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Optimization of Formic Acid-Formalin-Based Decalcification Protocol for Rat Calvarial Bone Histology

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ABSTRACT

Decalcification is crucial in histological processing, particularly for studying mineralized tissues like bone. The choice of decalcification method can significantly impact the quality of histological sections and the preservation of tissue morphology. This study aims to establish a standardized protocol for decalcifying rat calvarial bone using a formic acid-formalin-based decalcification solution. The protocol was systematically optimized and evaluated based on various parameters, including decalcification time, formic acid concentration, and tissue integrity preservation. The decalcification process was evaluated through comprehensive assessments, including gross physical examination, chemical analysis, and radiographic imaging techniques. Our result demonstrated that the 10% formic acid concentration proved most effective for decalcifying rat calvarial bone samples within eight days, excelling in mineral

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content removal while preserving specimen structural integrity. In contrast, the 5% concentration failed to complete decalcification within ten days, and the 15% compromised sample quality within eight days. Histological analyses confirmed the efficacy of the 10% formic acid concentration in maintaining tissue integrity and achieving optimal staining quality. The standardized protocol presented in this study provides an effective and reliable approach for achieving consistent and high-quality histological sections of rat calvarial bone. An ideal decalcification agent should effectively remove calcium salts, preserve structural integrity and molecular components, facilitate rapid yet minimally damaging decalcification, and ensure ease of handling for laboratory personnel. Further exploration of its applicability to different bone types or species is recommended to broaden its research utility.

1 Introduction

Teeth and bones fall into the group of the toughest tissues, being denser and less reactive chemically than other body tissues. The microscopic analysis is challenging due to the high calcium and phosphorus levels in the biological apatite that forms these tissues (AbouNeel et al. 2016). It is crucial to extract inorganic calcium from the organic collagen matrix, calcified cartilage, and nearby tissues to obtain well-defined bone sections. This process, referred as decalcification, is essential for successful analysis (Khangura et al. 2021). Decalcification removes calcium salts from mineralized tissues for histologic sectioning. But with balanced acidity, any acid can affect tissue stability. The impact depends on solution acidity and decalcification duration. Factors like solution strength, temperature, agitation, and tissue suspension affect decalcification speed (Callis and Sterchi 1998; Bancroft and Gamble 2008).

The samples should always be properly fixed before decalcification (González-Chávez et al. 2013). Various agents, such as weak and strong acids and chelating agents, are used for decalcification. Choosing the appropriate decalcification agents is crucial in handling mineralized tissues as it can impact the tissues' integrity and immunohistochemical properties (Savi et al. 2017). An ideal decalcification agent possesses several key characteristics such as it should effectively remove calcium salts from mineralized tissues without compromising the structural integrity of tissue or cellular details (Prasad and Donoghue 2013). Furthermore, it must preserve the antigenicity and molecular components of tissue, allowing for accurate histological and immunohistochemical analysis (Kim et al. 2016). An ideal agent should also facilitate a reasonably rapid decalcification process while minimizing tissue damage or distortion. It should also be easy to handle, ensuring safety for laboratory personnel and compatibility with subsequent staining techniques (Sanjai et al. 2012).

Methods include acid/manual, microwave, ion exchange resins, electrolytic, and ultrasonic decalcification (Skinner 2003). Precisely determining the endpoint is vital due to acids' harmful effects on tissues. The endpoint determination is achieved through physical, chemical, and radiographical techniques (Savi et al. 2017). Critical steps in the decalcification include meticulously assessing the specimen beforehand, ensuring thorough fixation, preparing slices of optimal thickness for fixation and processing, selecting an adequate volume of a suitable decalcifier changed at regular intervals, precisely determining the endpoint, and executing comprehensive processing according to a suitable schedule (Skinner 2003).

Since there is no ideal decalcification agent, the choice and standardization of the decalcification protocol depend on the specific bone type and the intended examination. This study aims to standardize a formic acid-formalin-based decalcification protocol for the histological evaluation of rat calvarial bone.

2 Materials and Methods

2.1 Specimen Preparation and Fixation

All experimental protocols adhered strictly to the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA Guidelines 2003). The study protocols were approved by the Institutional Animal Ethics Committee (IAEC) of the ICAR-Indian Veterinary Research Institute, ensuring compliance with ethical standards for animal research. Specimens used in this study were obtained from adult rats previously sacrificed in other approved experimental studies. The present study consisted of three groups, each containing six samples. A total of 18 rat calvarial bone samples were collected and underwent fixation in neutral buffered formalin for three days.

2.2 Decalcification Procedure

A formic acid-formalin-based solution was utilized for decalcification. Formic acid concentrations of 5%, 10%, and 15% were tested to optimize the protocol. The samples were randomly assigned into three groups – groups A (5% formic acid concentration), B (10% formic acid concentration), and C (15% formic acid concentration). The concentration of 40% formaldehyde was kept at 5% level. A decalcification solution was employed in a 100-fold volume relative to the specimen to ensure

adequate coverage. The decalcification solution was replaced daily, and the specimens were washed before replenishment with freshly prepared solutions to maintain optimal decalcification conditions. The endpoint of decalcification was monitored daily using chemical analyses and radiographic imaging techniques to assess the completion of the process (Skinner 2003).

2.3 Evaluation of Decalcification

Evaluation of the appearance, texture, and integrity of the specimens after each decalcification cycle was done. Daily chemical analyses were performed to track mineral content changes and verify decalcification progress. The calcium oxalate test was employed to determine the endpoint of decalcification. A 5 ml spent decalcification solution was mixed with 5 ml of concentrated ammonium hydroxide and 5 ml of saturated aqueous ammonium oxalate. Any white precipitate suggested calcium presence and incomplete decalcification (Shahid et al. 2023).

Daily radiographic imaging was employed to visualize the extent of mineral removal and determine the completeness of the process. The specimens were exposed at 25 KVp and 3 mAs for 10s. The tissue sections were processed, paraffin-embedded, and subjected to standard staining procedures for histological analysis. Routine staining using H&E and special staining using RGB Trichrome were employed to evaluate the tissue integrity (Mamachan et al. 2023).

After the staining was completed, a final grading system was employed, utilizing a scale from 1 to 4, and here,1 for poor, 2 for fair, 3 for good, and 4 for excellent, allowing for a detailed classification based on predefined criteria. Following this grading, the results were tabulated and analyzed using appropriate statistical methods to determine significant differences and optimal conditions for decalcification.

The statistical analysis employed in this study involved a One-Way ANOVA to assess the significance of the duration of decalcification across different groups. The Kruskal-Wallis test was also utilized to evaluate the significance of staining scores. A significance level of $p < 0.05$ indicated statistical significance, underscoring the rigor applied in determining the impact of varying decalcification durations and staining outcomes.

3 Results

The results highlighted that among the tested formic acid concentrations (5%, 10%, and 15%), the 10% formic acid concentration proved most effective for decalcifying rat calvarial bone samples. The mineral content was removed within 8 days while maintaining specimen structural integrity and texture. In contrast, the 5% formic acid concentration failed to complete decalcification within 10 days. Alternatively, the 20% formic acid concentration achieved decalcification in 5 days but compromised sample quality. By day 8, evident changes in appearance, texture, and integrity were noticed and the time for decalcification varied notably among the groups. Specifically, group B showed considerable variation compared to Groups C and A. Group A required significantly more days to achieve decalcification. In contrast, Group C achieved it significantly faster than Group B (Figure 1A).

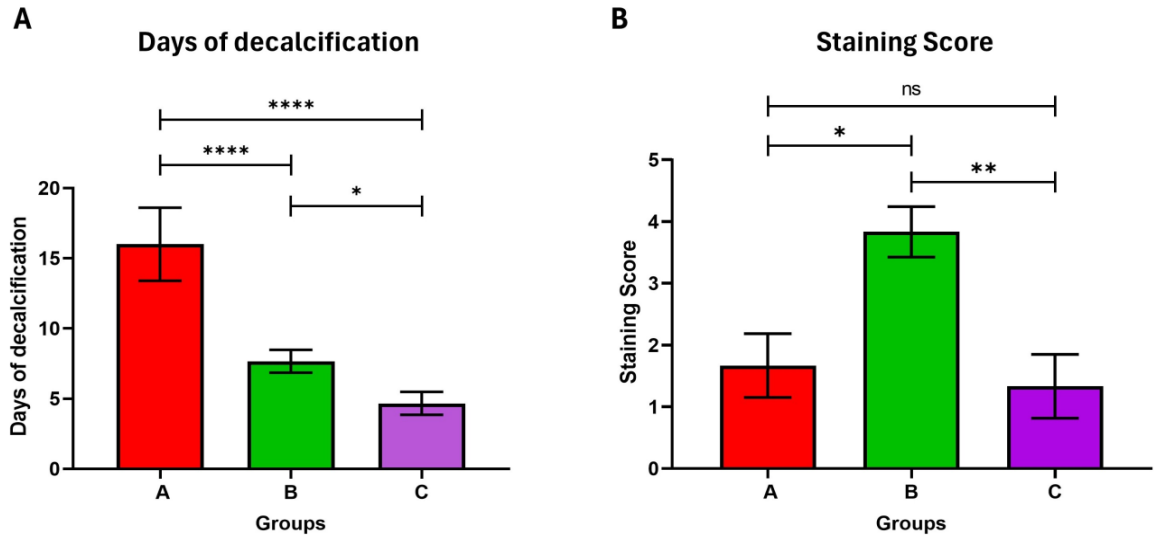

Figure 1 (A) The time for decalcification varied notably among the groups, with group B showing considerable variation compared to both groups C and A. Group A required significantly more days to achieve decalcification. In contrast, group C achieved it significantly faster than group B; (B) The staining score notably differed among the groups, with group B showing significantly higher scores than groups A and C, indicating that the 10% formic acid decalcified bone samples in group B achieved a better staining quality than those in groups A and C.

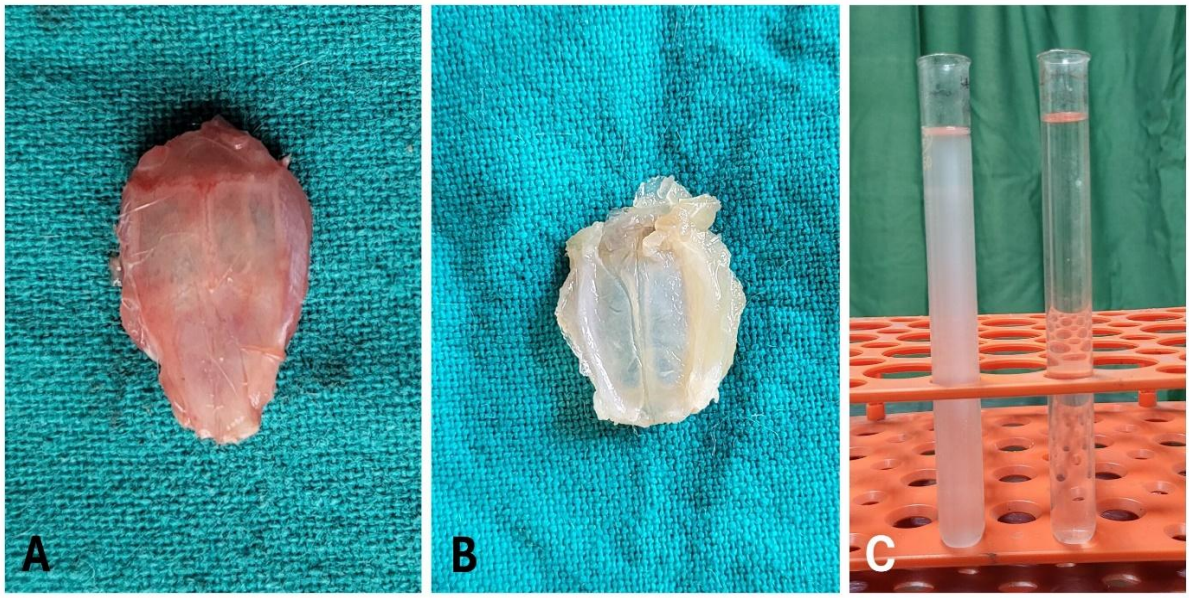

Figure 2 Upon decalcification, the gross observation of rat calvarial bone revealed, (A) Initially, the bone exhibits a rigid, opaque structure, while after the process, (B) it undergoes a discernible shift towards translucency and a softer texture due to the reduction in mineral content, (C) The presence of a white precipitate strongly suggests the residual presence of calcium, indicating incomplete decalcification (left), and the absence of residue, indicating complete decalcification (right).

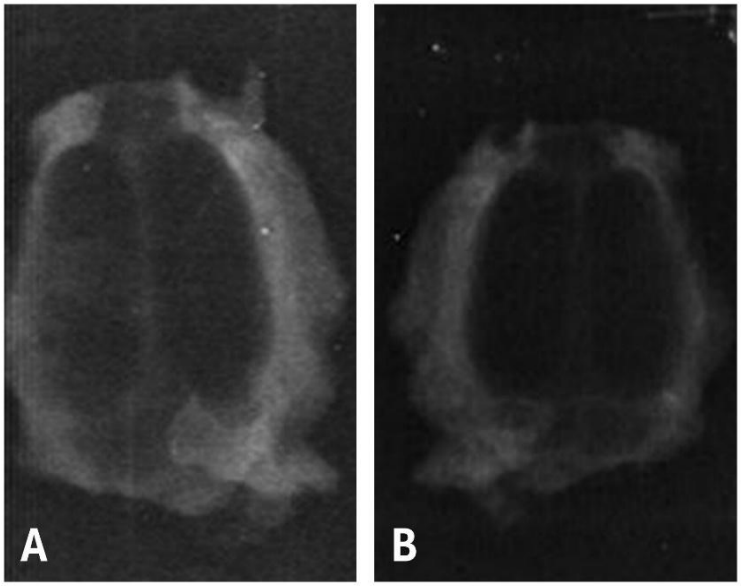

Figure 3 The detection of mineral loss in radiographic imaging from the initiation of decalcification solution application on day 1 (A) and until the complete decalcification (B).

Decalcification altered the appearance of rat calvarial bone, shifting it from a rigid, opaque structure to a translucent, softer form as mineral content diminishes (Figures 2A and 2B). This process modifies the texture of bone, making it more pliable while compromising its structural integrity, rendering it susceptible to bending or breakage under minimal stress. Consistently, the specimens treated with a 10% formic acid solution demonstrated the desired endpoint for decalcification. This was validated through rigorous daily chemical analyses, the calcium oxalate test, and radiographic imaging. The absence of any white precipitate in the calcium oxalate test and the clear observable reduction in mineral content in radiographic imaging confirmed the thoroughness and completeness of the decalcification process facilitated by the 10% formic acid solution (Figures 2C and 3).

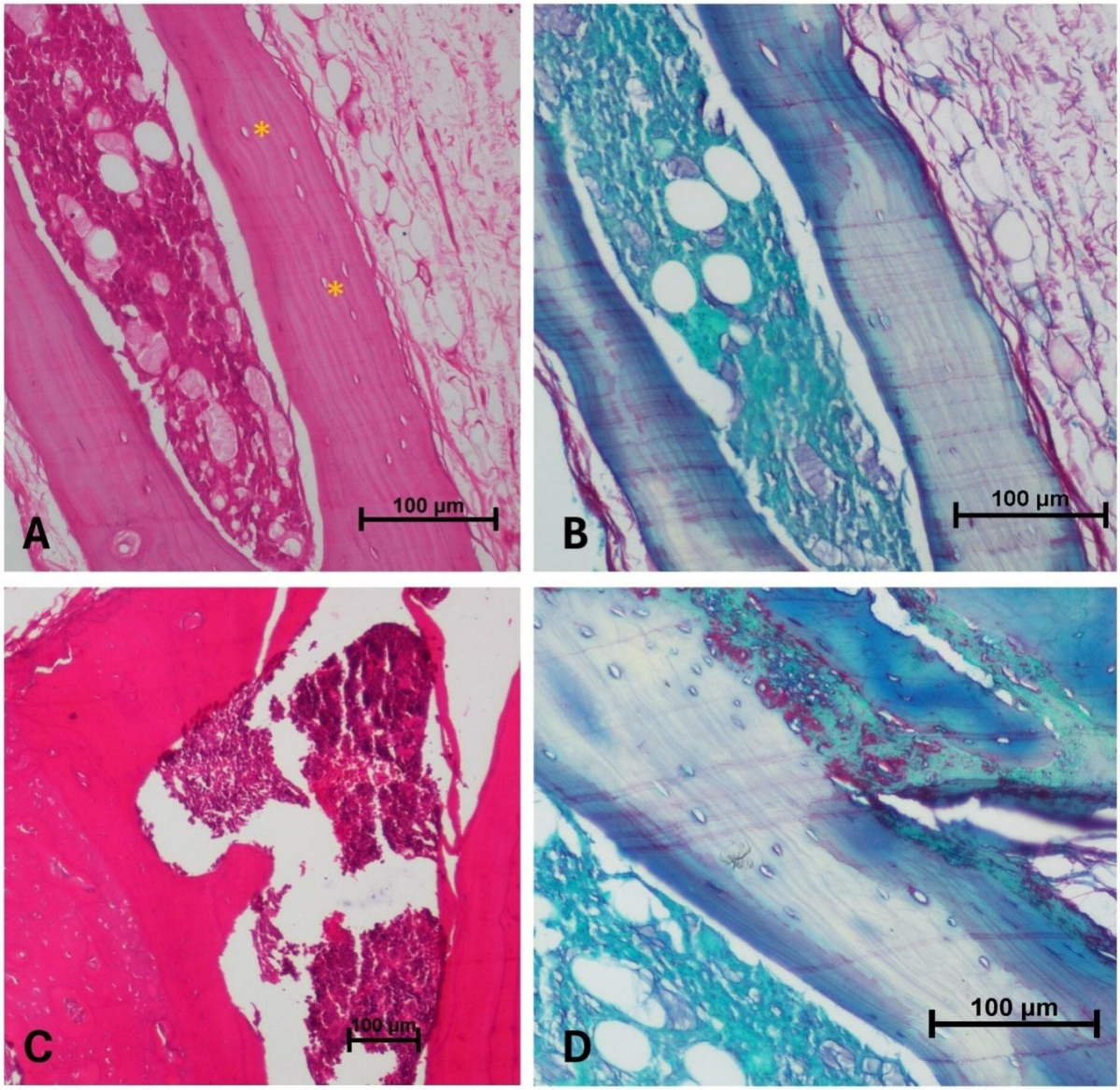

Figure 4 The effectiveness of the 10% formic acid concentration in maintaining tissue integrity while ensuring the integrity of the overall sample during (A and C) standard staining (H & E) and (B and D) specialized staining (RGB trichrome), (A) Osteocytes, are housed within small cavities called lacunae (orange colour) and appear as dark dots. The bone matrix appears pink due to its affinity to the eosin stain. (B) Red could potentially highlight osteoids and areas of active bone formation. Green emphasizes the bone matrix. Blue could represent areas with a high blood vessel density, indicating vascularization regions and nutrient supply within the bone tissue. (C) The section displayed a notable loss of tissue definition, hindering a clear distinction of structural components in samples due to the utilization of 20% formic acid for decalcification (D) The sections seemed inadequately stained, leading to diminished clarity in discerning the varied RGB grades, potentially causing a misjudgment or misinterpretation of the result in samples due to the utilization of 20% formic acid for decalcification.

Histological analysis, encompassing both standard staining (H&E) and specialized staining (RGB Trichrome), validated the efficacy of 10% formic acid. This concentration effectively maintained tissue integrity while preserving the overall bone sample structure (Figure 4A). The staining score notably differed among the groups, and among the tested groups, group B showed significantly higher scores than groups A and C. This indicates that the 10% formic acid decalcified bone sample in group B achieved better staining quality than those in groups A and C (Figure 1A). Upon H&E staining, decalcified rat calvarial bone revealed a dynamic microcosm of interconnected structures. Osteocytes, nestled within lacunae, punctuate the bone matrix. Trabecular and cortical bone

regions showcase distinct densities and arrangements, with trabecular bone forming a latticed network and cortical bone manifesting as denser outer layers. Interspersed blood vessels, vital for nourishment, course through the tissue, appearing as channels within the bone (Figure 4A).

Upon RGB staining, red coloured region could potentially highlight osteoids and areas of active bone formation, illustrating regions rich in collagenous matrix. The green hue emphasizes the matrix, and blue could represent areas with a high density of blood vessels, indicating regions of vascularization and nutrient supply within the bone tissue (Figure 4B). Conversely, the significant degradation of samples resulting from the use of 20% formic acid for decalcification notably compromised the quality of histological sections during staining, visibly impacting both standard staining (H&E) and specialized staining (RGB Trichrome) (Figures 4C and D). Statistical analysis of the collected data emphasized the significance of the 10% formic acid concentration, establishing it as the optimal condition for the efficient and effective decalcification of rat calvarial bone samples among the tested concentrations (5%, 10%, and 15%).

4 Discussion

Decalcification is performed in bone healing studies to make the bone tissue transparent or softer for better visualization and analysis under a microscope (El Khassawna et al. 2017). Bones naturally contain calcium salts, which make them hard and opaque, hindering the examination of cellular structures and detailed analysis of the healing process (Barrère et al. 2006). During decalcification, chemicals like ethylenediaminetetraacetic acid (EDTA) or hydrochloric acid remove calcium salts from the bone tissue. This process softens the bone and allows researchers to study the cellular and structural changes during bone healing, such as the formation of new bone tissue, blood vessels, and the behaviour of different cell types involved in the healing process (Choi et al. 2015). This helps in understanding the mechanisms of bone repair and aids in developing better treatments for bone injuries and diseases.

Common agents used for bone decalcification include EDTA, hydrochloric acid (HCl), formic acid, nitric acid, etc. (Khangura et al. 2021). EDTA is a widely used chelating agent that binds calcium ions, effectively decalcifying bones while preserving tissue structures for histological analysis. It is gentle, preserving antigenicity for immunohistochemistry and enabling a wide range of stains. However, it might prolong the decalcification process (several weeks) and can cause tissue shrinkage (Chow et al. 2018). Strong acids like HCl offer quicker decalcification but can damage tissue structures, impacting the quality of histological sections (Lindner et al. 2020). Formic acid is faster than EDTA and less damaging than HCl but might compromise some stains (Bogoevski et al. 2019). Each agent has its advantages and limitations, demanding a balance between speed, preservation of tissue structures, and the desired analyses in bone decalcification for research or clinical purposes. The speed of decalcification and the impact of decalcifying agents on tissue and staining properties are crucial factors that affect the choice of decalcification solutions (Sanjai et al. 2012). Faster agents such as nitric acid can damage tissue, affecting staining techniques. Hence, it is crucial to limit the exposure of tissue to decalcifying solutions as much as possible (Bancroft and Gamble 2008).

Several factors can influence the decalcification process in bone tissue studies. The primary factors include the choice of decalcification agent, the size and thickness of the bone specimen, temperature, pH of the decalcifying solution, and agitation (Dey 2023). The type of decalcification agent used significantly affects the rate and quality of decalcification. Stronger acids work faster but can damage tissue structures, while milder agents like EDTA preserve tissue integrity better but may take longer (Khangura et al. 2021; Chow et al. 2018; Lindner et al. 2020). The size and thickness of the bone specimen impact decalcification time. Smaller and thinner sections tend to decalcify faster due to increased surface area exposure to the decalcifying solution (Chow et al. 2018). Temperature plays a role as higher temperatures can speed up the decalcification process but may also degrade tissue structures or antigens of interest. Maintaining the appropriate pH of the decalcifying solution is crucial for effective decalcification. pH extremes can affect the process and the quality of subsequent histological analysis (Kapila et al. 2015). Agitation or stirring of the decalcifying solution can enhance the process by ensuring a consistent distribution of the decalcifying agent around the bone specimen, thereby speeding up the decalcification process (Dey 2023).

Monitoring the endpoint of bone decalcification is critical to prevent tissue damage or inadequate decalcification. To ensure optimal outcomes, regular visual inspections under a microscope track changes in tissue transparency and texture (Skinner 2003). Periodic X-rays assess the reduction in bone radiopacity as mineral content decreases. Chemical tests, such as calcium detection assays, gauge residual calcium levels in the solution, guiding the decalcification endpoint. Additionally, monitoring tissue hardness through gentle probing helps assess the progression of decalcification. These combined approaches allow adjustments in decalcification duration, solution concentration, or agent choice, ensuring precise control and stopping at the ideal endpoint for subsequent histological analysis while preserving tissue integrity (Dey 2023).

This study established a standardized decalcification protocol for rat calvarial bone histology using formic acid-formalin solutions. Among the tested concentrations, 10% formic acid emerged as the

most effective, achieving thorough mineral content removal within 8 days while preserving the specimen's structural integrity and texture. This concentration consistently demonstrated the desired endpoint, as confirmed by daily chemical analyses, calcium oxalate tests, and radiographic imaging. Histological analyses, including standard and specialized staining, further supported the efficacy of the 10% formic acid concentration in maintaining tissue integrity without compromising sample structure. Statistical analysis underscored the significance of this concentration, establishing it as the optimal condition for efficient and effective decalcification. However, further study should investigate the impact of the 10% formic acid concentration on specific cellular or molecular markers related to bone healing or pathology.

Conclusion

The study effectively standardized a decalcification protocol for rat calvarial bone using formic acid-formalin solutions, with 10% formic acid proving the most effective, ensuring comprehensive mineral removal within eight days while preserving tissue integrity. This standardized protocol offers a reliable method for researchers studying rat calvarial bone histology, ensuring consistent and high-quality results. Additional research investigating its applicability to other bone types or species may broaden its scope and utility in diverse research endeavours.

Ethical approval

Not applicable.

Data statement

The authors confirm that the data supporting the findings of this study are available within the article.

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Declaration of Interest

All authors declare that no commercial or financial relationships exist that could, in any way, lead to a potential conflict of interest.

Authors' contribution

All authors listed have made a substantial, direct, and intellectual contribution to the workand approved it for publication.

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