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TECHNICAL NOTE

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Frozen Human Bone: A Microscopic Investigation

ABSTRACT: The taphonomic effects of prolonged extreme cold and freezing on human bone have received little research attention. Questions of specific interest include whether previously frozen bone can be identified and whether freezing alters the structural integrity enough to prevent histological aging. There is no evidence from previous studies that freezing damages the structural integrity, and to date no research investigating the freezing process on bone microstructure has been undertaken. This research attempts to distinguish histologically previously frozen bone from nonfrozen bone by identifying patterned defects. To determine the effects of freezing in bone microstructure using light and scanning electron microscopy (SEM), several human bone sections were subjected to prolonged freezing and allowed to thaw before thin sectioning. Light microscopy failed to demonstrate statistically significant differences between frozen and nonfrozen specimens. SEM analysis revealed fractures, although these lacked pattern and did not occur systematically throughout the section. Evidence of microstructural changes caused by liquid expansion, however, was remarkable but did not alter the structural integrity of the microstructure. The results of this study suggest that freezing does not alter the process of histomorphological analysis.

KEYWORDS: forensic science, forensic anthropology, frozen human bone, bone histology, scanning electron microscope

Much research has focused on the fate of human bone when exposed to various external stressors, including taphonomic processes such as weathering, decomposition, and burning (1–6). These results may afford investigators the opportunity to analyze crime scene evidence in order to draw more accurate conclusions about perimortem events.

One taphonomic process receiving little attention is whether previous freezing can be ascertained in bone microstructure. Pertinent medicolegal questions include whether the decedent was frozen before removal from the crime scene and deposited at another location allowing the body to thaw and then decompose. Is it possible to determine histologically whether or not the remains have been frozen and thawed? Or does freezing damage histological integrity to an extent that impairs histological age assessment?

Few studies have analyzed the effects of freezing temperatures on human remains. These include detailed descriptions of soft tissue decomposition after a freeze–thaw event (7–9), none focused on bone. There is no previous research showing that freezing alters either the gross morphology or microscopic structure of the bone. This research will determine whether previously frozen bone would be histologically distinguishable by searching for patterned destruction (i.e., cracking, peeling, shrinkage, or expansion).

Microstructural analysis of frozen bone may identify change due to the expansion of moisture from blood vessels that permeate the bone to allow for communication and nutrient flow between bone cells. Freezing results in fluid expansion and may possibly force an increase in vessel diameter. This may be microscopically evident in a section of frozen bone as small fractures.

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Materials and Methods

Eleven human limb samples were obtained from surgical procedures at the University of Tennessee Medical Center (Table 1). Two of the limbs included all three lower limb elements (femur, tibia, and fibula). Eight of the limbs included only the tibia and fibula and one limb included only the femur. This resulted in the following totals: $N_{\text{Femur}} = 3$; $N_{\text{Tibia}} = 10$; and $N_{\text{Fibula}} = 10$. The researcher was blind to the age sex and ancestry of each of the individuals. From each sample, a large segment of each long bone midshaft (femur, tibia, or fibula) was obtained, and this segment was split into three equal segments measuring approximately 3–5 cm in length. This bone was removed from the muscle and skin covering without damage to the periosteum, endosteum, or marrow. These precautions would allow for a more realistic facsimile for freezing the entire limb with the marrow cavity intact.

TABLE 1—*Description of the specimens.*

Specimen	Side	Bones	Segments per bone
1	Right	Tibia, fibula, and femur	A—control; B, C—frozen
2	Right	Tibia and fibula	A—control; B, C—frozen
3	Left	Tibia, fibula, and femur	A—control; B, C—frozen
4	Right	Tibia and fibula	A—control; B, C—frozen
5	Left	Tibia and fibula	A—control; B, C—frozen
6	Left	Tibia and fibula	A—control; B, C—frozen
7	Right	Tibia and fibula	A—control; B, C—frozen
8	Left	Tibia and fibula	A—control; B, C—frozen
9	Right	Tibia and fibula	A—control; B, C—frozen
10	Left	Femur	A—control; B, C—frozen
11	Left	Tibia and fibula	A—control; B, C—frozen

Totals: 10 tibiae (30 segments), 10 fibulae (30 segments), and three femora (nine segments).

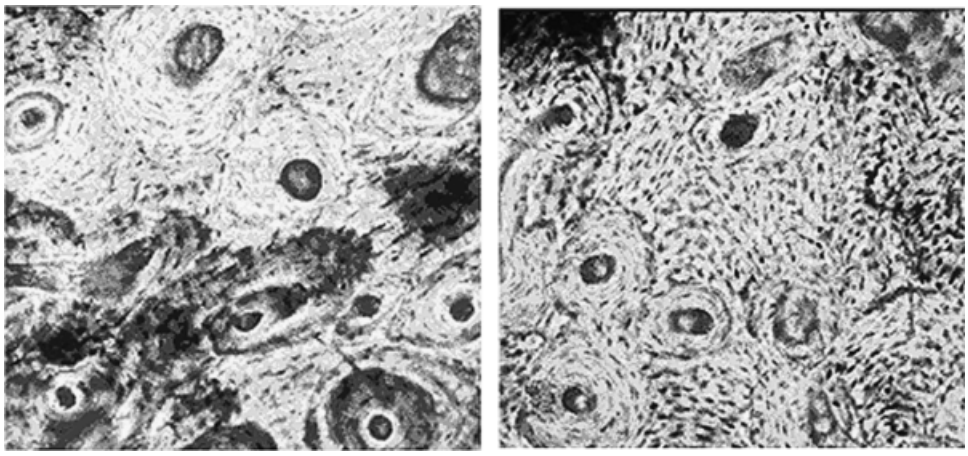

FIG. 1—*Microscopic view of nonfrozen human tibia (A) at ×100 magnification (right) and frozen human tibia (C) at ×100 magnification (left).*

Each bone segment was placed in a separate re-sealable plastic bag. For each sample, the segment denoted "A" for each bone was reserved as a control. The control segments were cleaned and analyzed in the same manner as the test groups but were not subject to other temperature variables. The remaining two segments designated bone "B" and "C" were placed in a Kenmore freezer at 0°C for 21 days to mimic an actual crime scene occurrence where an individual was frozen for 21 days and then removed from the freezer and placed in an outdoor area that had previously been searched.

Cleaning of the Sections

After freezing, each segment was subsequently cleaned by hand using water (without heat or detergent) and scalpel blades. This prevented any structural distortion due to heat or chemicals. The periosteum was removed and the inner marrow was flushed from the medullary cavity with a stream of cold running water. After cleaning, the samples were placed on absorbent paper to dry at room temperature.

Microstructure Analysis

The "A" and "C" samples were examined using light microscopy after thin sections were made using a Buehler Isomet 1000 high-concentration diamond blade (Buehler, Lake Bluff, IL). Each 0.6–0.8 mm section was attached to a glass slide and covered with a glass cover slip. Those specimens too small in diameter or too short in length to obtain an adequate thin-section were embedded in Buehler Epo-Thin epoxy resin (Buehler) for thin sectioning.

The slides were analyzed with an Olympus BX50 microscope (Olympus, Center Valley, PA) with image capture on an Olympus 35 mm camera with a Fugi color slide film and digitized using a Nikon Cool Scan 4000 slide scanner (Nikon, Melville, NY). In

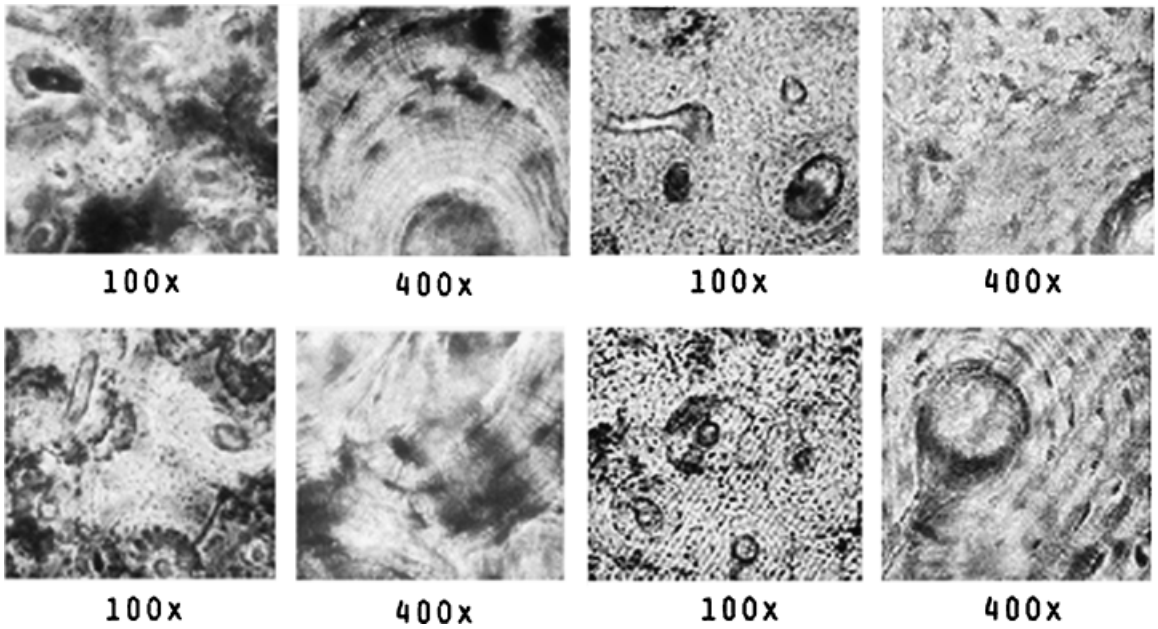

FIG. 2—*Photographic comparison of specimen #2 fibula nonfrozen (left, top) and frozen (left, Bottom) and specimen #2 tibia nonfrozen (right, top) and frozen (right, bottom) at ×100 and ×400 magnifications using light microscopy. These images show virtually no microstructural differences between the nonfrozen and frozen specimen of the same skeletal element.*

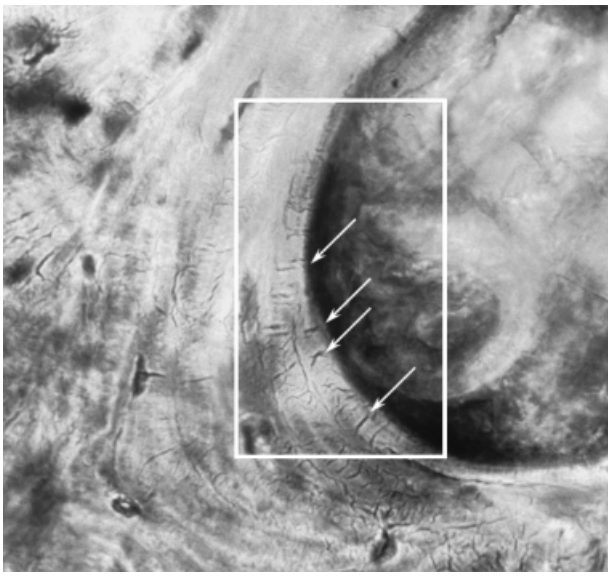

FIG. 3—*Specimen 3C fibula (frozen) under a light microscope (×400). The arrows point to cracks radiating from a Haversian canal.*

order to obtain base information about that particular sample, the control group was analyzed first. While consecutive thin sections are not identical, there is value in using a control from the same bone to orient the observer to particular nuances that may appear throughout the bone structure, such as general osteon size and approximate extent of secondary osteon population (Figs. 1 and 2). The control section was also used for comparison with the test sample to determine whether there were any major structural changes or evidence of microstructure deformation between the two.

Each specimen was examined at ×10, ×40, and ×200. A still image of each section was captured at ×10 and ×40 using the Olympus 35 mm camera (Olympus). These photographic slides were digitally scanned using a Nikon Cool Scan 4000 slide scanner. The images of sections "A" and "C" for all samples were compared.

The "B" sections along with the portions of the "A" sections that were left after thin sectioning were analyzed using the LEO 1525 Scanning Electron Microscope (LEO, Germany) at the University of Tennessee Department of Geological Science. For

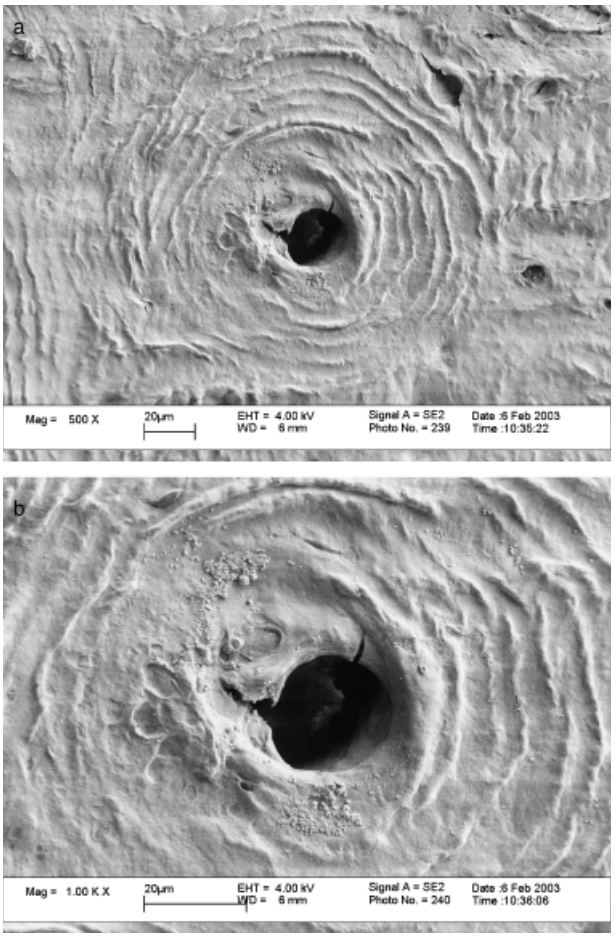

FIG. 5—*(a) Scanning electron microscosy image of 1C (frozen) fibula at ×500 magnification showing cracks originating within the Haversian system (b) SEM image of the same Haversian system as in (a) under ×1000 magnification.*

preparation, the samples were etched using nitric acid and placed in a vacuum dessicator for 96 h. Finally, the samples were dusted with 18 nm of gold palladium for scanning electron microscopy (SEM) analysis. Digital images of each sample were analyzed at magnifications between ×26 and ×2000.

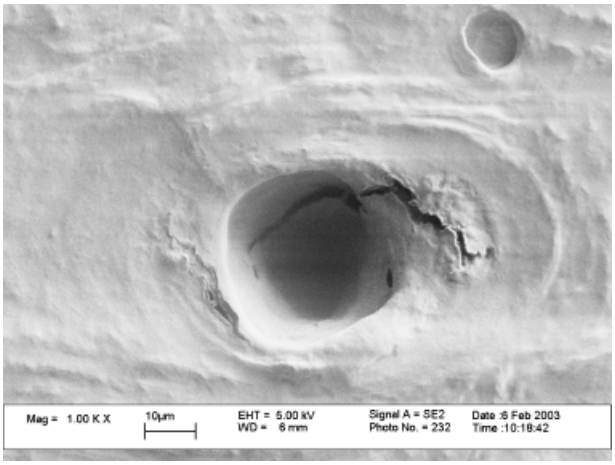

FIG. 4—*Scanning electron microscopy image of 1C (frozen) femur at ×1000 magnification showing a crack originating within the Haversian system.*

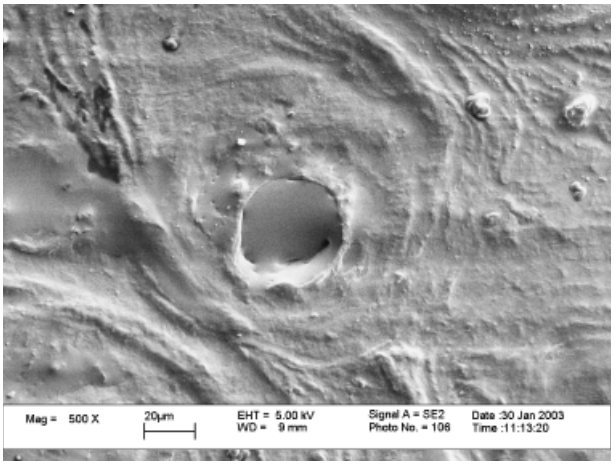

FIG. 6—*Scanning electron microscopy image of 3A femur (nonfrozen) at ×500 magnification showing no morphological variation.*

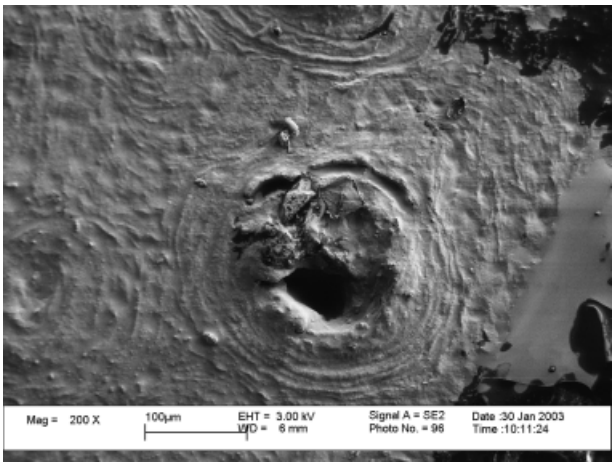

FIG. 7—*Scanning electron mircoscopy image of 5A fibula (nonfrozen) at ×200 magnification showing no morphological variation.*

Histomorphometric Analysis

To determine changes in the subunits of the thin sections, measurements were taken on 30 Haversian canals and 30 lacunae to determine whether the smaller structures were significantly altered due to fluid expansion during freezing. These measurements were compared with the nonfrozen control specimens. To facilitate comparison, a series of statistical tests were utilized to understand significance. This analysis used a *t*-test in SAS (10) to examine whether the difference in the average size of the Haversian canals between nonfrozen and frozen bone was significantly different from zero. *T*-tests were also performed using the average lacunae size between the frozen and nonfrozen bone.

Next, multivariate analysis of variance (MANOVA) was performed on the entire data set to determine whether there was a significant difference between Haversian canals size and lacunae size respectively, due to the treatment (frozen or unfrozen) or due to the type of bone (femur, tibia, or fibula).

Results

No patterned change in microstructure was discovered on any specimens using light microscopy. A few of the frozen sections, however, did exhibit some cracking (Fig. 3) around the center of the Haversian canal not evident on their nonfrozen counterparts.

The SEM images, however, showed cracking (Figs. 4 and 5) originating from Haversian systems in all frozen bones, although not every Haversian system in the sample was affected. This type of cracking was not noted on Haversian systems in controls (Figs. 6 and 7).

TABLE 2—*Results of t-test.*

	Haversian Data		Lacunae Data	
Statistics	Unfrozen	Frozen	Unfrozen	Frozen
N	690	690	690	690
Mean	0.056	0.056	0.014	0.014
Standard deviation	0.017	0.023	0.010	0.011
Standard error	0.000655	0.000860	0.000382	0.000402
DF				
Unequal	1286.9		1374.5	
Equal	1378.0		1378.0	
p-value				
Unequal	0.904		0.519	
Equal	0.904		0.519	

TABLE 3—*Results for MANOVA procedure.*

	Haversian Data		Lacunae Data	
Statistics	Frozen/ Unfrozen	Bone Type	Frozen/ Unfrozen	Bone Type
Degrees of freedom	1	2	1	2
Mean square	0.000006	0.003728	0.000044	0.001111
F-value	0.01	9.36	0.42	10.64
p-value	0.9034	0.0001	0.5158	0.0001

MANOVA, multivariate analysis of variance.

The statistical analyses are summarized in Tables 1–3. As noted by the *t*-test results, there is no significant difference in the size of the Haversian canals or lacunae between frozen and unfrozen bone. The MANOVA procedure concurs that there is no significant difference between frozen and unfrozen bone in terms of Haversian canals and lacunae size. However, the MANOVA procedure indicates that there is a significant difference in the size of Haversian canals and lacunae dependent on the osseous element analyzed. That is, the Haversian canal or lacunae sizes in femur, tibia, and fibula are significantly different from one another.

Discussion

Statistically, light microscopic analysis did not demonstrate significant differences in frozen and nonfrozen human bone. However, SEM analysis shows clear cracks originating from the Haversian systems of frozen sections. While this structural degradation presumably caused by liquid expansion is encouraging, each Haversian canal does not display cracking.

Additional studies should be performed on intact bones and at varying cross-sectional segments of the shaft of the bone. Theoretically, if a complete bone is frozen, it may react in a different manner than a segment, as the internal marrow will have nowhere to expand. The bone was sectioned into 3–5 cm sections to facilitate freezing of a number of specimens at the same time. Because of this process, both the proximal and distal potions of the bone were open and exposed, thus possibly reducing the effects of marrow expansion.

Statistical analyses of the Haversian canal and lacunae demonstrate the lack of significance of the freezing process on the microstructure. The *t*-tests show that the differences between the sizes of frozen and nonfrozen Haversian canals or lacunae were not significantly different from zero across the data set. Surprisingly, the MANOVA also indicated that there is a significant difference between both the Haversian canal sizes and the lacunae sizes based on which element is sampled.

Conclusions

Even though no consistent fracture pattern was noted, this research should not rule out some histologically identifiable changes associated with freezing. Noticeable changes were observed through SEM analysis due to freezing. Statistically, frozen or non-frozen demonstrates no significance in the size of the Haversian canals or lacunae. This indicates that there are no significant changes in the size of Haversian canals and lacunae due to the freezing process, although a clear correlation does occur between both Haversian canal size and lacunae size and the bone analyzed.

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