Evaluation of the Antimicrobial Effect on *Enterococcus Faecalis* of Bioceramic Cements with and without Silver Nanoparticles

Keywords

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ABSTRACT

Objective: To compare the antimicrobial effect of bioceramic endodontic cements with and without silver nanoparticles on Enterococcus faecalis. Materials and Methods: Six groups were evaluated (n=8), and divided according to the materials: MTA Flow + AgNP ; MTA Flow Group: Bio C Repair + AgNP; Bio C Repair; PBS Cimmo + AgNP; PBS Cimmo. The groups were maintained for 72 h in the suspension of Enterococcus faecalis in Simulated Body Fluid (SBF). Samples of 100 μL were removed from the suspension at 0, 24, 48 and 72 h and seeded in triplicate in a Petri dish. Colony forming units (CFU) were counted using a colony counter. All procedures described were performed in a laminar flow chamber. Two-way ANOVA followed by Tukey's post hoc test and paired t-test were used for statistical analyses ($\alpha = 0.05$). Results: The addition of silver nanoparticles resulted in a statistically significant difference for MTA and CIMMO PBS cements ($p<0.05$), with the lowest bacterial growth being shown by the MTA group. For all groups, only the times of 48 and 72 h presented results without differences. Conclusion: The addition of silver nanoparticles to bioceramic cements was efficient to promote an acceleration of bacterial death.

INTRODUCTION

The success of endodontic therapy is based on the maximum reduction of microorganisms presence in the root canal system, as these are largely responsible for the development and perpetuation of periapical conditions.[1] An important process for the success of intervention is the sealing of root canal system with guttapercha and filling cement. The main function of cement is to eliminate the voids between the solid material and the irregularities of dentinal walls. This process is preceded by maneuvers associating mechanical instrumentation and chemical substances. Even with the efficiency of such a protocol and the use of intracanal medication, the total elimination of microorganisms is not possible due to the complex anatomy of root canals.[2] In addition, there are still microorganisms that are resistant to canal shaping and to the action of medication used. The most resistant microorganism is *Enterococcus faecalis*.[3] When present in endodontic infections, it can make them refractory, preventing tissue repair and consequently treatment success. Thus, the use of cements for endodontic fillings that contain antimicrobial activity is considered an important factor in reducing the concentration of residual microorganisms.[4]

Mineral trioxideaggregate (MTA) is a bioactive endodontic cement composed mainly of calcium and silicate elements. It was approved by the Food and Drug Administration (FDA) for use in the United States in 1997.[5] Bioceramic cements are materials resulting from the combination of calcium silicates and calcium phosphates, and may also include particles of alumina, zirconia and bioactive glasses. Bioactive cements are considered advantageous mainly for two characteristics: the presence of calcium phosphate, a structure similar to hydroxyapatite (which improves the physicochemical bond between root dentin and cement), and their biocompatibility, which makes the periapical tissues do not reject the material.[6] As it is a material with excellent properties, several bioceramic products are currently being launched and studied. Among their properties are: high radiopacity, good sealing ability, tissue tolerance, chemical stability and zero contraction after setting.[7]

Adding an effective antiseptic substance against the microorganism *Enterococcus faecalis* in the composition of bioceramic cements in order to enable its elimination would be interesting.[8,9] Singh *et al*. (2016) assessed the antibacterial properties of endodontic cements against *E. faecalis*, and concluded that the bioceramic cement Endo Sequence BC Sealer was similar to MTA, having even better results when compared to resin-based and zinc oxide/eugenol cements.[10]

A good option to enhance the antimicrobial effect of endodontic cements would be the addition of silver nanoparticles, since silver is recognized for its antiseptic action. It is widely used in the medical field, in the manufacture of vascular and urinary catheters, as it prevents the attachment of microorganisms to the surface of such devices, among other applications.[11,12] As a nanoparticle, silver still has its broad spectrum and excellent biocompatibility, having a long history of use in medicine as an antimicrobial and anti-inflammatory agent.[13] It is characterized by clusters of insoluble silver atoms smaller than 100 nm in size. This feature is important because smaller particles give rise to larger specific surface areas and therefore reduce the concentration of particles needed for effectiveness.[9]

In restorative dentistry, the silver nanoparticle has been tested as an antiseptic substance with excellent results, having been incorporated into composite resins to reduce the accumulation of biofilm on the tooth surface.[14] In orthodontics, Ahn *et al*. (2009) included it along with silica nanofilaments to the adhesive system to improve the physical and antimicrobial properties in the fixation of brackets.[15] Kreth *et al*. (2008) incorporated silver ions into the Kerr Pulp Canal Sealer cement and tested it on *S. mutans*, obtaining an increase in the antiseptic action of this cement.[16] Lofti *et al*. (2011) demonstrated an antiseptic action similar to 5.25% sodium hypochlorite on the microorganism *E. faecalis*, but at much lower concentrations.[17] Onoda (2011) incorporated silver nanoparticles into several endodontic cements, obtaining a greater antiseptic action for zinc oxide and eugenol-basedcements.[18] Pinto (2013) compared the effects of the antimicrobial action on *E. faecalis* of white and gray MTA cements, when added to the liquid and powdered silver nanoparticles. It was concluded that the addition of the powdered form promoted anantimicrobial effect in a shorter time on the microorganism.[19]

It is known that micrometric silver particles are clog cell membranes, thus causing silver poisoning. So the use of silver nanoparticles, which are able to circulate in the membrane, is justified.[20] According to the kinetic and toxicity studies, the compositions of silver nanoparticles vary widely, as well as the descriptions of silver formulations used which in addition being divergent are limited, especially its solubility, size and aggregation. Good dispersion of silver nanoparticles is necessary for effective antibacterial activity and can influence their microbial toxicity.[21]

The toxicity of silver nanoparticles is directly associated with free silver ions.[22] Laboratory studies have reported that silver nanoparticles can induce oxidative stress thereby impairing the mitochondrial function of human cells.[20] Furthermore, silver can be detected in organs after the administration of massive doses of nanoparticles, mainly in the spleen and liver. Another concern is the ability of silver nanoparticles to cross the blood-brain barrier via transsynaptic transport and accumulate in the brain.[23]

Therefore, studies were carried out seeking confirmation of such concerns, however, silver nanoparticles, even at low concentrations, decrease some inflammatory cytokines and angiogenesis parameters, in addition to expressing anti-inflammatory properties. They are biocompatible with fibroblasts and keratinocytes. After 8 weeks, the silver present in the organs can be eliminated.[21]

Thus, the aim of the present study was to compare the antimicrobial effect of bioceramic endodontic cements Bio C Repair, MTA Flow, PBS Cimmo with and without powder silver nanoparticle on *Enterococcus faecalis* at 0, 24, 48 and 72 hours. The null hypothesis is that the addition of silver nanoparticles to bioceramic endodontic cements would not improve its antibacterial properties.

MATERIAL AND METHODS

SAMPLE SIZE

To determine the number of samples in this study, the Bio Estat 5.0 software was used, using the Student t-test for independent samples, significance level of 5% and Power of 90%, with an (n) of 8 samples per group being suggested, already adopting a 10% safety margin.

PREPARATION OF SPECIMENS

A flowchart of the study design is illustrated in Figure 1. Forty-eight specimens were made (n=8), with cement manipulation following the manufacturers' recommendations. A precision balance (Ohaus Corporation Pine Brook, New Jersey, USA) was used to adjust the weight of silver nanoparticle to be added to the cements of corresponding groups. The discripton of the materials used is found in Table 1. The distribution of the groups was as follows:

Group 1 – MTA+AgNP - MTA Flow cement plus 1% of the weight of the cement of silver nanoparticle powder (576832, Sigma-Aldrich BrasilLtda, São Paulo, Brazil);

Group 2 – MTA Flow cement (Ultradent Industry of dental products S/A, Indaiatuba, Brazil);

Group 3 – SE+AgNP - Bio-C Sealer Repair cement plus 1% of the weight of the cement poder of silver nanoparticle powder (576832, Sigma-Aldrich Brasil Ltda, São Paulo, Brazil);

Group4 – SE - Bio-C Sealer Repair cement (Angelus Industry of dental products S/A, Parana, Brazil);

Group 5 – CI+AgNP - PBS Cimmo cement plus 1% of the weight of the cement poder of silver nanoparticle powder (576832, Sigma-Aldrich Brasil Ltda, Sao Paulo, Brazil);

Group6 – CI - PBS Cimmo cement (MJS Industry of dental materials Ltda- ME, Pouso Alegre, Brazil);

The cements were manipulated on a glassplate (Prisma, Sao Paulo, Brazil) using a No. 24 spatula and inserted into a two-millimeter-diameter, 5-millimeter-high split Teflon mold (Metalcard, São Jose dos Campos, Brazil) with a plastic amalgam holder (Jon dental products, Sao Paulo, Brazil) and condenser (Condenser No. 3, Hu–Friedy, North Carolina, USA). After condensation of the cements, the molds were stored in plastic pots covered with wet gauze and kept at room temperature of 24ºC waiting for the final setting of material within 48 hours.

After setting, each specimen was removed from them old and placed in a polypropylene flask with a capacity of 1.5 mL (Eppendorf of Brasil Ltda., Sao Paulo, Brazil), packed in autoclavable envelopes (Sispack, Sao Paulo, Brazil) and sterilized in an autoclave (Cristofoli Equipamentos de Biosseguranca LTDA., Parana, Brazil) at 121ºC for thirty minutes.

PREPARATION OF SIMULATED BODY FLUID (SBF)

The simulated body fluid (SBF) was prepared for use following the protocol of Onoda (2011).[18]

CONTAMINATION OF SPECIMENS

One point eight mL of the bacterial suspension in PBS was transferred aseptically with an automatic pipette to each vial containing a sterilized specimen, establishing a 1:10 weight to volume ratio. The contaminated specimens were kept in

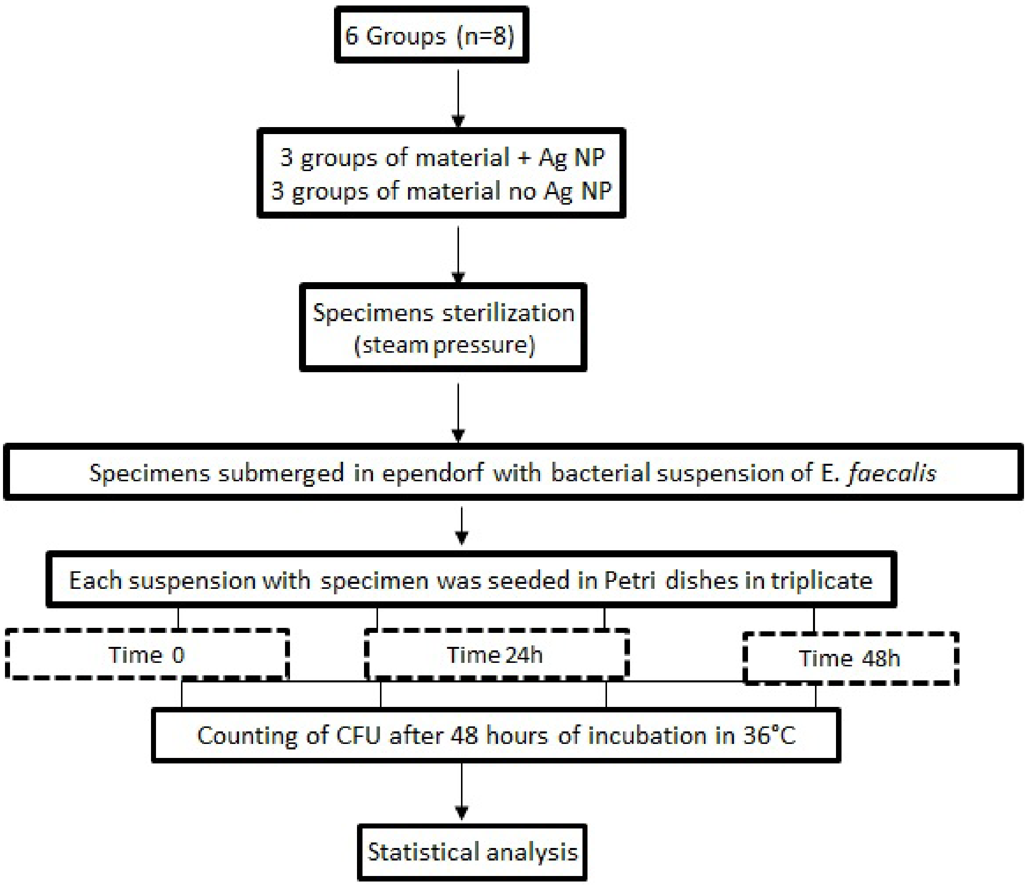

Figure 1: A flowchart of the study design

Table 1. Description of the materials used.

Product	Composition	Manufacturer
MTA FLOW	Tricalcium silicate and Dicalcium silicate	ULTRADENT DENTAL PRODUCTS
BC SEALER REPAIR	Tricalcium silicate, dicalcium silicate, tricalciumaluminate, calcium oxide, zirconium oxide, silicium oxide, polyethylene glycol andiron oxide	ANGELUS INDUSTRIA DE PRODUTOS ODONTOLÓGICOS S/S
PBS CIMMO	Calcium oxide, Calcium carbonate, magnesium oxide, dicalcium silicate, aluminium oxide, sodium oxide, potassium oxide, pozzolan	MJS INDUSTRIA E COMERCIO DE MATERIAIS ODONTOLÓGICOS Ltda. ME

an oven at 35°C for 72 hours. For positive control, 1.2 mL of the suspension was placed in a sterilized flask which was also incubated at 35°C for 72 hours. For the negative control, a sterilized vial containing 1.2 mL of SBF and one specimen from each group, in addition to a sterilized vial containing only 1.2 mL of SBF. All procedures described were performed in a laminar flow chamber.

BACTERIAL DEATH KINETICS

The kinetics of *E. faecalis* cell death was verified at each 24-hour interval of incubation. Aliquots of 100µl were taken from each vial containing a specimen, for decimal dilutions in sterile saline solution (10^{-1}, 10^{-2} and 10^{-3}). One hundred micro liters of each dilution were seeded, in triplicate, in Petri dishes containing Nutrien tagar (DIFCO) plus 0.001% bromothymol blue (Merck, Sao Paulo, Brazil) and incubated at 35°C for 48 hours. Colony forming units (CFU) were counted using a colony counter (CP 600 Plus, Phoenix, Araraquara, Sao Paulo, Brazil). After obtaining the CFU averages, the number of CFU/mL was calculated for each of the times.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS 20 program. Initially, the Kolmogorov-Smirnov test was performed with a significance level of 95% to determine data normality. In the direct contact test, intra group analysis was performed using the log of number of CFU/mL at each time point and the two-way ANOVA test followed by the TUKEY's *post hoc* test were applied. When comparing the difference in the amount of bacteria in each time interval: time zero-24 hours; 24-48 hours, for each of the samples from each group, the paired t-test was used. A significance level of 95% was considered.

RESULTS

The colony forming units of *Enterococcus faecalis* were quantified by milliliters (CFU/mL) and were seeded in triplicate in the direct contact test, as follows:

All negative control groups did not show growth on the plates during the entire experimental period. Positive control groups allowed plaque counting at 0 and 24 hours.

In view of the data presented, the addition of the silver nanoparticle resulted in a statistically significant difference for the MTA and CIMMO PBS cements, with the lowest bacterial growth being shown by the MTA group. When analyzing the times, within the same group, all comparisons were statistically significant, except for 48H x 72H, which had a result equal to zero. When the groups were compared, with the exception of the comparison of the SE x CI and CI+P x CI groups, all groups had no statistical differences. The means and standard deviation of the absolute numbers of the CFU/mL counts at the different times are shown in Table 2 and 3.

DISCUSSION

The microorganism *Enterococcus faecalis* has been identified as the most prevalent microorganism in persistent secondary apical periodontitis, in a percentage ranging from 14.06% to 77.8%. That speciesis a Gram-positive facultative anaerobe, which has characteristics that qualify it to survive in an inhospitable environment for most microorganisms, such as high temperature and extremely alkaline pH. They still have a large number of virulence factors and the possibility of staying alive, without proliferating, when deprived of nutrition.[1] Based on this information, it is clear why this microorganism was chosen for the present study.

It is important to note that studies on the antimicrobial action of new bioceramic cements are still scarce in the literature due to the recent launch of products on the market. It is then possible to use as a basis the works on the antimicrobial action of MTA against *Enterococcus faecalis*, which are still conflicting. Researches were carried out with different methods of exposure to the material and different standard strains. Using the inhibition halo, the work by Miyagak *et al.* (2006)[24] showed no inhibition while that of Holt *et al.* (2007)[25] showed inhibition.

Table 2. Mean and standard deviation (SD) for the absolute numbers of log 10 CFU/mL values at different times for the test groups.

	Material					
Time	**MTA+AgNP**	**MTA**	**CI+AgNP**	**CI**	**SE+AgNP**	**SE**
0	7.78±0.21	7.56±0.23	7.76±0.11	8.12±0.62	8.13±0.62	7.85±0.21
24	2.16±0.44	2.25±0.53	2.13±0.66	2.45±0.69	2.41±0.84	1.60±0.65
48	0	0	0	0	0	0
72	0	0	0	0	0	0

Table 3. Result of the difference in the bacterial growth in log 10 values (mean ±sd)

	Material						
Time	**MTA+AgNP**	**MTA**	**SE+AgNP**	**SE**	**CI+AgNP**	**CI**	**P**
T 0 -24	5.62±0.37Aa	4.79±0.63Ba	5.63±0.60Aa	5.67±0.91Aa	5.71±0.89Aa	6.25±0.70Ca	0.0001
T 0 -48	7.78±0.21Ab	7.56±0.23Ab	7.76±0.11Ab	8.12±0.62Bb	8.13±0.62Bb	7.85±0.21Ab	0.21
T 0 -72	7.78±0.21Ab	7.56±0.23Ab	7.76±0.11Ab	8.12±0.62Bb	8.13±0.62Bb	7.85±0.21Ab	0.21
T24-48	2.16±0.44Ac	2.25±0.53Ac	2.13±0.66Ac	2.45±0.69Ac	2.41±0.84Ac	1.60±0.65Bc	0.56
T24-72	2.16±0.44Ac	2.25±0.53Ac	2.13±0.66Ac	2.45±0.69Ac	2.41±0.84Ac	1.60±0.65Bc	0.56
T48-72	0	0	0	0	0	0	-

*** Different capital letters indicate statistical difference between groups on the same line p‹0.05.**
Different lower case letters indicate statistical difference in the same group according to time variation in the same column p‹0.05.

In direct contact tests, performed at different times, Al-Hezaimi *et al.* (2006) observed inhibition only when gray MTA was used, white MTA allowed bacterial growth.[26] Zhang *et al.* (2009) observed inhibition in up to four hours,[2] while in the work by Eldeniz *et al.* (2006) there was only delay in growth.[27] Bidar *et al.* (2012) did not observe any effect on the microorganism,[28] as well as Odabaş *et al.* (2011).[29] Given this, it seems pertinent to add some substance that will improve its actionon resistant microorganisms such as *Enterococcus faecalis*.[29]

According to the works presented here, the silver nanoparticle showed an effective antimicrobial action against *Enterococcus faecalis*, which is why it was proposed in this study[17,18,29] and because it has already been associated with endodontic cements with good results.[11,18,19,29]

The method of present study was following the research methodology of Onoda (2011), although it was carried out with different bioceramic cements.[18] The suspension prepared and used was SBF (Simulated Body Fluid), a solution that contains a concentration of ions similar to that of human blood plasma, thus creating a condition closer to the dental apex. It was developed by Kokubo *et al.* (1990).[30]

The concentration of 1% of cement weight of silver nanoparticles added to the tested materials was based on the study of Pinto (2013)[19] which in its preliminary tests made an experiment to identify the minimum inhibitory concentration (CIM) of such material on *Enterococcus faecalis*.

All bioceramic cements showed a reduction in bacterial growth of *Enterococcus faecalis* over time, and after 48 hours there was no growth. Analyzing the data of each groups eparately, it can be noticed that there was an antiseptic action of the materials during the entire period of experiment, with a statistically significant difference for all groups (p‹0.05).

The observation of data shows that the greatest variation in growth occurred between 24 and 48 h, which can be considered a natural antiseptic action of bioceramics. The variation between the time zero and 24 h was greater between the groups added with silver nanoparticle, which highlights the fact that the silver nanoparticle accelerates the antimicrobial action of endodontic cements presented here. The results of Odabaş *et al.* (2011),[29] who incorporated Silver-zeolite (2%) into MTA and performed a diffusion test on agar, resulted in the inhibition of *Enterococcus faecalis*. The effect increased as a function of time, while MTA alone showed no inhibition. The work also presented another very interesting data that, when performing the test to observe the release of silver ions from the MTA specimen associated with silver-zeolite, the authors observed a greater release of silver, which is in line with the results of this study, where the greatest action in the inhibition of microorganisms happened in the first 24 hours.

Our findings are also supported by the results of Pinto (2013).[19] In this study, the addition of silver nanoparticles to white MTA cement was evaluated to improve the antimicrobial action against *Enterococcus faecalis*. The direct contact test was performed using specimens of the materials: white MTA, gray MTA, white MTA + AgNP powder, white MTA + AgNP solution. The specimens were kept for 72 hours in the suspension of *Enterococcus faecalis* in SBF. The white MTA cement, with silver nanoparticles in powder, showed antimicrobial actionagainst *Enterococcus faecalis*, stopping bacterial growth from 24 h onwards.

The Bio C Repair group showed no statistical difference when compared to the other groups, a fact that it could be speculated on the possibility of being related to its composition, since it is composed of calcium silicates, calcium aluminate, calcium oxide, zirconium, iron oxide, silicondioxide and dispersing agent.

Onoda (2011) evaluated the antimicrobial capacity of endodontic cements added with silver nanoparticles: Endométhasone, Pulp Canal Sealer and AH Plus. At an interval of 24 hours, 1 mL aliquots was collected and plated for later counting of *Enterococcus faecalis* colonies. Data analysis showed that zinc oxide and eugenol-based cements are more effective in eliminating *E. faecalis* than AH Plus.[18]

Bringing these results to the daily routine of clinic, it is possible to conclude that better effective action in the first 24 h post-intervention is an important way to immediately eliminate microorganisms resistant to the endodontic procedure, reducing the chance of recontamination. However, more studies in biological complex systems, like animals, are needed to better understand the clinical effects of silver, since there is still no much information about its toxicity, crown staining and other possible adverse effects. Clinical studies in future might elucidate in fact its contribution on endodontic microbial control. The period of investigating the antimicrobial effect of *Enterococcus faecalis* on bioceramic cements could be another limitation of our study. Longer periods are suggested for future studies.

CONCLUSION

The silver nanoparticle was an important agent in the inhibition of Enterococcus faecalis associated with the bioceramic cements MTA Flow, Cimmo PBS and Bio C Repair. The results of the present study showed that the addition of silver nanoparticles to bioceramic cements was efficient to promote an acceleration of bacterial death.

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