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Research Article

COMPARING THE EFFECTS OF THREE DIFFERENT DECALCIFYING AGENT ON EXTRACTED TOOTH-AN IN VITRO STUDY

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A B S T R A C T

Background: Head and neck is a complex structure of both soft and hard tissues, soft tissues put forth little resistance to the histochemical techniques. But hard tissues possess unique characteristic of hardness and sensitive procedures in the histopathology laboratory. The hard tissue may arrive at the laboratory for a number of reasons. Various pathologies may be associated with this hard tissue for which they need to be processed and stained so as to assist the pathologist in their reporting. For this reason the preservation of hard tissue close to the living state is essential for understanding of cellular and subcellular structures and functions. Before any calcified tissue can be processed and sectioned conventionally, the calcium must be removed. This process is called "decalcification" which is a chemical process that helps in further sectioning of the histological specimen. This can be done using chemical solutions like acids and chelating agents for preserving the organic skeleton.

Aims and objective: In the present study comparison between three different decalcifying agents with respect to rate of decalcification, the effect of decalcifying agents on the dental tissues, and its influence on the staining characteristics are being studied. Various methods for end point determination of decalcification can be done by chemical, physical, radiological and by weighing the specimen. The mechanical and radiological method does not allow the course of the procedure to be followed strictly. Thus, the need of quantitative analysis of calcium estimation from the decalcifying solution arose which was carried out using atomic absorption spectrophotometer.

Material and Methods: 45 freshly extracted human premolar teeth were taken within the age group of 15-25 years. They were then separated randomly into three groups of 15 each for decalcification in three different solutions.. The three groups include Group A-5% nitric acid, Group B-5% Formalin-Formic acid, Group C-5% EDTA. End point of decalcification of solutions were assessed using the chemical method. Decant decalcifying solutions that were saturated with calcium ions were thereafter sent for quantitative calcium estimation using atomic absorption spectrophotometer to confirm if end point of decalcification determined was accurate. The decalcified teeth were then routinely processed, sectioned, and stained with hematoxylin and eosin stains and observed under microscope by two independent observers and grading was done.

Results: Considering preservation and staining characteristics of both hard and soft tissues, superior results were obtained with 5% EDTA followed by 5% nitric acid and formic acid - formalin which was according to the respective mean values obtained.

Conclusion: EDTA, though being the slowest decalcifying agent among the three agents used in the study, gave excellent results for soft-tissue integrity, and best quality of both soft-tissue and hard-tissue staining.

INTRODUCTION

Science has formed the basic component of life. Continuous advancement of science and synthesis of scientific knowledge has formed the rational basis for change in the basic component. Undoubtedly, modern man is labor of constant scientific research. Thus, over the years, scientific knowledge and investigations have become the part and parcel of every profession.

Vertebrate tooth-like projections and jaws first appeared in the fossils about 405 million years ago in a class of bony

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Paleozoic fishes called the "Placodermi". All vertebrates except Agnatha (jawless fishes) either posses teeth or have evolved from toothless ancestors. The primitive ancestors of the vertebrates were polydont, as would be expected, since teeth originated from numerous dermal denticles.[1]

Dentition of man has been the subject of intense histological investigations for the past many years. As we know that the head and neck is a complex structure of both soft and hard tissues, soft tissues put forth little resistance to the histochemical techniques.[2] But hard tissues possess unique characteristic of hardness and sensitive procedures in the histopathology laboratory.[3]

The hard tissue may arrive at the laboratory for a number of reasons. Various pathologies may be associated with this hard tissue for which they need to be processed and stained so as to assist the pathologist in their reporting. For this reason the preservation of hard tissue close to the living state is essential for understanding of cellular and subcellular structures and functions.

Teeth are the hardest tissues in the body.[4] It is due to tooth enamel, which is denser and chemically more inert than other body tissues[5]. Enamel has more amount of calcium with a ratio of 96-98%, followed by the dentin layer with 70%, while in cementum the ratio is close to that of bone and is 50 %[6]. Because of these large amounts of inorganic components in teeth, like the calcium and phosphorus, the biological apatite is very hard to prepare for microscopic examinations [7]. Thus the physical hardness, which is, a unique characteristic of these tissues makes it necessary to "soften" them by removing the mineralized component [8].

Before any calcified tissue can be processed and sectioned conventionally, the calcium must be removed. This process is called "decalcification" which is a chemical process that helps in further sectioning of the histological specimen. This can be done using chemical solutions like acids (nitric acid) and chelating agents (Ethylene diamine tetra acetic acid - EDTA), while preserving the organic skeleton [7, 9]

Various methods for end point determination of decalcification can be done by chemical, physical, radiological and by weighing the specimen. The mechanical and radiological method does not allow the course of the procedure to be followed strictly. Thus, the need of quantitative analysis of calcium estimation from the decalcifying solution arose which was carried out using atomic absorption spectrophotometer.

Atomic absorption spectrophotometer (AAS) is a spectroanalytical procedure for the quantitative determination of chemical elements employing the absorption of optical radiation (light) by free atoms in the gaseous state. AAS determines the presence of metals and measures their concentration in liquid samples. Thus the level of calcium that is removed from the hard tissues during the process of decalcification could be determined using this device.[10]
In the present study comparison between three different decalcifying agents with respect to rate of decalcification, the effect of decalcifying agents on the dental tissues, and its influence on the staining characteristics are being studied.

MATERIALS AND METHOD

Freshly extracted, non-carious, non-attrited, with no developmental anomaly human premolar teeth were taken within the age group of 15-25 years. The teeth were fixed in 10% formalin over a period of month. They were then separated randomly into three groups of 15 each for decalcification in three different solutions.. The three groups include Group A-5% nitric acid, Group B-5% Formalin-Formic acid, Group C-5% EDTA.

Each sample was kept in a small bottle and then the prepared decalcifying agent was poured in a ratio of 20:1. The exact time at the start of decalcification was noted. The procedure was carried out at room temperature that varied from summer (32 - 35ºC) to winter period (18-22 ºC).

In case of Group A & B, the solutions were changed every day. Whereas for Group C, solution was changed every alternate day since EDTA did not get saturated with calcium ions within 24 hours.

End point of decalcification of solutions in Group A & B were assessed using the chemical method as follows:

1. Strong liquor ammonia was added drop by drop to 5 cm^3 of decant of the used decalcifying agent until it turned alkaline to litmus.
2. If solution turned cloudy, it indicated the presence of calcium and fresh decalcifying solution was replaced in the sample bottle.
3. If solution did not turned cloudy, 5 cm^3 of saturated ammonium oxalate was added to the solution.
4. If solution remained clear for 30 min, it concluded that end point of decalcification had been reached. [11]

For EDTA due to absence of the free calcium ion in the solution, a more primitive method of feel of the tissue was followed to roughly estimate the end point of decalcification.

Decant decalcifying solutions that were saturated with calcium ions were thereafter sent for quantitative calcium estimation to confirm if end point of decalcification determined was accurate. From each group a 10 ml of solution at three randomly chosen days were sent for calcium estimation using atomic absorption spectrophotometer.

After the decalcification process had being completed, the teeth in Group A and Group B were washed under running tap water for 10 minutes whereas Group C decalcified teeth were washed for 2 hours and there after routine processing had been carried out.

Then paraffin embedding had been done in waxbath and blocks were prepared, sectioned into 5 microns and fixed on albumin slides.

The sections were then stained with routine hematoxylin and eosin and then they observed under the microscope (10x, 40x 0bjective) and were graded from 1 to 4 from 1-4 [1-poor, 2-fair, 3-good, and 4-excellent] based on the following criteria:

1. Ease of sectioning;
2. Hard-tissue staining;
3. Soft-tissue staining - both cytoplasmic and nuclear staining;
4. Soft-tissue staining - nuclear staining;
5. Soft-tissue attachment;
6. Soft-tissue shrinkage and;
7. Pulpal organization.

Atomic Absorption Spectrophotometeric Procedure

From each of the three decant decalcifying solution used in the study, 10 ml of sample solution was collected and sent for AAS estimation and procedure carried out was as follows.

Step 1: As sample solutions were in acid, so no more acid was added. It was observed that all solutions received were clear.

Step 2: Calcium (Ca) standards were prepared in 2% HCl (Standard of 1ppm-2 ppm & 4ppm)

Step 3: The sample solutions and standards were aspirated on atomic absorption spectrophotometer and absorbance were recorded. If the absorbance were high the samples were diluted with distilled water (containing no Ca)

Step 4: The calculation were then done as follows:

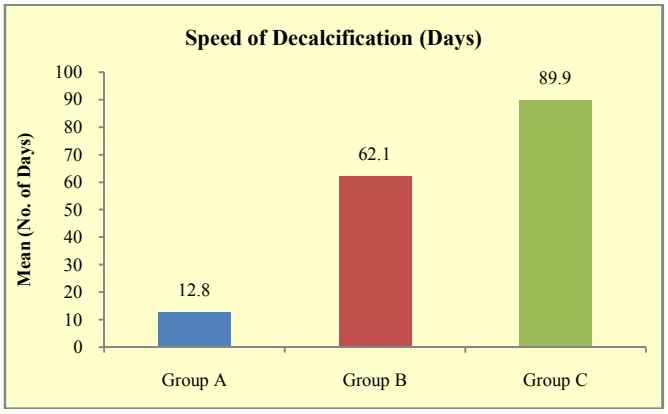
G ppm x dilution factor = ppm of Ca.

Statistical Analysis

Data collected were checked for accuracy and completeness and were coded and entered into the Statistical Package for Social Sciences (SPSS) software. Descriptive statistics for all studied variables and P-values by Chi-square test was used. P-value < 0.05 was considered to be statistically significant, while P-value < 0.05, P-value < 0.01, P-value < 0.001, was considered as Statistically Non-Significant.

RESULTS

The inter group comparison of speed or rapidity of decalcification was statistically significant (P-value < 0.05 using ANOVA) and was different for all the three groups. The number of days required was significantly more in Group C(5%EDTA) followed by Group B(5% Formalin-formic acid) and then Group A(5%nitric acid). **(**Graph no. 1)

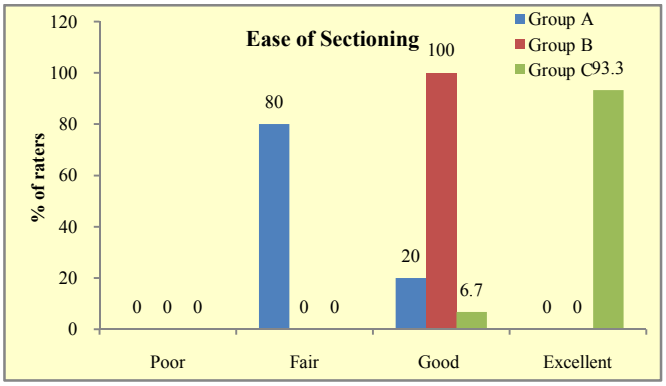


Graph 1 The inter-group comparison of speed of decalcification

The inter group comparison of ease of sectioning was compared and proved to be statistically significant (P-value<0.05 using Chi-square test). Ease of sectioning was "excellent" in Group C (5%) EDTA while it was "Good" in Group B (5% formalin-formic acid) as compared to Group A (5%nitric acid). (Graph no. 2)

For evaluation of hard tissue staining, soft tissue staining both cytoplasm and nuclear, soft tissue attachment, pulpal organization, soft tissue shrinkage, two observers (observer 1 & observer 2) had done the reading.

The inter-observer agreement (between observer 1 & observer 2) in evaluation of hard tissue staining, soft tissue staining both cytoplasm and nuclear, soft tissue attachment, pulpal organization showed significant (P-value = 0.001 using Kappa Statistic value) agreement in all the groups. (Table no. 1, Graph no. 3)

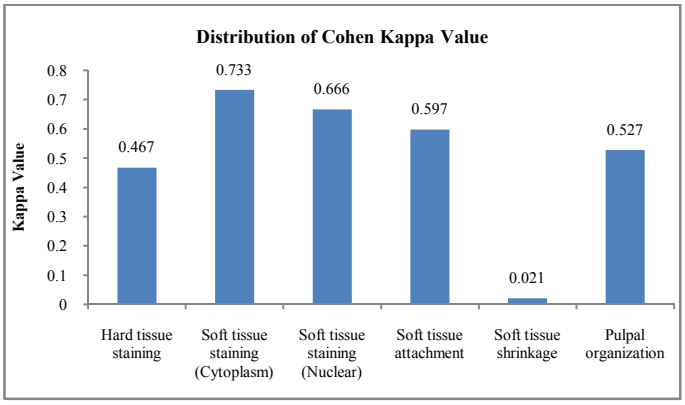


Graph 2 The inter-group comparison of ease of sectioning

Table 1 The inter-observer agreement in the evaluation of various staining characteristics (All groups combined)

Staining characteristics	All Groups Combined (n=45)	
	Cohen Kappa Value	P-value
Hard tissue staining	0.467	0.001***
Soft tissue staining (Cytoplasm)	0.733	0.001***
Soft tissue staining (Nuclear)	0.666	0.001***
Soft tissue attachment	0.597	0.001***
Soft tissue shrinkage	0.021	0.843NS
Pulpal organization	0.527	0.001***

Values are n (%). P-value<0.05 is considered to be statistically significant agreement. *P-value< 0.05, **P-value<0.01, ***P-value<0.001, NS: Statistically Non-Significant.



Graph no. 3 The inter-observer agreement in the evaluation of various staining characteristics (All groups combined).

While the inter-observer agreement (between observer 1 & observer 2) in evaluation of soft tissue shrinkage did not show significant (P-value = 0.843 using Kappa Statistic value) agreement in all the groups. (Table no. 1, Graph no. 3)

The inter-group comparison for hard tissue staining was significantly (P-value<0.05 using Chi-square test) different between Group A, Group B & Group C. Hard tissue staining was significantly "Excellent" in Group C as compared to Group A and Group B. (Table no. 2 , Graph no. 4)

The inter-group comparison for soft tissue staining (cytoplasm) was significantly (P-value <0.05, using Chi-square test) different between Group A, Group B & Group C. Soft tissue staining (cytoplasm) was significantly "Good" in Group A and Group C as compared to Group B. While it did

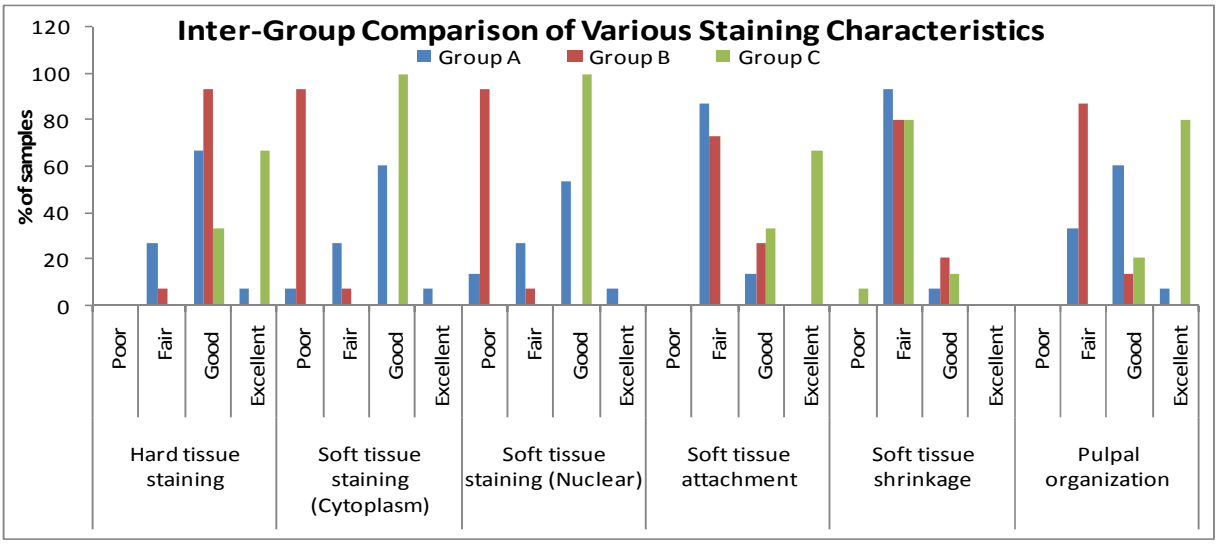
not differ significantly between Groups A & Group C.(Table no. 2, Graph no. 4)

between Group A, Group B & Group C. Soft tissue attachment did not differ significantly between Group A & Group B.

Table 2 The inter-group comparison of various staining characteristics

Characteristics	Group A 5% Nitric Acid (n=15)	Group B 5% Formalin-Formic Acid (n=15)	Group C 5% EDTA (n=15)	Inter-Group Comparisons (P-value)		
				Group A v Group B	Group A v Group C	Group B v Group C
Hard tissue staining						
Poor	0	0	0	0.177^{NS}	0.001^{***}	0.001^{***}
Fair	4 (26.7)	1 (6.7)	0			
Good	10 (66.7)	14 (93.3)	5 (33.3)			
Excellent	1 (6.7)	0	10 (66.7)			
Soft tissue staining (Cytoplasm)						
Poor	1 (6.7)	14 (93.3)	0	0.001^{***}	0.058^{NS}	0.001^{***}
Fair	4 (26.7)	1 (6.7)	0			
Good	9 (60.0)	0	15 (100.0)			
Excellent	1 (6.7)	0	0			
Soft tissue staining (Nuclear)						
Poor	2 (13.3)	14 (93.3)	0	0.001^{***}	0.028^{*}	0.001^{***}
Fair	4 (26.7)	1 (6.7)	0			
Good	8 (53.3)	0	15 (100.0)			
Excellent	1 (6.7)	0	0			
Soft tissue attachment						
Poor	0	0	0	0.361^{NS}	0.001^{***}	0.001^{***}
Fair	13 (86.7)	11 (73.3)	0			
Good	2 (13.3)	4 (26.7)	5 (33.3)			
Excellent	0	0	10 (66.7)			
Soft tissue shrinkage						
Poor	0	0	1 (6.7)	0.283^{NS}	0.475^{NS}	0.549^{NS}
Fair	14 (93.3)	12 (80.0)	12 (80.0)			
Good	1 (6.7)	3 (20.0)	2 (13.3)			
Excellent	0	0	0			
Pulpal organization						
Poor	0	0	0	0.011^{*}	0.001^{***}	0.001^{***}
Fair	5 (33.3)	13 (86.7)	0			
Good	9 (60.0)	2 (13.3)	3 (20.0)			
Excellent	1 (6.7)	0	12 (80.0)			

Values are n (%). P-values by Chi-square test. P-value<0.05 is considered to be statistically significant. *P-value< 0.05, **P-value<0.01, ***P-value<0.001, NS: Statistically Non-Significant.



Graph no. 4 The inter-group comparison of various staining characteristics

The inter-group comparison for soft tissue staining (nuclear) was significantly (P-value<0.05, using Chi-square test) different between Group A, Group B & Group C. Soft tissue staining (nuclear) was significantly "Good" in Group A as compared to Group B and also "Good" in Group C as compared to Group A & Group B) (Table no. 2, Graph no. 4)

The inter-group comparison for soft tissue attachment was significantly (P-value<0.05, using Chi-square test) different

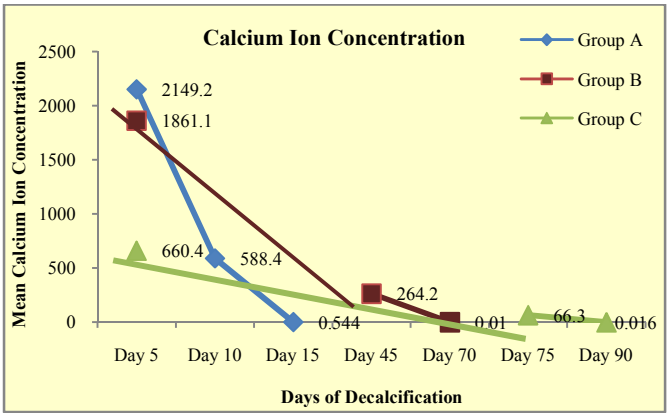
While it was significantly "Excellent" in Group C as compared to Group A& Group B. (Table no. 2, Graph no. 4)

The inter-group comparison for soft tissue shrinkage did not differ significantly (P-value<0.05, using Chi-square test) between Group A, Group B, Group C. (Table no. 2, Graph no. 4)

The inter-group comparison for pulpal organization was significantly (P-value<0.05, using Chi-square test) different between Group A, Group B, Group C. Pulpal organization was

significantly "Good" in Group A as compared to Group B and significantly "Excellent" in Group C as compared to Group A & Group B. (Table no. 2, Graph no. 4)

The inter-group distribution of calcium ion estimation by AAS was different for Group A, Group B & Group C for 5th , 10th , 15th , 45th , 70th , 75th , 90th days. (Graph no. 5)



Graph no. 5 The inter-group distribution of Calcium Ion Estimation

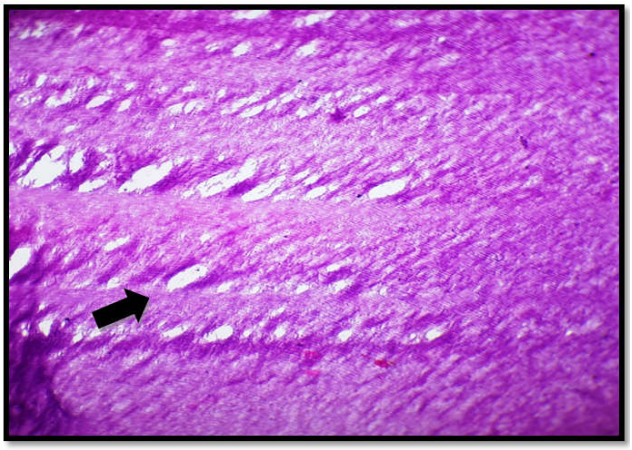


Figure 1 (a) Photomicrograph under 10x objective showing distortion of dentinal tubules in

Group A: 5% Nitric acid

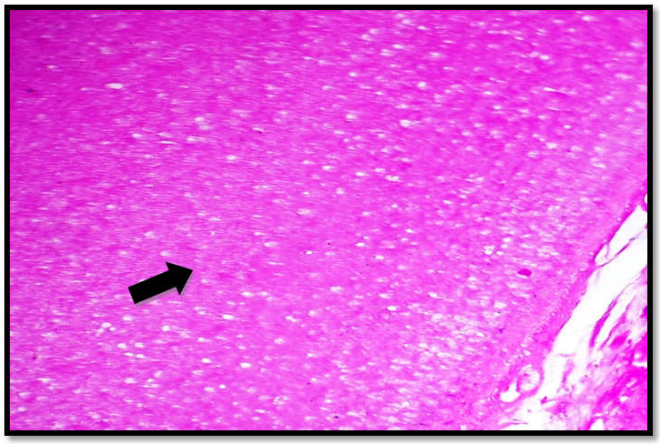


Figure 1 (b) Photomicrograph under 10x objective showing distortion of dentinal tubules in

Group B: 5% Formalin-Formic acid

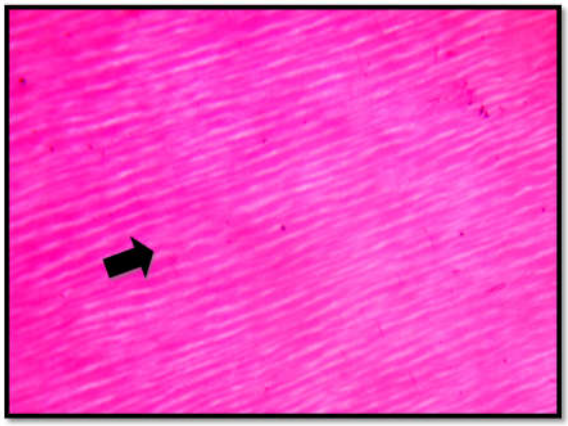


Figure 1 (c) Photomicrograph under 10x objective showing intact dentinal tubules in

Group C: 5% EDTA

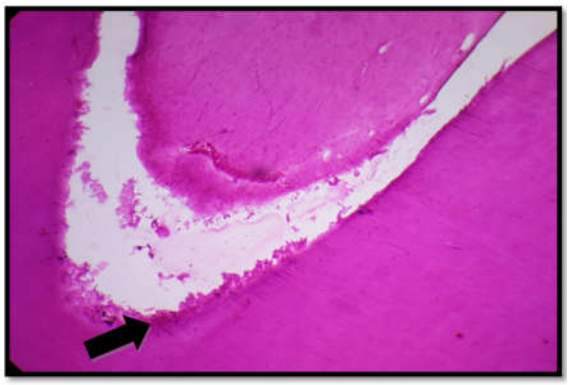


Figure 2 (a) Photomicrograph under 10x objective showing soft tissue (pulpal) detachment from hard tissue (dentin) in
Group A: 5% Nitric acid

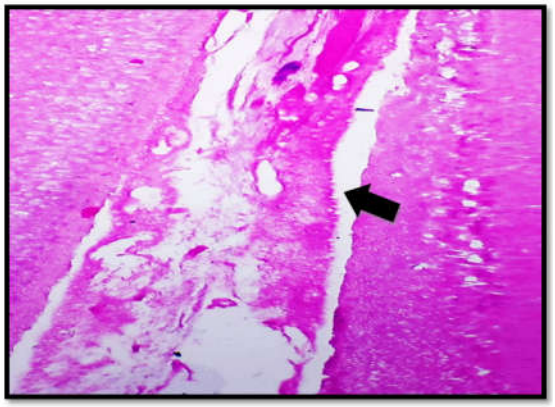


Figure 2 (b)Photomicrograph under 10x objective showing soft tissue (pulpal) detachment from hard tissue (dentin) in

Group B: 5% Formalin-Formic acid

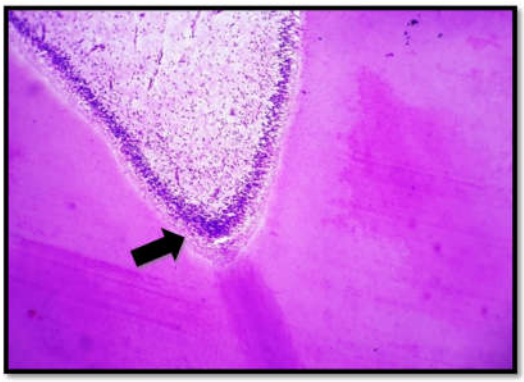


Figure 2 (c) Photomicrograph under 10x objective showing excellent soft tissue attachment in

Group C: 5% EDTA

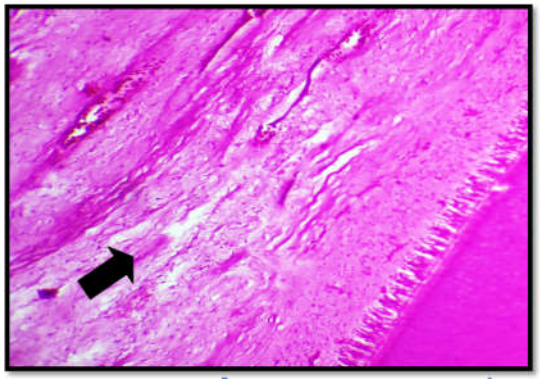


Figure 3 (a) Photomicrograph under 10x objective showing good pulpal organization in

Group A: 5% Nitric acid

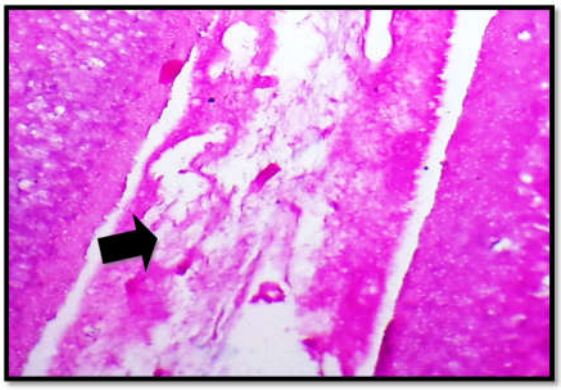


Figure 3: (b) Photomicrograph under 10x objective showing fair to poor pulpal organization in

Group B: 5% Formalin-Formic acid

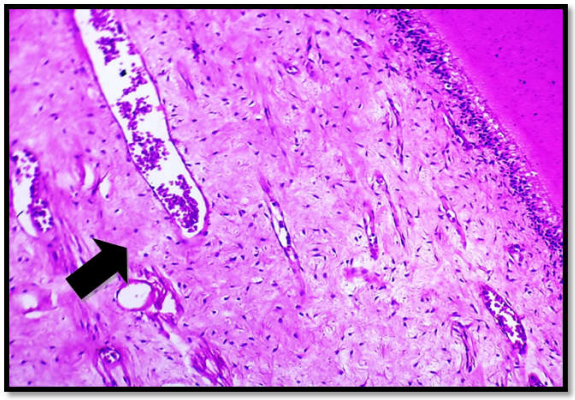


Figure 3 (c) Photomicrograph under 10x objective showing excellent pulpal organization in

Group C: 5% EDTA

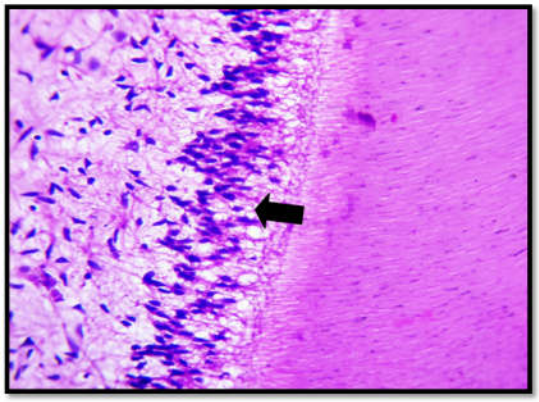


Figure 4 Photomicrograph under 40x objective showing excellent odontoblasts in

Group C: 5% EDTA

DISCUSSION

Decalcification is most important in the preparation of oral calcified tissues for microscopic examination. It is important from two stand points: first is the sections of teeth, bone, and surrounding tissues are difficult to obtain without removal of the calcium; and second is the effect of the various chemical decalcifiers upon the tissue components differ. Failure to decalcify tissue with large amounts of calcium salts will result in torn and ragged sections and damage to the cutting edge of the microtome knife. [(12)]

Preparation procedure of hard teeth tissues starts from its fixation[7].It has been shown by **Cook and Ezra Cohn (1962)**[13] that tissue damage during acid decalcification is approximately four times greater when the tissue is unfixed. Choice of a fixing reagent is dependent upon the tissue itself and the purpose for which it is to be preserved[7].

Fixation of teeth was prolonged as access to the pulp for fixative solution is limited to the apex which progressively becomes narrower with age. As teeth for the study was being collected over a period of one month, fixation of most of the teeth extended through this period ensuring adequate fixation.

It is sometimes recommended that the apex should be cut off to allow easier penetration or a hole may be prepared in the crown portion[14].A procedure that was not followed in the present study. As these practices are apt to disturb the position of the pulp and should be avoided as stated by **Cowdry (1978*)***.

Fixation with either 4% paraformaldehyde or 10% formalin seems to preserve the pulp tissue and maintain favorable conditions for examination and microscopic analysis of its cell components. Also, unbuffered formalin solution can better decalcify compared to buffered solution because acid component is not neutralized[15]. Thus in the present study we used 10% unbuffered formalin to fix the tissues because it is more readily available and may be stored for longer periods of time.

Many studies have been conducted to introduce new decalcifying agents and to modify the presently used agent. These purposed to meet and identify the most efficient decalcifying agent which ensured complete removal of calcium without causing any damage to tissue architecture as well as providing adequate staining characteristics[2,16].

Most authors have compared two to six decalcifying agents, sometimes varying the methods used and by employing a lot of permutations and combinations of the methods and agents, mainly to decalcify bone and teeth.[2,5,7,16,17,18,19,20]

In the present study, we used three different decalcifying agents, a strong acid - nitric acid, a weak acid-formalin & formic acid and a chelating agent - disodium salt EDTA. And then a comparison was made with respect to rate of decalcification, the effect of decalcifying agents on the dental tissues, and its influence on the staining characteristics.

Fresh stock of decalcifying agents was prepared just before placing the teeth within them and before each change. This was to prevent formation of yellow discoloration that has been observed when using the old nitric acid[14].

The established formulae of decalcifying agents were designed to strike a balance between desirable and undesirable agents. All decalcifying solutions used were adjusted to a

concentration of 5% as Cullings *et al* (1985) stated the stronger and more concentrated the acid, the harsher the effect.[21]

Callis & Sterchi (1998) stated that nitric acid can decalcify rapidly causing tissue swelling and damaging the tissue stainability[19]. Therefore a 5% solution of nitric acid was used in our study.

Studies by Clayden (1952) showed that formic acid more than 10% tends to be cloudy and masks the result. Therefore in our study a 5% solution of formic acid was selected, to which 5% formalin was added to permit simultaneous fixation and decalcification. However this additive increased the time required for decalcification[14].

EDTA is most commonly used in the form of its disodium salt. The rate at which EDTA will decalcify is pH dependent. As Preece Ann (1972) stated that because the alkaline solution of EDTA has a tendency to hydrolyze proteins to some degree and leads to tissue damage[22], so a 5% EDTA at a pH of 4.7 was used in our study.

One important criteria of an efficient decalcifying agent is the reasonable speed of decalcification[2]. To further enhance the speed of decalcification three criteria were followed in our study:

1. To ensure that all surfaces of the tooth decalcify simultaneously, it was placed on a gauze pack within the bottle containing the decalcifying solution.
2. Constant agitation at regular intervals was undertaken, thus stripping off the accumulation of particles on the surface, preventing re-precipitation and stagnation artifacts, removing carbon dioxide bubbles from the surface of the specimen & thereby allowing an intense contact between the tooth specimens & decalcifying agent.
3. To maintain the concentration of decalcifying agents for both strong and weak acid solution were changed daily while for chelating agents it was changed on alternate days. Thus ensuring that active reagent was not depleted. In the case of both nitric acid and formic acid-formalin, used for decalcification soluble calcium salts are formed whereas the chelating agent (EDTA) binds to calcium ions.

In our study speed of decalcification was more for 5% nitric acid followed by 5% formalin–formic acid while EDTA being the slowest.

Maurine William AB (1937) in his study reported that time for decalcification by 5% nitric acid was 7-9 days for single adult tooth[23]. But in our study the time taken varied from 12-15 days for single & double rooted tooth. (Graph no. 1)

Morse (1945) using formic acid-sodium citrate decalcifying solution observed that single extracted teeth underwent decalcification between 7-24 days[24]. However in our combination of formic acid-formalin decalcification occurred in 45-75 days. This slowing down of decalcification process is due to formalin, which is known for this property. (Graph no. 1)

On the other hand, EDTA has been observed to take 4 days to 8 weeks depending on composition of specimen and is well known for being a very slow decalcifying agent[25]. In our study it was observed that the tooth specimen placed in this chelating agent took 90-100 days. (Graph no. 1). It has been documented that specimens left in EDTA for over a prolonged period will not affect sectioning or staining of the same.

For the three decalcifying solutions used, the prolonged decalcification period may be due to variations in thickness of samples & the variations in room temperature, as the study was conducted in summer and winter periods for which a control temperature had not been maintained.

For ease of sectioning, it is necessary to determine the end point of decalcification. Various methods have been used for this purpose. In our study, end point of decalcification was done by the chemical test using saturated ammonium oxalate.

5% nitric acid were sectioned easily but were not being retained within the paraffin ribbon thus posing difficulty in their handling (Graph no. 2). This is in accordance to the results obtained by K Sanjai *et al* (2012) and Joshi Hemal *et al* (2014) who also faced problems in sectioning of tissues decalcified in 5% nitric acid[2,26].

While 5% formalin-formic acid gave good sectioning and 5% EDTA gave the excellent ease of sectioning by microtome knife (Graph no. 2). These observations were in accordance to the results obtained by Prasad Pratibha *et al* (2013) who noted that tissue decalcified in EDTA were easy to handle and not friable[8].

In our study, we observed and compared the effects of the three different decalcifying agents on hard and soft tissue staining (both cytoplasm and nuclear), soft tissue attachment, soft tissue shrinkage and pulpal organization (Graph no. 4).

With nitric acid it was expected, being a strong acid and rapid decalcifier to have more adverse affects on the subsequent staining characteristics of the tissue. However, in our study it was observed that they had a slightly better staining characteristic as compared to formic acid-formalin and gave better three differential shades of eosin (cytoplasm) almost comparable to EDTA. Satisfactory nuclear staining was observed with 5% nitric acid proving that fixation was adequate; however with EDTA nuclear staining was superior. (Figure 1a)

Though formic acid in combination with formalin has been documented to give good staining characteristics to both hard and soft tissue, this was not observed in our study. 5% formalin was used to accomplish gradual fixation along with decalcification process. However it prolonged the decalcifying procedure, causing swelling and affected the staining characteristics by producing a deep, unpleasant, brick red color, without producing the three preferred differential shades of eosin. It also affected the nuclear staining character. This proves that any acid, whether weak or strong has damaging effects on tissue stainability[14]. (Figure 1b)

It was observed that even though specimens of teeth that were placed in 5% EDTA for a prolonged period as compared to other decalcifying agents, it did not produce any detriment to the subsequent staining of both hard and soft tissue, both cytoplasmic & nuclear. (Figure 1c)

It was observed that with both 5%nitric acid and 5%formalin-formic acid, soft tissue attachment of pulp to dentinal wall was poor whereas with 5% EDTA excellent attachment was observed. This was in accordance with similar studies by Zappa *et al* (2005), K Sanjai *et al* (2012), Pratibha Prasad *et al* & R Sangeetha *et al* (2013)[2,3,5,8]. (Figure 2a,b,c)

The soft tissue detachment that was seen in both nitric acid and formalin-formic acid decalcifying agents may be accounted for, by the thickness of dentin that obstructed the inner pulpal tissue from attaining proper dehydration, causing separation of organic components of different densities-dentin & pulp. It is for this reason that some authors have suggested incorporation of slow dehydration process when treating hard tissues.

Butyl alcohol has been recommended by Morse (1945) for dehydration of hard tissue specimens as it permits slow dehydration without the serious hardening effect of ethyl alcohol as well as causes the clearing of the tissue in one step. Thus eliminating the necessity of clearing the tissue in other clearing agent which has a tendency to further harden the tissue[24].

Shrinkage of pulp from dentinal border and preservation of cellular details is dependent on fixation and the choice of decalcifying agent as well as processing technique[3,8]. On the other hand, adequate preservation of the soft tissues leaves the specimen incompletely decalcified. Thus, it is difficult to fulfill the requirements for simultaneous analysis of mineralized and non mineralized tissues[8]. In our study, soft tissue shrinkage from dentinal wall in teeth was seen in 5% nitric acid, formalin-formic acid, EDTA, with statistically insignificant results.

The pulpal organization with its extracellular matrix and histological zones were clearly distinct and excellent in teeth decalcified with 5% EDTA which was in accordance to K Sanjai *et al* (2012)[2]. Strong acid decalcification opens up the dentinal tubules quickly and serves as a pathway to the pulp tissue thus destroying the odontoblast architecture. In our study teeth placed in 5% Formalin-formic acid though weak in nature, showed more damage to the odontoblastic layer when compared to 5% nitric acid. The purpose of adding formalin to formic acid was to simultaneously accomplish both fixation and decalcification. However this prolonged the speed of decalcification and caused swelling of the tissue, thereby distorting the odontoblast architecture. (Figure 3a,b,c)

From the present results of the study, it was observed that 5% nitric acid was a rapid decalcifying agent that retained sufficient staining characteristic and tissue reactivity whereas 5% formalin-formic acid was a poor decalcifying agent in terms of time required for decalcification, staining characteristics and tissue reactivity. In case of 5% EDTA, though it took more extensive period than the other two agents to decalcify, the staining characteristics and tissue reactivity were excellent (Figure 4).

The 10ml of decant decalcifying solutions containing calcium obtained on the three consequent days for the individual agents used, had been aspirated into the flame at a temperature of 2000 - 3000ºC. The electrons in calcium were initially in their lower energy state and when the narrow beam of visible light was passed through the flame with a wavelength adjusted to 422nm that corresponded to an electron transition of calcium. When heated the calcium atoms get excited and their electrons go to higher energy levels. When the electrons fall back to lower levels, visible radiation is given off. The energy of the emitted photons corresponds to the energy difference of the calcium atom electron level. Thus the amount of radiation absorbed was then related to the concentration of calcium ions in the solution using Beers Law.[27]

Using AAS, we were able to estimate the concentration of calcium ions left behind in the decant decalcifying solution at different intervals that differed for the individual decalcifying agents used. This was done keeping in mind that strong acid decalcified rapidly whereas the weak acid and chelating agent took longer periods.

The first day, day 5 for observation by AAS, for decalcifying solutions were common for all the three groups. As observed from Graph no. 5, that the amount of calcium in the decant solution of day 5 for 5% nitric acid was much more (2149.2ppm) than 5% formalin-formic acid (1861.1ppm) and 5% formalin-formic acid showed much more calcium levels than 5% EDTA (660.4ppm) for the same said period. This reaffirms that nitric acid is a much rapid decalcifying solution when compared to formalin-formic acid and EDTA.

End points, as determined by chemical method using saturated ammonium oxalate was confirmed on days 15, 75 and 90 for 5% nitric acid, 5% formalin- formic acid and 5% EDTA respectively.10 ml of the decant solution for these three above mentioned days was put through the AAS to estimate the calcium level present and to reaffirm the end point obtained by the chemical method. When using EDTA, as decalcifying agent calcium ions remained attached and the availability of the free ions in solution were not available for chemical end point determination as per literature. Therefore a more primitive method of feel of tissue was followed in EDTA treated tissues so as to roughly estimate the end point of decalcification. However using AAS accurate end point estimation for EDTA could be determined based on the working of AAS. Thus calcium levels in solution on the above last three days were nil and proved that chemical end point determination was accurate for both the strong and weak acids used in the study.

End point determination is highly recommended while decalcifying any hard tissue. This is mainly to prevent tissue damage and impaired staining characteristics especially while using strong and rapid decalcifying agents.

CONCLUSION

It is evident that when urgent histopathologic reporting is required, use of strong acids (5% nitric acid) may be employed judiciously that preserve tissue integrity. EDTA may be strongly recommended as a good decalcifying agent when hard tissue, tooth, needs to be processed for academic or research purposes giving a excellent soft tissue integrity and quality of staining.

Nitric acid being a strong acid care needs to be taken in the handling and disposing of used solutions as it may lead to corrosion of skin and affects the container integrity and EDTA is a much safer alternative.

However other combination with formic acid, a weak acid, needs to be studied further as it is safer to handle than nitric acid and has been documented to be gentler on tissue.

However the further search for alternative decalcifying agent needs to be researched that would strike a balance between speed and maintenance of hard and soft tissue integrity ideal for histopathology study as well as being safe to handle and dispose.

During the course of our study few limitations were observed and may be considered for further research:

1. Radiographic ascertaining of structural density of the tooth may be performed prior to the process, to determine a suitable decalcifying agent.
2. Permutation and combinations of other gentle decalcifying agents such as formic acid-sodium citrate may be used as they provide brilliant nuclear stain and slight swelling of the cytoplasm and collagenous fibers.
3. Maintenance of a standard temperature may be used as this affects the rate of reaction.
4. Use of alternate dehydrating agents like butyl alcohol that have documented advantages over ethyl alcohol as this slows down the dehydration process that is required for decalcified hard tissues.

Thus a larger sample size can be studied keeping these above factors in mind.

Even though advance techniques have evolved that have advantages over conventional acid decalcification, the use of these acid decalcification methods which is time consuming and is difficult to monitor adequately, still proves to be convenient and reliable.

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