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Original

In vitro and *in vivo* analyses of the effects of desensitizing agents on dentin permeability and dentinal tubule occlusion

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Abstract: This study was done to assess the influence of the topical application of two different desensitizing agents on dentin permeability and dentinal tubule occlusion. Twenty-one rats provided 84 teeth: 36 for the *in vitro* and 48 for the *in vivo* investigation. The following agents were tested: Group 1, 2% potassium nitrate plus 2% sodium fluoride gel; Group 2, 5% sodium fluoride varnish; Group 3, 3% hydroxyethylcellulose gel (control). Cervical cavities were prepared and EDTA was applied to expose the dentinal tubules. After each treatment, Evans blue dye was applied to the teeth. Dentin permeability, scanning electron microscope (SEM) sections, and energy dispersive X-ray (EDX) were analyzed. One-way ANOVA was used to compare the data. There were significant differences ($P < 0.05$) among groups for dentin permeability, number of tubules/mm^2, tubule area and tubular diameter. Groups 1 and 2 (both *in vitro* and *in vivo*) showed open and partially occluded tubules. Group 3 had the most open tubules. EDX revealed similar composition for both experimental conditions. Within the limits of the study, 2% nitrate potassium plus 2% sodium fluoride gel and 5% fluoride varnish decreased the dentin permeability, resulting in partial tubular occlusion. (J Oral Sci 52, 23-32, 2010)

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Introduction

Life expectancy is increasing and patients are retaining their natural teeth for a longer time due to effective treatment strategies for caries and periodontal disease. Consequently, there is a higher risk of cervical dentin hypersensitivity (DH) as a result of physiological gingival recession with aging (1,2).

DH is defined as pain arising from exposed cervical dentin caused by chemical (erosive foods and drinks), thermal (hot and cold), mechanical (brushing), evaporative or osmotic stimuli applied to opened dentinal tubules. This is a common problem found in many adult populations, with a prevalence ranging from 8 to 57% (2-5).

Dentin exposure may be the result of abfraction, abrasion or erosion, and denudation of the root surface. It can occur as a result of gingival recession or non-surgical and surgical periodontal treatment (3-8).

The dentinal tubules play an important role in transferring stimuli and irritants to the pulp. The hydrodynamic theory of dentin sensitivity postulates a flow of fluid through the tubule as the transducing mechanism for hydrodynamic stimuli (5). It has been demonstrated that hypersensitive dentin has a larger number of wide, open dentinal tubules on the surface than does non-sensitive dentin (9). A rational approach to the control of pain arising from exposed dentin would thus be to block or reduce the diameter of the tubules.

Dentin desensitization may sometimes occur spontaneously as a natural decrease of dentin permeability. However, in most cases, treatment is still necessary. There are numerous treatments for DH. Application of anti-inflammatory agents, occluding dentinal tubular agents as well as root covering by periodontal surgery are treatment approaches to DH that reduce the excitability of the nerve fibers within the pulp (10-15).

Occluding dentinal tubular agents can create a barrier by precipitating proteins and calcium/phosphate ions on the surface or within the tubule orifices. The mechanism of action of the various chemical desensitizing agents is still not well understood, as these agents might have different behavior when applied to *in vitro* and *in vivo* conditions. Therefore, it is necessary to evaluate their effect in both conditions. Research with a variety of products has often shown contradictory results (1,2,12, 13,16-18). Moreover, the so-called "placebo effect" has to be taken into consideration for its significant role in clinical investigations. These reported treatments include a wide range of procedures depending on the nature of the dentin exposure and the associated symptoms (1).

Most of the studies on the effectiveness of important desensitizing agents were performed *in vitro*. However, animal models can reproduce oral characteristics, such as saliva, oral biofilm, temperature and diet. Dye diffusion in dentinal tubules is an additional method of analyzing the effect of desensitizing agents on dentin permeability. The purpose of this study was to compare the effects of the topical application of two different desensitizing products (potassium nitrate plus sodium fluoride gel versus sodium fluoridated varnish) on dentin permeability and dentinal tubule occlusion.

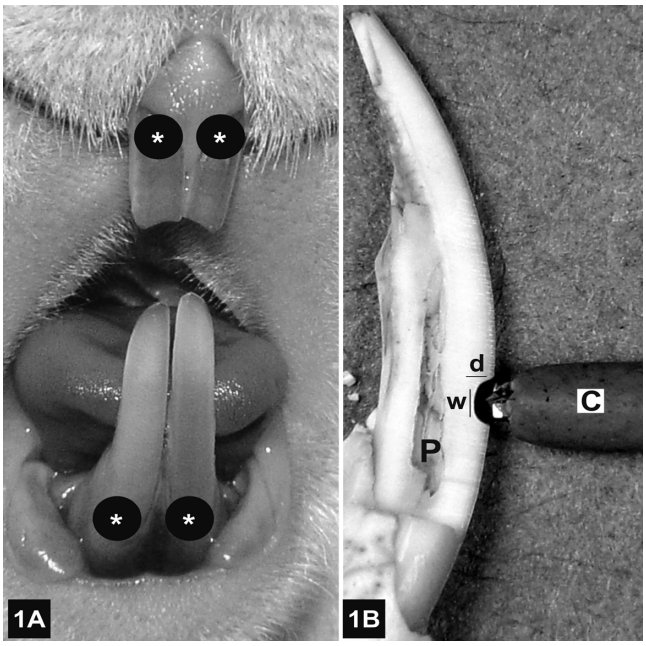

Fig. 1 Procedure resulting in exposure of dentinal tubules. (1A): Upper and lower incisors. (*) Cervical region where cavities were prepared. (1B): Cavity characteristics in lower incisor. (P) pulp chamber; (w) width of the cavity, 0.8 mm; (d) depth of the cavity, 0.3 mm; (C) # 245 carbide bur covered with epoxy resin: active tip with 0.3 mm.

Materials and Methods

This research was approved by the Ethics Committee for Teaching and Research in Animals of Ponta Grossa State University (protocol #6290/06) and followed the guidelines of the Brazilian College of Animal Experimentation (COBEA).

Twenty-one male rats (*Rattus norvegicus*; Wistar) (2-3 months old), weighing 200 to 300 grams, provided 84 teeth (upper and lower incisors) for the studies. For the *in vitro* study, 36 teeth were taken from 9 rats used for other laboratory experiments in the Department of Dentistry, Ponta Grossa State University. For the *in vivo* study, 48 teeth were obtained from 12 additional rats.

The teeth were randomly divided into three groups for each experimental condition (*in vitro* and *in vivo*) according to the desensitizing agents used: Group 1, (KNO_3 + NaF) 2% potassium nitrate plus 2% sodium fluoride gel (Dessensibilize® KF2%, FGM Produtos Odontológicos Ltda, Joinville, SC, Brazil); Group 2, (NaF) 5% sodium fluoride varnish (Fluorniz® , S.S. White, Rio de Janeiro, RJ, Brazil); Group 3, 3% hydroxyethylcellulose gel (control).

For the *in vivo* study, the animals were anesthetized with an intraperitoneal injection (ketamine 75 mg/kg and xylazine 10 mg/kg). Cavities of 0.3 mm depth and 0.8 mm width were prepared on the buccal surface of the upper and lower incisors (cervical region) using a standard (active tip with 0.3 mm) #245 carbide bur (S.S. White, Rio de Janeiro, RJ, Brazil) on a high-speed handpiece with copious tap water as coolant (Fig. 1). Prior to the treatments, the dentin of all the cavities was treated with 24% EDTA gel (Biodinâmica Química e Farmacêutica Ltd., Ibiporã, PR, Brazil) on tiny cotton pellets, which were replaced every 30 s for 3 min, in order to remove the smear layer and open the dentinal tubules, simulating dentin hypersensitivity.

For the *in vivo* study, the rats were anesthetized with halothane. The agents (tests and control) were topically applied to the cavities and kept for 10 min. This procedure was repeated once daily for 4 days. After each daily treatment, a cotton pellet saturated with 5% Evans blue dye solution was applied to the samples for 5 min.

At the end of the *in vivo* study, the animals were killed

by cervical dislocation after previous sedation with halothane, and the teeth were carefully extracted to avoid damage to their surface. The teeth were kept frozen (-20°C) until analyzed (24 h).

The same protocol was used for the application of the agents (tests and control) in the *in vitro* study; however, the teeth previously extracted were stored in saline solution (0.9% NaCl) at room temperature during the 4-day experimental period. The saline solution was changed daily in order to avoid bacterial contamination.

Dentin permeability analysis

Five percent (5%) by weight Evans blue (Merck Chemical Ltda, Darmstadt, Germany) solution was employed to analyze the dentin permeability of 42 teeth (6 per group *in vitro*; 8 per group *in vivo*). The samples were embedded in acrylic resin and sectioned longitudinally (bucco-lingual direction) using a water-cooled diamond saw (ISOMET 1000- Precision Saw, Buheler, model 112180, Lake Bluff, IL, USA). In order to remove the sludge and dentin within dentinal tubules, the specimens were ultrasonically cleaned for 10 min at 47°C.

The samples were photographed (Olympus™ BX41, Olympus, Tokyo, Japan) at ×40 magnification and images of 5.1 megapixels (digital camera, Olympus Camedia C-5060, Tokyo, Japan) were obtained. These stored digitalized images were not enhanced and no transformation procedures were carried out. The image analysis software used was Image Pro Plus™ Version 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA). Each image was calibrated individually with a standard scale (µm). Three measurements (upper, middle and lower parts) were taken for each image indicating the depth of the dye infiltration, and finally, the mean was calculated for each specimen. The same examiner performed all the measurements, after testing the reproducibility of the data.

Scanning electron microscopy (SEM)

A total of 6 teeth/group (*in vitro*) and 8 teeth/group (*in vivo*) were evaluated by SEM. All specimens were transversely sectioned using a water-cooled diamond saw (ISOMET 1000, Precision Saw Buehler, Lake Bluff, IL, USA). The sectioned specimens were washed with 20 ml of distilled water and ultrasonicated for 10 min. Dehydration was achieved to critical point dryness using a graded series of ethanol (25%, 50%, 70%, 90% and 100% for 10 min/each). Specimens were mounted on metal stubs, kept in a 37 °C stove for 24 h and stored in a vacuum silica-gel desiccator for 48 h. In order to perform the SEM analysis, the samples were sputter coated with 25 nm of gold for 10 min.

Nine images from each sample (central area) were obtained by SEM (Shimadzu SSX 550™, Shimadzu do Brasil Comércio Ltd, São Paulo, Brazil), operated at 20 kV at ×2,000 and ×5,000 magnification. The SEM photomicrographs were evaluated quantitatively and qualitatively. Quantitative analysis was performed by counting the number of dentinal tubules and obtaining an estimate per mm^2 (×2,000 magnification) considering a total area of 1,600 μm^2, the diameter of dentinal tubules (×5,000 magnification) and opened tubular area (×2,000 magnification) using the software Image Pro Plus™ Version 4.5.0.29. Qualitative evaluation (×2,000 magnification) considered dentin surface characteristics, intertubular and peritubular dentin, dentinal tubules and smear layer deposits. All the analyses were performed by one trained examiner.

Energy-dispersive X-ray (EDX)

Samples were examined by energy dispersive X-ray microanalysis (Shimadzu SSX 550™, Shimadzu do Brasil Comércio Ltd) in order to determine the presence of chemical elements in deposits found next to the dentinal tubules of each specimen. The spectrum was obtained at 20 Kv, spot size of 5 nm and the counting time was 300 s. This provided qualitative information of the presence of fluorine (F), sodium (Na), magnesium (Mg), silicon (Si), phosphorus (P), potassium (K) and calcium (Ca).

Statistical analysis

Intra-examiner reproducibility was assessed twice within 48 h to check the reproducibility of dentin permeability and tubular diameter measurements. Tubule number and tubule area were repeatedly compared using both manual and automatic methods (digital computer image analysis). The reproducibility was tested using the Bland and Altman procedure (19).

Comparisons among groups according to experimental condition (*in vitro* and *in vivo*) for tubule number, diameter and area as well as dentin permeability were tested by one-way ANOVA and Bonferroni post hoc test. The normality of the distribution of data was confirmed using the Shapiro-Wilks test. An alpha value of ≤0.05 was used to indicate statistically significant differences among the groups. All analyses were performed using a software program (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

Results

Reproducibility

Reproducibility for dentin permeability, tubule diameter (intra-examiner), dentin tubule number, and total tubule

area (comparing manual and automatic methods) were within the limits of agreement (Fig. 2).

Dentin permeability

The mean and standard error (SE) of the dentin permeability to 5% Evans blue dye is shown in Fig. 3. There was a significant difference among the groups in both experimental conditions (*in vitro*, $P < 0.0001$ and *in vivo*, $P = 0.0107$).

Scanning electron microscope (SEM)

The number of dentinal tubules per mm^2 (mean and SE) was significantly different among the groups (*in vitro*, $P = 0.0195$ and *in vivo*, $P = 0.0064$) according to one-way ANOVA and Bonferroni post hoc tests (Fig. 4).

The mean and SE of the dentinal tubule diameter are shown in Fig. 5. A statistically significant difference was found (*in vitro*, $P < 0.0012$ and *in vivo*, $P = 0.0022$).

The dentinal tubule area exhibited statistically significant differences among groups (*in vitro*, $P = 0.0001$ and *in vivo*, $P = 0.0011$), using one-way ANOVA and Bonferroni post hoc tests (Fig. 6).

The qualitative analysis showed the presence of opened and partially occluded tubules, peritubular and intertubular deposits and some smear layer in Group 1 and Group 2 (Figs. 7A, 7B, 8A and 8B). In Group 3, most of the dentinal tubules were opened, with no deposits on peritubular and intratubular dentin. A higher amount of smear layer was found in Group 1 and Group 2 than in Group 3 (Figs. 7C and 8C).

Energy-dispersive X-ray (EDX)

Energy-dispersive X-ray (EDX) analysis revealed peaks of chemical elements found on experimental and control samples. High peaks of calcium and phosphorus were observed in all groups (both experimental conditions). Group 1 showed traces of sodium, magnesium, silicon, and potassium; Group 2 showed fluorine, sodium and silicon; and Group 3 exhibited traces of sodium, magnesium and silicon (Figs. 9 and 10).

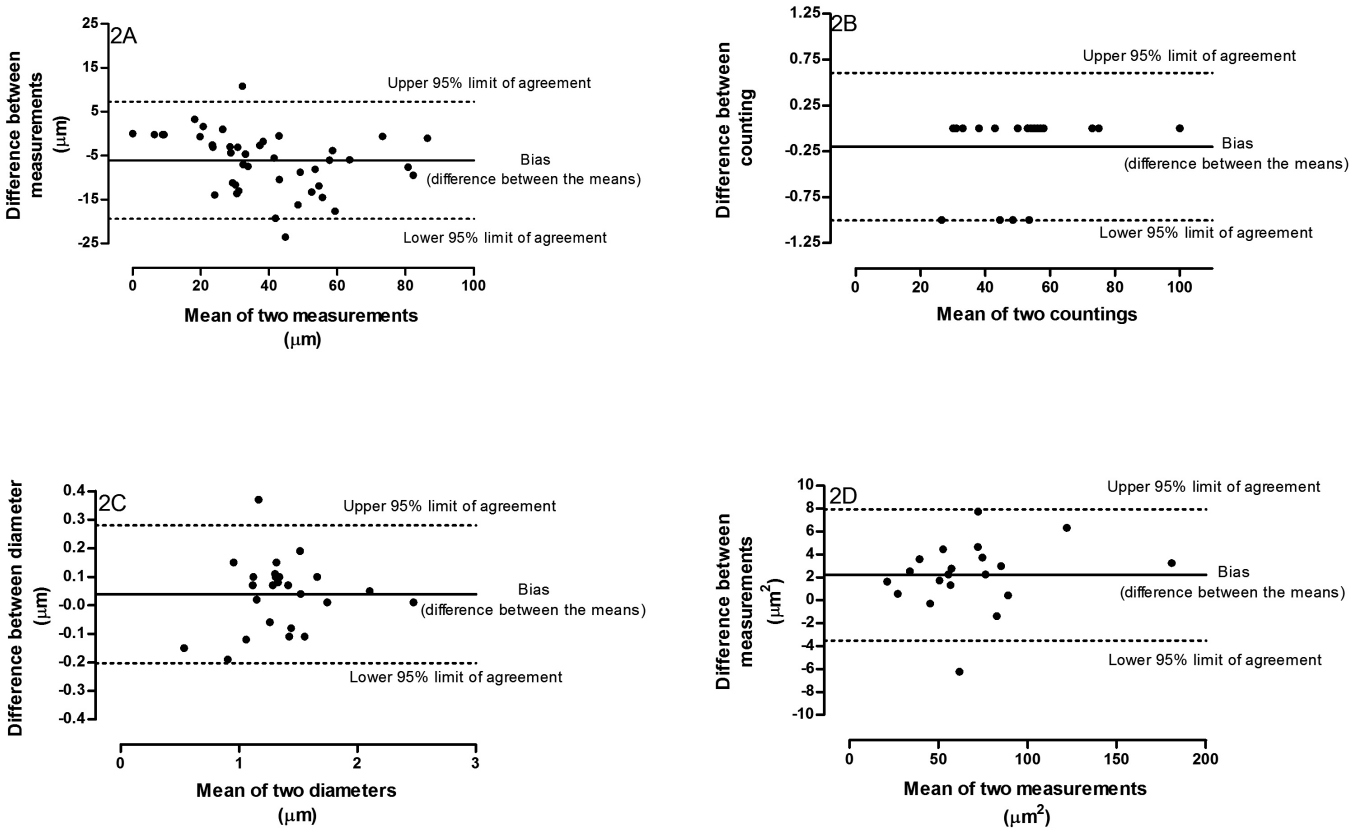

Fig. 2 Intra-examiner reproducibility. Bland-Altman plots of the data obtained by repeated analysis. (2A): Dentinal permeability measurements at two different time points. (2B): Tubule number and tubules (SEM) were repeatedly compared using both manual and automatic methods (digital computer image analysis). (2C): Tubule diameter (SEM) measurements at two different time points. (2D): Tubule area (SEM) was repeatedly compared using both manual and automatic methods. Intra-examiner reproducibility was within the limits of agreement. SEM, scanning electron microscope.

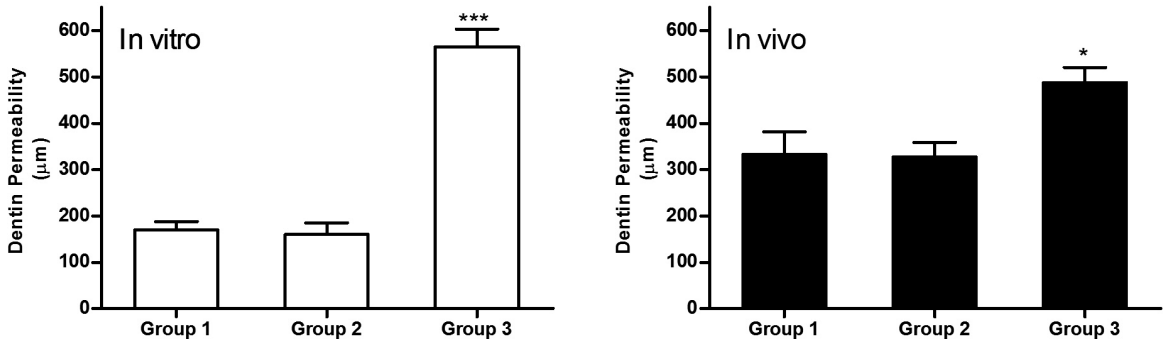

Fig. 3 Mean and standard error of dentin permeability (5% Evans blue dye) after treatment. Group 1, 2% Potassium nitrate with 2% Sodium fluoride gel; Group 2, 5% sodium fluoride varnish; Group 3, 3% hydroxyethylcellulose gel (control). *in vitro*: ***$P < 0.001$, statistically significant difference for Groups 1 and 2. *in vivo*: *$P < 0.05$, statistically significant difference for Groups 1 and 2. ANOVA and Bonferroni post hoc tests.

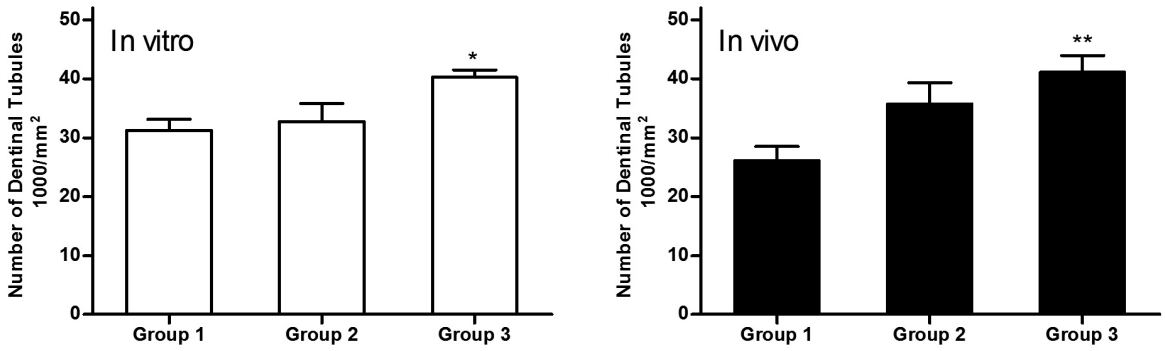

Fig. 4 Mean and standard error of the number of dentinal tubules (×1,000/mm^2) after treatment. Group 1, 2% Potassium nitrate with 2% Sodium fluoride gel; Group 2, 5% sodium fluoride varnish; Group 3, 3% hydroxyethylcellulose gel (control). *in vitro*: *$P < 0.05$, statistically significant difference for Group 1. *in vivo*: (**$P < 0.01$) statistically significant difference for Group 1. ANOVA and Bonferroni post hoc tests

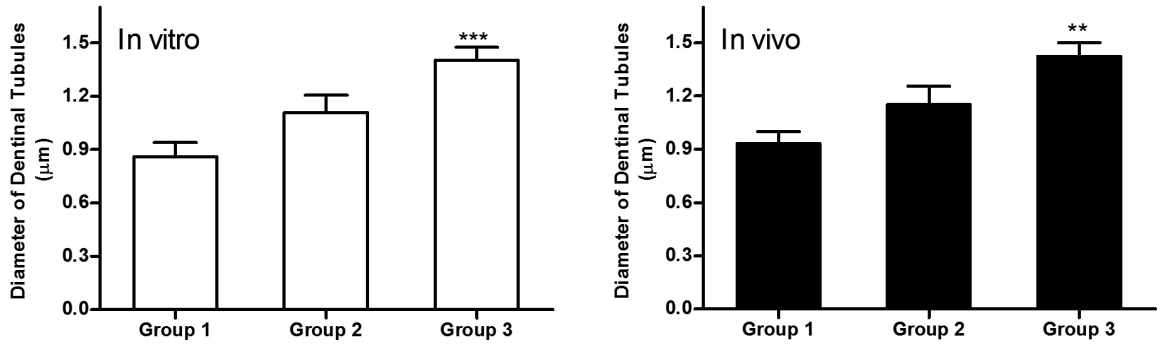

Fig. 5 Mean and standard error of diameter of dentinal tubules (μm) after treatment. Group 1, 2% Potassium nitrate with 2% Sodium fluoride gel; Group 2, 5% sodium fluoride varnish; Group 3, 3% hydroxyethylcellulose gel (control). *in vitro*: ***$P < 0.001$, statistically significant difference for Group 1. *in vivo*: **$P < 0.01$, statistically significant difference for Group 1. ANOVA and Bonferroni post hoc tests.

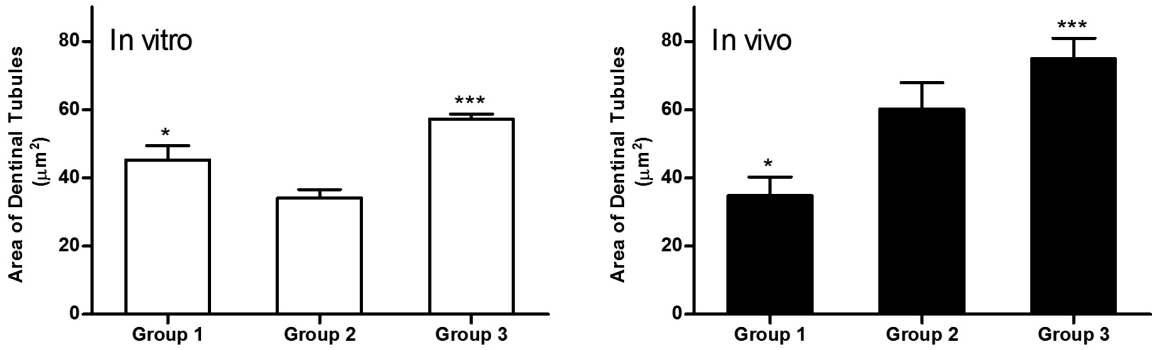

Fig. 6 Mean and standard error of area of dentinal tubules (μm^2) after treatment. Group 1, 2% Potassium nitrate with 2% Sodium fluoride gel; Group 2, 5% sodium fluoride varnish; Group 3, 3% hydroxyethylcellulose gel (control). *in vitro*: $*P < 0.05$, statistically significant difference for Groups 2 and 3; $***P < 0.001$, statistically significant difference for Group 2. *in vivo*: $*P < 0.05$, statistically significant difference for Groups 2; $***P < 0.001$, statistically significant difference for Group 1. ANOVA and Bonferroni post hoc tests.

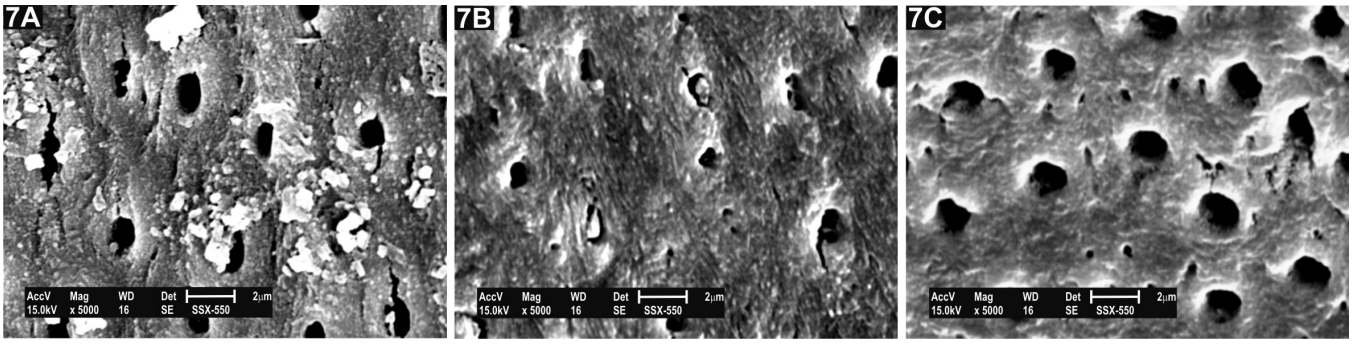

Fig. 7 SEM photomicrographs showing dentin surface after treatments *in vitro*. (7A): Group 1, 2% potassium nitrate plus 2% sodium fluoride gel. (7B): Group 2, 5% sodium fluoride varnish (7C): Group 3, 3% hydroxyethylcellulose gel (control). Groups 1 and 2 showed similar characteristics: the presence of opened and partially obliterated tubules, peritubular and intertubular deposits and some smear layer (7A and 7B). Group 3 had most of the dentinal tubules open, with no deposits on peritubular and intratubular dentin. A higher amount of smear layer was found in Groups 1 and 2 than in Group 3. Bar, 2 μm.

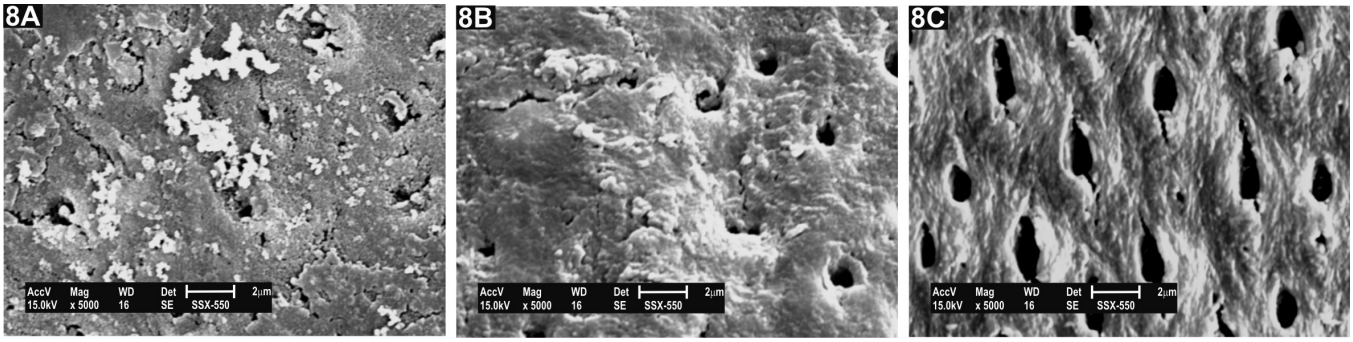

Fig. 8 SEM photomicrographs showing dentin surface after treatments *in vivo*. (8A): Group 1, 2% potassium nitrate plus 2% sodium fluoride gel. (8B): Group 2, 5% sodium fluoride varnish. (8C): Group 3, 3% hydroxyethylcellulose gel (control). Groups 1 and 2 showed similar characteristics: the presence of opened and partially obliterated tubules, peritubular and intertubular deposits and some smear layer (8A and 8B). Group 3 had most of dentinal tubules open, with no deposits on peritubular and intratubular dentin. A higher amount of smear layer was found in Groups 1 and 2 than in Group 3. Bar, 2 μm.

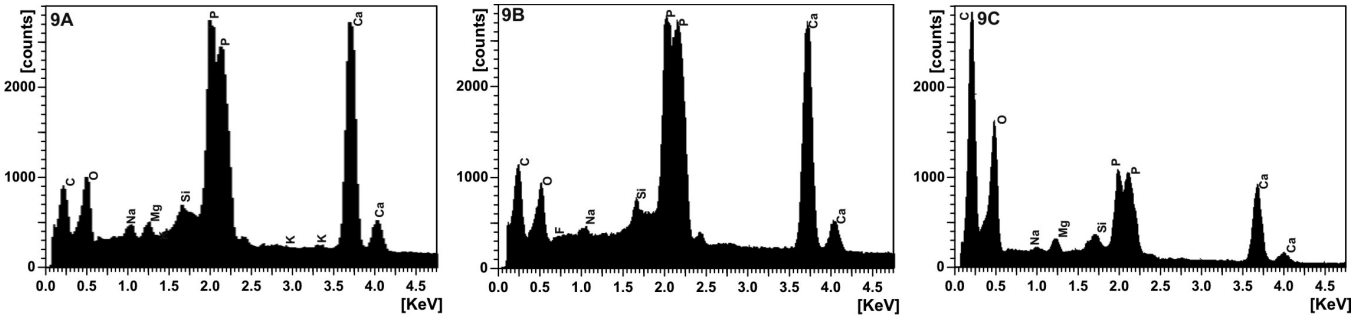

Fig. 9 Energy-dispersive X-ray (EDX) spectrum of *in vitro* samples. (9A): Group 1, 2% potassium nitrate plus 2% sodium fluoride. (9B): Group 2, 5% sodium fluoride varnish. (9C): Group 3, 3% hydroxyethylcellulose gel (control). EDX plots representing peaks of elemental ion concentration showed for Group 1 traces of Na, Mg, Si and K, Group 2 revealed F, Na and Si, and Group 3 exhibited Na, Mg and Si. Presence of C, O, P and Ca due to the background noise produced by mineral content of the underlying dentine.

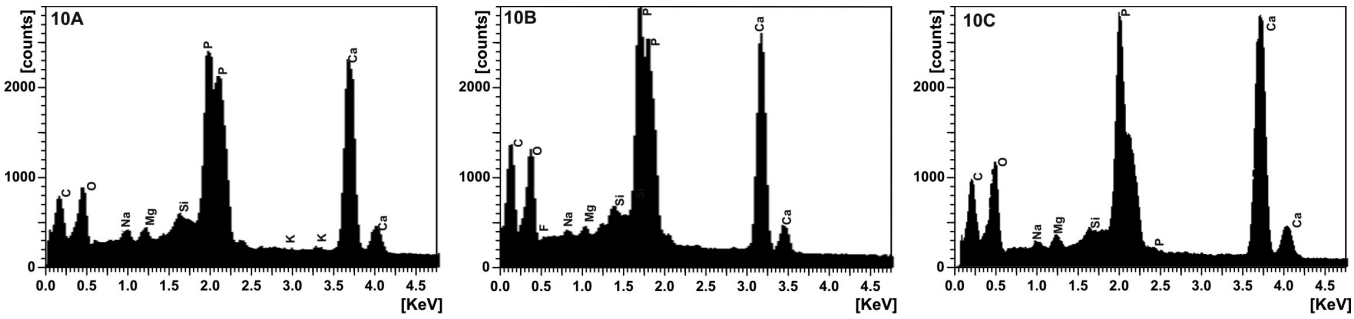

Fig. 10 Energy-dispersive X-ray (EDX) spectrum of *in vivo* samples. (10A): Group 1, 2% potassium nitrate plus 2% sodium fluoride. (10B): Group 2, 5% sodium fluoride varnish. (10C): Group 3, 3% hydroxyethylcellulose gel (control). EDX plots representing peaks of elemental ion concentration showed for Group 1 traces of Na, Mg, Si and K, Group 2 revealed F, Na, Mg and Si, and Group 3 exhibited Na, Mg and Si. Presence of C, O, P and Ca due to the background noise produced by mineral content of the underlying dentine.

Discussion

The present study used an animal model (rat) because of the ease of obtaining samples and reproducing oral characteristics similar to those of humans. The cervical area of the incisors was analyzed because it was more appropriate (higher number of dentinal tubules) than studying dentin permeability in the incisal tip (low permeability due to atubular tissue and pulp remnants), as has been demonstrated previously (20,21). The results of experiments concerning the permeability and tubular occlusion carried out on this animal model were similar to the results of previously published work (20).

The analysis of dentin permeability in this study using a blue dye showed that in both studies (*in vitro* and *in vivo*) there was greater dentin permeability in Group 3 (control). In the tooth specimens which received topical treatment with dentin desensitizing agents (Groups 1 and 2), there was lower permeability *in vitro* than *in vivo*. The reasons for different results are likely related to factors such as oral temperature, saliva flow, dental biofilm and mastication. These could interfere with the action of the desensitizing products. The higher dye diffusion in the control groups confirms these data. In the current study, dentin permeability was higher *in vivo* than *in vitro* specimens that were topically treated with desensitizing products (Groups 1 and 2). The results of the present study were different from those obtained by Vongsavan et al. (21), who observed minimal penetration of the dye *in vivo* and a higher penetration *in vitro*. They eliminated the external factors that could influence the dye diffusion when the dye was allowed to sit for 15 min. In the present study, the dye remained for 5 min daily for 4 days (a total of 20 min). Vongsavan and Matthews (22) showed better dye penetration *in vitro* than *in vivo*, probably due to pulpal pressure.

Considering the methodology used in this study, continuous eruption of the rat incisor could be a limitation for longer periods of experimentation. Therefore, treatment was done for only 4 days and the dentin was exposed on the buccal surfaces of the cervical area of rat incisors for DH analysis in this study (20). Rat incisors can thus be used to evaluate dentin permeability and dentinal tubule occlusion by desensitizing agents.

In this study, the number of dentinal tubules/mm^2 exhibited similar results for both experimental conditions (*in vitro* and *in vivo*). Teeth in Group 1 (*in vivo*) showed lower dentin tubule counts compared with the control group.

In the present study, a 0.3-mm cervical cavity was made, equivalent to one-fourth of the distance between the pulp and enamel. Forssell-Ahlberg et al. (20) observed a higher number of dentinal tubules than was found in the present study. This may have been due to the presence of desensitizing agents occluding tubules in the current results. Garberoglio and Brännström (23), Olsson et al. (24) and Dourda et al. (25) showed that the number of dentinal tubules ranged from 30,900/mm^2 to 37,000/mm^2 in human dentin, at a position halfway between pulp and enamel. Paes Leme et al. (8) observed 19,333 ± 2,770 tubules/mm^2 and 16,433 ± 782 tubules/mm^2, respectively, for potassium nitrate or strontium chloride containing dentifrice-treated dentin and those treated with fluoride varnish. Human cervical dentin has been reported to have 19,000 tubules per mm^2 in superficial dentin. As the halfway point between superficial dentin and the pulp is reached, the number of tubules increases to 30,000 tubules per mm^2 (23). Thus, the results found for desensitizing agent treatments in animals can be extrapolated to humans.

Dentin tubule diameter was smallest in teeth that were topically treated with 2% potassium nitrate plus 2% sodium fluoride gel (Group 1), followed by Groups 2 and 3. Similar results were found for *in vitro* and *in vivo* groups. These results obtained in Group 1 were probably due to the action of the desensitizing agents, creating a higher number of partially obliterated tubules. Rat incisors in the current study showed a mean dentin tubule diameter of 1.0 µm. These values were similar to those previously reported (20). Dentin tubule diameter, in the middle dentin, is similar for humans and rats according to Garberoglio and Brännström (23). Similar results were found in other studies (20, 23), although it is necessary to consider that in these studies, the dentin was not submitted to desensitizing agent treatment. Therefore, in the current study, dentin tubule diameter is expected to be lower in treated groups. The difference among studies may be related to the different chemical agents applied to the dentin.

Dentinal tubule area was evaluated by SEM at 2,000 times magnification (total area of 1,600 µm^2). It was possible to observe a significant difference among groups. These findings represent 3%, 2% and 5% (*in vitro*) and 2%, 4% and 6% (*in vivo*) of the dentin area occupied by tubules in Groups 1, 2 and 3, respectively. In humans, Dourda et al. (25) found 6% of the dentin area occupied by tubules at a distance halfway between pulp and enamel. Rat incisors showed 4% of the area occupied by tubules (20). The percentage of the area occupied by dentinal tubules in this study was probably lower due to the use of desensitizing agents, which occluded many tubule orifices.

Quantitative analysis of dentinal tubule number, diameter and area could be prejudiced due to changes in direction of the dentinal tubules and in the position of the SEM samples. The presence of the smear layer and the small analyzed area could also introduce some bias.

Similar characteristics were found in teeth that were topically treated with 2% potassium nitrate plus 2% sodium fluoride gel and 5% fluoride varnish. Partial to total dentin tubule occlusion could be seen. Paes Leme et al. (8) obtained similar results, in that potassium nitrate was not able to completely obliterate dentin tubules. However, Knight et al. (26) reported that 5% potassium nitrate toothpaste was able to occlude dentin tubules with crystals of different sizes and shapes. Although these studies did not analyze the chemical composition of the deposits, these precipitates could be due to the toothpaste abrasive agents (calcium carbonate) and not related to potassium nitrate.

The samples treated with fluoride varnish (Group 2) had dentin tubules that were partially occluded, and there were fewer deposits and less smear layer on peritubular and intertubular dentin. These data are in agreement with other reports (8,11,26). In those studies, fluorides were unable to occlude dentin tubules after a single application. Knight et al. (26), Pashley (11) and Paes Leme et al. (8) could not observe calcium fluoride crystals in dentin tubules, and consequently no reduction in dentin hypersensitivity. The majority of dentin tubules were open and there were no smear layer deposits in the Group 3 specimens that were treated with 3% hydroxyethylcellulose gel (control).

According to these results, both potassium nitrate and fluoride varnish were able to reduce dentin permeability when compared to the control group, suggesting that repeated applications could contribute to crystal formation, not only on the dentin surface but also inside dentinal tubules. This statement is supported by Mukai et al. (27) and Arrais et al. (14), who showed crystal precipitation on peritubular dentine and inside dentin tubules after acidulated

sodium fluoride application for 4 min.

The EDX microanalysis of samples showed only traces of desensitizing chemical active agents. However, Arrais et al. (7) did not find chemical evidence of the active ingredients of desensitizing agents after EDX analysis. The reasons for these different results are probably the variations in product formulation and concentration used in the present research.

The results of the present study should be interpreted with caution before extrapolating them to dental practice in humans, considering the nature and limitations of animal and *in vitro* experimentation.

In spite of the study limitations, the results showed significant difference among desensitizing agents in comparison with the control group. In conclusion, 2% nitrate potassium plus 2% sodium fluoride gel and 5% fluoride varnish decreased the dentin permeability, although they produced only partial dentin tubule surface occlusion. Repeated application of the desensitizing agents may possibly contribute to higher clinical effectiveness in dentin tubule occlusion.

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