A COMPARATIVE STUDY ON ROOT SURFACE DEMINERALIZATION USING CITRIC ACID AND TETRACYCLINE IN VITRO: A SCANNING ELECTRON MICROSCOPY STUDY

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Abstract- ***Citric acid and tetracycline HCl are the most common clinical root demineralization agents used in periodontal practice. In this comparative in vitro study the effects of Nd:YAG laser radiation on root surface was compared to that of citric acid and tetracycline HCl using SEM. A total of twenty one freshly extracted periodontally diseased single rooted premolar teeth were root planned in vitro. The proximal surface of each root was sectioned and divided to two equal surfaces of experimental and control. In group "A" Nd: YAG laser at power of 2 watts was used for 2 minutes at 0 pps. In group "B" citric acid PH= 1 was applied for 3 minutes & in group "C" 5% tetracycline HCl solution pH=3.3 was applied for 5 minutes.***

SEM results showed removal of smear layer, changes in surface characteristics and opening of dentinal tubules in all 3 groups when compared to controls. In Nd: YAG laser group signs of surface cracking, pits and craters were observed and removal of smear layer, was not complete at 2 watts power and tubular opening was minimal. In group "B" smear layer was completely removed, micro fractures were seen and large opening of tubules were observed. In group "C", more numbers of tubules were opened but openings were not as large as in group "B", smear layer was completely removed and experimental surfaces were smooth and no micro fractures were seen.

This study showed that Nd: YAG laser could be used for the purpose of root demineralization but it needs further investigations with modification in factors influencing laser effectiveness to be used as a common method of root surface demineralization .

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Key Words: ***Smear layer, dentinal tubules, citric acid, laser, tetracycline HCl, demineralization***

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INTRODUCTION

The periodontal diseases are a group of infectious diseases associated with a specific microorganism or a group of microorganism present in plaque and characterized by a progressive destruction of connective tissue fibers and alveolar bone and an apical migration of epithelial attachment (1). Studies have indicated that root surfaces of teeth which have been exposed to bacterial plaque are poor substrates for mammalian cells (2). For one possible explanation is that bacterial factors, such as endotoxins, accumulate and adsorb to the tooth surface where they remain even after mechanical debridement and thereby, prevent primary attachment of connective tissue cells (3). The traditional treatment of pathologically altered root surfaces has relied on mechanical removal of plaque and calculus, root-bound toxins, and contaminated cementum (4). Curettes and ultrasonic scalers have been the primary instruments used to accomplish these goals (5), but the ability to make the surfaces disease-free has been questioned (4). Instrumentation of the root surface, however, results in formation of a smear layer of organic and mineralized debris (1). The thickness of this smear layer usually ranges from 2-15 μm (6) and is thought to serve as a physical barrier between the periodontal tissues and the root surface and may inhibit deformation of new connective tissue attachment to the root surface (7). If the ultimate goal of periodontal therapy is complete restoration of the supporting structures lost during periodontal destruction through complete regeneration or new attachment, then the root surfaces must be devoid of any root/cementurn associated endotoxins, which are cytotoxic and the smear layer (7), which prevents regeneration or new attachment (7). For this reason topical chemotherapeutic agents have been used for both detoxification and enhancement of new attachment (8) and also attempt to overcome the most significant limiting factor to new attachment, namely the rapid rate of epithelial proliferation or down growth along the root surface (9). Demineralization (also referred to as 'decontamination' and 'detoxification' in literature) of the diseased root surfaces as an adjunct to mechanical root therapy

with various chemical agents has been performed as early as the latter half of the 19th century (10). Since then a large number of chemicals have been investigated to achieve this purpose. Among these chemicals citric acid (7) and tetracycline HCl (11), have shown to have additional effect of demineralizing the root surface, removing the smear layer and exposing collagen matrix of the mineralized radicular dentin. In contrast several animal studies (12), and clinical trials (13) utilizing citric acid or tetracycline, have failed to result in new attachment.

MATERIALS AND METHODS

The material for the present study consisted of twenty one freshly extracted periodontally diseased single rooted premolar teeth which were collected from Department of Oral Surgery. Following sectioning of teeth, they were divided into three groups. The test area was proximal surfaces. Each group comprised of seven specimens out of which one half portion served as control.

General criteria for selection of teeth for study

All the teeth selected were fulfilling the following criteria:

1. Presence of calculus and debris
2. Proximal attachment loss of greater than 5 min
3. Absence of dental caries
4. Absence of any kind of restoration
5. No history of oral prophylaxis within 6 months before the date of examination
6. Presence of mobility of grades II or III

Criteria for the boundaries of experimental surfaces

vertical boundaries the proximal surface of roots of the teeth were measured 6 mm below the line connecting the facial and lingual (palatal) cemento-enamel junctions and were marked to limit the boundaries of experimental portions in a vertical direction.

Horizontal boundaries

A vertical line representing the highest contour of the roots on both facial and lingual or palatal sides extending from the cemento-enamel junction to the apex were marked to form the boundaries of the experimental portion in a horizontal direction. 7th surface between these two lines measured and it was approximately 6 mm.

Mechanical debridement (instrumentation)

All marked exprimental portions of roots were scaled with a new Hu-friedy sickle scaler (No.15.30) and root planed with a new Hu-friedy Gracey curet (No.5-6) until an absolutely smooth surface resulted. The strokes were directed apico-coronary starting from left side to the right side and each stroke was extended from the line 6 mm below cemento-enamel junction terminating at cemento-enamel junction of the experimental surfaces. The areas were frequently flushed with water to avoid dryness of the instrumented surfices.

Sectioning procedure

The crown portion of each extracted tooth was sectioned following cemento-enamel junction as a guide line using a water cooled high speed diamond tapering fissure bur. Following the separation of crowns from the roots, a longitudinal sectioning was done at the line marked on facial and lingual or palatal surfaces of the roots extending apically from cemento-enamel junction up to the 6 mm marking and the two proximal halves were separated with a cross section at the termination of 6 mm vertical cut. The root blocks were measured individually at three dimensions as follow:

Length: 6 mm
Width :6mm
Depth : 2mm

Storage of the specimens

The specimens were washed and cleaned in an ultrasonic cleaner for 15 minutes (14) and then stored in sterile, clean labeled glass bottles containing distilled water separately until the further procedures.

Experimental design

The outer surface of each block was marked at midline to divide the width of the block into two 3 min halves, one half served a experimental portion and the other half as control portion

Criteria for grouping

All specimens were divided into three groups

1. Group A

This group consisted of seven experimental and seven control surfaces. Each experimental surface was 'LASED' with a pulsed Nd: YAG laser at the power of 2 watts, 10 pulses per second (pps) for 2 minutes (15). The control surface remained untreated.

2. Group B

This group consisted of seven experimental and seven control surfaces. Each experimental surface was rubbed with saturated citric acid solution (PH=1) for 3 minutes (16). The control surfaces remained untreated.

3. Group C

This group consisted of seven experimental and control surfaces. Each experimental surface was rubbed with 5% solution of tetracycline hydrochloride (PH= 3.3) for 5 minutes (2). The control surfaces remained untreated.

Laser treatment (group A)

A pulsed Nd: YAG laser was used in this study. The power of this laser ranged at 2 watts (15) and the distance from the tip of the beam to the surface of the specimen was 5 cm to perform a non-contact exposure. The angel of 'incidence' was kept at 90°. The adjustment was done in such a way as to direct the delivery of light to cover only the experimental portion. All experimental 'A' specimen surfaces were exposed by laser radiation for 2 minutes (15) at 10 pps (Approximately 100 milli-joules per pulse).

Table 1. Group A- Nd: YAG laser

Specimen No.	Control	Experiment
1	0	10
2	3	12
3	0	11
4	4	9
5	2	6
6	2	11
7	0	2

Citric acid conditioning (group B)

A saturated citric acid solution was freshly prepared prior to the experimentation by adding distilled water to the acid gradually until the PH reached 1 and the prepared solution was continuously stirred for 10 minutes at room temperature (16,17). The acid was then applied to each experimental 'B' specimen surface for 3 minutes (16,18,14), using cotton pellets which were changed several times within this period and then washed with distilled water.

Table 2. Group B- citric acid

Specimen No.	Control	Experiment
1	8	9
2	3	12
3	4	10
4	0	9
5	0	7
6	0	12
7	0	13

Tetracycline hydrochloride (HCl) conditioning (group C)

A tetracycline HCl solution was freshly prepared prior to the experimentation by adding the contents of a 500 mg capsule to 5 ml of distilled water to result in 5% solution and the solution was continuously stirred for 10 minutes at room temperature (17). The solution was then measured with pH meter and it showed a PH of 3.3. Subsequently the solution was applied on each experimental 'C' specimen surfaces for 5 minutes (18), using cotton pellets which were changed several times within this period and then was washed with distilled water.

Table 3. Group C-tetracycline HCl

Specimen No.	Control	Experiment
1	7	18
2	0	10
3	3	35
4	2	14
5	3	7
6	0	20
7	5	14

Preparation for scaning electron microscopy

After the experimental procedures, were completed all specimens from group A, B and C were fixed with 4% formaldehyde in 0.2 M phosphate buffer (pH= 7.2) at room temperature for 24 hours. Following fixation, the specimens were dehydrated using an ascending series of graded ethyl alcohol solutions with the following concentrations : 33%, 50%, 67%, 95% and 100% for 10 minutes at each concentration (14). The specimens were then air-dried for overnight (20). After the dehydration was completed the control portion of each specimen was separated from the experimental portion with the help of chisel blow at the junction of two portions to facilitate the cross sectional observation by scanning electron microscopy. The fractured experimental and the control specimens were then separately stored in a pair of labeled glass bottles. Prior to scanning electron Microscopy observations the fractured specimens were mounted on SEM stubs with silver paint and were kept for drying and then sputter coated with gold using sputter coater (14).

Scanning electon microscopy (SEM)

Twenty one experimental specimens and 21 control specimens were individually examined and photographed at magnification 2000 x and 5000 x with a scanning electron microscope (Model : JEOL-JSM-840A) to show the sectioned surfaces.

Table 4. Diameter of the largest tubule opened after experimentation (in μm)

Specimen No.	Group A	Group B	Group C
1	2.77	8.88	5.00
2	6.11	4.44	4.44
3	4.44	5.00	6.11
4	4.44	7.77	5.55
5	4.44	6.11	5.55
6	4.44	6.11	5.00
7	2.22	2.7	6.11

Table 5. Statistical results of tubular enlargement of experimental specimens

Group	Mean (μm)	Standard deviation
A	4.123	± 1.183
B	5.869	± 1.897
C	5.394	± 0.5725

RESULTS

The study consisted of 21 (twenty one) extracted periodontally involved single rooted diseased premolars collected 6 cm individual patients with an age group ranging from 25-55 years. The proximal root surfaces were scaled and root planed initially. Following the sectioning procedures the upper most portion of proximal root surface (6 mm in vertical length) was separated. The separated blocks of roots were divided into three groups each comprising of seven.

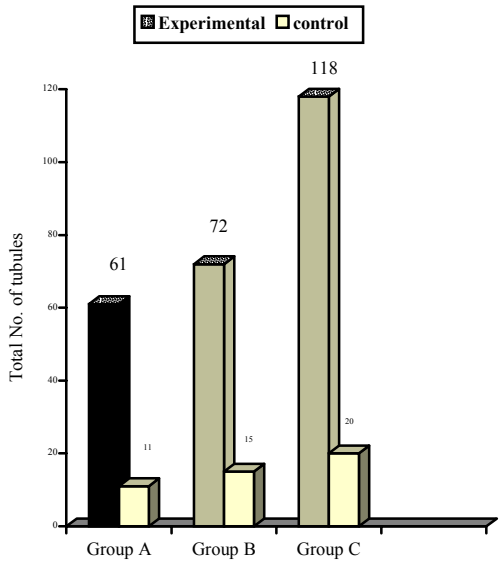


Fig. 1. Experimental surface covered by smear layer following in strumentation

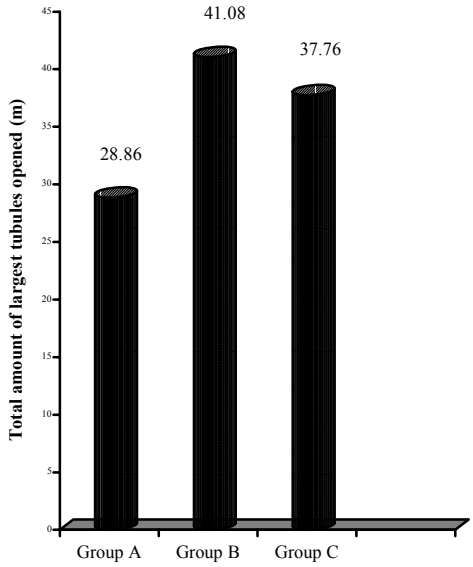
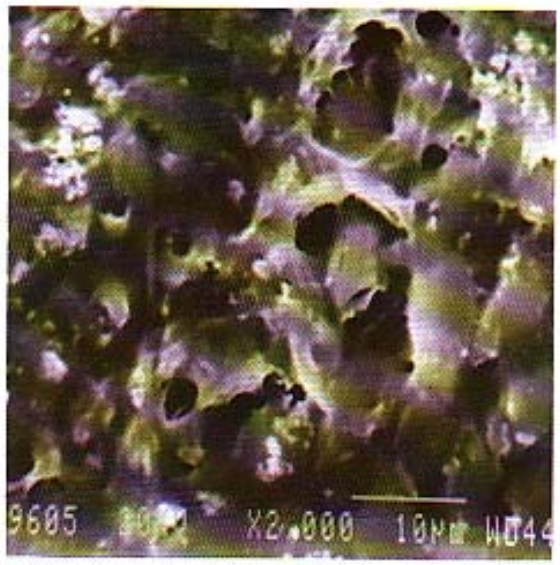
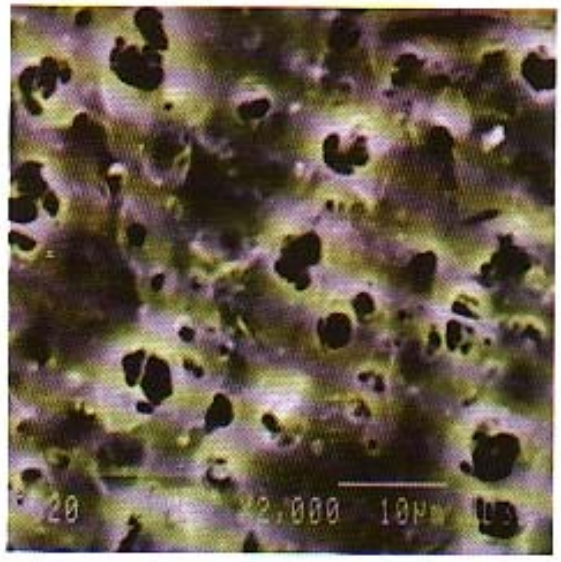


Fig. 2. Experimental surface following laser radiation (Group A)



Complete opening of tubles

Fig. 3. Experimental surface following citric acid conditioning (group B)



Large number of tubules opeened

Fig. 4. Experimental surface following tetracycline HCl conditioning (group C)

DISCUSSION

New attachment refers to reunion of connective tissue to a root surface deprived of its periodontal

ligament, usually by periodontitis (21). This reunion may occur by formation of new cementum with inserting collagen fibers (21). According to Kalkwarf (22) there are two factors which initially play the major role in new attachment phenomena: 1) Collagen adhesion, 2) Cemental repair. To have an environment suitable for such a phenomena to take place a diseased root surface must be converted to healthy surface free of deposits and with exposure of root dentin collagen matrix to attract the cells of gingival connective tissue which may be responsible elements in reproduction of a new periodontium. Instrumentation of diseased root surface is essential to create a disease free root surface but it also produces "smear layer" covering the entire root surface which has found to be composed of microorganisms, cementum fragments, plaques, calculus and cementum matrix components and ranges in thickness from 2-15 µm (6). Root surface conditioning following instrumentation has shown promising result in removal of smear layer and producing a surface free of deposits and bacterial accretion and also exposing the root detain collagen matrices. The present study was carried out in vitro to evaluate the results of root surface conditioning by using Nd: YAG laser radiation and to compare these results with those of citric acid and tetracycline hydrochloride (HCl) root conditioning. The result of this study has clearly demonstrated that irrespective of the methods or agents used in this study, root conditioning has a definite effect on removal of smear layer created by instrumentation. Unlike most other studies, this study was designed as such to eliminate chances of error to the maximum in differences between the control surfaces (root planed alone) and experimental surfaces (root planed and conditioned), because for each experimental area there was control on the same specimen surface so that factors such as the variation in extent of disease from one tooth to another, sharpness of instruments and number of strokes used for instrumentation were of no value in this study. Recent in vitro studies have shown that lasing root surface substance can give results even equal to that of curetts (23). A pulsed Nd: YAG laser at power of 2 watts with 10 (pps) for 2 minutes was selected based on the information given by Cobb, McCawly and Killoy (15) as the range of settings suggested by the manufacturers for assistance in root treatment (i.e. 1.50 to 2.00 watts). The results demonstrated a partial removal of smear layer, relatively narrow openings of dentinal tubules and surface topographical changes such as cracks, pits and crater like irregularities and a limited number of dentinal tubules were opened. These findings are in agreement with the results of previous investigations by Cobb et a1 (15), Morlock et al (24). Nd: YAG laser at power above 2 watts could even damage surface of titanium implants. So power setting is an important factor in minimizing laser damages (25).

The char layer created by laser radiation could possibly be avoided by using an air/water surface coolant (26). According to Cobb et al. The variables which may effect the result of laser radiation of the root surface are of two groups :

1) Tissue variables: such as color, density, thickness, water content and degree of mineralization.

2) Instrument variables: such as angel of beam, contacts, watts of energy and pulses per second.

This factors have been considered while performing flu experimental study.

Application of citric acid (pH= 1) for 3 minutes using a rubbing pressure on the surface resulted in a complete removal of smear layer and exposure of smoother root dentin surface with relatively wide dentinal tubular openings even up to 8.88 µm in diameter, which was consistent with previous studies by Garrett et al (16), Daryabegi et al (27), Lasho et al (8) and a large number of other investigators who have stated that citric acid produces a flared or funnel shape enlargement of the dentinal tubular opening. The factors influencing the effects of citric acid on root surface maybe concentration of the acid PH of the acid, duration of application and mode of application. Five percent solution of tetracycline hydrochloride (HCl) (PH= 3.3) was another agent used in this study and applied for 5 minutes on root planed surfaces with rubbing pressure. The results of application of this antimicrobial agent showed a complete removal of smear layer and also opening of numerous dentinal tubules which were significantly narrower in diameter in comparison to the citric acid group (Group “B”) but larger in diameter when compared to the tubules opened by Nd: YAG LASER radiation (Group "A"). Each root block specimen was divided into two equal halves, one half saved as experimental and another half was control portions. In each group the particular approach of conditioning was applied on the experimental portion only. All specimen were then prepared for scanning electron microscopy and the results observed subsequently on magnification of x 2000 and x 5000

Group A (Laser Treatment)

All the seven specimens showed changes in surface characteristics on the experimental portion except one which had little change after experimentation. All control portions exhibited a complete or semi-complete coverage of surfaces by smear layer. The dentinal tubular openings on the surface of central specimens were either absent or only a few openings were observed. These few numbers of tubular openings present were ranging from 2-4 on a given 4652 sq.µm surface area. The number of dentinal tubular openings present on experimental surfaces for a given 4652 sq.µm surface area ranged from 2-12 in number. The comparison between the controls and the experiments in regard to number of dentinal tubules opened on a given 4652

sq.µm surface area was statistically significant ($p<0.01$). The surface characteristics of experimental portions was typical of lased surfaces following mechanical debridement with an irregular shape and size of dentinal tubular openings which seemed more like depressions. The smear layer was only partially removed after lasing and surface cracking, pits and craters were observed in almost all specimens There were microfractures seen in six out of seven specimens.

Group B (Citric Acid treatment)

Specimens of this group exhibited absolute changes of surface characteristics between experimental and control portions. A typical surface smear layer was observed, covering six out of seven control portions. The dentinal tubular openings on the surface of the control portions were either absent or very few openings were observed in six out of seven control portions which were ranging from 3-4 in number on a given 4652sq.µm surface area. Only one control portion showed openings of eight tubules. The number of dentinal tubular openings present on experimental surfaces for a given 4652sq.µm surface area ranged from 7-13 in number. The number of tubular openings of experimental portions when compared to control portions of this group was statistically significant ($p<0.01$) for a given surface area of 4652sq.µm. The surface characteristics of experimental portions showed smooth surfaces in five out of seven specimens. The dentinal tubular openings were oval in shape but irregular in size. There were very large openings of tubules observed even up to 8.88 µm in diameter. The smear layer was removed in six out of seven experimental portions. Micro-fractures were observed in most of the portions but not large in number.

Group C (Tetracycline HCl treatment)

This group of specimens showed significant changes in surface characteristics between control and experimental surfaces. The surface smear layer covered six out of seven control portions completely and one control portion was covered partially. The dentinal tubular opening on the surface of control specimens were either absent or if present, only in few numbers ranging from 2-5 in six out of seven specimens (only one control specimen showed seven tubular openings) in measured 4652sq.µm surface area. The comparison between the controls and the experimental with regard to number of tubular openings was statistically significant ($p< 0.025$). The surface characteristics of experimental portions exhibited smooth surfaces with large number of open dentinal tubules most of which were oval in shape. The largest tubule measured, was 6. 11 µm in diameter. The smear layer was completely removed in six out of seven experimental specimens and partially removed in one experimental portion. There were no microfractures seen in experimental surfaces. The mean diameter of largest tubule in case of tetracycline was found to be 5.394 µm (±0.5725). The same measurement in case of citric acid was 5.869 µm (± 1.897). Any how this difference in size of largest tubules opened in case of tetracycline HCl and citric acid was not found to be significant ($p< 0.01$). The mean diameter of largest tubule opened in case of laser was found to be only 4.123 µm. The differences in diameter size of largest tubule in tetracycline (5.394 µm) and laser (4.123 µm) was found to be statistically significant ($p< 0.025$). The same difference between citric acid and laser was also found to be statistically significant ($p< 0.05$). The least diameter was in the case of laser followed by tetracycline and citric acid (4.123 µm, 5.394 µm and 5.869 µm respectively). But not much differences between tetracycline and citric acid were found in this statistical analysis. The results of tetracycline HCl root conditioning corroborated with the result of previous study by Hanes et al (18). The possible factors for variation in results of tetracycline HCl application on root planed surfaces may be similar to those of citric acid application. The pulpal reaction to the use of laser radiation on root surfaces due to thermal changes has been studied by several investigators. The study of Stern et al in 28 monkeys resulted in minimal pulpal changes and only a slight increase in the amount of cellular concentration due to thermal changes when there was an increase in energy density from 60 joules per sq.cm. to 111 joules per sq.cm was seen. Vascular changes and changes within the ground substance were also minimal in their study. They stated that the laser effect upon pulpal tissue might be an indirect effect, thus considerably a greater amount of energy would be necessary to cause irreversible pulpal changes. On the other hand the pulpal reaction to the use of low PH acids on the root surface has been questioned by several investigators (29,30). Garrett et al (16), with the use of transmission electron microscopy found morphologic changes extending to the depth of 4µm. Others (31) found acid effects ranging from 11 µm to 20 pm which indicated possible irritation of pulp tissues by acids. The result of study on animals by Ryan et a1 (32), also indicated inflammatory changes to the pulp due to application of acid on the root surface (only after 21 days post-operatively) which contradicted the result of previous study by Register (33) which claimed that there were no pulpal reactions to acid application. The antimicrobial effectiveness of the Nd: YAG laser radiation has been discussed by several investigators (15,34,35) They have stated that because the light of the Nd: YAG laser is adsorbed maximally by dark surfaces, this particular type of laser light can destroy the major group of periodontal pathogens namely black-pigmented bacteroides (34,35). Citric

acid also has been shown to have certain antimicrobial activities (36). Tetracycline hydrochloride other than having antimicrobial activity (17) has also an apparent substantivity to root surfaces (11) and inhibitory eflects on collagenase activity (38), which make it more desirable for use as a root surface conditioner in comparison to citric acid. Tetracycline HCl with concentrations ranging from 50 mg/ml to 150 mg/ml for any application time between 1-5 minutes has been shown to have maximum effect on smear layer and tubular opening (39). Since laser energy is adsorbed by root dentin, in clinical application there will be no harm to gingival soft tissue wall when it comes to contact with the lased root surface which it has been demonstrated in a study by Blomlof et al (31) and other animal studies; that application of low pH acids on the root surface may cause irritation and destruction of soft tissue cellular elements and marginal inflammation 31 which in turn prevents new attachment by application of acid on the root surfaces (40). The extent of demineralization has been shown in this study to be partial in the case of radiation of the roots and complete in the case of acid application. In this regard Tveit and Selvig (41) speculated that the completely demineralized outer surface of the root dentin would eventually be abraded away, while the partially demineralized root dentin might regain it's original degree of calcification. Use of Nd: YAG laser in combination with SRP has been shown to cause maximum reduction of IL-1β within 12 weeks which is a factor of bone demineralization (42). Another point of difference between results of laser radiation and the other two acids on the root planed surfaces was the diameter of the openings of dentinal tubules which was statistically significant ($p<0.025$ and $p<0.05$). Although wider tubular opening is indicative of higher peri-tubular dentin resorption and as a result more peri-tubular collagen matrix exposure, which is advantageous in accelerating connective tissue healing but if the coronal portion of the root is not absolutely covered by the flap tissue and it becomes exposed to the oral environment which occurs very likely in clinical practice and if the completely demineralized outer surface of the root is abraded away and there is not adequate undermined partially dermineralized zone of dentin to be calcified enough, the widened tubules could become filled with bacteria and result in carious softening or persistent inflammation of the contigecu gingival tissue. On the other hand it has been shown that if the demineralized root surface is exposed to oral environment, it is obvious that the severity of post-operative hypersensitivity is more if the tubular openings are wider in diameter and less likely if the tubular openings are narrower in diameter. In this point of view comparing the two acids used in this study, surfaces conditioned by tetracycline HCl showed a more favorable result when individual specimens were observed by scanning electron microscopy. However, the statistical results between the two acid groups (Group "B" and "C") in regard to degree of enlargement of tubular openings was not significant ($p< 0.01$). Number of dentinal tubules opened for a measured 4652 sq.µm surface area was statistically significant ($p< 0.01$ and $p< 0.025$), when experimental subgroups compared to control subgroups within each group. The results of present study suggests that use of Nd: YAG laser radiation has its own advantages over acids and maybe a possible substitute for acids in demineralization of the root surface. In conclusion the result of this study proved that conditioning of root surface following hand instrumentation can remove the surface smear layer and expose the underlying dentinal tubular openings and create a surface morphologically more suitable for new attachment to take place. This study supported the advantage of root conditioning in general, but evidences were not enough to suggest the use of "laser" radiation as a better method of root conditioning at certain power and duration used in this study. However, the changes observed on the root surface due to "LASER" radiation indicated that if there will be further modifications in factors influencing its effectiveness on root surface (eg: power, energy density, duration of exposure, distance from the surface etc.), there is a possibility of "laser" to become a substitute for biochemical agents in regard to root surface conditioning and in turn an assistance to regenerative therapy. In the end, it seems conceivable that laser method of root conditioning may become a more successful modality than the chemical ones in the pursuit of achieving regeneration in periodontal therapy.

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