ASSESSMENT OF WEAK AND STRONG ACID AS DECALCIFYING AGENT ON HUMAN EXTRACTED TEETH

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ABSTRACT:

Context: : nitric acid, perenyi's fluid, formic acid, formic-nitric acid, EDTA, decalcifying agent, teeth **Aims:** To assess the best decalcifying agent among nitric acid, perenyi's fluid, formic acid, formic nitric acid, EDTA for decalcification of teeth **Settings and Design:** The study was divided into three sets: set I, set II and set III. Each set contained 5 decalcifying solutions with recently extracted permanent 4 teeth i.e. one incisor, one canine, one premolar and one molar in each solution .i.e total 60 teeth. **Methods and Material:**, Decalcification agents used were NITRIC ACID, PERENIY'S FLUID, FORMIC ACID, FORMIC-NITRIC ACID and EDTA. Radio visiography was used for assessment of decalcification, and its end point. Processing and sectioning with staining of hematoxylin and eosin was done respectively for the specimen The stained sections were observed under a light microscope and grading was done. **Statistical analysis used:** The results were analysed using descriptive statistics. Discrete (categorical) data were summarized as in proportions and percentages (%) and quantitative data were summarized as Mean ± SD (standard deviation). Inter-rater agreements among the three observers were calculated by using Kappa Statistic. **Results:** The assessment was done for four parameters such as time of decalcification, ease of sectioning, soft tissue and hard tissue staining and loss of attachment. EDTA was best decalcifying agent while nitric acid was fastest. EDTA though slower decalcifying agent has proven to be the most efficient decalcifying agent among all.

Conclusions: EDTA is a good decalcifying agent, while nitric acid cause extensive tissue damage.

Keywords: decalcifying agent, Extracted teeth, Strong acid, weak acid

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Introduction:

Human teeth and bones are composed of hard and soft tissues. The mineralized tissue with maximum calcium content in the human body is the tooth enamel with a ratio of 96-98%.[1] For obtaining satisfactory paraffin or celloidin sections of bone, removal of inorganic calcium is a must from the organic collagen matrix and calcified cartilage and surrounding tissues. This is known as decalcification, which is carried out by chemical agents, either with acids to form soluble calcium salts or with chelating agents that bind to calcium ions. Mainly Decalcification is done in order to make teeth suitable for observing under microscope.[2]

Subjects and Methods:

The present prospective study was carried in the department of oral and maxillofacial pathology, career post graduate institute of dental sciences and hospital, Lucknow. Freshly extracted 60 permanent teeth (incisor, canine, premolars, molars) were collected from the department of oral surgery from career institute of dental sciences and hospital the patient age was between 40-45 years from whom the teeth were extracted. The teeth were free of attrition abrasion and erosion. Ethical approval was taken (Ethical No. CPGIDSH/578/17)

INCLUSION CRITERIA: Freshly extracted non carious 60 permanent teeth included one incisor, one canine, one premolar, one molar were included.

EXCLUSION CRITERIA: Deciduous teeth, Eroded, Attrition, Abrasion and Carious teeth were excluded.

The collected 60 teeth were placed in 10% formalin for 48 hours and were used to assess the effect of different decalcifying agents on hard and soft tissue in a histologic section

The agents used were NITRIC ACID, PERENIY'S FLUID, (The perenyi's fluid working solution was prepared by addition of 30 ml of 5% chromic acid 40ml of 10% nitric acid and 30 ml of absolute alcohol) FORMIC ACID, FORMIC-NITRIC ACID (Formaldehyde, nitric acid and distilled water are mixed in a ratio of 10ml,10ml,80ml, for preparation of final working solution of formic nitric acid) and EDTA.

This study was divided into three sets: set I, set II and set III.Respectively each set included 5 decalcifying solutions with 4 teeth i.e., one incisor, one canine, one premolar and one molar in each solution. Decalcification was done at room temperature by suspending the teeth in the container with the help of a thread by completely immersing the set of teeth in about 100 ml of the solution. Starting time of decalcification was observed and noted. The solutions were regularly agitated and was replaced by freshly prepared solutions every 24 h.

Here we have used radiovisiography (5100 Care stream) for assessment of decalcification, and the end point of decalcification was observed and confirmed by the following radiographic method.

The tooth were placed horizontally on a RVG sensor. The x-ray (GOMEX) beam was used perpendicular to the long axis of tooth and the results were obtained using dental imaging software. The radiographs were taken on a daily basis for all the solutions until complete radiolucency was reached.

PROCESSING: Post decalcification procedure, the samples were washed and cleared in distilled water, to remove the acid from it and then the tooth were sectioned accordingly.

The decalcification speed measurement of different agents was done in days.

The washed tooth section(samples) were transferred into the series of alcohol in increasing ratio of concentration for dehydration procedure. After the dehydration, clearing was executed in the samples with xylene solution. After clearing the tooth section was transferred into soft paraffin wax with a melting point of 46-48 degree respectively for 4 hours.

Then blocks of samples were made by usual embedding them into paraffin wax. Section of 7 micrometre thickness were taken from the blocked tissues by using a semi automatic Yorco rotatory microtome. These sections were moved on slides from the distilled water bath and then were dried.

All the sections were stained with routine staining Hematoxylin and eosin stain.

Qualitative assessment of decalcified sections was performed using a light microscope (Nikon Light Microscope, Eclipse 50i, Tokyo, Japan) and graded from 0 to 4 (0: no observation, 1: poor, 2: better, 3: good and 4: excellent) based on the following criteria: decalcification speed; ease of sectioning; staining characteristic including hard-tissue staining, soft tissue staining; soft-tissue attachment, and total score.

In our study for checking the reliability of data outcomes of decalcification, the complete data was accessed by three blind observers.

Results:

TABLES:

Table-1: Inter-rater agreement in outcomes of Decalcification features among the three observers

Interobserver Agreements	**Obs I vs**	**Obs I vs**	**Obs II vs**
Time of Decalcification (Days)	1.000	0.982	0.982
Ease of Sectioning	0.543	0.548	0.245
Hard Tissue Staining	0.614	0.591	0.273
Soft Tissue Staining	0.626	0.649	0.320
Soft Tissue Attachment	0.636	0.577	0.335

The mentioned values are Kappa statistics values

Table-2: Average time of Decalcification among the three observers for all the samples

Parameter	**Total**	
	Mean	SD
Time of Decalcification (Days)	47.61	32.26

Table-3: Average Scores of Qualitative Parameters of Decalcification for all the samples

Parameter	**Total**	
	Mean	**SD**
Ease of Sectioning	2.18	0.76
Hard Tissue Staining	2.86	0.83
Soft Tissue Staining	2.33	1.04
Soft Tissue Attachment	1.89	1.01
Total Score	9.17	2.65

Table-4: Comparison of time of Decalcification among the various tooth types

Incisor		**Canine**		**Premolar**		**Molar**		**Statistics**	**p-value**
Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**		
45.80	33.46	45.80	33.46	45.80	33.46	45.51	33.07	F=0.00	1.000

Table-5: Comparison of time of Decalcification between Weak & Strong Acids

Parameter	**Strong**		**Weak**		**Statistics**	**p-value**
	Mean	**SD**	**Mean**	**SD**		
Time of Decalcification (Days)	13.00	3.18	67.55	23.45	t=11.30	<0.001

Table-6: Comparison of Qualitative Parameters of Decalcification between Weak & Strong Acids

Parameter	**Strong**		**Weak**		**Statistics**	**p-value**
	Mean	**SD**	**Mean**	**SD**		
Ease of Sectioning	1.96	0.81	2.25	0.73	U=345	0.160
Hard Tissue Staining	2.38	1.06	3.11	0.52	U=252.5	0.003
Soft Tissue Staining	1.83	0.76	2.64	1.07	U=232.5	0.002
Soft Tissue Attachment	1.71	0.69	2.03	1.16	U=382.5	0.429
Total Score	7.88	2.61	10.03	2.34	t=3.337	0.001

Table-7: Comparison of time of Decalcification between Two Strong Acids

Parameter	**Nitric Acid**		**Perenyi's Fluid**		**Statistics**	**p-value**
	Mean	**SD**	**Mean**	**SD**		
Time of Decalcification (Days)	10.00	0.85	16.00	0.85	t=17.23	<0.001

Table-8: Comparison of Qualitative Parameters of Decalcification between two Strong Acids

Parameter	**Nitric Acid**		**Perenyi's Fluid**		**Statistics**	**p-value**
	Mean	**SD**	**Mean**	**SD**		
Ease of Sectioning	1.67	0.78	2.25	0.75	U=43.0	0.101
Hard Tissue Staining	2.08	1.08	2.67	0.98	U=48.0	0.178
Soft Tissue Staining	2.08	0.79	1.58	0.67	U=46.5	0.143
Soft Tissue Attachment	1.83	0.72	1.58	0.67	U=58.0	0.443
Total Score	7.67	2.96	8.08	2.31	t=0.384	0.705

Table-9: Comparison of time of Decalcification among three Weak Acids

Parameter	Formic- Nitric Acid		Formic Acid		EDTA		ANOVA	p-value
	Mean	SD	Mean	SD	Mean	SD		
Time of Decalcification (Days)	36.00	0.85	76.00	0.85	90.64	1.75	F=6350.9	<0.001

Table-10: Comparison of Qualitative Parameters of Decalcification among three Weak Acids

Parameter	Formic- Nitric Acid		Formic Acid		EDTA		Statistics	p-value
	Mean	SD	Mean	SD	Mean	SD		
Ease of Sectioning	2.33	0.78	2.08	0.67	2.33	0.78	chi sq=1.19	0.551
Hard Tissue Staining	2.92	0.67	3.08	0.29	3.33	0.49	chi sq = 3.67	0.160
Soft Tissue Staining	2.33	0.89	2.08	0.51	3.50	1.17	chi sq = 16.65	<0.001
Soft Tissue Attachment	1.50	0.52	1.25	0.75	3.33	0.78	chi sq = 22.02	<0.001
Total Score	9.08	1.88	8.50	1.31	12.50	1.38	F = 23.429	<0.001

Calculation Of Efficacy Score

Days of time of Decalcification	Score
9 - 2 5	1
26 - 40	2
41 - 57	3
58 - 75	4
76 & above	5

In the above table, The efficacies of various acids were measured by combining time of decalcification and ease of sectioning scores. For this, first scores were assigned to time of decalcification by factor analysis. The assigned scores after factor analysis had come out to be according to the above given table.

After adding the time of decalcification score with the case of sectioning score, we get the Efficacy Score.

Table-11: Comparison of Efficacy between Various Acids

Acid	Efficacy Score	
	Mean	SD
Nitric Acid	2.67	0.78
Pereny's Fluid	3.25	0.75
Formic-Nitric Acid	4.33	0.78
Formic Acid	6.75	0.87
EDTA	7.33	0.78
Statistics		Chi sq = 23.42
P-value		< 0.001

Table-12: Comparison of Deformity Quality among Weak & Strong Acids

Acid	Deformity Score	
	Mean	SD
Nitric Acid	6.00	2.30
Perenyi's Fluid	5.83	1.85
Formic-Nitric Acid	6.75	1.42
Formic Acid	6.42	1.08
EDTA	10.17	1.40
Statistics		Chi sq = 19.21
P-value		< 0.001

In table 1 The Kappa statistics showed maximum 100% agreement (k=1.00) between Observer1 & II for the parameter 'time of decalcification' and minimum agreement as 27.3% between observers II & II for the parameter 'Hard Tissue Staining',

In table 2, The average time of decalcification among the three observers for all the samples was 47.61±32.26 days. So there is a big variability present in time of decalcification. This was due to different natures of decalcification materials.

In table 3 For a single sample, score was taken as the agreed score of at least two observers

In table 4, the mean time of Decalcification was found to be almost equal for all the tooth types (p value is 1.000).

In table 5 the difference in time of decalcification between weak and strong acids was highly significant (p<.001). So the efficacy of weak acid was significantly better than the strong acid from the view of speed of decalcification.

In table 6, According to Mann Whitney test the difference in ease of sectioning score between weak and strong acids was not significant (p=.160). So the efficacy of weak acid was not significantly better than the strong acid from the view of case of sectioning

On comparing the difference in hard tissue staining score between weak and strong acids was found to be significant (p= .003). So the efficacy of weak acid was significantly better than the strong acid from the view of hard tissue staining.

On comparing the difference in Soft tissue staining score between weak and strong acids was found to be significant (p=.002). So the efficacy of weak acid was significantly better than the strong acid from the view of Soft tissue staining.

On comparing difference in Soft tissue attachment score between weak strong acids was not found to be significant (p=.429). So the efficacy of weak was not significantly better than the strong acid from the view of Soft attachment.

On comparing the difference in Total quality score between weak and strong acids was found to be significant (p=.001). So the efficacy of weak acid was significantly better than the strong acid from the view of Total quality.

In table 7, Among the strong acids, On comparing the time of decalcification between Acid and Perenyi's Fluid it was found highly significant (p<.001) So the efficacy of Perenyi's Fluid was significantly better than the Nitric Acid from the view of speed of decalcification.

In table 8, On comparing the difference in ease of sectioning score between Perenyi's Fluid and Nitric Acid was not significant (p=.101). So the efficacy of Perenyi's Fluid was not significantly better than the Nitric Acid from the view of ease of sectioning.

On comparing the Hard tissue staining score between Perenyi's Fluid and Nitric Acid was not significant (p=.171). So the efficacy of Perenyi's Fluid was not significantly better than the Nitric Acid from the view of Hard tissue staining.

On comparing Soft tissue staining score between Perenyi's Fluid and Nitric Acid was not significant (p=.143). So the efficacy of Perenyi's Fluid was not significantly inferior than the Nitric Acid from the view of Soft tissue staining.

On comparing Soft tissue attachment score between Perenyi's Fluid and Nitric Acid was not significant (p=.443). So the efficacy of Perenyi's Fluid was not significantly inferior than the Nitric Acid from the view of Soft tissue attachment.

On comparing the difference in Soft tissue attachment score between Perenyi's Fluid and Nitric Acid was not significant (p=.705). So the efficacy of Perenyi's Fluid was not significantly inferior to the Nitric Acid from the view of total quality.

In table 9, Among the weak acids, on comparing the time of decalcification between three weak acids it was found that the difference in time of decalcification among three weak acids was found to be highly significant (p<.001). So the efficacy of EDTA was maximum and of Formic-Nitric Acid was minimum from the view of speed of decalcification.

In table 10, Among the weak acids, on comparing the difference in case of sectioning score among three weak acids was not found to be significant (p=.551). So the efficacy of three acids did not significantly differ from the view of ease of sectioning.

Among the weak acids, it was found that the difference in hard tissue staining score among three weak acids was not found to be significant (p=.160). So the efficacy of three weak acids was not significantly differ from the view of hard tissue staining score.

Among the weak acids, the difference in Soft tissue staining score among three weak acids was found to be significant (p<.001). So the efficacy of three weak acids was significantly differ from the view of soft tissue staining score and have the order EDTA>Formic-Nitric Acid>Formic Acid.

Among the weak acids, the difference in Soft tissue attachment score among three weak acids was found to be significant (p<.001). So the efficacy of three weak acids was significantly differ from the view of soft tissue attachment score and have the order EDTA>Formic-Nitric Acid>Formic Acid.

Among the weak acids the difference in total quality score among three weak acids was found to

be significant (p<.001). So the efficacy of three weak acids was significantly differ from the view of total quality score and have the order EDTA>Formic-Nitric Acid>Formic Acid.

In table 11, On comparing the Efficacy score between various acids, it was found that the difference in Efficacy score among the three acids was highly significant (p<.001). So according to efficacy the acids can be arranged in order

EDTA Formic Acid >Formic-Nitric Acid<Perenyi's Fluid<Nitric Acid

EDTA Formic Acid >Formic-Nitric Acid<Perenyi's Fluid<Nitric Acid

In table 12, The quality of weak and strong acids in relation to the deformity produced is calculated by the deformity score which is the sum of three scores - Hard tissue staining score, Soft tissue staining score & Soft tissue attachment score.

On comparing the Deformity score among various acids.(which shows the less deformity) among various acids, it was found that the difference in deformity score among various acids was highly significant (p<.001). So according to deformity quality the acids can be arranged in order

EDTA> Formic-Nitric Acid> Formic Acid<Nitric Acid <Perenyi's Fluid

EDTA> Formic-Nitric Acid> Formic Acid>Nitric Acid>Perenyi's Fluid

DISCUSSION

In table 1, The result showed there was a proper agreement between observers thus there was no discrepancies on the part of observation.

Table 2 showed the average time of decalcification with a large variability present in time of decalcification. The similar results were obtained by Karpagaselvi, Sanjai. et al.[2] They also concluded that time taken for decalcification of neutral EDTA was maximum while nitric acid took least time for decalcification. The reason that could be attributed to this is EDTA is a chelating agent thus it takes higher time for removal of calcium from the specimen.

Table 3 shows all the qualitative parameter in respect of all the samples. The average ease of sectioning among all decalcifying agent did not show much variation while hard tissue staining shows a deviation of 2.86 ± 0.83 while average soft tissue staining score evaluated was 2.33±1.04. Soft tissue attachment showed a range of 1.89 ± 1.01 thus a significant variation seen in between soft tissue attachment and soft tissue staining. The result is in accordance with the study conducted by Joanna Zappa et al. found that teeth after decalcification had change in the form of shrinkage of tissue or decalcification.[3] During decalcification worst result was received during nitric acid decalcification while 10% EDTA showed best morphological images.

In other study by Sudha Jimson et al. similar result were obtained by them the reason that could be given that acidic demineralizer causes distortion of collagen fibres and deficiency in the affinity of histological stain while EDTA shows preservation and less distortion of collagenous tissue.[4]

Table 4 showed a descriptive summary of comparative time of decalcification among various tooth types. The inference achieved was that, the time taken for decalcification of teeth was found to be almost equal. In a study by Joanna Zappa et al. a slight change in the decalcification time of incisor, premolar and molar was noted, When subjected to different laboratories conditions.[5] In our study we have not altered the laboratories' condition thus the time of decalcification showed least variation among different types of tooth. The similar conclusion was achieved by Moulshree Kohli et al. that rate of decalcification is depended upon decalcifying agent temperature, agitation, microwave radiation, suspension and type and size of tissue.[6]

In table 5 there is a significant difference between strong acid and weak acid in decalcification time. In an similar study by Karpagaselvi Sanjay et al. it was reported that time taken for decalcification by EDTA was maximum while least for nitric acid.[2] The time of decalcification is determined by various factor temperatures. Pressure, agitation, electric current, microwave radiation, tissue suspension and size and type of tissue.[10] The reason that could be attributed to this is difference between the method of decalcification by EDTA and Nitric acid. The nitric is being a strong acid causes diffusion of calcium out of the tooth structure while EDTA acts by chelation of calcium ion..

In table 6. significant p value difference was seen in hard tissue staining and soft tissue staining, while in the case of sectioning and soft tissue attachment it did not show any significant P value on comparison of strong acid to weak acid and while overall conclusion regarding efficacy of weak acid was shown significant value(P= 0.001) was indicative of higher efficacy value of weak acid as compare to strong acid. The reason for the above could be the hard tissue and soft tissue staining showed a significantly better result in weak acid, similar result was found by Pratibha Prasad et al in their study.[7] As weak acid cause no chemical alteration of hard tissue and soft tissue while strong

acid cause changes in the soft tissue and hard tissue. The case of sectioning didn't differ much from strong acid and weak acid because our end point of decalcification was decided when complete mineral content was lost.[11]

In table 7,8 time of decalcification among two strong acid was calculated and significant differences was obtained between two acids showing (P value 0.001). In an similar study by Sudha Jimson et al. 5% nitric acid and HCL were used and nitric acid was found to be as faster decalcification agent as compared to HCL.[8] The reason for above finding could be attributed to 5% nitric acid an strong acid used in diagnostic laboratory that have a free H_2O ion concentration, associated with edema vacuolization rupture. The tissue stability and their effect in 5% nitric acid is due to the fact that quicker the decalcification , greater will be the injury and its effect on H&E section.[11]

Table 9 10 the time of decalcification is maximum for EDTA while least for formic nitric acid, there was significant p value less than 0.001 indicating that among weak acid also there was a difference between the efficacy of the acid. The efficacy of EDTA was found to be the maximum while least for the formic nitric acid. In a similar study by Archana Srinivasyaiah et al. it was found that EDTA and formic acid, irrespective of the method used EDTA have shown good overall structural detail.[9] In other study Sudha jimson et al. EDTA has shown to be best decalcifying agent, the reason could be that neutral EDTA is slower but details are preserved and the nuclear staining is good.[8] EDTA is a chelating agent slow in action and an excellent decalcifier for immunohistochemistry and electron microscope

In table 11 significant p value 0.001 was seen indicating of EDTA was the best decalcifying agent among strong acid and weak acid. The reason for the above finding could be that EDTA acts by the process of chelating while the strong acid causes chemical reaction between the hard tissue and acids. A similar result was obtained by Karpagaselvi Sanjai et al. it stated that neutral EDTA gives superior result due to mechanism of capturing metallic ion like calcium which binds to the chelating ions.[2] Thus it means that the calcium ions from the external layer of appetite crystal will be removed. When all calcium ions from the outer layer of appetite crystals was removed, they will be replaced from the deeper layer. In this way, crystal size decrease gradually producing an excellent preservation of tissue component.[8]

Table 12 The deformity score between nitric acid and EDTA were considerably different and shows a significant $p > 0.001$. The result is in accordance with Pratibha Prasad, et al which states that Perenyi's fluid in combination with other strong acid such as nitric acid and chromic acid showed maximum destruction, the reason could be given that, all strong acid can be accepted to be causing extensive tissue damage.[7] The stronger decalcifying agents causes its effects on H&E staining. The acid exposure to nucleus causes poor nuclear staining with cationic dyes such as Hematoxylin and the cytoplasm are over stained by briefest exposure to anionic dyes such as eosin. This effect can be of diagnostic significance .[12]

CONCLUSION:

An effective decalcifying agent must preserve the tissue architecture with a practical speed of decalcification for a rapid diagnosis. Thus to conclude, we can say that EDTA is a good decalcifying agent among strong acid and weak acid. which showed best morphological images while nitric acid cause extensive tissue damage.

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Conflicts of interest
There are no conflicts of interest.

FIGURES:

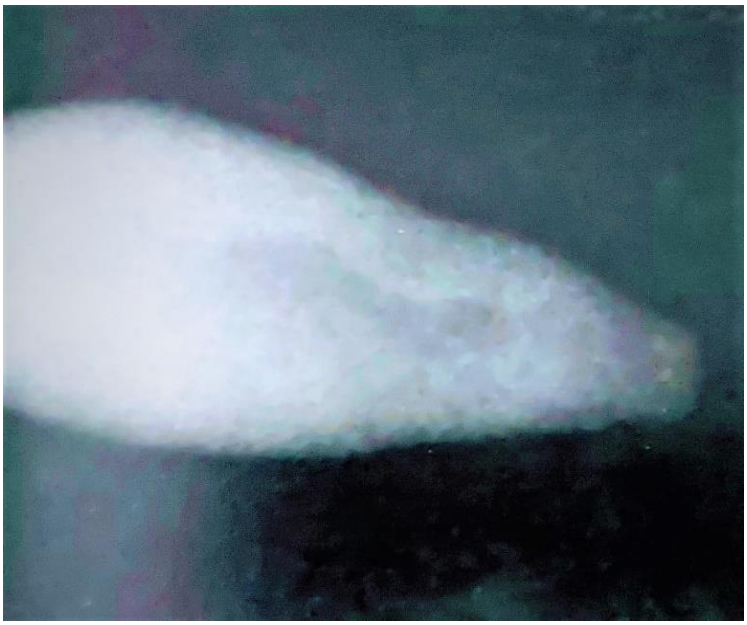

Fig 1 End point of decalcification of canine

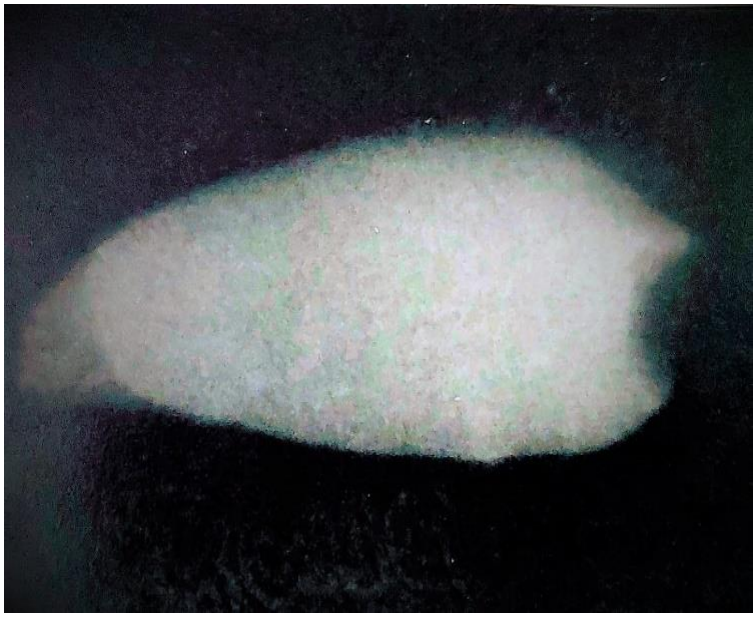

Fig 2 End point of Decalcification of Premolar

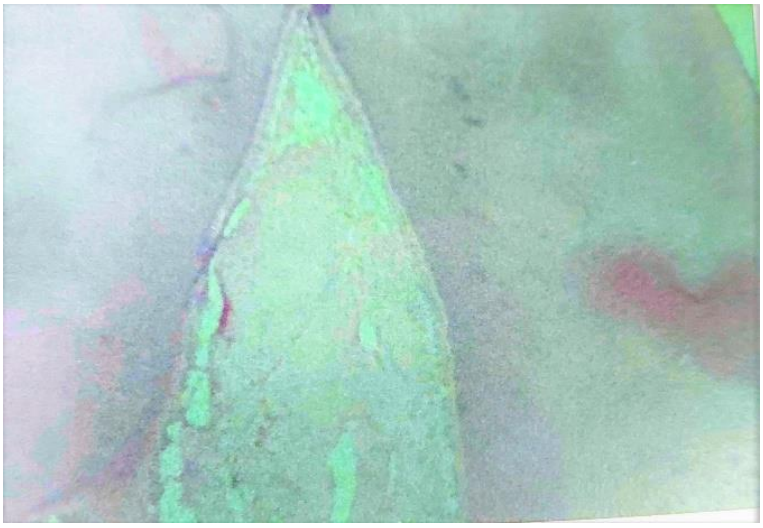

Fig 3 Decalcified section of Incisor in EDTA

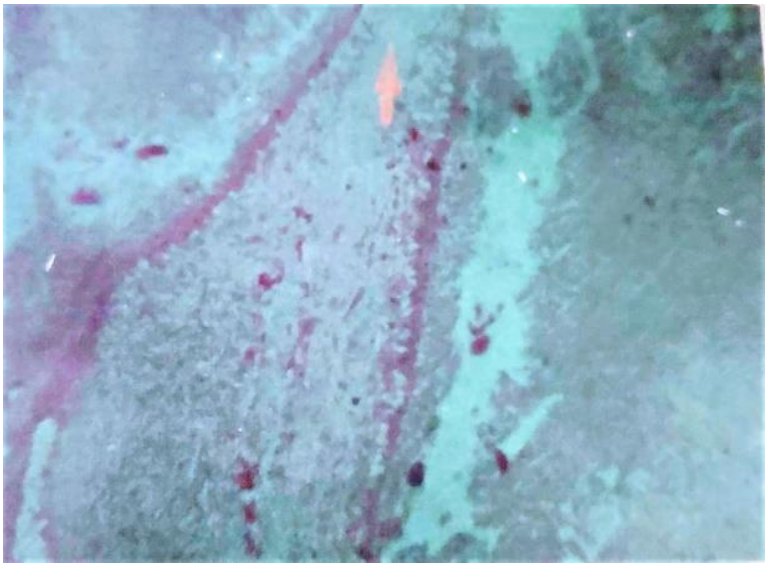

Fig 4 Decalcified section of Incisor in Perenyi's Fluid