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Comparison between conventional decalcification and microwave assisted decalcification in bone tissues

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Abstract

Background: In microscopic assessment of mineralized tissue, decalcification is an important step during tissue processing. In this present study conventional method of decalcification was compared with microwave assisted method of decalcification using a rat bone in 5% HCl.

Aims: To compare conventional decalcification with microwave assisted decalcification in a bone tissue. To decalcify bone tissue using microwave method of decalcification. To decalcify bone tissue using conventional method of decalcification.

Methods: A 2mm sized harvested hindlimbs and spine from a laboratory rat was subjected to both conventional and microwave assisted method decalcification using 5% HCl. The endpoint of decalcification was checked using the physical method. The specimens were then subjected to processing, sectioning and staining with haematoxylin and eosin. The stained sections were observed under a digital microscope.

Results: The results in the present study confirmed the fact that the microwave assisted method of decalcification was faster than that of the conventional method and produced a good histological picture and the microwave method took fifty minutes whilst the conventional method took twenty-four hours to decalcify.

Conclusion: We concluded that the microwave assisted method of decalcification was faster and better histological image were achieved when compared with the conventional method of decalcification.

Keywords: decalcification, chelating agent, calcium phosphate, microwave, incubation, hematoxylin, eosin, bone tissue

Introduction

Decalcification is a process of complete removal of calcium salt from mineralized tissues like bone and teeth and other calcified tissues. Study of fibrillar, cellular and sub cellular structures of mineralized tissues is only possible after the removal of the calcium apatite of these tissues by the process of demineralization. The physical hardness, which is, a unique characteristic of these tissues makes it necessary to "soften" them by removing the mineralized component (Prasad *et al*., 2013) [4]. Reduction of the time between receiving a specimen and reporting the diagnosis has been a main concern of pathologists throughout time. In doing so, there could be an overall decrease in health-care costs with simultaneous improvement in patient satisfaction, especially when there is a need to seek consultation and treatment in a centre/hospital away from the individual's residence or in cases where additional diagnostic measures are required (Hajihoseini *et al*., 2020) [24]. Decalcification of tissues is a routine process carried out in most laboratories by the use of various acids or chelating agents. The cutting of thin sections by ordinary methods is impossible in the case of tissues such as teeth, bone, teratomas containing bony tissue, lesions that have become partly calcified, odontomas and bony lesions. Such tissues must be treated to remove calcium phosphate by a process known as “decalcification”, thereby making the tissue soft enough to be cut by the microtome (Sangeetha *et al*., 2013) [2, 8, 21]. Decalcification of hard tissue is one of the most sensitive procedures in the histopathology laboratory when dealing with hard or calcified tissue. In the manual method of decalcification, hard tissues are placed in a decalcifying agent at room temperature with changes of the solution at regular intervals till the end point is reached. However, it is an inherently complex process, complicated by the trade off between time taken for the process and the quality of the sections produced (Prasad *et al*., 2013) [4]. Microwave decalcification is a novel technique compared to the manual method. In this method, hard tissues would be placed in the decalcifying agent in a microwave oven for intermittent periods with regular changes of the solution till the end point is reached. Microwave irradiation has shown to speed up the process of decalcification significantly–from days to hours (Keithley *et al*., 2001) [9]. The microwave oven has been used quite often for tissue processing, but there are very few studies describing its use in decalcification of bone. In this project we attempt to decalcify bone tissues using a microwave oven and comparing the process and results with conventional decalcification methods (Sangeetha *et al*., 2013). [2, 8, 21] Hard tissue specimens differ from their soft tissue counterpart, in that the former have to be decalcified before routine processing and staining. Several decalcifying reagents including acids and chelating agents have shown promising results. Processing would involve several steps including dehydrating the specimen and rehydrating them with the appropriate media to enable sectioning without tissue damage or distortion. Staining provides the tissue with the necessary differentiation to aid in diagnosis (Raj *et al*., 2016) [3]. The demand for faster processing is ever growing, especially in cases of malignancies where the diagnosis is time restrained. Conventional decalcification

employed in laboratories are labour intense and time consuming thereby delaying the report dispatch (Raj *et al*., 2016) [3]. Microwave assisted decalcification is the process of using microwave. The conventional decalcification is been compared with the microwave assisted decalcification to fast-track the process time. The staining qualities when hematoxylin and eosin is used. The microwave assisted decalcification will ensure that time be faster as compare to the conventional decalcification method. Microwave assisted decalcification of bone tissue during fixation and subsequent histochemical staining procedures would significantly reduce the time required for decalcification of bone tissue. Minimizing the incubation time in decalcification will reduces disruption of tissue morphology (Katoh, 2016) [1].
The aim of the study is to compare conventional decalcification with microwave assisted decalcification in a bone tissue

Materials and methods
Ethics statement
The study protocol was reviewed and approved by the Committee for Human Research and Ethics (CHRE) of the Department of Basic and Applied Biology, University of Energy and Natural Resources (UNER)Sunyani, Ghana (CHRE/CA/010/022)

Study area
The research was conducted at the Pathology Laboratory unit, Hope X change medical Centre, Kumasi to compare conventional decalcification with microwave assisted decalcification in a bone tissue.

Study design
This is an experimental descriptive study and purposive sampling technique aimed to compare the conventional decalcifying procedure with microwave-assisted decalcification with respect to morphology of bone tissue using hematoxylin and eosin stains for complete identification of the bone tissue.

Sample Size
Five (5) harvested undecalcified bone tissues of the laboratory rat for each method.

Laboratory procedure
Preparation of reagents
A 10% neutral buffer formalin was prepared from an aqueous formalin using a calibrated cylinder and a distilled water.

Experimental organs and samples
Five (5) Laboratory were purchased from Animal house at Kwame Nkrumah University Science and Technology, Kumasi and transported to the pathology laboratory at Garden City University College, Kenyasi-Kumasi in a semi-plastic and metallic cage. After the preparation of 10% neutral buffered formalin hindlimbs and spine tissue was harvested from a laboratory rat which was chloroformed. Each harvested bone was washed in a 5% buffer formalin to clean off blood on them. The harvested bone tissues were diced into 2mm thickness that gave us 10 bone sections and placed in the 10% buffed formalin. The bones were harvested at the Pathology unit, Department of medical laboratory technology,Garden City University College, Kenyasi-Kumasi.

Animal sacrifice and organ harvesting
Five (5) laboratory rats were chloroformed. Longitudinal abdominal incision was made and fresh undecalcified bone tissues were carefully harvested.

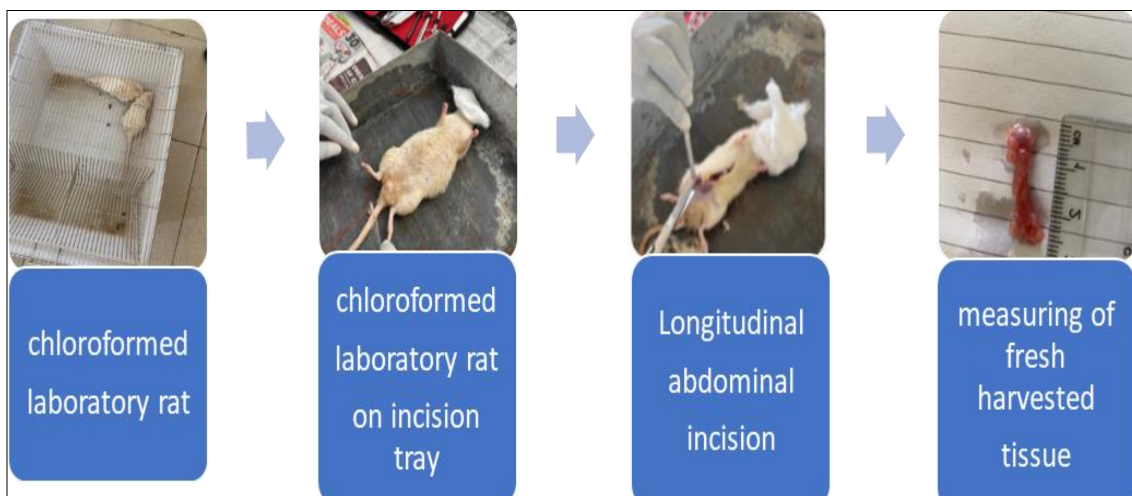

Fig 1

Conventional method of decalcification
A five (5) formalin fixed 2mm thick section bone tissue was washed 30 minutes before submerging in three 250ml pyrex squat beakers each containing 100ml of 5% aqueous hydrochloric acid (HCl) was placed at room temperature. The bone tissues placed in the decalcifying agents was monitored and the end point of decalcification was checked using the physical method known as pinning. A piece of bone was taken from the decalcifying agents. A needle was then piers in to the bone, if it goes through easily then decalcification would be completed. This process was repeated every hour until the needle was able to pass through the bones easily. Therefore, indicating that decalcification was completed. Within the first hour the bone tissues were still hard meaning that decalcification was not complete. The total time used was 24 hours 5% HCl.

Microwave assisted method of decalcification

A domestic microwave oven with fixed rotary plate with maximum power output of 700W and input voltage of 230V-50HZ was used. A glass beaker containing 100ml of distilled water was preheated for 5 seconds to warm up the magnetron. This will be replaced by a 100ml fresh distilled water and radiated to maintain the temperature around 43℃. The glass beaker was placed at different point in a microwave while radiating to determining the best position of the bone tissue during microwave decalcification. A 5-formalin fixed 2mm thick section bone tissue was washed for 30 minutes before submerging in three 250ml pyrex squat beakers each containing 100ml of 5% aqueous hydrochloric acid (HCl). Then the specimen was transferred in to the microwave and was irradiated for twenty cycles (where cycle means the number of seconds the backer containing the sample took) for 30 second each (three-minute interval) for a total time of fifty minute. At every cycle the temperature of the decalcifying solution was checked and then maintained at 43℃. The end product of decalcification was checked using the physical method known as pinning. A piece of bone was taken from the decalcifying agents. A needle was then piers in to the bone, if it goes through easily then decalcification would be completed. This process was repeated every fifty minutes until the needle was able to pass through the bones easily. Therefore, indicating that decalcification was completed.

Tissue Processing

After ensuring complete decalcification, the tissues were washed for 30 minutes following, the specimens were subjected to pre-processing procedures such as: selecting of tissue blocks based on the type and size of tissue. After this, the tissues were later subjected to an automated tissue processer following these protocols

Table 1

REAGENTS	TIME
70% Alcohol	45 min
80% Alcohol	45 min
95% Alcohol	45 min
100 % Alcohol	45 min
100 % Alcohol	60 min
100 % Alcohol	60 min
Xylene	60 min
Xylene	60 min
Molten paraffin	45 min
Molten paraffin	45 min
Molten paraffin	60 min
Molten paraffin	90 min

Embedding

After tissue processing, the tissues were then placed in a mold and embedded in a paraffin wax and allowed to cool on a cooling plate. After the tissues have been cooled and placed in a refrigerator to solidify.

Trimming and Sectioning

Tissue blocks were trimmed to expose the surface of the tissue and to get rid of the excess wax, after that the tissues were sectioned at 5µm thick using a microtome and ribbon section were obtained. The ribbon sections were picked up with a pair forceps and laid it on a water bath with a degree of 45℃. The best sectioned tissues were picked up on a slide.

Staining and mounting

Automated tissue Stainer and cover slipper following these protocols.

Table 2: Haematoxylin and Eosin staining

Stage	Regeant	Duration
1	Deparaffinize and rehydrate sections	30sec
2	Xylene	4×3min
3	100% ethanol	2×3min
4	95% ethanol	1×3min
5	80% ethanol	1×3min
6	70% ethanol	1×3min
7	Deionised water	1×3min
8	Harris Haematoxylin	1×3min
9	Tap water	1×5min
10	1% Acid alcohol	1×30sec
11	Tap water	1×5min
12	70% alcohol	1×3min
13	0.5% Eosin	1x1min
14	80% ethanol	1×5min
15	90% ethanol	1×3min
16	100%	3×5min
17	Xylene	3×2min

Results

Table 3: Decalcification status for microwave decalcification.

Cycles	Decalcification status
1st	NO DECALCIFICATION
2nd	
3rd	
4th	
5th	
6th	
7th	
8th	Partial decalcification.
9th	
10th	
11th	
12th	
13th	
14th	
15th	
16th	
17th	
18th	
19th	
20th	Completely Decalcified

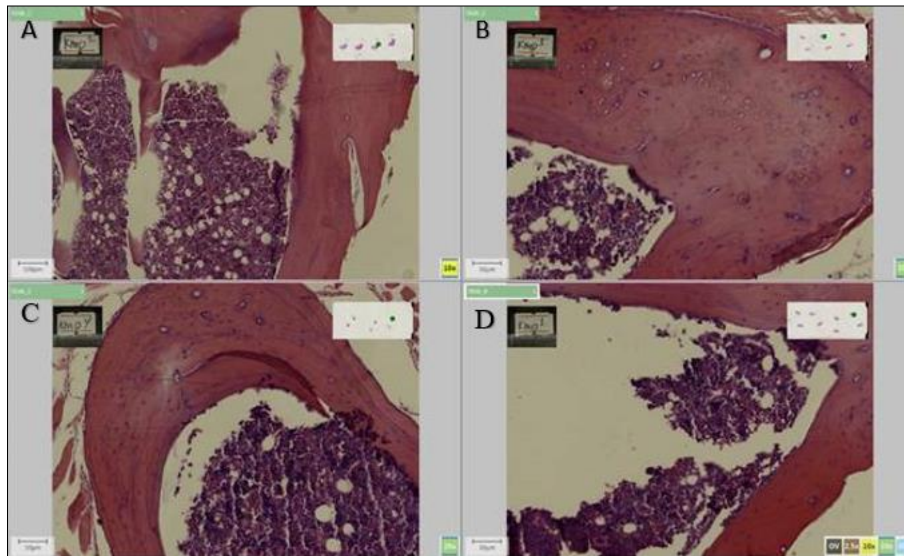

Fig 2: Bone tissues decalcified with the microwave method.

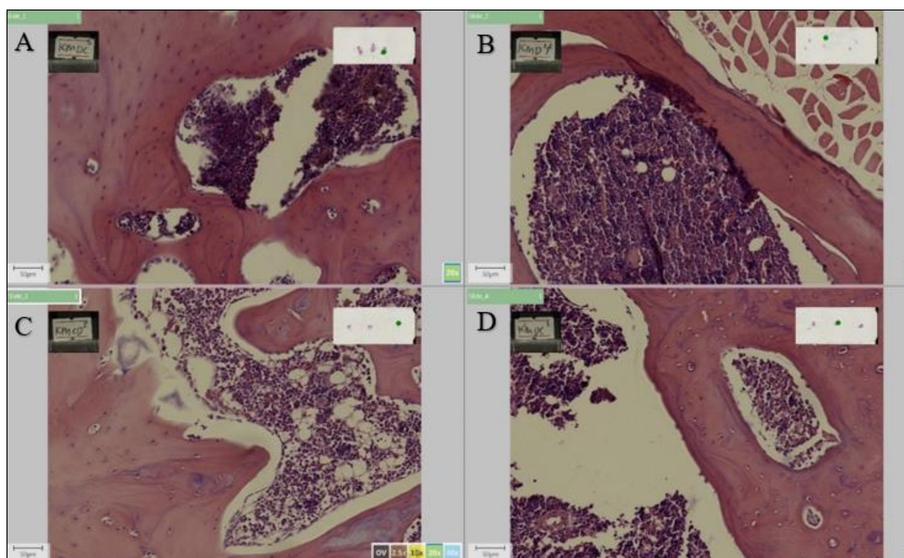

Fig 3: Bone tissue decalcified with conventional method

LEGEND: A= spine, B=HINDLIMBS, C= SPINE, D=HINDLIMBS

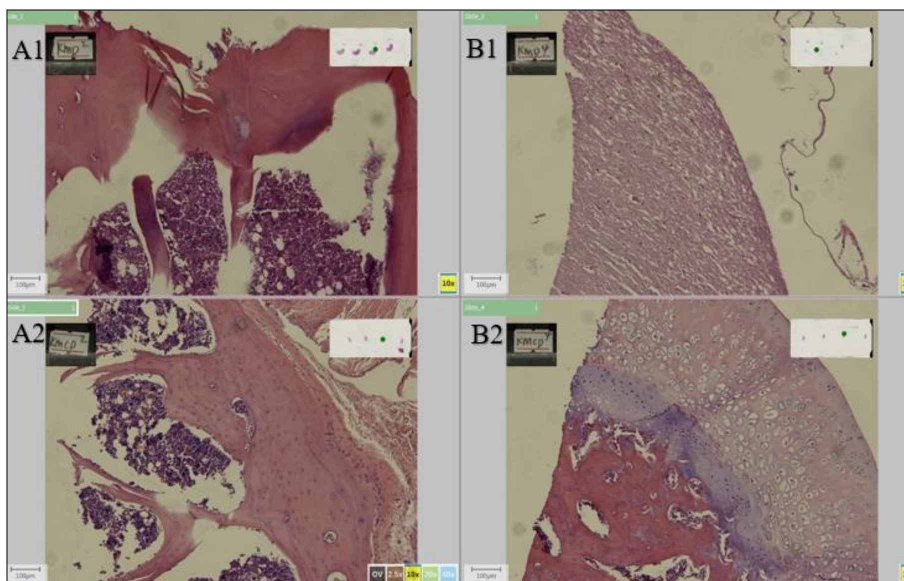

Fig 4: Compared tissues stained with hematoxylin and eosin.

LEGEND
A1 = Spine tissue decalcified with the microwave assisted method.
A2 = Spine tissue decalcified with the conventional method.
B1 = Hindlimb tissue decalcified with the microwave assisted method.
B2 = Hindlimb tissue decalcified with the conventional method.

Discussion

Regardless of the solution used, methods of decalcification share their characteristic of being accelerated when stirred either mechanically or electrolytically using electrodes (Pitol *et al*., 2007) [19]. Gonçalves and Olivério used an electric decalcification method with alternate current, increasing the decalcification rate in approximately 3 times compared to the conventional method (Gonçalves *et al.,* 1965) [17]. Pitol *et al*., (2017) in his study stated that there was 30-fold increase in the decalcification process compared to the conventional method when the material was irradiated in microwave oven using rat bones. A novel process using microwave oven was realized to quicken the decalcification process. The selection of the decalcifying agents was basically determined by the earnestness of the method and the possible use of microwave energy in histological technique which was first documented by Mayer's in 1970. The molecular kinetics then cause production of energy change, which last until radiation stops (Salih., 2020) [20]. According to a study done by Hajihoseini *et al.,* in 2020 [24] that use of a microwave accelerated the demineralization process of sheep hard tissues, regardless of the decalcifying agent and tissue type (bone or teeth). Microwave produces uniform increase in temperature throughout the specimen. Thus, microwave decalcification hastens the diagnostic time without compromising the diagnostic quality of the tissue. In our study, decalcification time reported for the microwave-enhanced decalcification took fifty minutes with 5% HCl. It was clear that microwave decalcification method for the bone tissue was faster and was completely decalcified. The microwave decalcification protocol employed in the present study is based on a modification of Sangeetha *et al*. (2013) [2, 8, 21], protocol as elaborated in methodology.

Furthermore, decalcification of the bone with the conventional method took twenty-four (24) hours whereas the microwave oven method took fifty minutes. A study, conducted by Salih et al., in 2020. Reported decalcification times for microwave-enhanced decalcification and the conventional decalcification procedure was five and eight hours with 5% HCl respectively. Another study done by Srinvasyaiah *et al.,* in 2016 has shown a reduction in time with respect to decalcification of tooth. This means that the decalcification process of microwave is faster than that of the conventional method. The energy produced by microwaves generated in a domestic oven interacts with dipolar molecules by imparting kinetic energy and altering the electric fields. This energy induces a dielectric field leading to a rapid oscillation of dipolar molecules, generating heat that is rapidly distributed homogeneously within the tissue (Sangeetha *et al.,* 2013) [2, 8, 21]. Gruntz *et al.,* and Sangeetha *et al.* (2013) [2, 8, 21], in their observed that microwave decalcified tissue displayed a superior histopathological picture in comparison to the conventionally decalcified tissue. Raj *et al.* (2016) [3], observed that conventional decalcification staining displayed quality. Cunningham *et al.* (2017), in his study concluded that microwave decalcification provides an efficient and reliable means of processing human temporal bones for histological and histochemical de-amination and also stated that the decalcification time was reduced with no apparent adverse effects on structural preservation or antigenicity (Cunningham *et al*., 2007). Pitol *et al.* (2007) [19] again stated that good morphological preservation of the bone tissue.

Conclusion

We concluded that the microwave assisted method of decalcification was faster and better histological morphology were achieved when compared with the conventional method of decalcification.

Recommendation

We recommend that other decalcifying agents should be used. Also, different time should be allocated to tissue with high calcium content other. These results should be supported by further studies with larger sample sizes and a wider choice of decalcifying agents.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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