba, São Paulo BRAZIL	
Corresponding Author Secondary Information:		
Corresponding Author's Institution:	State University of Campinas	
Corresponding Author's Secondary Institution:		
First Author:	Lauter Eston Pelepenko, Msc	
First Author Secondary Information:		
Order of Authors:	Lauter Eston Pelepenko, Msc	
	Fernanda Romeiro De Gennaro, DDS	
	Marco Antonio Hungaro Duarte, PhD	
	Josette Camilleri, PhD	
	Adriana de Jesus Soares, PhD	
	Marina Angélica Marciano, PhD	
Order of Authors Secondary Information:		
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Abstract:	Objective Evaluate colour stability, chemical element migration in contact with dentine of calcium silicate-based cements and evaluate whether the addition of zinc oxide (ZnO) to formulations containing bismuth oxide intending to prevent dental discolouration. Materials and methods Thirty-five bovine teeth were prepared and filled with MTA HP, Biodentine, MTA Flow, MTA Flow+5% of ZnO and an experimental biomaterial. Teeth were sealed with composite and stored in separate flasks immersed in tap water at 37oC. Colour assessment was performed with a spectrophotometer before filling, 24 hours, 28 days and 90 days after filling. Colour change (ΔE) and luminosity (L) were calculated. Representative samples were sectioned for stereomicroscopy. Elemental mapping in scanning electron microscope (SEM) and energy dispersive spectroscopy (EDS) was evaluated. Statistical analysis was performed using nonparametric Kruskal–Wallis and Dunn test (p<0.05). Results Higher L values were found for MTA HP, Biodentine and experimental biomaterial in comparison to MTA Flow at 90d (p<0.05). The addition of ZnO inhibited dental staining of MTA Flow after 90d of contact with dentine. The mapping of elements revealed migration of bismuth and silicon in dentine.	

	Conclusions MTA Flow exhibited potential for dental discoloration, which was inhibited by the ZnO addition. Experimental biomaterial containing bismuth oxide and ZnO, had similar luminosity values to that of MTA HP containing calcium tungstate and Biodentine containing zirconium dioxide as radio-opacifiers. Clinical relevance The bismuth oxide containing material MTA Flow, has potential for dental discoloration, which is inhibited by addition of ZnO. Biomaterials with other radiopacifiers does not discolour tooth structure.
Suggested Reviewers:	Hal Duncan Hal.Duncan@Dental.tcd.ie

Anexo 2 – Carta submissão ao periódico *Journal of Applied Oral Science*.

Journal of Applied Oral Science

Physicochemical properties of high-plasticity reparative biomaterials

Journal:	*Journal of Applied Oral Science*
Manuscript ID	Draft
Manuscript Type:	Original Article
Please use keywords available at http://decs.bvs.br/.:	Endodontics, Dental Cements, X-Ray Microtomography

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Anexo 3 - Relatório do *Turnitin*

AVALIAÇÃO DAS PROPRIEDADES FÍSICO-QUÍMICAS E ANTIMICROBIANAS DE BIOMATERIAIS À BASE DE SILICATO TRICÁLCIO

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