SOME PRACTICAL CONSIDERATIONS ON DECALCIFICATION OF DIFFERENT TYPES OF BONES.

By

Zainab, .S.Othman; Moussa, M.H.G. and Mosallam, El-S.M.

Department of cytology and histology faculty of Veterinary Medicine Cairo University

ABSTRACT

This study was carried out to obtain the best technique for preparation of histological sections of bone with minimal artifacts, preserved tissue and high staining affinity. Different bones from adult female rats were gathered and fixed in 10% neutral buffered formalin (10%NBF) then decalcified in 5 different decalcifying agents. Decalcifiers contained strong acids revealed rapid decalcification when compared with that contained weak acids and neutral ethylene-diaminetetraacetic acid (E.D.T.A), however, weak acids and E.D.T.A. showed well-preserved tissue with high staining affinity.

Key words:

Bone - Fixation- Decalcification - Decalcifies-Chelating agent.

INTRODUCTION

Bone is a specialized connective tissue, its extracellular matrix is calcified, incarcerating the cells that secreted it. The main bulk of bone is approximately 70% mineral and 30% organic components by weight. Bone cells, as opposed to marrow cells, are relatively sparse. The minerals of bones are mainly calcium and phosphate combined with hydroxyl ions to form hydroxyapatite crystals. The mineral is approximately 38% calcium and thought to be deposited as amorphous calcium phosphate in the mineralization phase **(Bancroft and Gamble, 2002)**. Only 10-20% of the bone matrix mass is water. 67% of the dry weight is made of inorganic mineral salts (mainly hydroxyapatite), 30 – 40 % is collagen, and the remainder (about 5%) is non - collagenous protein (NCP) and carbohydrates. The ratio of collagen to NCP is unique for bone, with collagenous protein comprising 90 % of the organic matrix compared to 10 - 20% in other tissues **(Robey and Boskey, 1996).** The association of minerals with collagen fibers is responsible for the hardness and resistance

of bone tissue **(Mescher, 2010)**. In order to obtain satisfactory paraffin or celloidin sections of bone, inorganic calcium must be removed from the organic collagen matrix, calcified cartilage and surrounding tissues. This is called decalcification **(Bancroft and Gamble, 2002)**. Decalcification is carried out by chemical agents, either with acids to form soluble calcium salts or with chelating agents that bind to calcium salts **(Drury and Wallington, 1980; Bancroft and Gamble, 2002 and Skinner, 2003)** resulting only in removal of the mineral phase of bone and leaving the organic osteoid. The preservation of tissue structures and their interrelations depend on the quality and velocity of demineralization. This process usually involves immersion of the specimens in acid solutions for weeks or months **(Goncalves and Olivèrio, 1965)**. An appropriate choice of decalcifying solution greatly reduces the production of artifacts **(Charman and Reid, 1972 and Weatherford and Mann, 1973).**

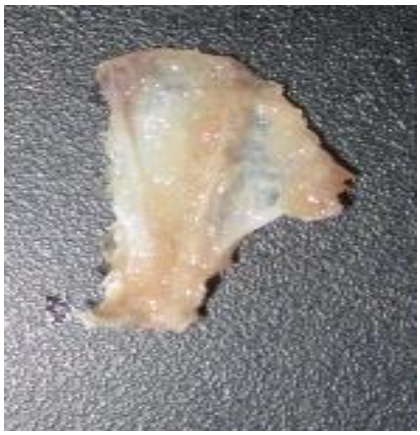
MATERIALS AND METHODS

Different types of bones (scapulae, femurs and knee joints) had been gathered from adult female rats of 250 g body weight. These bone specimens had been fixed in 10% neutral buffered formalin (10%NBF) for 48 hours then decalcified in the following 5 different decalcifying agents.

1- Perenyi's fluid (Perenyi, 1882)		
▪ **10 per cent nitric acid**	**40 cm^3**	
▪ **Absolute alcohol**	**30 cm^3**	
▪ **0.5 per cent chromic acid**	**30 cm^3**	
Mix shortly before use.		
2- Jenkin's fluid (Culling,1975)		
▪ **Absolute alcohol**	**73 cm^3**	
▪ **Chloroform**	**10 cm^3**	
▪ **Distilled water**	**10 cm^3**	
▪ **Glacial acetic acid**	**3 cm^3**	
▪ **Hydrochloric acid**	**4 cm^3**	
3- Formic acid-formalin (Gooding and Stewart, 1932)		
▪ **90 per cent formic acid**	**5-10 cm^3**	
▪ **Formalin**	**5 cm^3**	
▪ **Distilled water**	**to 100 cm^3**	
4- 5%Trichloroacetic acid aqueous solution freshly prepared		
5- Ethylene-diaminetetra acetic acid (EDTA) saturated solution		
▪ **EDTA disodium salt**	**250 g**	
▪ **Distilled water**	**1750 cm^3**	
E.D.T.A. solution is often cloudy. It is neutralized to pH 7 by the addition of approximately 25 g of sodium hydroxide which will clear it.		

After immersing the bone specimens in the decalcifying agents, we had to test the completion of decalcification. There are several methods to obtain the completion of decalcification.

Radiological test:



Scapula of rat before decalcification

Scapula of rat tested radiologicaly, before decalcification

Chemical (Calcium oxalate) test:

Principle:

Detection of calcium by the precipitation of insoluble calcium hydroxide or calcium oxalate.

Solutions:

Concentrated ammonia / saturated aqueous ammonium oxalate.

Method:

5ml of decalcifying fluid was taken and detection of PH was applied using Litmus paper.

Saturated ammonia drop by drop was added until the solution became neutral to litmus, after addition of each drop shaking should be applied.

5ml of saturated ammonium oxalate was added with well shaking.

Allowing standing for 30 minutes.

RESULT

If precipitate (Calcium hydroxide) forms after the addition of ammonia……..Considerable amount of calcium is present.

If precipitate occurs after the addition of ammonium, oxalat… less calcium is present.

If fluid remains clear for 30 minutes.It is safe to assume that decalcification is complete.

Physical test:

The physical test was used by gentle manipulation and bending of bone specimens.

After completion of decalcification, bone specimens of rats (scapulae and femurs) were sectioned into cross sections but Knee joints were bisected longitudinally. Then bone specimens washed in 2 changes of 70% alcohol overnight before processing carried out. Processing of the bone specimens was carried out as any soft tissue but increase the time for each change of alcohol and benzene to be 3 hours for each change to ensure a good penetration of reagents to bone tissue. It depends mainly on the bone thickness.

The samples were dehydrated in ascending series of ethanol, cleared in benzene and were embedded in paraffin. Sections of 6 Um thick were obtained using a rotatory microtome and stained by.

Harris haematoxylin and eosin,

Crossman's trichrome stain and Schmorl's picro-thionin stain as outline by **Bancroft and Gamble (2002).**

Stain but Schmorl's picrothionin stain was excluded from this work as it gave bad results with paraffin sections

RESULTS

The time needed for the used bone specimens to be completely decalcified:

Decalcifying agent	Scapula	Femur	Knee joint
Perenyi's fluid	5hours	2 days	1 day
Jenkin's fluid	6hours	3 days	2 days
5%Trichloroacetic acid	7hours	3 days	1.5 days
Gooding and Stewart solution	6hours	1 month	2.5 days
Neutral E.D.T.A.	12hours	1-3weeks	6 days

Nitric acid (Perenyi's fluid):

Sections of scapulae stained with H&E showed deep or brilliant red color and yellowish tint at the periphery with partially obscured nuclear details and less clear bone canaliculi and cement lines Fig. (1). Femur sections showed faint staining affinity to eosin with clear basophilic bone canaliculi and cement lines. Haversian canals were clear and the periosteum was preserved and showed its usual structure. With crossmon's trichrome stain bone sections showed deep greenish bone matrix with slightly reddish shadow .Elongated basophilic nuclei were scattered between the bony lamellae Fig. (2).

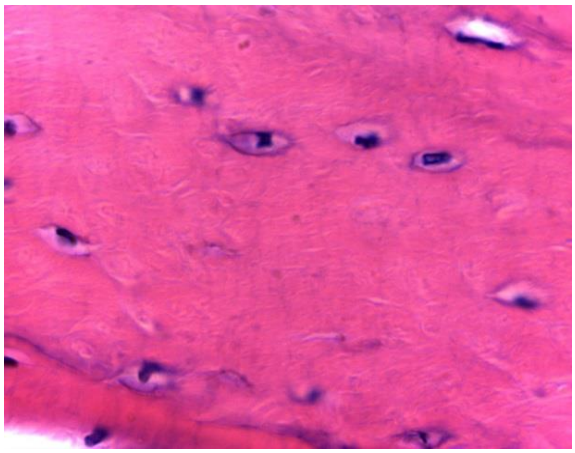


Fig.(1): :Section of rat scapula previously fixed in 10%NBF then decalcified in perenyi's fluid and stained with H&E stain showing deep or brilliant red color and appeared yellowish at the periphery with partially obscured nuclear details and less clear bone canaliculi and cement lines, X 800.

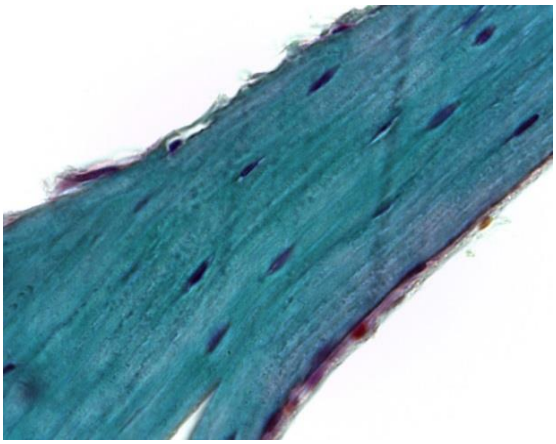


Fig.(2): Section of rat scapula previously fixed in 10 % NBF then decalcified in perenyi's fluid an stained with Crossman's trichrome stain showing deep greenish bone matrix with slightly reddish shadow .Elongated basophilic nuclei scattered between the bony lamellae, X320.

HCL (Jenkin's fluid):

Sections that had been decalcified in Jenkins fluid and stained with H&E stain showed undesired rosy red color of the bone matrix with yellowish red background Fig. (3) and less clear bone canaliculi. Dark basophilic cement lines appeared clearly.

With Crossman's trichrome stain, sections showed improper stained bone matrix with uneven staining affinity of collagen to the light green stain. Violet color tinge was noticed scattered randomly in the bone matrix Fig. (4).

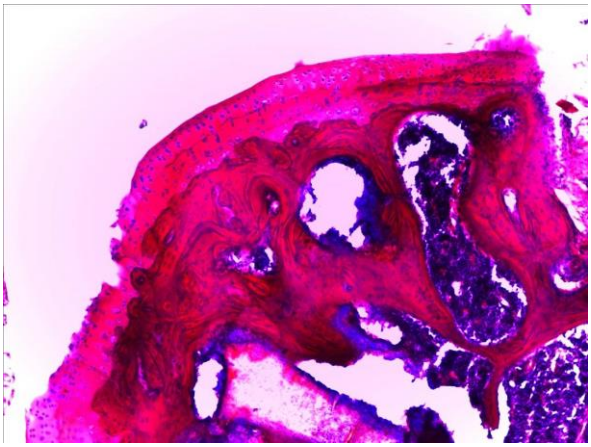


Fig.(3): Sections of rat knee joint decalcified in Jenkin's fluid and stained with H&E stain revealing improper stainind affinity to stain, X150.

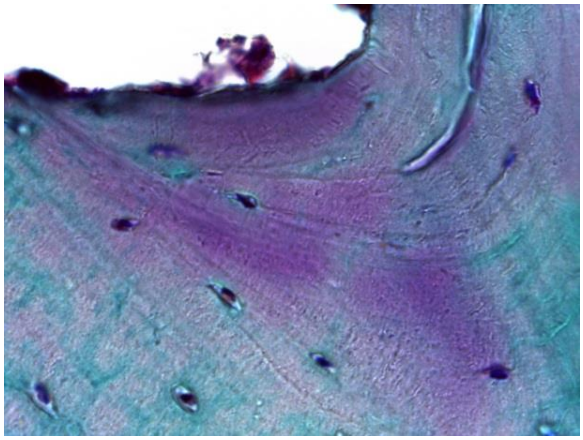


Fig.(4): Sections of rat scapula decalcified in Jenkin's fluid and stained with Crossman's trichrome stain showing uneven staining affinity of collagen to light green. Violet color tinge was noticed scattered randomly in the bone matrix, X800.

5%Trichloroacetic acid:

Sections that had been decalcified in 5%trichloroacetic acid and stained with H&E stain revealed high affinity of collagen fibers of bony lamellae to eosin with well apparent basophilic reversal lines that appeared parallel to the longitudinal axis of bone. Basophilic bone canaliculi appeared very fine and radiating out from the lacunae Fig. (5). the periosteum and marrow elements revealed their normal histological structure. With Crossman's trichrome stain, sections showed good affinity to both light green and acid fuchsine, typical staining affinity of bone matrix, bony lamellae, periosteum and the associated skeletal muscles were observed Fig. (6).

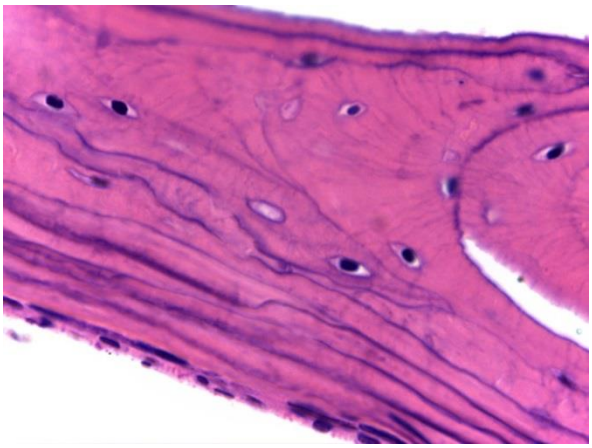


Fig.(5): Section of rat scapula previously decalcified in 5%trichloroacetic acid and stained with H&E stain revealing high affinity of collagen fibers to eosin with well apparent basophilic reversal lines and fine canaliculi radiating out from lacunae, X800.

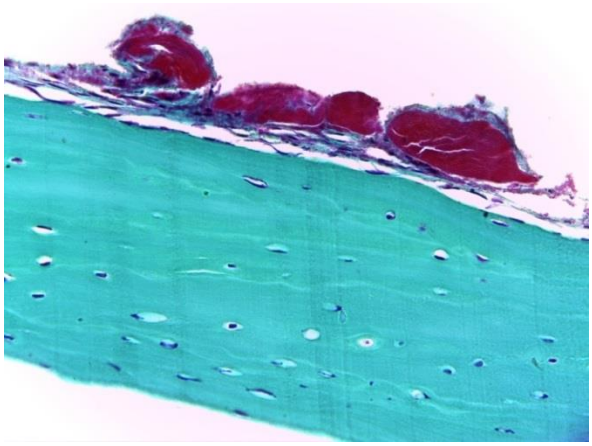


Fig.(6): Section of rat scapula previously decalcified in 5%trichloroacetic acid and stained with Crossman's trichrome stain showing typical staining affinity of bone matrix, bony lamellae, periosteum and the associated skeletal muscles were observed, X320.

Formic acid (Gooding and Stewart solution):

Sections that had been decalcified in Gooding and Stewart solution when stained with H&E stain showed good affinity of bone matrix with well observed basophilic cement lines and fine bone canalicuil radiating out from the lacunae. Fig. (7). With Crossman's trichrome stain, sections showed good affinity of collagen fibers to light green Fig. (8) and well stained bone matrix, periosteum and the associated skeletal muscles.

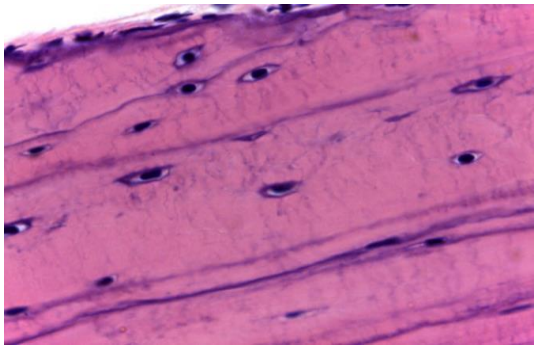


Fig.(7): Section of rat scapula previously decalcified in formic acid and stained with H&E showing good affinity of bone matrix with well observed basophilic cement lines and fine bone canalicuil radiating out from the lacunae, X800.

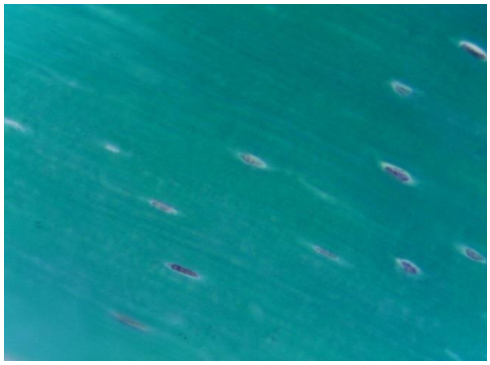


Fig.(8): Section of rat femur previously decalcified in formic acid and stained with Crossman's trichrome stain showing good staining affinity of collagen fibers of bony lamellae to light green stain, X800.

Ethylenediamine tetra acetic acid (EDTA) :

Bone sections that had been decalcified in neutral EDTA and stained with H&E stain showed good staining affinity that exhibited rosy-red bone matrix and lamellae with clear basophilic cement lines and bone canaliculi Fig. (9). the bone marrow elements and the osteocytes nuclei were normal. With Crossman's trichrome stain the bone sections revealed typical staining affinity of bone matrix and collagenic lamellae to light green stain with preserved periosteum and well stained associated skeletal muscles with acid fuchsin Fig. (11).

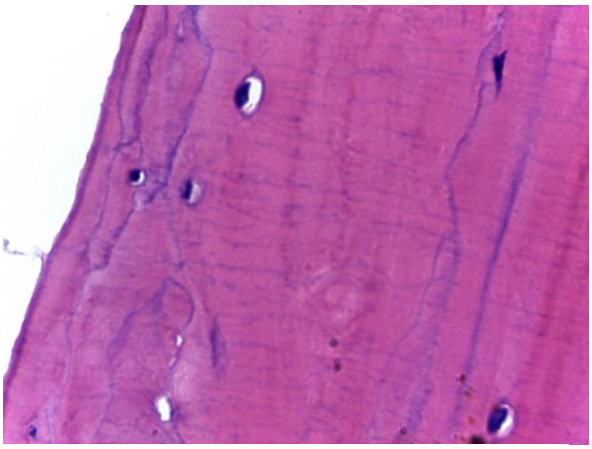


Fig.(9): Section of rat scapula previously decalcified in neutral EDTA and stained with H&E stain showing good affinity of bone tissue to stain and rosy-red bone matrix and lamellae with clear basophilic cement lines and bone canaliculi, X800.

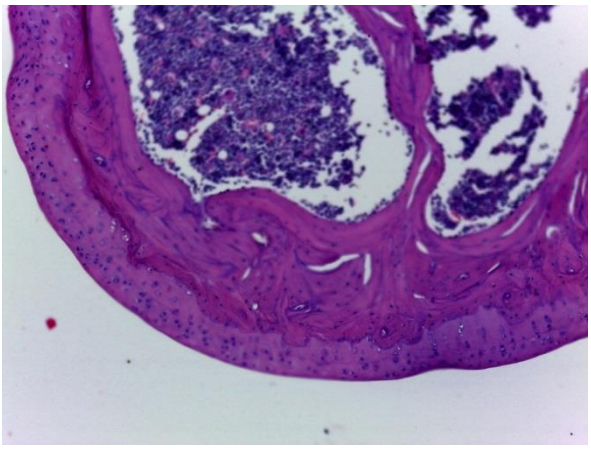


Fig. (10): Section of knee joint of rat previously decalcified in neutral EDTA and stained with H&E stain showing good affinity of bone lamellae and bone marrow cells to stain, X150.

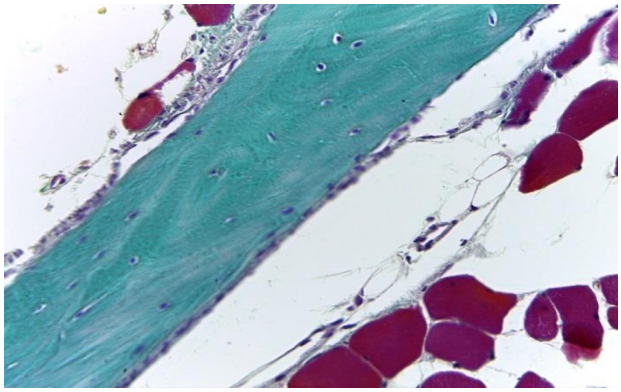


Fig.(11): Section of rat scapula previously decalcified in neutral EDTA and stained with Crossman's trichrome stain showing typical staining affinity of bone matrix to light green stain with preserved periosteum and well stained associated skeletal muscles with acid fuchsin, X320.

DISCUSSION

In agreement with **Drury and Wallington (1980)**, before bone or any calcified tissue can be processed and sectioned with a routine microtome, the calcium salts must be removed "decalcification" to soften the tissue. This is carried out by treatment with acids that react with calcium to form soluble calcium salts or chelating agents to take up calcium ions. Failure to de-calcify tissue with large amounts of calcium salts will result in torn and ragged sections and damage to the cutting edge of the microtome knife. 10 % neutral buffered formalin is satisfactory for routine diagnostic purposes. A finding agrees with **Sheehan and Hrapchak, 1987**. No harmful effect could be noticed on fixed tissue, as also described by **Drury and Wallington (1980)**. In our study, we used the perenyi's fluid, Jenkins fluid and 5 % Trichloroacetic acid ,formic acid as acids and used neutral EDTA as a chelating agent. Acid demineralizers are significally more rapid in action. This finding was recorded by **(Page, 1977 and Eggert and Germain, 1979)** but they cause structural distortion and deficiency in affinities for stains as **Fejerskov (1971)** and cause damage to the tissue as **Fejerskov (1971), Schajowicz and Cabrini (1955)** mentioned. According to **Luna (1960),** the increased acidity of the decalcifier and the length of the decalcification period will affect the stainability of the tissue.

Nitric and hydrochloric acids are strong acids, they decalcify rapidly but if used for longer than 48 hours serious deterioration of stainability can result due to swelling and maceration of the collagen fibers of bone lamellae. Schajowiez and Cabrini (1955) and Fejerskov (1971) described the same finding. Nitric acid decalcifies quickly but causes damage to tissue. Due to this fact, it is used mainly for the rapid decalcification of small pieces of bone **(Drury and Walligton, 1980 and Bancroft and Gamble, 2002)** to permit rapid diagnosis within 24 hours or less **(Bancroft and Gamble, 2002)**. Decalcification should not extend beyond 48 hours **(Drury and Wallington, 1980)**. Formic acid is the only weak acid extensively used as a decalcifier. In the present study, formic acid was used with additive (formalin). Formalin is added to effect simultaneous fixation and decalcification as Gooding and Stewart (1932) mentioned it. In agreement with **Drury and Wallington (1980), Bancroft and Gamble (2002),** formic acid is gentle and slower than HCL or nitric acids. It was less damaging to tissue structures and staining**.** The formalin 10% formic acid mixtures simultaneously fixes and decalcifies and is

recommended for very small bone pieces or Jamshid needle biopsies **(Bancroft and Gamble, 2002)**.We agree with this finding as this solution if used for very small bone pieces, it will save the time to avoid the prolonged immersion needed for large bone specimens .In addition, it saves the morphological structure and the tissue exhibit an excellent staining. **Verdenius and Alma (1958)** reported that, the decalcification develops more quickly in nitric acid, whereas in trichloroacetic acid it takes considerably longer. In formic acid decalcification is strikingly slow. These findings agreed with that in our study, the decalcification of scapula, femur and knee joint of rat is more rapid in perenyi's fluid, Jenkin's and 5% Trichloroacetic acid than Gooding and Stewart and Neutral EDTA. In an attempt to reduce the commonly encountered artifacts of tissue shrinkage and adverse staining results obtained with rapid decalcification in strong mineral acids such as nitric acid many laboratories have utilized the much slower decalcification achieved with formic acid or EDTA **(Mawhinney, Richardson and Malcolm, 1984 and Sheehan and Hrapchak,1987)**. We accommodate with this opinion as no harmful effect could be noticed in tissues in addition to good affinity to H&E and Crossmon's trichrome when bone decalcified in formic acid and EDTA, it is resulted from the gentle effect of weak acid (formic acid) and chelating agent on tissue when compared with tissues decalcified in strong acids. The same results reported by **Warshawsky and Moore (1965)** who mentioned that, using a diluted solution of EDTA buffered with NaOH (PH 7.2) turned the medium isotonic and less aggressive to the tissues. This decalcification method, though efficient, is time consuming (nearly 3weeks), depending on the specimen size **(Laboux, Dion, Chaves, Ste-Maria and Nanci, 2004)**. EDTA is a relatively slow decalcifying agent, while at the same time it preserves well the structural integrity of the tissue **(Brain 1966; Charman and Reid, 1972 and Bancroft and Gamble, 2002)**. Acid demineralizers cause distortion of the collagen fibers and deficiency in the affinity of histologic stains for tissue structures, which has not been reported in connection with the use of EDTA **(Fejerskov, 1971 and Culling, Allison and Barr, 1985)**. Treatment beyond the end of decalcification will not improve cutting qualities and will adversely affect staining **(Drury and Wallington, 1980)** and the histologic and cytological detail will be harmed **(Sheehan and Hrapchak, 1987)**. An exception to this is with the use of EDTA, where treatment appreciably beyond the time of complete decalcification causes no apparent harm to the tissue or it's staining **(Drury and Wallington, 1980)**. We are agreed with these findings as we find

that treatment beyond the end of decalcification will not improve cutting qualities and will adversely affect staining and have a bad effect on the tissue as it may cause dissolving of tissue when left in strong acid after the end achieved. An ample volume of decalcifying solution is necessary; we used about 150 ml for each specimen of bone. This opinion is reported by **Sheehan and Hrapchak (1987)** who reported that, about 100 times the volume of the tissue is a good approximate amount, and this large volume is necessary since the mineral content of a good-sized piece of bone will soon neutralize the small amount of acid present in the solution, however, **Drury and Wallington (1980)** mentioned that, an ample volume of decalcifying solution should always be used, at least 30-50 times the volume of the tissue**.** Another factor that should be taken in mind is the change of solution and its freshness. Skinner (2003) who mentioned that, in order to ensure "freshness" or availability of specific ions, or lower pH fluid, the solution should be changed on a regular basis explains this point. This allows the calcium ions to more freely migrate out of the material and into the surrounding decalcifying fluid.

CONCLUSION

Tissue thickness should not exceed 4-5 mm to ensure adequate fixation and decalcification in short time. Fixation for 48 hours is satisfactory and adequate volume of fixative should be available. 10%NBF is satisfactory for bone fixation before decalcification process. It preserves the tissue structures and exhibits an excellent staining affinity of tissue to both H&E and Crossman's trichrome stain.

Hydrochloric acid (Jenkin's fluid) causes swelling of collagen fibers of bony lamellae and impairment of staining.

Perenyi's fluid could be used for specimens that will be decalcified within few hours for rapid diagnosis and careful monitoring of the decalcification end point should be done as over decalcification may lead to tissue dissolve.

The decalcifying agents of choice that give good results with minimal harmful effect on bony tissue are 5% Trichloroacetic acid, Formic acid and Neutral EDTA.

5%Trichloroacetic acid should be used for bone specimens that will be decalcified within few days (2-3 days) and tissue should be prefixed in 10% NBF.

Formic acid after Gooding and Stewart and neutral EDTA should be used for small specimens of

bone. Adequate volume of decalcifying agent and freshness of solution are important factors for decalcification process to allow excess of free ions and ensure the low PH of the solution to permit the release of calcium ions from the bone.

Radiological test is unsuitable for tissues of low bone density as bones of rats. So, it should be replaced by other tests. Adequate processing time should be allowed to ensure the penetration of solutions to the tissue. 3 hours per each change is satisfactory dependent on the tissue thickness.

The stain of choice is H&E stain that permit the appearance of the detailed structures of bone as basophilic fine canaliculi and basophilic cement line. In addition to the osteocytes in their lacunae and the acidophilic bone lamellae. So, it should be used instead of Schmorl's picrothionin stain that give undesirable results with paraffin sections.

Crossman's trichrome stain also gives results with bone specimens however, could not show the fine canaliculi and the cement line.

REFERENCES

Bancroft, J. D. and Gamble, M. (2002): Theory and Practice histological techniques. 5th ed., chapter 14, 269 - 299.

Brain, E. B. (1966): The preparation of decalcified sections. Charles C. Thomas, Springfield, IL: 69 -143.

Charman, J. and Reid, L. (1972): The effect of decalcifying fluids on the staining of epithelial mucins by Alcian Blue. Stain Technol.

Culling, C. F. A. (1975): Handbook of Histopathological and Histochemical Techniques, 3rd ed., Butterworths, Boston, MA, 63 -72.

Culling, C. F. A.; Allison, R. T. and Barr, W. T. (1985): In cellular pathology technique, Butterworth& Co, P414. Cited after Shah, Goh, Karunanithy, Low, De and Bose (1995).

Drury, R. A. B. and Wallington, E. A. (1980): Carleton's histological technique. 4thed. New York, Toronto Oxford University Press. 138 -152.

Eggert, F. M. and Germain, J. P. (1979): Rapid demineralization in acidic buffers. Histochemistry, 59:215-224.

Fejerskov, O. (1971): The effect of different demineralizing agents on oral mucous membrane. Scand J. Dent. Res., 79:172-182.

Goncalves, R. P. and Olivèrio, L. G. (1965): Electrical decalcification of bonnet. Mikroscopie, 20:154-156.

Gooding, H. and Stewart, D. (1932): A comparative study of histological preparations of bone which have been treated with different combinations of fixatives and decalcifying fluids. The Laboratory Journal, 7:55.

Laboux, O.; Dion, N., Chaves, V. A., Ste -Maria, L. G. and Nanci (2004): Microwave irradiation of ethanol-fixed bone improves preservation, reduces processing time, and allows both light and electron microscopy on the same sample. J. Histochem. Cytochem, 25:1267-1275.

Luna, L. G. (1960): Manual of Histologic Staining Methods of The Armed forces Institute of Pathology, 3rd ed. McGraw Hill, New York, NY;1-6.

Mawhinney, W. H. B.; Richardson, E. and Malcolm, A. J. (1984): Control of rapid nitric acid decalcification. J. Clin. Pathol, 37:1409 -1415.

Mescher, A. L. (2010): Junqueira's basic histology Text and Atlas,12th ed., Mc Graw Hill,121-128.

Page, K. M. (1977): Bone and the preparation of bone sections. In: Theory and Practice of histological techniques, edited by Bancroft, J.D. and Stevens, A., Churchill Livingstone, London, New York, 223 -248.

Perenyi, J. (1882): über eine neue Erhärtungsflussigkeit. Zoologischer Anzeiger, 5:459.

Robey, P. G. and Boskey, A. L. (1996): The biochemistry of bone. In: Marcus R., Feldman D., Kelsey J., Eds: osteoporosis. Academic Press, San Diego, CA: 95 -183.

Sheehan, D. C. and Hrapchak, B. B. (1987): Theory and Practice of histotechnology, 2nd ed. Columbus, Ohio: Battelle press: 89 - 96.

Schajowicz, F. and Cabrini, R. L. (1955): The effect of acids (decalcifying solutions) and enzymes on the histochemical behavior of bone and cartilage. J. Histochem. Cytochem, 3:122-129.

Skinner, R. A. (2003): Decalcification of bone tissue. In: Hand book of Histology Methods for bone and Cartilage, edited by Y.H. An and K.L. Martin. Humana Press Inc., Totowa, NJ, 167-184.

Verdenius, H. H. W. and Alma, L. (1958): A quantitative study of decalcification methods in histology. J. Clin. Pathol, 11:229 -236.

Warshawsky, H. and Moore, G. (1965): A method of fixation and decalcification of rat incisors for electron microscopy. Anat.Rec, 151:431.

Weatherford, T. W. and Mann, W. V. Jr. (1973): A method of microscopic evaluation of effects of demineralizers on complex carbohydrates of rat tissues. J. Microscopy, 99: 91-100.