ORIGINAL PAPER

EVALUATION OF THE EFFECTIVENESS OF VARIOUS TREATMENT PROCEDURES ON DENTINE MICROSTRUCTURE *IN-VITRO*

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ABSTRACT

Introduction: Investigation of dentine microstructure *in-vitro* using scanning electron microscopy (SEM) allows comparing effects of various treatment methods of dentine hypersensitivity (DH).

Objectives: The aim of this study was to investigate the microstructure of dentine *in-vitro* after application of fluoride varnish, low-level laser (LLL) (810 nm) irradiation, ethanolic extract of propolis (EEP) application, and joint LLL (810 nm) irradiation and EEP application using SEM.

Material and methods: 32 specimens of permanent teeth were divided into four groups. Samples of the first group were treated with fluoride varnish, second group with EEP, third group with LLL (810 nm) irradiation, and fourth 4 group with joint LLL (810 nm) irradiation with EEP application. Four samples were taken from all groups and immersed in 6% citric acid. From each of the four groups, 4 other samples were placed in oral fluid for a week, and were brushed twice a day with a soft toothbrush. Then, samples were studied by the SEM.

Results: The utilization of fluoride varnish as well as EEP on the surface of the exposed dentine promotes formation of acid-resistant film. After using LLL irradiation, dentine tubules with a double structure were found. After joint use of EEP and LLL irradiation, the entrances of dentinal tubules were completely blocked, and only single-opened entrances to the dentinal tubules were identified.

Conclusions: The joint usage of EEP and LLL irradiation provides the highest permeability of components to the dentinal tubules, which was proved by the results of SEM.

Key words: propolis, fluoride, dentine hypersensitivity, low-level lasers.

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INTRODUCTION

According to results of epidemiological studies, dentine hypersensitivity (DH) is a widespread pathological condition [1, 2]. Studies in India indicate that more than 32% of patients complained of DH [3, 4]. The American Dental Association has reported that approximately 30 to 60 million Americans complain of hypersensitivity of tooth hard tissues [5]. According to results of a study conducted among many European countries, it was determined that DH occurs in more than 42% of the adult population [6].

Among the existing theories of origin of DH, the most common is the hydrodynamic theory. This theory indicates rapid displacement of the dentine fluid within dentinal tubules in any direction as a result of the action of stimulus on the tooth, which leads to the activation of sensory nerves [7]. The hydrodynamic hypothesis of

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hypersensitivity was proposed more than a hundred years ago [7], but supporting evidence have been obtained only in the 60s of 20th century [8]. Cold air waste test, which is directed on the exposed dentine, is one of the most common clinical screening methods for DH, as air has both thermal and evaporative properties [9, 10].

Scanning electron microscopy (SEM) data suggests that there are differences between hypersensitive and insensitive dentin. Sensitive dentine has more open dentinal tubules, which are larger in diameter. In addition, another study based on replication models of hypersensitive and insensitive dentine showed that in hypersensitive dentine, the smeared layer is thinner and differs in structure from insensitive dentine, and probably less calcined than the latter [11].

This information confirms the hydrodynamic theory. More open and wide tubules on the dentine surface increase the permeability of dentinal fluid through the dentine and, therefore increase the possibility of transmitting further pain. It is the width of tubules that is an important factor, as doubling the diameter of the tubule leads to a 16-fold increase in the flow rate of dentinal fluid in tubules [12].

Therefore, today there are many ways to treat DH, but despite this fact, they have their drawbacks. That is why there is an urgent need to obtain a prolonged effect of reducing pain in the presence of DH, and improve quality of life.

Scientists in many countries around the world are studying the effectiveness of low-level laser treatment (LLLT) in the treatment of DH. *In-vitro* and *in-vivo* studies have used diode lasers with a wavelength in range of 635-830 nm [10].

Propolis-based bee products are widely used in dentistry, including DH treatment. The effectiveness of treatment is due to the content of high levels of flavonoids in propolis, which in turn interact with the dentine to form crystals that 'stick' to the dentine surface and clog holes in dentinal tubules [13]. There is information that natural rubber-like substances in propolis have a mechanism of action similar to the mechanism of action of dentinal adhesive systems [14].

Today, fluoride drugs are common in DH treatment. A large number of publications are devoted to application of fluorine, which effectively eliminates the symptoms of DH for a short time [15, 16].

To study and explain the types of treatment of DH, it is necessary to assess the microstructure of dentine teeth with hypersensitivity using SEM method.

OBJECTIVES

The aim of this study was to evaluate and compare dentine microstructure *in-vitro* after application of fluoride varnish, LLL (810 nm) irradiation, EEP application, and joint LLL irradiation (810 nm) irradiation and EEP application with SEM.

MATERIAL AND METHODS

To study the microstructure of dentine teeth with hypersensitivity and the impact of the proposed treatments, the method of SEM was applied.

Ethical and bioethical committee of the Kharkiv National Medical University (minutes No. 6 of October, 4, 2017) approved the study protocol and related consent forms. The study was conducted in the laboratory of electron microscopy of VN Karazin Kharkiv National University (cooperation agreement No. 0301-149). Microstructures of the samples were studied using SEM with a scanning microscope J-840 (Jeol, Japan) with an accelerating voltage of 20 kV, in the range of magnification from 100 to 15,000 times.

In total, 32 permanent teeth (18 molars, 14 premolars) with signs of dentine hypersensitivity, extracted in patients according to the orthopedic indications were examined. Patients from whom teeth were extracted had periodontal diseases. According to their case histories, they have complained of DH, with short-term, sharp pain in the teeth arising from stimuli, typically thermal, evaporative, tactile, osmotic, or chemical.

The extracted teeth were thoroughly washed from the blood, cleaned of soft tissues with an excavator, and plaque and pellicle were removed with a toothbrush. Disinfection of the studied teeth was performed by means of 0.05% solution of chlorhexidine bigluconate.

If necessary, the cervical area of the teeth was treated with abrasive paper to remove cement residues from the surface for 30 seconds for each tooth. The cervical area of the teeth, which was planned to be studied, was divided into two equal parts using a vertical shallow groove, which was applied with a diamond bur and a turbine tip in the middle of the vestibular surface of the teeth. After that, the samples were left for 10 minutes in distilled water, the teeth were dried with an air gun of the dental unit for 1 minute, and treated with 37% orthophosphoric acid in the form of a gel for 30 seconds to remove the smear layer. After that, the orthophosphoric acid was washed off with distilled water for 2-3 minutes. The samples were dried with an air gun of the dental unit for 1 minute.

According to the study, the left side of the cervical zone of the vestibular surface of the teeth of all samples was control; in order to keep it untreated with drugs, it was tightly covered with teflon tape. All samples were randomly divided into four groups according to the method of exposure (8 samples in each group).

The first group consisted of 8 samples that were treated with fluoride varnish Ftoroplen (Latus, Ukraine), one-component varnish of air drying on the basis of polymeric film formation. As an active agent contains a preparation of sodium fluoride and calcium fluoride, which helps to eliminate hypersensitivity of the teeth. Varnish was applied to the surface of the tooth sample by means of microbrush to create a monolayer. After

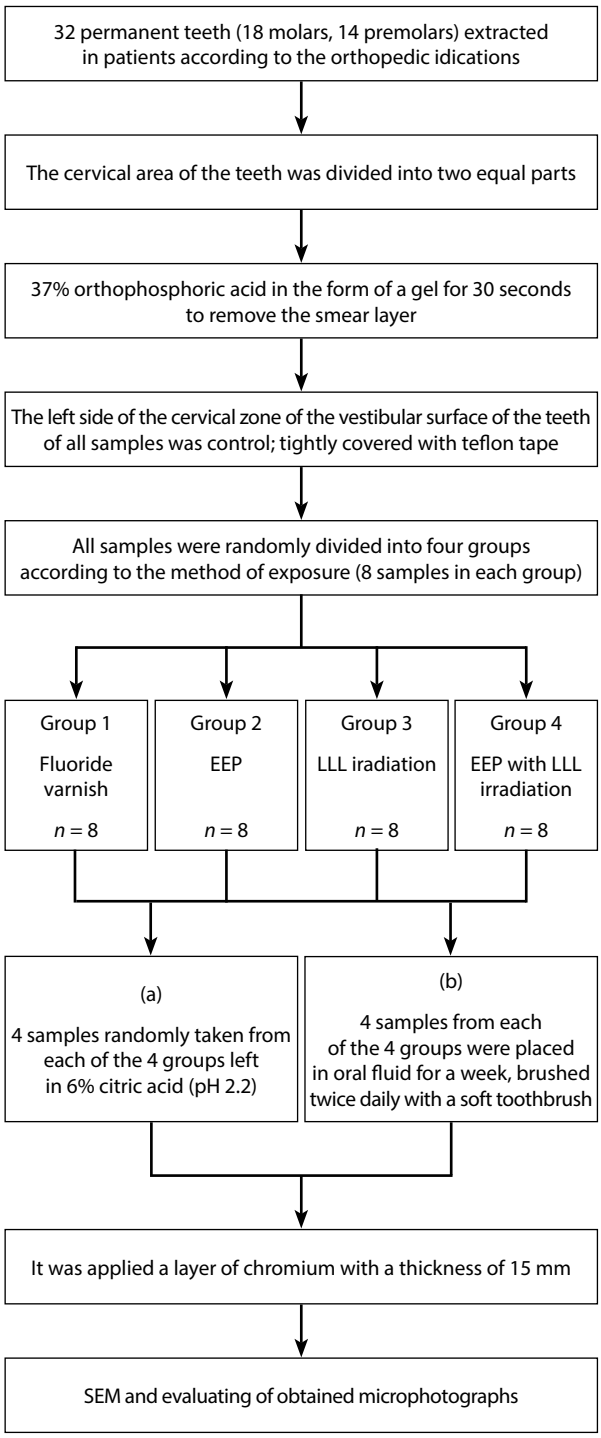

FIGURE 1. Summary of experimental design to prepare teeth specimens for different methods of treatment and scanning electron microscopy (SEM) observations

application, the varnish was dried with compressed air for 1.5-2 minutes. According to the instructions, the procedure was performed twice.

In the second group, EEP (Ternopharm LLC, Ukraine, Ternopil) was applied to the cervical surface of 8 tooth samples. One vial contained EEP (propolis tincture, 1 : 10; extractant, ethanol 80%) 25 ml, with other excipients missing. The tincture was applied by rubbing it using a micromotor with a rubber cup for 60 seconds. This procedure was performed twice, with an interval of 2 minutes.

In the third group, the vestibular surface of 8 tooth samples was treated with LLL irradiation using a laser therapeutic device Lika-Therapist M (Photonics Plus, Ukraine, Cherkasy), consisting of an electronic unit and detachable remote handles. We used a remote handle, which operates in infrared optical range, with a wavelength of 810 nm and a maximum power of 100 mW. A tip was directed perpendicular to the cervical area of the vestibular surface of the tooth, and the irradiation was performed for 3 minutes. The procedure was performed once; the dosimetry was 6 J/cm^2.

In the fourth group, EEP was applied to the vestibular surface of 8 tooth samples by rubbing it using a micromotor with a rubber cup for 60 seconds. This procedure was performed twice, with an interval of 2 minutes. Then, the samples were treated with LLL irradiation using a laser therapy device Lika-Therapist M (wavelength 810 nm and maximum power 100 mW). The peripheral tip was directed perpendicularly to the cervical region of the vestibular surface of the tooth, and the irradiation was performed for 3 minutes. The procedure was performed once; the dosimetry was 6 J/cm^2.

After all the above manipulations, 4 samples were randomly taken from all groups and left in a Petri dish with 6% citric acid (pH, 2.2) for 1 minute to test the resistance of the proposed agents to acid and determine their acid-fast characteristics. The samples were then washed in distilled water for 60 seconds, dried, and prepared for microscopic examination.

Four tooth samples from each of the four groups not involved in an earlier study were placed in previously collected oral fluid for a week, brushed twice daily with a soft toothbrush, simulating brushing in the mouth. The patient's oral fluid was collected after extraction of the teeth into individual test-tubes, which were stored in a refrigerator in 2-8°C. The tooth samples were then washed in distilled water for 60 seconds, dried, and prepared for microscopic examination.

For further microscopic examination, each of the groups was divided into 2 subgroups. The first subgroup of each study group included samples of teeth treated with citric acid. Letter 'A' was added to the group number to indicate a subgroup. The second subgroup of each study group included tooth samples that were immersed in oral fluid for a week and brushed twice daily with a soft toothbrush. Letter 'B' was added to the group number to indicate this subgroup.

Since the samples did not have sufficient electrical conductivity, as required by the research method, a conductive layer was formed on their surfaces. To create a conductive layer on the samples using thermal evaporation method in high vacuum, a layer of chromium with a thickness of 15 nm was applied. Morphological structure of the sample surface was studied with scanning elec-

tron microscopy in the mode of secondary electrons, using a Jeol JSM-840 microscope. The selected sample was mounted on a holder and placed in the working chamber of a scanning electron microscope (Figure 1).

Microrelief of the dentine was studied in the mode of secondary raster emission at a voltage of 10-30 kV and an increase of 15-15,000 times. Volume of the image was provided by large depth of focus of the electron microscope as well as the effect of shading the contrast relief in secondary electrons.

After microscopic examination, the obtained microphotographs were evaluated. Much attention was paid to condition of dentinal tubules, particularly visual assessment of their blockage by the active substance. The total number of dentinal tubules on the area of 2.5×10^{-9} m^2 as well as the number of open and closed tubules after the impact on tooth samples were calculated using method of direct counting.

Statistical processing of the obtained results was performed with Microsoft Excel and IBM SPSS Statistics trial (USA). Statistical analysis was performed using descriptive statistics. Arithmetic mean, standard deviation, minimum and maximum values of the indicator in the group were calculated and analyzed, and distribution of the studied features on the proximity to normal distribution (Gaussian distribution) was evaluated using single Kolmogorov-Smirnov normality test and Shapiro-Wilk test.

RESULTS

After studying the tooth samples of all studied groups using SEM method, the structure of dentine was evaluated, including the presence and condition of such structural units as dentinal tubules and main substance. Microphotographs evaluated dentinal tubules, their numbers, and diameters if they were traced in the images.

When studying microphotographs of these parts of tooth samples of all four groups that remained untreated, i.e. without signs of drug interventions obtained at a magnification of 500 times, pores with a diameter of ≈ (2 – 5) x 10^{-6} m were visualized on the dentine surface. They were probably cross-sections in the horizontal direction of the dentinal tubules of tooth samples. Dentine tubules on the transverse section had a rounded or oval shape (Figure 2). The microscopic image (Figure 3) at a magnification of 15,000 times showed a homogeneous structure of dentine and the entrance to the dentinal tubule of oval shape. The opening of the dentinal tubule was free.

Microscopic images of untreated tooth surfaces of other groups were similar. The number of dentinal tubal holes on the untreated parts of samples in each of the four groups on the surface area of 2.5×10^{-9} m^2 was calculated using direct counting method (Table 1).

Microscopic images of the surfaces of the samples of group 1A showed that the varnish layer was formed,

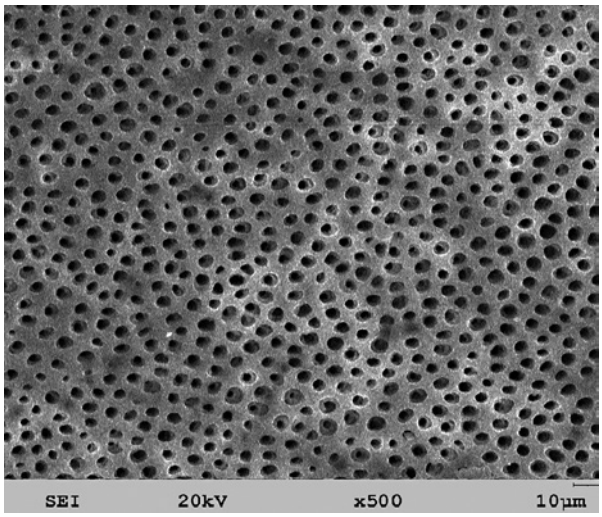

FIGURE 2. Dentine structure of untreated part of group 1A sample (magnification ×500)

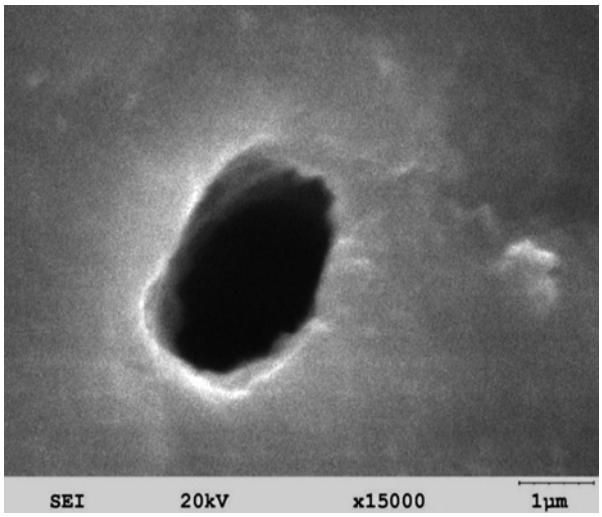

FIGURE 3. Dentine structure of untreated part of group 1B sample (magnification ×15,000)

TABLE 1. Average number of dentinal canal holes on untreated surfaces of tooth samples in four groups on an area of 2.5×10^{-9} m^2

	Group			
	1	2	3	4
Average number	147.24 ± 13.26	141.11 ± 11.47	142.91 ± 11.78	145.64 ± 13.47

preserved, and inhomogeneous, represented by rounded and flat areas, was bulky and loose (Figure 4).

Microscopic images of the samples of teeth of the group 1B revealed that the resulting layer was heterogeneous, and represented by rounded and flat areas (Figure 5).

On some samples of the group 1B, the film did not completely close the entrance to dentinal tubules, and single-free entrances to the dentinal tubules were observed (Figure 5). Large crystals of polygonal shape, single or united in conglomerates, were visualized on the surface of plots. These crystals covered the dentine surface unevenly, and in some areas, these layers were completely absent.

Microscopic images of the group 2A showed a formed film with a smooth surface. The structure of EEP provided a special pattern on the surface of the film, i.e., stripes in the form of polygons with varying degrees of congestion (Figure 6).

Microscopic images of the samples of group 2B showed that there were holes on the dentine's surface, some of them opened, but most of them closed (Figure 7). The number of opened and closed holes of dentinal tubules on the area of 2.5×10^{-9} m^2 was calculated on microphotographs. As a percentage, the average of closed dentinal tubules was 54.25 ± 1.71%, and the average of opened dentinal tubules was 45.75 ± 1.71% (Figure 7).

Microscopic images of samples of the group 3A showed pores on the dentine surface, which were probably entrances to dentinal tubules. In the near-surface layer, the size of diameter of dentinal tubules was $(4.0\text{-}5.0) \times 10^{-6}$ m, on depth of a dentinal tubule $\approx 5 \times 10^{-7}$ m, which sharply decreased, showing $(1.5\text{-}3.0) \times 10^{-6}$ m (Figure 8).

Dentine tubules have a double structure, which was clearly visible in the photomicrographs (Figure 8).

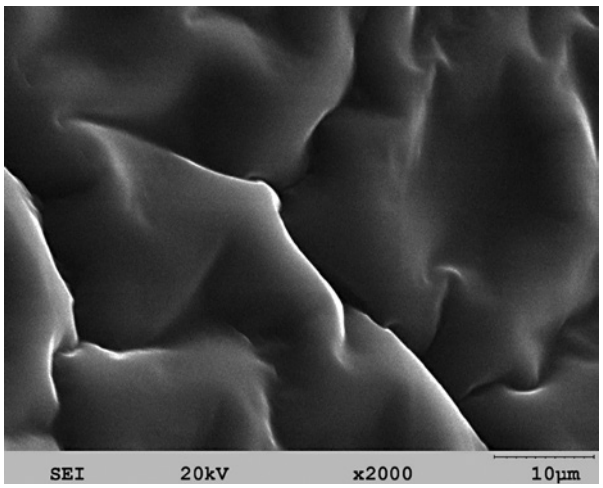

FIGURE 4. Dentine structure of group 1A sample (magnification ×2,000)

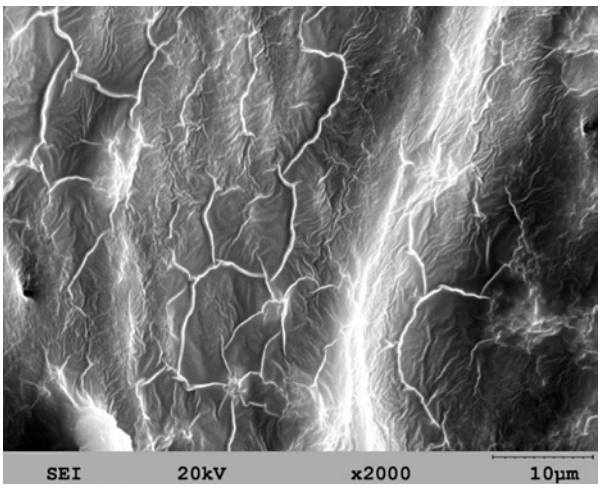

FIGURE 5. Dentine structure of group 1B sample (magnification ×2,000)

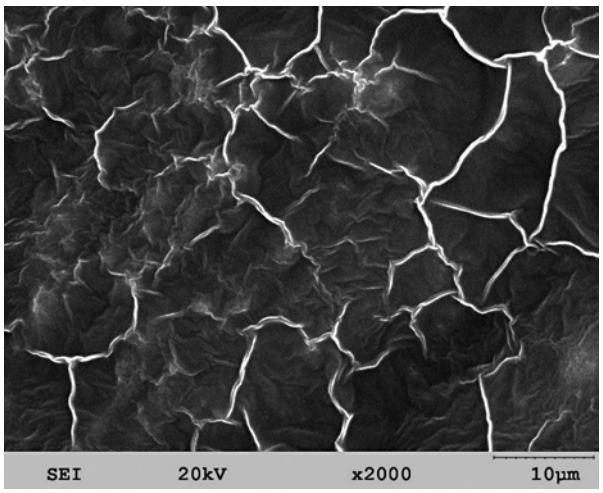

FIGURE 6. Structure of dentine of treated part of group 2A sample (magnification × 2,000)

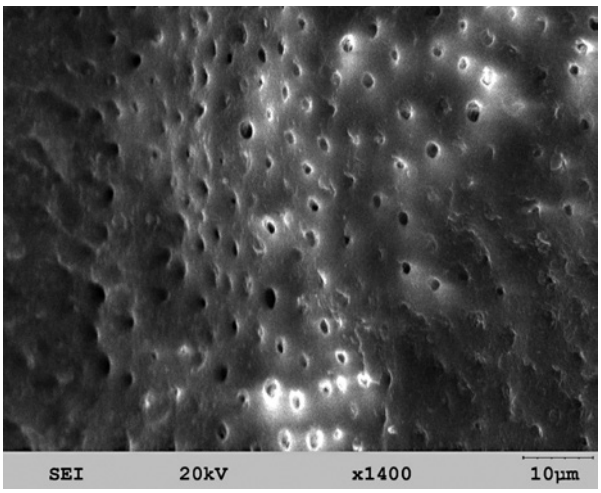

FIGURE 7. Structure of dentine of treated part of group 2B sample (magnification × 1,400)

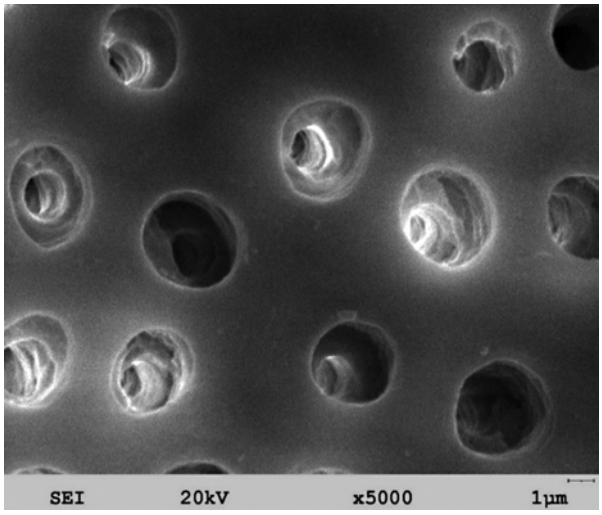

FIGURE 8. Structure of dentine of treated part of group 3A sample (magnification ×5,000)

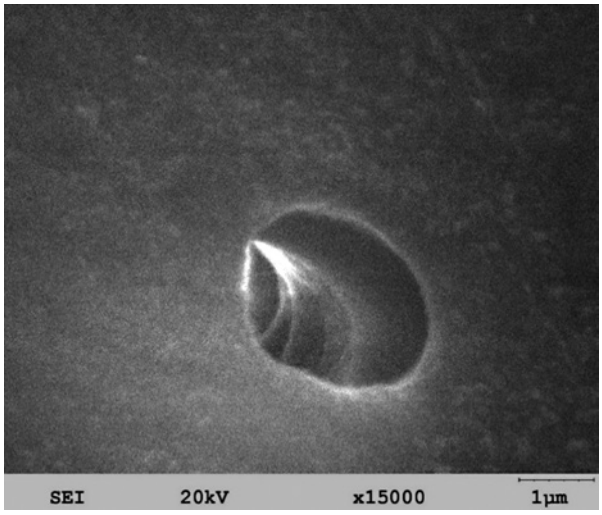

FIGURE 9. Structure of dentine of treated part of group 3A sample (magnification ×15,000)

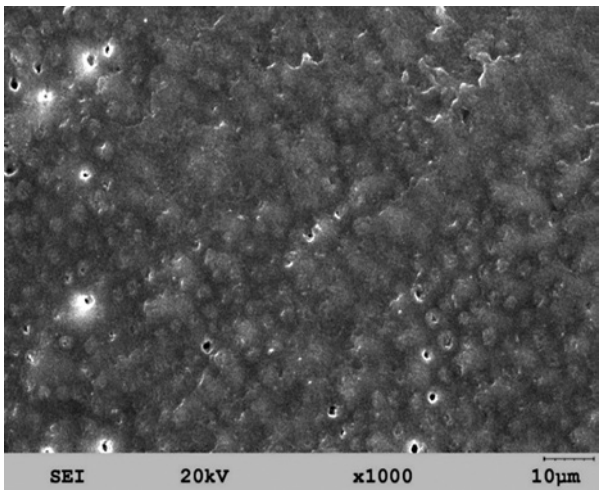

FIGURE 10. Structure of dentine of treated part of group 4A sample (magnification ×1,000)

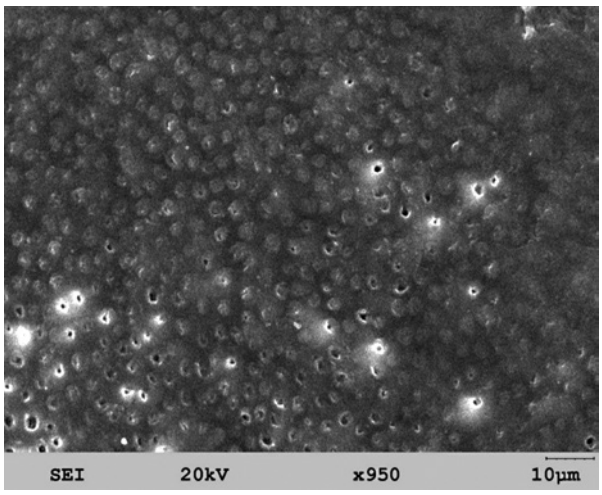

FIGURE 11. Structure of dentine of treated part of group 4B sample (magnification ×950)

The microscopic image at a magnification of 15,000 times (Figure 9) revealed the entrance to the dentinal tubule oval with double walls. The dentine surface of tooth samples of the group 3B was similar to the surface of tooth samples of the group 3A.

Microscopic images of the samples of the group 4A showed openings, i.e., entrances to dentinal tubules, which were tightly closed. In the field of a view, there were single openings of dentinal tubules that remained open (Figure 10).

Microscopic images of the tooth samples of the group 4B showed holes, which were also tightly closed. There were more open holes in the field of view compared with the samples that were in citric acid (Figure 11).

The number of closed and opened entrances to the dentinal tubules in both the subgroups of the group 4 was calculated with microphotography of the area of 2.5×10^{-9} m^2 using direct counting method.

TABLE 2. Average number of opened and closed entrances to dentinal tubules in subgroups of 4 groups of samples on an area of 2.5×10^{-9} m^2

Entrances to the dentinal tubules	Subgroup	
	A	B
Opened	6.45 ± 1.11	15.18 ± 4.09
Closed	93.55 ± 1.11	84.82 ± 4.09

On average, closed dentinal tubules were 93.55 ± 1.11% after treatment of tooth samples with 6% citric acid, and 84.82 ± 4.09% after storage of samples in oral fluid for a week and daily cleaning with a soft toothbrush (Table 2).

DISCUSSION

Photomicrographs of parts of dentine samples of hypersensitive teeth that were not treated by any method showed the presence of pores with a diameter $(2.0\text{-}5.0) \times 10^{-6}$ m, which were the entrances to the dentinal tubules. This coincides with data of a study, which conducted microscopic examinations of teeth with dentine hypersensitivity [17]. The method of direct calculation was applied to calculate the average number of open dentinal tubules in the dentine of hypersensitive teeth, which was 144.22 ± 12.68 in the area of 2.5×10^{-9} m^2.

After treating the tooth with fluoride varnish and 6% citric acid, it was seen that the layer formed was mostly preserved, but heterogeneous in structure, sometimes represented by rounded and flat areas, was bulky and loose. The obtained data indicate the resistance of the surface treated with fluoride varnish to the influence of acid, which agrees with the results obtained by other scientists [18]. After applying fluoride varnish and brushing tooth samples with a soft brush for a week, a thin layer of varnish and single-open holes were found on the tooth surface.

In the second group, the dentine surface after the use of EEP and subsequent treatment with 6% citric acid was covered with a preserved film, with stripes in the form of polygons of varying degrees of congestion. This allows us to conclude that EEP on the surface of tooth sample is resistant to acid. We can assume that EEP has such properties due to the content of various components, primarily wax. Similar results were obtained by Arabnejad *et al.* [18], where the researchers evaluated the microstructure of dental dentine after a separate action on tooth samples of saline, 30% propolis extract, fluoride varnish, and combined use of the above factors with citric acid. According to the results, the authors observed the property of propolis and fluoride varnish to close the entrances to the dentinal tubules, and the acid resistance of tooth samples treated with propolis extract and fluoride varnish [18].

Chen *et al.* also noted a greater resistance of propolis to citric acid in their study, which evaluated and compared the microstructure of dentine after application of novamine, arginine-calcium carbonate, and propolis extract in combination with citric acid [19].

Microscopic specimens showed that some of the samples treated with EEP were kept in the oral fluid for a week and cleaned with a soft brush. The number of opened and closed holes of dentinal tubules in the area of 2.5×10^{-9} m^2 was calculated on microphotographs. The percentage of closed dentinal tubules was 54.25 ± 1.71%, and the percentage of opened dentinal tubules was 45.75 ± 1.71%. According to an *in-vitro* study, Kripal *et al.* claimed that up to 61.75% of dentinal tubules were occluded after application of 15% propolis extract [20].

Microphotographs of dental samples after application of LLL irradiation and their treatment with 6% citric acid revealed entrances to the dentinal tubules with a double structure. The diameter of dentinal tubules was $(4\text{-}5) \times 10^{-6}$ m, at a tubules' depth of $\approx 5 \times 10^{-7}$ m that sharply decreased and amounted to $(1.5\text{-}3.0) \times 10^{-6}$ m. The dentine surface of tooth samples kept in oral fluid and cleaned with a soft toothbrush for a week was similar to the surface of the samples that were treated with citric acid.

Scientists explain the double structure of dentinal tubules after the use of LLL irradiation by the fact that the surface layer of dentine melts first because it absorbs low energy. With increasing energy, the surface layer of dentine melts and collapses again, so a two-layer structure is formed in the dentinal tubules [21].

Micrographs of dental samples after joint use of EEP and LLL irradiation as well as exposure to 6% citric acid, revealed closed entrances to the dentinal tubules. In the field of view, single openings of dentinal tubules remain open. Microscopic images of parts of the tooth samples treated with EEP in combination with LLL irradiation, kept in the oral fluid for a week and cleaned with a soft toothbrush showed holes that were also tightly closed. On average, after treatment of tooth samples with 6% citric acid, 93.55 ± 1.11% of dentinal tubules were closed, and after storage of samples in oral fluid for a week and daily cleaning with a soft toothbrush, they remained closed only in 84.82 ± 4.09% of dentinal tubules.

Scientists compared the tincture of propolis on the influence of adhesives showing that it can form bonds with the surface dentine [14, 19]. At the time when LLL irradiation melts the surface layer of the tubules, EEP obstructs their entrances [21]. In the available literature, we did not find information on the joint use of EEP and LLL irradiation and its' effect on dentine microstructure.

CONCLUSIONS

According to the results of the present study, all methods demonstrated dentinal tubule occlusion. However, it was established that joint usage of EEP and LLL irradiation allows the entrances of the dentinal tubules completely closed, and only isolated opened entrances to the dentinal tubules were identified. This fact makes DH treatment more effective in comparison with traditional methods. Our results of the research are beneficial for the further application of this treatment method of DH in practical dentistry.

CONFLICT OF INTEREST

The authors declare no potential conflict of interests with respect to the authorship and/or publication of this article.

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