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Comparison of Different Decalcifying Solutions on Human Teeth

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Keywords
Teeth decalcification, HCL, formic acid, nitric acid, neutral EDTA, decalcifying solution, pulp integrity.

Abstract
Introduction: In histopathology practice, decalcification is an important procedure during processing of a specimen. Number of decalcifying solutions were used till now but the potential and effect of it on the tissue and speed of decalcification are the basic pillars for the selection of a decalcifying solution. Some of the solutions eliminate the calcium rapidly and efficiently but they are detrimental for staining characteristics.
Aim: The research study was done for the evaluation of the time taken for decalcification of four decalcifying solutions and their worsening effect on staining characteristics.
Materials and Methods: four different decalcifying solutions namely, 37 % HCl, 5% Nitric acid,5 % Formic acid, 14% Neutral Ethylene Diamine Tetra Acetic acid (EDTA)were used for decalcification of 30 freshly extracted teeth. Decalcification end point was assessed by means of chemical method. Then teeth were processed routinely and stained H & E stain. Results: Neutral EDTA was the most innocent to the tissues architectural preservation and 37 % HCL was the least one. Conclusions: Neutral EDTA, in spite of being the slowest solution among all, gave very good results with respect to structural integrity, and excellent quality of staining.

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1. Introduction

Head and neck involve both soft and hard tissues which is quite complex architecture with respect to histochemical techniques. Soft tissues show mild resistance to the histopathological procedure, where the calcified tissues need complex, precise technique for diagnosis purpose.1 In histopathology practice, hard tissue examination is often a requisite such as in case of teratomas, bone, teeth, lesions those are partially calcified, odontomes and other bony lesions.1

Teeth are the hardest structure among all the tissues in the body. Because of a large amount of inorganic content in teeth, it is pretty hard to prepare a section for examination under a microscope.2Hard tissue preparation for microscopic evaluation include preparation of decalcified section and ground section. The soft tissues such as pulp, can be preserved only in decalcified section which is lost in ground section. 3,4

Decalcification is the process of removal of inorganic component such as calcium from the tissue, by means of various chemicals like acid and chelating agents and with the preservation of organic contents.2 So, the best decalcifying solution should be reasonably fast with the preservation of organic components and should not affect subsequent staining qualities. The time taken for decalcification and the worsening effect on the tissue, are two crucial criterions for the selection of a decalcifying solution. Some of the agents remove calcium ions thoroughly but they hamper the staining quality and may adversely affect the organic contents.1

Selection of decalcifying solution is done with respect to urgency of case, extent of investigation ,extent of mineralization, and staining technique to be performed.

Decalcifying solutions can be categorize as three main groups: strong acids, weak acids and chelating agents.

Strong acids such as nitric acid, hydrochloric acid,formalin-nitric acid and perenyi'sfluid are used as an aqueous solutions with concentration of 5-10%.Formic acid, picric acid, formic acid- formalin are weak organic acids. Of these, formic acid is used mostly as a primary decalcifier. Most commonly used chelating agent is EDTA (Ethylene Diamine TetraaceticAcid). Although EDTA is named as 'acid', it does not perform as acids but binds metallic ions, calcium and magnesium. More concentrated acid solutions perform decalcification more speedily but are detrimental to the tissues. Few other factors like temperature, agitation, suspension can also affect the rate of decalcification.

Determination of end point of decalcification is essential because incomplete decalcification makes microtomy of specimens difficult and over decalcification is undesirable as it may affect the staining procedure. There are few methods for end point determination for decalcification, with two considered as most decisive. One is radiography, with the use of X-ray unit and the chemical method. Few other method used are the weight loss, physical test and bubble test.

This research study was done to evaluate four decalcifying solutions for their speed of decalcification and their effect on subsequent staining procedure . Two strong inorganic acids (37 % hydrochloric acid, 5% nitric acid) and a weak organic acid (5% formic acid) and a chelating agent (14 % neutral EDTA) were included as a decalcifying agent for the study.

2. Materials and Methods:

A prospective study to compare and contrast among different decalcifying solutions was carried out in the Department of Oral Pathology and Microbiology. Patients advised for prophylactic extraction which were periodontally weak, impacted or because of orthodontic treatment purpose, were included in the study. Whereas the patients associated with pathological condition were excluded from the study.

The sample of the research study consisted of 40 freshly extracted teet collected from the Department of Oral and Maxillofacial Surgery.

The teeth were divided into 4 different groups. (Group A, Group B, Group C, Group D)

Group – A 37 % HCl

Group - B 5% Nitric acid

Group - C 5 % Formic acid

Group - D 14% Neutral Ethylene Diamine Tetra Acetic acid (EDTA)

Total 40 freshly extracted teeth including anterior and posterior teeth were included in the study and were kept in 10 % formalin for fixation for the period of 48 hours

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and divided into four groups. Different decalcifying agents were freshly prepared and the teeth were subjected to decalcification in different decalcifying solutions groups. Teeth were subjected to decalcification in 200 ml of solution at normal room temperature.

Solutions were agitated repeatedly and were replaced by fresh solutions every day till completition of decalcification. Decalcification end point was governed by chemical method. After the completion of decalcification process, teeth were subjected to washing under running tap water for the period of 24 hours for neutralization of the remaining acids and then processed routinely. Sections of 4µm thickness were obtained and staining was carried out.

Then the sections were examined with the light microscope and were subjected to score according to selected parameters as follows:Soft tissue details (1-poor, 2-fair, 3-good); Hard tissue detail (1-poor, 2-fair, 3-good); Quality of staining (1-poor, 2-fair, 3-good); Speed of decalcification(1-slowest,2-slow,3-fast,4-fastest)

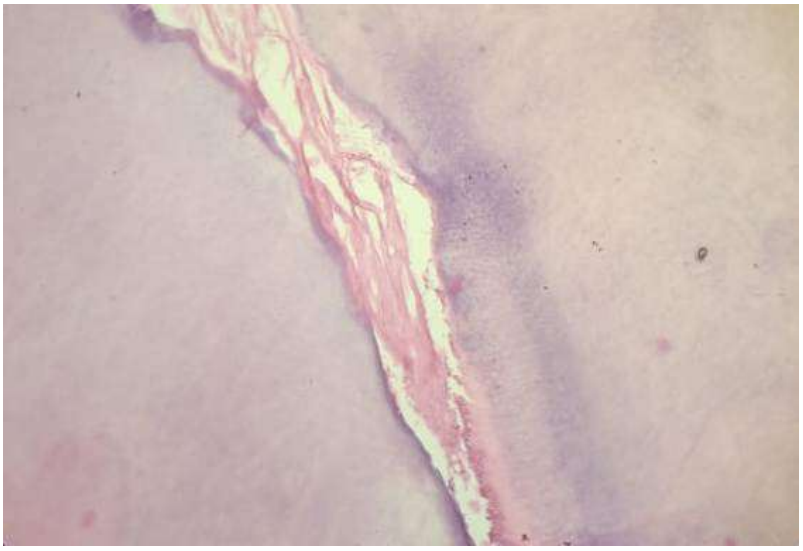

Figure 1: Photograph of tooth decalcification done in HCL (H and E stain, 10 X).

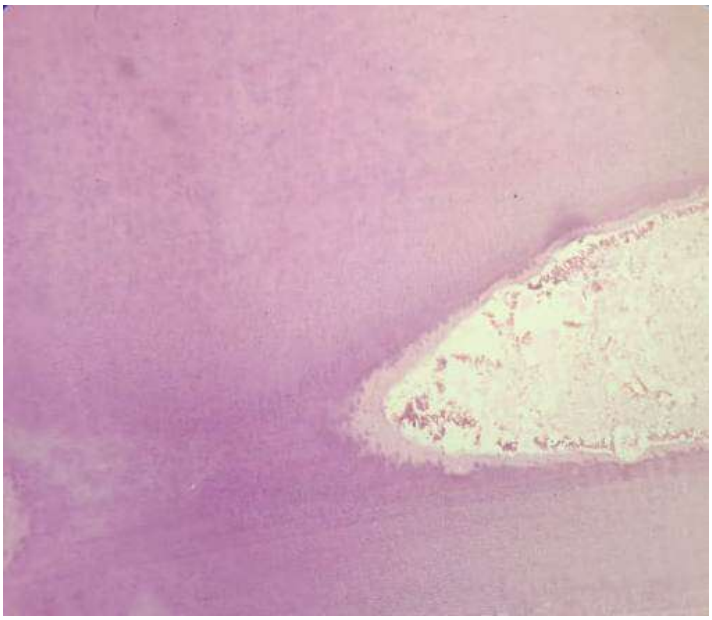

Figure 2: Photograph of tooth decalcification done in Nitric acid (H and E stain, 10 X).

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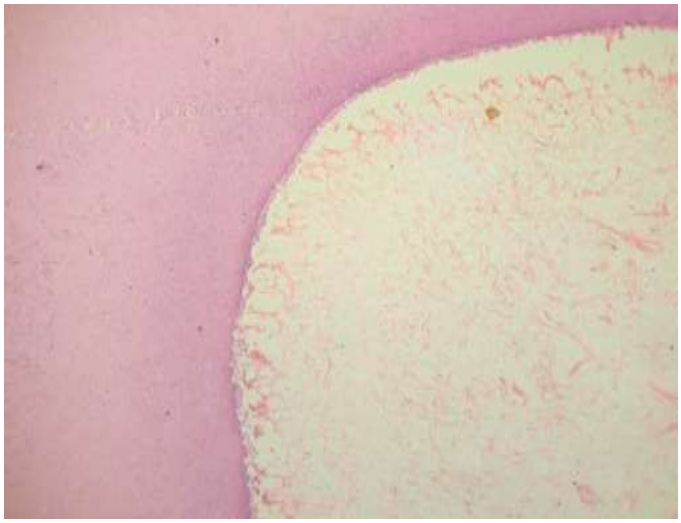

Figure 3: Photograph of tooth decalcification done in Formic acid (H and E stain, 10 X).

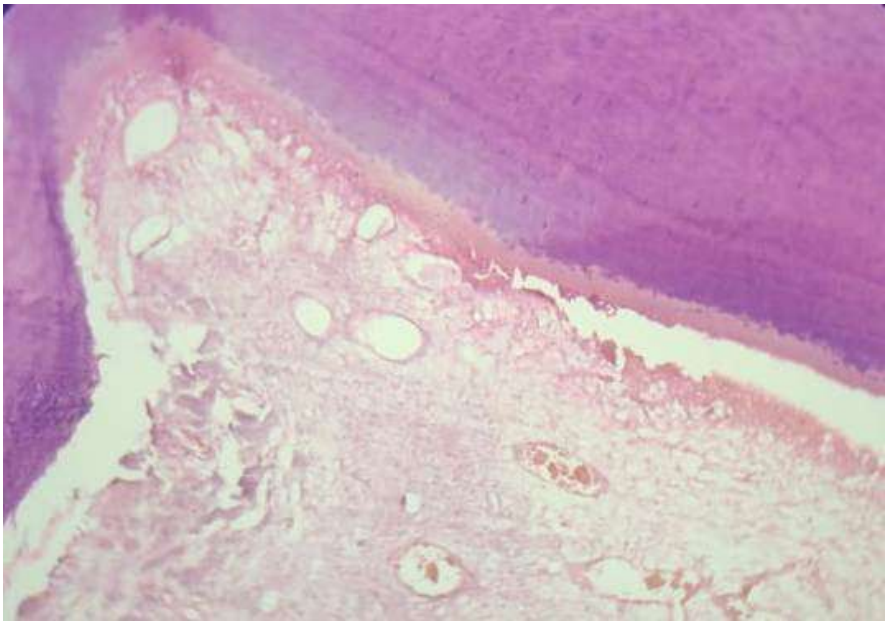

Figure 4: Photograph of tooth decalcification done in EDTA (H and E stain, 10 X).

3. Results

Total 40 freshly extracted teeth were used for the process of decalcification in four decalcifying solutions and were grouped as: Group A (Hydrochloric acid; Figure 1), Group B(Nitric acid; Figure 2), Group C (Formic acid; Figure 3), Group D(EDTA; Figure 4).After routine processing, 4 µm sections were stained using H & E stain and the 40 slides were evaluated by 3 qualified oral pathologists independently on the basis of the criteria laid down and graded according to parameters. The results of this assessment were then tabulated and subjected to statistical analysis and conclusions were drawn

Table 1represents the results obtained in relation to hard tissue preservation, soft tissue preservation, overall staining and time taken for decalcification in four different solutions. Mean value score for hard tissue preservation, soft tissue preservation, overall staining and time for group A is 2.20, 2.00, 2.10, 1.10 , for group B is 2, 1.90,1.10,2.20, for group C is 2.70,2.40,2.80,5.40, and for group D is 2.60, 2.50, 2.80, 53.40 respectively.Table 2 represents the cross tabulation of hard tissue preservation. Highest score 3

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was obtained in 3,1,7 and 7 number of samples respectively in Group A,B,C and D. Table 3 represents the cross tabulation of soft tissue preservation between group A,B,C and D. The number of samples obtained highest rank 3 are 3,2,4 and 5 respectively in group A,B,C and D.

Table 4 represents the cross tabulation of overall staining between group A,B,C and D. The number of samples obtained highest rank 3 are 1,2,8 and 8 respectively in group A,B,C and D.

Table 5represents the cross tabulation of rate of decalcification (time) between group A, B, C and D. Mean days taken for decalcification were are 1,2,5 and 53 respectively in group A,B,C and D.

Table 1: Mean Value Of Score Obtained In Four Groups.

	Hard tissue preservation	**Soft tissue preservation**	**Overall staining**	**Time(days)**
Group A	**2.20**	**2.00**	**2.10**	**1.10**
Group B	**2.00**	**1.90**	**2.10**	**2.20**
Group C	**2.70**	**2.40**	**2.80**	**5.40**
Group D	**2.60**	**2.50**	**2.80**	**53.40**

Table 2: Hard Tissue Preservation In Group A, B, C And D.

		Hard tissue preservation			
		1	**2**	**3**	**Total**
Solution	**A**	1	6	3	10
	B	1	8	1	10
	C	0	3	7	10
	D	1	2	7	10
Total		3	19	18	40

Table 3: Soft Tissue Preservation In Group A, B, C And D.

		Soft tissue preservation			
		1	**2**	**3**	**Total**
Solution	**A**	3	4	3	10

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	B	3	5	2	10
	C	0	6	4	10
	D	0	5	5	10
Total		6	20	14	40

Table 4: Overall Staining in Group A, B, C And D

		Overall staining(score)			**Total**
		1	**2**	**3**	
Solution	A	0	9	1	10
	B	1	7	2	10
	C	0	2	8	10
	D	0	2	8	10
Total		1	20	19	40

Table 5: Rate of Decalcification (Time In Days) in Group A, B, C And D.

		N	**Mean**	**Minimum**	**Maximum**	**P value**
Time(days)	A	10	1.10	1	2	
	B	10	2.20	2	3	
	C	10	5.40	5	7	<0.0001*
	D	10	53.40	52	55	
	Total	40	15.53	1	55	

4. Discussion:

Change is constant ' and many time inevitable, in this fast moving world, it is yet difficult to accept when the age-old procedures and techniques that are considered gold standard are challenged. Currently, change is what we need to go green and combat global warming. Being a responsible pathologist are we prepared for change, and can we imagine a more efficient decalcifying agent in our laboratory!! 5

Hard tissue preservation in original living state is a mandatory component for better understanding and interpretation of cellular as well as subcellular structures. Cutting quite thin sections by routine technique is difficult in the case of tissues containing calcified component within them such as teeth, bone

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and bony lesions. Such specimens must be subjected to removal of inorganic component by "decalcification", so that tissue become somewhat soft for easy cutting. 6

Decalcification is an essential histochemical technique to carryout research related structure of tooth, pulp calcification, and also to judge the pulp response. Failure to decalcify tissue or improper decalcification leads to ragged and torn tissue sections and may damage to microtome knife also.7

It is an important procedure from two aspects: first, sections of hard tissues such as teeth, bone, and surrounding tissues are difficult to prepare without decalcification; and second , the effects of the various decalcifying agents on the tissue architecture. 2

Decalcifying agents basically can be classified in three categories as strong acids, weak acids and chelating agents. In an attempt to include the commonly used agents from each category, 5 % nitric acid and 37 % hydrochloric acid as strong acid, 5 % formic acid as weak acid and 14 % EDTA as a chelating agent was included in our study.

In the study, it was initiated to determine the potential of four different decalcifying agents based on hard tissue preservation, soft tissue preservation, overall staining quality and rate of decalcification. A total of 40 extracted teeth were taken and were divided in four groups according to four different solutions. The decalcification was achieved within one day by 37 % HCl which was the fastest one where 14 % EDTA took longest time, that is 53 days. 5% nitric acid took 2 days, whereas 5%formic acid took five days.

In terms of efficiency of solutions with respect to hard tissue preservation, most favorable results were with the most time taking decalcifying agent, neutral EDTA. Eventhough 5 % formic acid also showed satisfactory results equivalent to that of neutral EDTA followed by 5 % nitric acid and 37% HCl. Preservation of soft tissue was found to be best with neutral EDTA and 5 % formic acid and fair results were obtained with 5% nitric acid and 37 % HCl gave poor results.

Decalcification by means of strong acids, open up the dentinal tubules rapidly and thus destroys the pulpal soft tissue readily. This was the main cause for fraying in the dentinal tubules(poor hard tissue preservation) along with destruction of odontoblastic layer(poor soft tissue preservation)was seen in section obtained using strong acid.8

Specimen decalcified with neutral EDTA and 5% formic acid showed the highest number of tissue sections with satisfactory staining followed by 5 % nitric acid and 37% HCl.

The most obvious feature of decalcification by strong acids is ruination of staining properties. This is again dependent on the solution pH and time taken to decalcify. Thus, faster the decalcification , the greater will be the injury and its effect on H & E staining.9

The overall structural integrity of the tissue was best preserved in samples decalcified in neutral EDTA. Cellular structure could be well recognized in all the 5 % formic acid demineralises tissue section, whereas they were not satisfactory in many sections prepared by 5 % nitric acid and 37 % HCl.

At gross examination, best quality obtained with neutral EDTA may be because of the removal of calcium ions which binds to the chelating component. This means that the calcium ion will be pull out from the external layer, they will be replaced by deeper layer ions. so the crystals size decreases gradually, results in the nearly best preservation of tissue architecture.2

Chelating agents (EDTA) for decalcification might bypass poor preservation and stain ability problem because that is the only agent which form the chelate with calcium ions and gradually depletes Hydroxyapatite crystals from the outer layer. Although the greatest disadvantage is being slowest of all, with time taken for decalcification is up to several weeks . 10

Strong acids as nitricacid and hydrochloric acids are used mainly with dense bony tissue because they remove bulk of calcium rapidly. So , these acids also negatively affect the cellular morphology. Although strong acid can carry out decalcification very rapidly, but causes serious ruination of stainability.8

Organic acids as formic acid are chosen where delicate soft tissue preservation is of prime concern. Organic acids performs slowly than mineral acids, and will require time to decalcify throughly .11

In our study, group IV containing EDTA followed by group III containing 5% formic acid gave

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comparatively satisfactory results with respect to of soft tissue component preservation and alsoless worsening effect on staining quality with eosin and hematoxylin. Other groups in which 5% nitric acid gave rapid and fair results whereas 37% HCl gave fastest but worst results overall.

5. Conclusion:

"Slow , steady wins the race".12

The results of the present study lead to a proposition that EDTA was the most considerate among all , although being slowest. When urgency is not a constraint , choise of neutral EDTA is advisable for its best tissue architectural preservation and least worsening effect on staining as well as for immunohistochemistry purpose.

However when the aim of decalcification is the diagnosis and the surgeon is reliant on the pathologist for the biopsy report, the balance has to be maintained and the solution that nearly satisfy both the parameters of time and tissue integrity was 5% formic followed by 5% nitric acid.

37% HCl decalcify at the fastest speed but overall worst results were obtained among all four groups.

To conclude, harder the tissue is, its quite hard to decalcify and Slower the decalcification, results would be better.

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