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ANALYSIS OF PHYSICOCHEMICAL PROPERTIES OF AH PLUS CEMENT ASSOCIATED WITH ANTIMICROBIAL SUBSTANCES[1]

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ABSTRACT

HA Plus is the endodontic cement considered the gold standard, which will perform the function of the union between the gutta-percha interfaces and dentin walls, however, its action against microorganisms is limited. Ambroxol (ABX) is a VII metabolite of bromhexine and has known pharmacological mucokinetic and expectorant actions. N-acetylcysteine is a potent antioxidant derived from cysteine, being a non-antibiotic compound, but with antimicrobial action. Thus, the objective of the present study is to evaluate HA Plus associated with 5% N-acetylcysteine and HA Plus associated with 5% Ambroxol, regarding physical properties (radiopacity, setting time, flow, solubility, compressive strength) and antimicrobial activity. 1 - To evaluate radiopacity, we will use metal rings in which the experimental cements will be inserted and maintained, these will be X-rayed and processed manually, to be later digitized and analyzed in the Digora 1.51 program. 2 – Setting time: the experimental cements poured into metal rings will be subjected to marking with vertical pressure, using Gilmore needles. 3 – Flow: the cement will be spatulated in the center of a glass plate where another will be placed on it, both weighing 100g, and, after 10 minutes from the beginning of spatulation, the weight will be removed and the larger and smaller diameter of the cement will be measured with the help of a digital caliper, and the average of the two diameters will be considered the cement flow. 4 – Solubility: 30 acrylic teeth will be filled with the experimental cements. The analyses will be made by comparing the volume in mm^3 by the images obtained in micro-computed tomography (micro-CT). Antimicrobial action: under the metal rings filled with the test cements and control group will be induced *in vitro* biofilm of *Enterococcus faecalis*. After the 21-day period, the samples will be stained Live/Dead Technique-In Vitro Gen, and then evaluated under the Confocal Laser Scanning Microscope. Under the cement blocks, the adhesion of *Ent. faecalis will be analyzed*. The results will be submitted to the D'Agostino and Pearson tests for verification of normal distribution.

Keywords: HA plus. Ambroxol. N-acetylcysteine. MicroCT. SEM. Biofilm.

[1] Final Report presented to FAPESP (Scientific Initiation, Process 2018/26299-6)

Revista ARACÊ
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RELATED TO THE SCIENTIFIC PROJECT

The work plan and execution schedule covers the period between February 2019 and June 2019.

In the months of February, March and April, the radiopacity and setting time experiments were carried out. In May and June, the runoff, solubility and antimicrobial capacity test was carried out.

To evaluate the radiopacity, we used metal rings in which the experimental cements were inserted and maintained, these were radiographed and processed manually, and later they were digitized and analyzed in the Digora 1.51 program. For the setting time, the experimental cements poured into metal rings were submitted to vertical pressure marking, using Gilmore needles. Regarding the flow, the cement was spatulated in the center of a glass plate where another was placed on it, both weighing 100g, and, after 10 minutes from the beginning of the spatulation, the weight was removed and the largest and smallest diameter of the cement were measured with the aid of a digital caliper, and the average of the two diameters was considered the cement flow. For solubility: 30 acrylic teeth were filled with the experimental cements. The analyses were performed by comparing the volume in mm^3 by the images obtained in micro-computed tomography (micro-CT). Under the cement blocks, the amount of adhesion of *Ent. Faecalis* after 2 hours. And the results were submitted to the D'Agostino and Pearson tests to verify normal distribution.

RELATED TO ACADEMIC BACKGROUND

In March and April 2019, the student participated in the Academic League of Pediatric Dentistry at FOB/USP.

Since February 2019, the student has been participating as a member of the Organizing Committee of the 32nd Bauru Dental Congress, at the Faculty of Dentistry of Bauru, which was held in May 2019.

In September 2019, the student participated in the SBQPO, presenting the work in question.

During this period, the cements evaluated were AH Plus + 5% N Acetylcysteine, AH Plus + 5% Ambroxol and AH Plus (control group).

Revista ARACÊ
ISSN: 2358-2472

MATERIALS AND METHODS

From February to April 2019, the experiment and analysis of radiopacity and setting time were carried out.

Regarding the radiopacity analysis, metal rings of 10 mm internal diameter and 1 mm thickness were used, according to the ISO 6876/2001 standard (ISO, 2001). 3 (three) specimens were made for each cement. The freshly spatulated cements were inserted into the rings and kept in an oven at 37°C and 100% humidity until complete set. Then, the samples were checked for thickness with the aid of a digital caliper (Mitutoyo Corp, Tokyo, Japan). Samples with failures were deleted and redone. The samples were X-rayed on occlusal film D (Kodak Comp, Rochester, New York, United States) together with an aluminum scale with variations from 2 to 16 mm (2 mm increments). An X-ray device was used following the specifications recommended by ISO 6876/2001, 60kV, 10mA, 0.3 seconds and 30cm focus-film distance (ISO, 2001). The radiographs were processed manually using a developer and fixation solution (Kodak, São José dos Campos, São Paulo, Brazil). After processing, the radiographs were digitized and analyzed using the Digora 1.51 program (Soredex, Helsinki, Finland). The determination of radiopacity in aluminum millimeters was carried out according to DUARTE *et al.* (2009):

$$\frac{DRM - DRPAA}{DRPAS - DRPAA} \times 2 + \text{mmAl pitch below value} = \text{radiopacity in mmAl}$$

DRM – Radiographic density of the material
DRPAA – Radiographic density of the aluminum pitch below
DRPAS – Radiographic density of the upper aluminum pitch

To determine the setting time of the cements, the ASTM C266/08 standard was followed. The spatulated cements were immediately poured into metal rings of 10 mm in internal diameter and 2 mm thick. Three (3) specimens were used for each cement. The specimens were kept in an incubator at 37°C ± 1°C temperature and 95% ± 5% humidity during the test. After 180 seconds from the beginning of spatulation, the specimens were submitted to vertical pressure marking using Gilmore needles. To determine the initial setting time, a 113.4g needle was used, then the 453.6g needle was used for the analysis of the final setting. The times, in minutes, elapsed from the beginning of spatulation to the moment when it is not possible to see the marking of each needle on the surface of the samples, representing the initial and final setting of the cements, were recorded.

Revista ARACÊ
ISSN: 2358-2472

In the periods of May and June 2019, the solubility experiment, the analysis of the flow and antimicrobial capacity of the experimental cements were carried out.

The flow test was carried out according to ISO 6876/2001 (ISO, 2001). A total volume of 0.5mL of cement was spatulated and placed in the center of a glass plate. After 3 (three) minutes from the beginning of spatulation, another glass plate of mass 20 ± 2g was adapted on the plate containing the cement and on both a weight corresponding to 100g. After 10 (ten) minutes from the beginning of spatulation, the weight was removed and the larger and smaller diameter of the cement were measured with the aid of a digital caliper (Mitutoyo MTI Corporation, Tokyo, Japan). The mean of the two diameters was considered the cement flow. Three (3) measurements were taken for each cement variable.

The volumetric alteration was evaluated volumetrically using micro-computed tomography (micro-CT) (CAVENAGO, *et al.*, 2013)element. Each specimen was scanned twice. Thirty acrylic teeth (n=10) with a retrocavity were used. The cavities were filled with the cements and scanned in micro-CT (SkyScan 1174v2; SkyScan, Kontich, Belgium) with 50 kV and 800 µA.

In the months of July, August, September, October, November and December 2019:

For the analysis of microbial adhesion, metal rings of 10 mm internal diameter and 1 mm thickness were made, according to the ISO 6876/2001 (ISO, 2001) standard of the test cements and control group. On the discs, *a biofilm of* Enterococcus faecalis *was induced in vitro*, using strain (ATCC 29212) according to the work of Guerreiro Tanomaru et al., at a cell density of 3.2 X 107 colony-forming units per µL. Plates from 24 culture wells were used, where each well had 1 slide, 0.9 mL of sterile BHI and 0.1 mL of inoculum, and were kept under agitation in an incubator at 37°C (Q816M20; Composed of Quimis Cientıficos Ltda, Diadema, SP, Brazil) for 2 hours, after this period, the blocks were washed in PBS to eliminate the unadhered microorganisms and stained with LIVE/DEAD dye that remained for 15 minutes and analyzed under a laser scanning microscope. For the analysis of the antimicrobial action, metal rings of 10 mm internal diameter and 1 mm thickness were made, according to the ISO 6876/2001 (ISO, 2001) standard of the test cements and control group. On the discs, *a biofilm of* Enterococcus faecalis *was induced in vitro*, using strain (ATCC 29212) according to the work of Guerreiro Tanomaru et al., at a cell density of 3.2 X 107 colony-forming units per µL. Plates from 24 culture wells were used, where each well had 1 slide, 0.9 mL of sterile BHI and 0.1 mL of inoculum, and kept under agitation in an incubator at 37°C (Q816M20; Compost of Quimis Cientıficos Ltda, Diadema, SP, Brazil)

Revista ARACÊ
ISSN: 2358-2472

for 21 days. To avoid nutrient deficiency, BHI culture medium was completely replaced every 48 hours, without the addition of new microorganisms.

After the 21-day period, the samples were stained with 50 µl of Syto 8/Propidium iodide solution (Live/Dead Technique-In Vitro Gen) for 10 minutes. Syto8 is a nucleic acid-selective fluorescent (green) dye, indicated for staining living and dead cells (general dye). On the other hand, propidium iodide aims to identify the microbial population with an affected cell membrane, or dead cells, with red fluorescence. Upon entering cells, red fluorescence decreases the fluorescence of Syto-8, leaving the dead cells a red or yellow fluorescence. After staining, the samples were evaluated under the Laser Scanning Confocal Microscope (Leica, Mannheim, Germany) at 100x magnification to obtain a panoramic view. This analysis was based on the total biofilm volume and the percentage of live and dead bacteria in the colonized area in each section of the biofilm. For biofilm analysis, the images obtained (files with .lif extension) were analyzed in the ImageJ software using the LOCI and ComStat2 plug-ins, available at: http://www.comstat.dk/. The proportion of the volume of live bacteria (General probe-Syto9) and dead bacteria (populationalprobe-Iodide propium) was evaluated using the Daime software available at: http://www.microbialecology.net/daime-download.

EXPERIMENTAL GROUPS

During this period, the cements evaluated were AH Plus + 5% N Acetylcysteine, AH Plus + 5% Ambroxol and AH Plus (control group).

For the preparation of the experimental cements, the electronic analytical balance (Gehaka AND-GR-202, Tokyo, Japan), with precision of up to thousandths of a gram will be used, and the cement handling, we will follow the ratio of 1 gram of powder to 0.3 mL of liquid.

RESULTS

Table 1 - Median values (minimum and maximum) of final and initial setting time of the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups.

Groups	Start time	End Time
AH Plus control	360.0(357.0-364.0)[a]	580.0(576.0-585.0)[a]
AH Plus + Ambroxol	340.0(339.0-341.0)[b]	598.0(594.0-606.0)[b]
AH Plus + N-acetylcysteine	298.0(290.0-302.0)[c]	488.0(484.0-500.0)[c]

Revista ARACÊ
ISSN: 2358-2472

*Different lowercase letters showed a statistical difference between groups (comparison between lines) P>0.05.

Table 2 - Median (minimum and maximum) flow values of the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups.

Groups	Runoff
AH Plus control	19.97 (16.99 - 23.04)[a]
AH Plus + Ambroxol	19.30 (19.11 - 19.81)[a]
AH Plus + N-acetylcysteine	16.80 (10.27 - 16.98)[a]

*Different lowercase letters showed a statistical difference between groups (comparison between lines) P>0.05.

Table 3 - Median values (minimum and maximum) of radiopacity of the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups.

Groups	Radiopacity
AH Plus control	7,194(5,874-9,308)[a]
AH Plus + Ambroxol	6,087(5,557-6,682)[b]
AH Plus + N-acetylcysteine	5,564(4,996-8,225)[b]

*Different lowercase letters showed statistical difference between groups (comparison between lines) P>0.05

Table 4 - Median values (minimum and maximum) in cubic millimeters of initial and 30-day solubility of the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups.

Solubility	immediate	30 days
AH Plus control	3017(2329-4280)[aA]	2867 (2179-4225)[aB]
AH Plus + Ambroxol	2965(2432-3840)[aA]	2867 (2179-4225)[aB]
AH Plus + N – acetyl	3158(2024-4551)[aA]	3265(4552-2013)[aB]

*Different letters showed a statistical difference between groups (comparison between lines) P>0.05

Table 5 – Results of bacterial adhesion and biovolume of E. faecalis on the surface of cement blocks after 2 hours.

Cement	AH Plus	AHP + Ambroxol	AHP+ N-acetyl
Adhesion (% Live Bacteria)	72.50 (9.11 – 100)[A]	48.42 (0.98 – 100)[A]	30.27 (0.57 – 98.57)[A]
Biovolume	841, 9 (92.7 – 35207)[A]	1453 (103.5 – 29400)[B]	5732 (604.9 – 30580)[AB]

*Different letters showed a statistical difference between groups (comparison between columns) P>0.05

Table 6 - Result of the percentage of viable bacteria after 21 days of cultivation on experimental cement blocks and biofilm biovolume.

Cement	Control	AH Plus	AHP + Ambroxol	AHP+N-acetyl
% Live bacteria	97.52 (92.9 – 99.54)[A]	22.38 (0.09 – 100)[B]	56.47 (0.13 – 92.98)[B]	21.22 (0.007 – 87.01)[B]
Biovolume	59053 (13435 – 320928)[A]	8376 (71.1- 254488)[A]	2433 447.7 – 29891.3)[B]	9260 (981.9 – 3.0580)[A]

*Different letters showed a statistical difference between groups (comparison between columns) P>0.05

Table 1 shows the median (minimum and maximum) values of final and initial setting time for the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups. The

Revista ARACÊ
ISSN: 2358-2472

experimental groups associated with n acetylcysteine and ambroxol had a shorter initial setting time, with a statistically significant difference between all groups (P>0.05).

Regarding the flow shown in Table 2, there was no statistical difference between all the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups (P>0.05).

Table 3 shows the median values (minimum and maximum) of radiopacity of the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups, where the AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups showed a statistically significant difference when compared to the AH Plus control group.

Table 4 shows the values in cubic millimeters of initial and 30-day solubility of the AH Plus, AH Plus + Ambroxol and AH Plus + N-acetylcysteine groups.

Table 5 shows the results of bacterial adhesion and biovolume of E. faecalis on the surface of the cement blocks after 2 hours.

FORM OF ANALYSIS OF THE RESULTS

The results will be submitted to the D'Agostino and Pearson tests for verification of normal distribution. In case of absence of normality, the non-parametric Kruskal-Wallis test will be used. If normality is present, the parametric ANOVA test will be used. For all tests, a significance level of 5% will be considered.

DISCUSSION

AH plus is a gold standard endodontic cement, excellence in biocompatibility.

In this study, two different antimicrobials were selected to be associated with HA plus, ambroxol and N-acetylcysteine.

Ambroxol is the VII metabolite of bromhexine and has pharmacological action in addition to an antimicrobial, kinetic mucus and expectorant. N-acetylcysteine is a derivative of cysteine and an excellent antimicrobial.

The association of HA plus with Ambroxol, in relation to the initial setting time, had a shorter time in minutes, which is very interesting as to the clinical applicability, both for the handling and for the initial stability of the cement inside the cavity, the flow of the experimental cement had no statistical difference when compared to the HA plus, which is important for the fluidity to complete the entire cavity, radiopacity had a median statistical value of 6.087 when compared to HA Plus 7.194, where it was within the standards specified by the standards.

Revista ARACÊ
ISSN: 2358-2472

The association of HA plus with N-acetylcysteine, in relation to the setting time, also had a decrease in minutes in the initial pre-setting time, which is important, the flow had no statistical difference when compared to the AH Plus in our control, the radiopacity had a median of 5.564 when compared to the HA Plus 7.194 where it was within the pre-established standards.

DIFFICULTIES AND USE OF THE TECHNICAL RESERVE

During this period, no funds were spent to pay for the materials necessary to carry out the research.

CHANGES TO THE INITIAL PLAN

The analysis and statistical analysis of the radiopacity, flow, setting time and solubility experiment was advanced to the first semester, and as for the antimicrobial, it was completed in the second half of 2019.

WORK PLAN AND SCHEDULE OF THE FOLLOWING STAGES

From the second stage of the scientific initiation scholarship, the antimicrobial analysis and statistical analysis of the experimental cements will be developed (July, August and September 2019), and finally antimicrobial analysis and statistical analysis and submission of the final report to FAPESP (October, November and December 2019).

Revista ARACÊ
ISSN: 2358-2472

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Revista ARACÊ
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