Tooth decalcification using different decalcifying agents – A comparative study

Amberpreet Kaur Khangura, Shally Gupta, Anubha Gulati, Simranjit Singh
Department of Oral Pathology & Microbiology, Dr. Harvansh Singh Judge Institute of Dental Sciences and Hospital, Panjab University, Chandigarh, India

Abstract

Background: In microscopic assessment of mineralized tissue, decalcification is an important step during tissue processing. The present study was attempted to compare the efficacy of various decalcifying agents and to evaluate the most efficacious decalcifying agent.

Aims and Objectives: The aim was to study and compare the time taken for complete decalcification of the specimen by six different chemical agents; to study and compare the effect of various decalcifying agents on cellular and nuclear changes of hard and soft tissues; to study and compare the effect of various decalcifying agents used on the staining intensity with Ehrlich’s Hematoxylin and Eosin stain and to determine the ideal decalcification technique.

Materials and Methods: The six decalcifying agents, namely 5% nitric acid, 8% formic acid, formalin-nitric acid, 5% trichloroacetic acid, neutral ethylenediaminetetraacetic acid (EDTA) and Perenyi’s fluid were used to decalcify 30 human permanent teeth (5 teeth in each solution). The endpoint of decalcification was evaluated by chemical (calcium oxalate test) as well as radiographic methods. The specimens were then subjected to processing, sectioning and staining with hematoxylin and eosin. The stained sections were observed under a light microscope and grading was done.

Results: The results in the present study confirmed the fact that the time required for complete decalcification process was least in Perenyi’s fluid, 5% trichloroacetic acid and highest in 14% EDTA. Teeth decalcified in 5% trichloroacetic acid, 8% formic acid, formalin-nitric acid and 5% nitric acid were easy to section. Sectioning was most difficult for teeth decalcified in Perenyi’s fluid and 14% EDTA. The overall structure details as well as staining characteristics were best in teeth decalcified by 5% trichloroacetic acid and neutral EDTA and worst in teeth decalcified by Perenyi’s fluid.

Conclusion: Five percent trichloroacetic acid was showing the most efficient result as it balances both tissue integrity and time factor suggesting that it can be used as a stable decalcifying agent for routine histopathological diagnosis.

Keywords: Decalcification, decalcifying agents, histopathology

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/jomfp.jomfp_203_21

How to cite this article: Khangura AK, Gupta S, Gulati A, Singh S. Tooth decalcification using different decalcifying agents – A comparative study. J Oral Maxillofac Pathol 2021;25:463-9.
INTRODUCTION

Teeth are composed of both organic as well as inorganic content. Tooth enamel is the most mineralized tissue of the human body. It consists of 96% of inorganic content as well as 4% of organic content and water by weight. Bulk of the tooth is made up of dentin, it consists of 70% of inorganic content and 30% of organic content by weight. The inorganic material of both enamel and dentin is mainly composed of calcium phosphate arranged as the hexagonal hydroxyapatite \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \). The organic matrix of enamel is made up of noncollagenous proteins (90% amelogenins and 10% nonamelogenins) and enzymes. Nonamelogenins in enamel are ameloblastin, enamelin and tuftelin. The inorganic matrix of dentin is composed of collagenous and noncollagenous proteins. The collagenous proteins are type I as well as type V collagenous fibrils. The noncollagenous proteins are dentin phosphoprotein, dentin matrix protein 1, dentin sialoprotein (DSP), osteopontin and osteocalcin. The central core of dentin is made up of soft tissue known as pulp. Pulp is a specialized connective tissue composed of fibers, cells, blood vessels, nerve terminations as well as ground substance.\(^1\)

Ground sections are very useful for the study of inorganic components of teeth, but decalcification is mandatory to study the organic components. Decalcification is the removal of inorganic calcium from the organic collagen matrix, calcified cartilage as well as surrounding tissues, so it is a routinely used technique in most histopathology laboratories for the microscopic evaluation of calcified tissues. Decalcification can be carried out by various methods such as the use of heat, vacuum, electric current as well as chemical agents. Among them, the chemical agents are the most commonly used for routine histopathological analysis. Three types of chemical agents can be used, they are strong inorganic acids, for example, nitric acid and hydrochloric acid; weak organic acids, for example, formic acid, acetic acid and picric acid and chelating agents, for example, EDTA.\(^2\)

Chemical agents usually react with calcium present in teeth to form soluble calcium salts or act as chelating agents which form a complex with calcium. Factors that influence the rate of decalcification include concentration of decalcifying agent, temperature, agitation as well as suspension.\(^2,3\)

Strong inorganic acids are very harsh chemicals that can damage the soft-tissue structure as well as negatively affect cellular integrity, but on the other hand, strong acid usually takes less time to complete decalcification procedures so the strong acids can be used for the decalcification of highly mineralized cortical bone specimen. Weak organic acids take 1–10 days for the completion of the decalcification process and should be used for the decalcification of dense cortical or large bones. Chelating agents do not act like inorganic or organic acids but bind metallic ions, predominantly calcium as well as magnesium. They are very slow decalcifying agents and do not damage tissue nor affect their stainability.\(^4,5\)

Chemical method of decalcification is practiced regularly and gives good structural details, but it takes a longer time to decalcify than the microwave method. A study conducted by Pitol et al.\(^6\) using microwave method concluded that there is a 30-fold increase in decalcification speed with microwave method as compared with a conventional method. Decalcification endpoint test usually validates the process of decalcification. There are several methods for testing the completion of decalcification such as physical methods (needle and bubble test); radiographic method and chemical method (calcium oxalate test).\(^2\)

Hence, the aim of this study was to evaluate the rate of decalcification of six decalcifying agents (5% nitric acid, 8% formic acid, Perenyi’s fluid, formalin-nitric acid, 5% trichloracetic acid and 14% EDTA) as well as their effect on the histopathological characteristics of dental hard tissues.

MATERIALS AND METHODS

The study had been approved by the Research Degree Committee of the Dental Institute. The study was conducted in the department of oral and maxillofacial pathology. Freshly extracted, noncarious, nonattrited, 30 human permanent teeth including incisor, canine, premolar and molar were obtained from the patients and fixed in 10% formalin for 24 h. After that, the specimens were exposed to different decalcifying agents such as 5% nitric acid, 8% formic acid, Perenyi’s fluid, formalin-nitric acid, 5% trichloracetic acid and 14% EDTA. All decalcifying agents were procured from Loba Chemie Pvt. Ltd., Mumbai, Maharashtra, India. A combination of single as well as the multi-rooted tooth was placed in all the decalcifying agents.

The decalcification was carried out at 37°C by placing the decalcifying agent in the containers with tooth samples in an incubator (NSW-151, Narang Scientific Works Pvt. Ltd. New Delhi, India). The decalcifying solutions were replaced with fresh solutions on every 5th day. The endpoint of decalcification was evaluated by chemical...
After verifying the completion of decalcification by chemical as well as radiographic method, the teeth were removed from the respective decalcifying solutions and washed under running tap water for 10 min. The speed of decalcification of different agents was measured in days. After that, the decalcified teeth were processed, sectioned and stained with hematoxylin and eosin. 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: speed of decalcification; ease of sectioning; staining characteristic including hard-tissue staining, cytoplasmic staining and nuclear staining; soft-tissue integrity including soft-tissue attachment, soft-tissue shrinkage and pulp organization; overall histological appearance and total score.

RESULTS

Parameter 1 (speed of decalcification): it was fastest with Perenyi’s fluid followed by 5% trichloroacetic acid, 5% nitric acid, formalin-nitric acid, 8% formic acid and 14% EDTA [Table 1]. Parameter 2 (ease of sectioning): during microtomy, it was observed that sectioning was most difficult for teeth decalcified in Perenyi’s fluid and EDTA [Graph 1]. Parameter 3 (staining characteristics): best staining characteristics were obtained with neutral EDTA followed by 8% formic acid and 5% trichloroacetic acid [Figures 1-3]. Poor staining quality was seen in samples decalcified with Perenyi’s fluid. [Graph 2 and Figure 4]. Parameter 4 (soft-tissue integrity): with respect to soft-tissue integrity, teeth decalcified with EDTA and 5% trichloroacetic acid gave excellent results [Graph 3]. Parameter 5 (overall histological appearance): it was excellent with EDTA; even 5% trichloroacetic acid and 8% formic acid gave overall good histological appearance [Graph 4]. Parameter 6 (total score): among the six decalcifying agents, 5% trichloroacetic acid followed by 8% formic acid and EDTA proved to be the best with all the parameters considered in the study [Table 2].

DISCUSSION

Decalcification is the regularly used technique in most of the histopathology laboratories. The process of decalcification is mandatory to study the organic components of the teeth. Inorganic calcium must be removed from the organic collagen matrix, calcified cartilage and surrounding tissue. The process of decalcification is carried out by chemical agents, either with acids to form soluble calcium salts or with chelating agents that bind to calcium ions. Acid decalcifiers are further divided into two groups: strong (inorganic) and weak (organic) acids. Strong inorganic acids are nitric and hydrochloric acid. Weak organic acids are formic acid, acetic acid and picric acid. Properties of a good decalcifying agent generally include complete removal of calcium, cause minimal damage to cells and tissues, cause nonimpairment to subsequent staining and decalcifie at a reasonable speed. In addition to chemical agents, the other factors which may play an important role in the process of decalcification are fixation, concentration of decalcifying agent used, temperature, pressure, agitation, electric current, microwave radiation, tissue suspension and size and type of tissue. Microwave decalcification is a new technique which speeds up the process of decalcification significantly.
from days to hours. A study conducted by Ahmad Danish Rehan et al.\cite{10} included 30 premolar teeth for decalcification with routine chemical method as well as microwave method. The three solutions used were diluted nitric acid (5%), formic acid (5%) and EDTA (14%). They found that the microwave method using nitric acid was the fastest decalcifying method needing just about 4 days for premolars, compared with routine decalcification. They also concluded that the overall structural details and good staining characteristics were better in teeth decalcified by 5% nitric acid in comparison to EDTA and formic acid in both the methods used. However, nitric acid showed good staining details in the microwave method in comparison to the conventional method.\cite{10,11}

The selection of decalcifying agents depends on four factors such as urgency of case, mineralization stage, aim of research as well as staining technique. The decalcifying agents should maintain the balance between rapid decalcification and preservation of morphology. Many studies have been carried out by the researchers to introduce new decalcifying agents and to modify the presently used agents to meet the criteria of the most efficient decalcifying agent which ensures complete removal of calcium without causing any damage to tissue morphology and provide adequate staining characteristics.\cite{2}

In the present study, we attempted to compare the rate of decalcification of six decalcifying agents (5% nitric acid, 10% formic acid, Perenyi’s fluid, formalin-nitric acid, 5% trichloroacetic acid and 14% EDTA) and their effect on the histopathological characteristics of dental hard tissues as well as to find the most efficacious decalcifying agent which can maintain the balance between the rapid decalcification and preservation of morphology in the urgency of case.

Strong acids such as nitric acid, hydrochloric acid, Perenyi’s fluid and formalin-nitric acid complete the decalcification rapidly but can seriously damage tissue stainability. In our study, we found that nitric acid took approximately 7–10 days, Perenyi’s fluid took 5–8 days and formalin-nitric acid took 10–13 days for tooth decalcification [Graph 1]. These acids gave worst results in terms of staining characteristics and soft-tissue integrity [Figures 4–6]. Weak acids such as formic acid, trichloroacetic acid and picric acid are somewhat gentle and take more time to complete the decalcification than strong acids. According to our findings, formic acid took approximately 21–25 days and trichloroacetic acid took 6–9 days for tooth decalcification. These acids gave good results in terms of staining characteristics and soft-tissue integrity.

Chelating agent,

| Agent                | n  | 1     | 2     | 3     | 4     | 5     | 6     | Significance |
|----------------------|----|-------|-------|-------|-------|-------|-------|-------------|
| Perenyi’s fluid      | 5  | 9.6000|       |       |       |       |       | 1.000       |
| Formalin-nitric acid | 5  |       | 16.700|       |       |       |       | 1.000       |
| Nitric acid          | 5  |       |       | 19.000|       |       |       | 1.000       |
| EDTA                 | 5  |       |       |       | 21.500|       |       | 1.000       |
| Formic acid          | 5  |       |       |       |       | 25.000|       | 1.000       |
| Trichloracetic acid  | 5  |       |       |       |       |       | 26.500| 1.000       |

EDTA: Ethylenediaminetetraacetic acid

Figure 2: Tooth decalcified using trichloracetic acid (H&E, ×20)

Figure 3: Tooth decalcified using formic acid (H&E, ×20)
i.e., EDTA, is commonly used for tooth decalcification. EDTA is nominally “acid”, it does not act like inorganic or organic acids but binds metallic ions, namely calcium and magnesium. It is a very slow agent but does not damage tissue or its stainability. In our study, we found that EDTA took approximately 90–100 days for tooth decalcification, but it is a good decalcifying agent in terms of staining characteristics and soft-tissue integrity [Figure 1].

The speed factor of decalcifying agents in the present study was the highest with Perenyi’s fluid and lowest by neutral EDTA. Perenyi’s fluid took approximately 5–8 days for tooth decalcification. This finding was in accordance with...
In terms of efficacy of agents with respect to soft-tissue attachment, soft-tissue shrinkage and pulp organization, trichloroacetic acid gave good results as it showed minimal soft-tissue shrinkage and minimal loss of tissue. Even formic acid gave good results and showed minimal soft-tissue shrinkage as well as minimal loss of tissue architecture. The pulp organization was clearly distinct and excellent in teeth decalcified with EDTA and 5% trichloroacetic acid, these findings are in accordance with the study conducted by Karpagaselvi Sanjai et al.\(^{[12]}\) In

the study conducted by A Choube et al.\(^{[8]}\) however, in the study conducted by Karpagaselvi Sanjai et al.\(^{[12]}\) the speed factor of decalcifying agents was highest with 5% nitric acid and lowest by neutral EDTA decalcifying solution and Perenyi’s fluid took approximately 50–60 days for tooth decalcification.\(^{[12,13]}\)

During microtomy, it was noted that sectioning was most difficult for teeth decalcified in Perenyi’s fluid and EDTA. These findings concur with those of A Choube et al.\(^{[8]}\) whereas Karpagaselvi Sanjai et al. found that the teeth decalcified with neutral EDTA responded the best to microtomy.\(^{[12]}\)

In terms of efficacy of agents with respect to staining characteristics, excellent results were actually obtained with the slowest decalcifying agent, i.e., EDTA [Figure 1]. Even 5% trichloroacetic acid [Figure 2] as well as 8% formic acid [Figure 3] showed good staining characteristics which was in accordance with the study conducted by Karpagaselvi Sanjai et al.\(^{[12,14]}\) The study conducted by Gupta et al.\(^{[2]}\) however, concluded that formal-nitric acid gives the superior hard- and soft-tissue staining in comparison to formic acid–EDTA, these findings are in contrast to the findings of our study.

According to our study, among the six decalcifying agents, trichloroacetic acid followed by formic acid and EDTA proves to be the best with all the parameters considered in the study. 5% trichloroacetic acid showed the most efficient result as it balances both tissue integrity and time factor, suggesting that it can be used as a stable decalcifying agent for routine histopathological diagnosis.

**CONCLUSION**

Here, we propose that in case of urgent requirement, use of acids such as 5% trichloroacetic acid can be employed, as it gives better results and also maintains the soft-tissue integrity. When time is not a factor, the use of neutral EDTA as well as 8% formic acid may be advocated for their excellent soft-tissue integrity and quality of staining.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**

1. Nanci A. Ten Cate’s Oral Histology-E-Book: Development, Structure, and Function. India: Elsevier Health Sciences; 2017.

2. Gupta S, Jawanda MK, Sm M, Bharti A. Qualitative histological evaluation of hard and soft tissue components of human permanent teeth using various decalcifying agents – A comparative study. J Clin Diagn Res 2014;8:6:G69–72.
3. Sheehan DC, Hrapchak BB. Bone. In: Theory and Practice of Histotechnology. 2nd ed. Columbus: Battelle Press; 1980. p. 89-117.
4. Savi FM, Brierly GI, Baldwin J, Theodoropoulos G, Woodruff MA. Comparison of different decalcification methods using rat mandibles as a model. J Histochem Cytochem 2017;65:705-22.
5. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 5th ed. Edinburgh: Churchill Livingstone; 2002.
6. Leonardo PD, Henrique CF, Orive LL. Microwave-induced fast decalcification of rat bone for electron microscopic analysis: An ultrastructural and cytochemical study. Braz Dent J 2007;18:153-7.
7. Drury RA, Wallington EA. Carleton’s Histological Technique. 5th ed. Oxford: Oxford University Press; 1980. p. 199-205.
8. Choube A, Astekar M, Choube A, Sapra G, Agarwal A, Rana A. Comparison of decalcifying agents and techniques for human dental tissues. Biotech Histochem 2018;93:99-108.
9. Culling CF. Handbook of Histopathology and Histochemical Techniques. 3rd ed. London: Butterworths and Co.; 1974. p. 63-72.
10. Rehan AD, Saigal S, Bhargava A, Kumar U, Thakur P, Kausar T. Comparison of microwave decalcification with conventional decalcification method by using different decalcifying agents. Int J Res Med Sci 2017;5:3126-8.
11. Sangeetha R, Uma K, Chandavarkar V. Comparison of routine decalcification methods with microwave decalcification of bone and teeth. J Oral Maxillofac Pathol 2013;17:386-91.
12. Sanjai K, Kumarswamy J, Patil A, Papiiah L, Jayaram S, Krishnan L. Evaluation and comparison of decalcification agents on the human teeth. J Oral Maxillofac Pathol 2012;16:222-7.
13. Prasad P, Donoghue M. A comparative study of various decalcification techniques. Ind J Dent Res 2013;24:302-8.
14. Mawhinney WH, Richardson E, Malcolm AJ. Control of rapid nitric acid decalcification. J Clin Pathol 1984;37:1409-13.
15. Zappa J, Cieslik-Bielecka A, Adwent M, Cieslik T, Sabat D. Comparison of different decalcification methods to hard teeth tissues morphological analysis. Dent Med Probl 2005;42:21-6.
16. Mattuella LG, Bento LW, Vier-Pelisser FV, Araiyo FB, Fossati AC. Comparative analysis of two fixating and two decalcifying solutions for processing of human primary teeth with inactive dentin carious lesion. Rev Odonto Ciênc 2007;22:99-105.
17. Singh S, Sircar K. Evaluation of efficacy of various chemicals for decalcification of dental hard tissues – An in-vitro study. J Orofac Sci 2010;1:5-10.