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Original Research Article

Comparison of various decalcifying agents to evaluate their efficacy

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ABSTRACT

Background: Bone is a biological complex structure primarily comprising collagen and minerals. It is important to demineralize these mineralized tissues to remove their calcium apatite crystals for analysing the sub-cellular, cellular, and fibrillar architecture. Six demineralizing agents' efficacy was examined by assessing their duration, ease of handling tissue, staining, and histological criteria. The present study aimed to evaluate six commonly used demineralizing agents to identify the best decalcifying agent.
Methods: Twenty resected hard tissue specimens (1 cm × 1 cm x 1 cm) from the archives were used in the study. These segments were decalcified by solutions namely 10% nitric acid, 10% formic acid, 14% ethylene di amine tetra acetic acid (EDTA), a mixture of formic acid and hydrochloric acid (formic + HCL) 4% each, and a mixture of formic acid and nitric acid 4% each (formic + HNO_3), 10% formal nitric acid further subjected to radiographic endpoint test.
Results: The present study confirmed the fact that samples treated with EDTA showed the best overall impression in terms of tissue integrity and histology followed by 10% formal nitric acid which gave fairly good cellular details and was also rapid in the action.
Conclusions: Based on the present study findings, we suggest that 10% formal nitric acid is the better decalcifying agent available, considering time and tissue integrity as two main factors.

Keywords: Bone, Decalcification, Ethylene di amine tetra acetic acid, 10% formal nitric acid, 10% formic acid

INTRODUCTION

Bone is a biological composite structure that provides it with great strength, toughness, and lightweight needed for its mechanical activities.[1] The structure of bone can be investigated by treating demineralized bone since it contains numerous hierarchical levels of structural organization.[2,3] It is required to "soften" these tissues by eliminating the mineralized components due to the particular physical hardness it provides. In-depth histology studies of human teeth and bones have lasted for a long time. To perform histological studies on pulp stones, immature enamel, dentin, and cementum, the mineralized components must be eliminated through the decalcification procedure.[4] To analyze fibrillar, cellular, and sub-cellular structures, calcium apatite crystals from mineralized tissues have to be removed. It is vital to examine these areas of dental hard tissues, bony lesions, teratomas with bony tissue, odontomas, and numerous other soft tissues that may become rigid owing to modifications like metastatic and dystrophic processes to perform various clinical research and to study its developmental processes. Additionally, the diagnosis of complicated osteocartilaginous pathologies depends on immunohistochemical and molecular analyses which is the need of the hour.[5] Decalcification is required before tissue processing, but available protocols often lead to nucleic acids and proteins being altered and thus compromising the diagnosis.

In the histopathology laboratory, decalcification of hard tissue is one of the technique-sensitive processes. As decalcification of bone and teeth is a regularly required procedure in oral pathology, it is of utmost importance. Without decalcification, it is impossible to cut thin sections of hard tissue using conventional methods; such

tissues must be treated to eliminate calcium phosphate by this procedure, making the tissue pliable enough to be cut using a microtome. Some significant factors that affect the choice of decalcifying solutions include the rate of decalcification, the impact of decalcifying agents on the tissue, and its staining qualities. While some substances entirely and quickly eliminate calcium ions, they have a negative impact on staining properties and may even harm the organic constituents. The effectiveness of these compounds in decalcifying hard tissues has only been investigated in a relatively small number of investigations. Since formic acid and nitric acid are the most widely used weak and powerful demineralizing acids, respectively, they were both chosen. As 10% formal nitric acid and ethylene diamine tetra acetic acid (EDTA) is the mildest decalcifying agents known to science, they were selected.[6] Mixtures of strong and weak acids have also been put to the test, including a 4% mixture of formic and hydrochloric acid and a 4% mixture of formic and nitric acid. Therefore, the present study was conducted to test the efficacy of various decalcifying agents which will further aid in a better understanding of hard tissues.

METHODS

The present study comprised twenty healthy participants aged above twenty years. Contrarily, those with known bone metastasis, benign tumors, or malignancies of bone were excluded from the study. Twenty resected hard tissue specimens (1 cm x 1 cm x 1 cm) for each solution were obtained from the archives of the King George's Medical University faculty of dental sciences department of oral pathology and microbiology, Lucknow.

Conventional decalcification procedure

These specimens were decalcified using solutions containing 10% nitric acid, 10% formic acid, 4% each of formic + HCL, and 4% each of formic acid+HNO_3, 14% EDTA, and 10% formal nitric acid (Table 1). Beginning with fixing, the decalcification was carried out subsequently. 10% neutral buffered formalin (10% NBF) was used as a fixative. Following the decantation of the fixative, the smaller biopsy specimens were sieved out after 24 hours. The bigger specimens underwent numerous washings with distilled water after being divided into tiny portions of 1 cm x 1 cm x 1 cm. Each sample of tissue was given a label before putting in a Coplin jar with 100 ml of a decalcifying agent. The precise moment when decalcification began was recorded. Every day, the pH and temperature of the solutions were noted.

Using the radiographic approach, the endpoint of decalcification for all acids and EDTA was calculated. (Figure 2C, 2D) The tissue was examined for signs of sufficient decalcification six hours later. Tissues that had undergone decalcification were maintained for routine processing after being rinsed in running tap water for 30 to 45 minutes.

Table 1: The demineralizing agents used and their composition.

Demineralizing agents	Composition
10% nitric acid	10 ml- conc. nitric acid 90 ml- distilled water
10% formic acid	10 ml- formic acid 90 ml- distilled water
4% formic and 4% hydrochloric acid (HCL)	4 ml (formic acid) + 46 ml (distilled water) 4 ml (conc. nitric acid) + 46 ml (distilled water)
4% formic and 4% nitric acid (HNO_3)	4 ml (formic acid) + 46 ml (distilled water) 4 ml (conc. HCL acid) + 46 ml (distilled water)

Tissue processing and staining

The processing of the tissue continued using the following reagents: formalin (2 hours, 1 change), alcohol (70%, 80%, 96%- 1.5 hours each and 3 jars of 100%- 1 hour each), xylol (1.5 hours, 1 change), and paraffin (2 hours 2 changes; temperature default value- 62°C). Cycles of 6-hour exposure to decalcifying agents were repeated until the tissue was sufficiently decalcified/softened. Blocks made of paraffin were ready. Using a semi-automated rotary microtome, tissue sections were cut at 3-5 m thickness and then transferred onto a clean glass slide. To show the structure of the decalcified sections, Harris Hematoxylin was used. Regressive staining was carried out for 20-30 minutes, then viewed under a light microscope.[7,8] The effectiveness of several decalcifying agents used in the study were assessed using the x-ray method, which is claimed to be the most accurate way of determining the endpoint of decalcification.[9]

Evaluation of results

Sections were evaluated by an expert histopathologist. The quality of the decalcification procedure and staining result were assessed and evaluated by certain guidelines. Firstly, the time is taken for decalcification.[6]

Secondly, the impact on processing was then evaluated based on the development of ribbons, the scoring or splitting of sections during cutting, and the convenience of dealing with the sections and were ranked as easy, difficult, and very difficult. Thirdly, The intensity of hematoxylin and eosin staining the cytoplasm and nucleus was used to assess the staining effect and given a staining grade of adequate, understanding, or overstain.[7,10] Finally followed by the impacts on the histological details of the tissue, which are influenced by a variety of factors, such as fixation, processing, cutting technique, staining duration, etc.[4] Two parameters were chosen, osteoblasts lining the bony trabeculae which were graded as present or absent and osteocyte retraction within the lacunae assessed as present or absent. The adoption of standardized

methodologies and advised parameters ensured the consistency of all the aforementioned variables.

Statistical analysis

Data were tabulated and the outcomes were analyzed. Mean, SD and percentage were calculated for each case. For multiple group comparisons, one-way ANOVA was performed, and the Chi-square test was determined to assess categorical data. The p value <0.05 was considered to be statistically significant.

RESULTS

Duration

The fastest method, 10% formal nitric acid, took barely 1.5 days (36 hours) to decalcify the sample, whereas EDTA took the longest, 21 days (504 hrs). 10% formic acid required 20.20 days (485 hours), 10% nitric acid took 3.3 days (80 hours), whereas 4% formic + HCL and 4% formic + HNO_3 took 13.12 days (315 hours) and 12.5 days (300 hours), respectively (Table 2).

Table 2: The various parameter used for evaluating the decalcifying agents.

Parameters		Nitric	Formic	Nitric + formic	HCl + formic	EDTA	10% formal nitric acid	P value
Duration	Hours	80	485	300	315	504	36	-
	Days	3.33	20.20	12.5	13.12	21	1.5	
Based on the ease of section cutting	Easy	60.0	0.0	40.0	20.0	100%	80.0	0.004*
	Difficult	0.0	80.0	60.0	20.0	0	20.0	
	Very difficult	0.0	20.0	0.0	60.0	0	0	
Intensity of hematoxylin staining of nucleus	Adequate	60.0	66.6	60.0	33.3	93.3	60	0.19
	Understained	40.0	40.0	40.0	40.0	6.6	40	
	Overstained	0.0	0.0	0.0	6.66	0	0	
Intensity of eosin staining of nucleus	Adequate	53.3%	60.0%	60.0%	53.3%	93.3	60	0.27
	Overstained	46.7%	40.0%	40.0%	46.7%	6.6	40	
Osteoblast lining bony trabeculae	Present	60.0%	90.0%	66.7%	53.3%	95%	70%	6.58
	Absent	40.0%	10.0%	33.3%	46.7%	5%	30%	
Osteocyte retraction within lacunae	Present	66.7%	60.0%	66.7%	73.3	20%	40%	7.50
	Absent	33.3%	40.0%	33.3%	26.6	80%	60%	

*P value <0.005 was considered statistically significant.

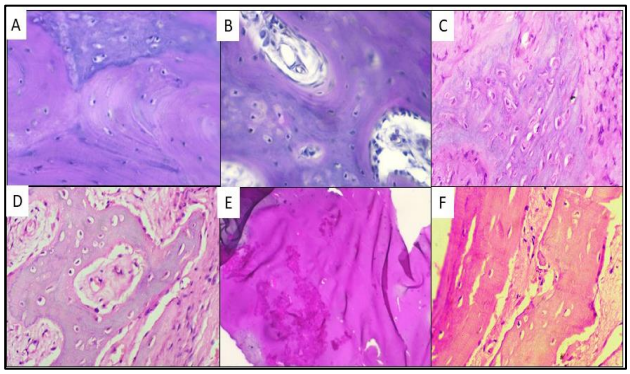

Figure 1: Photomicrographs depicting effects on histological details by various decalcifying agents.
(A) and (B) representing the adequate staining quality, osteoblastic cells lining the trabeculae with osteocytes in the lacunae using 10% formal nitric acid (C) and (D) depicting the best staining property with hematoxylin and eosin with osteoblastic rimming and osteocytes present in lacunae using 14% EDTA. (E) and (F) showing the overstaining with tear and folding of a tissue section with loss of histological details such as osteoblast rimming and osteocyte retraction with 4% formic + HCL acid.

Based on the convenience of sectioning: Friability and the degree of difficulty in handling the tissues were assessed based on ease of cutting sections without any cleaves. Observations revealed that EDTA and 10% formal nitric acid were the easiest to use, followed by 10% nitric acid. formic + HNO_3, and 10% formic acid decalcified samples were more friable and challenging to handle. Nearly 90% of the tissues had shown extremely adverse outcomes using 4% formic + HCL acid, making the section cutting cumbersome (Figure 2). The tissues were unworkable and incredibly friable (Table 2, Figure 1E and F).

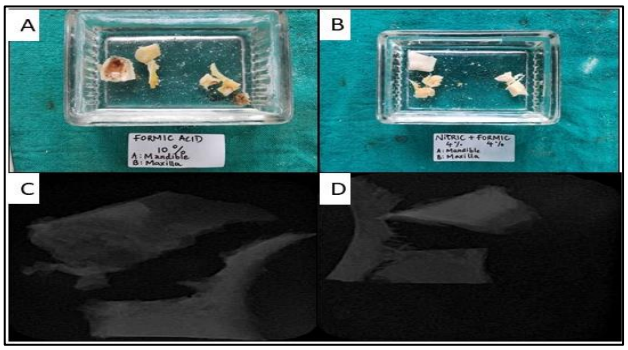

Figure 2: (A) and (B) depicting the hard tissue specimen in various decalcifying agents followed by (C) and (D) showing the transparency of bony tissue subjected for radiographic end point test.

Intensity of hematoxylin and eosin staining: The largest percentage (95%) of H and E sections without any over or under staining were observed in specimens decalcified with EDTA. 10% formal nitric acid, 10% nitric acid (60%) and formic+HNO_3 acid (60% each) with appropriate staining, 10% formic acid with the most adequate staining (66.6%) produced the next-best results. The specimens that had been decalcified with 4% formic + HCL acid displayed the most over- and under-stained sections (Table 2 Figure 1).

Effects on histological detail of tissues: Osteoblasts were examined for effect brought on by decalcification procedures. With the exception of 4% Formic + HCL acid (53.3%), where osteoblasts were least noticeable, the lining of osteoblasts was evident in nearly all of the sections decalcified in solutions. In EDTA (95%), 10% formic acid (90%), and 10% formal nitric acid (70%), the presence of osteoblast lining was more obvious (Figure 1). Nitric acid (60%) and formic+HNO_3 (66.7%) cases had mediocre outcomes (Table 2). The average number of osteocytes showing retraction was analysed to determine whether or not osteocytic retraction was present within the lacunae. When compared to other solutions that demonstrated retraction of more than 60%, it was noticeably less in the case of EDTA (80%) followed by 10% formal nitric, 10% formic acid, and 4% formic + HNO_3 acid (60.0%) (Table 2).

In samples treated with EDTA, the overall tissue integrity as seen under the microscope was well preserved, and they largely met all the histological requirements. Cellular structures were fully evident in all of the EDTA sections, but not in almost half of the 4% formic + HCL acid sections. 10% formal nitric acid and 10% formic acid provided remarkable cellular detail, with the former acting relatively quickly (Figure 1).

DISCUSSION

Decalcification of the bone is a laborious process taking days to weeks. The speed and quality of the demineralization process determine how well the tissue structure is preserved. The urgency of the procedure heavily influences the choice of decalcifying agent and approach.[9] The first criteria duration was in parallel with the study done by Geoffrey Brown and Bancroft, which revealed that 10% formal nitric acid (with formaldehyde fixative) was the fastest decalcifying agent, requiring just 1.5 days to decalcify 5 mm of bone followed by 10% nitric acid.[9] In the current study EDTA-based decalcification process took 21 days (504 hours) which was due to its chelating property. Additionally, compared to other acids, it produced better results for DNA and RNA next-generation sequencing and for in situ hybridization procedures, indicating that this chelating agent should be prioritized for samples used for these techniques.[11,12]

The second criteria was dependent on how efficiently sections were made using a microtome; EDTA (100%) was the easiest of all, while nitric acid (60%) produced samples that were easily sectioned, simple to handle, and weren't friable. The samples that could be easily sectioned, handled easily, and produced sections that were mostly intact and unbroken were provided by 10% formal nitric acid (80%), the fastest decalcifying agent. Both results supported a prior study which found superior sections decalcified by formal nitric acid.[7] Samples decalcified by 10% formic acid were sectioned which graded as moderate difficult (80%) and very difficult (20%) i.e. more friable, posing difficulty in their handling. This was followed by a combination of formic + HNO_3 acid (40%). However, the formic + HCL acid case was the least favourable, with the majority of tissues (20%) posing moderate difficulty and (80%) severe difficulty in sectioning. The tissues were very fragile and challenging to handle. The poor sectioning of the latter could be due to hydrochloric acid's significant impact on tissue friability.

The most notable impact of decalcifying agent is the impairment of staining characteristics. This depends on how acidic the solution is and how long it will take for the calcium to decalcify.[7,9,10] Thus, the quicker the decalcification, the greater will be the injury and its effects on hematoxylin and eosin staining.[4] After exposure to acid, the nucleus stains poorly with cationic dyes like hematoxylin, and even a brief exposure to anionic dyes like eosin causes the cytoplasm to overstain. Such under-stained nuclei are incorrectly perceived as non-viable, which may have diagnostic importance. Therefore, after acid decalcification, staining procedures must be carefully carried out.[4,14] The largest percentage (93.3%) of H and E sections with acceptable staining were found in specimens decalcified with EDTA, followed by 10% formic acid (66.6%). The next-best results were provided by 10% formal nitric acid, formic + HNO_3 acid and 10% nitric acid (60%), respectively. Specimens decalcified with 4% formic + HCL acid displayed the greatest number of over and under-stained sections. Nitric acid can decalcify more quickly but severely impair stainability, as discussed in Stevens et al (1990) and Callis and Sterchi (1998).

Finally, the histological section impressions are subjective and influenced by a variety of factors, including fixation, processing, sectioning technique, staining timings, etc.[9] It is crucial to obtain the sections with the fewest processing modifications possible for histological investigation. Fixation as well as the decalcifying agent selected may have an impact on the presence of osteocytes in the lacunae, shrinkage of pulp away from the dentinal wall, and damaged odontoblastic layer.[14] Stronger acids function more quickly than EDTA and formic acid, but they require continuous monitoring since they pose a higher risk of tissue injury due to protein hydrolysis leading to maceration or the dissolution of the soft tissue components and the potential loss of all histological information.[15] In our study, the effects on the histological details of the tissue were evaluated by looking for osteoblasts lining the bony trabeculae and osteocytes within lacunae in all decalcified specimens. In contrast to other solutions,

osteocyte retraction was noticeably less in EDTA (80%) and 10% formal nitric acid (60%), intermediate in 10% formic acid, and strongly evident in a 4% formic + HCL acid. On the contrary, osteoblasts lining in bony trabeculae was marked in EDTA (80%) and 10% formal nitric acid (60%). The results were least noticeable when 4% formic + HCL acid were combined. Intermediate results were seen with 10% formic acid. Formic + HCL acid, which are a combination of a strong acid and a weak acid, are likely to produce significant tissue damage at 4% each. According to a study by Miquelestorena-Standley et al use of hydrochloric acid can save the time needed for decalcification, but it is also known to alter morphology of protein and nucleic acid and cause damage to the tissue.[15] In their study, immunohistochemistry, and in situ hybridization were done and false-negative results were obtained whereas in our study, hydrochloric acid treated samples showed altered staining. Reduced DNA and RNA purity affect the ability to identify predicted molecular changes (mutations and translocations). According to earlier investigations, altered antigenicity was shown following hydrochloric acid decalcification, but it was maintained using EDTA or formic acid.[17-19] Findings of altered DNA and RNA integrity with hydrochloric acid were consistent with other studies. Singh et al observed a decrease in DNA and RNA yield as well as increased cycle thresholds in various tissue samples decalcified with a strong acid.[12] HCL was therefore inappropriate for use in immunohistochemistry. This issue might be avoided by decalcification using chelating chemicals like EDTA. On the other hand, decalcification in EDTA has minimal to no impact on tissues. It solely interacts with calcium ions and gradually reduces the size of the hydroxyapatite crystal's outer layer. The main drawback of EDTA decalcification is its slow progress; depending on the level of mineralization, incubation durations can reach several weeks. Moreover, EDTA remains difficult to use with specimens because the solution is rapidly saturated and needs to be changed frequently.[20]

In samples treated with EDTA, the general tissue integrity was well preserved even under the microscope. Cellular features could be clearly observed in every tissue section that had undergone EDTA demineralization, but not in more than half of the sections that had undergone 4% HCL + formic acid demineralization. 10% nitric acid and 4% formic + HNO_3 combination were intermediate in action while 10% formal nitric and 10% formic acid supplied reasonably good cellular details and the former was also swift in its action.

CONCLUSION

Although EDTA is now the best decalcifying agent available, it is recommended for immunohistochemistry and special staining techniques where time is not a major consideration. Time of diagnosis, tissue integrity, and histological criteria must all be balanced throughout. The current work has therefore demonstrated that formal nitric acid is not only a quicker decalcifying agent but also produces acceptable quality of sections with appropriate staining. Therefore, formal nitric acid 10% can be utilized with time and tissue integrity being the two prime considerations.

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