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DECALCIFICATION:

It is the process of removal of calcium salts from the bone and calcified tissues by suitable method according the specimen, time and the equipment. Decalcification is a routine procedure with the purpose of making a calcified tissue compatible with the embedding media for cutting micro slides and subsequent staining.

CRITERIA OF A GOOD DECALCIFYING AGENT:

- Complete removal of calcium
- Absence of damage to tissue cells or fibres /Minimal tissue damage
- Non impairment of subsequent staining technique/ shouldn't interfere the staining reaction
- Reasonable speed of decalcification

FACTORS AFFECTING THE RATE OF DECALCIFICATION:

1. Concentration of decalcifying agent:
    - Large volume of the fluid compared with the volume of tissue- 20 to 1 is recommended to avoid total depletion of the acid or chelator by their reaction with calcium.
    - Fluid should be changed several times during the decalcification process
2. Temperature:
    - Increased temperature accelerates decalcification but also increases the damaging effects of acids on tissue. 18º C -30º C is acceptable.
3. Agitation:
    - Gentle agitation may increase the rate slightly by influencing fluid exchange within as well as around tissues.
4. Suspension:
    - Fresh decalcifier should have ready access to all surfaces of the specimen.
    - Enhances diffusion and penetration into the specimen and facilitate solution, ionization and removal of calcium.

THERE ARE FOUR METHODS OF DECALCIFICATION

1. Simple dilute mineral acids: a. Weak acid. b. Strong acid.
2. Ion exchange resins with acid decalcifying fluids
3. Electrolytic decalcification
4. Chelating agents
5. Surface decalcification

1. **Simple dilute mineral acids:** This method of decalcification dissolves the calcium salts in an acid solution called decalcifying fluids used either singly or mixture such as nitric acid, formic acid and trichloro acetic acid.

   Principle: Acid releases calcium from its chemical combinations with phosphates and carbonates in bone through ionic exchange giving soluble calcium salt.

   Two types: 1. Strong Inorganic Acids 2. Weak Organic Acids

a.) Nitric acid, HCL (Inorganic acid): Nitric acid solution (5-10%). Fastest decalcifier. Strong acid recommended for the decalcification of small pieces of bone which must be processed rapidly. Over-exposure to nitric acid destroys nuclear staining. • Used in urgent biopsy specimens.

Procedure:

1. Fix the selected block of bone for 2-3 days in buffered neutral formalin.
2. Place a mixture of 95ml distilled water and 5ml of nitric acid.
3. Change nitric acid solutions daily until bubbles cease to evolve from the tissues(1-3 days,depending on the size and consistency of the bone block)
4. Wash in 3 changes of 90% alcohol.
5. Dehydrate,clear in xylene or benzene and embed in paraffin

Advantages

1. Rapid in action 2. Gives better nuclear staining
3. Causes very little hydrolysis 4. Rapid Diagnosis for Needle & Small Biopsy Specimens.

Disadvantage

- Old yellow nitric acid cause yellow coloration of tissue, treated by using 1% urea. (transfer tissues directly to 70% alcohol). Yellow discolouration owing to formation of nitrous acid, this accelerates decalcification but also stains and damage tissues.
- Tissue left for long time causes damage to tissue

Precaution

- Formation of nitrous acid checked temporarily by addition of 0.1% urea to the conc nitric acid.
- It's the fastest decalcifier, but end point must be carefully watched .

b. Formic acid, Picric acid & T.C.A (organic acid & weak acid): Formic acid (5%) it is much slower than nitric acid but considerable less damaging to tissue structures and staining.

• 10% Formic acid in D.W is recommended for (2days) to small pieces of bone and for (20days) for to larger pieces of dense compact bone.

• T.C.A. used as 5%aqueous solution rather quicker in action than the same concentration of formic acid with good staining results.

Procedure

1. Specimens should be decalcified in (5-10%) HCl or formic acid mixture (24- 48)hrs.
2. Change to fresh solution each day until decalcification is complete. It may take 24 hours up to days or months depending on size of the specimens.
3. Once the decalcification is complete, rinse specimens in water briefly and transfer to ammonia solution to neutralize acids left in specimens for 30 minutes .
4. Wash specimens in running tap water thoroughly up to 24 hours.
5. Routine paraffin embedding.

2. Ion exchange resins with acid decalcifying fluids:

It is a process by which one type of ion is absorbed into a solid material and replaced by an equivalent quantity of another ion of the same charge. The Ion-Exchange Decalcification incorporates a strong cationic Ion-Exchange resin in a weak acid solution to remove calcium ions from bone, while replacing them with hydrogen ions. The Ion-Exchange Decalcification does not require strong concentrated acid solutions.

Uses:

- Remove the calcium ions from the fluid
- Ensures a more rapid rate of solubility of the calcium from the tissue
- Reduction in the time of decalcification.

Advantages :

1. Well preserved cellular detail
2. Faster decalcification
3. Elimination of the daily solution change
4. Resin can be reused by removing excess acid.

Procedure:

- Tissue is placed in a bottle in a mixture of 10% or 20% resin and formic acid.
- Resin used is ammonium form of sulphonated polystyrene resin.
- The volume of fluid is 20 – 30 times that of the specimen.
- After use, resin may be regenerated by washing twice with dilute N/10 HCl , followed by 3 washes in distilled water.

3. **Electrolytic or Electrophoretic decalcification:** First described in 1947. There are using a(+ve) and (-ve) electrons. The bone is raped by a platinum wire which is the cathode & the anode is form of carbon wire. The decalcifying solution consist of equal parts of 80% formic acid & 10% HCL acting as electrolyte with 6volts for (1hrs). When the current passed, calcium will be removed to the anode and the temp will rise up to (30-45)C.

Principle: Attraction of the calcium ions to a negative electrode in addition to the solution of the calcium in the electrolyte.

Advantage: 1. Shortened time for complete decalcification.
2. Better preservation of soft tissue details.

Disadvantage: Limited no. of specimen processed at a time.

Procedure:

1. A glass jar containing the acid decalcifying solution in which is the electrode assembly and bone specimen, bone specimen is suspended by a platinum wire anode in the jar.
2. Decalcifying fluid : 88% formic acid 100ml + HCl 80ml • Distilled water 820ml
3. Current, causes an electric field between the electrodes, enables the calcium ions to migrate rapidly from the specimen (anode) to the carbon electrode (cathode).
4. Temperature of the reaction- 30" to 45" C.
5. Solutions changed after 8 hours of use to ensure maximum speed of decalcification.
6. Tissues are rinsed well in alkaline water & immersed in lithium carbonate before staining.
7. Lithium carbonate treatment of a cut section will neutralize any remaining acid in the tissue

4. Chelating agents: First described by HILLMAN & LEE (1953). Chelating agents are organic compounds that are capable of binding with certain metals. They are typically very slow acting and gentle, making them good fixatives for electron microscopy and immunohistochemistry studies. Ethylene diamine tetraacetic acid (EDTA) is the most common chelating agent used for decalcification. It binds with calcium ions and gradually removed. Care should be taken when specimens contain cartilage because overexposure can remove proteoglycans and weaken staining.

Advantages:

1. Tissue isn't hardened after EDTA decalcification.
2. The tissue easier to cut by microtome.
3. Some enzymes are still active.
4. It shows a minimum of artefact
5. Section stained by most techniques with first class results.
6. Slow process that does not damage tissues or their stainability, also pH sensitive.
7. Excellent bone decalcifier for immuno histochemical or enzyme staining & electron microscopy.

Disadvantages-

- slow process as calcium is removed layer by layer from the hydroxyapatite lattice.

5. **Surface decalcification**: Needed when partially decalcified bone/unsuspected mineral deposits in soft tissue are found during paraffin sectioning. After finding a calcification, the exposed surface in a paraffin block is placed face side down in 5% HCL for 1 hour or 10% formic acid for 15 to 60 minutes. Rinsed to remove the corrosive acids & re sectioned.

Neutralization

Various neutralizing treatments used after acid decalcification including:

1. Overnight washing in tap water or other alkaline solution and washing in the changes of 70% alcohol for (12-18)hrs before continuing the dehydration process. This is to remove any acid.

2. For frozen sectioning should be washed in water or stored in formal saline before being frozen.

End-Point of Decalcification :

Methods for testing the completion of decalcification:

1. Radiological method.
2. Physical method.
3. Chemical method.
4. Bubble test

1. Radiological method:

It is the best method, very quick it is not practically used because it is not available and very expensive.

2. Physical method:

It is a very bad method, damage the cells structure. The physical tests include bending the specimen or inserting a pin, razor, or scalpel directly into the tissue, or twisting the sample by fingers

3. Chemical method:

It is the suitable method used in the routine histopathology lab. The following solutions are needed to chemically test for residual calcium.

Principle : - Ammonium Oxalate react in alkaline pH with calcium salts to give calcium oxalate .

4. Bubble test:

- Acids react with calcium carbonate in bone to produce carbon dioxide , seen as a layer of bubbles on the bone surface.
- Bubble test is subjective & unreliable, tiny bubbles indicate less calcium present.