

The effect of 30% ethanolic extract of Indian propolis on replica of human dentin compared against commercially available desensitizing agent: A methodological SEM study *in vitro*

Sudhir Hongal, Nilesh Arjun Torwane, Pankaj Goel, Byarakele Chandrashekar

Department of Public Health Dentistry, People's Dental Academy, People's University, Bhopal, Madhya Pradesh, India

Submission: 22-07-2013

Accepted: 21-11-2013

Published: 18-03-2014

ABSTRACT

Objective: This study evaluated the ability of 30% ethanolic extract of Indian propolis on dentinal tubule occlusion comparatively against CPP-ACP containing desensitizing agent GC tooth mousse. **Methodology:** The specimens were prepared from 30 freshly extracted sound human third molars stored in 10% formalin (pH 7.0) at a room temperature. From each specimen, a sectioned sample (5 mm length × 5 mm width × 3.5 mm depth) was obtained including the cervical area. Samples were smoothed and wet-polished with 1000- and 1200-grit aluminum oxide abrasive paper and diamond pastes, in order to stimulate the clinical aspect of hypersensitive dentin cervical surfaces. All the specimens were randomly assigned to three groups ($n = 10$), according to dentin surface treatments. Negative control: Untreated specimens ($n = 4$) and pretreated with 6% citric acid ($n = 6$); Test Group: 30% ethanolic extract of Indian propolis ($n = 10$); Positive Group: GC Tooth Mousse ($n = 10$). All the specimens were prepared for SEM analysis. **Results:** GC tooth mousse promoted tubule occlusion by crystal-like deposits in the lumen of the tubules. While propolis created a thin, smooth layer over dentin surface. **Conclusion:** According to the SEM analysis, both desensitizing agent were able to occlude the dentinal tubules.

Key words: Dentin hypersensitivity, dentinal tubule occlusion, desensitizing agents, GC tooth mousse, propolis

INTRODUCTION

Dentin hypersensitivity is a short, sharp pain that occurs due to the exposure of dentin as a response to chemical, thermal, or osmotic stimuli that cannot be justified as originating from any other types of dental defect or pathology.^[1] It is neither a recent problem nor a rare one. However, it remains a poorly understood area and consequently there appears to be no effective or permanent treatment for this painful clinical condition. Although different theories have been put forward to explain the mechanism behind dentinal hypersensitivity, it is still not clearly defined that how stimuli affects to the external dentin surface to stimulate nerve fibers.^[2-6]

Address for correspondence:

Dr. Nilesh Arjun Torwane,
Department of Public Health Dentistry, People's Dental Academy,
People's University, Bhopal, Madhya Pradesh, India.
E-mail: nealdip@gmail.com

Access this article online

Website:
www.phcogres.com

DOI: 10.4103/0974-8490.129026

Quick Response Code:



At the present time, the most widely accepted theory for dentinal hypersensitivity is the hydrodynamic theory proposed by Brannstrom.^[7,8] Based on this theory, open dentinal tubules permit fluid flow through the tubules, which results in stimulation of the nerve endings in the dental pulp. Clinically, replicas of teeth with dentinal hypersensitivity have shown various numbers of open or partially occluded dentinal tubules under Scanning Electron Microscope (SEM).^[9-11]

According to the laws of hydrodynamics, any decline in dentinal fluid movement causes a reduction in dentinal hypersensitivity. Corresponding with this theory, Pashley investigated that physiological or pathological formation of intratubule crystals from the dentinal fluid or saliva crystals, invasion of bacteria in the tubules, formation of sclerotic dentin, and formation of reparative dentin might reduce dentinal hypersensitivity.^[3]

Treatment and prevention of dentinal hypersensitivity involves two fundamental approaches. In the first approach,

tooth is treated with a chemical agent (e.g. Potassium nitrate) that penetrates into the dentinal tubules and depolarizes the nerve synapse; this causes reduction in sensitivity due to prevention of pain impulses.^[12,13] In the second approach, a chemical or physical agent is applied to the tooth surface that creates a deposition layer and mechanical occlusion in dentinal tubules to reduce the sensitivity by prevention of dentinal fluid flow.^[2,14] In spite of the fact that, both approaches are effective in treatment of hypersensitivity, the duration of relief is greatly inconsistent. Also, the hypersensitivity symptoms occur repeatedly due to factors such as abrasion of a tooth, variations in acidic challenges in the oral cavity, and/or reduction in the coating material.^[2,15-17]

Therefore, there is a need for a material that will chemically adhere to the dentinal surface, and will significantly diminish the chance of reopening dentinal tubules caused by prolonged contact with acidic foods, oral fluids, and/or forceful tooth brushing.

Recaldent™ is a special milk-derived protein containing amorphous calcium phosphate (ACP) and casein phosphopeptide (CPP). ACP-CCP complex creates a firm binding with the biofilm on teeth and form calcium and phosphate reservoir, which is combined into both enamel and dentin surfaces. Due to its ability to block opened tubules it is recommended in dentin hypersensitivity.^[18]

Propolis is a naturally occurring, relatively safe resinous material produced by honey bees that has been widely used since ancient era^[19] and presents a complex composition depending basically on the plant sources accessible to the bees, possessing a various kind of biological and pharmacological activities, attracting the concern of many researchers.^[20] Propolis has been shown to have antimicrobial, antitumor, anesthetic, anti-inflammatory, antiviral, and healing properties.^[21,22] Many studies have indicated that propolis can treat and manage the dental caries,^[23] precipitate and alleviate the healing of oral tissues,^[24] reduce the pulpal inflammation^[25] with no major side effects, and there is no contraindication to be used in human. Propolis prevents the formation of water-insoluble glucans needed by cariogenic *Streptococci* to adhere to the tooth structure; propolis also enhances the micro-hardness of enamel surface and has a notable effect on dentinal hypersensitivity.^[26-29] A study conducted by Giamlia *et al.* showed that both 10% and 30% propolis showed effect on human enamel.^[27]

Earlier studies have reported use of replicas in order to observe normal and hypersensitive patterns of dentin surface^[11,30-32] as well as to assess the effect of the desensitizing agents on dentin substrate.^[33]

Regarding the case that merely few investigations have evaluated the outcome of propolis extracts on dentinal surface. This study was conducted to evaluate the ability of 30% ethanolic extract of Indian propolis on dentinal tubule occlusion comparatively against commercially available CPP-ACP containing desensitizing agent GC tooth mousse.

METHODOLOGY

Source of data

A total of 30 freshly extracted human third molars stored in 10% formalin (pH 7.0) were used to prepare the specimens. The teeth were obtained after informed consent of the patients and under the approval by the Ethics Committee of Peoples University, Bhopal, India.

Selection criteria for teeth

Inclusion criteria

1. Third molar tooth indicated for extraction due to impaction
2. Teeth with intact root surfaces
3. Tooth surface unaltered by extraction procedure
4. No previous history of professional periodontal treatment.

Exclusion criteria

1. Previous history of periodontal treatment.
2. Teeth with caries or root canal therapy or apical lesion or roughness of root surfaces.
3. Teeth with developmental anomalies such as concrescence, fusion etc.

Study design

After removal of gross debris, the teeth were placed in deionized water for 24 h before beginning the experiment. The teeth were sectioned in mesio-distal direction using a water-cooled diamond saw (SwapTop™ 6 ½” Diamond Trim Saw, 230 V). A sectioned sample from each buccal surface with 5 mm length × 5 mm width × 3.5 mm depth was obtained including the cervical area. Each fragment was ground (600-grit) flat (125 rpm) on a polishing machine to remove enamel and expose the underlying dentin cervical area. To simulate the clinical dentin hypersensitivity, 1000- and 1200-grit aluminum oxide abrasive paper and diamond pastes were used to wet polish the exposed dentin surfaces. Four dentin specimens were kept absolutely untreated and other remaining (26) were etched with 6% citric acid (pH 2.1) for 2 min and rinsed with distilled water, the purpose of this was to increase the diameter and to make sure the complete opening of dentinal tubules, according to the experimental approach proposed by Pashley *et al.*^[34] Finally, the specimens were

ultrasonicated^[35,36] by using ultrasonic cleaner for 30 min to make sure the complete removal of the residual smear layer and were placed in distilled water until they require for treatment [Figure 1].

Randomization: All the specimens were randomly assigned to three groups [Table 1].

- Negative control: Included four untreated specimens (as a gross specimen) and six pretreated with 6% citric acid with no additional treatment (as negative control) (*n* = 10)
- Positive group: Treated with GC Tooth Mousse for 4 min (*n* = 10)
- Test group: Treated with 30% ethenolic extract of Indian propolis for 4 min (*n* = 10).

The treated specimens were rinsed with distilled water 30 min later. This was done to simulate the clinical

condition in which the patient must avoid the ingestion of any kind of food or liquid for at least 30 min after the application of these agents.^[37]

After receiving the desensitizing treatments, all the treated samples were etched again with 6% citric acid pH 2.1 for 1 min. This was done to evaluate the resistance of the ultimate occlusive effect of the investigated materials to an acidic condition, similar to that found in the oral cavity.^[38] All specimens were then rinsed with distilled water for 15 s and dried for 24 h.

Scanning electron microscopy

Finally the specimens were prepared for scanning electron microscopic analysis. Each sample was sputter coated (JEOL, JFC-1600), with a thin gold layer and was examined under Scanning Electron Microscope (JEOL, JSM-6390A). Photomicrographs of representative dentin

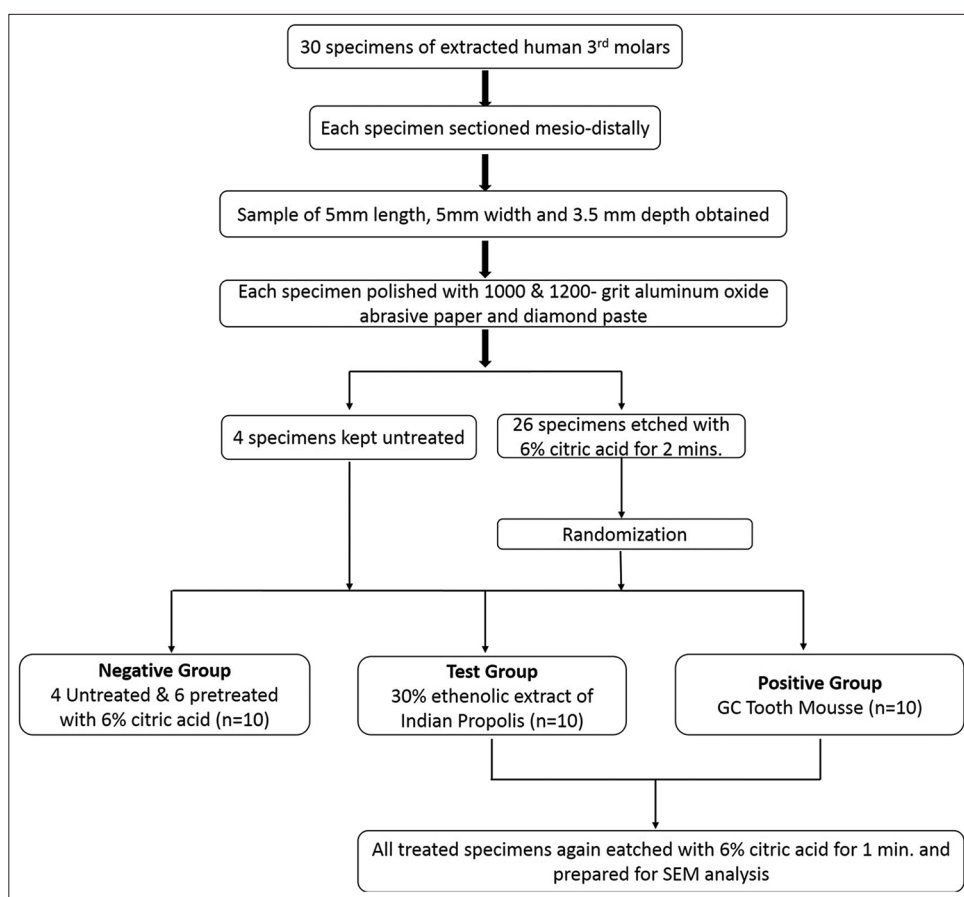


Figure 1: Study Design

Table 1: Desensitizing agents used to treat the dentin surface

Treatment groups	Desensitizing agent applied	Composition	Manufacturer
Test	Indian Propolis	30% Ethenolic extract of Indian Propolis	Hi Tech scientific natural, India
Positive	GC tooth mousse	Casein phosphopeptide (CPP)-amorphous calcium phosphate (ACP)	GC Corporation Tokiyo, Japan

CPP= Casein phosphopeptide, ACP= Amorphous calcium phosphate

surface areas were obtained at standard magnification between 200× and 6000×.

During the SEM analysis of each specimen, the images were obtained from the center and side of each specimen to maintain the standardization in analysis.

RESULTS

Negative control group

Untreated gross specimens: SEM Photomicrographs for absolutely untreated gross specimens in control group are shown in Figure 2a and b. The thick smear layer covering the majority of the dentin surface can be observed. Examined untreated areas presented an irregular appearance with most of the closed dentinal tubules.

Specimens pretreated with 6% citric acid: Photomicrographs for the pretreated specimens with 6% citric acid in the control group are shown in Figure 3a and b. The citric-acid-treated areas presented a smooth appearance and revealed open tubules orifices. Furthermore, no smear coating on dentin, and no smear plugs blocking tubules were noticed.

The SEM observations revealed that the desensitizing agents presented various forms of tubular occlusion.

Positive group

Figures 4a and b are micrographs of dentinal surfaces treated with CPP-ACP containing desensitizing agent-GC Tooth Mousse. An approximately 2-3 μm thick, granular layer of Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) crystals covered most of the treated dentin surface compared to 30% Indian propolis extract. Some crystals had approximately same diameter of the

tubules while some tubules were partially obliterated by the CPP-ACP crystals. The open dentin tubules were barely seen.

Test group

The SEM micrographs were used to observe the morphology surface of the samples after treatment with 30% ethanolic extract of Indian propolis solution [Figure 5a and b]. The SEM micrographs revealed that many of the dentin tubules showed complete occlusion. In addition, the surface of the specimen was covered smoothly by 1 μm thick resinous layer of 30% ethanolic extract of Indian propolis. Also, the dentin surface was more homogeneous compared to CPP-ACP crystals. This may be because of two reasons: obliteration of dentin tubules and deposition on dentin superficially. In some areas, a decrease in the diameter of the dentinal tubules along with partial obliteration can be observed. However, some complete openings of the dentinal tubules can also be seen.

DISCUSSION

Studies related to desensitizing agents have shown that the treatments nowadays used to block the dentin hypersensitivity pain could be augmented to achieve easy, quick, noninvasive, and substantial relief of patient discomfort.

The primary focus of the present *in-vitro* investigation was to sequentially evaluate the dentinal surfaces treated and not treated with desensitizing agents. This strategy allowed the observation of not only the interaction of desensitizing agents with dentin, but also their ability of occlusion and deposition in open dentinal tubules.

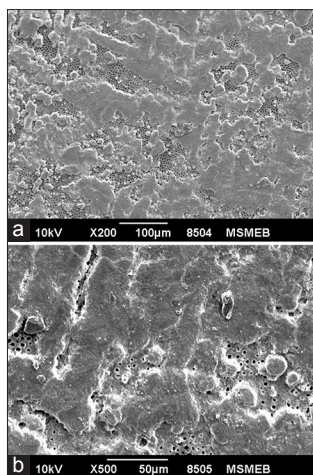


Figure 2: (a) SEM micrograph of the untreated dentin surface. (b) Smear layer covering the majority of the dentin was observed. (2a: ×200; 2b: ×500)

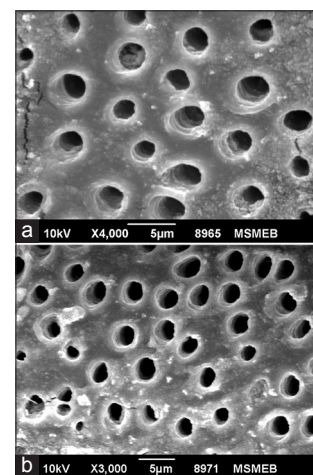


Figure 3: (a and b) SEM micrograph of specimen treated with 6% citric acid. No smear layer and smear plugs were observed. Open dentinal tubules can be seen. (3a: ×4,000; 2b: ×3,000)

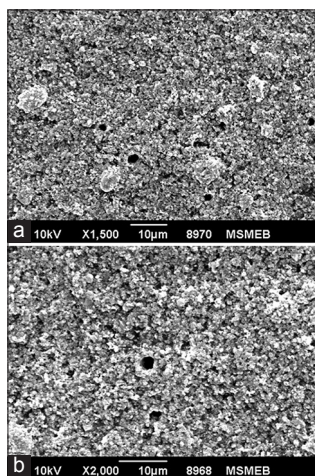


Figure 4: (a) SEM micrograph of CPP-ACP containing GC tooth mousse treated dentin. (b) Obliteration of CPP-ACP crystals into dentin can be seen. (3a: $\times 1,500$; 2b: $\times 2,000$)

In this study, desensitizing agents occluded the dentinal tubules by crystalline precipitation or by deposition of a resin. Hence, if hypersensitivity is caused by opening of tubules at the dentinal surface, then desensitizing agents may enhance a reduction in the number of open tubules or in their diameter could reduce fluid movement within the tubules (according to hydrodynamic theory), which may result in abate dentine hypersensitivity.^[39]

The human dentin replicas obtained from the extracted third molars were used in our study. The replicas are an essential investigation tool for analyzing modifications of dentin surface morphology during clinical research on dentin hypersensitivity. Use of replica technology may allow indirect records of the various effects among the dentinal substrate and desensitizing agents.^[37] According to Oyama and Matsumoto, the use of replicas in longitudinal studies of hypersensitive cervical areas may overcome the difficulty of obtaining clinical samples.^[32] This investigation revealed that significant details of dentin surfaces treated with desensitizing agents may be observed in SEM micrographs at the comparatively low magnification of $500\times$. This noninvasive method may be useful in clinical investigations in replacement for dentin biopsy^[36] or teeth extraction for direct observation.^[32]

Use of citric acid after the application of desensitizing agent was done to simulate the resistance to acid challenge produced by acidic foods and drinks in the oral environment.^[38] The results of the study did not indicate any notable difference among the two test groups after application of citric acid; this consolidate the hypothesis that all tested desensitizing agents offered a certain resistance to the acid challenge.

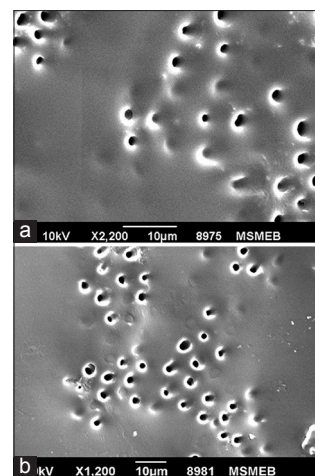


Figure 5: (a) SEM micrograph of 30% ethanolic extract of Indian propolis-treated dentin. (b) Smooth, regular, and homogenous layer of propolis resin covering the dentin surface can be seen

The gross untreated specimens of the control group showed a thick smear layer on majority of the dentin surface. Most of the dentin tubules were covered by large plugs of smear. However, control group specimens that were pretreated with 6% citric acid and ultrasonicated in order to remove smear layer (which could alter the underlying dentin) presented open tubules and tubule density [Figure 3a and b] similar to those described for sensitive areas.^[34-36] In addition, the examination of specimen allowed confirmation of the simulated sensitive cervical areas for control group, superficial tubule occlusion, and intratubular precipitation for treated groups.

Combination of CPP-ACP into various oral health care products such as mouth rinses, sports drinks, and sugar-free chewing gums has been effectively decreased enamel erosion.^[40] Dentin surface treated with GC Tooth mousse showed substantial crystal-like deposits within the tubule lumen. Nevertheless, in few zones, the layer of amorphous calcium phosphate present on the dentin, covered the orifices of dentinal tubules [Figure 4a and b]. GC Tooth Mousse contains casein phosphopeptide (CPP) that carries amorphous calcium phosphate (ACP). When the peptide complex binds to plaque or the tooth surface it is said to deliver bio-available calcium and phosphate for remineralization, resulting in occlusion of dentin tubules.^[18] It has been suggested that the remineralization action of CPP-ACP includes deposition of ACP on the tooth surface that buffers free calcium and phosphate ions.^[41] By sustaining a condition of super saturation with respect to the hydroxyapatite, these ions reduce demineralization and boost remineralization. CPP-ACFP exhibit a superior remineralization effect than single CPP-ACP. This may be attributed to a combination of CPP-ACP

and fluoride occurred in co-localization of calcium and phosphate ions with fluoride ions at the enamel surface, possibly as CPP-ACPF nanocomplexes.^[42] This reaction provides the treated dentin a granular appearance, especially at the tubule opening.

Investigation on the properties of propolis for oral conditions has indicated that it has an anti-inflammatory action and stimulates the formation of reparative dentin,^[22] which may reduce the dentin permeability. Thus, it can be extrapolated the benefit of propolis in dentin. Propolis is a lipophilic sticky material, not soluble in water so it has low solubility and act as adherent to prolong the time of contact with the tooth structure and increases the resistance to acid solubility of the enamel.^[43] The 30% ethanolic extract of Indian propolis produced a more homogenous dentin surface, probability due to the obliteration of the superficial dentin tubules [Figure 5a and b].

The obliteration of tubules when the 30% Indian propolis extract was used can be due to the interaction of components of propolis, how flavonoids may interact with the dentin, thus forming crystals that reduce fluid movement within dentin and, consequently, reduce dentin sensibility. This theory was based the study by Sabir *et al.*,^[25] in which direct pulp capping was performed with propolis-derived flavonoids and mild and moderate inflammation was seen in the pulp chamber at Week 2 and 4, and partial dentin bridge formation was detected beneath the pulp-capping material at Week 4.

Another hypothesis for the better obliteration of dentin tubules after treatment with 30% Indian propolis extract, could be that it is more fluid, that permits the easiest entrance in the tubules and therefore a better interaction with exposed dentin. Propolis has different therapeutic properties without causing major side effects^[21-25] and can be a good option in the treatment of patients with dentin sensitivity.

At the end of this study, we were able to observe how a single application of desensitizing agents affected the dentinal tubules and dentin surfaces.

The several interesting findings of the study are as follows:

- The products tested showed different patterns of dentinal tubule occlusion, and deposition of both GC Tooth Mousse crystals and resinous layer of 30% Indian propolis in open dentinal tubules
- The GC Tooth Mousse containing CPP-ACP crystals were having granular irregularities on dentin surface whereas; 30% Indian propolis was uniformly spread all over the dentin surface
- The CPP-ACP crystals covered most of the dentinal tubules than compared to propolis resin.

Limitations of the study

The study is single-period and observational that is not able to show the maintenance of the precipitated material so further long term investigations are required. Our *in-vitro* model only evaluated the outcome related to occluding effect of 30% Indian propolis extract and no statistical comparison between groups in terms of the depth of dentinal tubules occlusion has been done. Further studies to investigate the durability of occlusion, hydraulic conductance and morphological changes in dentinal tubules with different concentrations of propolis extracts are needed. Also, more *ex-vivo* and *in-vivo* investigations should be carried out to explain the true action and advantageous effects of propolis for the treatment of dentin permeability.

CONCLUSION

The results of present study confirmed by SEM analysis of dentin samples, demonstrated that, both GC Tooth Mousse and 30% ethanolic extract of Indian propolis were able to occlude dentinal tubules by different modes, which suggests that, use of natural propolis can provide a safe and noble option for the treatment of dentin hypersensitivity.

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Cite this article as: Hongal S, Torwane NA, Goel P, Chandrashekar B. The effect of 30% ethanolic extract of Indian propolis on replica of human dentin compared against commercially available desensitizing agent: A methodological SEM study *in vitro*. *Phcog Res* 2014;6:113-9.

Source of Support: Nil, **Conflict of Interest:** None declared.