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REVIEW ARTICLE

Decalcification: An Amenable Process in Oral Histology

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ABSTRACT

In histopathology, decalcification of calcified tissues plays a highly essential role in determining any hard tissue pathology by means of adequate tissue processing protocols. Before the examination of the tissue thoroughly to thrive for a final diagnosis, tissues go through a sequential curriculum to be differentiated clearly. To prepare tissues for microscopic examination, the elimination of calcium from hard tissues is an essential step so that acceptable sections can be obtained and used further. Decalcification is carried out by various decalcifying methods incorporating a variety of decalcifying agents, including acids and chelating agents. However, as it sometimes takes a long time lag and decreases the staining qualities of the specimen, many ventures have been made to find methods for accelerating this procedure.

Keywords: Acid, Bones, Decalcification, EDTA, Nitric Acid, Tissue.

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INTRODUCTION

Hardest tissues of the body consist of an enormous amount of inorganic components: calcium and phosphorus, which makes them chemically inert and denser than other tissues. In comparison with soft tissues, hard tissues are more resilient towards histopathological procedures, and thus they need much more receptive and multifaceted protocols in diagnostic histopathology.[1] In the case of bone, to augment decalcification, calcium (inorganic) ought to be detached from the unrefined collagen fiber matrix and adjoining structures.[2] This process is termed decalcification and is required because it's really difficult to prepare micro sections. Also, decalcification is a prerequisite to visualize pulp histology as the pulp chamber resides within a closed chamber surrounded by dentin over around. In such scenarios, cutting through hard tissue becomes mandatory.[1] The existence of calcium in the tissues damages the microtome knife required for cutting the tissue.[3] There has been no "universal" solution for decalcification existing for the histopathology laboratory.[4-6] The consequence of characters of the fundamental pathology of the bone on the decalcification procedure can also be assessed.[7-9]

In histopathology, there is a continuous conflict between the rapidity of decalcification that is mandatory against the eminence of the sections that are desired.[10-12] Decalcification should be the prior and foremost step of tissue processing before the fixation of hard tissues. Rapid fixation is inaccessible to obtain in such cases due to the presence of hard structures such as enamel, dentin, and bone.[2,13] Tissue stability is majorly affected by the buffering capacity of acids that are further dependent on the acidity of agents and the time duration of the decalcification process.[5] Concentration, temperature, decalcification solution, degree of agitation affects speed of decalcification and also leads to an untoward impact on staining techniques. Decalcifying agents including acids and chelates perform it. Subsequently, poor results in decalcification can be caused by over-exposure of the entitled agent and scarce control

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measures.[13,14] In this review, the method for decalcification and the successful monitoring of the process is enumerated, and some accepted options for the choice of reagents are provided.

OBJECTIVE OF DECALCIFICATION

1. To completely eliminate calcium following fixation
2. To make it suitable for further sectioning.
3. Decalcification ensures sections' adequate softness, enabling smooth cutting with a microtome knife. This is because, if not thoroughly decalcified, the microtome knife can be damaged.[15]

CHOICE OF DECALCIFIER IS INFLUENCED BY

Case Urgency
Extent of calcification and minerlization
Staining Procedures compulsions
Degree of exploration [2]

TECHNIQUE OF DECALCIFICATION

Decalcification techniques are divided into the following steps

1. Choice of a section of tissue
2. Choice of Fixatives and Fixation
3. Decalcifying agents and decalcification
4. Acid removal after decalcification
5. Tissue handing out
6. Staining[13]

Choice of Section of Tissue

Equipment for obtaining tissue	Sharp knife and/or Bone Saw
Section thickness	4-5 mm[8]

Choice of Fixatives and Fixation

It not only ensures but protects bone from degradation caused by decalcifying agents as sections macerate and stain poorly if not fixed adequately. Fixative should penetrate bone properly, and an adequate amount of it should be used to enhance fixation. Neutral buffered formalin is an ideal fixative for bone. Besides this, Zenker's fluid, bouin's fluid, Muller's fluid can also be used in special cases, such as in case of bone marrow[16,17]

Decalcifying Agents and Decalcification

There are diverse decalcifying agents such as acids and chelating agents. Additional mixtures contain reagents, like buffer salts, chromic acid, formalin or ethanol, anticipated to neutralize the objectionable inflammation effects that acids have on tissues.

There are basically two major categories of Decalcifiers – Acids and chelating agents [2]

An appropriate decalcifying agent should ensure complete removal of calcium, no effect on staning protection to cells and fibers, rational pace of decalcification.[13]

Acid Removal After Decalcification

Removal of acids after completion of decalcification should be complete and must be neutralized chemically. For chemical neutralization, sodium bicarbonate or lithium carbonate can be immersed into the decalcified bone. It can also be achieved by appropriate dipping in alcohol for approximately one day or rinsing in water for half an hour before starting dehydration step in tissue processing.[15]

Tissue Processing and Staining

Processing tissues containing small quantities of bone must be done daily, whereas larger tissues require extraordinary measures. In the case of hard tissues, much firmer and harder paraffin is often preferred to support bony sections. In molten wax, tissue cassettes containing bone or teeth must be placed for a much longer duration than normal tissue processing, i.e., 2 hours.

DIFFERENT METHODS OF DECALCIFICATION

1. Acid decalcification
2. Ion substitute method
3. Electrical ionization
4. Chelating methods
5. Surface decalcification[13]

METHODS OF DECALCIFICATION

Acid Decalcification

A variety of acid solutions are used to decalcify bone or teeth or, in addition subsequently gelled with a neutralizer. Neutralizer seems to dissolve cellular edema. Treating issues with acid results in tissue damage and artifacts. The stronger or more concentrated the acid, the harsher the effects. Therefore, a balance has to be struck between the urgency of the specimen and the deleterious effects of acid. With their slow decalcification rates, the weak acids show a higher degree of maintained antigenic reaction with better staining quality and preserved morphology.[10,11,18,19] (Figure 1)

Following are the usually used decalcifying solutions –

Aqueous Nitric Acid (Nitric Acid, Distilled Water)

Precautions must be taken while using this solution because there is a much higher susceptibility to tissue damage due

to the formation of urea which also gives yellow color. But at the same time, it has excellent staining properties and a fast duration of action

Nitric Acid Formaldehyde (Nitric acid, Formalin, Distilled Water)

Advantages –
Rapid action
Good nuclear staining
Washing with water is not required
Formalin protects the tissues from maceration

Formic Acid Solution

Formic acid (90%) - 100 mL
Distilled water - 900 mL

In this solution, the decalcification is slow. Increasing the concentration can have deleterious effects on the maceration of tissue inspite of being advantageous in relation to rapid action.[2]

Gooding & Stewart's fluid (1932)

Formic acid (90%)	100 mL
Formalin	50 mL
Distilled Water	850 mL

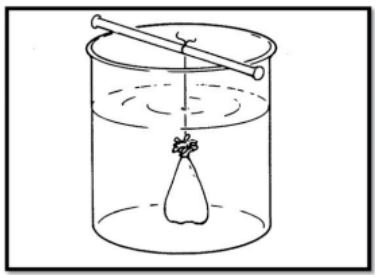

Figure 1: Acid Decalcification Method

Commercial Acid Decalcifiers: The exact composition of these reagents is unknown, but most are thought to contain a fairly high concentration of HCl. They are rapid in action but may interfere badly with subsequent staining. The reaction of formaldehyde and HCl acid may give rise to the formation of a potent carcinogen, bischloromethyl ether (BCME), but this is said to take place only in the vapor phases of these reagents and not the solutions.[13]

Ion Exchange Method

This method facilitates more frequent removal of calcium ions thus establishing an increased solubility pace of this ion from the selected tissue immersed in the decalcifying liquid.

The resin, commonly an ammonium form of sulfonated polystyrene, is layered on the bottom of the container to a depth of approximately 1-cm, and the specimen is allowed to rest on it. (It should be > 10% of the bulk of the decalcifying agent.) The liquid quantity by this technique should be 20 to 30 times the bulkiness of the specimen.

Disadvantages: Resin is limited to those decalcifying fluids not containing mineral acids; formic acid is recommended. The tissue regeneration can be enhanced after its application by dipping and rinsing with hydrochloric acid in the dilute form twice and then washing 3 times in distilled water. This procedure allows the resin to be used over a very long period without renewal. Complete decalcification can only be determined by X-ray.

The assets include
Faster decalcification
Preserved tissue morphology
Increased duration of resin[13]

Electrolytic Method

In the electrolytic method, calcium ions which majorly contribute to the hardness of tissues, attract towards negatively charged cathode in an electrolytic medium, which is hydrochloric and formic acid. The calcium ions move towards the cathode. Rapid decalcification is achieved, but the heat produced may damage the cytological details. The technique relies on the solution of Ca ions in the electrolyte and their attraction to the cathode. This method is not used commonly. Here the anode and cathodes are Cu plates. (Figure 2)

The rapidity of the process may be due to the heat generated. There is acid decalcification first by a decalcifying agent like formic acid - HCl acid, which is kept for 1 to 4 hrs, liberating free actions. (Ca^{+}) to the cathode

Formic acid (90%)	100 cc
HCl	80 cc
Dist. water to make	1000 cc[13]

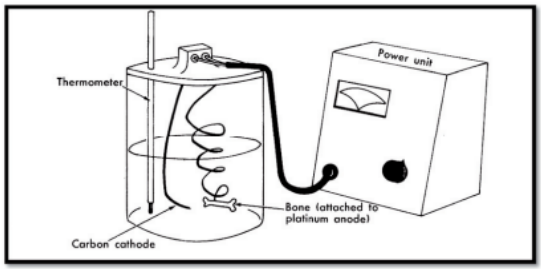

Figure 2: Electrolytic decalcification apparatus

Chelating Agents

The basic mechanism of chelates is to incorporate metallic ions. There is a formation of a soluble complex in non-ionized form. This is by far the unsurpassed technique because it maintains the morphological and cytological details of biopsies, most importantly in the case of bone marrow

EDTA Solution: (Sequestrene or Versene)

EDTA (disodium salt) 55 gms
Formalin 100 mL
Dist. water 900 mL

Disadvantages: This method is slower than other methods because the rate of extraction of calcium ions from hydroxyapatite crystals is through coating by a coating technique

Advantages: More convenient with least amount of artifacts, gives appropriate results can be stained by different staining techniques. In addition to this, it is used in electron microscopy.

Surface Decalcification

If a small area of calcification is left and may be apparent while a building block made of paraffin is being trimmed it is decalcified by inverting the block in HCl (5%) for about 1 hour or drops of 5% HCl can be added at the specific spot. This is less drastic than returning the tissue to aqueous solutions. We should be cautious during the collection of starting sections as the only top layer of approximately 30µm is decalcified. Another measure to take is to rinse the block in water to avoid erosion by the effect of acid. Once decalcified, the acid's exterior surface should be removed with water from the tissue. If there is any delay before processing, the tissue should be returned to formal saline. Some advocate neutralizing the acid before processing, but this may not be required as processing fluids may wash it out.

Nucleic acids are particularly intolerant to acid decalcification, so hematoxylin staining may be less effective. Staining time may be prolonged, or a stronger hematoxylin such as Ehrlich's may be used. Acid dyes, especially eosin, tend to stain more strongly; hence shorter time is required. Methyl-green-pyromin staining of nucleic acids is also severely affected. Artifacts produced during decalcification of hard tissues by acids is due to the production of Co_2 gas, which causes the separation of hard and soft tissues termed as tearing.[2]

COMPONENTS ON WHICH DECALCIFICATION DEPENDS ON (Figure 3)

Figure 3: Factors affecting rate of decalcification

METHODS OF DETERMINING DECALCIFICATION OR ENDPOINT

1. X-ray (the most accurate way)
2. Chemical testing (accurate)
3. Physical testing (less accurate and potentially damage of specimen)

The tissue should be left in decalcifying fluids for the minimum time possible due to the harmful effects of acid on the tissue. Hence the determination of the endpoint of decalcification is necessary.

Manually, the 'feel' of the tissue may give a guide to whether decalcification is complete. Probing the tissue with a needle should not be done. Judicious bending of the tissue may be done after a period of time. The approximate time required for decalcification depends on the tissue structure and size of the block. Small biopsies can be examined after 24 hours, and other specimens daily after two to three 24 hours changes of fluid. Accurate determination of endpoint may be determined using either chemical tests or X-rays.

Radiography is the most efficient test for the endpoint and is invaluable in detecting foreign bodies in specimens. Periodic

X-rays of the specimen can be taken every day. Several specimens can be exposed on the same x-ray if care is taken for over-identification.

Drawback: Minimal amount of bone (30%) should be present for positive

X-ray. i.e., negative x-ray need not mean complete decalcification.

Chemical Test It depends upon the identification of calcium in the decalcifying solution by sampling the fluid change following completion. This method cannot be used following EDTA decalcification.

CONCLUSION

Decalcification is a straightforward process but to be successful requires a careful preliminary specimen assessment.

REFERENCES

1. Bhat N, Thakur K, Bhardwaj N, Singh R. Comparison of 10% Nitric Acid, Edta and 10% Formic Acid for Tooth Decalcification. Adv. Med. Dental Health Sci. 2019; 2(1):4-7
2. Callis GM, Bancroft JD. Theory and Practice of Histological Techniques.6th edition 2008; 338-360
3. Module Cytology and histology
4. Sangeetha R, Uma K, Chandavarkar V. Comparison of routine decalcification methods with microwave decalcification of bone and teeth. Journal of Oral Maxillofacial Pathology. 2013;17:386-91.

5. Dey P. Decalcification of bony and hard tissue for histopathology processing. Basic and Advanced Laboratory Techniques in Histopathology and Cytology.1st edition. 2018;35-9
6. Prasad P, Donoghue M. A comparative study of various decalcification techniques. Indian J Dent Res off Publ Indian Soc Dent Res. 2013;24:302-8
7. Naresh, KN, Lampert I, Hasserjian R, Lykidis D, Elderfield K, Horncastle D et al. Optimal processing of bone marrow trephine biopsy: The Hammersmith Protocol. J Clin Pathol. 2006; 59:903-11.
8. Sanjai K, Kumarswamy J, Patil A, Papaiah L, Jayaram S, Krishnan L. Evaluation and comparison of decalcification agents on the human teeth. J Oral Maxillofac Pathol.2012; 16:222-7.
9. Waerhaug J. Decalcification of bone and teeth under vacuum; a rapid method for producing hard tissue preparations. J Dent Res.1949; 28:525.
10. Kapila SN, Natarajan S, Boaz K, Pandya JA, Yinti SR. Driving the mineral out faster: Simple modifications of the decalcification technique. J Clin Diagn Res. 2015; 9:93-7.
11. Vries I, Siebers AG, Burgers L, Diepenbroek C, Link M, Groenen P et al. How we do: Optimizing bone marrow biopsy logistics for sign-out within 2 days. J Hematop. 2016; 9:67-71.
12. Athanasou NA, Quinn J, Heryet A, Woods CG, McGee JO. Effect of decalcification agents on immunoreactivity of cellular antigens. J Clin Pathol. 1987; 40:874-8.
13. Culling CF, Allison RT, Barr WT. Cellular Pathology Technique.4th ed. 1984; 408-30.
14. Callis G, Sterchi D. Decalcification of Bone: Literature Review and Practical Study of Various Decalcifying Agents, Methods and Their Effects on Bone Histology. The Journal of Histotechnology.1998; 21:49-58.
15. Lillie, R.D.Histopathologic technique and practice histochemistry.3rd edition 1965
16. Afreen Nadaf MS, Radhika MB, Lalita J, Thambiah, Paremala K, Sudhakara M. Decalcification: A simpler and better alternative. Journal of Dentistry and Oral Bioscience. 2011; 2:10-3.
17. Mathews B J, Mason G I. Influence of decalcifying agents on immunoreactivity of formalin-fixed, paraffin embedded tissue. Histochemical journal.1984; 16:771-87.
18. Mukai K, Yoshimura S, Anzai M. Effects of decalcification on immunoperoxidase staining. Am J Surg Pathology. 1986;10:413-19.
19. Mullink H, Henzen- Logmans SC, Tadema TM, Mol JJ, Meijer CJ. Influence of fixation and decalcification on the immunohistochemical staining of cell specific markers in paraffin embedded human bone biopsies. J Histochem. Cytochem. 1985; 33:1103-9