Original Research

THE QUANTITATIVE AND RADIOGRAPHIC ASSESSMENT OF RATE OF DECALCIFICATION OF TEETH BETWEEN ELECTROPHORETIC AND ROUTINE METHOD, USING WEAK AND STRONG ACIDS: A COMPARATIVE STUDY

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ABSTRACT

Teeth are highly calcified structure of body. The process of decalcification is routinely carried out to study the structure of bone, teeth and other soft tissues of teeth like pulp. It is also done to evaluate the biological response of dental pulp against restorative materials and microbial activity. Failure to decalcify the tissue with large amounts of calcium salts will result in bad sectioning and damage to the microtome knife. Because of the technique-sensitive procedure, decalcification of hard tissue is carried out in different ways depending on urgency of final diagnosis. Routine decalcification of dental tissue may take weeks to month depending upon decalcifying solution and procedure used and is represented by: $Ca_{10}(PO_4)_6(OH)_2 + 20H^+ \leftrightarrows 10Ca^{2+} + 6H_3PO_4 + 2H_2O$. Various methods have been developed to accelerate the rate of decalcification like agitation, raise of temperature, increasing surface area and even use of electric current. In electrophoretic decalcification electric current attracts the Ca^{++} ions toward cathode and increases rate of decalcification. In the present study twenty formalin fixed teeth were used to evaluate the rate of decalcification by electrophoretic decalcification in different acids and their effect on staining of soft and hard tissue of teeth. Progress and end point of decalcification was confirmed by quantitative and radiographic methods respectively. Arithmetic mean of time taken for both decalcifications methods were calculated and then compared statistically. Then tissue were kept for routine tissue processing and stained with hematoxylin and eosin staining protocol. In the electrophoretic decalcification method teeth decalcified three times faster as compared to routine decalcification in both

acids keeping temperature of solutions within biological limits. Moreover inorganic acid decalcify teeth faster than organic acids used in this study.

Key Words: 7.5% Formol Nitric Acid, 10% Gooding and Stewart's Fluid, electrophoretic decalcification, graphite electrodes.

INTRODUCTION:

Biopsy mean bios (life) and opsis (vision): vision of life, is an obtainment of tissue from a living organism for the diagnosis of various periapical and malignant lesions under the microscope (Berwal *et al.*, 2014; Mota-Ramírez *et al.*, 2007). With advancement of science and technology in detection of disease and sophistication in diagnosis, biopsy is being used with increasing frequency as a diagnostic tool even in dental profession (Nitul, 2011). As head and neck contain both soft and hard tissues and their lesions need technique- sensitive methodology for interpretation and diagnosis (Sanjai *et al.,* 2012).

The morphology of oral tissue and identification of pathologic conditions are important for histologic details. Before the study histo-pathologic details under microscope, tissue must be processed through routine tissue processing which includes fixation, dehydration, clearing, impregnation, embedding, sectioning and staining (Charnia *et al.*, 2010).

Fixation of most of soft and hard tissue including bone and tooth are done with 10 % neutral buffered formalin (a 4% solution of formaldehyde) to stop autolysis prior to sample reaching the pathology laboratory (Oliver *et al.*, 2004; Cullings,1974; Rosebush *et al.,*2010). But the presence of calcium salts in tissue prevents the preparation of good sections by routine tissue processing methods (Cullings, 1974).

Pathological and developmental processes of dental tissues, bone and calcified tissues due to dystrophic and metastatic processes can only be studied after the process of demineralization or decalcification (Prasad and Donoghue, 2013; Rolls, 2010). To obtain a good quality paraffin section which can preserve all the essential microscopic elements from calcified tissue, decalcification (removal of minerals) is carried out (Rolls, 2010; Cullings, 1974; Prasad and Donoghue, 2013).

Decalcification is commonly employed in most histopathology laboratories for the microscopical examination of bone and other calcified tissues (Sterchi, 2013; Mawhinney *et al.*,1984; Cullings, 1974; Sangeetha *et al.*, 2013). The incomplete removal of these

salts results in torn and artifactual sections, in-effect damaging the microtome knifeôs cutting edge (Cullings, 1974). Various acids that form soluble calcium salts and chelating agents are used in the process of decalcification (Prasad and Donoghue, 2013; Sterchi, 2013). Strong acids though give faster results with poor quality of sections and chelating agents provide the best quality sections with slow decalcification rate (Prasad and Donoghue, 2013).

An ideal decalcifying agent aids in completely removal of calcium, with minimum damage to tissue cells, no impairment to staining and has reasonable speed (Cullings, 1974). Several factors influence the rate of decalcification, like concentration, volume and temperature of solution, type of tissue e.g. bone, dentine, enamel and cementum, size of specimen i.e. surface area of tissue, agitation and electric current in electrophoretic decalcification (Cullings, 1974, Sterchi, 2013, Singh , 2003). In electrophoretic method it is supposed that under the influence of an electric field, the liberated Ca^{++} ions by the decalcifying solution will be removed more rapidly and this will increase rate of decalcification (Verdenius and Alma, 1958).

The present study was conducted to assess the rate of decalcification of the pre-molar teeth in 7.5 % formol Nitric Acid and Gooding and Stewart's fluid by electrophoretic method and compared with routine decalcification. Effect of acids and electric current were also assessed on soft and hard tissue microscopically.

The aim of this study was to evaluate the time taken and to study effect of acids on the soft and hard tissue of teeth in the electrophoretic decalcification process.

MATERIALS AND METHODS

Twenty freshly extracted premolar teeth, which had been extracted for orthodontic purpose, were taken. These teeth were then immediately fixed in 10% neutral buffered formalin (Cullings, 1974, Sterchi, 2013) for 24-48 hours. To facilitate easy fixing of pulp (soft tissue) of teeth the apical third was removed with the help of air rotor. These teeth were grouped in four groups of five each, as in Table 1

The teeth were then processed for decalcification in Gooding and Stewartôs and 7.5%Nitric Acid +10% Formalin in different glass jar for routine decalcification at room temperature of 28^0C. For electrophoretic decalcification, teeth were then decalcified in two different customized plastic containers for different solutions, which were assembled with graphite electrodes for anode and cathode. In electrophoretic decalcification the direct

electric current was supplied by regulated DC power supply unit calibrated from 0.1 volt to 15.0 volts and 0.01 ampere to 2.00 ampere. (BAKU Model No. BK-1502 DD). Direct current of 5.0 Volts and 450mA was suitable for 7.5% Nitric Acid +10% Formalin (formol nitric acid) solution and direct current of 12.0 Volts and 250mA was suitable for Gooding and Stewartôs solution were fixed in our study. As these values of voltage and current for solutions raised the temperature of solutions by maximum of 5^0C i.e. 33^0C from 28^0C that was within biological limit.

End point of decalcification

The progresses of both the procedures were tested using quantitative mass loss of each tooth and then radiographic method after every six hours to confirm the end point of decalcification. Electronic weighing machine and RVG (Dr. SUNI) and X-RAY (Gnatus IOPA) were used.

Temperature of solution

The temperature of laboratory was 28^0C and solutions were 28^0C for routine decalcification solutions and 31^0C (mean) for electrophoretic were noted with the help of digital thermometer of 0.1^0C calibration.

Post decalcification

The tissue is washed in running tap water (which had alkaline pH) for 3-4 hours

Processing of decalcified teeth

Then all the decalcified tissues were subjected to normal tissue processing. The decalcified teeth were then sectioned to a thickness of 4□m using microtome. (Model number Yorko YSI060).

Staining of tissue

The decalcified sections were stained by Hematoxylin and Eosin (H & E) and observed under light binocular microscope (Nikon Eclipse E100).

The efficacy of electrophoretic methods used in the present study was evaluated on the basis of time taken for decalcification of teeth (based on radiographic estimation of endpoint) and effect of staining on soft and hard tissue.

Table 1. Distribution of sample for study, Potential difference & Current

Method → / **Decalcifying Solution↓**	**Routine decalcification**	**Electrophoretic decalcification**	**Potential difference**	**Ampere**
10% Gooding and Stewart's solution	5 Teeth	5 Teeth	12.0V	0.25 A
7.5% Formol Nitric Acid solution	5 Teeth	5 Teeth	5.0V	0.45A

RESULTS AND OBSERVATIONS

1. Time taken for Decalcification

Time taken for decalcification of premolars in routine decalcification method using 7.5% Nitric Acid + 10 % Formalin was 66 hours to 80 hours with mean of 76 hours at temperature 28^0C. Decalcification of premolars completed in 120 hours to 150 hours for Gooding and Stewartôs solution with average of 138 Hours. While time taken for decalcification of premolar by electrophoretic decalcification was 24 hours to 32 hours with mean of 27 hours for7.5% Nitric Acid + 10 % Formalin and Decalcification of premolars completed in 72 hours to 90 hours for Gooding and Stewartôs solution with average of 84.5 hours when maximum temperature of solution was 33^0C. (Initial temperature of solution was 28^0C) The end point of decalcification of each tooth was confirmed by radiographic method as it is most accurate method than chemical and manual methods.

2. Physical changes during decalcification

2.1 Color changes

Color change of teeth present in routine decalcification in 7.5% Nitric Acid + 10 % Formalin were yellowish which were more intense in routine method than electrophoretic decalcification in same solution. Bleaching of dentine were observed in Gooding and Stewartôs solution, which were more in routine decalcification than electrophoretic decalcification.

2.2 Change in mass

Initial mass loss was faster in electrophoretic methods using 7.5% Nitric Acid + 10 % Formalin followed by routine decalcification in 7.5% Nitric Acid + 10 % Formalin, electrophoretic Gooding and Stewartôs solution and least in routine decalcification using Gooding and Stewartôs solution (as shown in figure. 1).

2.3 Pattern of decalcification by radiographic examination

Radiograph was taken when there was no reduction in mass of teeth and it was observed that most of decalcification had been completed in roots of all teeth except in furcating area, enamel of all the teeth had been completely removed but decalcification in dentine of crown was partial, which actually decided the time taken for complete decalcification. The pattern of decalcification (as in figure 2) was same all the sample except the rate of decalcification (as in figure 1).

3. Sectioning/(Microtomy)

Though end point of decalcification were confirmed radiographically yet sectioning of tissues was easier in teeth decalcified in 7.5% Nitric Acid + 10 % Formalin than in Gooding and Stewartôs solution which was significant practically.

4. Staining and Histologic Changes

The H&E stained sections were then observed for hard and soft tissues staining at 10X and 40X using Nikon Eclipse 100 microscope. The H&E staining of sections did not show any significant difference at cellular level for soft and hard tissue in all sections but teeth decalcified in formol nitric acid using electrophoretic method showed minimal tissue damage.

The biominerals of bones and teeth in vertebrates is apatite and is of special interest to human because in calcified tissue there is substitution of ions in calcium apatite of biological origin (Puura and Nemliher, 1999; Dorozhkin, 2012). Chemically, apatites are carbonate hydroxy apatite $[Ca_{10-x}(PO_4)_{6-n}(CO_3)_nOH_y]$ to carbonate-fluoro-apatite $[Ca_{10x}(PO_4)_{6-n}(CO_3)_nF_y]$. X-ray diffraction analysis in real apatite show substitution of hydroxide ions by fluoride, and phosphates by carbonates creates a wide range of carbonate-apatite (Puura and Nemliher, 1999).

Figure 1 : % of mass loss during decalcification; GSR – Gooding and Stewart's routine ; GSE – Gooding and Stewart's Electrophoretic; NAR - Nitric Acid and Formalin routine ; NAE – Nitric Acid and Formalin Electrophoretic

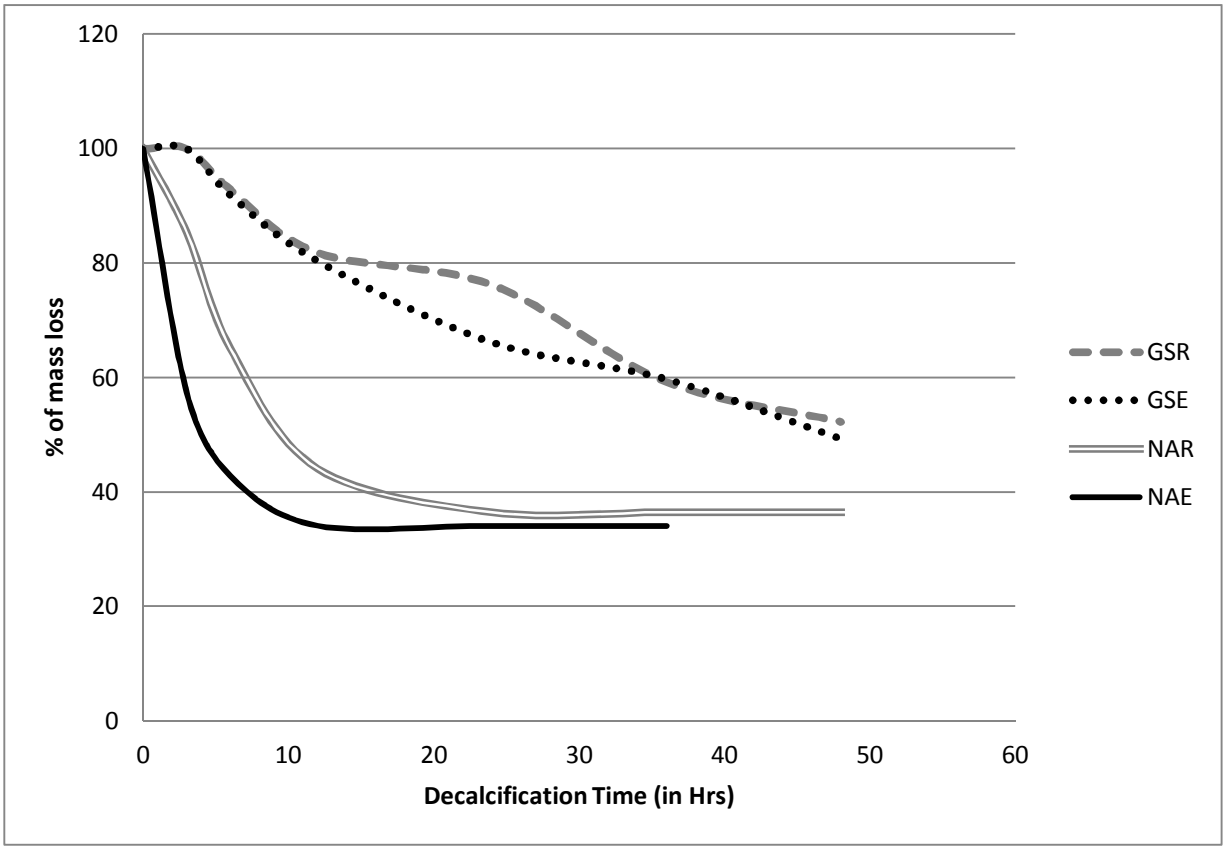
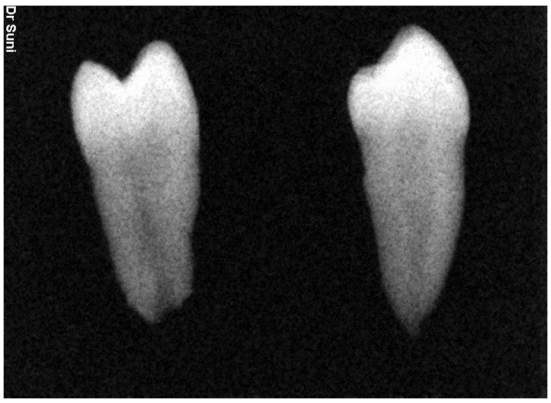
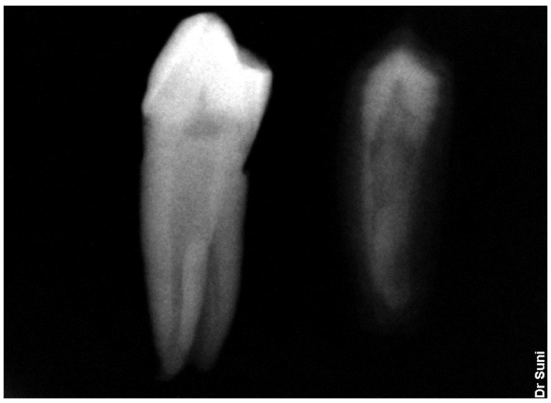


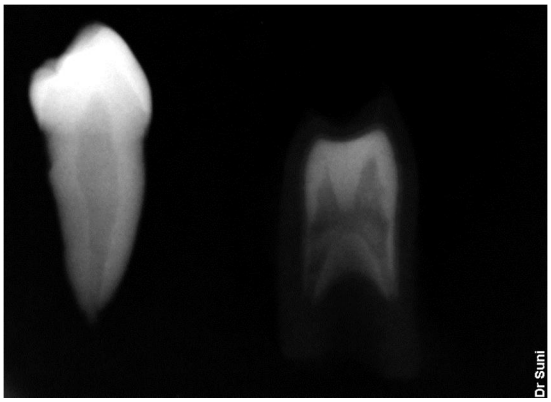
Figure 2 RVG showing pattern of decalcification in crown and root of teeth in Electrophoretic method using 7.5 % HNO_3 + 10% formalin solution.



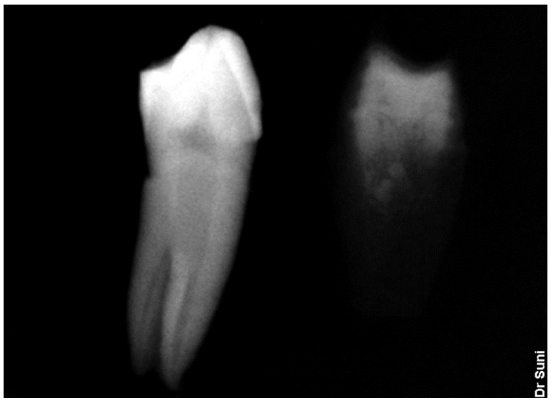
Before Decalcification



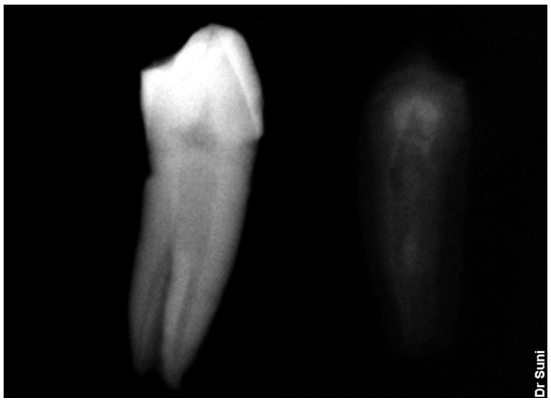
12 Hours of Decalcification



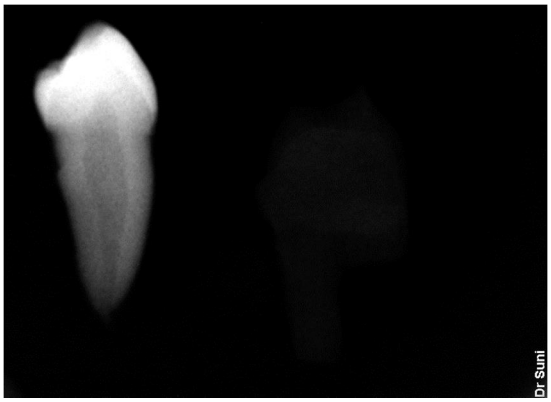
18 Hours of Decalcification



24 Hours of Decalcification



27 Hours of Decalcification



30 Hours of Decalcification

DISCUSSION

Thus dissolution of calcium appatite crystal in an acid (weak and strong) is termed decalcification which leads to removal of calcium and other minerals from calcium appatite of biological origin. Sometimes use of different technique may be required with the available chemicals to attain a reasonable speed of decalcification like agitation, temperature and electric current. In the present study we used electric current to increase the rate of decalcification. But rate of decalcification in a tooth vary because of:

1. Composition of tooth

Teeth are composed of three mineralized tissue namely enamel, dentine, cementum and one non mineralized tissue in central core namely pulp which comprises of fibers, cells, blood vessels, nerve terminations and ground substance. (Gupta *et al.*, 2014, Kumar (2011), Antonio (2014)) Inorganic components of enamel, dentine and cementum they vary in percentage of mineralization and thickness. Enamel which has 96% inorganic component and 4% organic content having maximum thickness of 2.5-3.0 mm on occlusal surface limited only to crown portion of teeth. Cementum being avascular having approximately 45% to 50% hydroxyapatite by weight, having highest fluoride content of all mineralized tissue and the remaining portion is collagen and non collagenous matrix protein and covers the root of teeth. The third component of tooth which forms the bulk of tooth is dentine which range in thickness from 3.0 mm to 10.0 mm or more depending on surface to surface, crown and root portions and amount or reactionary dentine formed. Mature dentin is made up of approximately 70% inorganic material, 20% organic material and 10% of water (Kumar (2011), Antonio (2014), Berkovitz *et.al.*(2011)). Thus during decalcification different tissue shows different decalcification rates and patterns (as shown in radiographic pattern in the present study) as they varies in mineralization and composition with in a tooth.

2. Reactivity of acids

Nitric acid being strong acid having ionization constant (27.79) decalcified the tooth more rapidly than Formic acid ($HCO_2H + H_2O \rightleftarrows H_3O^+ + HCO_2^-$) having ionization constant (1.8×10^{-4}) which is much less than that of nitric acid. Yellow discoloration of teeth were observed in nitric acid because of calcium nitrate formed as : $CaSO_4 + 2HNO_3 \rightleftarrows Ca(NO_3)_2 + H_2SO_4$ (Jadhav *et al.*,2009) but the yellow discoloration does not affect histological examination as explained by Moore (1994).

3. Effects of electric current

Electric current forces migration of calcium ions towards cathode and allows further calcified tissue to react with decalcifying solutions. Thus electric current enhances the rate of decalcification within limit. Gelfand *et.al.,* (1952) regarded that the acceleration of decalcification was due to ñforced migrationò of calcium ions. Study carried out by Ducey and Shippy (1950), Dolan (1951) and Verdenius and Alma (1958) using alternating current had same results. Dorozhkin (2012) theoretically explained that rate of decalcification in electric field was initially due to increase rate of reaction and in later stages it was due to the rate of diffusion.

4. Effects of temperature

Due to influence of electric current within an electrolyte there were collisions of opposite directing ions within solution and on electrode that cause raise of temperature within solutions , which depends upon voltage and ampere allowed to pass through the solutions (Gelfand *et.al.,*1952) Various studies had proved that raised temperature of decalcifying solution also increase rate of decalcification. But in this study we controlled temperature of solution within biological limit by adjusting current flow through the electrodes that is why we were able to prevent tissue damage as it was observed in previous study by Ducey and Shippy (1950), Dolan (1951) and Verdenius and Alma (1958) where temperature of solution raised up to 40-50^{o}C. The rate of decalcification as observed in the present study by electrolytic method was three times faster as compared to routine decalcification and it was in accordance with study carried by Verdenius and Alma (1958) with piece of bone.

More rises in temperature, which was previously observed (up to 40^{0}C-50^{0}C) in study done by Gelfand *et al..* (1952) in electrolytic decalcification of bone, was not observed in this study because the voltage and ampere were controlled using controlled power supply, though the rise in temperature of electrolyte by 5°C also support heat production while electric current passes through electrolytes as previously explained in literatures and also by Gelfand *et al.* (1952) and Verdenius and Alma (1958).

There were no significant difference in microtomy of decalcified teeth of both methods, but microtomy of electrophoretic decalcified teeth were easier in deeper section which can be explained as the electric current allow forced migration of ions from deeper core of tissue to solution as already supported by Gelfand *et.al.*(1952).

The staining of sections was better in case of electrolytic decalcified teeth than routine decalcified teeth because acid

exposure of teeth was for less duration in electrophoretic decalcification.

CONCLUSIONS

The present study concluded that it was urgency of report or diagnosis which mainly decides the choice of decalcifying agents and method. We observed that electrophoretic decalcification was threefold faster method of decalcification in 7.5% HNO_3+10% formalin and Gooding and Stewartôs solution than routine method in the same solution. The accelerated rate of decalcification in electric field was due to easy migration of ions from tissue to solution than reactivity of acid. These results concluded that electric current only allowed removal of calcium ions from solutions and from tooth structure (dentinal tubules in dentine), moreover in deeper layer of calcified tissue it easily facilitates removal of calcium ions and prevents remineralization. Temperature of decalcifying solution can be maintained with in biological limit by controlling current and potential difference between electrodes. Further studies will be needed on other weak decalcifying solutions and chelating agent of decalcification to evaluate the effect of electric current on decalcification rate.

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Approval was taken, for the study, from the ethical committee of PDM Dental College and Research Institute, Bahadurgarh (Haryana). A written informed consent was taken from each patient.

Conflict of interest: None

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