

## Original Article

# Comparison of routine and microwave-assisted decalcification of bone with or without teeth: A histologic study

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## ABSTRACT

**Background:** The aim of this study was to compare conventional and microwave-assisted decalcification of sheep bone with and without teeth and to detect any difference in tissue detail preservation, staining quality, and rate of decalcification.

**Materials and Methods:** In this method analysis study, twenty-four specimens consisting of 12 blocks of mandibular molars with their surrounding bone and 12 blocks of mandibular osseous tissue were allocated into two microwave or routine decalcification groups using 5% nitric or formic acid as decalcifying agents. In addition to decalcification rate, a number of variables were used to assess staining quality and tissue detail preservation which were compared between the two groups using Mann-Whitney test ( $P < 0.05$ ).

**Results:** Time to complete decalcification was significantly reduced in the microwave-treated samples as compared to the conventional method, regardless of the decalcifying agent ( $P = 0.025$ ). For both acids, most variables related to staining quality and tissue detail preservation were similar between the techniques ( $P > 0.05$ ). Patchy staining in bone samples and tissue tears in bone + teeth specimens were more common in the routine method when using nitric acid (NAc) ( $P = 0.046$ ) and formic acid (FAc) ( $P = 0.046$ ), respectively. In comparing acids, the performance of FAc was slightly inferior to that of NAc, especially for specimens containing both tooth and bone.

**Conclusion:** The use of microwave technology can accelerate decalcification of bone and teeth of sheep mandibles and at the same time preserve tissue structure and staining quality. Further studies are required to help select the best demineralizing agent, especially in specimens containing bone and teeth.

**Key Words:** Bone and bones, decalcification technique, microwaves, tooth

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## INTRODUCTION

Reduction of the time between receiving a specimen and reporting the diagnosis has been a main concern of pathologists throughout time. In doing so, there could be an overall decrease in health-care costs with simultaneous improvement

in patient satisfaction, especially when there is a need to seek consultation and treatment in a center/hospital away from the individual's residence or in cases where additional diagnostic measures are required.<sup>[1]</sup>

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Routine histologic processing of soft tissues involves formalin fixation, dehydration, clearing, and paraffin embedding followed by obtaining thin sections for microscopic evaluation.<sup>[2]</sup> The average time for this procedure has been estimated to take 21–24 h,<sup>[3]</sup> which would delay the final diagnosis for at least one day: a lapse of time that may be critical for treatment in some cases. For hard tissue sectioning, an additional decalcification step is required to soften the specimen to a state similar to the consistency of paraffin to facilitate accession of thin 3–10 µm sections. Depending on the type, thickness, and structural features of the specimen, conventional decalcification may take from 25 to 45 h, which is added to the usual tissue processing and staining times, further hindering patient treatment.<sup>[4,5]</sup>

Pathologists are constantly under pressure to provide accurate and dependable diagnoses within the shortest amount of time, which could be extremely stressful, especially considering the time it takes for a sample to develop into a microscopic slide ready for observation.<sup>[6]</sup> Therefore, if the duration of processing could be shortened, the pathologist may have more time and less stress resulting in increased accuracy.<sup>[1,3,4]</sup>

For this purpose, various methods have been used during the years to hasten histoprocessing including frozen sections, rapid manual tissue processing, and heating which have had considerable shortcomings.<sup>[1,3,4]</sup> Microwaves cause agitation of molecules leading to vibration and generation of heat that is uniformly distributed within an object.<sup>[6-8]</sup> They have been used to reduce the turnaround time in pathology laboratories for decades during different stages of tissue processing from the initial fixation step to the final staining of specimens.<sup>[6,9]</sup> However, the number of studies concentrating on microwave-assisted decalcification of oral hard tissues is limited, especially those with simultaneous evaluation of teeth and bone in a single block. Therefore, we aimed to compare the conventional and microwave decalcification of sheep teeth and their supporting osseous tissues through evaluation of tissue detail preservation, staining quality, and rate of decalcification.

## MATERIALS AND METHODS

This cross-sectional method analysis study was approved by the ethics committee of our university (IR.shahed.REC.1395.33). A total of three fresh mandibles were collected from adult healthy male sheep weighing 65–75 kg, immediately after

being sacrificed in a slaughterhouse. All surrounding soft tissues were removed, and the samples were immersed in 10% buffered formalin. Using a cutting machine with irrigation, 24 sections were cut at distances of 4 mm from the posterior mandible and fixed in 10% fresh buffered formalin for 72 h. The specimens included 12 bone samples and 12 molars with their encasing alveolar bone (each block containing one tooth), which were weighed, labeled, and randomly allocated to 4 treatment groups as follows: (1) routine decalcification with 5% nitric acid (NAc), (2) routine decalcification with 5% formic acid (FAc), (3) microwave-assisted decalcification with 5% NAc, and (4) microwave-assisted decalcification with 5% FAc.

In the routine method (considered as the gold standard), each specimen was thoroughly washed with tap water and immersed in the respective acid (10 times its volume) at room temperature, after which the exact time was recorded. The solutions were changed every 3 days, and complete decalcification was determined by the calcium oxalate method.<sup>[10]</sup>

For the microwave-assisted method, a domestic microwave oven (Samsung MW123ST) with 2450 MHz operating frequency and 1000 W power output was employed according to that explained previously.<sup>[10]</sup> Briefly, the magnetron was warmed by heating 100 ml distilled water for 5 s. This was repeated with fresh distilled water to sustain the temperature at 41°C–43°C which took 30 s. The best position of the container was assessed by relocating it to different spots during irradiation. Similar to the routine method, the specimens were washed and placed in jars containing the decalcifying solution. This was followed by positioning the containers on the spot that was previously determined in the microwave oven and irradiation for 15 s, every hour for a total of 8 times/day while maintaining the temperature at 41°C–43°C.

Four-micrometer sections were stained with hematoxylin and eosin after washing and routine processing of all decalcified specimens (confirmed by calcium oxalate method). All sections were scanned under a microscope attached to a computer, and three observers (2 oral and maxillofacial pathologists and a dental student) simultaneously analyzed the live images projected on the monitor. Any disagreements were resolved by consensus. Using the criteria proposed by

Sangeetha *et al.*,<sup>[11]</sup> assessment of staining quality was performed by determining tears and crushes, yellow discoloration, and patchy staining. For tissue detail preservation, empty osteocyte lacunae, odontoblastic layer damage, and pulp shrinkage (empty space between pulp and dentin) were recorded [Figure 1].

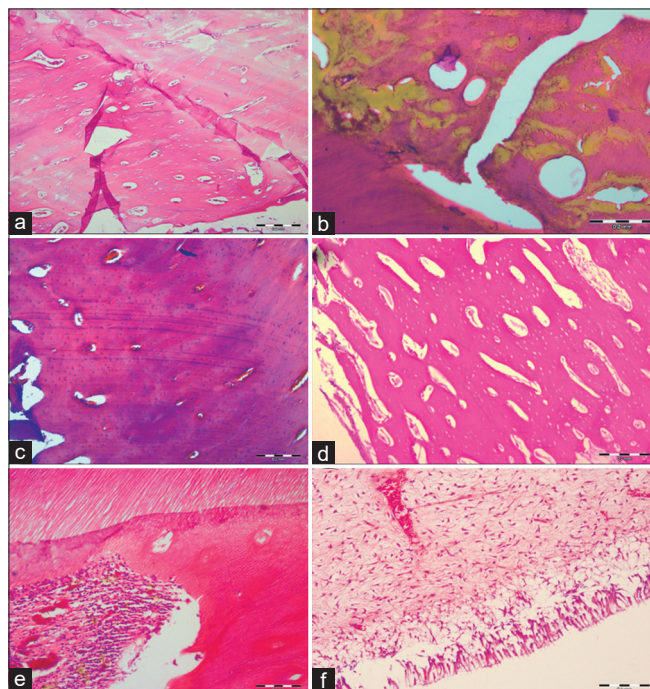
Data were analyzed using Mann–Whitney-U-test, and statistical significance was defined as  $P < 0.05$ .

## RESULTS

### Duration of decalcification

Our findings indicated that the use of a microwave significantly accelerated decalcification by both NAc and FAc for jawbones with and without teeth:

Decalcification of bone specimens with NAc was completed in 12 days using the routine method, while it was reduced to 2 days by the microwave-assisted technique ( $P = 0.025$ ). Bone samples immersed in FAc decalcified in 18 days and when placed in a microwave this process took 3 days, which was also significantly different between the groups ( $P = 0.025$ ).



**Figure 1:** Microscopic images of specimens with issues in staining quality and tissue detail preservation representing: tears/crushes (a), yellow discoloration (b), patchy staining (c), empty osteocyte lacunae (d), pulp shrinkage (e), and odontoblastic layer damage (f). Note that more than one issue may be found within a sample (hematoxylin and eosin staining, scale bars represent the indicated length in millimeters).

When working with bone samples containing teeth, decalcification in NAc occurred within 18 and 3 days using the routine and microwave-assisted methods, respectively ( $P = 0.025$ ). The same specimen type (bone + teeth) placed in FAc decalcified in 21 days without the use of microwave, which was significantly decreased to 5 days after placement in a microwave ( $P = 0.025$ ).

Specimens consisting of both osseous and dental tissues generally took longer to decalcify than those composed of just bone.

### Comparison of decalcifying agents for decalcification duration

Bone tissues with and without teeth decalcified faster in NAc as compared to FAc, regardless of the use of microwave technology ( $P = 0.025$ ), meaning that in pressing situations, where time is of essence, NAc may be a better choice.

### Histological variables

#### Staining quality compared between convention and microwave-assisted methods

As demonstrated in Table 1, when considering bone specimens, tears/crushes and yellow discoloration (%) were similar between the two decalcification methods with both acids, showing no significant differences ( $P > 0.05$ ). However, patchy staining was significantly more common among the routine decalcification specimens with NAc as compared to the microwave-assisted technique ( $P = 0.046$ ). This indicates that if NAc is to be used for decalcification, the microwave-assisted method would have a lower chance of producing patchy staining.

In the bone-containing tooth samples, staining quality did not differ among the decalcifying agents and methods with the exception of tears which were significantly more prevalent when using the routine method with FAc ( $P = 0.046$ ).

#### Tissue detail preservation compared between convention and microwave-assisted methods

In bone samples with and without teeth, all variables related to tissue detail preservation were similar between the two decalcifying methods with both acids, showing no significant difference in any of the comparisons ( $P > 0.05$ ).

#### Comparison of decalcifying agents for histological variables

Among the staining quality variables, only patchy staining was found to be more common when using FAc and the microwave method in comparison to

**Table 1: Staining quality of study samples for routine and microwave-assisted decalcification using nitric and formic acids (values represent mean ranks)**

Staining quality variables	Bone				Bone + tooth			
	NAc	P	FAc	P	NAc	P	FAc	P
Tears/crushes (%)								
Routine	4.83	0.077	4.00	0.507	4.67	0.127	5.00	0.046
Microwave	2.17		3.00		2.33		2.00	
Yellow discoloration (%)								
Routine	3.50	1	4.00	0.317	3.83	0.637	3.00	0.317
Microwave	3.50		3.00		3.17		4.00	
Patchy staining (%)								
Routine	5.00	0.046	2.86	0.369	2.00	0.068	3.00	0.513
Microwave	2.00		4.17		4.50		4.00	

NAc: Nitric acid; FAc: Formic acid

NAc and the same method in bones ( $P = 0.046$ ). Tissue preservation details compared between the two acids showed pulp shrinkage to be more prevalent in the FAc + routine method ( $P = 0.046$ ). Damage to the odontoblastic layer was also more often observed when FAc was employed with the microwave technique ( $P = 0.05$ ). Comparisons of other variables between the two decalcifying agents were not significantly different ( $P > 0.05$ ).

## DISCUSSION

According to the results obtained in the present study, the use of a microwave significantly accelerated the demineralization process of sheep hard tissues, regardless of the decalcifying agent and tissue type (bone, bone + teeth). In histologic comparison of the two methods, microwave-assisted decalcification did not alter the staining quality of the microscopic slides and showed less patchy staining and tissue tears, when using NAc for bone and FAc for bone + tooth specimens, respectively. Between the two decalcifying agents, FAc took longer to decalcify both types of hard tissues with both techniques. Similarly, based on some of the histologic variables, it showed inferior staining quality of bone samples and tissue preservation detail of bone + teeth specimens in comparison to NAc when using the same method. In general, where bone was accompanied by teeth, a longer demineralization rate was observed no matter which technique or acid was used.

There has been considerable development in dental research within the past decade, and both clinical and laboratory studies have contributed to this advancement. Consequently, in recent years, oral and maxillofacial pathology laboratories not only

carry the burden of diagnosing lesions and providing patient reports but also spend a significant amount of time processing tissues for studies conducted by researchers in this field. Considering the nature of the discipline, a large portion of the specimens received as research samples in these laboratories are hard tissues of the oral and maxillofacial regions, especially those of animals.<sup>[12-16]</sup> Therefore, in order to offer the best possible service to both researchers and clinicians, the need for timely processing and reporting is apparent.

A variety of animals have been used in different areas of dentistry to study treatment methods, diseases, and biocompatibility of dental materials. Depending on the study purpose, availability of animals, ethical issues, cost, and simplicity, the researcher selects a suitable animal for investigation. In each individual species, there are a number of similarities in anatomical, genetic, and structural aspects between the contemplated animal and humans.<sup>[17,18]</sup> Sheep have served as models for research in periodontics, endodontics, maxillofacial surgery, osseous turnover, and subjects related to bone remodeling function.<sup>[18-20]</sup> This is due to similarities in premolar/molar periodontium,<sup>[21]</sup> various aspects of periodontitis characteristics,<sup>[19]</sup> anatomic and histologic aspects of incisors,<sup>[18]</sup> enamel and dentin microstructure, hardness and modulus of dentin,<sup>[22]</sup> pattern of bone ingrowth,<sup>[20]</sup> and Haversian bone tissue microstructure and histological structure size<sup>[23]</sup> between these species. According to comparisons between sheep and *Homo sapiens*, sheep were shown to have higher enamel inorganic carbon and lower degree of enamel and dentin mineralization,<sup>[24]</sup> thinner enamel, higher bone density,<sup>[18]</sup> demonstrations of both plexiform and Haversian osseous tissues,<sup>[23]</sup> lower Young's modulus and hardness of enamel,<sup>[22]</sup> and differences

in a number of anatomical features of some teeth.<sup>[18,25]</sup> These can lead to differences in demineralization results between sheep and human hard tissues. When comparing studies of different species or extrapolating results of animal studies to humans, all differences should be considered.

Several methods using various agents have been employed through time to accelerate the generation of slides from tissue samples.<sup>[1,4]</sup> Among them, the use of microwaves has gained popularity due to its reduction in time, cost, exposure to toxic reagents, and denaturation of nucleic acids.<sup>[4,5,7]</sup> Microwave-assisted decalcification has been previously studied in tissues taken from different locations, and most of these studies have attested to the good quality of the resulting microscopic slides.<sup>[4,7,11,26]</sup> However, aside from the fact that the number of studies in the oral cavity is limited, most of them have evaluated bone and teeth separately and not within the same block. Considering the different levels of mineral content within tooth and bone, when both these tissues are to be processed together, they demonstrate different decalcification times leading to varying consistencies, causing difficulties in sectioning. Due to the fact that a large body of recent research in dentistry requires histologic analysis of teeth alongside bone,<sup>[12-16]</sup> we evaluated section quality after routine and accelerated decalcification using blocks containing both tissues.

Similar to previous studies,<sup>[4,11,26]</sup> we found decalcification time to be significantly shorter when employing the microwave method, regardless of the decalcifying agent. More time was needed when the samples contained bone and tooth compared to exclusive bone specimens. Considering that the size of the tissue blocks was similar in both the groups, the increase in decalcification time may be due to differences in calcification levels and the size, morphology, and composition of apatite crystals in dental tissues compared to bones.

Similar to former investigations,<sup>[11,26]</sup> microwave-assisted decalcification did not alter staining quality and, when compared to routine decalcification, even demonstrated better results with significantly less patchy staining and tears in bone and bone + tooth samples, respectively, which was in agreement with the results obtained by Sangeetha *et al.*<sup>[11]</sup> Tissue detail was also adequately preserved in both techniques with no significant differences confirming previous findings.<sup>[11]</sup> It is noteworthy

that most studies on this subject who used bone and teeth were either on human tissues<sup>[4,11]</sup> or did not mention the tissue of origin.<sup>[26]</sup> In addition, teeth and bone were evaluated separately and not in a single block<sup>[4,11]</sup> or the investigation specifically concentrated on teeth.<sup>[26]</sup> A study on rat maxillae and mandibles, similar to our results, found a significantly faster decalcification time in the microwave method and reported no significant effect on the morphology of the specimens. Their findings were probably based on observation and not scoring; there was no mention of the assessment method.<sup>[27]</sup> Likewise, an investigation evaluating rat and cat teeth and bones reported faster demineralization using the microwave technique, and the reason was attributed to thermal effects and not to microradiation impact. Microscopic evaluation was not performed in this study.<sup>[28]</sup> Both investigations utilized different decalcifiers than that used in the current investigation.

An interesting observation in the present research was that when comparing the two decalcifying agents, alteration of a number of important histologic features was more prevalent with FAc as compared to NAc, especially in the tooth part of the bone + tooth specimens. Similarly, other studies<sup>[26,29,30]</sup> have also found FAc to perform worse than NAc in some aspects related to tissue quality. This is in contrast to the fact that NAc is a strong decalcifier and has been recognized as being damaging to tissues and can lead to impaired staining, especially compared to weaker acids such as FAc which has been suggested to provide better histological detail.<sup>[11,31,32]</sup> A number of factors may contribute to the discrepancies between the results of these studies. The most obvious includes different laboratory conditions such as room temperature and light, acid manufacturer and concentration, microwave type and model, processing equipment, etc. In addition, different tissue samples could also have a role which includes differences in species of the specimens as well as the presence of bone and teeth in the same or different blocks. Furthermore, subjective factors related to the histopathologic processing technicians and observers responsible for reporting the histopathologic findings could also contribute to the aforementioned differences.

Specimens with bone and teeth are made of diverse tissue structures with different mineralized contents, organic compositions, and apatite crystal sizes.<sup>[33]</sup> Enamel, dentin, bone, and cementum approximately contain 95%, 60%–70%, 65%, and 50% inorganic

material, respectively.<sup>[34]</sup> In addition to these, tooth + bone specimens also contain pulpal soft tissue and bone marrow with minimal calcification. While tissues with higher mineral content (i.e., enamel) may take longer to decalcify after being placed in acids, others have already been decalcified and their organic content is being unnecessarily exposed to acid, leading to tissue damage. Therefore, more diversity in tissue calcifications of the different components of a specimen can lead to higher tissue damage in general.<sup>[29,35]</sup> For ideal demineralization of hard tissues, a balance should be obtained between the erosive effects of acids (solution acidity, penetration rate, etc.) and the amount of time they are in contact with a specimen. The longer the duration of exposure continues, there is more chance of already demineralized tissues being deteriorated, i.e., over-decalcified.<sup>[29,31,35]</sup> A theory that may help explain the better histologic performance of NAc compared to FAc in teeth-containing samples could be that weaker acids such as FAc may have a milder effect on tissues and appear to cause less damage, but they take longer and therefore expose the tissues to a longer period of acid contact, possibly resulting in more damage to the less-calcified tissues. Consequently, in explaining our findings and those of similar studies,<sup>[26,29,30]</sup> it seems that the balance between exposure time and erosive effects of the acids in producing tissue damage, was in favor of time, which, as stated above, could be the result of our specific laboratory, performer, and specimen conditions. A similar theory was used to justify the worse “ease of sectioning” found after demineralization in a mild decalcifier compared to a stronger one.<sup>[31]</sup> However, it should be strongly emphasized that these are merely hypotheses, and the exact reason for this observation requires further research and confirmation by future studies. Larger sample sizes and evaluation of different species may also be beneficial.

According to our findings and those reported elsewhere,<sup>[26,29,30]</sup> it seems that when faced with a choice for decalcifying specimens containing teeth, NAc could be considered before FAc, when selection is to be made between these two acids. Needless to say that these results should be supported by further studies with larger sample sizes and a wider choice of decalcifying agents.

## CONCLUSION

Based on our findings, the decalcification of oral and maxillofacial hard tissue specimens can be accelerated with a microwave without alteration of histologic

details. In addition, when selecting a decalcifying agent, especially for specimens containing teeth, we suggest that FAc not be considered as the first choice. However, we maintain that conventional decalcification should remain the gold standard for comparison purposes in studies evaluating new methods and where time would not be a limitation for pathologic reports. Further studies are suggested before microwave-assisted decalcification could be used as a routine processing method in oral and maxillofacial laboratories.

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## Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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