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Decalcification: A Simpler and Better Alternative.

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ABSTRACT

Background: Decalcification of calcified tissues plays an important part in histological techniques. However, as it often takes a long time and some procedures decrease the staining qualities of the specimen, many attempts have been made to find methods for accelerating this procedure and ensuring good staining properties. One of the factors that regulates decalcification is temperature. Controlled increase of temperature yields decalcification at a faster rate and also retains the basic molecular arrangement. The aim of the study is to formulate a simpler and better alternative for conventional decalcification. **Methods:** Thirty freshly extracted periodontally compromised molar teeth without evidence of dental caries were used for decalcification in three groups. In each group 10 teeth were used. Group A: 5% HNO_3 was used. Group B: 10% HNO_3 and 10% formalin was used. Group C: 10% HNO_3 and 20% formalin was used. A constant temperature of 55^0C was maintained. Complete decalcification was checked using X-ray method. The teeth were sent for routine processing and stained using Haematoxylin and Eosin. **Results:** Decalcified teeth of Group C containing 10 % HNO_3 and 20% formalin proved to be advantageous completing decalcification faster among the 3 groups while maintaining good tissue details. **Conclusion:** In the present study, it was observed that, regardless of the employed fixative solution, preservation of pulp architecture was best, when a combination of 10% HNO3 and 20% formalin was used as a decalcifying agent.

Keywords: Decalcification; Teeth; Temperature; Simple method.

INTRODUCTION

Teeth belong to the category of hardest tissues due to tooth enamel, which is denser and chemically more inert than other body tissues. Because of large amounts of inorganic components in teeth, calcium and phosphorus, biological apatite is very hard to prepare for microscopic examinations. On the other hand, complex tooth structure and first of all the extraordinary hardness of enamel makes this organ very difficult to process and section for observation under microscope.[1]

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Human teeth, as well as alveolar bone, must be decalcified during processing for histological analysis because of its structure. Rapid fixation of all dental elements is difficult to obtain because penetration of the fixating agent through such structures as enamel, dentin and bone is a slow process. In such cases, the tissues in the center of the specimen may undergo some alterations before fixation is completed. The most seriously affected tissue is perhaps the pulp.[2]

The goal of decalcification is to remove calcium salts from the mineralized tissues, preparing them for further sectioning of the histological specimen. Any acid, even if properly buffered, affects tissue stability. These effects depend on the solution's acidity and duration of the decalcification process. In addition, the faster the action of the decalcifying agent, the greater the damage to the tissue. The rapidity of decalcification may also lead to untoward effects to the staining technique performed subsequently. The factors influencing the speed of decalcification include decalcifying solution concentration, temperature, stirring and tissue suspension.[2]

Decalcification is performed by chemical solutions, which employ acids (acids may be divided into strong and weak acids or chelates.[3-5] It is ought to be emphasized that fixative agents that contain acid in their composition, such as formalin (that contains formic acid), may also be able to act as decalcifying agents if the acid component is not neutralized.[3]

Decalcification is commonly employed in most histopathology laboratories for the microscopic examination of bone and other calcified tissues. Plastic processing without decalcification may produce superior results in terms of eliminating shrinkage and for demonstrating osteoid versus mineralized matrix but may give poor cytological detail and is a much longer process. The diagnosis of non-metabolic diseases of bone such as infections and tumours requires good cellular morphology and a quick result to allow rapid therapeutic intervention for optimal patient care.[3]

Most unsatisfactory results with decalcification can be attributed to overexposure to the agent used due to inadequate control procedures. The commonly used control procedures using the ammonium oxalate chemical test[5] and its modifications are accurate with experienced hands[6] but can be otherwise easily abused. Furthermore, although control using radiographs is simple and accurate in principle; it proves to be expensive for smaller laboratories and is only realistic for use at 24 hr intervals. Normal mechanical methods of "testing"

using "bending or probing' cause tissue artifacts and are not considered by any serious bone laboratory for control purposes.[3]

Many alternative decalcification regimen have been proposed,[4] but most of them have at least a few unsatisfactory characteristics. In an attempt to reduce the commonly encountered artefacts of tissue shrinkage and adverse staining results obtained with rapid decalcification using strong mineral acids such as nitric acid, a rapid method of decalcification was devised which gave excellent and reproducible results.[3]

MATERIALS AND METHODS

The study was carried out on 30 freshly extracted periodontally compromised molar teeth without evidence of dental caries. These were then sectioned bucco-lingually. All extracted teeth were fixed in 10% neutralized formalin solution.[3] different decalcifying agents were prepared which were slight modifications using nitric acid as the main component. The teeth were then divided into 3 groups depending on the solutions in which the teeth were decalcified. These solutions were formulated with slight modifications of the regularly used decalcifying agents.

Group A: 5% HNO_3 (n=10)

Group B: 10% HNO_3 and 10% formalin (n=10)

Group C: 10% HNO_3 and 20% formalin (n=10)

Teeth were decalcified in 100 ml of decalcifying solution maintained at a constant temperature of 55ºC in an incubator. The decalcifying solutions were changed every three hourly and were stored in 10% formalin overnight. The process continued till complete decalcification was obtained. Radiographic method was used to determine the end point of decalcification. The specimens were kept in running tap water for 24 hours to neutralize the acids. Thereafter the teeth were dehydrated in an increasing ethanol series, cleared with chloroform, soaked in liquid paraffin and embedded in solid paraffin. The paraffin-embedded tissues were serially sectioned in a semiautomatic "Microm-HM 340E" rotary microtome. Serial sections of 4-µm-thickness were obtained and stained with hematoxylin and eosin. All specimens were mounted using DPX and were observed under light microscope.

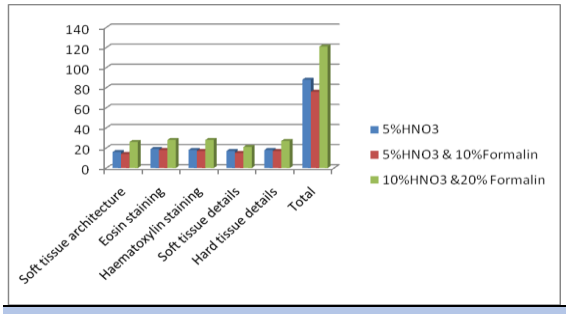
The photomicrographs of teeth showing all the components - enamel, dentin, cementum, pulp and surrounding tissues were compared. It was assumed that basing the results on morphological analysis, it was possible to establish the best teeth decalcification methods, which were indispensable for routine histopathological diagnosis. Elements of the tooth structure were estimated qualitatively, considering their microscopic structure after decalcification.

A score of 1 to 3 was given to estimate the time of decalcification. Hard tissue staining, soft tissue staining for eosin, soft tissue staining for hematoxylin, soft tissue architecture and cellular details were graded, using a score of 1 to 4. 1 was taken to be poor and 4 were very good, and for time taken for decalcification 1 was slow and 3 were fast.

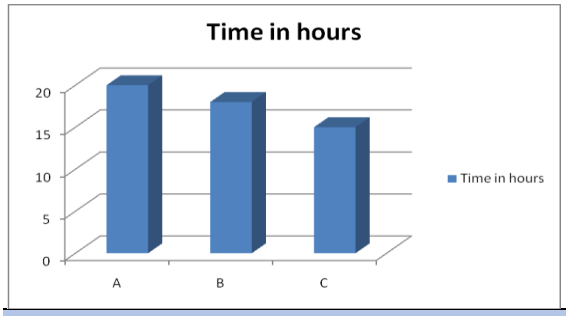
RESULTS

The best results i.e clarity of morphological details were obtained after decalcification in 10% HNO_3 and in 20% Formalin (group C) than the other groups. Pulp structure and organic dentin matrix, cementum were very well preserved [Figures 1, 2]. Group A specimens showed better results than the group B. [Graph 1]

In terms of time taken for complete decalcification, specimens from group C completed decalcification in 15 hours, group B took 18 hours and group A took longer time of 20 hours [Graph 2].



Graph 1: Comparison of parameters used to assess the cellular and staining properties of teeth in group A,B and C.



Graph 2: Time taken for decalcification for specimens of group A, B and C.

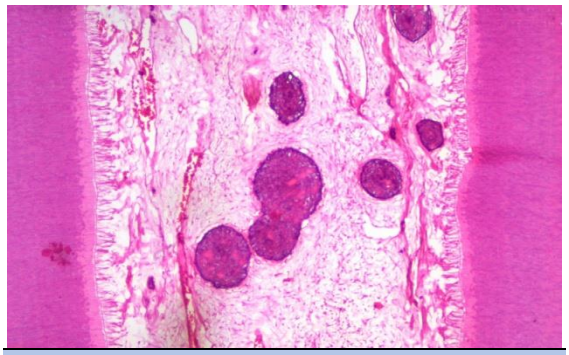


Figure 1: Photomicrograph (4X) of the specimen from group C showing good architectural and cellular details with good eosin and hematoxylin staining of hard and soft tissues.

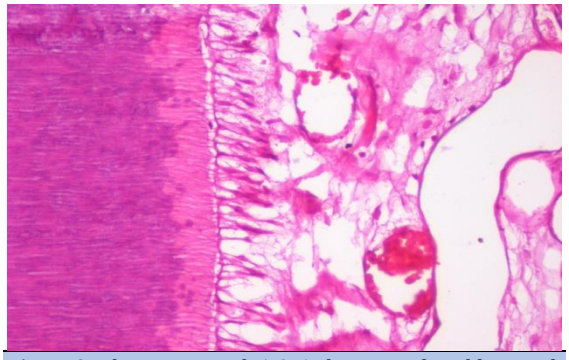


Figure 2: Photomicrograph (20X) showing odontoblasts with its processes entering the predentin and dentin. Globular calcifications are seen.

DISCUSSION

Decalcification is most important in the preparation of oral calcified tissues for microscopic examination. It is important from two standpoints: first, sections of teeth, bone, and surrounding tissues are difficult to obtain without removal of the calcium; and second, the effect of the various chemical decalcifiers upon the tissue components differ.[6] Preparation procedure of hard teeth tissues is started from its fixation.[3]

Choice of a fixing reagent is dependent upon the tissue itself and the purpose for which it is to be preserved. Special reagents prepare the tissue for study of specified elements or reactions and chemically prepare for the application of selective stains. Formaldehyde solution is an excellent general fixing reagent and permits the subsequent use of a large variety of staining methods. Many questions may be raised as to the advisability of its use. However, it is an agent which may be handled by anyone not acquainted with the details of the special agents.[6]

Fixation with either 4% para-formaldehyde or 10% formalin seems to preserve the pulp tissue and maintain favorable conditions for examination and microscopic analysis of its cell components. Nevertheless, 10% buffered formalin is more commonly used because it is more readily available and may be stored for longer periods.[2] Thus we used 10% neutral buffered formalin to fix the tissues. The decalcification methods, independently of the demineralizing agent used, have in common the facts that the process is accelerated when the solution is shaken, mechanically or electrolytically. Goncalves & Oliverio used an electric decalcification technique, with alternate chain, increasing the decalcification velocity. According to these authors, this process promotes -molecule shaking, resulting in an increase of the decalcification process. The results of this work, producing molecular vibration induced by microwaves, a similar effect was observed.[7]

Clearly, in 1978, explained that the acceleration process promoted by microwaves is due to the energetic portion of the electromagnetic spectrum that interacts with dipolar molecules provoking a fast oscillation, in this way, increasing the intra and intermolecular movements of the water and of the polar portion of the protein chain, increasing the temperature and resulting in its coagulation. It was observed that, independently of the demineralized agent used, the microwaves accelerated the process and the morphology was preserved. Some authors suggested that the microwaves can induce an elevation of the temperature, increasing the decalcification process by decalcified agent diffusion (Boon & Kok, 1998; Balatona & Loget, 1989; Vongsavanea et al., 1990; Tornero et al. 1991). At the same time, an increase of the temperature is interesting, but a higher temperature elevation (60 °C) is a disaster for the morphologic characteristics preservation (Balatona & Loget; Boon & Kok; Tornero *et al.).*[7] Therefore in our study we used a constant temperature of 55°C maintained in an incubator as not all labs have the facility of a microwave. We observed that by using a raised temperature, decalcification was carried out at a faster rate than by the conventional method. Decalcification was faster for group C specimen.

We also observed that architectural [Figure 3] and cellular [Figure 4, 5] and details were maintained in good condition and we obtained good hematoxylin and eosin staining of the tissues. This showed that even at 55º C the staining properties were unaffected and cellular details were maintained.

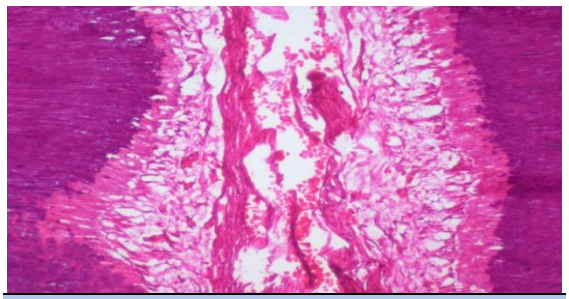


Figure 3: Photomicrograph (4X) of the specimen from group A showing fair architectural and cellular details with satisfactory eosin and hematoxylin staining of hard and soft tissues.

The size of the specimen also dictated the time taken for decalcification, incisors completing decalcification at a faster rate than the molar teeth. Murayama and his colleagues (1937) reported that with increasing temperature a gradual decrease in the time necessary for decalcification in nitric acid and trichloracetic acid was observed. These findings were in rough agreement with our findings.[8]

Strong mineral acids such as nitric and hydrochloric acids are used with dense cortical bone because they remove large quantities of calcium at a rapid rate. As might be expected, these strong acids also damage cellular morphology. Mineral acid decalcifiers are not recommended for delicate tissues such as bone

marrow. Because they are not as aggressive, organic acids such as acetic and formic acid are better suited to bone marrow and other soft tissues. Organic acids act more slowly than mineral acids, and will require extended treatments to decalcify cortical bone. Formic acid in a 10% concentration is the best all-around decalcifiers. Some commercial solutions combine formic acid with formalin to fix and decalcify tissues at the same time.

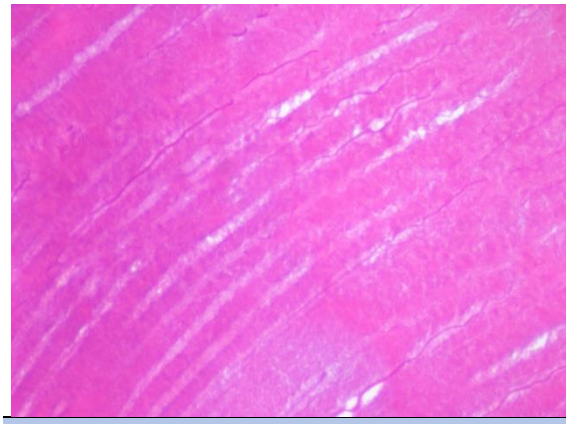


Figure 4: Photomicrograph (40X) showing odontoblastic process in the dentinal tubules.

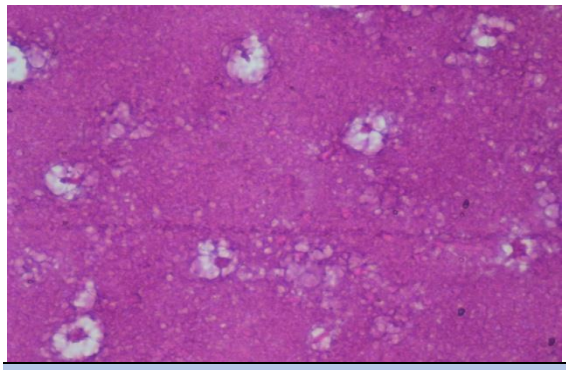


Figure 5: Photomicrograph (40X) showing cementocytes with cytoplasmic extensions within the lacunae..

In our study, group C containing 10% nitric acid along with 20% formalin gave better preservation of soft tissue architecture and also good staining characteristics with eosin and hematoxylin when compared with the other two groups.

CONCLUSION

In the present study, it was observed that, regardless of the employed fixative solution, preservation of pulp architecture was best, when a combination of 10% HNO_3 and 20% formalin was used as a decalcifying agent. The specimen in this group presented a greater accuracy of anatomical details and better preservation of tissue matrix and cells. 4µm sections could be easily obtained using this technique. The use of controlled temperature speeds up the decalcification procedure and this technique could be employed for routine specimen for faster processing and better preservation of tissue details in the case of hard tissue.

Further research is required to corroborate the results obtained with the solutions formulated in our laboratory and the temperature employed in our study.

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