Mechanical properties of a glass ionomer cement incorporated with Amazon plant extract

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To evaluate the mechanical properties (MP) and antimicrobial activity of a glass ionomer cement (GIC) incorporated with an antimicrobial agent from the aerial parts of Dioscorea altissima. MP were: syneresis & imbibition; solubility; elasticity module; surface microhardness and fluoride release, and the anti-Streptococcus mutans (Smut) activity was accessed by microdilution broth assay and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) analysis. Syneresis & imbibition did not change over time for both groups, but EG showed lower values at days 7th and 30th. The control group (CG)'s and the experimental group (EG)'s weights were similar before the solubility assay, but after seven days, CG exhibited weight gain in comparison to EG. The elasticity module had no significant differences between groups. The CG showed lower surface microhardness compared to the EG. CG and EG had similar behaviors regarding fluoride release. EG showed diminished Streptococcus mutans count in comparison to CG. EG showed similar or better mechanical properties and an improvement in the antibacterial activity than the original cement.

Keywords: Glass ionomer cement, Plant extract, Dental materials, Mechanical properties, Dioscorea altissima

INTRODUCTION

The introduction of new materials to be incorporated in minimal invasive techniques (MIT) is an alternative to diminish the cost of caries treatment, which is one of the widespread oral conditions in children, adolescents and adults. New propositions as MIT are being used for the treatment of caries lesions. This approach foresees the maintenance of the dentin that was affected by caries once the diminishing of the bacteria amount is achieved, resulting in the restoration of the pulp inflammatory process and in the dentin remineralization.

Glass ionomer cement (GIC), a polyalkenoate cement, shows important properties such as adhesion on tooth structure, fluoride release and biocompatibility. A significant anti-cariogenic action is associated to the fluoride release property and, for that reason, is part of the usual clinical protocol regarding MIT1,2. However, GIC shows low mechanical properties, which make its use more restricted as basis material.

Previous studies have shown that GIC allows the incorporation of substances that provide better action against cariogenic bacteria, without modifying its properties3). Amazon plant extracts were reported to be tested against Streptococcus mutans, S. sanguis4) and Enterococcus faecalis5,6). Dioscorea altissima Lam., a species from the yam family (Dioscoreaceae), has significantly inhibited S. mutans in a one-microorganism planktonic culture7). In the present work, the extract of the species was incorporated in GIC and the alterations over mechanical properties were assessed.

The present work aims to characterize of mechanical properties of GICs incorporated with an organic extract obtained from the aerial parts of D. altissima.

MATERIALS AND METHODS

Plant material and extract preparation

The aerial organs of Dioscorea altissima Lam. (Dioscoreaceae) were collected in the Amazon rain forest under Brazilian government licenses (no. CGen/ MMA#12A/2008 and no. MMA/ICMBio/SISBIO#14895). The exsicate of the specimen was deposited at the UNIP Herbarium under the number [A.A.O. 3812 (UNIP)]. The extract, designated in this report as EB1779, was obtained by a 24 h maceration—which is described as the contact of the plant with the solvent for a specific period—with a mixture of dichloromethane and methanol (1:1). The extract was dried under vacuum and then lyophilized and was kept in the freezer at −20°C until use8).

GIC preparation

Vidrion R® (SSWhite Duflex, Rio de Janeiro, Brazil) was selected to be used in the assay due to its characteristics as a chemical cure. It is composed of sodium, calcium, aluminium, fluorosilicate, barium sulfate, polyacrylic acid and pigments. The experimental GIC was manipulated such that it received a 2%w/w of the dried organic extract EB1779 using geometric techniques handled in a mortar and pestle, which provided a better sample homogenization. A concentration of 2% w/w was chosen to be used for the evaluation based on a previous study9). The pharmaceutical approach to prepare the GIC was to take a 2 g portion of the plant extract and add it to 98 g of the GIC. The time of manipulation and
Surface microhardness analysis

Pieces from the CG and the EG (n=10, n total=20; piece dimension 6×3 mm) were extracted from the stainless steel matrix after an initial setting and were then used in the surface microhardness assay (ISO 3824, 2.11). Each piece of the CG/EG was fixed with sculpture wax in a round acrylic support measuring 10 mm high×8 mm diameter made by Gerber, São Paulo, Brazil. The pieces were positioned in the center of the acrylic support in a specific demarcation made by the manufacturer. After fixation, the pieces were sanded with a polisher, using sandpaper with a granulation of 1200 for 30 s under water refrigeration on a flat surface. In order to standardize the sanding axis, each sample was adapted to a standard device that was idealized and developed by our team. A final polishing was performed with a metalographic cloth, humidified with lubricant and impregnated with diamond paste granulated at 1 µm diameter. After that, the pieces were taken to an ultrasonic camera. The initial surface microhardness was subsequently assessed by means of a microdrometer (Future Tech-FM-300, Kawasaki, Japan) with a Knoop-like indenter at static charge of 25 g for 5 s. Five indentations were realized on the surface of the pieces, each of which was separated by a distance of 100 µm.

Fluoride release assay

The dimensions of the pieces of the CG and the EG (n=12, n total=24) were defined as 10 mm diameter×2 mm height. The pieces were made using a stainless steel matrix that was specially developed for the assay. After an initial setting, the pieces of the CG and the EG were extracted from the stainless steel matrix, drilling was performed in the central region of each sample and a silk thread was inserted in the drilling in order to keep the pieces in suspension in the plastic reservoirs containing 18.0 mL of distilled water in an incubator at 37°C during the course of the experiment. The fluoride liberation measurements were made using a fluorometer (BioTek Instruments, Winooski, VT, USA) that was calibrated before each measurement with two sodium fluoride standard solutions prepared at 1 and 10 ppm as required by the manufacturer. For the measurement, a solution named TISAB III (Total Ionic Strenght Adjustment Buffer, Analion, Ribeirão Preto, Brazil) was added in the liquid at 1:10 (final volume) in order to keep the fluoride ion free. Fluoride measurements were made at 6, 12, 24 h, 3, 7, 14 and 28 days. During the readings, the solution was stirred with the aid of a magnetic stirrer. The results were obtained in µgF/mm². This unit expresses the amount of fluoride that was released relative to the surface of the pieces of the CG and the EG.

Antibacterial analysis

GIC’s were prepared as previously described. Groups of five pieces were prepared, and were divided into two groups named control and experimental groups (CG and EG, respectively; n=5, n total=10). Streptococcus mutans ATCC™ 25175® (Smut) was used in the execution of the experimental model. The microorganism culture...
was obtained from loops containing the lyophilized microorganisms (CultiLoops®). Each initial culture (here so-called mother-plaque) was maintained for one month and served as a source of fresh bacteria collection, obtained at each procedure. In this way, the bacteria were strictly in the same passage (3rd passage) during the experiment. Smut was grown on Brain Heart Infusion Agar (BHIA; Oxoid®) medium in cycles of 48 h at 36.0°C. For the experiments, the respective broth media were used and prepared as indicated by the manufacturer (BHIB; Oxoid®17).

From the culture, sufficient amount of bacteria was withdrawn to prepare the bacterial suspension with BHIB medium, whose final concentration was 1.0×10⁸ and 1×10⁴ CFU/mL. The assay was performed in 96 flat bottom-well microplate (Coastar®), and 200 µL were transferred to each well. After that, the GIC’s pieces were transferred to each corresponding control or experimental set of wells. Experimental contamination control was made by adding 200 µL medium without bacteria to 4 wells in the plate, GIC’s contamination was controlled by adding GIC’s pieces in medium without bacteria (one for each GIC group) and bacteria growth control was made by adding 200 µL of the bacteria suspension in two wells in the plate. After that, plate was incubated for 24 h at 36.0°C. Bacteria inhibition was evaluated by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT assay)14), with adaptations to planktonic culture bacterial measurements15). So, 100 µL of the treated suspension media were transferred to a 96 flat bottom-well microplate to each corresponding well. Ten microliter of MTT solution diluted to a final concentration of 0.5 mg/mL was added to each well. Plate was left incubating at 36.0°C for 4 h, and 100 µL of dimethylsulfoxide (DMSO) were added to each well in order to dilute formazan salt that was obtained after biochemical reaction in viable bacteria mitochondria. Plate was left in microplate shaker for 10 min under light protection. Plaque was read in a plate reader (Biotek®) at 595 nm. Data were collected and statistically analyzed in the GraphPad Prism 7.0 package, by the use of unpaired Student’s t-test, considering significance if α<0.05. Bacterial results were also visually evaluated by subculturing 2.0 µL of each control and experimental bacteria suspensions in BHIA medium prepared in Petri dishes that were left to grow under incubation of 48 h at 36.0°C.

Statistical analysis
The following statistical analyses were used in the present work: Shapiro-Wilk for homoscedasticity; non-parametric Friedman and Mann-Whitney (Bioestat 5.0); parametric two-way repeated measures ANOVA and one-way ANOVA followed by Bonferroni’s or Tukey’s post-test (SPSS 2.1); test t for homogeneous variances; and unpaired t-test with Welch correction. Results from the antibacterial assay were achieved by two-tailed Student’s t-test. Significance was considered to be p<0.05, if differences occurred among the means.

RESULTS

Syneresis and imbibition assays
Shapiro-Wilk indicated that there was no homoscedasticity in the variances, thus, the Friedman and Mann-Whitney test was performed. The results for imbibition, considering the variable weight, showed that there was no significant differences over time for the CG (p=0.993) or the EG (p=0.934). In relation to the differences among the groups at a fixed time, we observed that the CG and the EG means were statistically similar at the beginning (p=0.112) and after 24 h (p=0.106). Nonetheless, at the 7th day, the CG showed a higher weight compared to the EG (p=0.056). After 14 days, both groups showed no significant differences (p=0.106), and after 30 days, the CG showed a higher weight compared to the EG (p=0.024), which is shown in Table 1. In relation to syneresis, an evaluation of the diameter alterations among the groups showed that there were no significant differences in the group factor (p=0.92) or over time (p=0.17), and there was no interaction between group and time (p=0.27). Thus, the diameter parameter was not different between the control and experimental groups over time as shown in Table 2.

Solubility assay
The initial weight obtained was significantly different from the weight obtained after 7 days of water immersion for both the CG and the EG (p=0.000). An interaction between the group and time was observed (before and after) (p=0.025). The weight of the CG was statistically similar to the weight of the EG before the assay. After 7 days, the CG showed a lower weight compared to the EG, and both the CG and the EG showed a significant weight gain after 7 days compared to the weight before the assay as shown in Table 3.

Elasticity module assay
The statistical analysis showed that there were no significant differences between the groups (p>0.05) according to the test t, as shown in Table 3.

Surface microhardness assay
The samples showed homoscedasticity variance (p=0.115), and thus, a test t for homogeneous variances was chosen. The results showed that there was a significant difference between the two groups (p=0.0001), which means that the CG showed a lower surface microhardness compared to the EG as shown in Table 312).

Fluoride release
The results showed that there was no significant differences in fluoride release between the CG and the EG (F=0.615, p=0.449). However, there were significant differences in fluoride release over time (F=3.465, p=0.000), independent of the group that was analyzed, as shown in Table 4.

Antibacterial assay
Results showed that GIC incorporated with 2% EB1779
Table 1  Weight (mg) obtained for glass ionomer cement Vidrion R® (CG) and glass ionomer cement Vidrion R® incorporated with 2% organic extract obtained from Dioscorea altissima (Dioscoreaceae; EG), as the base for calculating imbibition observed for both groups over time. The Friedman and Mann-Whitney test was performed, p<0.05

| Groups | Start | 24 h | 7 days | 14 days | 30 days | Friedman p-value |
|--------|-------|------|--------|---------|---------|-----------------|
| CG     | 80.00 (4.30) Aa | 80.00 (2.90) Aa | 80.00 (4.30) Aa | 80.00 (2.90) Aa | 80.00 (2.90) Aa | 0.993 |
| EG     | 70.00 (5.10) Aa | 80.00 (4.90) Aa | 75.00 (5.20) Ba | 80.00 (4.90) Aa | 75.00 (5.20) Ba | 0.934 |
| Man Whitney p-value | 0.112 | 0.106 | 0.056 | 0.106 | 0.024 | — |

Different letters indicate significant differences by the Friedman and Mann-Whitney test. Capital letters in vertical and lower case in horizontal.

Table 2  Mean (standard deviation) of the differences in diameter (mm) for calculating syneresis in the glass ionomer cement Vidrion R® group (CG) and the glass ionomer cement Vidrion R® incorporated with 2% aqueous extract obtained from Dioscorea altissima (Dioscoreaceae) group (EG) over time, n=12. Repeated measures ANOVA was performed followed by Bonferroni’s post-test, α<0.05

| Groups | Start | 24 h | 7 days | 14 days | 30 days |
|--------|-------|------|--------|---------|---------|
| CG     | 4.87 (0.13) Aa | 4.85 (0.07) Aa | 4.86 (0.08) Aa | 4.91 (0.14) Aa | 4.90 (0.08) Aa |
| EG     | 4.86 (0.13) Aa | 4.87 (0.06) Aa | 4.91 (0.09) Aa | 4.85 (0.09) Aa | 4.88 (0.07) Aa |

Similar letters indicate statistical similarity by repeated measures-ANOVA, p>0.05. Capital letters in vertical, lower case in horizontal.

Table 3  Results obtained from solubility assay where the glass ionomer cement Vidrion R® group (CG) and the glass ionomer cement Vidrion R® incorporated with 2% aqueous extract obtained from Dioscorea altissima (Dioscoreaceae) group (EG) were tested before and after 7 days. (*) A two-way ANOVA repeated-measures and Tukey post-test were adopted for evaluation. The results obtained from the Elasticity module assay for the CG and the EG were assessed by t-test (**). The results obtained from the surface microhardness assay for the CG and the EG were evaluated by a t-test (**). The means and standard deviation, in Knoop, of both the CG and EG were determined in the surface microhardness assay. Significances were considered if α<0.05.

| Groups | Solubility assay* (n=12) | Elasticity module assay** (n=10) | Surface microhardness assay*** (n=10) |
|--------|---------------------------|---------------------------------|--------------------------------------|
|        | Before | After | Mean(SD) | Before | After | Mean(SD) | Before | After | Mean(SD) |
| CG     | 80.10 (2.42) Ab | 82.40 (2.76) Ba | 19.19 (5.32) A | 143.59 (61.28) B |
| EG     | 80.60 (2.01) Ab | 83.60 (1.71) Aa | 23.11 (5.18) A | 328.96 (106.45) A |

Solubility assay=p<0.05; different letters indicate significant differences. Capital letters in vertical, lower case in horizontal. Elasticity module assay=p>0.05; similar lower case means no significant differences between the two groups. Surface microhardness assay=p<0.0001; different letters indicates significant differences.

Table 4  Results obtained from the fluoride release assay performed for the glass ionomer cement Vidrion R® group (CG) and the glass ionomer cement Vidrion R® incorporated with 2% aqueous extract obtained from Dioscorea altissima (Dioscoreaceae) group (EG). Two-way repeated measures ANOVA followed by Bonferroni’s post-test, significant if α<0.05, is given. Means (standard deviation) in µgF/mm² are given.

| Groups | 6 h | 12 h | 24 h | 3 days | 7 days | 14 days | 28 days |
|--------|-----|------|------|--------|-------|--------|--------|
| CG     | 5.24 (0.25) Aa | 2.17 (0.3) Ab | 1.1 (0.19) Ac | 0.84 (0.12) Ad | 0.69 (0.09) Ad | 0.52 (0.05) Ad | 0.33 (0.05) Ad |
| EG     | 5.09 (0.43) Aa | 2.13 (0.32) Ab | 1.02 (0.19) Ac | 0.82 (0.12) Ac | 0.68 (0.07) Ac | 0.50 (0.04) Ac | 0.31 (0.05) Ac |

Different letters indicate significant differences by repeated measures ANOVA, p<0.05. Capital letters in vertical, lower case in horizontal.
Fig. 1 Results obtained from the antibacterial analysis done with glass ionomer cement Vidrion R (CG) incorporated with the organic extract from the aerial organs of Dioscorea altissima Lam. (Dioscoreaceae; EB1779; EG) at 2%. Antibacterial assay was performed with Streptococcus mutans ATCC 25175, diluted to A: 1×10^2 colony forming units/mL and B: 1×10^3 colony forming units/mL. Results were analyzed by two-tailed Student’s t-test (α<0.05).

Fig. 2 Bacterial growth evaluation after treatment with glass ionomer cement Vidrion R (CG) incorporated with the organic extract from the aerial organs of Dioscorea altissima Lam. (Dioscoreaceae; EB1779; EG) at 2%, compared to bacterial growth control (BGC). Antibacterial assay was performed with Streptococcus mutans ATCC 25175 in microdilution broth assay and subcultured in Brain Heart Infusion agar medium as shown.

DISCUSSION

In the present study, the alterations of the GIC’s mechanical properties after incorporation of an antibacterial plant extract were evaluated. The introduction of antimicrobials in GIC is highly indicated for the use in MIT, once its original properties are preserved or improved.

Plant extract was incorporated into GIC’s powder, considering this is more appropriate than incorporating it to the liquid portion of GIC, which could compromise its solubility. The use of antibiotics in GIC was previously done before, as described by Pinheiro et al.⁹, who have incorporated 1% of metronidazole, 1% of ciprofloxacin and 1% of cephalexin into the powder as well, corroborating our technique.

Present findings show that there was no alteration in weight over time, when considering the cements separately. However, there was an increase in the weight of the conventional cement in the 7th and in the 30th days. The improvement of weight in the conventional cement may indicate that the experimental cement suffered less of an imbibition effect after 30 days, which is a beneficial additional factor to be considered in the development of a new GIC that has a property of diminishing the sorption of cement water⁷. In the present work, the control group weight was lower than the experimental weight on the 7th day, indicating that the control group is more soluble than the experimental group. The diameter was not altered whatsoever.

Syneresis, imbibition and solubility assays showed that the incorporation of the plant extract kept the cement more stable in relation to the original formula and that the experimental formula is less soluble than the original in the initial periods. Syneresis and imbibition are phenomena that happen in the first
hours of cement exposition to the oral environment, particularly in the first 24 h, when the cement has not yet reached its final cure. The variation of weight and dimension is particularly relevant in the evaluation of both mechanical properties. Dimensional alterations may lead to the structural loss of the material, consequently diminishing its ability to seal, despite other mechanical and physical properties.

Considering the elasticity module, the experimental GIC was not statistically different from the conventional GIC. No alterations were observed in the elasticity module after adding the plant extract, which was beneficial. These findings corroborate with the requirement of preserving the GIC’s deformation equivalent to that of the lost dentin. Brescian et al.16 affirmed that the incorporation of antimicrobials into GIC diminishes the resistance related to compression, which is directly related to the elasticity module. Deepalakshmi et al.17 and Tüzünər et al.12 observed that cement incorporated with chlorhexidine or propolis also diminishes this property. The unconformity of these results with those observed in the present work may be explained by the fact that these authors have incorporated the antimicrobials into the liquid, instead of incorporating them into the powder13.

GICDA, the experimental cement, showed a higher surface microhardness compared to the conventional cement. It seems that the presence of the extract improved the surface microhardness, which means that it may lead to a diminished abrasion of the material. El-Tatari et al.18 evaluated GICs incorporated with Salvadora persica and observed that the antimicrobial activity was satisfactory but reported an impairment of the physical properties of the material, unlike our experimental GIC.

Although the process of hypermineralization of dentin has not been thoroughly elucidated to date, it is suggested that this phenomenon can be induced by large amounts of fluoride in the water and in dental materials. The higher intensity of fluoride release from GIC occurs in the first 24 h and significantly decreases in the first week. Additionally, the fluoride release phenomenon is related to the antibacterial activity of GIC and to the low pH related to the setting reaction of GIC11. In the present experiment, a higher fluoride release was observed in the first 24 h for both the GIC and GICDA cements, and a decrease, that was statistically significant, was observed in the subsequent periods of observation, as expected19-21.

The incorporation of antimicrobial agents to dental materials is a reality today, as it is the association of natural products to dental materials, which is a promising alternative of introducing new products into the market. Present findings described that the incorporation of EB1779 to GIC’s have significantly inhibited bacterial growth, in comparison to the regular GIC. Further studies related to the isolation of the active component needs to be performed, as well as elimination of the color influence of the extract over GIC, which is not accepted today. In the meantime, a chromatographic prospection of the main classes of the chemical compounds was made, and the presence of phenolic compounds and terpenes were observed (data not shown). Although no chemical studies related to D. altissima were identified in the literature, the occurrence of steroids glycosides22, phenolic compounds20 and steroidal saponins24 and norclerodane diterpenoids25 is largely reported for other species of Dioscorea. No reports on toxicity effects of D. altissima were found. A dry extract of D. villlosa were tested in acute and subchronic toxicity models in vivo but no evidence was found26. Species of Dioscorea attenuated cardiotoxicity induced by drugs as doxorubicin, in vivo27. The experimental GIC, which was incorporated into the extract obtained from D. altissima, showed a very good stability over the conventional cement, particularly in relation to syneresis, imbibition and solubility, higher surface microhardness, and a similar pattern of fluoride release and elasticity module was observed.

The experimental GIC demonstrated a strong potential to be further introduced as a dental material to be used in MIT, once no significant interferences with the mechanical properties of the original cement has been shown and that the antibacterial properties of the cement were significantly improved. Based on present achievements, the introduction of natural products as a strategy to improve the dental materials properties is feasible.

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