Research Article

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Efficacy of Various Decalcifying Agents on Human Teeth

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ABSTRACT

Aim: To evaluate the rate of different decalcifying agents and also their effect on staining characteristics on dental hard tissues.

Materials and methods: 50 freshly extracted non-carious teeth were taken, fixed in 10 % formalin for 48 hours and were kept for decalcification in three different solutions and were grouped accordingly. Group A: 5 % HNO_3, Group B: 10 % HNO_3, Group C: 10 % HNO_3 and 10 % formalin was used. Endpoint decalcification was evaluated using a radiographic method, and teeth were routinely processed and stained with Haematoxylin and Eosin stain. Different parameters were used to assess the decalcification process and were graded.

Results: Overall, the solution containing 5% HNO_3 scored the highest proving to be advantageous among the three groups.

Keywords: Decalcification, decalcifying agents, HNO_3 solution, HNO_3 + Formalin solution, Human teeth.

INTRODUCTION

Pierre de Coubertin proposed the Olympic motto "citius, altius, forties" which is Latin for faster, higher, and stronger. With this basis, we too have adopted the same in our quest for a decalcifying agent Human dentition and bone has been the subject of intense histological investigations for many years.[1] The head and neck is a complex structure of both soft and hard tissues. Soft tissues put forth little resistance to the histochemical techniques. Lesions affecting hard tissues need an intricate, technique-sensitive methodology for interpretation and diagnosis. Rephrase tissue is an inherently difficult tissue to work with histologically. The technique for demonstration includes hard-tissue grinding and decalcification. Preservation of hard tissues close to the living state is essential for an understanding of cellular and subcellular structures and functions. 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, odontomes 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.[2]

However, it is an inherently complex process, complicated by the tradeoff between time taken for the process and the quality of the sections produced. Where the stronger acids provide the fastest result with the poorest quality of sections, chelating agents provide the slowest result and the best sections.[1]

For many years, scientists have tried to introduce new decalcifying substances or tried to modify

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known decalcification agents, in order to meet the criteria of a good decalcifying agent which

i. Ensures complete removal of calcium;
ii. Causes minimal damage to cells and tissues;
iii. Causes non-impairment to subsequent staining and;
iv. Decalcifies at a reasonable speed.

Most authors have compared two to four decalcifying agents, sometimes varying the methods used and by employing a lot of permutations and combinations of the methods and agents, mainly to decalcify bone.[3]

So, we present here the comparative evaluation of different decalcifying agents with respect to the rate of decalcification, the effect of decalcifying agents on the dental tissue, and its influence on the staining characteristics. The quest for at most near-ideal decalcifying agent begins.[3]

The aims and objectives of the study were:

1. To evaluate the fastest decalcifying agent;
2. To evaluate the decalcifying agents based on its effect on the organic content and integrity of the soft tissue

MATERIALS AND METHOD

The study was conducted on 50 freshly extracted anterior and single-rooted premolar teeth without evidence of dental caries. Teeth were fixed in 10% formalin solution for a minimum 48 hours. Different decalcifying agents were prepared, which were slight modifications using nitric acid as the main component.

Teeth were divided into 3 groups depending on solutions in which teeth were decalcified:

- Group A: 5% HNO_3.
- Group B: 10% HNO_3.
- Group C: 10% HNO_3 and 10% Formalin.

Teeth were decalcified in 100ml of decalcifying solution at room temperature. Decalcifying solutions were replaced by newer fresh solutions every day until complete decalcification was obtained. The radiographic method was used to determine endpoint of decalcification. **(Figure 1)**

Specimens were kept in running tap water for 24 hours to neutralize the acids and continued with routine processing, paraffin wax infiltration, and embedding. Sections of 4µm thickness were obtained and stained with Hematoxylin and Eosin stain. The stained sections were mounted using DPX and observed under a light microscope and evaluated for the following parameters and were graded from 1-4. (1-poor, 2-fair, 3-good, 4-excellent)

Parameters used to assess the decalcification process were:

- Speed of decalcification
- Ease of sectioning
- Soft tissue details
- Hard tissue details
- Quality of staining

Speed of decalcification (in hours) was graded as:

- Slow (48 hours or greater than that)
- Medium (24 to 48 hours)
- Fast (24 hours or less than that)

• Time taken for decalcification based on radiographic estimation of the end-point.
• Ease of sectioning: assessed based on ribbon formation, scoring/splitting of sections during cutting and the ease with which the sections could be handled.
• Quality of staining: assessed by the intensity of Hematoxylin staining of the nuclei and intensity of Eosin staining of the cytoplasm, graded as adequate or over stained or under stained.
• Soft and hard tissue details:
• Dental pulp: the presence of all the four zones of the pulp and the amount of separation of pulp from the surrounding dentin.
• Dentin: assessed based on the presence of vapour bubbles, fraying in the dentinal tubules and destruction of the odontoblast architecture.
• Cementum: The loss of Cementum architecture and loss of attachment from surrounding dentin or Periodontal Ligament (PDL) were assessed.
• Periodontal ligament: examined for the amount of separation from surrounding hard tissues, namely tooth and bone.
Overall cumulative scoring was done for all three decalcifying agents.

RESULTS

Evaluation of speed of decalcification of various decalcifying solutions revealed that group B (10% HNO_3 solution) decalcified teeth faster with the meantime taken 20 hours (16 – 24 hours) while group C (10% HNO_3 + 10% formalin) decalcified teeth slowest with the meantime taken 63 hours (52 – 71 hours). Meantime taken for decalcification by group A (5% HNO_3 solution) was 35 hours (24 – 45 hours). **[Graph 1]**

Ease of sectioning was very good with 10% HNO_3 (group B) solution while there was crumbling of tissue decalcified in 10% HNO_3 + 10% formalin (group C) solution while sections decalcified using 5% HNO_3 (group A) solution gave intermediate results. In terms of efficacy of agents with respect to soft tissue details, hard tissue details and quality of staining, excellent results were actually obtained with sections of teeth decalcified using 5% HNO_3 solution (group A) and fair with that of 10% HNO_3 + 10% formalin (group C) solution.

Soft-tissue attachment and soft-tissue shrinkage revealed the worst results in sections decalcified using 10% HNO_3 solution wherein 5% HNO_3 solution gave good results as it showed minimal soft-tissue shrinkage and minimal loss of tissue. The pulp organization, with its extracellular matrix and histological zones, were clearly distinct and excellent in teeth decalcified with 5% nitric acid. **[Graph 2]**

Overall, 5% HNO_3 solution scored over the other agents, and 10% HNO3 + 10% Formalin solution scored the least. The overall tissue integrity as seen under the microscope was well preserved in samples decalcified with 5% HNO_3 solution, and they fulfilled all the histological criteria fairly well. Cellular structures could be well appreciated in all sections of teeth decalcified with 5% HNO_3 solution whereas they could not be seen in nearly half of the sections of teeth decalcified using 10% HNO_3 + 10% Formalin solution. **[Table I].**

Table I: Cumulative scores of the decalcifying agents based on various parameters.

Decalcifying agent	Ease of sectioning	Soft tissue details	Hard tissue details	Quality of staining	Total
5 % HNO_3	3	4	4	4	15
10% HNO_3	4	3	3	3	13
10% HNO_3 + 10% Formalin	2	2	2	3	9

DISCUSSION

Before bone or any calcified tissue can be processed and sectioned with a routine microtome, the calcium salts must be removed by a process called "decalcification." Failure to decalcify tissue with large amounts of calcium salts will result in torn and ragged sections and damage to the cutting edge of the microtome knife.[4]

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 effects of the various chemical decalcifiers upon the tissue components differ. Preparation procedure of hard teeth tissues is started from its fixation.[5]

Choice of a fixing reagent is dependent upon the tissue itself and the purpose for which it is to be preserved.[5] Fixation with either 4% paraformaldehyde or 10% formalin seems to preserve the pulp tissue and maintain favourable conditions for examination and microscopic analysis of its cell components. Also, unbuffered formalin solution can better decalcify compared to buffered solution because the acid component is not neutralized. Thus in the present study, we used

10% formalin to fix the tissues because it is more readily available and may be stored for longer periods of time.[6]

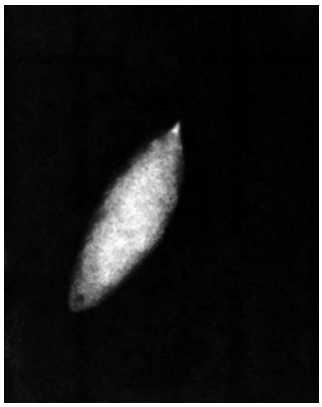

Fig 1: Radiograph of canine tooth showing endpoint decalcification.

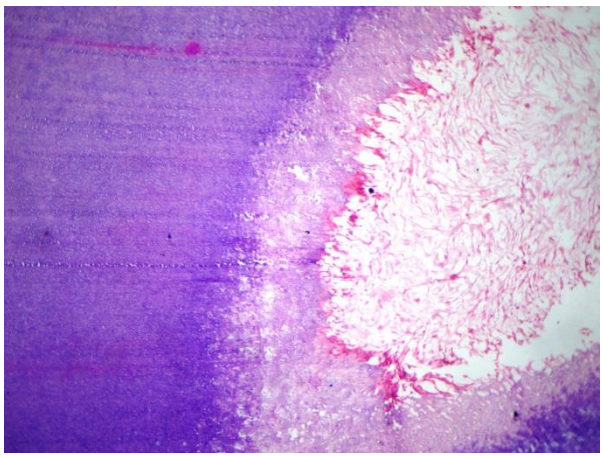

Fig 2: Photomicrograph of specimen from 10% HNO3 solution (group B) showing pulp, predentin as well as dentin. Separation of pulp tissue is seen. (40x).

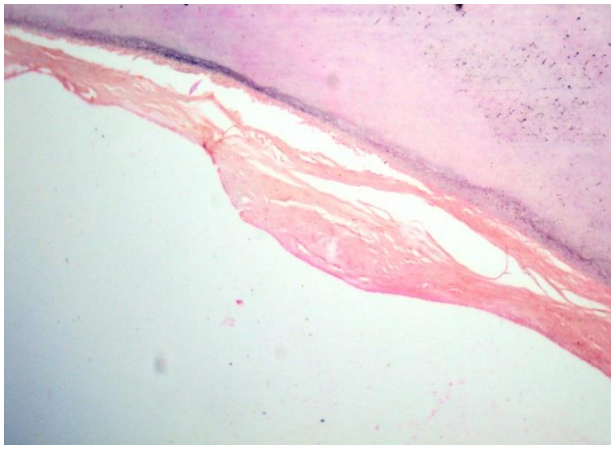

Fig 3: Photomicrograph of specimen from 10% HNO3 group B showing separation of Peridontal ligament. (40X).

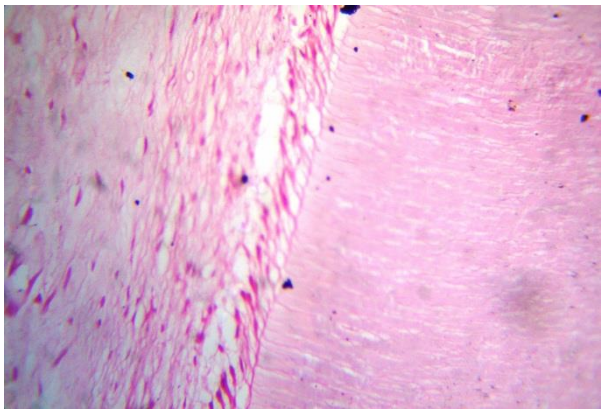

Fig 4: Photomicrograph of specimen from 5% HNO3 solution (group A) showing pulp, odontoblasts with its processes entering into dentinal tubules. (40x)

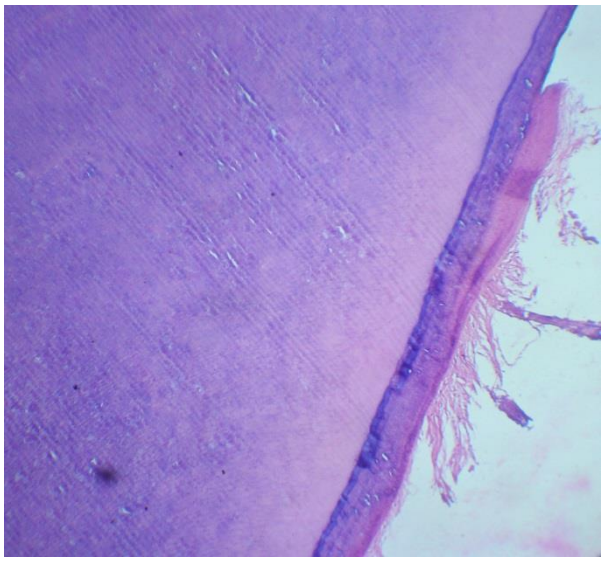

Fig 5: Photomicrograph of specimen from 5% HNO3 solution (group A) showing dentinal tubules, cementum and PDL. (40x)

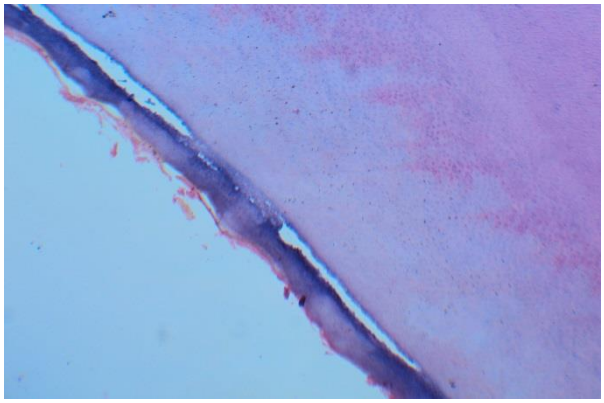

Fig 6: Photomicrograph of specimen from 10%HNO3 + 10% Formalin (group C) showing detached cementum and Periodontal ligament. (40x)

Many alternative decalcification regimens have been proposed[7], 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.[8] Fixating agents that contain acid in their composition, such as formalin (that contains formic acid), are also able to act as decalcifying agents if the acid component is not neutralized.[6]

So, in the present study, we have chosen three groups of decalcifying solutions 5% nitric acid, 10% nitric acid and combination of 10% nitric acid + 10% formalin for comparing their efficacy.

More concentrated acid solutions decalcify more rapidly. This is in accordance with results obtained in our study where teeth decalcified using 10% HNO3 solutions decalcified faster as compared to the other groups.

The fastest decalcifying agents, 10% nitric acid provided samples that were readily sectioned were easy to handle and produced sections, most of which were intact and not shattered. Samples decalcified by 5% nitric acid were also sectioned easily but were posing a difficulty in their handling. This is in accordance with the results obtained by Sanjai et al. (2012) who also noted crumbling of tissues decalcified in 5% nitric acid. Samples decalcified by 10% nitric acid + 10% formalin posed some difficulty in sectioning. The tissues were also difficult to handle and friable.[3]

PDL separation from surrounding dentin was observed more in samples decalcified by 10% HNO3 + 10% Formalin solution followed by 10% HNO3 solution. PDL was very well preserved in samples decalcified with 5% HNO3 solution. Similarly, Zappa et al. (2005) found that formic acid and nitric acid produced the worst result in terms of soft tissue attachment and soft tissue shrinkage.[9] Pulp separation from dentinal border and preservation of cellular details is dependent on fixation, and the choice of decalcifying agent.[1] The destruction of odontoblasts, vapour bubbles and fraying in dentinal tubules in the present study, was negligible in case of 5% HNO_3 solution. Greatest severity was noted in case of 10% HNO_3 solution. **[Fig. 2, Fig. 3]** The authors suggested that strong acid decalcification opened up the dentinal tubules quickly and served as a pathway to the pulp tissue, thus destroying or separating the pulp from dentin. This was the reason why fraying in the dentinal tubules along with any destruction of the odontoblast architecture was seen in sections obtained after using strong acids.[10] A particularly intriguing finding is the presence of all the layers of pulp zones in all the samples decalcified using 5% HNO_3 solution. **[Fig. 4]**

The separation of cementum from dentin and loss of tissue architecture was checked. The present study found that specimens decalcified using 5% HNO_3 showed no cementum destruction and samples treated with 10% HNO_3 + 10% Formalin showed maximum destruction while 10% HNO_3 showed an intermediate level of destruction. The cementum destruction can also be explained on the basis of lytic effects of the acids.[1] **[Fig. 5, Fig. 6]**

Specimens decalcified with 5% HNO_3 solution showed the highest number of H and E sections with adequate staining (not over or under stained) followed by 10% nitric acid and 10% nitric acid + 10% formalin solution.

The most pronounced effect of acid decalcification is the impairment of staining properties. This is dependent on the solution acidity, and the time it will take to decalcify. Thus, the quicker the decalcification, the greater will be the injury and its effects on Hematoxylin & Eosin. I am following acid exposure nucleus stains poorly with cationic dyes such as Hematoxylin and cytoplasm over-stains by the briefest exposure to anionic dyes such as Eosin. This effect can be of diagnostic significance if such under stained nuclei are falsely interpreted as being non-viable. For this reason, staining procedures must be performed carefully following acid decalcification.[1]

The overall tissue integrity, as seen under the microscope, was well preserved in samples treated with 5% HNO_3. Cellular structures could be well appreciated in all the 5% HNO_3 demineralized tissue sections, whereas they could not be seen in many sections prepared by 10% HNO_3 + 10% Formalin solution demineralization. Our result obtained is in accordance to that obtained by Nadaf

et. al. (2011) who also found that samples decalcified using 5% HNO_3 solution showed better results than that of 10% HNO_3 + 10% Formalin solution.[5]

Regardless of the solution used, methods of decalcification share their characteristic of being accelerated when additional factors are employed. The present study was done with respect to different decalcifying agents only, and none of the factors was employed, thus standardizing the procedure. Future studies, in which the factors can be varied, can be done.

CONCLUSION

"Slow but steady wins the race". The final impression led to the proposition that 5% HNO_3 solution was indeed the best decalcifying agent among the three groups, although being slow. However, with a time constraint, the use of 10% nitric acid is advocated. To conclude, harder the structure, hardest to decalcify. Slower the process, better the results.

Further research is required to corroborate or confront the results obtained with the solutions employed in our study.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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