# Comparative evaluation of the efficacy of the mechanical agitation-assisted decalcification of human permanent teeth: A histological analysis

MOHAMMAD SHAHID[1], PREETHESH SHETTY[2], TONY PAUL[3] and RAKSHA BHAT[2]

[1]A.J. Shetty Institute of Dental Sciences, Mangaluru, Karnataka 575004; [2]Department of Conservative Dentistry and Endodontics, AB Shetty Memorial Institute of Dental Sciences (ABSMIDS), Nitte (Deemed to be University), Mangaluru, Karnataka 575018, India; [3]Germans Trias I Pujol Research Institute (IGTP), Badalona, 08916 Barcelona, Spain

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**Abstract.** The decalcification of teeth constitutes a persistent challenge for pathology and histology research laboratories. The conflict concerning the duration of the procedure and the fineness of the sections render it fundamentally challenging. The present study aimed to introduce a novel method for decalcifying teeth using a magnetic agitator. For this purpose, 192 non-carious premolars extracted for orthodontic treatment were collected and fixed immediately with 10% formalin. The apical tips of all the samples were removed. A total of four decalcifying agents (DAs) were selected for decalcification: 5% Nitric acid (group I), 10% HCl (group II), 10% formic acid (group III) and 14% EDTA (group IV). The samples were decalcified separately by the same solution, one with and one without agitation with a magnetic agitator. The solutions were changed daily to accelerate decalcification. The decalcification rate (DR) was higher in group I, followed by groups II, III and IV. There was a statistically significant difference between the groups as regards the DR. Agitation significantly reduced the decalcification time for all the DAs used. Tissue preservation following decalcification was sufficient with the group IV solutions, average with those used in groups II and III, and poor with the group I solutions. Tissue preservation was improved in the sections subjected to agitation than in those without. On the whole, the results of the present study suggest that 14% EDTA can decalcify at an accepted rate and provides satisfactory tissue details for decalcifying healthy premolars with an agitation speed at 100 rpm. However, further studies are required to rule out the effects of this DA on carious, malformed teeth associated with pathologies, normal and pathological bone.

*Correspondence to:* Dr Preethesh Shetty, Department of Conservative Dentistry and Endodontics, AB Shetty Memorial Institute of Dental Sciences (ABSMIDS), Nitte (Deemed to be University), Deralakatte, Mangaluru, Karnataka 575018, India
E-mail: preethesh_shetty@yahoo.co.in

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## Introduction

Teeth are considered the hardest tissue of the body, primarily due to tooth enamel, which is denser due to a higher concentration of inorganic content. The inorganic component within enamel exists as hydroxyapatite crystals, rendering it difficult to prepare for microscopic analysis. The exceptional hardness of enamel poses a challenge when it comes to the intricate process of preparing and segmenting it for microscopic examination (1). The traditional procedure for preparing bone or tooth specimens for histopathological examination involves the grinding of the specimen to the desired thickness or decalcification. The selection of the technique for tooth examination is determined by the initial clinical diagnosis, the urgency of the case and the extent of investigation required (2).

Mineralized/ground sections of bone serve as valuable materials for micro-radiographic and histomorphometric studies, as well as for polarized and fluorescent light microscopy. Similarly, ground sections of teeth provide a means with which to explore enamel, dentin, cementum and any defects. To delve into diverse pathological and developmental processes, the examination of demineralized sections of other dental tissues and bones becomes essential. As a result of changes caused by dystrophic and metastatic processes, several additional soft tissues may also become rigid, necessitating decalcification. These cases require decalcification before the histopathologist may provide results on hard tissue biopsies (3,4).

The decalcification of teeth remains a persistently challenging aspect in the realms of pathology and histology research laboratories. The inherent complexity arises from the delicate trade-off between the duration of the procedure and the quality of the resulting sections. Chelating agents (CAs) yield the highest quality sections but take longer to prepare; by contrast, potent acids deliver rapid results, but compromise the quality of the sections (3). A century ago, pioneering investigators began the study of thin ground sections of acid-soaked bone. Since then, several decalcifying agents (DAs) have

been described and utilized for bone and teeth decalcification. These agents can be broadly categorized as acids, which form soluble calcium salts and CAs, which bind to surface calcium ions. While acids swiftly decalcify teeth, they can also harm the surrounding tissues. On the other hand, CAs maintain tissue integrity, but require an extended duration for decalcification (2). As a result, there is a need for a decalcification agents (DAs) that can both rapidly decalcify and uphold tissue integrity. Commonly employed acidic DAs, include potent acids such as aqueous nitric acid, formal nitric acid, and a mixture of nitric acid and chromic acid. Additionally, weak acids, such as aqueous formic acid, a mixture of formic acid and formalin, and buffered formic acid are used (2). Ethylenediaminetetraacetic acid (EDTA) and EDTA-Tris are commonly-used CAs (5).

Any acid, regardless of its buffering, has the potential to negatively affect the tissue staining characteristics. The issue becomes more pronounced with the increased solution acidity (lower pH) and longer decalcification periods. Cell nuclei are particularly susceptible, failing to properly take up basic dyes, such as haematoxylin compared to soft tissues not exposed to acids (2). Staining with acidic dyes also has some effects to a certain extent; for example, eosin, an acidic dye, can lead to an undesirable deep brick-red stain. To mitigate these effects on haematoxylin and eosin (H&E) staining, the use of the appropriate DAs, conducting a decalcification endpoint test, post-decalcification acid removal and slight modifications to the staining procedure are recommended (4,6).

CAs, such as EDTA, help mitigate the adverse effects of acidic DAs. They not only maintain tissue integrity, but also yield excellent staining results, albeit at a slower pace. Consequently, CAs have been used in research and situations where tissue and cell architecture are of paramount importance (2). Nonetheless, prolonged exposure to any DA can affect the staining properties. An ideal DA should theoretically achieve rapid and complete decalcification, be gentle on tissues, retain staining characteristics, and preserve antigens for immunohistochemical studies (1). However, a practically perfect DA remains elusive.

Over the years, numerous methods have been proposed to address these challenges. These approaches encompass combinations of DAs and other reagents, such as alcohol and phloroglucinol. Combinations such as hydrochloric acid-nitric acid, formalin-nitric acid and formalin-formic acid have been investigated. Techniques to expedite decalcification include a reduction in pH levels, an increase in temperature, the use of vacuums and electrolytic devices, ion the exchange of resin, sonication (the use of sonic vibrations), microwaves and agitation, along with commercially available DAs (5). While several studies have acknowledged that agitation accelerates the decalcification process, only a few have compared teeth decalcification with and without agitation. Moreover, a number of studies that involve agitation have manually induced it, typically by hand (2-6). Thus, the present study compared the efficacy of various decalcification agents, namely nitric acid ($HNO_3$), hydrochloric acid (HCl), formic acid (HCOOH) and EDTA using a mechanical agitation device. The present study examined the effects of these agents on the organic content and tissue integrity, as well as the resulting staining characteristics of decalcified teeth. Primarily, the present study aimed to present a more simplified and cost-effective alternative method which can be used for decalcification.

## Materials and methods

*Source of data*. The present study consisted of prospective samples of 192 non-carious maxillary and mandibular single-rooted premolars, recently extracted during standard orthodontic treatments. Informed consent was procured from all participants, ensuring their approval to contribute their biological samples for the research. Individuals >30 years of age, and those with carious, attritted, abraded, eroded, or periodontally affected teeth, were excluded from the study. Ethical clearance was obtained from the Institutional Ethical Committee, AJ Institute of Medical Sciences and Research Centre (Approval no. AJEC/REV/D/34/2015-16).

*Sample size*. The present study consisted of four groups, each containing 48 samples. Group 1, 5% $HNO_3$; group 2, 10% HCl; group 3, 10% HCOOH; and group 4, 14% EDTA. Each of these groups was further divided into two subgroups as follows: Group A, without agitation; and group B, with agitation.

*Decalcification process*. The decalcification process comprised a systematic four-step procedure. Commencing with the formulation of decalcification agents, the subsequent phases are delineated. Sample subdivision ensued, resulting in the categorisation of specimens into four distinct groups, predicated upon the variant decalcification agents employed. Specifically, these groups encompassed the utilization of 5% $HNO_3$, 10% HCl, 10% HCOOH and 14% EDTA (Merck Specialities Pvt. Ltd.). To ensure congruence with laboratory standards, the concentrations of these agents were meticulously adjusted, and each formulation was meticulously prepared utilizing distilled water. Subsequent to the formulation phase, an aqueous rinse was administered to the specimens. The apical tip of the specimens was meticulously excised through the employment of a diamond disc (Brasseler USA, Savannah, GA), subsequently followed by immersion in a 10% formalin solution, with this immersion process extending for a duration of 48 h. The actual decalcification process was enacted over a duration of 6 h, in tandem with consistent agitation of the specimens. Following this phase, the specimens were allowed to remain immersed in the decalcification agent throughout the ensuing overnight period. After the passage of 24 h, the initial decalcification agent was substituted with a freshly prepared solution of the same composition. During this phase, the specimens were again subjected to agitation of 100 rpm until the process of decalcification was deemed complete. For the subgroup of specimens undergoing decalcification without agitation, immersion in the respective agents transpired for an equivalent overnight duration, devoid of any agitation. The decalcification process was rigorously documented, with each decalcification agent being utilized for a continuous duration of 24 h.

*Mechanical agitation*. The agitation was performed on the specimens at 100 rpm (Power Regulator, Rotek India). The speed was adjusted at 100 rpm. The stirrer moves in a circular motion, improving the dehydrating agent's efficacy. Agitation allows fresh molecules of the dehydrating agent to be in

continuous contact with teeth and aids in reducing the time required for decalcification.

*Decalcification endpoint test*. In this preliminary test, specimens were gently bent using finger pressure to gauge their flexibility. Once this stage was attained, a 27 gauge fine needle (Hindustan Syringes & Medical Devices Ltd.) was employed to conduct a needle prick test at three locations: the crown, the cemento-enamel junction and the root. The completion of decalcification was finally confirmed through a chemical test involving the preparation of 5% ammonium hydroxide and 5% ammonium oxalate stock solutions. Equal volumes of ammonium hydroxide and ammonium oxalate solutions were meticulously combined, resulting in the formulation of a functional ammonium hydroxide/ammonium oxalate working solution. A pipette was delicately introduced into the decalcifying solution, housing the specimen, and an approximate volume of 5 ml of this solution was meticulously extracted from beneath the specimen. The acquired solution was subsequently transferred into a designated test tube. An additional 10 ml of the established ammonium hydroxide/ammonium oxalate working solution was then introduced into the test tube, followed by a comprehensive mixing procedure. This amalgamation was then allowed to stand undisturbed for the duration of an overnight period. The determination of decalcification completion was contingent upon the absence of any discernible precipitate on two consecutive assessment days. To validate this condition, the aforementioned assessment was iteratively conducted at intervals of 2 to 3 days.

*Processing and embedding of specimens*. The procedural sequence commenced with the dehydration process of specimens utilizing a successive gradient of alcohol solutions, with each immersion lasting a duration of 30 min. Subsequent to the dehydration phase, the specimens underwent clearing via xylene treatment, followed by their subsequent embedding in paraffin wax medium (Surgipath Paraplast High Melt, Leica Biosystems Nussloch GmbH). A precision microtome (MICROM model HM340E) was employed to meticulously section the specimens, yielding sections with a thickness of ~5 $\mu$m. These sections were then carefully positioned onto glass slides, and the ensuing staining process encompassed the application of haematoxylin (Merck Life Sciences Pvt. Ltd.) and eosin (Thermo Fisher Scientific, Inc.). The preparation of dewaxed tissue sections for microscopic analysis involved rehydration through a series of alcohol dilutions, followed by thorough washing in running tap water. Subsequently, the sections were stained with haematoxylin for 3 min at room temperature, rinsed in water, and then briefly immersed in 1% acid alcohol for contrast enhancement. After further washing in tap water, the sections were stained with 1% eosin for cytoplasmic coloration for 2 min at room temperature. Following this, the sections underwent washing in running tap water, dehydration in alcohol solutions, and clearing with xylene before being mounted using dibutyl phthalate polystyrene xylene (MilliporeSigma) for histological examination. This meticulous procedure ensured the proper preparation and preservation of the dewaxed sections for accurate histological analysis.

*Assessment of section quality*. A meticulous evaluation of the section quality was undertaken, and any sections not meeting the requisite quality standards; namely the teeth with decay, cracks, abnormalities which would hamper with the results like sclerotic dentin were excluded from further consideration. The retained sections underwent comprehensive scrutiny utilising a light microscope (BX 41, Olympus Corporation), during which pertinent images were captured for subsequent analytical endeavours. The histological assessment encompassed a thorough evaluation of the staining quality pertaining to both the cytoplasmic and nuclear constituents. Parameters including soft tissue shrinkage and organisational coherence were rigorously appraised. Furthermore, a detailed analysis of the dental pulp was conducted to ascertain the presence of all delineated zones and to quantify the extent of separation from the surrounding dentinal structure. In parallel, the dentinal framework underwent meticulous scrutiny, specifically targeting indicators such as vapor bubbles, alterations within the dentinal tubules, and any observable degradation of the odontoblastic layer. The architectural integrity of the cementum was likewise evaluated, with a keen focus on its structural arrangement and potential detachment from the underlying dentin. Upon the completion of the staining, a final grading process was executed, employing a quantified scale ranging from 1 to 4. This grading system encompassed four levels as follows: 1, poor; 2, fair; 3, good; and 4, excellent, enabling a nuanced classification based on the predefined criteria. Subsequent to the grading process, the acquired results were systematically tabulated, paving the way for a comprehensive statistical analysis.

*Statistical analysis*. Following the decalcification of specimens, the results were compiled, and statistical analyses were performed using SPSS 17.0 software (SPSS, Inc.) using ANOVA followed by post-hoc Tukey's test. The histological grading was analysed using the Fischer's exact test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

## Results

Upon the decalcification of the specimens, the results were compiled, and subjected to statistical analysis as described above. Based on the table, the decalcification process was swiftest with 5% $HNO_3$ and slowest when using 14% EDTA. The mean number of days taken for decalcification by the different agents was as follows: 32 days with 5% $HNO_3$, 36 days with 10% HCl, 40 days with 10% HCOOH and 80 days with 14% EDTA. All these outcomes differed significantly, with a P-value of 0.001. An inter-group comparison was performed using Tukey's post hoc test, and a p-value ≤0.05 was obtained, which was statistically significant (Table I).

In the inter-group comparison, it was observed that 14% EDTA, compared with 5% $HNO_3$, had a mean difference of 48 days. Similarly, when compared with 10% HCOOH, there was a mean difference of 40 days and the comparison with 10% HCl revealed a mean difference of 44 days. When 5% $HNO_3$ was compared with 10% HCOOH, there was a mean difference of 8 days, and the comparison with 5% HCl revealed a mean difference of 4 days. When 10% HCOOH

Table I. Number of days required for decalcification with and without agitation.

| Groups | No. of samples | Mean no. of days | Standard deviation | P-value |
|---|---|---|---|---|
| EDTA | 24 | 80 | 2.00 | 0.001 (H.S) |
| Nitric acid | 24 | 32 | 2.16695 | |
| Formic acid | 24 | 40 | 2.14679 | |
| Hydrochloric acid | 24 | 36 | 2.58760 | |
| EDTA with agitation | 24 | 50 | 1.91107 | 0.001 (H.S) |
| Nitric acid with agitation | 24 | 20 | 1.47442 | |
| Formic acid with agitation | 24 | 25 | 2.43168 | |
| Hydrochloric acid with agitation | 24 | 23 | 2.83866 | |

EDTA, ethylenediaminetetraacetic acid; H.S, highly statistically significant (P<0.01).

Table II. The mean difference in the number of days between the four groups.

| | | | | 95% Confidence interval | |
|---|---|---|---|---|---|
| Groups | Mean difference | Standard error | Significance value | Lower bound | Upper bound |
| EDTA | | | | | |
| Nitric acid | 48.00000 | 0.64550 | 0.001 (H.S) | 46.3110 | 49.6890 |
| Formic acid | 40.00000 | 0.64550 | 0.001 (H.S) | 38.3110 | 41.6890 |
| HCl | 44.00000 | 0.64550 | 0.001 (H.S) | 42.3110 | 45.6890 |
| Nitric acid | | | | | |
| Formic acid | -8.00000 | 0.64550 | 0.001 (H.S) | -9.6890 | -6.3110 |
| HCl | -4.00000 | 0.64550 | 0.001 (H.S) | -5.6890 | -2.3110 |
| Formic acid | | | | | |
| HCl | 4.00000 | 0.64550 | 0.001 (H.S) | 2.3110 | 5.6890 |

EDTA, ethylenediaminetetraacetic acid; HCl, hydrochloric acid; H.S, highly statistically significant (P<0.01).

was compared with 10% HCl, there was a mean difference of 4 days (Table II).

From the aforementioned findings, the speed of decalcification was most rapid with 5% $HNO_3$ and slowest with 14% EDTA. The mean number of days taken by 5% $HNO_3$ for decalcification was 20 days, 23 days for 10% HCl, 25 days for 10% HCOOH and 50 days for 14% EDTA. All the results were highly statistically significant, with a p-value of 0.001. The inter-group comparison was performed using Tukey's post hoc test, and a P-value ≤0.05 was considered to indicate a statistically significant difference (Table I).

In the inter-group comparison, it was observed that 14% EDTA, compared with 5% $HNO_3$, had a mean difference of 30 days. Similarly, when compared with 10% HCOOH, there was a mean difference of 25 days, and a comparison with 10% HCl revealed a mean difference of 26 days. When 5% $HNO_3$ was compared with 10% HCOOH, there was a mean difference of 5 days, and the comparison with 5% HCl revealed a mean difference of 3 days. When 10% HCOOH was compared with 10% HCl, there was a mean difference of 2 days (Table III).

The histological grading was statistically analysed using Fisher's exact test (Table IV). From the results obtained, it was shown that the histological sections exhibited excellent staining and soft tissue integrity for EDTA, good cellular details for HCOOH, fair cellular details for HCl and a poor tissue integrity for $HNO_3$ (Fig. 1). The different was significant (Table IV). Of the 24 samples in 14% EDTA, 21 sections exhibited excellent results, and three good results. Of the 24 samples in 10% HCOOH, 21 sections exhibited promising results, and three were fair. Of the 24 samples in 10% HCl, 18 sections exhibited fair results, and six were poor. However, all the 24 samples in 5% $HNO_3$ depicted poor results. Fisher's exact test value was found to be 172.064, and the P-value was 0.001, which was highly significant. (Table IV).

## Discussion

The selection of the most appropriate DA is dependent on the decalcification speed and the diagnostic quality of the sections. Each method is associated with its own set of advantages and disadvantages. For example, chelation with EDTA has been shown to provide superior tissue preservation and a clear histological impression; however, its extended processing time renders it impractical in routine diagnostic practice (2,7,8). This was demonstrated by previous studies which compared the efficacy of HCOOH, $HNO_3$ and EDTA as DAs (5,9).

Table III. The mean difference in the number of days between the four groups.

| | Mean difference | Standard error | Significance value | 95% Confidence interval | |
|---|---|---|---|---|---|
| | | | | Lower bound | Upper bound |
| EDTA | | | | | |
| Nitric acid | 30.00000 | 0.64221 | 0.001 (H.S) | 28.3196 | 31.6804 |
| Formic acid | 25.00000 | 0.64221 | 0.001 (H.S) | 23.3196 | 26.6804 |
| HCl | 26.83333 | 0.64221 | 0.001 (H.S) | 25.1529 | 28.5138 |
| Nitric acid | | | 0.001 (H.S) | | |
| Formic acid | -5.00000 | 0.64221 | 0.001 (H.S) | -6.6804 | -3.3196 |
| HCl | -3.16667 | 0.64221 | 0.001 (H.S) | -4.8471 | -1.4862 |
| Formic acid | | | | | |
| HCl | 1.83333 | 0.64221 | 0.027 (S) | .1529 | 3.5138 |

EDTA, ethylenediaminetetraacetic acid; HCl, hydrochloric acid; H.S, highly statistically significant (P<0.01); S, significant (P<0.05).

Table IV. Comparison of groups with histological grading using Fisher's exact test.

| Groups | Histological grading, n (%) | | | | Total | Fisher's exact test value | P-value |
|---|---|---|---|---|---|---|---|
| | Poor | Fair | Good | Excellent | | | |
| EDTA | 0 (0) | 0 (0) | 3 (12.5) | 21 (87.5) | 24 (100) | | |
| Nitric acid | 24 (100) | 0 (0) | 0 (0) | 0 (0) | 24 (100) | | |
| Formic acid | 0 (0) | 3 (12.5) | 21 (87.5) | 0 (0) | 24 (100) | 172.064 | 0.001 (H.S) |
| HCl | 6 (25) | 18 (75) | 0 (0) | 0 (0) | 24 (100) | | |
| Total | 30 (31.3) | 21 (21.9) | 24 (25) | 21 (21.9) | 96 (100) | | |

EDTA, ethylenediaminetetraacetic acid; HCl, hydrochloric acid; H.S, highly statistically significant (P<0.01).

Bumalee *et al* (8) established EDTA to be the most efficient DA following a histological evaluation with $HNO_3$, HCOOH and sodium citrate, the results of which are in accordance with the results obtained in the present study. Potent acids, such as HCl and $HNO_3$ can decalcify rapidly, causing tissue damage and deleterious changes in tissue morphology and stainability (5,10). HCOOH (a weak acid) is the preferred DA, which is less damaging to tissues, while being relatively slower in action (3,9). As deduced in the present study, the acid decalcification process could be accelerated with certain superficial modifications.

Acid decalcification is the method which is most commonly used in the majority of laboratories. Among the key factors which influence acid decalcification are the concentration of the acid, temperature and agitation. Decalcification procedures can be accelerated by the use of additional factors, such as an increased temperature and the mechanical agitation of the decalcifying fluid. Such simple modifications have the potential to increase the rate of decalcification. Physical agitation has a dual function by increasing the diffusion of fluids into specimens and preventing the fluid layer around the specimen from becoming saturated with calcium, thus promoting the chemical reaction in acid decalcification (1,2,11).

In the present study, the effectiveness of four DAs was compared in terms of their decalcification rate (DR), their effects on dental tissues and the staining characteristics, both with and without agitation. Among the DAs, 5% $HNO_3$ exhibited the most rapid DR, followed by 10% HCl, and 10% HCOOH, with 14% EDTA exhibiting the slowest DR. Furthermore, as regards the effect on dental tissues and their staining characteristics, the optimal results were obtained with the slowest DA, i.e., 14% EDTA. By contrast, 5% $HNO_3$ produced less satisfactory outcomes. Introducing agitation considerably accelerated the decalcification process, reaching the endpoint in just two-thirds of the time compared to the control block.

The quality of decalcified sections and their rate of decalcification is dependent on factors such as the concentration of the DA, temperature, agitation, microwave radiation, tissue suspension, and size and type of tissue (2).

Numerous studies have been undertaken to assess the effectiveness of diverse DAs for the demineralization of bones. Cho *et al* (12) conducted a study employing skull specimens spanning various developmental stages. They ascertained that the utilization of 0.1 M EDTA at a temperature of 42˚C significantly curtailed the temporal requisites for achieving complete demineralization, concurrently upholding

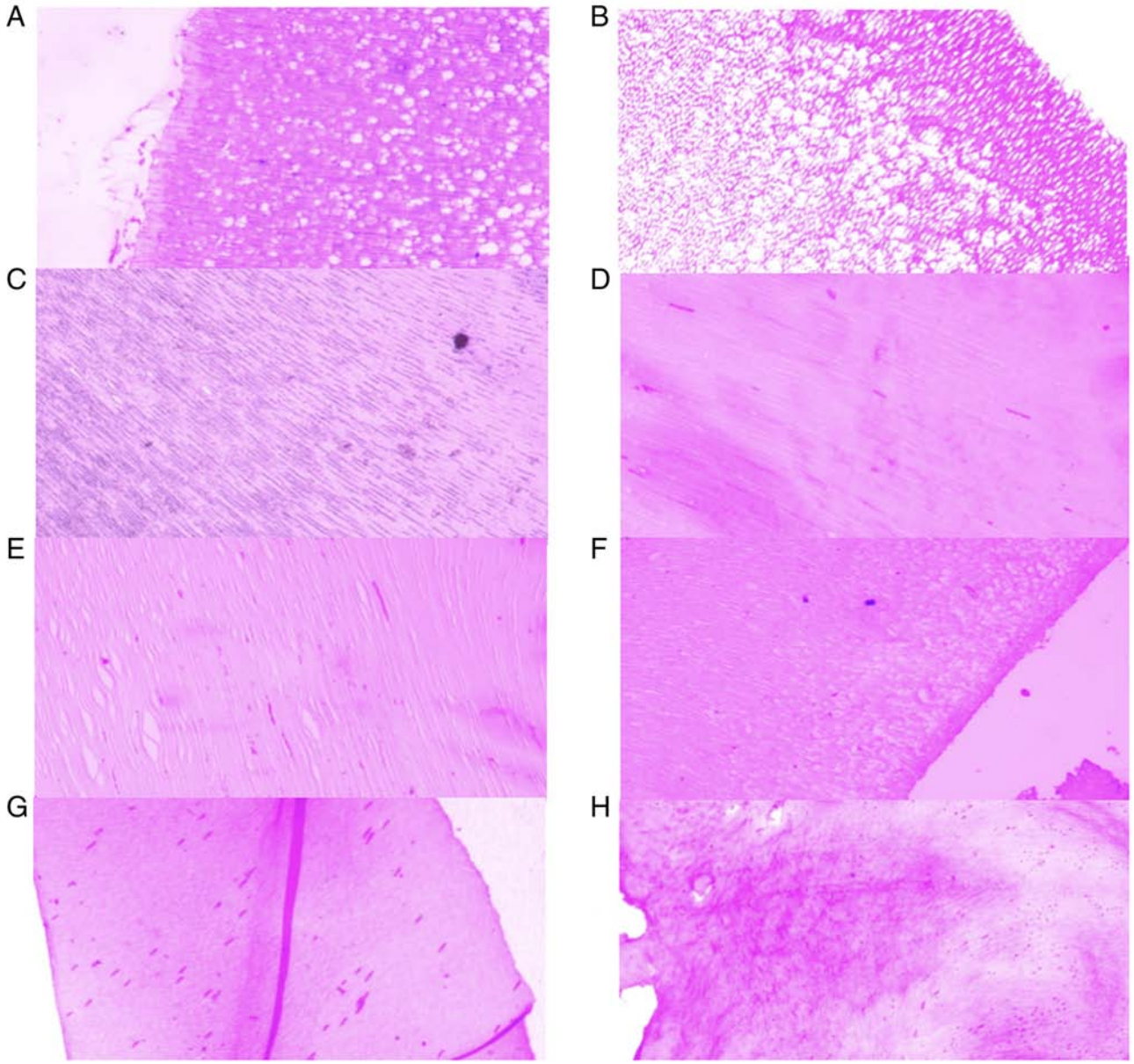


Figure 1. Histological images depicting the groups with and without agitation. (A) Use of EDTA without agitation, and (B) after agitation. Use of hydrochloric acid (C) without agitation, and (D) after agitation. Use of formic acid depicting moderate cellular details (E) without agitation and (F) after agitation. Use of nitric acid depicting poor tissue integrity (G) without agitation, and (H) after agitation. No notable changes were observed before and after agitation (x10 magnification).

the essential histological attributes of the tissue (12). Prasad and Donoghue (3) examined six frequently employed demineralization agents, culminating in the determination that 10% formal nitric acid facilitated the most expeditious decalcification, while EDTA yielded the most favourable overall histological manifestations. Savi *et al* (13) arrived at the determination that 10% formic acid emerged as the preeminent DA for human mandibular osseous specimens (13). Ali *et al* (4) advocated for the application of 10% HCOOH as the superior DA when compared to 5% trichloroacetic acid in accordance with the results of the present study. Sanjai *et al* (14) conducted a comparative analysis of six DAs, finding 5% $HNO_3$ to be the swiftest, neutral EDTA to be the slowest, and neutral EDTA to yield superlative outcomes in terms of soft-tissue integrity, while 5% $HNO_3$ yielded the least favourable outcomes. Sangeetha *et al* (15) juxtaposed customary decalcification against microwave-assisted decalcification using 5% $HNO_3$, 5% HCOOH and 14% EDTA with regard to expedition, the preservation of tissue architecture and the efficacy of staining. Their study encompassed 30 premolar teeth and 30 condylar specimens, each subset subjected to manual and microwave methods employing each respective decalcification solution. The outcomes indicated that microwave-assisted nitric acid decalcification exhibited the swiftest performance, requiring only 2 days for condyles and 4 days for premolars. EDTA and HCOOH demonstrated commendable histological presentations irrespective of the applied method, whereas $HNO_3$ routinely yielded deficient cellular detailing (15). Their findings are similar to those of the present study. Sangeetha *et al* (15) concluded that microwave-assisted decalcification surpassed conventional decalcification in expeditiousness, while microwave-assisted nitric acid decalcification outperformed routine

nitric acid decalcification in terms of tissue preservation and staining efficacy. In a separate study, Mawhinney *et al* (16) observed that HCOOH or EDTA are frequently employed by laboratories to mitigate tissue contraction and deleterious staining consequences encountered with rapid demineralization utilizing potent mineral acids such as nitric acid.

Waerhaug (17) determined that the implementation of vacuum-based decalcification yielded a substantial reduction in the temporal requirement for this procedure, achieving a compression of time to a mere one-tenth of the standard duration. Moreover, the rate of this process exhibited a direct correlation with the level of vacuum applied. Notably, the study by Verdenius and Alma in 1958 unveiled that the introduction of agitation during the decalcification process led to a notable reduction in the time necessary to attain the intended outcome, effecting a curtailment by approximately two-thirds when contrasted with control conditions (9). Their study delved into the expedience of the decalcification process across different chemical contexts. Their findings underscored that $HNO_3$ facilitated the most expeditious decalcification, whereas trichloroacetic acid induced significantly slower rates. Notably, the application of agitation yielded an acceleration of the decalcification process (9). Exploring diverse conditions and methodologies, Lucas (18) conducted a study wherein they demonstrated that decalcification executed at 37˚C exhibited commensurate swiftness with electrolysis. Moreover, the agitation of the fluid at this temperature yielded outcomes akin to the electrolysis method when applied to rib specimens (18). Sangeetha *et al* engaged in the use of assorted DAs and chelating solutions to demineralize bone tissue with optimal efficiency in terms of temporal expenditure, preservation of cellular integrity, and minimal disruption of subsequent staining procedures (15).

Drawing from the findings of the present study and other studies, as aforementioned, decalcification occurs most rapidly with 5% $HNO_3$ and slowest with 14% EDTA. The histological sections prepared with EDTA are of superior quality, while those with $HNO_3$ are subpar. The introduction of agitation reduces the process by 39%. Despite the longer duration period required by 14% EDTA, it appears to be the most effective DA when agitation is employed. The limitation of the present study was a recording of time. It could have been overcome using hours rather than days as a parameter. Further studies are thus required to standardize the protocol.

In conclusion, of the four DAs examined in the present study, 14% EDTA was found to be the most optimal due to its ability to preserve tissue details relatively. When the agitation method was compared with the routine immersion method of decalcification, the former enhanced the DR, irrespective of the DA used. Section quality and ease of sectioning were also better with the agitation technique regardless of the DA used, as it enhances the DR, prevents prolonged exposure of the tissue to the acid solution and evenly decalcifies the tissues. Finally, we suggest daily changes of 100 ml of 14% EDTA solution with agitation to decalcify healthy premolar teeth. However, the amount of solution varies according to size and type of specimen. The decalcification time can also fluctuate depending on many factors. Thus, it is essential for each laboratory to establish a standardized protocol tailored to its specific needs.

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## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on request.

## Authors' contributions

MS and PS were fundamentally responsible for the conception and design of the study. TP, PS and RB substantially contributed to the analysis and interpretation of the data, and to the drafting of the article or revising it critically for important intellectual content. MS and PS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Informed consent was procured from all participants, ensuring their approval to contribute their biological samples for the research. Ethical clearance was obtained from the Institutional Ethical Committee, AJ Institute of Medical Sciences and Research Centre (Approval no. AJEC/REV/D/34/2015-16).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Fleming WE, Weedon AE and Casson HE: Decalcification of bone and teeth for histological section. Aust J Dent 55: 209-211, 1951.
2. Khangura AK, Gupta S, Gulati A and Singh S: Tooth decalcification using different decalcifying agents-A comparative study. J Oral Maxillofac Pathol 25: 463-469, 2021.
3. Prasad P and Donoghue M: A comparative study of various decalcification techniques. Indian J Dent Res 24: 302-308, 2013.
4. Ali S, Bansal P, Bhargava D, Choudhary A, Hasti A, Chandravarkar V, Mishra M and Singh S: Evaluating the efficacy of two decalcifying agents on hard and soft tissues of human permanent teeth. J Evolution Med Dent Sci 10: 2396-2400, 2021.
5. Khan MI, Khare A, Shamim Khan S, Mahendra A, Nasir A and Khan A: Tooth decalcification: A correlation between weight loss in a decalcified tooth with different decalcifying agents. J Oral Biol Craniofac Res 12: 748-752, 2022.
6. Castania VA, Silveira JW, Issy AC, Pitol DL, Castania ML, Neto AD, Bel EA and Defino HL: Advantages of a combined method of decalcification compared to EDTA. Microsc Res Tech 78: 111-118, 2015.
7. Choube A, Astekar M, Choube A, Sapra G, Agarwal A and Rana A: Comparison of decalcifying agents and techniques for human dental tissues. Biotech Histochem 93: 99-108, 2018.

8. Bumalee D, Lapthanasupkul P, Songkampol K, Srimaneekarn N, Kitkumthorn N and Arayapisit T: Qualitative histological evaluation of various decalcifying agents on human dental tissue. Eur J Dent: Oct 11, 2022 (Epub ahead of print).
9. Verdinius HH and Alma L: A quantitative study of decalcification methods in histology. J Clin Pathol 11: 229-236, 1958.
10. Abrantes AA, Rafacho A, Rivero ER, Mariano FV, Siqueira FM and Gondak RO: Tissue integrity, costs and time associated with different agents for histological bone preparation. Microsc Res Tech 80: 344-349, 2017.
11. Gupta S, Jawanda MK, Sm M and Bharti A: Qualitative histological evaluation of hard and soft tissue components of human permanent teeth using various decalcifying agents-a comparative study. J Clin Diagn Res 8: ZC69-ZC72, 2014.
12. Cho A, Suzuki S, Hatakeyama J, Haruyama N and Kulkarni AB: A method for rapid demineralization of teeth and bones. Open Dent J 4: 223-229, 2010.
13. Savi FM, Brierly GI, Baldwin J, Theodoropoulos C and Woodruff MA: Comparison of different decalcification methods using rat mandibles as a model. J Histochem Cytochem 65: 705-722, 2017.
14. Sanjai K, Kumarswamy J, Patil A, Papaiah L, Jayaram S and Krishnan L: Evaluation and comparison of decalcification agents on the human teeth. J Oral Maxillofac Pathol 16: 222-227, 2012.
15. Sangeetha R, Uma K and Chandavarkar V: Comparison of routine decalcification methods with microwave decalcification of bone and teeth. J Oral Maxillofac Pathol 17: 386-391, 2013.
16. Mawhinney WH, Richardson E and Malcolm AJ: Control of rapid nitric acid decalcification. J Clin Pathol 37: 1409-1413, 1984.
17. Waerhaug J: Decalcification of bone and teeth under vacuum; a rapid method for producing hard tissue preparations. J Dent Res 28: 525, 1949.
18. Lucas RB: Observations on the electrolysis method decalcification. J Pathol Bacteriol 64: 654-657, 1952.

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