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Research Report

Effects of manufacturing methods of abalone gel as a desensitisation material on the closing of dentinal tubules

Sri Budi Barunawati,[1] Wayan Tunas Artama,[2] Suparyono Saleh,[3] Siti Sunarintyas[4] and Yosi Bayu Murti[5]
[1]Doctoral Program, Faculty of Dentistry, Universitas Gadjah Mada
[2]Department of Biochemistry, Faculty of Veterinary Medicine, Universitas Gadjah Mada
[3]Department of Prosthodontics, Faculty of Dentistry, Universitas Gadjah Mada
[4]Department of Biomaterial, Faculty of Dentistry, Universitas Gadjah Mada
[5]Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada
Yogyakarta – Indonesia

ABSTRACT

Background: *Abalone (Haliotis varia Linnaeus) shells possess a high arginine content and are expected to be an alternative desensitisation material that is both insoluble and able to properly close dentinal tubules. Different methods of manufacturing abalone gel affect the molecular weight, hydrophilic or hydrophobic properties, and protein content of the lysis.* ***Purpose:*** *This study aimed to determine the effects of different manufacturing methods on the dentinal tubule closure of abalone desensitisation gel.* ***Methods:*** *This study involved the extraction of abalone shells followed by preparative and thin-layer chromatography. The drying of the samples was carried out by the precipitation, drying, and addition methods. The research was divided into eight treatment groups, each consisting of three samples (F1, F2, F3). Each sample was applied to two study subjects' post-extracted third molars, which were cut into disc shapes and subsequently etched with 6% citric acid. The percentage of dentinal tubule occlusion was calculated by Image J (NIH, USA) software. Data were analysed using three-way ANOVA.* ***Results:*** *The results showed that there were significant differences ($p < 0.05$) both in terms of the effects of the samples with deposition and addition on the occlusion of the dentinal tubules and in terms of the interactions between the samples with drying and addition. There was no significant difference ($p > 0.05$) in terms of the interactions of the samples' three manufacturing methods.* ***Conclusion:*** *The manufacture of abalone gel as a desensitisation material requires a minimum of two interactions between the sample-making method and the addition, deposition, and drying methods. The best method was deposition.*

Keywords: *abalone gel; arginine; hypersensitivity dentin; tubule occlusion*

Correspondence: Sri Budi Barunawati, Doctoral Program, Faculty of Dentistry, Universitas Gadjah Mada, Jl. Denta No. 1, Sekip Utara, Yogyakarta 55281, Indonesia. Email: barunawati@ugm.ac.id

INTRODUCTION

The tooth preparation procedures that are necessary to the manufacture of fixed denture restorations have the potential to trigger discomfort afterwards, particularly in the forms of dentin hypersensitivity and pulp irritation.[1] Full crown preparation results in the exposure of 1 cm^2 of dentin, containing more than 3 million tubules/cm^2. The dentinal pulp complex deposits a tertiary layer of dentin under the severed tubules during the cavity preparation procedure, which forms a prepared dentinal wall, preventing the entry of bacteria into the pulp.[2] This response by the pulp is influenced by many factors, including: remaining dentin thickness (RDT), heat friction and bur vibration generated during crown preparation, dentin drying, local anaesthetic effects, gingival haemostatic material, and the moulding and cementing of the restoration. The increase in complications related to endodontic treatment after the fixed denture fabrication procedure is generally due to the preparation of teeth that are too deep. This results in the thickness of the dentin remaining above the surface of the dentin-pulp complex on the prepared teeth being less than 2 mm, which can endanger the vitality of the pulp.[3]

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The current biological approach to dentin hypersensitivity therapy is to mimic the natural desensitisation process, which triggers the spontaneous closure of the dentinal tubules.[4] The disadvantages of current hypersensitivity therapy, such as fluorine, hydroxyapatite, strontium, zinc chlorides, and potassium oxalate, are the short durability and poor effectiveness of their therapeutic effects, namely that the desensitisation material has only a short effect due to the daily brushing process, dissolution in acidic beverages, and closure of the tubules is incomplete. Several studies have shown that, to date, there is no agreement on a gold standard for desensitisation materials.[5]

The latest technological development in desensitisation materials is based on the understanding of the natural processes of reducing dentin hypersensitivity such as that effected by the arginine contained in saliva. An important component of this latest desensitising agent is arginine, an alkaline amino acid with a physiological pH (i.e. 6.5–7.5). Arginine is physically absorbed into the surface of calcium carbonate in vivo, forming positively charged agglomerates that bind negatively to the dentin on the exposed surfaces of the dentin layer and inside the dentinal tubules. The interaction of arginine and calcium carbonate in vivo triggers phosphate, calcium, and carbonate deposition on the surface of the dentin and in the dentinal tubules.[6]

When a desensitisation agent is applied to exposed dentin, the arginine and calcium carbonate in the saliva work together to accelerate the natural mechanism of closure, meaning that the desensitisation agent binds to the negatively charged surface of the dentin, forming a dentin-like mineral deposit as a covering over the dentinal tubules and a protective layer on the dentin's surface.[4] One instance of marine biota that contains high amounts of the amino acid arginine is abalone. Abalone has an expensive shell that contains iodine, zinc, iron, potassium, vitamin A, vitamin E, vitamin B12, omega-3 fatty acids, and magnesium.[7] The dominant part of the abalone is its muscles, which attach to the shell and legs. One species of abalone that is bred is *Haliotis varia* Linnaeus; arginine is the highest average of its amino acid contents.[8] Some studies also mention that abalone meat has a high mineral content along with its protein content, especially the amino acid arginine.[9] Abalone brings such health benefits as being anti-inflammatory, accelerating bone growth, containing vitamins and minerals, and containing omega-3 fatty acids, which are antioxidants.[7]

The method of manufacturing desensitisation gel from abalone shell extraction take place over several steps. Some characteristics of a good desensitisation material are its ability to properly close dentinal tubules and not be easily dissolved by saliva or acid in the oral cavity.[5] Therefore, this study aims to investigate the effects of different sampling methods from abalone shell extraction on dentinal tubule closure.

MATERIALS AND METHODS

The abalone clam used in this study was a male, the third offspring (F3) from a parent of natural origin (F0) cultivated by the Center for Marine Fisheries Research, Gondol, Buleleng, Bali. Species identification – as *Haliotis varia* Linnaeus, 1758 – was carried out at the Animal Systematics Laboratory, Faculty of Biology, Gadjah Mada University.

The extraction procedure was carried out using abalone clam meat that had been frozen at a temperature of −26°C. As much as 50 g was taken and washed, dried, cut into pieces, and blended with 150 ml of 0.1 N HCl. This solution was then centrifuged in 15 ml tubes for 20 minutes at a speed of 5,000 rpm. One tablet-shaped protease inhibitor was added to each 15 ml tube for samples undergoing the addition method. Then, 60 ml of a solution consisting of 0.5 M Tris (hydroxymethyl aminomethane), 30 mM 2-Mercaptoethanol, and 2 mM EDTA disodium dihydrate was added to the resulting supernatant. The resulting mixture was then centrifuged in 15 ml tubes for 20 minutes at a speed of 5,000 rpm. This supernatant was taken and freeze-dried for 24 hours. The results of which were dissolved into 18 ml of a 50 mM acetate buffer eluent (pH 5.0) and then filtered with filter paper.[10]

Preparative chromatography was then carried out after the extraction process with the stationary phase of a Sephadex G-25 and the mobile phase of the acetate buffer 50 mM (pH 5.0).[10] The instrument used was a glass column with a height of 60 cm x 2 cm. A 20-g Sephadex G-25 matrix was packed in a column, and 150 ml of acetate buffer were added and allowed to condense for 24 hours. The acetate buffer eluent was added until the glass column was full and allowed to drip until within ± 2 cm from the deposited G-25 Sephadex. Then, 3 ml of abalone extract were dropped circularly along the column wall. The results of fractionation incorporated as much as 8 ml into a tube with a flow rate of 1 ml per minute.

The grouping of fractionation active substances with qualitative thin layer chromatography (TLC) tests performed with the stationary phase were TLC silica gel 60 F254 and the mobile phase was n-butanol 3.5 ml, acetone 3.5 ml, glacial acetic acid 1 ml, and distilled water 1 ml. The tests of active substances in each fraction was carried out by taking every 10 µl of liquid per fraction and dropping it on TLC silica gel and placing it into a chamber containing a mobile phase. The grouping of active substances was made visible by painting ninhydrin so that three fractions (F1, F2, F3) were obtained for each group of protease inhibitors and without protease inhibitors. The results of the F1, F2, F3 fractions were dried using two methods: freeze-drying for 24 hours and oven-drying at 40°C for 24 hours.[11] The centrifuge and non-centrifuge methods were performed using cold centrifuge procedures at 4°C. A 20 mg samples obtained by both oven- and freeze-drying procedures were dissolved into 500 µl of acetate buffer

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solvent and cold centrifuged in five-minute intervals at 7,000 rcf, 9,000 rcf, 11,000 rcf, 13,000 rcf, 15,000 rcf, 17,000 rcf, and 19,000 rcf. The supernatant, which yielded as much as 200 µl, was put into a microtube and dried in an oven at 40°C for 24 hours, producing a precipitate. At this stage, 24 sample groups were obtained, namely the F1, F2, F3 samples, in the following groups: NPI FD S (without protease inhibitor, freeze-drying, centrifuge), NPI O S (without protease inhibitor, oven, centrifuge), PI FD S (protease inhibitor, freeze-drying, centrifuge), PI O S (protease inhibitor, freeze-drying, centrifuge), NPI FD NS (Without protease inhibitor, freeze-drying, without centrifuge), NPI O NS (without protease inhibitor, oven, without centrifuge) PI FD NS (protease inhibitor, freeze-drying, without centrifuge), and PI O NS (protease inhibitor, freeze-drying, without centrifuge). To obtain dry samples before they were dissolved into CMCNa, all samples were dried in a 40°C oven for 24 hours. To make a sample into gel, 5 mg of dry sample were dissolved into 250 µl of a 1% CMCNa solution. The samples were then stirred until all of them were dissolved.

For this study, three methods of dry sampling were categorised for observing dentinal tubule occlusion: 1) with and without the addition of protease inhibitors, 2) with and without the use of a centrifuge, and 3) oven-drying at 40°C and freeze-drying for 24 hours. In vitro research was carried out on 48 third molars (two subjects for each sample), eight treatment groups, and 24 samples (two samples for each treatment group). The caries-free third molars were extracted from healthy adult patients according to a protocol that has been accepted by the Research Ethics Commission of the Faculty of Dentistry, Gadjah Mada University, No. 001126/KKEP/FKG-UGM/EC/2017.

Teeth were cleaned and stored in a PBS solution (pH 7.4) at 4°C with no more than one month to be used as research subjects. Specimens of Disc-shaped dentin with a thickness of 1 mm was prepared by cutting parallel to the dental axis on a cement enamel junction (CEJ) using a slow-speed bur with water spray (Isomet, Buehler Ltd., Lake Bluff, IL, USA). The formation of a standard smear layer on the surface of the dentin was made using 600-grit silicon carbide paper for 30 seconds under a constant flow of water, followed by 1200-grit silicon carbide paper.[5]

This study was divided into eight treatment groups with each group consisting of three samples (F1, F2, F3); each sample was applied to two research subjects, which were caries-free, extracted third molars. Subjects had 6% citric acid applied to them for two minutes to ensure the opening of the pulp suffix from the dentinal tubules. The surfaces of the subjects were observed with an optical microscope (Olympus, Tokyo, Japan) to examine the exposed dentinal tubules. Twenty-four of the treatment samples were treated with an application of abalone desensitisation gel to the dentin surface with a microbrush, left for seven minutes and washed with distilled water. The subjects were stored in artificial saliva at 37°C, and the procedure was repeated for seven days.[12]

After seven days, the subject was dried and stored in a desiccator. The specimens were mounted on aluminium stubs and sputter-coated in platinum before examination using a scanning electron microscope (JEOL, JSM-6510LA, Japan). For each region of each subject, typical fields were photographed at a magnification of 500x. Quantitative analysis of dentinal tubule closure on the surface of the dentin was performed by an assessment of the occlusion or non-occlusion of dentinal tubule closure, which was measured using Image J (NIH, USA) software.[13] The percentage of dentinal tubule closures was determined by dividing the total number of closed dentinal tubules by the total area of the subject in the SEM image area. Data were analysed using the three-way ANOVA method. The probability level for statistical significance was set at $\alpha = 0.05$.[14]

RESULTS

Results showed that the percentage of dentinal tubule closure was different in each treatment group with different manufacturing methods, as shown in Table 1 and Figure 1. The data in Table 1 shows the highest incidence of dentinal tubular closure, seen in the PI O NS treatment group (98.692 ± 0.306). The lowest incidence of dentinal tubular closure was seen in the NPI FD NS treatment group (91.817 ± 1.386). Figure 1 shows that the treatment group without the precipitation method (NS) had a higher rate of closure

Table 1. The mean and standard deviation of dentinal tubule occlusion (%)

Sample method		Deposition	
Drying	Addition	Centrifuge (S)	Without centrifuge (NS)
Oven (O)	Protease inhibitor (PI)	94.825 ± 1.327	98.692 ± 0.306
	Without protease inhibitor (NPI)	94.108 ± 1.054	97.492 ± 2.004
Freeze-drying (FD)	Protease inhibitor (PI)	96.767 ± 1.247	97.183 ± 0.934
	Without protease inhibitor (NPI)	91.817 ± 1.386	95.850 ± 1.236

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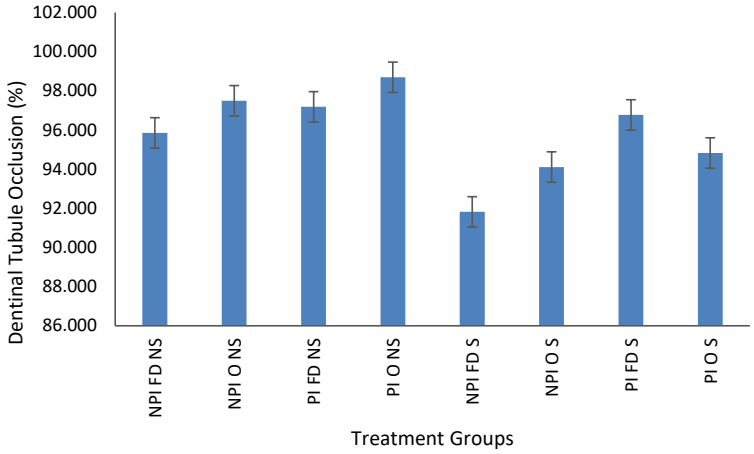

Figure 1. Average closing of dentinal tubules from eight treatment groups.

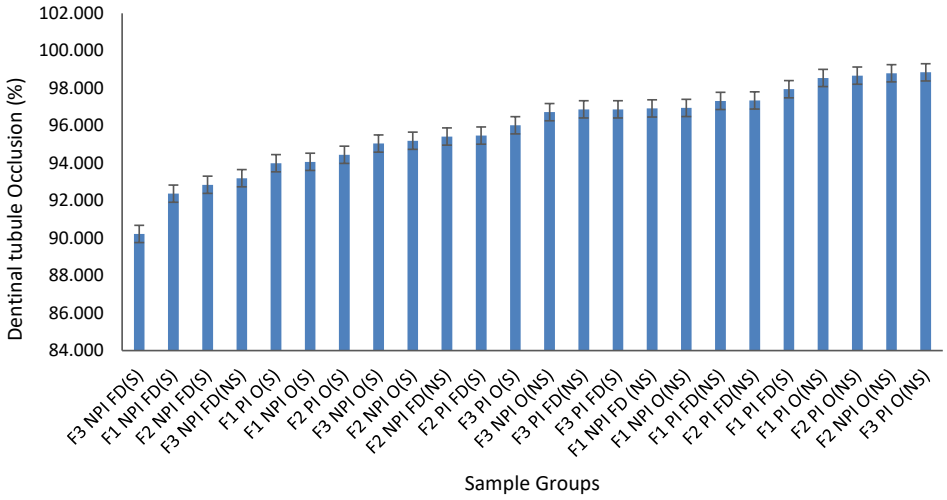

Figure 2. Percentage of dentinal tubule closure in each sample. Notes: F1: first fraction; F2: second fraction; F3: third fraction; NPI: without the addition of protease inhibitor; PI: with addition of protease inhibitor; FD: drying with freeze-drying; O: drying with oven; S: centrifuge; NS: without centrifuge.

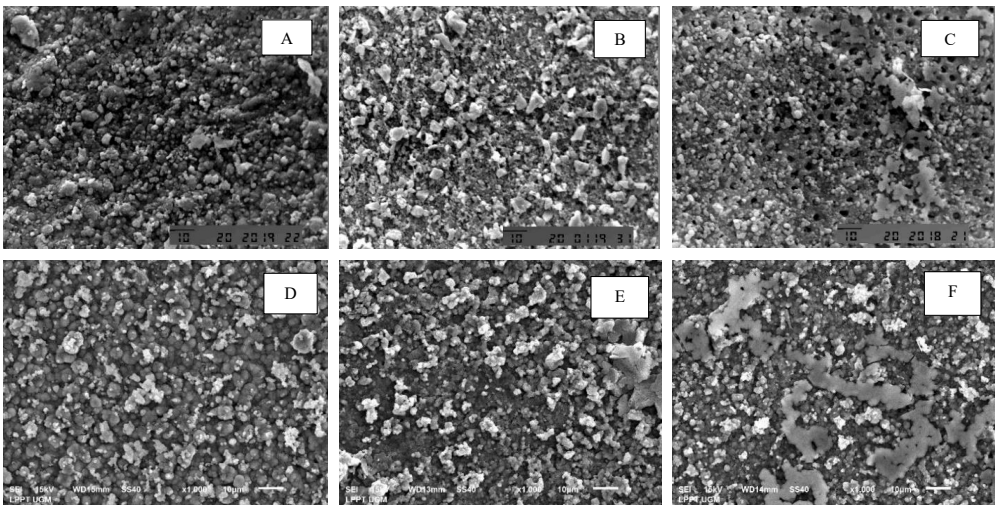

Figure 3. Closure of the dentinal tubules is seen in samples without the addition of protease inhibitors and without a centrifuge. (A), (B), (C): drying by freeze-drying. (D), (E), (F): drying with an oven (1000x magnification). Figure C shows more open dentinal tubules than closed dentinal tubules.

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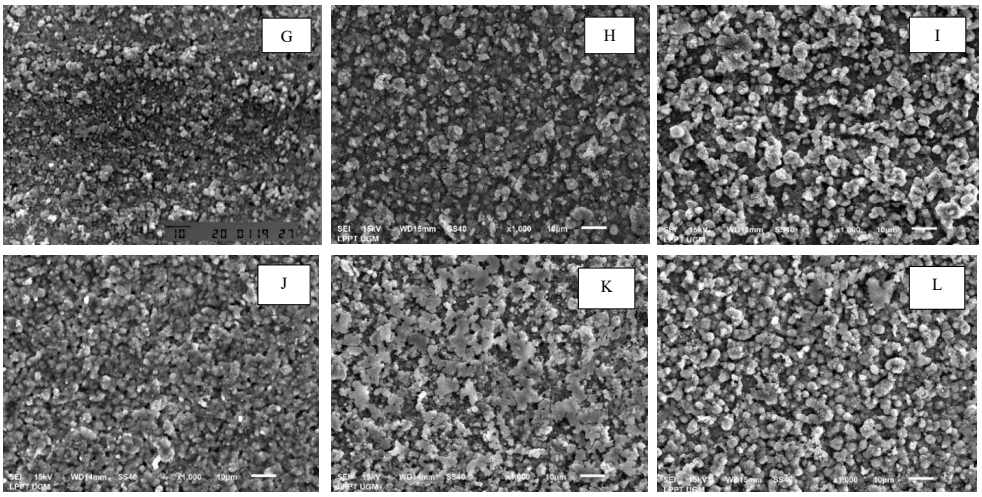

Figure 4. Closure of the dentinal tubules is seen in samples with the addition of protease inhibitors and without a centrifuge. (G), (H), (I): drying with freeze-drying. (J), (K), (L): drying with an oven (1000x magnification).

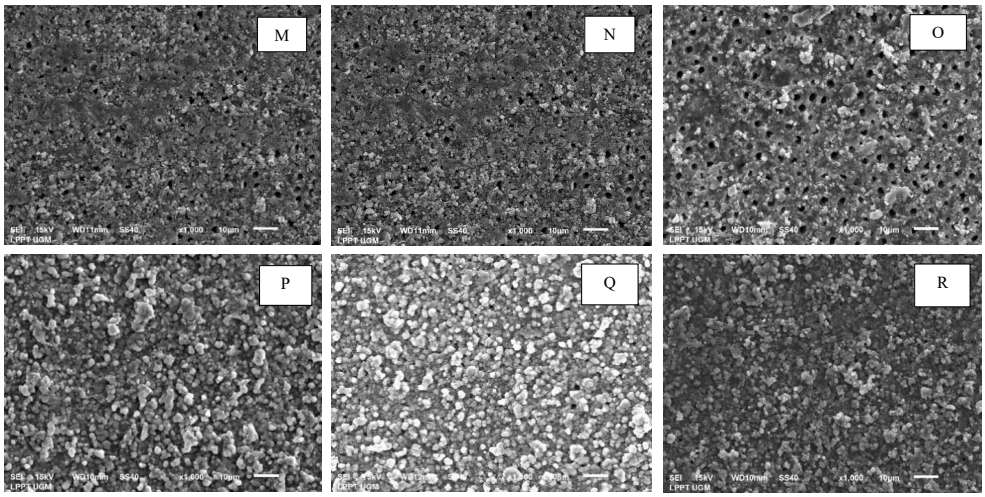

Figure 6. Closure of the dentinal tubules is seen in samples by the addition of protease inhibitors and by centrifuge. (S), (T), (U): drying with freeze-drying. (V), (W), (X): drying with an oven (1000x magnification).

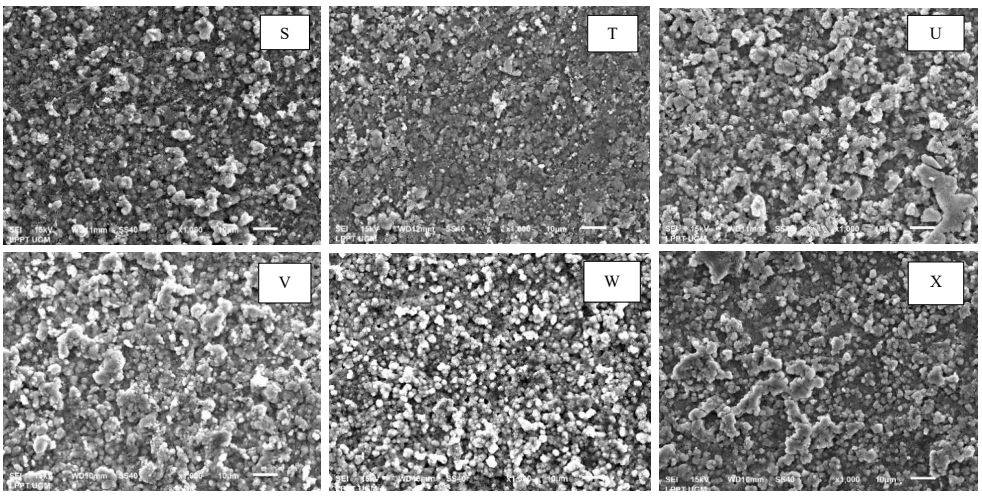

Figure 5. Closure of the dentinal tubules is seen in samples without the addition of protease inhibitors and by centrifuge. (M), (N), (O): drying with freeze-drying. (P), (Q), (R): drying with an oven (1000x magnification). Figures M, N, and O show more open dentinal tubules than closed dentinal tubules.

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than that in which the precipitation method was used (S). Incidence of closure in the treatment group with the addition method (PI) was higher than in the group without (NPI). The treatment group in which oven drying was used (O) was higher than that in which freeze-drying was used (FD).

The percentage of dentinal tubule closure in 24 samples, which comprised three samples (F1, F2, F3) for each group of eight treatment groups, is shown in Figure 2. The results showed that the percentage of dentinal tubule closure was different for each sample within the eight different treatment groups shown in Figure 2. The highest dentinal tubular closure was seen in the F3 PI O NS sample. The lowest dentinal tubular closure was seen in the F3 NPI FD S sample. Closure of the dentinal tubules was observed with a scanning electron microscope (SEM), as shown in Figures 3, 4, 5, and 6 (at 1000x magnification). The SEM figure displays the different percentages of dentinal tubule closure in each sample.

The results of the three-way ANOVA test (Table 2) showed that there was a significant difference ($p < 0.05$) in dentinal tubule closure between the samples prepared with the centrifuge deposition method and those without (G1). There was no significant difference ($p > 0.05$) in dentinal tubule closure between the samples prepared using the freeze-drying method and those using oven-drying (G2). There was no significant difference ($p > 0.05$) in dentinal tubule closure between the samples prepared by adding protease inhibitors and those without protease inhibitors (G3). There was no significant difference ($p > 0.05$) in dentinal tubule closure between the interaction of the method of making the deposition sample (G1) and drying sample (G2). There was a significant difference ($p < 0.05$) in dentinal tubule closure between the interaction of sampling methods between deposition (G1) and addition (G3). There was a significant difference ($p < 0.05$) in dentinal tubule closure between the interaction of sampling methods with drying (G2) and addition (G3). No significant difference ($p > 0.05$) in dentinal tubule closure was seen in the interactions of the sampling methods with deposition (G1), drying (G2), or addition (G3).

DISCUSSION

One effective way to overcome dentin hypersensitivity is to develop a new material that can close dentinal tubules well; last for a long time; not be dissolved by saliva, acidic food, or drink; and not be lost to abrasion or erosion. Arginine is just such an alternative ingredient. Natural proteins play a role in the formation of teeth, such as dentin phosphophoryn, dentin matrix protein-1 (DMP-1), which is rich in glutamic acid, and phosphoprotein, which facilitates the nucleation of hydroxyapatite (HAp) and modifies the formation of the mineralised collagen fibres in dentinal tubules.[15] Abalone is a natural, alternative desensitisation material due to its high arginine content, its anti-inflammatory properties, its rich in mineral content, and other factors.

The centrifuge procedure for abalone extraction produces deposits with high molecular weights, resulting in small to medium molecular weights remaining in the supernatant. The primary concern with dentin regeneration is the realisation of mineralisation between the fibres in the collagen matrix. Collagen requires a material with a molecular weight ranging from 6–40 kDa. However, the molecular weight of L-arginine is 174.2 kDa, which is much higher.[16] This study used the supernatant of abalone extraction, which resulted in more material with low-to-moderate molecular weight in the sample. Tubule closure with samples that were prepared using the centrifuge method was less than those that were prepared without the centrifuge.

Oven-drying and freeze-drying for 24 hours evaporated the acetate buffer solvent. The oven-drying procedure was carried out at 40°C for 24 hours, which eased the evaporation of the buffer without damaging the protein content. This is consistent with research that has found that the amino acid profile in the oven-drying process produces hydrophobic residues, whereas freeze-drying produces hydrophilic residues – both without damaging the protein content.[17] Neither the freeze-drying nor the oven-drying procedure affected the protein composition of the samples, and there was no significant difference in tubule closure.

Table 2. The results of three-way ANOVA

Source of Variance	Sum of Squares	Free Degrees	Average Square	F	*Sig.*
G1	0.012	1	0.012	48.358	0.000
G2	7.465	1	7.465	0.028	0.869
G3	0.001	1	0.001	3.712	0.057
G1 * G2	0.001	1	0.001	2.667	0.106
G1 * G3	0.011	1	0.011	42.679	0.000
G2 * G3	0.007	1	0.007	26.836	0.000
G1 * G2 * G3	0.000	1	0.000	1.176	0.281
Error	0.023	88	0.000		
Total	88.240	96			
Corrected Total	0.055	95			

Notes: G1: With centrifuge (S) and without centrifuge (NS) depositional treatment groups; G2: Freeze-drying (FD) and oven-drying (O) treatment groups; G3: With added protease inhibitors (PI) and without protease inhibitors (NPI); (*): interaction.

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The current study showed that the mean closure of dentinal tubules with oven-drying was higher because samples that underwent freeze-drying had a hollower structure with hydrophilic properties, meaning that when they attached to the dentin surface, they became more soluble. The dentin matrix is principally composed by mineralised collagen fibres, which are hydrophilic; it therefore becomes difficult to wet or infiltrate the surface of dentin that has been etched with hydrophobic material, although some studies do mention having been done using a hydrophilic monomer 2-hydroxyethyl methacrylate or ethanol wetting technique; however, these desensitisation materials could not cover the dentinal tubules for long.[15] This is reinforced by the fact that the patient's oral cavity is filled with flowing saliva. A desensitisation material will dissolve if it is not able to sufficiently bind to dentin.[18] Amino acids, as the basic building blocks of proteins, play a biochemical role in the remineralisation process of etched dentin. Arginine, as a desensitisation material, is an amino acid that is positively charged under physiological environmental conditions if it forms a complex with calcium carbonate as a source of calcium; thus, it can close exposed tubules well and remain resistant to acids. They are thus believed to be constitute a long-lasting prevention of dentin hypersensitivity.[15]

Protease inhibitors catalyse the hydrolytic breakdown of proteins into peptides or amino acids, produce protein degradation, and deactivate or inhibit protease in the cell lysis buffer or cell extract.[19] This research shows that samples that included protease inhibitors kept the protein content of the samples from going into lysis. The longer the peptide chain, the higher the ability to bind hydroxyapatite and collagen. Peptides bind to the positively charged side of demineralised hydroxyapatite and collagen fibres via electrostatic interactions. Then, nucleation templates occur to absorb calcium ions through negatively charged groups or secondary structures, which allows calcium ions to join with phosphate ions to form deposited minerals on the dentinal tubules.[18] This study showed that the samples with added protease inhibitors had a greater effect on dentinal tubule closure than those without. This is consistent with the opinion of Kleinberg (2002), who asserted that the combination of arginine and calcium carbonate would form a positively charged complex that would bind to the surface of the negatively charged dentin and inside the dentinal tubules. The alkali nature of the arginine compound with calcium carbonate would also facilitate the deposition of calcium and phosphate from saliva or dentinal fluid in permanently closing the dentinal tubules.[20]

Table 2 shows that the deposition method (G1) had a significant effect ($p < 0.05$) on dentinal tubule closure. However, the drying (G2) and addition methods (G3) had no significant effect ($p < 0.05$) on dentinal tubule closure. It positively asserted that the manufacture of samples by the G1 method alone can affect dentinal tubule closure. The results of the three-way ANOVA analysis showed the presence of interaction factors. Interaction factors that significantly influenced ($p < 0.05$) dentinal tubule closure were the G1–G3 and G2–G3 methods. This shows that the most important factor was the G1 method, while the G2 method in this case did not have a significant effect on tubule closure. The G2 method needs to be carried out in conjunction with the G3 method in order to significantly influence tubule closure, whereas the G1 method did not require the presence of the other methods to influence tubule closure. The interaction of the G1 and G3 methods affected the ability of the abalone gel to close dentinal tubules. Deposition using the centrifuge separated the results of abalone extraction with low-to-moderate molecular weight more widely than without the use of the centrifuge. Adding protease inhibitors extended peptide bonds, thereby further increasing the ability of the abalone gel to bind hydroxyapatite in the dentinal tubules. The interaction of the G2 and G3 methods also showed a significant difference ($p < 0.05$). The most suitable drying process is that which does not damage the protein content, as it further enhances the ability of the abalone gel to adhere to the walls of the dentinal tubules due to its insoluble, hydrophobic nature. The interaction of the G1and G2 methods was not significant ($p > 0.05$) to tubule closure, meaning that the presence of the G2 method did not support the G1 method. The interaction of G1, G2, and G3 was not significant ($p > 0.05$) because the G2 method did not significantly influence dentinal tubule closure.

The results of the current study showed that manufacturing abalone gel for desensitisation by causing dentinal tubule closure requires a minimum of two interactions of sample-making methods, such as the interactions of the methods of deposition (G1) and addition (G2), or the methods of drying (G2) and addition (G3). The best method for manufacturing abalone desensitisation gel samples is the deposition method (G1).

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