End-Point Determination In Acid Decalcification Of Bone: A New Novel Method

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***Abstract*- Background:** Calcium oxalate test is currently the chemical method employed in testing endpoint of decalcification in acid decalcification. However, there are challenges with this existing method used; firstly, the method takes a long duration of 15-30 minutes to complete; secondly, the method uses ammonia which is a highly irritating gas with a sharp suffocating odor necessitating its use in a fume hood; thirdly, exposure to ammonia gas through breathing, swallowing or skin contact causes burning and irritation of the contact surfaces, coughing and can result in blindness, lung damage or death; fourthly, ammonia loses potency over time and lastly, containers of ammonia may explode when exposed to high heat. Additionally, when a high concentration of ammonia is mixed with air, it can explode when ignited. Accordingly, there is a need to device a new chemical method for testing endpoint of decalcification in acid decalcification.

Objective: The purpose of this study was to introduce a new novel chemical method involving optimized chemical reagents and procedure, allowing the determination of endpoint of decalcification of bone in acid decalcification method.

Design: Experimental research design was conducted.

Data analysis: asymmetric measures of agreement for binary data was used to analyze the results of endpoint of determination using two chemical methods, calcium oxalate test and a new novel method, sodium carbonate test.

Setting: The research study was conducted in a teaching histology/histopathology laboratory at Murang'a University of Technology.

Method: Bone samples that were harvested from the radius bone of a rabbit and fixed in 10% neutral buffered formalin were used in this study. The fixed bone samples were decalcified in 5% aqueous nitric acid solution for a period of 2-7 days and tested for end-point of decalcification using both calcium oxalate test method and the new novel method, sodium carbonate test method. Formation of a cloudy precipitate and clear solution in both methods indicated incomplete and complete decalcification respectively.

Results: there was agreement between the results of calcium oxalate and sodium carbonate test methods in determination of endpoint of decalcification in acid decalcification of bone, in that, the bone samples that showed incomplete and complete decalcification in the two test methods were similar.

Conclusion: According to this study, the findings suggests that the new novel method, sodium carbonate test method is a suitable alternative to the existing calcium oxalate test method in routine histopathological determination of endpoint of decalcification in acid decalcification. Additionally, the new novel method proves to be the best with quick time of getting results and overall safety.

***Index Terms*-** decalcification, endpoint determination, ammonium oxalate test, sodium carbonate test

I. Introduction

Decalcification refers to the process of complete removal of calcium ions from a previously fixed calcified tissue. Decalcification is important so as to soften the calcified tissues to facilitate preparation of tissue sections from them for microscopic examination and study[1,2,3,4,7,8,9,10,13, 14].

Calcium is abundant in nature and the human body, primarily in bone and teeth, where it exists as calcium phosphate, hydroxyapatite and magnesium whitlockite. In bone, calcium serves as an essential element for bone growth and preservation of bone mineral density. Bone provides structural support and strength that is necessary for locomotion and protection [4, 8, 10, 12, 14].

Calcification is generally a normal physiological process, as occurs in bone and teeth, but can also be a pathological process, as in the case of deposition of calcium in blood vessels and lungs and arteries due to diseases like atherosclerosis and tuberculosis respectively [8, 12].

Bones are among the hardest tissues found in humans, because they are composed of large amounts of inorganic compounds primarily calcium ions/salts. Thin sections of bone are very difficult to prepare for microscopic examination hence require use of chemical acids to remove these inorganic compounds. Therefore, histological analyses of these highly mineralized calcium ions/salts is achieved through the process of decalcification [3, 15].

Various methods have been employed for decalcification such as electrophoresis, use of ion-exchange resins and chemical agents. Among these, chemical agents, mainly acids and chelating agents, are commonly used to remove these calcium ions/salts. The acids react with calcium in bone to form soluble calcium salts and chelating agent bind calcium to form a complex [2, 4, 5, 6, 8, 9, 11, 13, 14, 15].

Traditionally, determination of endpoint of decalcification has been done using physical, chemical and radiographic methods [5, 10, 13, 14, and 15].

II. Materials and Methods

This is an experimental descriptive study that was aimed at introducing and evaluating the performance of a new novel method, sodium carbonate test method, in determination of endpoint of decalcification in acid decalcification of bone. Its performance was compared with the existing method, calcium oxalate test method, which is considered the gold standard in this case.

2.1 Sample selection

All the bone samples used in this study were collected from rabbit radius bone; the radius bone was cut into 10 pieces ranging from 1-10 cm in size using a bone saw.

2.2 Sample processing

The 10 bone samples were fixed in 10% neutral buffered formalin for 72 hours.

2.3 Decalcification

The 10 bone pieces were then decalcified at room temperature by immersing them in 5% aqueous nitric acid decalcifying fluid (which was 20 times their volume) for a range of 2-7 days depending on the size of the bone tissue. The exact date and time at the start of decalcification was noted. Each day, all the 10 bone samples were chemically tested for endpoint of decalcification using calcium oxalate test method and the new novel method of sodium carbonate test. Each time the endpoint had not been reached; the tissue was removed from the decalcifying fluid, rinsed in running distilled water and placed in a fresh change of decalcifying fluid. This step was repeated until decalcification was found to be complete.

2.4 Testing endpoint of decalcification using chemical method

The following chemical procedures were used for each of the 10 bone samples to determine whether decalcification was complete or incomplete.

2.4.1 Calcium oxalate test method [5]

1. A piece of blue litmus paper was introduced into a clean test tube.
2. 5 ml of the spent decalcifying fluid was decanted into the test tube turning the litmus to pink colour.
3. Strong liquid ammonia was added drop by drop until the solution became alkaline, turning litmus to blue colour.
4. The solution was observed for any cloudiness which meant that calcium was present, indicating need for further decalcification which was achieved by placing the bone undergoing decalcification into fresh decalcifying fluid for 1-4 hours depending on the size of the bone. Testing endpoint of decalcification was repeated again.
5. If the solution did not become cloudy in step 4 above, 1ml of saturated ammonium oxalate was added into the test tube and left to react for 30 minutes. If the solution turned cloudy, decalcification was not completed and the bone was placed in fresh decalcifying fluid for 1-4 hours to undergo further decalcification. If the solution was clear after the 30 minutes, it was presumed that decalcification was complete, and the bone samples underwent post-decalcification processing.

2.4.2 Sodium carbonate test method

This method has FIVE steps:

1. 2mls of the used acid decalcifying solution was decanted into a clean and dry test tube,
2. 3mls of 20% aqueous sodium carbonate solution was added into the test tube while shaking slightly until effervescence stopped. This step neutralized the acid and provided excess hydroxyl ions that reacted with any calcium ions present to form calcium salts that would precipitate turning the solution cloudy.
3. Neutralization was confirmed by addition of a piece of red litmus paper which changed colour to blue.
4. The reaction mixture was observed for presence or absence of turbidity. Turbidity indicated that calcium was still being released by the bone tissue and decalcification has not ended while a clear solution indicated that calcium was no longer being released and decalcification was presumed to be complete.

2.5 Post-decalcification

After decalcification was complete for all the 10 bone samples, they were washed in running tap water for half an hour and then subjected to normal tissue processing starting with 70% alcohol.

III. Results

Both calcium oxalate test method and the new novel method, sodium carbonate test method, showed agreement for bone samples that had completed decalcification and those that had not completed decalcification for each of the 7 days that testing endpoint of decalcification had been performed.

IV. Discussion

A chemical method is disclosed herein which is used to check whether calcium has been removed from bone tissues that are undergoing the process of calcium removal, a process called decalcification. In this case, the decalcification is done using a 5% aqueous dilution of nitric acid. The above description of the procedure for sodium carbonate test is given by way of example, and not limitation. Given the above disclosure, one skilled in the art could devise variations that are within the scope and spirit of the invention disclosed herein. This method has the following advantages; first, it takes a duration of 2 minutes to complete the steps; second, it is inexpensive as it does not require to be used within a fume hood; third, it uses an odorless chemical that is stable under normal conditions of use and storage; fourth, the chemical used is usually not very toxic in small amounts and fifth, the chemical used is not flammable or combustible. This method invention is a protocol for end-point determination of decalcification performed using strong acids. The principle of the protocol is based on reaction of aqueous sodium carbonate salt with the acid used in decalcification. The calcium ions present in

the acid react with the alkaline aqueous sodium carbonate solution to form a turbid solution which indicates calcium is still being released by the tissues undergoing decalcification. This indicates decalcification should continue.

V. Conclusion

According to our study, the proposed new novel method, sodium carbonate test method, proves to be the best in comparison to the existing calcium oxalate test method, which is considered the gold standard in this case. Therefore, this finding indicates its suitability as an alternative method for determining endpoint of decalcification in bones undergoing acid decalcification in a histopathology laboratory. However, further studies are needed to explore performance of this new novel method for other tissues like teeth and pathologically calcified tissues like lungs and blood vessels that are undergoing acid decalcification.

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Conflict of Interest: The authors declare no conflict of interests.

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Ethics statement: This study fulfills ethical requirements of laboratory animal care and use.

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