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ORIGINAL ARTICLE

Effect of Formic Acid-Formalin Concentration on Decalcification Rate and Histological Quality of Bone Tissue in Northern, Tanzania

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ABSTRACT

Background: Decalcification is essential for processing and microscopic evaluation of bone tissue. Formic acid–formalin (FAF) is commonly used in Tanzania, but the optimal concentration for balancing decalcification speed, ease of sectioning, and histological quality has not been adequately established.

Objective: To compare the quality of H&E-stained bone sections following decalcification with 10%, 15%, and 20% FAF at the Pathology Laboratory of Kilimanjaro Christian Medical Centre (KCMC), Northern Tanzania.

Methods: A cross-sectional study was conducted from April to June 2021 using 80 bone specimens, comprising 40 archived patient specimens and 40 cadaveric specimens. Each specimen was divided into three pieces, producing 240 tissue pieces that were randomly allocated to 10%, 15%, and 20% FAF. Decalcification time, ease of sectioning, and microscopic quality of H&E-stained sections were assessed. Data were analysed using SPSS version 20.

Results: Decalcification was fastest with 20% FAF (42.0 days), followed by 15% FAF (51.2 days) and 10% FAF (56.3 days). Sectioning was difficult for 78.8% of pieces decalcified with 10% FAF, moderate for 75.0% with 15% FAF, and easy for 82.5% with 20% FAF. Good microscopic quality was observed in 61.3% (49/80) of pieces decalcified with 20% FAF, while more than half of the pieces treated with 10% and 15% FAF had satisfactory microscopic quality.

Conclusion: Among the concentrations evaluated, 20% FAF provided the fastest decalcification, easier sectioning, and better histological quality. The findings support the use of 20% FAF for routine bone decalcification in pathology laboratories in Tanzania.

BACKGROUND

Decalcification is an essential step routinely performed for pathological observation of bone and bone-containing tissue.[1] Problems arise during tissue sectioning and processing because of the mineral content in a densely packed organic extracellular matrix structure consisting of both collagenous materials.[2] Tissue decalcification is the process of removing inorganic calcium from the organic collagen matrix, calcified cartilage, and surrounding tissues to obtain satisfactory paraffin sections of bone.[3] Bone has two phases: organic and inorganic.[4] The organic phase of bone consists of blood vessels and bone-specific proteins, which allow bone to interact with its surrounding environment. The inorganic phase of bone is the mineralized part of bone, which is made up of hydroxyapatite.[5]

Calcium and phosphate ions are part of the hydroxyapatite molecule that forms the crystalline structure of bone. Calcium is one of the key components giving a bone its mechanical properties and crystalline matrix.[5] To perform paraffin histology on bone, it is vital to remove the calcium from the hydroxyapatite matrix so that the bone can be sectioned by microtomy.[6]

Decalcification is generally carried out by chemical agents, either with inorganic acids to form soluble calcium salts or chelating agents that bind to calcium ions.[7] Tissue is submerged in a decalcification solution that causes calcium ions to dissolve into the solution. Mineral acids (nitric and hydrochloric acid) and organic acids FAF use hydrogen ions to break the bond between calcium ions and hydroxyapatite. This then causes the dissolution of hydroxyapatite into calcium ions that form salt and allows inorganic phosphate to become soluble, which is shown in the following equation.[8]

$$Ca_{(10)}(PO4)_6(OH)_{2(s)} + 8H^+_{(aq)} = 10Ca^{2+}_{(aq)} + 6HPO_4^{2-}{}_{(aq)} + 2H_2O_{(l)}$$

The selection of an appropriate decalcification solution in pathology laboratories depends on several factors,

such as the preservation of cellularity, urgency of the specimen requiring decalcification, integrity of the soft tissue adjacent to the bone, and financial costs.[9] The ideal decalcification solution has to rapidly decalcify the bone while not interfering with the gross tissue morphology.[1] Currently, there is an intensive search for decalcifying solutions with a profile close to ideal, i.e. a decalcifier that does not damage cell structures or their components or genetic material and that preserves tissue antigenicity.[10] For this reason, the importance of preserving tissue quality for histological analysis has resulted in the vast majority of solutions based on 1 of the 4 most commonly used acids in decreasing order of strength: nitric, hydrochloric, formic and Ethylenediaminetetraacetic acid (EDTA)[1] The concentration and volume of the active reagent, including the temperature at which the reaction takes place, are always important. Other factors that contribute to how fast bone decalcifies are the age of the patient, type of bone, size of specimen, and solution agitation.[3]

Although 10% formic acid, formalin, and 5% nitric acid are the decalcifying agents most commonly used in pathology laboratories in Tanzania, their use is largely based on routine laboratory practice rather than locally generated scientific evidence. To date, there is limited comparative research evaluating the effects of these commonly used concentrations on decalcification time and the preservation of histological tissue quality under local laboratory conditions. This lack of evidence creates uncertainty regarding whether the current practice provides the optimal balance between rapid decalcification and high-quality histological outcomes. This represents an important gap, since the choice of decalcification protocols in local laboratories continues to rely on convention and the specific bone type or intended examination, rather than on concentration-specific evidence generated locally.

Therefore, this study aimed to determine the effect of formic acid–formalin concentration (10%, 15%, and 20%) on the rate of decalcification and the histological quality of bone tissue, to inform an evidence-based, optimal decalcification protocol for routine laboratory practice.

METHODS

Study Population and Study Area

The study population comprised bone specimens extracted from archived tissue at the pathology laboratory of KCMC Hospital, and cadaveric bone specimens of the upper and lower limbs from the KCMC University Anatomy Laboratory, which handles specimens from approximately 50 cadavers annually for teaching and research purposes. The study was conducted at the Pathology Laboratory of KCMC Hospital in Kilimanjaro, Northern Tanzania.

Inclusion Criteria

Bone specimens with at least 5 mm thickness obtained from patients and from cadavers, and archived bone specimens extracted from patients and cadavers.

Exclusion Criteria

Inadequately fixed bone specimens and bones from patients diagnosed with osteomalacia or osteoporosis

Study Design

A cross-sectional study was conducted at the Pathology Laboratory of KCMC Hospital in Kilimanjaro, Northern Tanzania, from April to June 2021. This design was selected because of the short time available for the study. Specimen collection, including retrieval of archived patient bone specimens from the KCMC Pathology Laboratory and cadaveric bone specimens from the KCMC University Anatomy Laboratory, was undertaken during this period, after which decalcification, tissue processing, and staining were carried out and monitored until completion for each concentration group.

Data Collection Methods

Data were collected through direct observation, recording the time required for complete decalcification, the ease of tissue sectioning, and the microscopic quality of H&E-stained bone sections following decalcification with 10%, 15%, and 20% formic acid–formalin (FAF).

Study Procedures

All laboratory procedures, including specimen processing, decalcification, tissue preparation, and H&E staining, were performed by one laboratory scientist who also assessed the ease of sectioning during microtomy. The resulting tissue sections were subsequently examined microscopically by a pathologist in the Pathology Department at KCMC Hospital, who assessed the histological quality of the stained tissue sections.

Sample Collection

A total of 80 bone specimens were obtained, 40 cadaveric upper (20) and lower limb (20) bone specimens from the KCMC University Anatomy Laboratory, cut into small fragments (3–5 mm) using a hacksaw and hammer, and 40 archived remnant bone specimens from patients at the KCMC Pathology Laboratory, similarly cut into small fragments (3–5 mm). All fragments were kept in 10% neutral buffered formalin (NBF) and transported to the pathology laboratory. [9,11]

Fixation and Grossing

All 80 bone specimens were fixed in 10% NBF, with a fixative volume of20 times the volume of the bone specimens [12]. Then washed in water for 30 minutes before decalcification. Fixation protects the cellular and fibrous elements of bone from damage caused by the acids used as decalcifying agents. Each bone tissue was grossed into three pieces of equal thickness (5mm), yielding a total of 240 tissue pieces and each piece was placed into a separate individual labelled tissue cassette, recording the sample number and the concentration of the decalcifying agent to which that piece was allocated (80 pieces per concentration group 10%,15% and 20% FAF) of the sample and the concentration of the decalcifying agent that was used to decalcify the bone tissue in the particular tissue cassette.

Decalcification

Bone tissue specimens in cassettes were decalcified in 1000ml of 10%, 15%, and 20% FAF in separate containers. The volume of decalcifying fluid was at least 20 times that of the specimen.[13] The starting time for decalcification was noted, and the room temperature

was recorded regularly. The decalcifying solutions were changed every three days[14] to prevent saturation of the decalcifying solutions with calcium, which may reduce the rate of removing calcium from the bone tissue. The endpoint of decalcification was determined daily by both physical methods, such as gentle bending of the bone, and chemical methods. On completion of decalcification in acid, the specimens were rinsed in running tap water for 4 hrs before histological processing.

Tissue Processing, Embedding, and Microtomy

Bone specimens were processed in a carousel tissue processor by dehydrating through graded ethanol solutions, clearing in xylene, and infiltrating with paraffin wax. After processing, specimens were embedded in paraffin wax blocks using a modular paraffin embedding centre. Embedded paraffin blocks were sectioned at a thickness of 5 µm using a manual precision microtome with a microtome blade and collected onto poly-l-lysine microscope slides and oven-dried at 60°C for 20 min. The steps for tissue processing, paraffin embedding, and microtomy are instructed in standard operating procedures (SOPs).

Staining and Mounting

Each paraffin tissue section was stained with H&E, and the specimens were dewaxed and rehydrated through xylene and graded ethanol, respectively. Bone tissue and cellular morphology were analyzed by H&E; hematoxylin (H) stains for cell nuclei, and Eosin (E) was used to stain cellular cytoplasm as instructed by SOPs. The stained slides were mounted using Dibutyl phthalate Polystyrene Xylene (DPX) mountant prior to histological evaluation using a light microscope.

Data Analysis

Double data entry, cleaning, and analysis were performed using SPSS version 20 (IBM Corp., Armonk, NY, USA), and results were presented in tables and graphs. Descriptive statistics were used to summarize data on the quality of microscopic tissue sections, with categorical variables summarized as frequencies and percentages, and numerical variables summarized as mean (standard deviation).

Ethical Consideration

Ethical approval for this study was obtained from the Kilimanjaro Christian Medical University College Research Ethics and Review Committee (Certificate No. UG 01/2021) before the beginning of the study. Archived and cadaveric specimens were handled in accordance with institutional policy for research use of such material, and patient identifiers were not used at any stage of the study.

RESULTS

Bone Specimen Characteristics

A total of 80 bone specimens were obtained: 40 cadaveric upper (20) and lower limb (20) bone specimens from the KCMC University Anatomy Laboratory, cut into small fragments (3–5 mm) using a hacksaw and hammer, and 40 archived remnant bone specimens from patients at the KCMC Pathology Laboratory.

Time Taken to Complete Decalcification

Comparing the time taken to complete decalcification of the 10%, 15%, and 20% FAF decalcifying agents (Table 1), decalcification was achieved within an average of 42.0 days by 20% FAF; whereas decalcification was achieved within an average of 56.3 days by 10% FAF.

Ease of Tissue Sectioning

The results for easiness of tissue sectioning obtained were observed in the following order for ease of cutting: 10%<15%<20% FAF, whereby 78.8 % of all samples that were decalcified in 10% FAF were categorized as difficult, 75% of all samples that were decalcified in 15% FAF as moderate and 82.5% of all samples that were decalcified in 20% FAF as easy during tissue sectioning. Table 2 and Figure 1 show grades of tissue sectioning.

Quality of microscopic tissue sections after H & E staining

The majority of bone specimen samples, 49(61.3%), decalcified using 20% FAF had good microscopic section quality, whereas over half of the samples decalcified by 10% and 15% FAF had satisfactory microscopic qualities. Table 3 and Figure 2a, 2b & 2c show the quality of microscopic sections after H & E staining.

FIGURE 1: Comparison of Ease of Tissue Sectioning with Concentration of Formic Acid-formalin

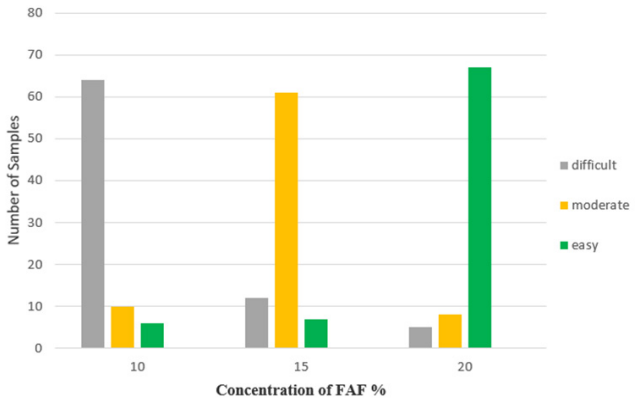


FIGURE 2a: Histologic Section of Bone Tissue Demonstrating A Good-Quality Section Decalcified by 20% FAF (H&E stain, ×200)

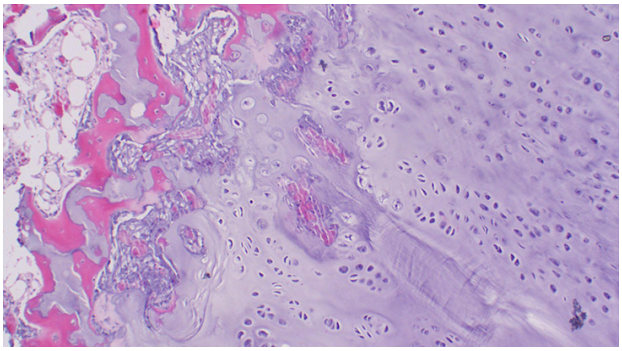


FIGURE 2b: Histologic Section of Bone Tissue Demonstrating a Satisfactory Quality Section Decalcified by 15% FAF (H&E stain, ×200)

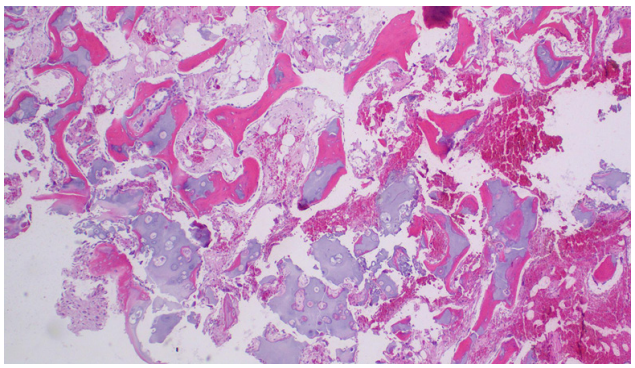


FIGURE 2c: Histologic Section of Bone Tissue Demonstrating a Bad-quality Section Decalcified by 10% FAF (H&E stain, ×200)

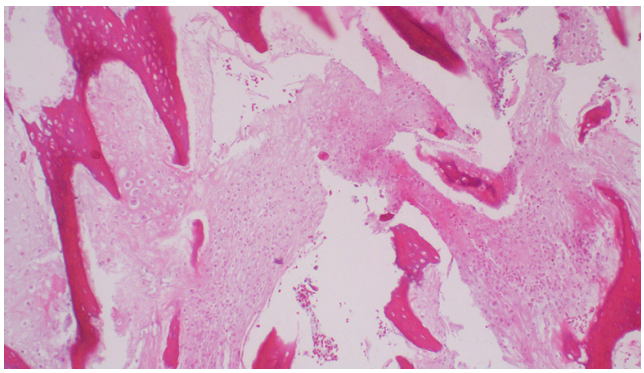


TABLE 1: Time (in Days) Taken to Complete Bone Decalcification (N=240)

Concentration of FAF	n	Mean Time Taken (Days)	SD	SE
10%	80	56.3	1.9	0.216
15%	80	51.2	2.2	0.245
20%	80	42.0	1.9	0.220

TABLE 2: Assessing the Ease of Bone Tissue Sectioning with Increasing Formic Acid-Formalin Concentration (N=240)

	Concentration of Formic Acid-Formalin						Total	
	10%		15%		20%			
Easiness of tissue sectioning	n	%	n	%	n	%	n	%
Easy	7	8.8	8	10	66	82.5	81	33.75
Moderate	10	12.5	60	75	8	10.0	78	32.5
Difficult	63	78.8	12	15	6	7.5	81	33.75
Total	80	100	80	100	80	100	240	100

TABLE 3: The Quality of Microscopic H&E-Stained Tissue Sections Decalcified Using 10%, 15%, and 20% FAF

Quality of H&E Staining	Concentration of FAF 10% n (%)	15% n (%)	20% n %)
Bad	16(20.0)	7(8.8)	5(6.3)
Satisfactory	43(53.8)	45(56.3)	26(32.5)
Good	21(26.3)	28(35.0)	49(61.3)
Total	80(100)	80(100)	80(100)

Abbreviation key: FAF, Formic Acid-Formalin; H&E, hematoxylin and eosin

DISCUSSION

Decalcification time is critical for timely diagnosis and treatment planning. In this study, bone samples decalcified in 20% FAF, the fastest concentration, took an average of 42 days to complete decalcification. This is comparable to findings by Abrantes et al [9], who reported an average of 40 days for rat femur specimens. The time taken to complete decalcification using 20% FAF in this study was longer than that reported by Kapila et al[13], who achieved decalcification in 11 days for tooth samples; this difference may reflect both the nature of the specimen and the more sophisticated decalcifying methods used in that study, which involved heating and achieved rapid decalcification despite the high mineral content of teeth. Samples decalcified in 10% FAF, the slowest concentration in this study, took an average of 56.3 days, considerably longer than the 18 days reported by Hajihoseini et al[15] for sheep mandibular bones. This difference likely reflects the use of hard, highly calcified human bone in the present study compared with sheep mandibular bone.

Striking the right balance between decalcification speed and histological quality is essential for accurate and timely diagnosis. In this study, 20% FAF produced the best histological results, while 10% FAF produced the worst, likely because the prolonged decalcification time required with 10% FAF adversely affects subsequent H&E staining[13]. These findings are consistent with those of Bhat et al [16], and Hajihoseini et al[15] who similarly found that 10% formic acid produced the poorest H&E staining results. Maloth et al [17],likewise reported that stronger FAF concentrations, and combinations of formic acid with other strong acids, achieve superior histological outcomes.

These findings have direct clinical relevance for laboratories in Tanzania and similar low-resource settings. Faster decalcification with preserved histological quality shortens the turnaround time for bone pathology results, which is important for the timely diagnosis of bone lesions and metabolic bone disease, and for supporting immediate clinical decision-making. Adoption of 20% FAF in place of the currently more widely used 10% concentration could therefore reduce diagnostic delay in laboratories with a caseload and equipment comparable to KCMC's.

Study Strength and Limitation

To our knowledge, this is the first study in Tanzania to comparatively evaluate the effects of three concentrations of formic acid–formalin (FAF) (10%, 15%, and 20%) on both decalcification time and the quality of H&E-stained bone tissue sections. Another strength of the study is the relatively large sample of 80 bone specimens obtained from cadavers and patients, which allowed comparison of the performance of the different FAF concentrations under the study conditions.

The study was limited by the lack of demographic and specimen-related information for the bone samples, including sex, age, weight, duration of storage, and specimen size. Consequently, it was not possible to determine whether variations in these characteristics influenced the decalcification process, histological quality, or contributed to variability in the study findings. Therefore, the results should be interpreted in consideration of these methodological and specimen-related limitations.

CONCLUSION

The findings of this study indicate that 20% formic acid formalin (FAF) is the most effective decalcifying agent, providing an optimal balance between decalcification efficiency, tissue sectioning ease, and microscopic quality. Given the routine need for bone decalcification in histopathological diagnostics, 20% FAF is recommended as the optimal concentration for use in standard laboratory practice. The selection of an appropriate decalcifying agent must strike a balance between decalcification speed and the preservation of histomorphological integrity to ensure diagnostic accuracy.

Recommendations

Based on the findings of this study, we recommend the use of 20% formic acid–formalin (FAF) as a decalcifying agent in routine histopathology, with prolonged rinsing in water for at least 4 hours following decalcification to improve tissue integrity and H&E staining quality. Future studies should include larger sample sizes and investigate optimization strategies, such as mechanical agitation using a magnetic stirrer, to determine whether these approaches can further accelerate decalcification while preserving tissue architecture and staining quality.

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