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Review Article

Decalcification- The Path to Hard Tissue Visualization

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Abstract

The decalcification procedure is a process of completely removing the calcium salt from the bone and teeth which are mineralized tissues, and also some other calcified tissues. Through this procedure mineralized component is removed, and the physical hardness also gets soft. Mineral components from the dentin and cementum should be removed. By using a microscope, we can observe pulp, immature enamel, dentin, and cementum histologically. This review tries to encompass the various procedures which help in diagnosing pathological hard tissue lesions.

Keywords: Decalcifying Agents; Acids; Endpoint Decalcification; Fixatives; Buffers; Chelating Agents; Resins; Microscope

Introduction

Biopsies that are obtained from the head and neck region often show complexity as they include both tissues which are soft and hard. As compared with soft tissues, the biopsy samples, including bone, teeth, and also soft tissue with deposits of calcium salts (calcified tissue), often need a more complex method such as decalcification to make them available for histopathological diagnosis.

The decalcification procedure completely removes the calcium salt from the bone and teeth which are mineralized tissues, and also some other calcified tissues [1].

Through this procedure mineralized component is removed, and the physical hardness also gets soft.

Mineral components from the dentin and cementum should be removed. By using a microscope, we can observe pulp, immature enamel, dentin, and cementum histologically.

Calcium salts prevent the preparation of good sections, which are present in the soft tissue.

Decalcification of bone and teeth has a special significance in oral maxillofacial pathology as it is one of the required procedures which is routinely done [4].

Appropriate preparation is required to study the structures histologically with a microscope.

The average thickness of the tissue specimen should be thin (4-6μ).

Decalcification is necessary to facilitate the smooth cutting of bone and other calcified tissue during the preparation of sections.

The hard calcified tissue is cut into small pieces.

The tissue should be thoroughly washed before the decalcification procedure to remove the excess fixative.

Chemical agents used for decalcification, are chelating agents that bind to calcium ions or acids to form soluble calcium salts.

Choosing a decalcifier

It depends on four interdependent factors

- The case urgency
- Mineralization degree
- The status of the investigation
- Required techniques for staining.

Criteria of a good decalcifying agent

- It completely removes calcium from the tissue.
- Doesn't damage tissue cells or fibers.
- It does not impair the subsequent staining techniques.
- Reasonable fast decalcification [2].

Factors that affect the decalcification procedure

The decalcifying agent's concentration

A more concentrated acid solution decalcifies faster than less concentrated, but it causes harm to the tissue.

Example: Alcohol, buffers decrease the rate of decalcification as they protect the tissue.

For avoiding acid or chelator depletion of reaction with the calcium, fluid with a large volume than the tissue volume (20:1) should be recommended.

Temperature

Decreasing the temperature causes acceleration of the decalcification as well as its causes increasing the damaging effects on tissue by acids.

- 18°Celsius - 30°Celsius is preferable.
- Decreasing temperature decreases the reaction rate.
- To prevent excessive temperature that damages tissues.

Electrolytic methods, microwaves, and sonication cause heat production, and careful monitoring is required.

Agitation

- Agitation speeds up decalcification.
- By low-speed rotation, stirring, rocking, and also bubbling air into the solution, fluid agitation will be achieved.
- The method of Sonication is required, which vigorously agitates both fluid and specimen.

Suspension

All the surfaces of a specimen should be able to make contact with the decalcifying fluid.

- It enhances and facilitates solution, ionization, and removal of calcium by penetration and diffusion into the specimen.
- Separation of bone samples is done and then suspended with a thread into the fluid mostly preferred within a cassette.
- For bag suspension, a cassette will be provided without having to prepare tags, for identification.

Techniques of decalcification of bone

Bone and its constituents can be demonstrated by many techniques. These techniques vary depending on whether it is the demonstration of decalcified bone or mineralized bone.

- **Decalcified bone:** Paraffin, transmission electron microscopy (TEM), or frozen [3].
- **Mineralized bone:** Frozen, paraffin, scanning, plastic, or transmission electron microscopy.

Techniques of decalcification

There are different stages in the decalcification

Stages in decalcification

- Selection of tissue
- Fixation
- Decalcification
- Neutralization of acid
- Thorough washing.

Selection of tissue

Bone

By using a fine-toothed hacksaw or bone saw thin slices of bone are prepared. Bone slices should not be more than 4-5mm in thickness for required fixation and complete removal of the calcium, after completion of decalcification and washing, the cut surfaces of tissue should be trimmed again to remove the areas which were damaged by the saw.

Calcified tissue

Thin slices can be obtained by cutting with a sharp knife. However, when it is complex, then a saw is used.

Fixation

The bone should be cut into small pieces of 2-5mm thickness. Proper or adequate fixation of all bones and the calcified tissue specimen is necessary before proceeding or subjecting them to any decalcification. A general rule for fixation applies to bone and tissues with calcium. Complete fixation is required as it protects bone and its surrounding soft tissues from the damage effects of acid decalcification. The fixative chosen should preserve the tissue elements to be demonstrated. When the tissue is unfixed, the tissue damage during acid decalcification is about four times greater. There are few fluids having fixing and decalcifying action, e.g., Bouin's decalcifying solution and formic acid-formalin.

Fixative

Usually, a routine fixative such as 10% neutral buffered formalin is used for paraffin and also non-tetracycline labeled bone. The best fixative for bone marrow is Zenker formol.

- Do not use alcohol-based fixatives, if the bone is decalcified using acids because alcohol slows or prevents decalcification.
- Do not use fixatives that contain mercury (B5, Zenker's Susa) and chloroform (Carnoy's).

The fixation will be faster if

- Bone is reduced in its size.
- Bone is opened
- More skin, the soft tissue which is surrounded by the lesion are removed.

Decalcification

- Decalcification is achieved by using various methods
- Various methods of decalcification

Acid decalcifiers

Strong (inorganic) acids

Nitric acid

- Aqueous nitric acid
- Formal nitric acid
- Perenyi's fluid
-

Aqueous hydrochloric (HCI)

Weak/dilute organic acids.

Formic acid

- Aqueous formic acid
- Formic acid-formalin
- Formic acid-sodium citrate

Others: Acetic and picric acid

- Ion exchange resins
- Chelating agents
- Electrophoretic technique
- Microwave technique

Acid decalcifiers

Acids react with calcium and form soluble calcium salts.

Any acid (though well buffered) causes damage to tissue as well as its Stainability.

The damage increases when decalcifying acid is stronger or concentrated and also longer duration of exposure of the tissue to the decalcification agent.

Thus, any decalcifying agent has adverse effects and can also be reduced by the decalcification end-point test, post-decalcification acid removal, and stain procedure adjustment. (e.g.by prolongation of staining time or use of stronger hematoxylin such as Ehrlich).

Acids are used as a simple solution or as mixtures with also other reagents like buffered solutions or fixatives [6].

Classification of acid decalcifiers

- Strong (inorganic)
- Weak(organic) acids.

Strong acids

- Example: Hydrochloric acid, Nitric acid.
- They are useful as simple aqueous solutions
- They rapidly decalcify.
- If they are used longer than 24-48hours they cause damage to tissue stainability and tissue swelling.
- They are used for rapid diagnosis within 24 hours by using a needle and small biopsy specimens.
- Useful in heavily or large mineralized- cortical bone specimens by the decalcification and they are monitored by the procedure of endpoint test.

Advantages

They cause rapid decalcification.

Disadvantage

- They cause swelling of the tissue and also damage tissue.
- They can interfere with staining if used for more than 24-48hours

Decalcifying solutions are

- Aqueous nitric acid (5-10%)
- Perenyi's fluid
- Formalin-nitric acid.

Nitric acid

- Formula: 5% in distilled water.

1. Act Rapidly.
2. Impair staining is seen by exceeding the endpoint.

Hydrochloric acid

- Formula: 5-10% into the distilled water.

1. Rapidly acting.
2. Impair staining is seen by exceeding the endpoint.

Perenyi's fluid

- Formula: 40ml of 10% nitric acid, 30ml of 0.5% chromic acid, and 30ml of absolute alcohol.

1. Relatively Rapidly it acts.
2. Impair staining is seen by exceeding the endpoint.

Von Ebner's solution

- Formula: 50ml of sodium chloride saturated solution, 42ml of distilled water, 8ml of hydrochloric acid.

1. Rapidly it acts.
2. Impair staining is seen by exceeding the endpoint.

Weak acids

E.g.: acetic, formic, picric

Formic acid is used most widely as one of the primary decalcifiers.

In small needle biopsies or bone pieces then, Formalin-10% formic acid mixture is recommended.

Picric and acetic acid cause swelling of tissue and are not used.

The above fixatives were used only in urgent cases and in minimal calcification.

Decalcifying solutions are

- Aqueous formic acid
- Formic acid- formalin
- Buffered formic acid

Formic acid

- Formula: 10% distilled water
- It is an effective and simple decalcifier.

Evans and Krajian:

- Formula: 25ml of Formic acid, 10g of Sodium citrate, 75ml of Distilled water.

1. Formic acid decalcifier buffered with citrate.

Kristensen

- **For**mula: 18ml of formic acid and 3.5g of Sodium formate, 82ml of distilled water.

1. Formate is buffered with a Formic acid decalcifier.

Gooding and Stewart

- **Formu**la: 5-25ml of Formic acid, 5ml of 40%formaldehyde, 75ml of Distilled water.

1. A formalin is added to formic acid decalcifier
2. Claims that it fixes and decalcifies.

Ion exchange resins

- Removes calcium ions from fluids.
- Reduced at the time of decalcification.
- Procedure: Ammonium form of sulfonated polystyrene resin is the resin, which is layered to a depth of approximately half-inch at the bottom of the container and also the specimen was allowed to rest on it.
- The volume of fluid: 20-30 times the bulk of the specimen [7].
- For the determination of the endpoint of decalcification, an X-ray is used as a chemical test that cannot be applied.

Advantages

- Decalcifies faster.
- Preserves the tissue structures very well.
- More prolonged use of this resin.

Disadvantages

- Resins can be used only with decalcifying fluid not containing mineral acids.
- Strong acids cannot be used, and only formic acid (weak acid) is used.
- The end of decalcification cannot be assessed by chemical tests.
- For determination of the endpoint, an x-ray may be needed.

Electrophoretic decalcification

It was first described in the year of 1947.

The process is of calcium ions attracted to a negative electrode and also the calcium solution in the electrolyte [9].

Advantages

- Decreases the time of the decalcification.
- Better in preserving the details of the soft tissue.

Disadvantage:

- Several specimens are limitedly processed at a time.
- Cumbersome process and expensive.
- The heat generated during the process may damage the tissue.

Technique: 6-volt DC supply is required.

- The electrolyte used is equal parts of 8 percent hydrochloric acid and 10 percent formic acid.
- Decalcification should be checked by x-rays every 2-3 hours.
- Temperature 40-45°C.

Microwave technique

It accelerates the decalcification process but requires monitoring of time and temperature.

These advantages are overcome by using special microtomes solely used for histological techniques.

Advantages

- Rapid decalcification.
- Useful for tissues with a high content of calcified material.

Disadvantages

- Time and temperature are to be managed carefully.
- Requires a microwave.
- Expensive technique.

Buffer mixtures

Citrate - citric acid buffer

- 7% citric acid-5ml
- 7.5% ammonium citrate - 95ml
- 1% zinc sulphate - 0.2ml
- Chloroform a few drops.
- Calcium ions are soluble at ph. 4.5, so buffer solutions are used for decalcification
- Generally, they are slow.
- They do not cause any damage to cells or tissues.

Chelating agents

- A generally used chelating agent is EDTA.
- Certain metals have the power of binding.
- Like calcium and magnesium, they bind to metallic ions.
- It may take 6-8 weeks or longer to decalcify dense cortical bone, whereas for Small bone spicules it takes less than a week.
- For enzyme staining immunohistochemical and also for electron microscopy, it is an excellent bone decalcifier.

  E.g.: -formalin-EDTA, EDTA (aqueous).

Advantages

- It shows minimal artifacts.
- Sections are stained by most of the techniques with one of the best results.

Disadvantages

It is a slower process [5].

Neutral EDTA

Formula: 250g of EDTA disodium salt, 1750ml of Distilled water, bringing the pH to 7.0 by the addition of sodium hydroxide.

- Slow acting but causes minor damage to the tissue. No effect is seen in the Conventional stains.

Endpoint of decalcification

These are methods to determine the procedure of decalcification: -

- **Physical test:** Inaccurate and also causes damage to the tissue artifact, needling, probing, bending, slicing, or squeezing tissue can cause artifacts.

  Example: Needle tracks, false-positive micro-fractures of fine trabeculae.
- **Chemical test:** By the following test, it determines the presence of calcium which releases from the bone.
  - **Calcium oxalate test:** It is done by precipitation of insoluble calcium hydroxide or calcium oxalate by the detection of calcium in acid solutions.
  - **Solutions:** Concentrated saturated aqueous ammonium oxalate and ammonium hydroxide.
  - **Method:** 5ml of fluid is taken from the bottom of the container
  - By adding a small piece of litmus paper or using a ph. Meter it with a magnetic stirrer.
  - By adding ammonium hydroxide drop by drop until litmus, then it indicates the solution is neutral (ph. 7).
  - By adding 5ml of saturated ammonium oxalate and then shake well and next allow it to stand for 30min.
  - **Result:** Appearance of turbidity indicates the presence of calcium.
  - The absence of turbidity indicates complete decalcification and tissue should be removed immediately [8].
- **Bubble test:** To produce carbon dioxide, acids react with calcium carbonate in the bone, which is seen as a layer of bubbles on the bone surface and when there is less calcium content then the bubbles become tiny and smaller.
- **Radiography:** Most sensitive test.
  - If not decalcified properly, mineralized areas are easily identified, by using a handheld magnifier, tiny calcifications are best viewed

- It is not applicable when the tissue is fixed with mercuric chloride as it is opaque to x-ray.

Neutralization of acid

Following the process of immersion in mineral acids, after decalcification is complete, the acids present in tissues should be removed or neutralized chemically

Methods of chemical neutralization

- It can be done by treatment with a weak alkali by immersing decalcified bone which is either 5-10% aqueous sodium bicarbonate solution or saturated lithium carbonate solution for many hours.
- Another method is for 12-18% hours of washing of decalcified tissues in two changes of 70% alcohol, which improves staining in most cases. Following next step is dehydration which is continued in the usual way.

Thorough washing

- **Purpose:** It is done before processing to remove the acid, which can interfere with staining reactions.
- **Method:** washing should be done for 3-4 hours in alcohol or overnight in water.

After thorough washing, tissues can be routinely processed.
(i.e., by dehydration and clearing).
Then as usual tissue is embedded.

Surface decalcification

- **Definition**: Surface decalcification is the process of removal of a small focus of calcification on the surface of a paraffin block [10].
- **Indication**: Required when a bone is partially decalcified or unsuspected calcium or else mineral deposits in the soft tissues were detected in a small area during paraffin sectioning. Blocks containing unsuspected calcium deposits can be treated in the same manner; thus, avoiding deblocking, rehydration, and decalcification of tissue.

Method

- After finding a focus of calcification on the surface of the paraffin block, it is trimmed with a scalpel or knife to expose the tissue.
- In 1 to 5% HCL, and 10% formic acid solution for 15-60minutes the exposed tissue surface of the block is then placed face down.
- Then later it is rinsed with water for removing the corrosive acids and then followed by a sectioning procedure done in the usual manner.
- A few micrometers of calcium are removed by the acid from the tissue surface.

Advantages

This technique will prevent knife damage and torn tissue sections.

Conclusion

Decalcification is the procedure that successfully needs

A careful primary examination of the specimen. Thorough fixation is required. For fixation and processing prepare the ideal thickness of sections. The suitable decalcifier should be selected with adequate volume and should be changed regularly. Determination of the endpoint of decalcification should be done carefully.

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