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Effectiveness Of Various Decalcification Agents On Qualitybeef Rib Bone Tissue (Bos Taurus): A Comparative Study Of Formic Acid, Nitric Acid, And Their Combination With Edta

Alifah Putri Safina[1]
[1]Universitas Pelita Harapan
Charissa Virginia Serhalawan[2]
[2]Universitas Pelita Harapan
Christian Pairan[3]
[3]Universitas Pelita Harapan
Jessica Rose Whaite[4]
[4]Universitas Pelita Harapan
Fransisca Probo Setyoningrum[5]
[5]Universitas Pelita Harapan
Diana Intan Gabriella Lusiasa[6]
[6]Universitas Pelita Harapan

Email correspondence:Fransisca.setyoningrum@uph.edu

Abstract

Decalcification is the process of removing calcium salts and other minerals from hard tissue such as bone to produce histological specimens that can be sectioned and observed effectively. Selecting the right decalcifying agent is crucial because it must soften the tissue without damaging its morphology. This study aimed to compare the effectiveness of various decalcifying agents on the quality of beef rib bone tissue (*Taurus boss*) using formic acid, nitric acid, and a combination with ethylenediaminetetraacetic acid (EDTA). The study was conducted in a comparative experimental manner using six treatment groups, namely 5% formic acid, concentrated formic acid, 15% nitric acid, 15% nitric acid + 10% EDTA, concentrated nitric acid + 10% EDTA, and concentrated nitric acid. The effectiveness of decalcification was evaluated based on the level of bone hardness using the needle method, changes in tissue morphology, and the duration of treatment. The results showed that 5% formic acid and concentrated formic acid were not able to

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soften bones optimally. 15% nitric acid produced soft bones after five days of immersion, while the combination of 15% nitric acid and 10% EDTA did not show optimal results. The combination of concentrated nitric acid and 10% EDTA produced slightly soft bones with minimal tissue damage compared to concentrated nitric acid without EDTA. In contrast, the use of concentrated nitric acid without EDTA caused tissue shrinkage, residue formation, and morphological damage. Based on the research results, the effectiveness of decalcification was influenced by the type and concentration of decalcification agent, with 15% nitric acid and a combination of 10% concentrated nitric acid–EDTA showing better results than other treatments.

Keywords: Decalcification; beef rib; formic acid; nitric acid; EDTA, histotechnology.

INTRODUCTION

Decalcification is a procedure carried out with the aim of removing calcium salts and other minerals found in hard tissues such as bones and teeth.[1]In histotechnology studies, analysis of bone specimens is an important part that is taken into account to support the process of establishing a diagnosis of diseases such as Osteosarcoma or bone tumors.[2]The high levels of calcium salts in bone specimens pose a dilemma for anatomical pathology laboratory technicians in determining a decalcification formula that maintains histological quality without damaging the sample. The precision of the decalcification process is a key determinant of the quality of the specimen. Bone that is less malleable risks compromising the quality of microtome sections, producing defective histopathological specimens, and complicating medical interpretation.[3]

To date, the selection of decalcification agents in cytohistotechnology laboratories often faces a dilemma between speed and tissue structure retention. Nitric acid (HNO_3) is one of the strong acids most frequently used for routine decalcification due to its very rapid demineralization rate through the formation of soluble calcium salts.[4]However, its aggressive nature carries a high risk of triggering sudden shrinkage, cell swelling, and even damage to the details of the cell nucleus if the immersion time exceeds the optimal limit.[3], [5]. In contrast, Ethylenediaminetetraacetic acid (EDTA) as a chelating agent works gently by binding calcium ions on the surface of the bone matrix.[3]. The use of EDTA is known to be able to maintain the integrity of tissue microstructure, but requires a much longer exposure time, even up to weeks.[4], [5].

Most previous decalcification research generally used small laboratory test animals, such as the femur or mandible of rats (Rattus norvegicus).[5]. There are still few studies evaluating the

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performance of decalcification agents on bone specimens with denser and thicker cortical densities such as commercial veterinary tissue. Bovine ribs (Costae) have structural characteristics, cortical thickness, and trabecular density (structures forming spongy tissue) that are much more complex than rodent bones. Therefore, evaluating the effectiveness of various decalcification agents on bovine ribs is essential to determine the extent to which each agent is able to maintain the integrity of the histological structure of the tissue while providing efficiency in the demineralization process.[4], [5].

This study aims to analyze the effectiveness of various decalcification agents on the quality of beef rib tissue (Bos taurus) through a comparative study of the use of formic acid, nitric acid, and a combination with EDTA. Specifically, this study aims to compare the histological tissue quality of beef ribs after the decalcification process using the three treatments and to determine the most effective decalcification agent in maintaining the integrity of the tissue structure.

The hypothesis of this study is that there are differences in the effectiveness of formic acid, nitric acid, and a combination of EDTA on the quality of beef rib bone tissue (Bos taurus). Each decalcifying agent is expected to produce different levels of histological structure preservation, thus affecting the quality of the resulting tissue.

MATERIALS AND METHODS

This study was a comparative experimental study with six decalcification agent treatment groups. The study focused on beef ribs as the primary sample, which were treated uniformly in each group with measured size and thickness to avoid variation in results. This study design was designed to allow for consistent comparison of the effectiveness of each decalcification agent, thus providing a more valid picture of the differences between treatments.

The samples used in this study were beef ribs obtained from the Malabar market in Tangerang. The ribs were cut uniformly into 5 cm x 3 cm x 1 cm pieces using a sterile bone saw to ensure morphological uniformity. Each treatment group used six pieces of beef ribs as replications, allowing for consistent comparison and enhancing the validity of the study. This study used six treatment groups distinguished by the type of decalcifying agent and immersion time. A description of each treatment group is presented in Table 1.

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Table 1.Decalcifying agent treatment groups used in the study.

No	Decalcifying Agent	Duration	Results
1	Formic Acid 5% (200ml)	5 days	 Bones remain hard
2	Concentrated Formic Acid (200ml)	5 days	 Gaping bones, still hard
3	Nitric Acid 15% (200ml)	5 days	 Soft bones
4	Nitric Acid 15% (100ml) + EDTA 10% (100ml)	5 days	 Bones remain hard, there are crystals
5	Concentrated Nitric Acid (100ml) + 10% EDTA (100ml)	3 days	

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			The bones are a little soft and contain crystals.
6	Concentrated Nitric Acid (200	Less than 24 hours	 Hard, shrinking, there is residue

The quality of decalcification results was evaluated based on several observation parameters, including bone hardness, tissue morphology, decalcification duration, and final score. The assessment was conducted to determine the effectiveness of each decalcification agent in producing optimal beef rib bone quality. The assessment criteria used in this study are presented in Table 2.

Table 2.Criteria for assessing the effectiveness of decalcification agents on beef ribs (Bos taurus)

Picture	Assessment aspects	Score
	• The level of bone hardness using the needle method with a puncture depth of 2.3 inches/mm (2), the bone becomes soft. • Bone morphology (2), permanent boneintact. • The duration of time required is 5 days.	Final score (4) = Good

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	• Bone hardness level using needle method with kpuncture depth 1.6 inch/mm (1), the bone becomes slightly soft. • Bone morphology (2), permanent bone intact. • The duration of time required is 5 days.	Final score (3) = Not good enough
	• Bone hardness level using needle method puncture depth 0.6 inch/mm, bone is still hard. • Bone morphology(0), the bones experience shrinkage. • The duration of time thatless than 24 hours are needed O'clock.	Final score (0) = Bad

The data analysis in this study is presented in a comparative descriptive manner, emphasizing the results of visual observations and bone hardness tests in each treatment group. Determination of the 'soft' category was carried out using the needle method (Assessment), namely by inserting a needle into the bone surface. Bone is considered soft if the needle can penetrate easily (score 2), whereas if the needle penetrates only slightly, it is categorized as

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slightly soft (score 1), and if the needle does not penetrate at all, the bone is still considered hard (score 0). In addition, bone morphology was also observed visually to determine whether the bone remains intact, has shrunk, or gaped. Bone that remains intact with a soft texture is categorized as soft, while bone that shrinks or gapes but remains hard is not included in these categories. The duration of decalcification time was recorded to assess the reaction speed of each agent. By combining the needle test, morphological observations, and duration, a clear comparative picture was obtained regarding the effectiveness of each decalcification agent.

RESULTS

The results of observations on six decalcification treatments on beef ribs are presented in Table 1 above. Overall, only two of the six treatments successfully softened the bone: the 15% nitric acid group (treatment 3) and the concentrated nitric acid group combined with 10% EDTA (treatment 5).

The 5% formic acid treatment (group 1) showed no significant changes in bone hardness after 5 days of treatment. In the concentrated formic acid group (group 2), morphological damage occurred in the form of gaping or cracked bones on the surface, but the interior of the bone remained hard. This indicates that concentrated formic acid is only able to decalcify the outermost layer without sufficient penetration into the interior.



(Group 1)



(Group 2)

Treatment with 15% nitric acid (group 3) successfully softened the bone after 5 days. However, in group 4 (15% nitric acid + 10% EDTA), the bone remained hard despite the combination of the two agents, indicating that low nitric acid concentrations were not sufficient to activate the synergistic effect with EDTA.

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(Group 3)



(Group 4)

The concentrated nitric acid + 10% EDTA group (group 5) showed the best results, with the bone softening in just 3 days, faster than the other successful treatments. Conversely, the concentrated nitric acid without EDTA (group 6) produced the most damaging results: the bone hardened and significantly reduced in size, with residues and even partial tissue loss.



(Group 5)



(Group 6)

DISCUSSION

Decalcification Mechanism and Factors Affecting Success

Decalcification is the process of removing minerals from hard tissue, especially beef ribs, to soften the tissue so that it can be cut using a microtome without damaging its histological structure. The main mineral that makes up bone is hydroxyapatite crystals (Ca10(PO4)6(OH)2) which provide strength to the bone. When decalcification is carried out with an acidic solution, hydrogen ions (H^+) dissolve the hydroxyapatite through the reaction $Ca_{10}(PO_4)_6(OH)_2 + 20H^+ \rightarrow 10Ca^{2+} + 6H_3PO_4 + 2H_2O$, thus causing calcium ions to be released from the beef rib matrix and the tissue becomes softer. The success of the decalcification process is greatly influenced by the type of solution, concentration, pH, size of the beef rib sample, soaking time, and the ability of the solution to diffuse into the tissue. Therefore, to ensure that histological preparations remain of high quality, the decalcifying agent must be able to balance the rate of mineral dissolution while

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maintaining tissue morphology.[6].

The Effect of Formic Acid on the Decalcification Process of Beef Rib Bones

The results of the study showed that the use of 5% formic acid was not able to decalcify beef ribs optimally, while concentrated formic acid only caused the outer part of the beef ribs to crack on the outside, while the inside remained hard. This shows that increasing the solution concentration does not always result in more effective decalcification, if the penetration of the solution into the tissue is still limited.[7].

Chemically, formic acid is a weak acid that only undergoes partial ionization ($HCOOH \rightleftharpoons H^+ + HCOO^-$) so it produces fewer H^+ ions than mineral acids. Thus, the dissolution of hydroxyapatite in beef ribs is slower because the amount of H^+ increases in concentrated formic acid, thus accelerating surface demineralization. However, the solution is still unable to diffuse optimally to the inside of the beef ribs. This condition causes the surface of the bone to crack, while the inside still contains minerals.[8].

The Effect of Nitric Acid on the Decalcification Process of Beef Rib Bones

The use of 15% nitric acid can provide better results than formic acid because it can soften beef ribs evenly within five days. This is because nitric acid is a strong acid that can undergo complete ionization ($HNO_3 \rightarrow H^+ + NO_3^-$) thus producing many H^+ ions and accelerating the dissolution of hydroxyapatite in beef ribs. However, the use of concentrated nitric acid without EDTA causes the tissue to become brittle, shrink, and still leave residues (group 6).[9], [10]This condition indicates over-decalcification, a condition in which the acid not only dissolves minerals but also begins to damage the collagen matrix, resulting in a decrease in the quality of histological preparations of beef ribs. Therefore, the use of nitric acid must be adjusted according to the concentration and soaking time to ensure a balance between decalcification rate and tissue preservation.[11].

The Effect of EDTA Addition on the Decalcification Process of Beef Rib Bone

EDTA (Ethylenediaminetetraacetic acid) is a chelating agent that works by binding calcium ions (Ca^{2+}) that have been released from hydroxyapatite through the reaction $Ca^{2+} + EDTA^{4-} \rightarrow [Ca\text{-}EDTA]^{2-}$. The binding of calcium ions helps maintain the mineral release process so that decalcification of beef ribs can be carried out more effectively. In this study, the combination of

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15% nitric acid and 10% EDTA did not produce optimal decalcification because the beef ribs had not fully softened.[12]In contrast, the combination of concentrated nitric acid and 10% EDTA yielded quite good results because it accelerated the dissolution of hydroxyapatite while binding the released calcium ions. This combination resulted in a faster decalcification process without causing significant tissue damage, thus indicating a balance between decalcification effectiveness and preservation.[13].

General

Overall, our research results indicate that decalcification effectiveness is influenced by the type of decalcifying agent, the solution concentration, and the mechanism of action of each agent. Formic acid works more slowly because it is a weak acid, while nitric acid is more effective because it produces a greater number of H^+ ions. However, the use of concentrated nitric acid without EDTA increases the risk of overdecalcification, which can damage the collagen matrix and reduce the quality of histological preparations of beef ribs. The results showed that the combination of concentrated nitric acid and 10% EDTA is the most effective treatment for decalcification because EDTA can bind released calcium ions. This combination can accelerate the decalcification process while maintaining tissue quality, resulting in better preparations for histopathological examination.[6].

CONCLUSION

This study concluded that the effectiveness of the decalcification process on beef rib bone tissue (Bos taurus) is largely determined by the type, concentration, and mechanism of action of the decalcification agent used. The experimental results showed that the use of 15% nitric acid with a 5-day immersion duration and a combination of concentrated nitric acid with 10% EDTA with a 3-day immersion duration were the most optimal treatments. The combination of concentrated nitric acid and 10% EDTA was considered the best decalcification agent because it was able to accelerate mineral dissolution while maintaining the morphological integrity of the tissue through a balance between demineralization and preservation. In contrast, the use of formic acid (5% and concentrated) proved ineffective in softening beef rib bone completely. Furthermore, the use of concentrated nitric acid without a chelating agent combination is highly discouraged because it causes significant damage to the histological structure of the tissue due to over-decalcification.

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