ORIGINAL ARTICLE

Effect Of Strong Organic Acids On Dentinal Tubules Of Teeth- A Scanning Electron Microscopic Study

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ABSTRACT

Introduction: The head and neck is a complex structure of both soft and hard tissues. Natural teeth are the most durable of all tissues, being denser and chemically more inert. This study is an attempt to observe changes caused by strong inorganic acids (hydrochloric acid, nitric acid and sulphuric acid) to human maxillary first premolar teeth under scanning electron microscope. Changes were also noted at different time periods to determine the best decalcifying agent among these acids. **Material and Methods:** A total of 40 human maxillary first premolar teeth were collected. All the teeth were placed in 10% formalin (immediately after extraction). After fixation in formalin for 24- 48 hours, teeth were immersed in their respective acids 10 in 37% HCl, 10 in 65% HNO_3, 10 in 96% H_2SO_4 and 10 normal teeth were immersed in normal saline. **Discussion:** We conclude that 37% HCl took the minimum time to dissolute the teeth immersed and also showed the maximum change in number and diameter of dentinal tubules as compared to time taken by other two acids. But the structural changes were more in 65% HNO_3 as compared to other two acids. **Conclusion:** Work done on presence of intertubular dentin, occlusion of dentinal tubules and presence of crystals make this study unique. Additional studies with larger sample size would further help to assess the structural changes in dentin of the acid treated tooth.

Keywords: Teeth; Dentine, Strong Organic Acids, Scanning Electron Microscope

How to cite this article: Daksh Sharma, Shreedhar Balasundari, Kamboj Mala, Natarajan S. Effect of strong organic acids on dentinal tubules of teeth- a scanning electron microscopic study. International Journal of Contemporary Medical Research. 2015;2(2):445-452

Source of Support: Nil

Conflict of Interest: None

INTRODUCTION

Head and neck is a complex structure of both soft and hard tissues.[1] Natural teeth are the most durable, being denser and chemically more inert.[2] When teeth first form they are composed of soft tissue resembling the consistency of a jelly fish. As the body matures, this soft tissue mineralizes by a process of incorporating minerals into its specialized soft tissue, making teeth and bone hard and strong.[3] Soft tissues pose little resistance to histochemical techniques. Presence of high inorganic content like calcium and phosphorus in teeth, make it very hard to prepare for microscopic examinations.[2]

Decalcification is done to make teeth suitable for viewing under microscope. The aim is to remove calcium salts from mineralized tissue using chemical solutions like acids and chelating agents, while preserving the organic portions.[1] It is carried out by chemical agents, either with acids to form soluble calcium or with chelating agents that bind to calcium ions.

Acids decalcifiers are divided into 2 groups:

1. Strong inorganic acids: like HNO_3 and HCl. They decalcify rapidly and cause considerable change in tooth immersed in them.
2. Weak organic acids: like formic acid, picric acid and acetic acid. Studies have been done on teeth using these acids.[4]

Dentinal tubules extend through entire thickness of the dentin from the DEJ to the pulp. The tubules follow an S-shaped path from the outer surface to the perimeter of pulp in coronal dentin. They are tapered structures measuring approx 2.5 µm in dia near the pulp, 1.2 µm in mid portion of dentin & 900 nm near DEJ. In the coronal parts of the young premolar and molar teeth, number of tubules range from 59,000 to 76,000 per sq mm at the pulpal surface, with approx half as many per sq mm near the enamel.

Dentinal tubules are delimited by a collar of more highly calcified matrix called peritubular dentin.[5] Peritubular dentin is a mineralized deposit formed centripetally in the dentinal tubules with advancing age, so that the tubular diameter is smaller in teeth from older persons.[6] It extends for about 1 to 2 µm.[7] To observe these minute structures of dentinal tubules normally and the ultra structural changes after being subjected to decalcification, SEMs are used.

As Thomas Huxley said "*In a scientific work, those who refuse to go beyond fact, rarely get as far as fact*". Thus, limiting ourselves only to light microscopy would mean to handicap ourselves. Looking beyond and trying to study the surface details and ultra structural changes caused by the strong organic acids on normal teeth with the help of SEM is what we have done in our study.

Thus, this study is an attempt to observe changes caused by strong inorganic acids (HCl, HNO_3 and H_2SO_4) to human maxillary first premolar teeth under SEM. Changes were also noted at different time periods to determine the best decalcifying agent among these acids.

MATERIALS AND METHODS

Source of Data

The study was conducted on extracted teeth and only the non carious maxillary first premolar teeth which were extracted for orthodontic or periodontal reasons were used. These teeth were free of any restorations. A total of 40 human maxillary first premolar teeth were collected.

Method to observe changes under naked eye

All teeth were placed in 10% formalin (immediately after extraction). After fixation in formalin for 24- 48 hrs teeth were immersed in their respective acids 10 in 37%HCl, 10 in 65% HNO_3, 10 in 96% H_2SO_4 and 10 normal teeth were immersed in normal saline. Changes in teeth were observed after specific time intervals in all acids. Changes were clicked and recorded in Nikon 255 camera. Teeth which had sufficient amount of crown portion left after acid immersion were kept in plastic containers containing distilled water. These teeth were used for observing and analyzing changes in density and diameter of dentinal tubule.

Method to observe changes under SEM

The SEM specimens were coated with gold palladium coating by EMITECH Sputter for two minutes and were fed into the (ZEISS Evo Special edition) Scanning Electron Microscope, after mounting over the metallic stub. Each specimen was scanned at the lesional surface accelerating voltage of 10kV. The chosen magnification was x3000. The areas selected for photography were those where maximum width of the dentinal tubules was visible. Only the images of the representative areas were photographed. Maximum fields in the areas of interest on the SEM specimens were observed. As the teeth specimens were acid treated, the clicked surfaces showed uneven changes at fixed magnification.

The normal samples (control group) were scanned first to form a base for the interpretation of samples with lesions. Then the acid immersed samples were subjected to a preliminary scan to note the changes. Based on preliminary trial scan and review of literature, the following features were looked for in the teeth of HCl, HNO_3 and H_2SO_4.

1. Diameter and density of dentinal tubules.
2. Distinctness of peritubular and intertubular dentin
3. Presence of crystals within the tubules and its nature.

The data obtained was entered in a master chart for further interpretation and analysis.

Inclusion criteria

1. Crown surface closer to pulp of maxillary first premolars
2. Age: 20-65 yrs
3. Teeth extracted for orthodontic and periodontal purposes

Exclusion criteria

1. Carious teeth
2. Restored teeth

STATISTICAL ANALYSIS

Continuous data was summarized in Mean ± SD while discrete (categorical) in %. All analyses were performed on STATISTICA statistical software (Windows version 6.0).

RESULT

Morphological changes

The morphological changes in teeth of three groups (37% HCl, 65% HNO_3 and 96% H_2SO_4) over the periods (0.5 hr to 96 hrs) were summarized in Table 1. The teeth were immersed separately in different containers containing the 3 different acids. At various intervals, the samples were taken out of the container and examined for any morphological changes; photographed and placed back in the containers. The specimens were under observation until they had completely dissolved or precipitated completely. Among acids, the 100% dissolution of teeth without any remnants was evident earliest in 37% HCl at after 8 hrs; 65% HNO_3 showed 100% dissolution without any remnants after 15 hrs and 96% H_2SO_4 showed complete dissolution after 96 hrs.

Normal dentinal tubule diameter and density: Closer to the pulp was measured by SEM and summarized in Table 2.

Change in diameter of dentinal tubules and density of dentinal tubules/field: The changes in diameter and density of dentinal tubules of Group 2 (37% HCl), Group 3 (65% HNO_3) and Group 4 (96% H_2SO_4) were observed up to complete dissolution. Observations are summarized in Table 3.

Type of morphological changes like Intertubular dentin, Occluded tubules and Presence of crystals (calcification) closer to the pulp of all four groups up to complete dissolution (except Group 1) were observed. Distribution of Intertubular dentin and distribution of Occluded tubules were assessed according to four type i.e. (-) denotes absent, (+) denotes fewly present, (++) denotes moderately present, (+++) denotes significantly present. They are summarized in Table 4.

DISCUSSION

Teeth are the hardest tissues in the body, consisting of enamel, dentin and cementum, and have a highly mineralized extracellular matrix.[11] Unlike soft tissues, which can be easily sectioned and analyzed, teeth require complete demineralization in order to prepare proper sections for histological analysis. The process of decalcification is done to study the structure of tooth, pulp calcifications, and also to evaluate the biological response of dental pulp to restorative material.[1]

There have been studies conducted on diameter and density of dentinal tubules in human permanent teeth when viewed under light microscopes, scanning electron microscope and transmission electron microscope. But to best of our knowledge there has been no study on effects of strong inorganic acids on dentin with density and diameter changes in dentinal tubules in human permanent teeth under scanning electron microscope. Therefore the present study was undertaken to observe the changes in dentin and within dentinal tubules in maxillary first premolars. We also observed the changes in intertubular dentin, occlusion of dentinal tubules and the presence of crystals. Changes were also noted at different time periods to determine the better decalcifying agent among these acids.

There have been few studies conducted over the morphological changes when teeth were immersed in strong inorganic acids. The changes were seen for specific time duration until the teeth dissolute or there was complete fragmentation of teeth as in case of 96% H_2SO_4. Jadhav et al worked with 37% HCL, 65% HNO_3 and 96% H_2SO_4 and came up with the conclusion that 37% HCL took minimum time in tooth dissolution followed by 65% HNO_3 and lastly by 96% H_2SO_4.[10] In an another study by Alessandra Mazza et al comprising of four acids 37% HCL, 65% HNO_3, 96% H_2SO_4 and Aqua Regia (chloroazotic acid-HCL/Nitric acid 1:3), it was found that 96% H_2SO_4 took the maximum time to

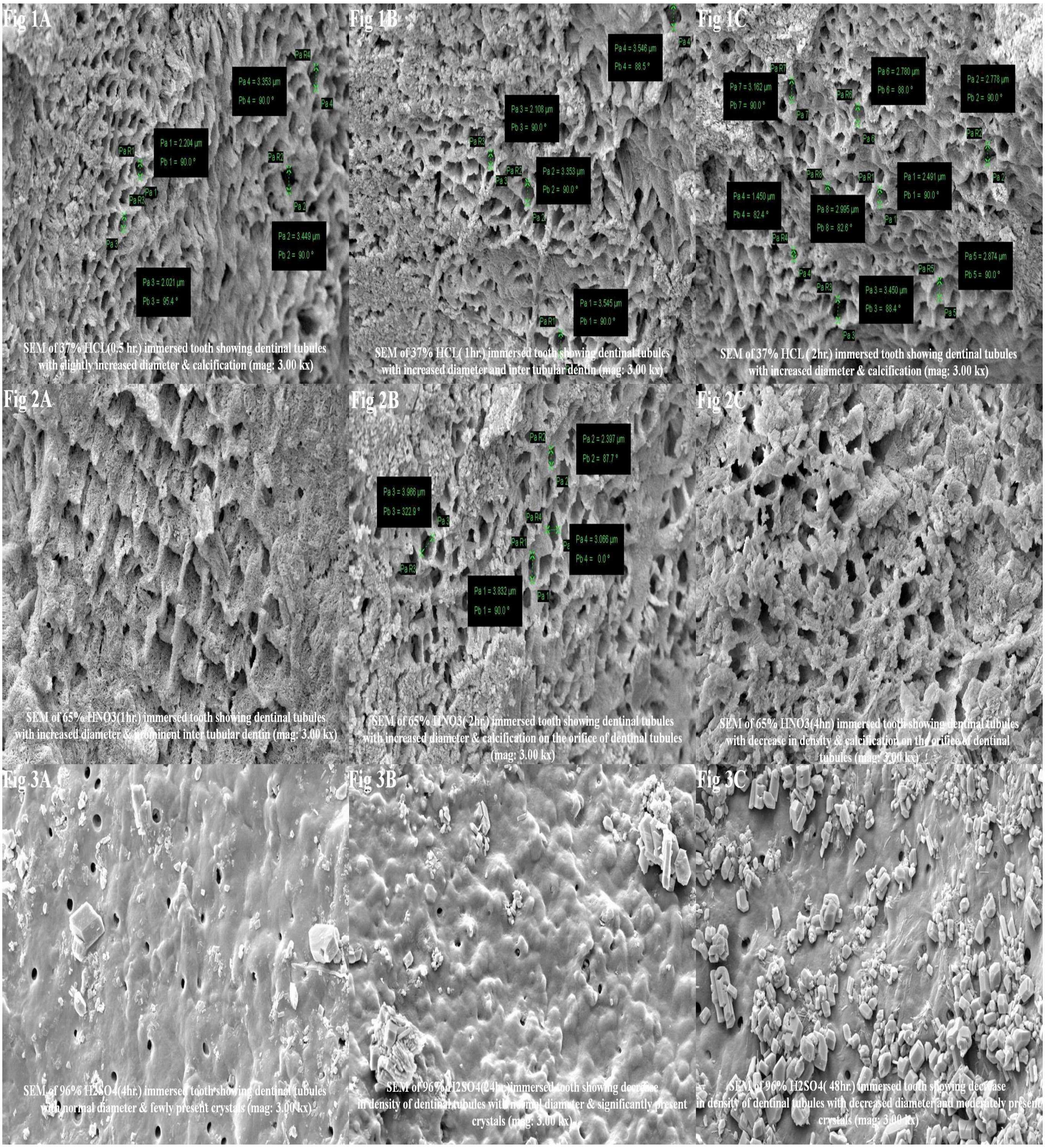

Figure-1: Scanning electron micrographic figures of teeth immersed in 37% HCl, 65% HNO_3, 95% H_2SO_4 at various hours to monitor the changes in the dentinal tubules of teeth.

dissolve the teeth immersed among the four inorganic acids.[9] Our results are in agreement with the above studies and we found complete dissolution of teeth immersed in 37% HCl in 8 hrs, 65% HNO_3 in 15 hrs and 96% H_2SO_4 in 96 hrs respectively.

We used Scanning Electron Microscopy (SEM) in place of light microscope as SEM is a powerful method for the investigation of surface structures. Electrons in SEM penetrate into the sample within a small depth, so that it is suitable for surface topology, for every kind of samples. This

Table-1: Morphological changes in teeth of three groups over the periods

Observation periods	Group 2 (37% HCl)	Group 3 (65%HNO_3)	Group 4 (96% H_2SO_4)
0.5 hr	Presence of effervescence in solution in 10/10 (100%) with color alterations in 7/10 teeth (70%)	Presence of effervescence in 8/10 teeth (80%) with yellow color deposit over teeth in 10 /10 teeth (100%)	No Change
1 hr	Transparency in 7/10 teeth with color alterations in 10/10 teeth	Yellow colored residue deposited over tooth in 8/10 teeth (80%) and transparency present.	No Change
2 hr	Disintegration of apical third of root with crack extending from incisal surface to middle third of crown in 7/10 teeth.	Disintegration of apical third without any crack on the crown in 8/10 teeth.	No Change
4 hr	Disintegration of middle third of root in 8/10 teeth.	Disintegration of middle third of root in 10/10 teeth	Presence of white precipitate seen in 4/10 teeth (40%)
8 hr	Complete dissolution in 10/10 teeth without any remnants	Splitting of teeth in 9/10 teeth (90%)	Presence of white precipitate seen in 7/10 teeth (70%)
15 hr	-	Complete dissolution in 10/10 teeth without any remnants.	Increase in white precipitate seen in 10/10 teeth with partial dissolution of apical third of root present in 3/10 teeth
20 hr	-	-	Partial dissolution of apical third of root seen in 4/10 teeth (40%)
24 hr	-	-	Partial dissolution of apical third of root seen in 6/10 teeth. Tooth was recognized in 10/10 teeth. White precipitate increased in 6/10 teeth
48 hr	-	-	White precipitate was increased in 8/10 teeth with fragmentation of teeth in 6/10 teeth. Tooth was recognizable in 8/10 teeth.
72 hr	-	-	White precipitate was increased in 10/10 teeth with fragmentation of teeth in 7/10 teeth. Tooth was still recognisable in 4/10 teeth.
96 hr	-	-	Complete dissolution in 10/10 teeth.

Inference: 37% HCL took minimum time to dissolute the teeth followed by 65% HNO_3 and lastly by 96% H_2SO_4

Table-2: Normal Dentinal Tubule Diameter And Density Measured By SEM

Tooth number	Chosen areas	Diameter of dentinal tubules (µm)	Density of dentinal tubules/field (no)
1.	Closer to the pulp	2.1	258
2.	Closer to the pulp	2.6	122
3.	Closer to the pulp	2.4	123
4.	Closer to the pulp	2.5	52
5.	Closer to the pulp	1.8	418
6.	Closer to the pulp	2.2	322
7.	Closer to the pulp	1.9	103
8.	Closer to the pulp	2.1	245
9.	Closer to the pulp	1.8	324
10.	Closer to the pulp	2.2	185

Inference: Tubule diameter ranges from 1.8 to 2.6 µm and number/field from 52.0 to 418.0.

Table-3: Summary (Mean ± SD) Of Changes In Diameter (µm) Of Dentinal Tubules And Changes In Density/Field (No.) Of Three Groups Over The Periods

Observation periods	Diameter (µm) of dentinal tubules			Density/field (no.) of dentinal tubules		
Groups	**Group 2 (37% HCL)**	**Group 3 (65% HNO_3)**	**Group 4 (96% H_2SO_4)**	**Group 2 (37% HCL)**	**Group 3 (65% HNO_3)**	**Group 4 (96% H_2SO_4)**
0.5 hr	2.44 ± 0.29	No change	No change	98.70 ± 6.78	No change	No change
1 hr	3.42 ± 0.15	2.87 ± 0.17	No change	110.90 ± 7.31	22.10 ± 1.73	No change
2 hr	3.83 ± 0.19	3.30 ± 0.16	No change	33.80 ± 2.30	55.00 ± 1.49	No change
4 hr	2.13 ± 0.40	3.60 ± 0.12	2.11 ± 0.15	28.60 ± 3.03	34.10 ± 1.66	29.70 ± 1.34
8 hr	Complete dissolution	2.37 ± 0.17	2.10 ± 0.19	Complete dissolution	16.10 ± 4.43	14.60 ± 2.55
15 hr	-	Complete dissolution	2.49 ± 0.09	-	Complete dissolution	31.70 ± 7.29
20 hr	-	-	2.30 ± 0.12	-	-	26.40 ± 7.46
24 hr	-	-	1.89 ± 0.12	-	-	26.20 ± 7.36
48 hr	-	-	1.93 ± 0.12	-	-	6.80 ± 1.87
72 hr	-	-	1.40 ± 0.08	-	-	2.00 ± 1.05
96 hr	-	-	Complete dissolution			
% change (pre to final reading)	**12.7% (in 3.5 hrs)**	**17.4% (in 7 hrs)**	**33.6% (68 hrs)**	**71.0% (in 3.5 hrs)**	**27.1% (in 7 hrs)**	**93.6% (68 hrs)**

Inference: Maximum diameter change according to time taken was seen in 37% HCL > 65% HNO_3 > 96%H_2SO_4.
Inference: Maximum change in number of tubules/field according to time taken was seen in 37% HCL, followed by 65% HNO_3 and lastly by 96%H_2SO_4.

technique provides a large depth of field, which means the area of the sample that can be viewed in focus at the same time is actually quite large. According to Tencate, normal dentinal tubule diameter is approximately 2.5 µm in diameter near the pulp.[5] Our SEM findings were also in agreement as the diameter of dentinal tubules of all samples ranged from 1.8 to 2.6 µm with mean (± SD) 2.16 ± 0.28 µm at closer to the pulp. Density of dentinal tubules/field (no.) of all samples ranged from 52.0 to 418.0 with mean (± SD) 215.20 ± 117.57. Correlation analysis revealed a significant ($p>0.05$) and negative (inverse) correlation ($r= -0.65$, $p<0.05$) between diameter of dentinal tubules and density of dentinal tubules/field. This finding of ours is new

Table-4: Frequency distribution of Changes in Intertubular dentin, Occluded tubules and Presence of Crystals among the four groups

Groups	Intertubular Dentin			Occluded Tubules			Presence of Crystals	
	+	++	+++	+	++	+++	Yes	No
Group 1(n=10)	10(100%)	0 (0%)	0 (0%)	8(80%)	2(20%)	0 (0%)	0(0%)	10(100%)
Group 2(n=10)	7 (70%)	3 (30%)	0 (0%)	6(60%)	4(40%)	0 (0%)	9(90%)	1(10%)
Group 3(n=10)	8 (80%)	2 (20%)	0 (0%)	2(20%)	8(80%)	0 (0%)	8(80%)	2(20%)
Group 4 (n=10)	0 (0%)	10 (100%)	0 (0%)	1(10%)	8(80%)	0 (0%)	2(20%)	0(0%)
χ^2 value (DF=3)	24.21			15.61			28.60	
P value	P<0.001(Highly Significant)			0.016(Significant)			P<0.001(Highly Significant)	

in the field of dentinal tubules as previous authors counted the number of dentinal tubules in mm^2.
During decalcification of teeth in acid there are findings that the peritubular dentin to a large extent is dissolved. Decalcification of the peritubular dentin preceded that of the intertubular regions, not only in areas where the tubules opened on the experimental surface but also in areas where they were orientated parallel to this surface, indicating that the peritubular zone represents the most easily demineralized part of dentin. The peritubular zone became completely demineralized throughout most of the lesion. The tubule diameter measured on decalcified sections thus gives an excessively high value.[6] Our findings were in agreement as there is increase in diameter of dentinal tubules in all the three acids for certain time periods i.e 1hr, 2hr in 37% HCL, 1hr, 2hr, 4hr in 65% HNO_3 and 15hr in 96% H_2SO_4. After certain time period i.e. (4 hr in HCL, 8hr in HNO_3) there was decrease in diameter of dentinal tubules. There was a unique finding in case of 96% H_2SO_4 that changes in diameter of dentinal tubules were not persistent as in the other two acids. The maximum mean value of number of dentinal tubules per field was seen in 37% HCL in 1 hr (110.90) of immersion whereas the minimum number per field was seen in 96% H_2SO_4 in 72 hr (2).
Changes in proportion of intertubular dentin fewly present was significantly ($p<0.001$) higher in normal tooth, 37% HCL and 65% HNO_3 as compared to 96%H_2SO_4 while moderately present was significantly higher in 96% H_2SO_4 as compared to normal tooth, 37% HCL and 65% HNO_3.Similarly, comparing the proportion of Occluded tubules between the four groups. In other words, the proportion of Occluded tubules fewly present was significantly higher in normal tooth and 37% HCL as compared to 65% HNO_3 and 96% H_2SO_4 while moderately present was significantly higher in 65% HNO_3 and 96%H_2SO_4 as compared to normal tooth and 37% HCL. Further, comparing the proportion of presence of crystals between the four groups, the presence of crystals was significantly higher in 37% HCL, 65% HNO_3 and as 96% H_2SO_4 compared to normal tooth. These findings of SEM are new in its kind.
Through our study we conclude that 37% HCL took the minimum time to dissolute the teeth immersed and also showed the maximum change in number and diameter of dentinal tubules as compared to time taken by other two acids. But the structural changes were more in 65% HNO_3 as compared to other two acids. Our study was one of its kind as we worked on untouched and unrevealed effects of three commonly used strong acids on dentinal changes of human permanent maxillary first premolars.

CONCLUSION

The changes of dentin under effect of strong inorganic acids were best appreciated on SEM because of its large depth of field and range of magnification which is relatively wide and allows the investigator to easily focus on an area of interest. To the best of our knowledge there has been no work done previously to assess the presence of intertubular dentin, occlusion of dentinal tubules and presence of crystals in observing the changes of strong inorganic acids on teeth.

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