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An Evaluation of Embalmed Cadaveric Human Tissue in the Investigation of Multiple Freeze and Thaw Cycles on the Histological Morphology of Human Bone

by

Aaron Perkins

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ABSTRACT

Our understanding of the myriad of possible taphonomic agents acting on remains in a forensic context has dramatically increased over the last two decades (Haglund and Sorg, 2002); however, the effects of cold temperature on the microstructure of bone tissue are still not well understood. Recent research on unembalmed human bone has investigated these effects (Tersigni, 2002, 2007). Although tentative, this research has shown that freezing does affect bone tissue at the microscopic level. Considering the potential significance of these findings for forensic evaluation and identification of found human remains, Tersigni's research highlights the need for additional research. The research reported in this thesis provides an important step forward in the development of methodological approaches to the study of temperature effects on human bone.

ACKNOWLEDGMENT

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the strength of your character and your deep patience, and I am very fortunate to have you in my life. Thank you.

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CHAPTER I:

INTRODUCTION and BACKGROUND

The goal of the research reported in this thesis is to evaluate the utility of using human research materials that are more accessible to researchers (i.e. embalmed human bone tissue) than unembalmed, or fresh, human bone. The framework of forensic anthropology was used to examine this topic through a taphonomic experiment that examined the effects of multiple freeze and thaw cycles on the microstructure of embalmed human bone.

Forensic anthropology is the application of osteological knowledge from biological anthropology to address questions in the medical and legal (*medicolegal*) aspects of investigations involving found human remains. The circumstances surrounding these discoveries can relate to the commission of a crime, but often do not. Forensic anthropologists, similar to biological anthropologists, specialize in gleaning information from human skeletal remains. This evaluation can provide estimates of approximate age at time of death, the interpretation of biological sex, and estimates of living stature, as well as detecting and documenting idiosyncratic variation from the skeleton (personal individualization). The evaluation of these estimates and interpretations is used to develop an *osteobiography*, which can provide the foundation for the eventual identification of the remains.

Investigators examining human remains found in medicolegal contexts involving possible criminal acts consider the taphonomic state of the remains in their assessment. *Taphonomy* is the study of the biological, chemical, and cultural treatment of remains around the time of death (perimortem), after death (postmortem), and during recovery and analysis (Sorg and Haglund, 2002). Taphonomic data on found remains are compiled into *taphonomic profiles* (Nawrocki, 1996), which document any aspects or features that may indicate the history of the body's treatment, and this information can be relevant to establishing the existence of a crime. Taphonomy may aid forensic investigators in the determination of the time since death (*postmortem interval*), the characteristics of the original deposition or interment environment, and the differentiation of the features of foul play from those reflecting normal human variation or animal activity (Ubelaker, 1997: 77).

Taphonomy developed within the field of paleontology as an approach to understand the relationship between the burial environment and the preservation of fossil assemblages. Beginning in the 1920s, under paleontologists such as Rudolf Richter and Johannes Weigelt, actualistic research was conducted on modern death assemblages to model the effects of burial conditions on the preservation of biological tissue. The findings were used as a framework for the development of decomposition models and were used to understand better the varied preservation of paleontological depositions (Senckenberg, 2010; Richter, 1928; Weigelt,

1989). Ivan Yefremov (Efremov, 1940) expanded this approach by considering the deceased organism's contribution to the creation and preservation of its death assemblage. Credited with the inception of the field (Ubelaker, 1997: 77), Yefremov proposed the field of taphonomy (Efremov, 1940: 85) to encompass the study of the depositional history of biological remains.

The beginnings of actualistic research studies in forensic taphonomy reflect the initial adoption of the principles of taphonomy into archaeology and anthropology (Sorg and Haglund, 2002; Lyman, 1994). Research was most commonly conducted in hot, arid, or dry environments where historically important forensic facilities were located (Komar, 1998: 57) or where research results would be applicable to both paleoanthropological and forensic questions. As a result, the majority of the initial anthropological taphonomic research was concerned with animal chewing (e.g. Sutcliffe, 1971), butchering and trampling (e.g. Haynes, 1983), and gross macroscopic changes on bone caused by the effects of hot weather and sun exposure (e.g. Brain, 1967; e.g. Behrensmeyer, 1978; e.g. Miller, 1975; Ubelaker, 1997). The macroscopic and microscopic indicators of fire were also extensively studied over the past three decades (Mayne Correia, 1997; Thompson, 2009).

Actualistic studies on the effects of cold temperature on human tissue are sparse, excluding those conducted for medical research on the integrity of previously frozen bone for donation and transplant (Tersigni,

2002). Taphonomic research of cold temperature in the forensic sciences focuses on the different rates of desiccation and decomposition between previously frozen and fresh carcasses (e.g. Micozzi, 1986; e.g. Micozzi, 1991; e.g. Micozzi, 1997; e.g. Perper *et al*., 2006), weathering differences between warmer climates and Northern climates (e.g. Marceau, 2007), and soft tissue alteration (e.g. Zugibe and Costello, 1993; e.g. Schäfer and Kaufmann, 1999; e.g. Baraibar and Schoning, 1985). Research on the alteration of bone histological features caused by cold temperature is limited to Tersigni's Master's thesis research and subsequent technical note (2002 and 2007, respectively), which explore the microscopic damage caused by a single freeze and thaw of human bone. Her results, although preliminary in nature, provide another method to glean data from human skeletons, and illustrate that histological change can occur as a result of freezing bone tissue. The findings are relevant to the medical field, which regularly freezes bone for research and implant therapy (e.g. D'Aloja *et al*., 2001; Goldberg and Stevenson, 1987; Bauer and Muschler, 2000).

In forensic anthropological research it is often difficult to source research materials, particularly fresh human bone. One example of the challenges in sourcing human remains is represented by the University of Tennessee's Forensic Anthropology Center (often referred to as the "Body Farm") where human bodies are used in short or long-term experiments in decomposition and taphonomy (Mann et al., 1990). These relatively rare facilities pose significant challenges to the institutions and individuals

involved due to local through to federal regulations, as well as varying community, cultural, and societal tolerances to decay research (D'Elia, 2009). One result of this reality is that forensic researchers have looked for viable alternatives to using human bodies in decomposition and taphonomy research. Perhaps the most common of these substitutions is the use of adult *Sus scrofa*, domestic pigs, as human analogues, as these animals have internal (gut fauna, bone microstructural features and cortical thickness) and external features (minimal fur, chest cavity size, and fat distribution) similar to adult humans (Schoenly *et al.*, 2006; Marceau, 2007; Martiniaková *et al*., 2007; Urbanová and Novotny, 2005; Cattaneo *et al*., 1999). As a result, pigs have now become an accepted replacement for human bodies in many research projects. In fact, the surprisingly large amount of taphonomic research conducted in the past decade has been greatly enabled by the ability to use pigs for the investigations of many aspects of decomposition and taphonomy that are applicable to the interpretation of found human remains in various forensic contexts.

Taphonomic research conducted with fresh human bone is uncommon. Although there are appropriate sample pools available at various institutions, the most accessible of sample sources are usually individuals who have donated their bodies for teaching and research purposes, and these individuals are embalmed as part of this process. Access to fresh, unembalmed materials that have been donated for research purposes is more difficult to obtain and, when available, the number of

individuals is noted to be smaller than the embalmed pool. Therefore, an evaluation of whether embalmed human bone tissue responds to freezing in the same manner as observed in Tersigni's research on unembalmed human bone, or not, could have a significant impact on the selection of human materials for examination and use in future research. The research reported in the following chapters investigates the extent to which the histological integrity of embalmed cadaveric human bone may be compromised by multiple freeze and thaw cycles in a regulated temperature environment.

The results of the research reported in this thesis will be used to understand better the phenomenon of the cold temperature treatment of bone tissue, with the ultimate goal of providing additional sources of information for forensic investigators. This research tests the applicability of embalmed cadaveric bone as a replacement for fresh bone in cold temperature taphonomy studies through the evaluation of the histology of previously frozen and thawed embalmed cadaveric femora.

CHAPTER II:

REVIEW

Fundamental knowledge of bone microstructure, the role and organization of water in human bones, and the processes of how water freezes under normal atmospheric pressure underlie the hypotheses and methods of this thesis. Bone microstructure is a particularly difficult topic to grasp without a basic review of histological features to orient the reader (Beauchesne and Schoning, 2006). The following chapter establishes a context for the research, including brief discussions of bone development and microstructural features, water's dual form in human bone, ice formation, and previous forensic research of the effects of cold temperature on human tissue. Detailed accounts, descriptions, and definitions of bone development and the physics of ice formation can be found in greater detail in Scheuer *et al.* (2000) and Petrenko and Whitworth (2003), respectively.

Bone Development, Growth, and Histology

Bone tissue is created through either intramembranous ossification or endochondral ossification. Intramembranous ossification is the process associated with the development of the majority of the bones of the skull and flat or irregularly shaped postcranial bones (Scheuer and Black, 2004).

In this model, bone tissue is formed within the *mesenchyme,* an embryonic connective tissue. In contrast, endochondral ossification is the process associated with the formation of the *diaphysis*, or shaft, of a long bone, which is the area of interest in this study.

The formation of a long bone begins when the mesenchymal tissue surrounding a cartilaginous model of a long bone differentiates into a dense, irregular connective tissue called the *perichondrium*. Cartilage is deposited in the inner layer of the perichondrium through a process known as *chondrogenesis,* and the size of the cartilage model increases appositionally as additional cartilage is secreted (Scheuer and Black, 2004). *Osteoblasts*, the cells responsible for the initial stage of bone tissue formation, secrete *osteoid*, an unmineralized bone tissue precursor, around the perichondrium, forming layers of bone around the model. These layers of bone (*lamellae*) form the superficial bone of the shaft. Under these bone layers, the cartilage cells hypertrophy and secrete vascular endothelial cell growth factor, which stimulates the vascularization of the model from the perichondrium and encourages the cartilage cells to undergo apoptosis and calcify (Scheuer and Black, 2004). The blood vessels that vascularize the model deliver hematopoietic and osteoprogenitor cells into the model's centre. The osteoprogenitor cells differentiate into osteoblasts and use the calcified matrix left by the cartilage cells as a foundation to deposit osteoid and form bone trabeculae (*woven bone*). Subsequent remodeling of the woven bone creates the *cortical* (or *compact*) bone of the shaft.

Histological comparisons of cortical bone were used to examine the effects of freezing and thawing during this study. A cross section of a normal histological slide of cortical bone exhibits canals, tunnels, oblong pits, and parallel and circular lines alluding to the dynamic nature of bone formation and lifelong remodeling processes (Figure 1). The histological morphology of cortical bone reflects the numerous factors that influence microstructural organization: disease processes, activity levels, age, sex, atrophy or dystrophy, hormones, and genetics (Frost, 1987; Frost, 1990a). The following pages define and describe key histological terms used in the remainder of this study.

Primary osteons (Figure 2, feature A) have an oval shape with a central canal. Blood vessels trapped by osteoid deposited underneath the periosteum form the initial version of this structure. As the osteoid is remodeled into mature bone, the perivascular space is reduced, and a canal is formed around the vascular channel. Primary osteons are not demarcated from the circumferential lamellae (Figure 2, feature B), which are the surrounding bone layers laid down underneath the periosteum.

Secondary osteons (Figure 2, feature C), also called Haversian systems, are the end product of bone tissue remodeled over the lifetime of an individual (Wolff, 1892; Currey, 1964; Currey, 2003).[1] Mature cortical

[1] The literature is inconsistent in the use of the term "Haversian system." Some authors, most notably Currey (e.g. 2003), stress the exclusive use of the term to refer to secondary osteons. Other authors (e.g. Martini *et al.*, 2003) include primary osteons within the definition of this term. This paper uses the former, more widely used, definition of Currey (2003).

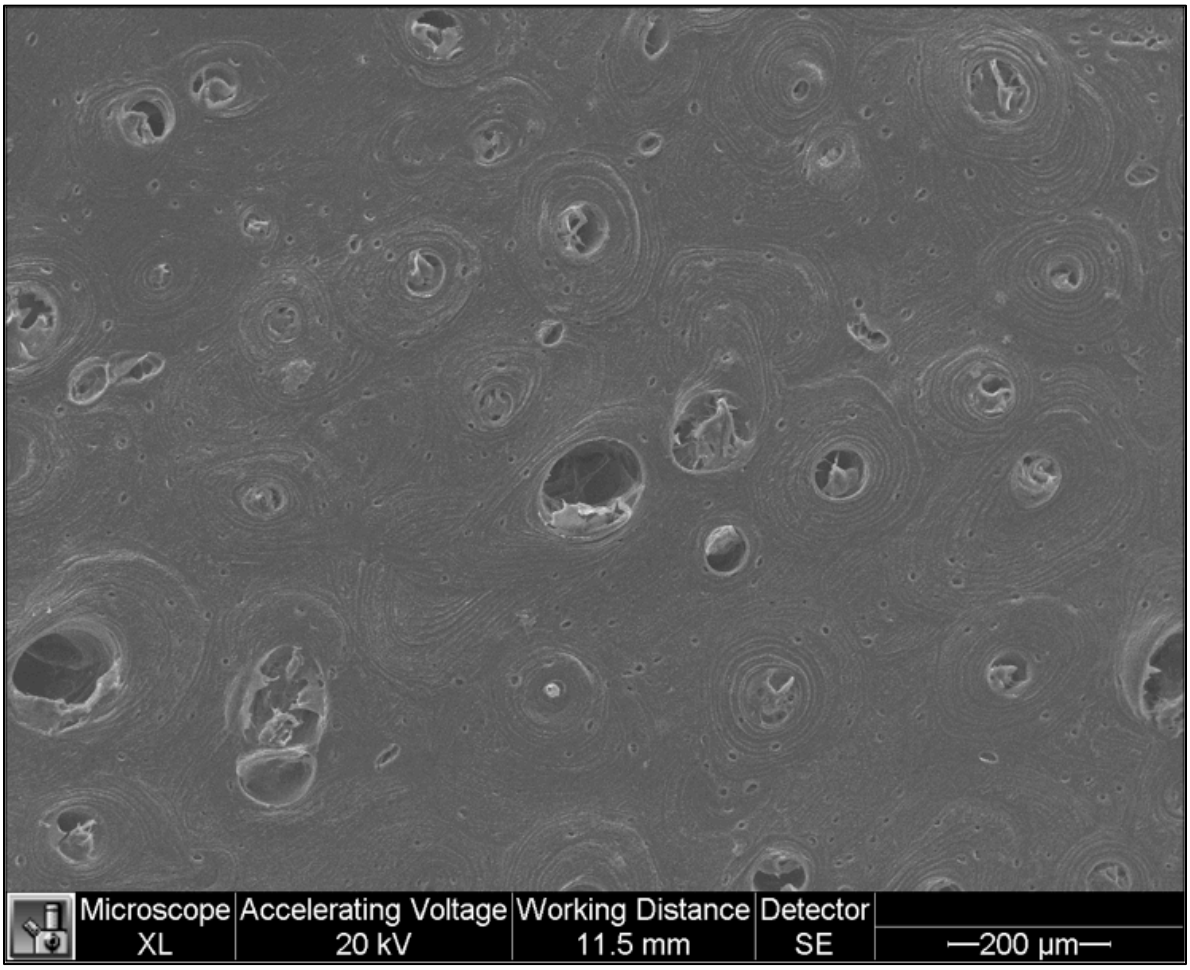

Figure 1: The microstructural organization observed in a transverse slice of human bone. The study of the organization of these structures can elucidate information on the individual including the postmortem history of their remains. (Image source: G(l) from this study. Original magnification: 100x.)

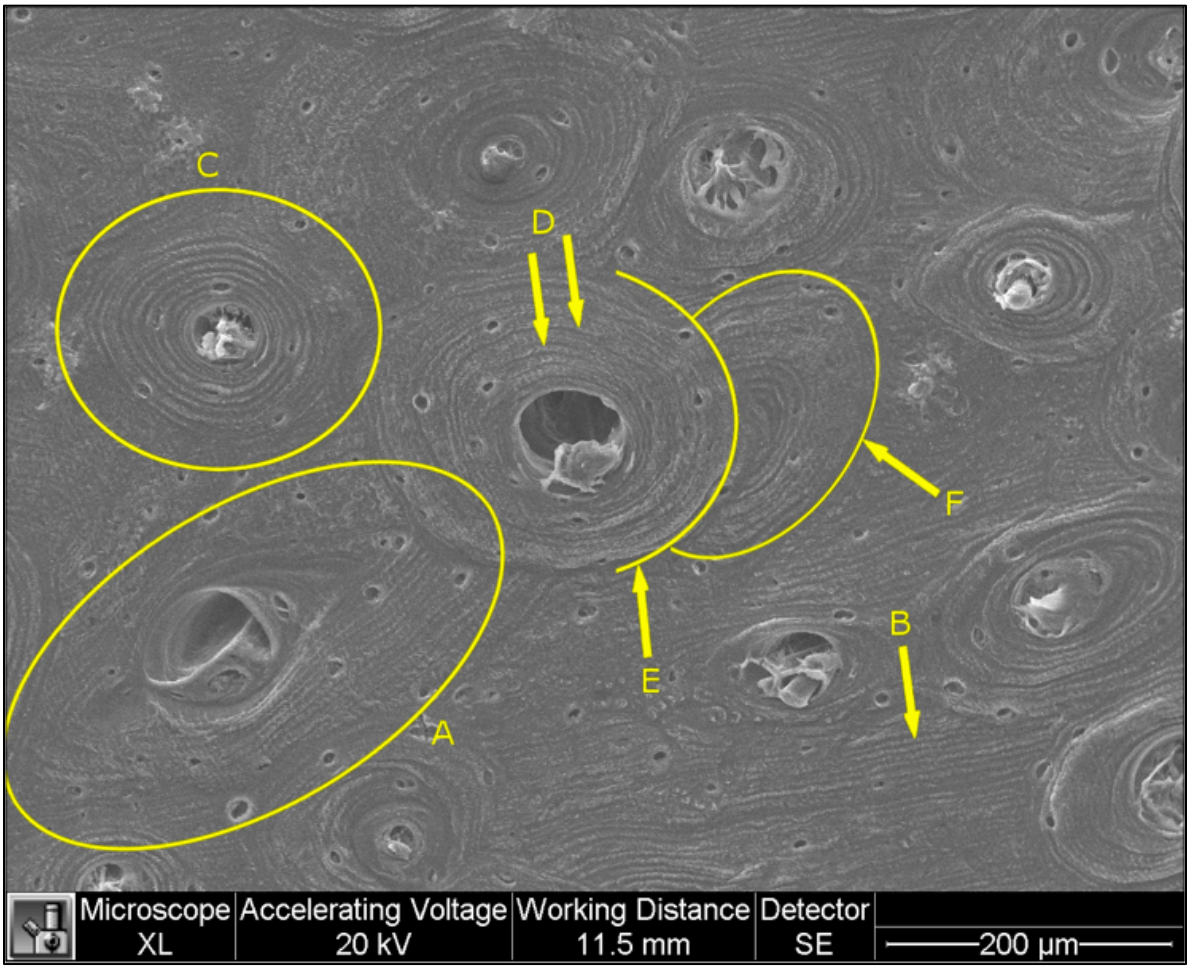

Figure 2: Histological features in a prepared slide of scanning electron microscopy (SEM) imaged human bone. Feature A: a primary osteon that is partially obscured on the right side by a secondary osteon. Feature B: parallel circumferential lamellae. Feature C: a secondary osteon. Feature D: concentric lamellae encircling a secondary osteon. Feature E: the line partially highlights the right 40% of a cement line. The secondary osteon associated with the cement line indicated by "E" is newer than the adjacent secondary osteon, which was affected during the creation of the newer osteon and became a fragmentary secondary osteon (Feature F). (Image source: C(r) from this study. Original magnification: 350x.)

bone is remodeled by a basic multicellular unit (BMU) composed of several hundred osteoblasts and 9-10 *osteoclasts,* multinuclear cells that resorb bone (Jaworski *et al.,* 1981). Osteoclasts are recruited to the remodeling area by chemical indicators. They demineralize the matrix and digest collagen fibers in a roughly circular and longitudinal tunnel (Hert *et al*., 1972). Osteoblasts follow the osteoclasts, using the fibers and demineralized matrix as a material source for osteoid. Osteoid is laid down from the edge of the tunnel inwards, forming circular rings of bone around the central canal (concentric lamellae; Figure 2, feature D). The initial site of osteoid deposition is referred to as the cement line (Figure 2, feature E) and it demarcates the structure from the surrounding bone tissue. Blood and nerve tissue follow the osteoclastic removal of bone tissue and the central canal forms when the perivascular space is reduced following the osteoid mineralization (Scheuer and Black, 2004). The blood vessels and nerves in these canals transport hematopoietic cells from the *medullary cavity* in the centre of the bone to the body, as well as receive and interpret information from the brain to initiate local remodeling in response to nutritional and functional stress (Knothe Tate, 2003).

In response to stressors, cortical bone tissue is remodeled by BMUs throughout life. The remodeling of bone tissue increases the number of secondary osteons (up to the age of 50; *see* Walker *et al*., 1994), destroys other primary and secondary osteons (creating fragmentary osteons (Figure 2, feature F)), and increases the amount of *pore space* (areas for vascular

fluid in the bone) (Currey, 1964; Frost, 1987). The phenomenon is well understood (Frost, 1990a; Frost, 1990b; *but see* Robling and Stout, 2008: 153-154), and methods to estimate the age at death have been developed based on the correlation between the number of secondary osteons and age (e.g. Kerley, 1965; Kerley and Ubelaker, 1978; Jee *et al.*, 1991; Stout, 1992).

A lacuna is an oblong pit found in the lamellae of bone tissue (Figure 3, feature A). Within each lacuna is an osteocyte, a cell that differentiated from an osteoblast trapped during the deposition of osteoid. The cytoplasmic extensions of the osteocytes are housed in the canaliculi (Figure 3, feature B), which are small anastomosing canals used to communicate with neighboring osteocytes, exchange waste products and nutrients, and maintain the surrounding bone tissue (Scheuer and Black, 2004).

Water and the Freezing Process

Water in the Body

Water is ubiquitous in the human body. This substance is found in all the body's structures in varying degrees and it accounts for approximately 70% of the weight of a middle-aged adult (Martini *et al.*, 2006: 4). Although there is variance with the amount of water between bone types and within individual bones (Timmins and Wall, 1977), water

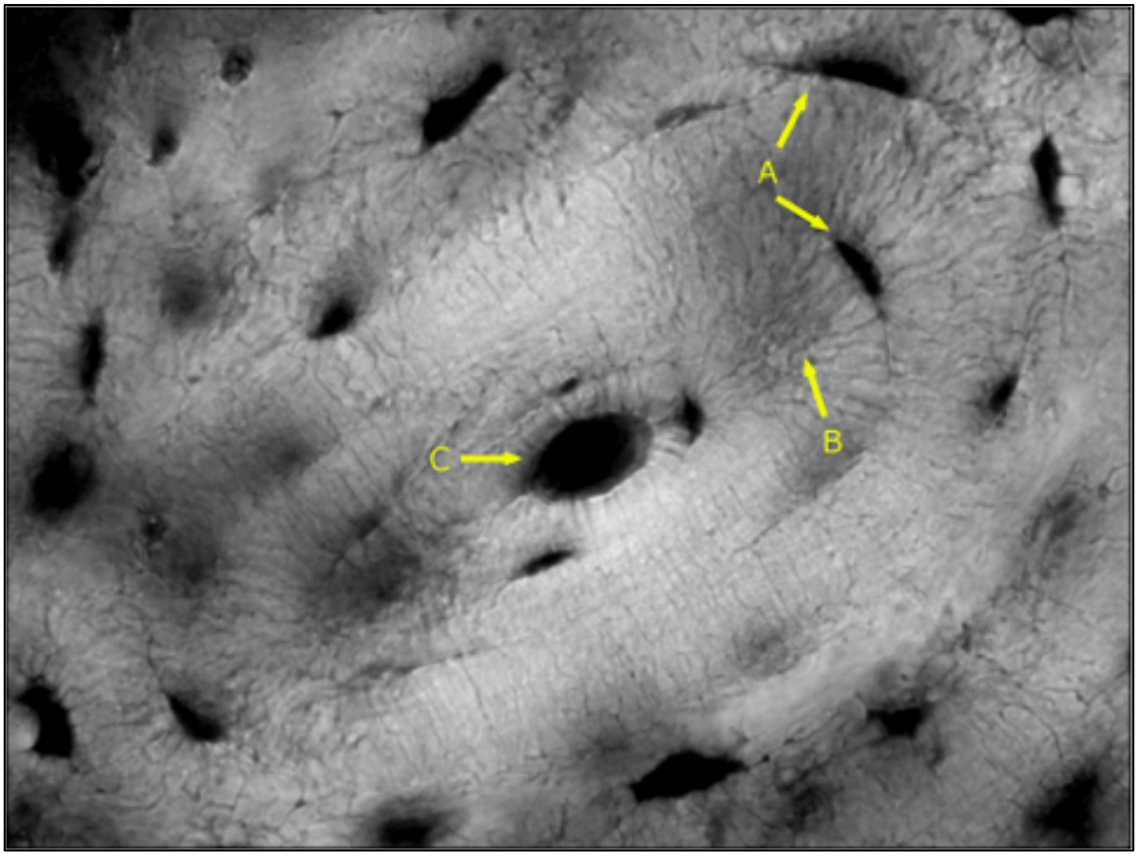

Figure 3: A secondary osteon in a cross section of a pig's femur. Feature A: two lacunae. Feature B: canaliculi radiate from a lacunae in feature A. Feature C: the central canal of this Haversian system. (Source: from this study. Light microscopy. Originally 400x magnification.)

generally accounts for 25% of a bone's weight. It is predominantly found in the vascular system of the bone, which is made up of the primary osteon canals and Haversian canals, Volkmann's canals (canals which connect Haversian canals transversely), the capillaries and capillary wall pores, and the epithelial fenestrae of the marrow cavity vessels (Knothe Tate, 2003; Wilson *et al.*, 2006: 3722). The abundance of water in tissue is related to its unique chemical structure: an oxygen atom with two covalent bonds, one to each hydrogen atom (H_2O) (Petrenko and Whitworth, 2003: 1). This covalent bond permits other elements to "share" the hydrogen atom, allowing it to bind with another water molecule or inorganic molecules in the bone tissue.

Two forms of water are found in the human body: bound and unbound. Bound water is found in the crystal-lattice structure of hydroxyapatite as a substitute for calcium or other elements. The ability of water to incorporate into this structure prevents the formation of less biologically suitable lattices and provides the hydroxyapatite a structure more adaptable to tensile stress (Wilson *et al.*, 2006: 3730). The function of this bound water plays a key role in the overall strength of the bone, and the removal of bound water from the hydroxyapatite significantly reduces the ability of the bone to resist stress and deformation (Nyman *et al.*, 2006; Wilson *et al.*, 2006). Hydroxyapatite bound water is relatively stable and is not affected by embalming procedures (Blanton and Biggs, 1968; Boskey *et al.*, 1982; Edmondston *et al.*, 1994).

Serving two roles, unbound water is present within the vascular system as bodily fluid and within cells of the bone tissue. In one capacity, water acts as a buffer in the space between the collagen and the mineral apatite, mitigating damage caused by unilateral sheer forces (Wilson *et al.*, 2006: 3730). Consequently, historical and archaeological bone is more prone to fractures during excavation or analysis than fresh bone. A more significant proportion of bone water is found in the pore spaces as bodily fluid, and it acts as a conduit for nutrient and waste exchange (Timmins and Wall, 1977). This system permits local bone tissue remodeling in response to internal and external stimuli (Knothe Tate, 2003).

Frozen Water

Ice 1h is formed when water is cooled to 0°C under normal atmospheric pressure (Hobbs, 2010).[2] As a result of its constituents other than water, bodily fluid freezes at -0.5°C (Storey and Storey, 1996). When pure water is cooled to a temperature of 3.98°C its density increases to a maximum of ~1.0 g/cm^3, and when water is further cooled its density decreases up to 9%. The result is an expansion of water as it cools. This phenomenon is known to cause infrastructural damage (e.g. burst pipes) as the temperature decreases (Petrenko and Whitworth, 2003: 11-12). When water freezes, the covalent bonds provide ice with a high degree of

[2] The "1h" designation indicates ice that has formed in a hexagonal lattice with each one of the six "points" containing an oxygen molecule at its tip and two shared hydrogen atoms each in a covalent bond with a neighboring oxygen molecule. This form of frozen water is the most common on earth (Petrenko and Whitworth, 2003: 11).

plasticity, which underlies the phenomenon of frost heaving. Because ice is able to withstand pressure, as well as crack and deform without falling apart, ice can creep into open spaces without losing structural integrity (Petrenko and Whitworth, 2003: 184). In soil, ice moves into warmer areas and subsequently thaws and refreezes, causing an expansion of the surrounding matrix as the density of the cooling water decreases and its volume increases (Michalowski and Zhu, 2007: 430; Petrenko and Whitworth, 2003: 305).

The application of ice formation dynamics within bone tissue has not been thoroughly explored in forensic taphonomy. Clinical research has explored the effects of freezing and thawing tissue to understand its effect on bone cell survival, collagen fiber organization, and the preservation of tendon and ligament integrity for transplant and donation (Goldberg and Stevenson, 1987). The disruption of normal cellular organization as a result of freezing bone tissue is well known (Lillie, 1965; Mellors, 1959) and this knowledge has been applied successfully in at least one forensic case (Zugibe and Costello, 1999; Bruno, 1993). The mineral component of bone has been shown in clinical studies to be impervious to the application of freeze and thaw cycles (Goh *et al*., 1989; e.g. Suto *et al.,* 2010; McElderry *et al*., 2011; Andrade *et al*., 2008); however, the more recent findings of Tersigni's research (2002; 2007), discussed below, strongly suggest that bone tissue integrity can be compromised by a single freeze event. Given what is known about the ability of water to continuously expand into

previously unfrozen surfaces upon thawing and refreezing, it is plausible that multiple freeze and thaw cycles of human bone would result in microstructural damage as the volume of bodily fluids changes according to temperature while moving into newly exposed areas.

Previous Cold Temperature Research

Considerable progress has been made in the last two decades on a myriad of potential environmental taphonomic effects (Ubelaker, 1997: 81), but the effect of cold temperature on bone tissue is an area requiring further research. Information on cold temperature taphonomy is sparse and is not a unified endeavour, which is surprising given that it is not uncommon for bodies to be concealed in freezers or frozen as a result of natural causes (Schäfer and Kaufmann, 1999: 149-150; e.g. Tabata *et al.*, 2000).

Previously Frozen Soft Tissue

The preservative effect of cold temperature on soft tissue is well-known. Notable archaeological and historical examples of preserved remains include those from the Scythian tombs in Siberia (e.g. Artamanov, 1965), Ötzi the Tyrolean "Iceman" (e.g. Bereuter *et al.*, 1997), Kwäday Dän Ts'ìnchi in British Columbia (Beattie *et al.*, 2000), and several 19th century circumpolar ship-hands and explorers (e.g. Beattie and Geiger, 1988;

Micozzi, 1991: 12). Bodies that are sufficiently cooled harden as the fluids in the vascular system freeze and can be preserved indefinitely in this state with only minor changes to skin colour over time (Perper *et al.,* 2006). Bodies frozen immediately after death can regain postmortem lividity when thawed rapidly in a saline bath at room temperature and exhibit few macroscopic differences that can be used to distinguish them from fresh cadavers (Kozawa *et al*., 2010; but *see* Schoning, 1992).

In a forensic context, the pattern of decomposition and dismemberment is one indication of whether a body has been previously frozen. Although variance in climate and ecological resources produce biological variation between and within biomes, decomposition in warmer climates follows a well-established pattern (Clark *et al*., 1997):

1. *Fresh:* no discoloration.
2. *Putrid:* drying of appendages and area around orifices, skin discoloration, and skin slippage.
3. *Bloating:* swelling of body, especially abdomen, marbling of skin tissue, and discoloration of body.
4. *Destruction*: rupture of abdomen and release of gasses, complete hemolysis, and partial skeletonization (exposure of bony elements).
5. *Skeletonization:* predominantly skeleton remains with minimal desiccated ligament remaining (or none at all).

The primary decomposition agent in warmer climates is putrefaction as the enteric bacteria digest the body internally (*autolysis*), and the gasses produced as a byproduct of the digestion (methane, ammonia, and hydrogen sulfide) bloat and eventually rupture the abdomen, allowing for increased insect access and colonization of the body (Micozzi, 1991; Micozzi, 1997: 172; Micozzi, 1986).

In previously frozen cadavers, the autolysis process has been interrupted and the decomposition pattern of the organism is more closely tied to external agents. The order of limb disarticulation, for example, remains the same between fresh and previously frozen cadavers, but in cold temperatures the integrity of the ligaments and tendons is impaired, which allows easier limb removal by carnivores (Micozzi, 1986). Cold temperature also mitigates or entirely precludes the internal signs of decomposition, most notably the bloating of the abdomen (Micozzi, 1986: 860). When the internal temperature of a cadaver reaches 4°C or below, the proliferation rate of the enteric bacteria is effectively zero (Micozzi, 1991: 40-41; but *see* Stokes *et al.,* 2009, and Wagster, 2005). A minimal but unsustainable amount of internal decomposition continues in frozen cadavers, but this process is strongly muted (Stokes *et al*., 2009).[3]

Previously frozen soft tissue is distinguishable from fresh tissue through an examination of the microstructural integrity of soft tissue. Although the overlying epidermal tissue of a previously frozen body appears

[3] In general, biological activity is drastically retarded below 10°C (Stokes *et al*., 2009). For this reason, bodies retrieved immediately after death are stored between 7-10°C.

and palpates normally, the normal state of the cellular constituents has been irreparably disrupted (Bereuter *et al*., 2006). In the interest of establishing a baseline for forensic inquiries, thawed dog brain, lung, liver, small intestine, and kidney (Baraibar and Schoning, 1985), as well as human heart and lung (Schäfer and Kaufmann, 1999), have been histologically examined. Although differences in the specific damage profiles of each tissue were noted, both studies found that previously frozen soft tissue generally exhibits extracellular fluid accumulation as a result of cellular rupture and/or cellular shrinkage. The former occurs if a cell cannot shrink in response to external osmotic pressure whereas cellular shrinkage occurs when it can (Baraibar and Schoning, 1985: 445). In either case, the intercellular connections are stressed by the expansion of bodily fluids and the result is increased extracellular space and cellular disorganization. Histological examination of these previously frozen tissues notes fissures or fractures in the extracellular areas, presumably as an artifact of the expansion of the ice (Schäfer and Kaufmann, 1999: 153-154).

A review of the literature yields only one application of the histological indicators of previously frozen tissue to the investigation of a crime. Zugibe and Costello (1999) hypothesized that the unique decomposition of a body found in a roadside ditch in September 1983 was the result of being frozen and thawed. The forensic pathologist noted no distention or bloating and classified the odor as abnormal (Zugibe and Costello, 1999: 1406). During their histological examination of liver and

heart samples, the researchers noted nucleic distortion, as well as irregularly shaped and increased extracellular spaces (Zugibe and Costello, 1999: 1406). The investigators concluded that the body had been previously frozen based on the disorganization of the cellular components combined with the unusual odor and absence of enteric activity. After Richard Kuklinski was arrested for another crime, he confessed he had stored the body examined by Zugibe and Costello in a walk-in freezer for two years in an attempt to obfuscate estimations of the postmortem interval (Bruno, 1993: 216).

Previously Frozen Hard Tissue

Tersigni has published the sole exploration in the field of forensic science on the effect of freezing and thawing on human bone (Tersigni, 2007), and her study warrants a detailed review as it is the inducement for this research project. Tersigni (2002; 2007) tested whether segments of bone treated with cold temperature would exhibit microstructural damage similar to the damage profile observed in previously frozen soft tissue (e.g. Schäfer and Kaufmann, 1999). She posited that unbound water in the pore spaces of the bone would cool and subsequently expand, damaging the bone around the Haversian canals (Tersigni, 2002: 8). To test her hypothesis, she froze 3-5 centimeter mid-diaphysis segments of femora, tibiae, and fibulae from unembalmed cadaveric samples in a freezer at 0^{0}C for 21 days. Bone segments were thawed in a field and retrieved after an

unreported length of time (Tersigni, 2007: 17). Samples were cleaned with water and dried at room temperature on paper towels. After the samples were dry, the bone segments were cut with a diamond saw into 0.6-0.8 millimeter slides and either prepared for light microscopy with further grinding (Tersigni, 2002) or etched with nitric acid, dried for 96 hours in a vacuum desiccator, and dusted with gold-palladium for SEM (Tersigni, 2007). Images were taken at resolutions ranging between 26x and 2000x magnification. The maximum diameters of 30 Haversian canals and 30 lacunae were recorded for multivariate analysis of variance (MANOVA) statistical tests (Tersigni, 2002).

Analysis of the previously frozen bone segments identified small cracks at the periphery of some of the Haversian canals in the previously frozen samples (Figure 4), but not to a degree with statistical significance (Tersigni, 2007). The control bones did not exhibit microstructural damage. The MANOVA statistical test applied to the Haversian canal and lacunae diameters found no relationship between treatment method (i.e. previously frozen or control) and maximum diameter of the features. Images captured using the scanning electron microscope exhibited clearer evidence of the microstructural damage than the samples imaged using light microscopy (Figure 5), but no consistency or patterning in the microstructural cracks were discerned (Tersigni, 2007: 19).

Each novel experiment in taphonomy inherently generates new research questions. Tersigni's technical note (2007) and Master's thesis

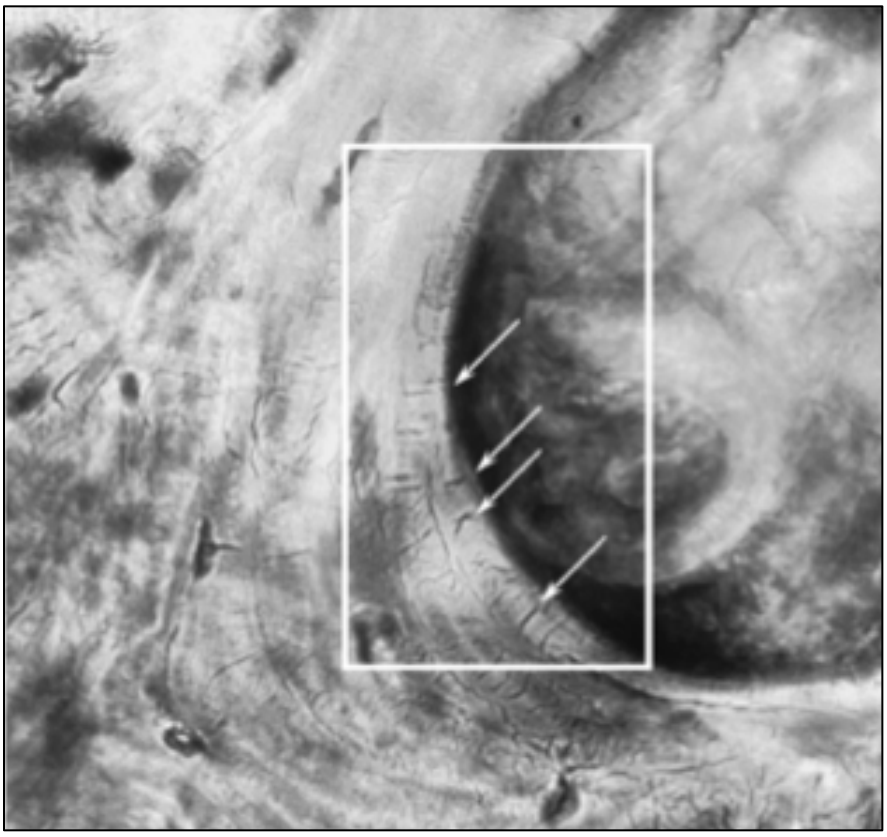

Figure 4: Arrows point to small cracks at the edge of a Haversian canal in this human tibia viewed under light microscope. (Original magnification: 400x. Figure used with permission; Tersigni, 2007.)

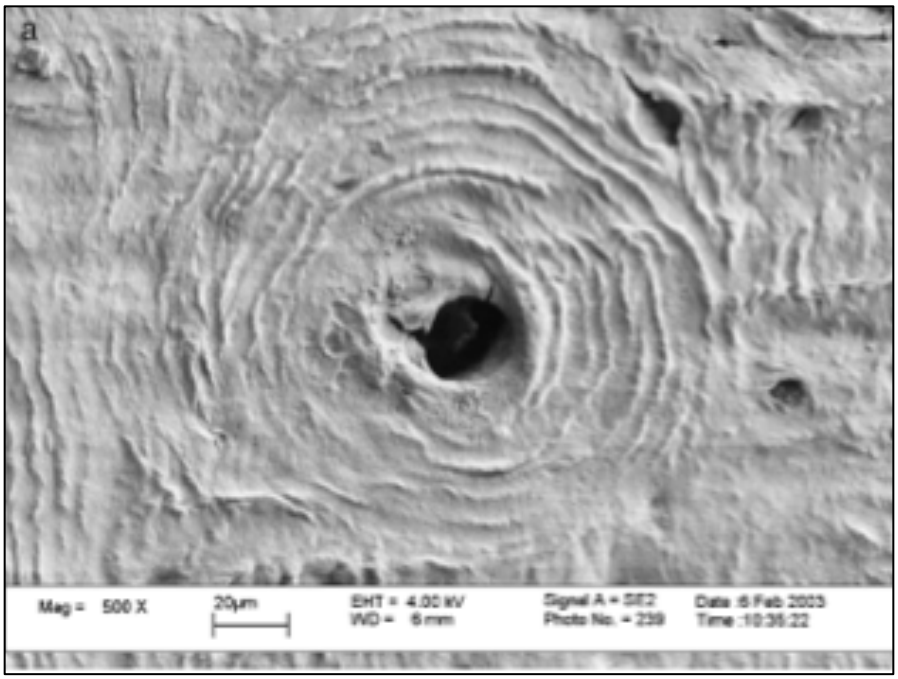

Figure 5: Two prominent microstructural cracks can be seen at the periphery of this Haversian canal from a previously frozen fibula. (Original

(2002), although preliminary in nature, provide a foundation for future researchers to approach questions of cold temperature taphonomy through the exploration of the variables in her research design. During my undergraduate degree, I conducted two exploratory studies using pig long bone as an analogue for human femora to increase the initial information on this topic.

The first study addressed the phenomenon of freeze and thaw damage by freezing and thawing unembalmed pig femora and humeri five subsequent times. Whole, partially fleshed bone was used to address the caveat of using segmented bones, which may have reduced the intracanal pressure in Tersigni's samples. The bones were cooled to -30°C in a commercial freezer and thawed to a temperature of 20°C five subsequent times. Light microscopy was employed to assess the microstructural integrity of the samples. None of the control bones exhibited microstructural cracks. In some of the previously frozen samples, cracks were observed at the periphery of the Haversian canals with a similar morphology to those in Tersigni's (2002) study; however, the cracks in this study were longer, suggesting that crack length might be correlated with temperature, time, or the method of freezing and thawing (Figure 6).

A second exploratory study was conducted to test whether a natural freezing and thawing treatment, involving the exposure of bone tissue to an unregulated outdoor environment, would induce microstructural cracks at the periphery of the Haversian canals. The research sought to establish if

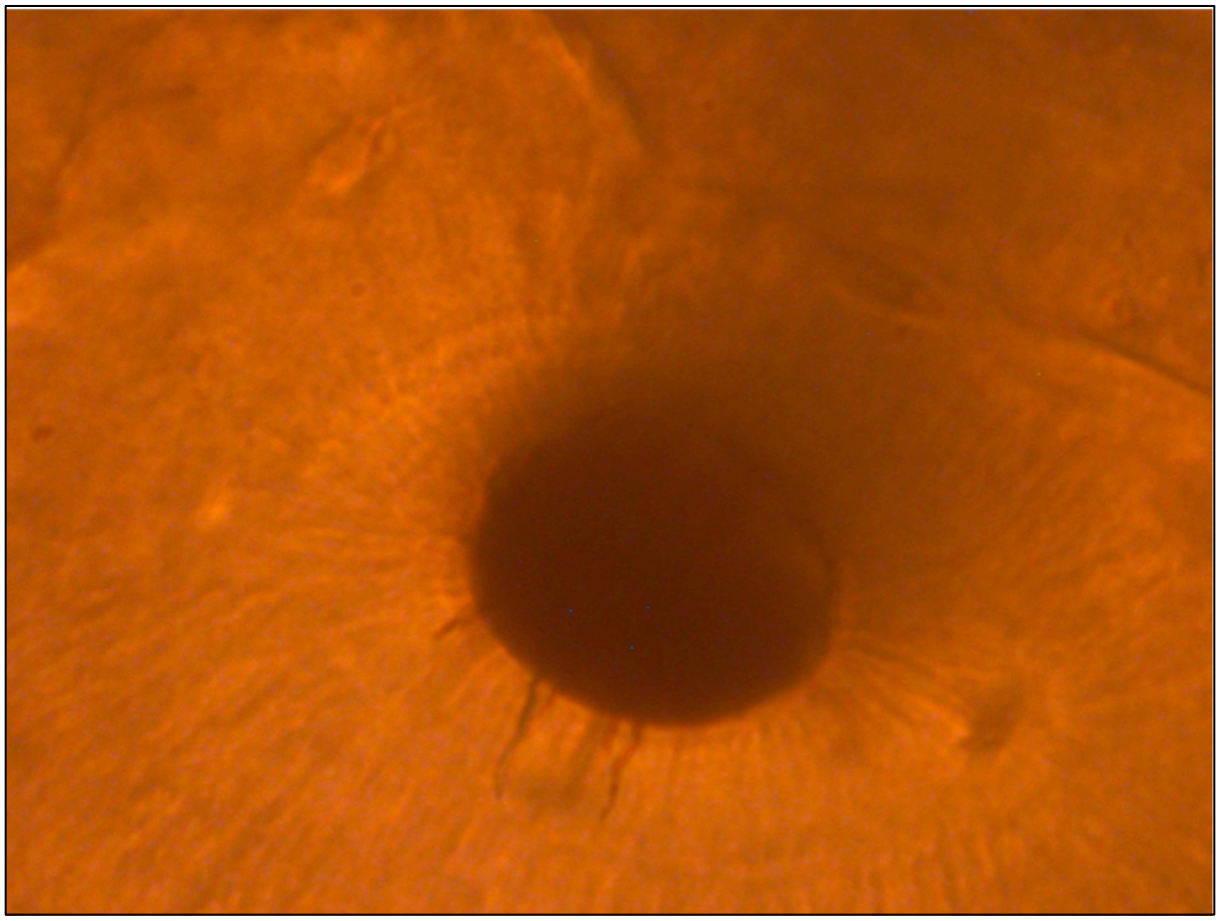

Figure 6: A Haversian canal showing several cracks at its periphery from the initial exploratory study involving pig femora and freeze and thaw cycles. (Source: from this study. Light microscopy. Original magnification: 630x)

crack length was related to the number of freeze and thaw cycles by examining the histological morphology of the bone samples between each freeze and thaw cycle. If crack length was increasing, the findings could be further developed into "weathering stages," similar to those developed by Behrensmeyer (1978) for macroscopic sun weathering of cortical bone. Unfortunately, six of the fourteen femora were lost during the course of the experiment due to human and carnivorous activity so the scope of the study was reduced to two freeze and thaw stages. A histological comparison of the control bones and the bones exposed to a single 14 day exposure to cold with plus-zero temperature exhibited no histological indicators of being previously frozen (Figure 7). The histology of these bones were indistinguishable from the control group. During this initial freeze and thaw cycle, the average ambient air temperature dropped below zero on only two occasions, which could account for lack of diagnostic markers. In the second group, which was exposed to a much greater fluctuation of temperature, including a total of 15 days with an average ambient air temperature below zero, microstructural cracks were found at the periphery of some of the Haversian canals, and these were substantially larger than those observed in prior experiments (Figure 8). It is hard to draw any meaningful conclusions from the second study, as each test group consisted of two bones, but the results suggest that multiple freeze and thaw cycles can result in the formation of larger crack lengths than those observed during a single freeze event.

The microstructural damage caused by freezing bone tissue is hypothesized by Tersigni to be the result of the cooling and expansion of unbound water in the bodily fluid (Tersigni, 2007: 19). The role of unbound water is of particular interest to this study as the sample population consists of embalmed cadavers, which retain an unknown amount of unbound water in the vascular system of the bone (Jason Papirny, March 23, 2011, personal communication). Since fresh bone is difficult to obtain for research purposes, this study evaluates the feasibility of using embalmed human bone for the analysis of freeze and thaw damage.

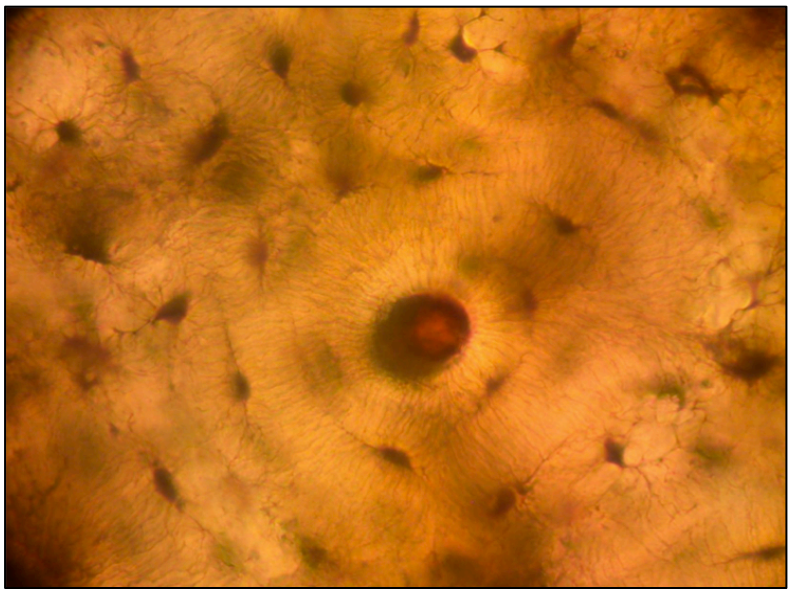

Figure 7: A Haversian system from a test bone that underwent one freeze and thaw cycle in the second exploratory experiment. There were no histological differences between this group and the control group. (Image source: from this study. Light microscopy. Original magnification: 400x.)

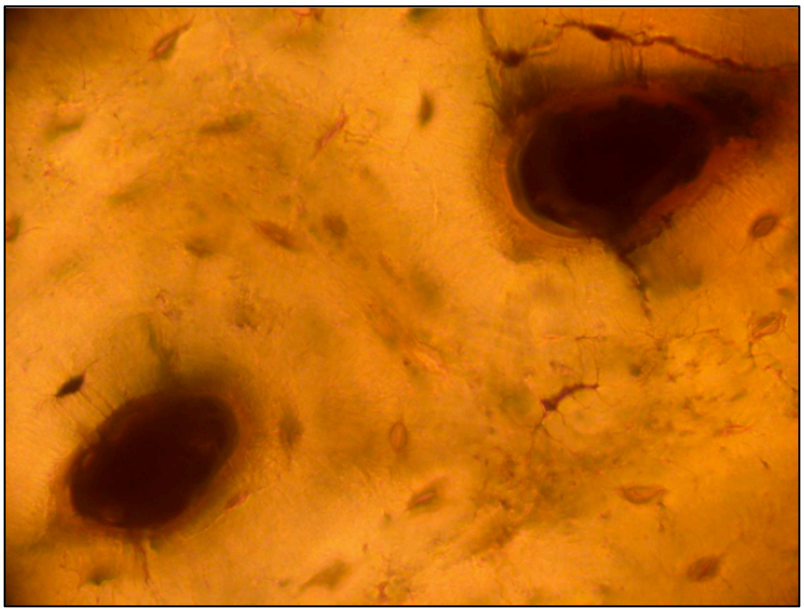

Figure 8: Two secondary Haversian systems in an osteon band (Mulhern and Ubelaker, 2001) in the second group of previously frozen bones in the second study. Both Haversian systems exhibit cracks at the periphery of the Haversian canals. (Image source: from this study. Light microscopy. Original magnification: 630x.)

CHAPTER III:

MATERIALS AND METHODS

Materials

Sample Information

Twenty-two human femora from eleven individuals were procured from the Division of Anatomy's Anatomical Gifts Program at the University of Alberta.[4] The embalmed cadavers in the Anatomical Gifts Program are used as teaching aids for the anatomy, dentistry, and medical students for a two-year period before being cremated.[5]

The femur was chosen as the research target bone for several reasons. Although there is inconsistency in the literature on its degree of preservation in medicolegal contexts (*see* Janjua and Rogers, 2008: 17), the overall size and robustness of the femur provide it with a relatively high level of preservation in archaeological contexts (Waldron, 1987). Of the samples provided by the program, the age at time of death was a concern, as bone porosity increases with age, particularly in females (Russo *et al*., 2002). In females, lower limb bones are more resistant to age-related cortical width reduction and general bone wasting than the upper limb bones (Doyle *et*

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[4] Ethics approval for research with human subjects was granted from the Arts, Science, and Law Research Ethics Board under *Pro00011949* on March 3rd, 2010 and renewed under *Pro00011949* on February 10th, 2011.

[5] The embalming fluid recipe and embalming procedures are listed in Appendix C.

al., 2011). In addition, paired femora exhibit no statistically significant differences in their cortical bone density or histomorphology, excluding femurs with arthroplasty-related implants (Torchia and Ruff, 1990; Rosenbaum *et al.*, 2006). Thus, the femur was chosen because either femur of a healthy individual is homologous, and it was also chosen to mitigate complications stemming from age-related cortical changes.

Limited demographic information was available for each individual. Age, sex (Table 1) and cause of death (Table 2) were provided. Information on ancestry was not provided or recorded. The age-at-death of the individuals range from 58-96 years, and the average age at the time of death was 81.6 years. Eleven cadavers were provided to this researcher, all of which were female except for individual J. During dissection, three prosthetic femoral heads were discovered, which indicated hemiarthroplasty had been undertaken at some point in these individuals' past. Femoral head transplants impact the load transfer of weight during activity and cause the cortical bone to reduce in thickness and develop abnormal histomorphology in the medial aspect of the mid-diaphysis over time (Torchia and Ruff, 1990; Rosenbaum *et al.,* 2006). Therefore, these femora were removed from the study group. The femora and data associated with individual K were removed during the study after a request by the family was made for the return of the remains.[6] The finalized study group was comprised of seventeen human femora from ten individuals.

[6] The research was not a factor in this request.

Table 1: Sample Demographic Information

Identification	Status	Left Femur	Right Femur	Age	Sex
A	Control	Yes	Yes	96	Female
B	Frozen	No	Yes	94	Female
C	Control	Yes	Yes	93	Female
D	Control	Yes	Yes	58	Female
E	Control	Yes	Yes	71	Female
F	Frozen	Yes	No	75	Female
G	Control	Yes	No	93	Female
H	Frozen	Yes	Yes	76	Female
I	Frozen	Yes	Yes	95	Female
J	Frozen	Yes	Yes	65	Male
K	Frozen	n/a	n/a	n/a	Female

Table 2: Recorded Cause of Death

Individual	Cause of Death
A	Pneumonia, dementia
B	Multi-organ failure, dementia, dehydration
C	Congestive heart failure, hypertension, pneumonia
D	Shy Drager syndrome, renal cardiac decompensation
E	Artheroscelerotic cardiovascular disease
F	Carcinoma of bladder
G	Respiratory arrest, pneumonia, dementia
H	Pulmonary disease and hypertension, ACD
I	Myocardial infarction, ACD, dementia
J	n/a
K	n/a

Initial Dissection and Preparation

The cadavers were stored and disarticulated at the Dr. H. E. Rawlinson Dissecting Laboratory in the Division of Anatomy at the University of Alberta. The femora were in their final months of use as teaching aids and were in various states of dissection. In all samples, the femora retained the majority of the overlying soft tissue except for the epidermis and dermis. The hip joint was disarticulated through incisions

around the femoral head and through the ligamentum teres, and the knee joint was disarticulated through a series of incisions around the femoral condyles. A scalpel and a pair of dissecting scissors were used to remove all of the soft tissue except for the periosteum.

Each cadaver was designated with a capital letter chosen at random. Each femora was tagged with two aluminum tags, one wrapped around the anatomical neck and one wrapped above the epicondyles, with its designation and side scribed into the metal, as well as written in permanent ink. For example, cadaver A's left femur would be identified as A(l). Femora were randomly sorted into either a control group (final n=9) or one of three test groups (n=2, n=3, and n=3).[7]

Application of Cold Temperature

After the femora were cleaned and tagged, they were placed anterior side up in a small floor freezer in the Dr. H. E. Rawlinson Dissecting Laboratory. To monitor base line temperature to examine potential deviations from this temperature, and to document planned freeze and thaw cycles, an EL-USB-2 temperature logger (Lascar Electronics) was placed inside the freezer alongside the bones. Each of the three test groups underwent a different number of freeze and thaw cycles (Table 3). For example, the two femora that constitute the first test group (J(l) and H(r))

[7] Individual K's femora were removed partway through the study: one from the first test group and one from the second test group. (The initial groups were control (n=9), test group 1 (n=3), test group 2 (n=4), and test group 3 (n=3).

Table 3: Freeze and Thaw Schedule

Individual		Freeze 1	Thaw 1	Freeze 2	Thaw 2	Freeze 3	Thaw 3
A	Left	C	C	C	C	C	C
	Right	C	C	C	C	C	C
B	Right	Y	Y	Y	Y	N	N
C	Left	C	C	C	C	C	C
	Right	C	C	C	C	C	C
D	Left	C	C	C	C	C	C
	Right	C	C	C	C	C	C
E	Left	C	C	C	C	C	C
	Right	C	C	C	C	C	C
F	Left	Y	Y	Y	Y	Y	Y
G	Left	C	C	C	C	C	C
H	Left	Y	Y	Y	Y	N	N
	Right	Y	Y	N	N	N	N
I	Left	Y	Y	Y	Y	Y	Y
	Right	Y	Y	Y	Y	N	N
J	Left	Y	Y	N	N	N	N
	Right	Y	Y	Y	Y	Y	Y
K*	n/a	N	N	N	N	N	N
	n/a	N	N	N	N	N	N

C (green) = control.
Y (blue) = in freeze and thaw cycle.
N (yellow) = not in freeze and thaw cycle.
* Individual K was removed from the study after the first freeze and thaw event.

were cooled in a freezer at -20°C for forty-eight hours and then removed from the freezer and thawed uncovered in the laboratory at 19.5°C for forty-eight hours. The bones in the second and third test group were removed and permitted to thaw during this time but were placed back in the freezer for up to two more freeze and thaw cycles after this initial thaw event (Table 4).

Table 4: Freeze and Thaw Timeline

Event	Date and Time Range	Total Frozen Time	Total Thaw Time
Freeze 1	May 13, 6 pm to May 15, 6 pm	48 h	0 h
Thaw 1	May 15, 6 pm to May 17, 6 pm	48 h	48 h
Freeze 2	May 17, 6 pm to May 19, 6 pm	96 h	48 h
Thaw 2	May 19, 6 pm to May 21, 6 pm	96 h	96 h
Freeze 3	May 21, 6 pm to May 23, 6 pm	144 h	96 h
Thaw 3	May 23, 6 pm to May 25, 6 pm	144 h	144 h

Physical Preparation

The femora were taken to the Department of Anthropology's Palynology Laboratory for preparation for scanning electron microscopy (SEM). Femora were washed with distilled room temperature water and without chemicals. The proximal and distal ends of the femora were removed with a hacksaw, and the marrow was flushed from the medullary cavity with running room temperature water and prodded with a Q-tip. Bones were dried on paper towels with the ambient room temperature of 19.5°C.

Three segments of bone, each measuring three centimeters in length, were removed from the mid-diaphysis with a hacksaw (Figure 9). Each of these segments was cut transversely using a Leica SP1600 microtome into pieces measuring 2500 µm in length. To mitigate distortion and preclude the formation of artifacts due to the heat generated by the cutting process, the speed of the saw blade was kept low and water was continuously poured over the sample for cooling.

Chemical Preparation

Chemical preparation of the bone tissue was conducted in the Advanced Microscopy Facility (AMF) in the Department of Biological Sciences. Following the laboratory's standard protocol for biological materials, the hexamethyldisilazane (HMDS) methodology was used to prepare a small amount of control bone to determine the suitability of using the HMDS methodology (Appendix A). The preferred methodology for this study is critical point drying, which is a common methodological choice for preparing samples for SEM in biological anthropology (Marshall *et al*., 2003: 312), but the AMF did not have the required equipment. The HMDS methodology is a suitable alternative and mitigates artifactual damage due to the low surface tension of HMDS (Bray *et al*., 1993).

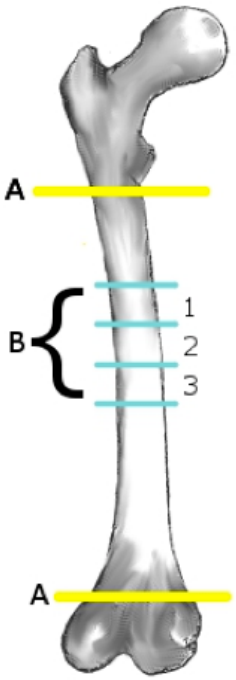

Figure 9: Representation of a femur, posterior side. "A" indicates the initial transverse cuts. Subsequent cuts, indicated by "B" were made to prepare a suitable size bone stub for the microtome. (Image modified from Figure 252, Gray's Anatomy (1918).)

The results of using HMDS on the bone tissue were problematic (Figure 10). In the majority of the samples, fat had not been fully dissolved, causing a biofilm to precipitate on the sample's surface. As a result of this biofilm, large areas of the bone surface became overexposed when the beam was focused, and the grease prevented the bone stubs from securely

adhering to the SEM stage (Arlene Oatway, April 8, 2010, e-mail correspondence with technician). The precipitation of formalin onto the surface during the overnight drying stage caused large crystals to form that precluded histological examination (An, 2003).

To fix these methodological problems, several alternative methodologies were tested on small pieces of bone before a suitable procedure was established. The final methodology includes two notable changes from the HMDS methodology: 1) the use of hydrochloric acid (HCl) to etch the bone surface, and 2) the use of acetone (CH_3COCH_3) to dissolve the fat in the sample (Degidi *et al.*, 2003; Kubek *et al.,* 2010). The revised methodology used for the entirety of the study is presented in Appendix B.

Methods of Analysis

The prepared stubs of bone were imaged on a Phillips / FEI LaB6 ESEM machine in the AMF. Images were captured at resolutions ranging from 80x to 5000x using the onboard photo-capture software. When necessary, images were modified for brightness and contrast in Adobe Photoshop, and originals of all images were kept. The anterior region-of-interest (ROI) on the superior surface of the bone stub was chosen as the primary site for qualitative analysis. In a study of 28 femoral cross sections of men aged 20-89 years, Villa and Lynnerup (2010) established that ROI choice does not measurably affect the outcome of histological analyses. The

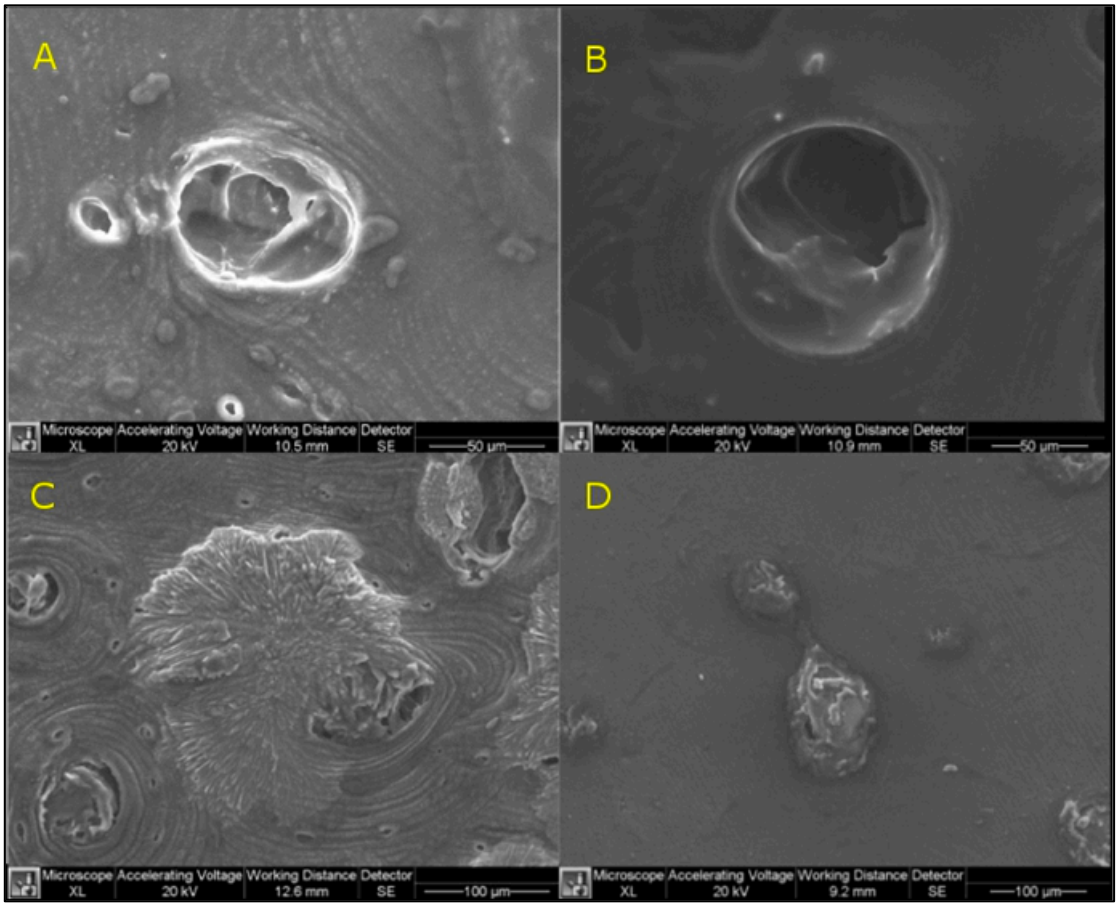

Figure 10: A composite of four images taken using the initial HMDS methodology on several control pieces of bone. The brighter areas around the periphery of the Haversian canal in Figure A is diagnostic of a build-up of static charge as a result of poorly dried or sputter-coated organic tissue. Figure B illustrates a greasier area of the bone surface. This prevented a proper focus on the relatively larger histological features (e.g. Haversian systems) and precluded identification of smaller features (e.g. lacunae). The jagged-edge feature in the middle of Figure C is likely the product of precipitated salts from the formalin solution sputter coated (An, 2003: 234). Lastly, Figure D illustrates an example of the tissue adherence problems encountered before the introduction of acetone. Often tissue would remain in the Haversian canals, preventing any detailed observation of the Haversian canal edges.

Image source (all from this study): A: E(r) (orig. 1500x). B: H(l) (orig. 1000x). C: E(r) (orig. 650x). D: D(l) (orig. 500x).

medial and lateral ROIs were photographed for comparison. Within the anterior ROI, the histology was primarily examined in the "middle cortical bone" section, a somewhat arbitrary region comprised solely of mature cortical bone (Garland, 1987: 110-113), to avoid the conflation of freeze and thaw damage with artifacts introduced during the preparatory stage (Figure 11). Images were compared with those found in the literature, most notably Tersigni (2007), to establish a "normal" and compatible histological appearance.

Statistical analysis (ANOVA model 1) was planned for the microstructural cracks originating from the Haversian canals if the results were appropriate for this type of analysis. ANOVA statistical tests compare the equality of means among several samples that have a factor or factors that influence each independently (Khazanie, 1990). An analysis of the variance in the length of cracks may elucidate a means to differentiate between the numbers of freeze and thaw cycles based on the presumably varying means of crack length.

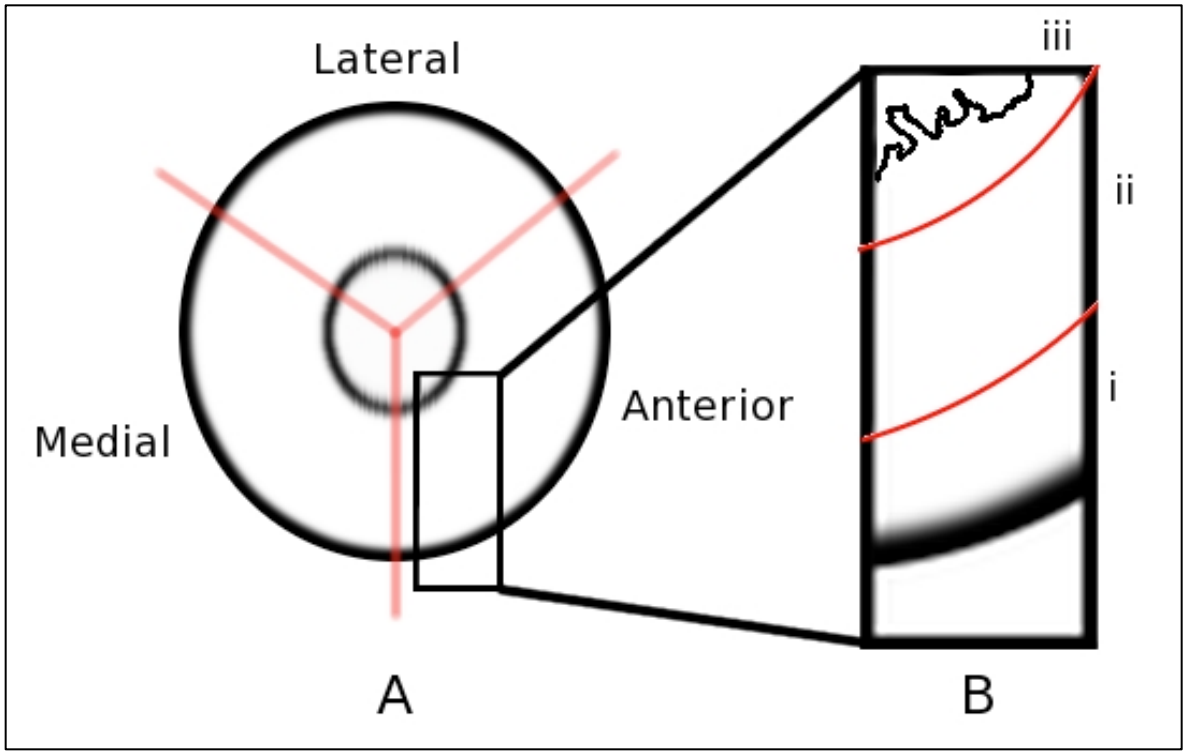

Figure 11: Image A shows the ROI designations used in this study in a transverse section of bone. Anterior was used for comparisons between the previously frozen bones and the control bones. Image B shows an enlarged area of the bone surface to highlight how the areas of the bone surface were defined. (i) is the outer cortical bone zone, and it accounts for the area of bone primarily comprised of external circumferential lamellae; (ii) is the middle cortical zone from which the comparisons of Haversian canals took place; and, (iii) is the lower cortex, or the internal surface of bone, which is marked by trabecular bone.

CHAPTER IV:

RESULTS

The effects of multiple freeze and thaw cycles on the microstructure of embalmed human femora were examined in this study. Non-control human femora were sorted into three groups and were cooled in a floor freezer to an ambient air temperature of -20°C and thawed up to three times (Chapter 3, table 4; Appendix D). The control bones and the previously frozen bone sections were examined using SEM, and the groups were compared to identify any qualitative differences or diagnostic histological features that could assist in differentiating previously frozen bone tissue from control bone tissue. Based on the findings of previous research (Tersigni, 2007), the canals of the Haversian systems in the middle cortical region were primarily investigated with the intent of locating and measuring cracks that formed in response to the application of cold temperature.

A careful examination of the Haversian systems in both the control group and the three test groups yielded no discernible qualitative differences between the samples (Figures 12 through 15). The Haversian canals across all groups presented smooth and unbroken edges, and there were no indications of damage caused by an increase in intracanal pressure due to expanded, cooled fluid. The inner and outer cortical areas of bone were investigated to identify any previously overlooked histological features

indicative of the application of cold temperature, but no features unique to the previously frozen bone groups were identified. No cracks were observed to compare so ANOVA statistical analysis was ruled out.

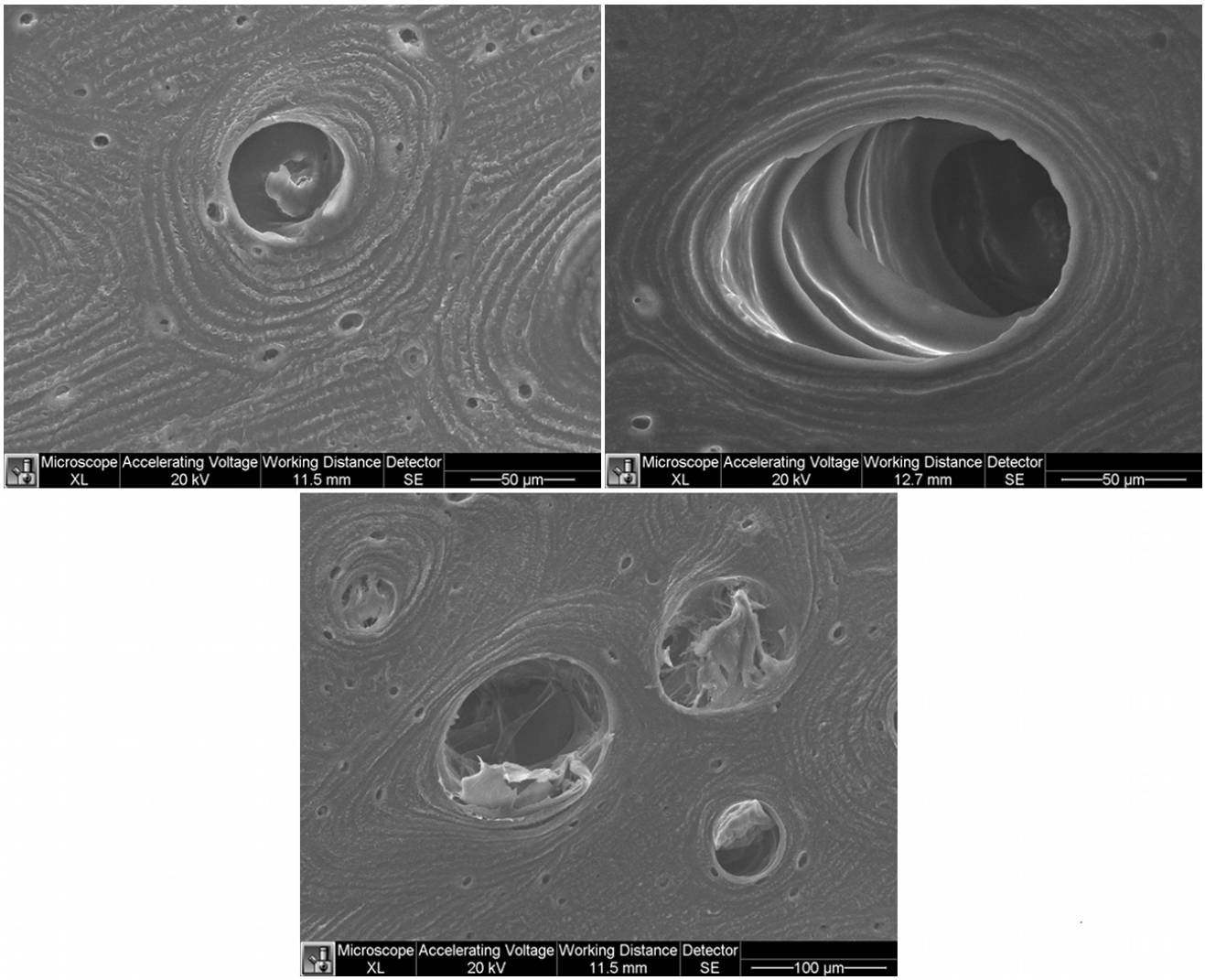

Figure 12: The control group specimens C(r), D(l), and G(l) (beginning at the top left and moving clockwise) at 1000, 1000, and 650 times original magnification, respectively. The Haversian canals in the control sample exhibited smooth, unbroken edges consistent with undamaged, normal bone. Soft tissue was present in the majority of the Haversian canals in all the groups.

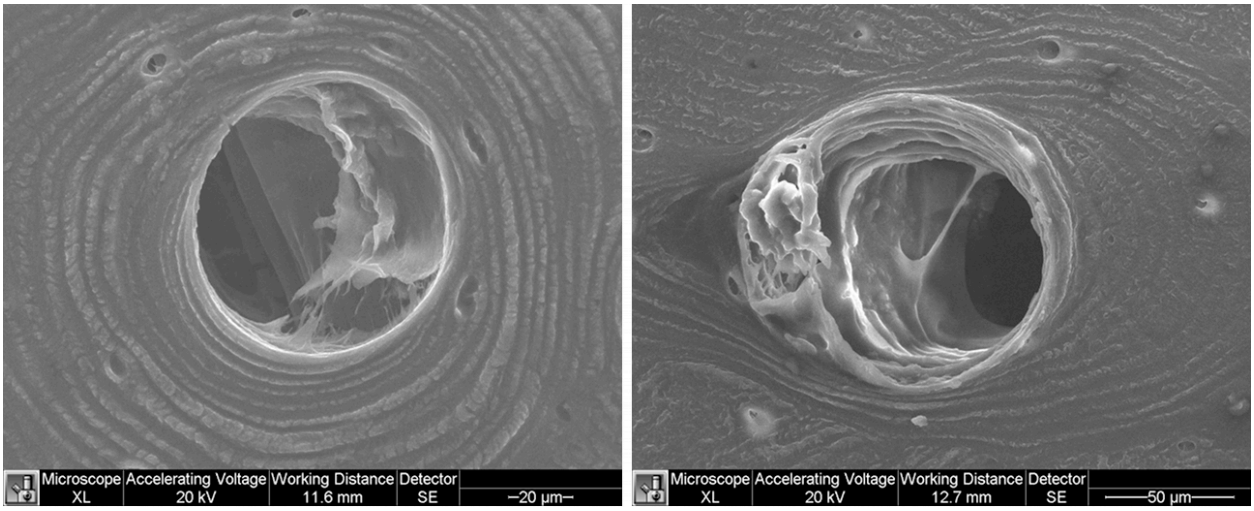

Figure 13: Two Haversian canals from the Group 1 specimens H(r) and J(l), which were frozen and thawed one time, at 1500 and 1000 times original magnification, respectively. No markers diagnostic to this group were identified.

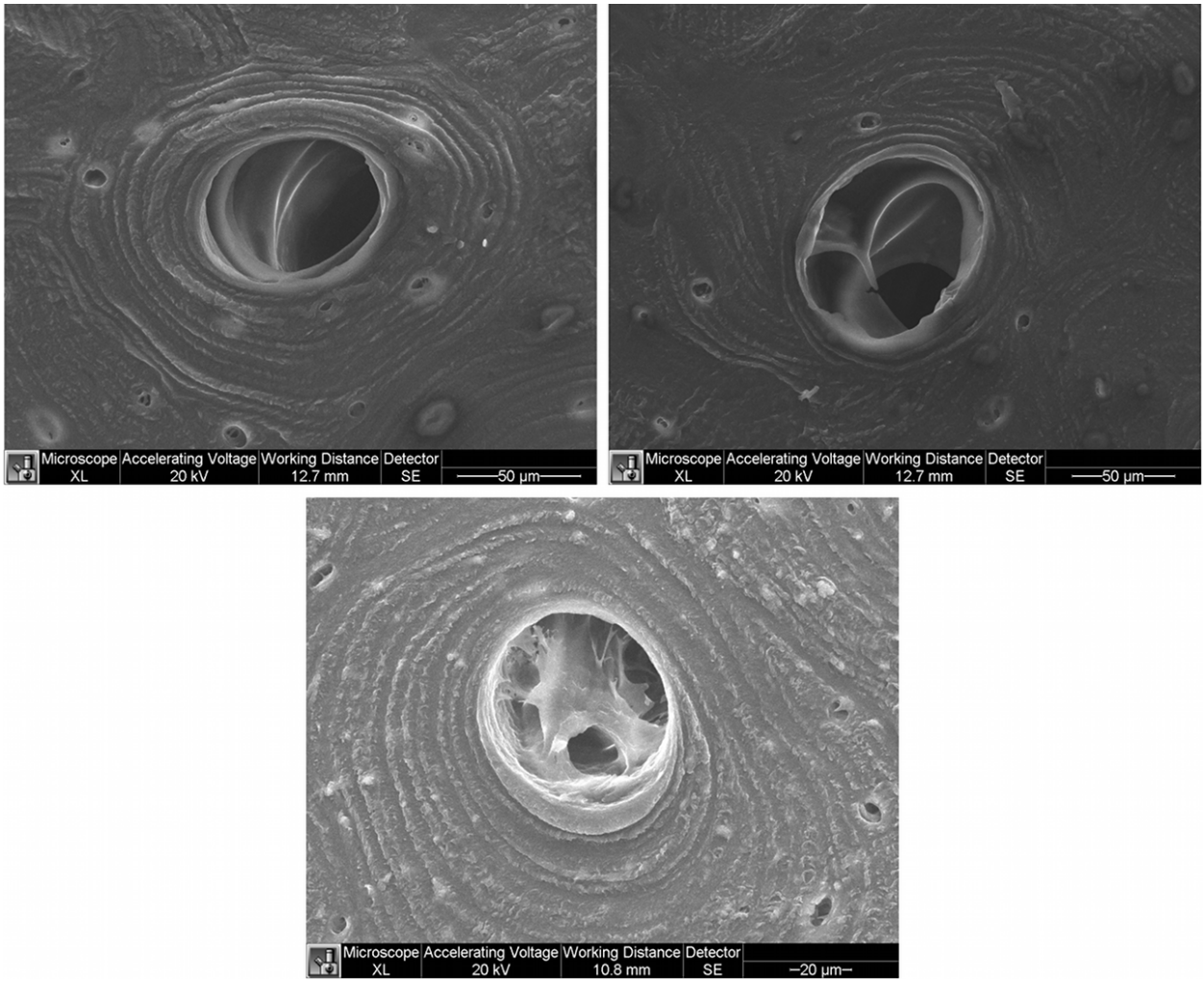

Figure 14: Three Haversian systems from the Group 2 specimens B(r), H(l), and I(r), which were frozen and thawed two times, at 1200, 1000, and 1500 times original magnification, respectively. No evidence of damage caused by freeze and thaw cycles was observed.

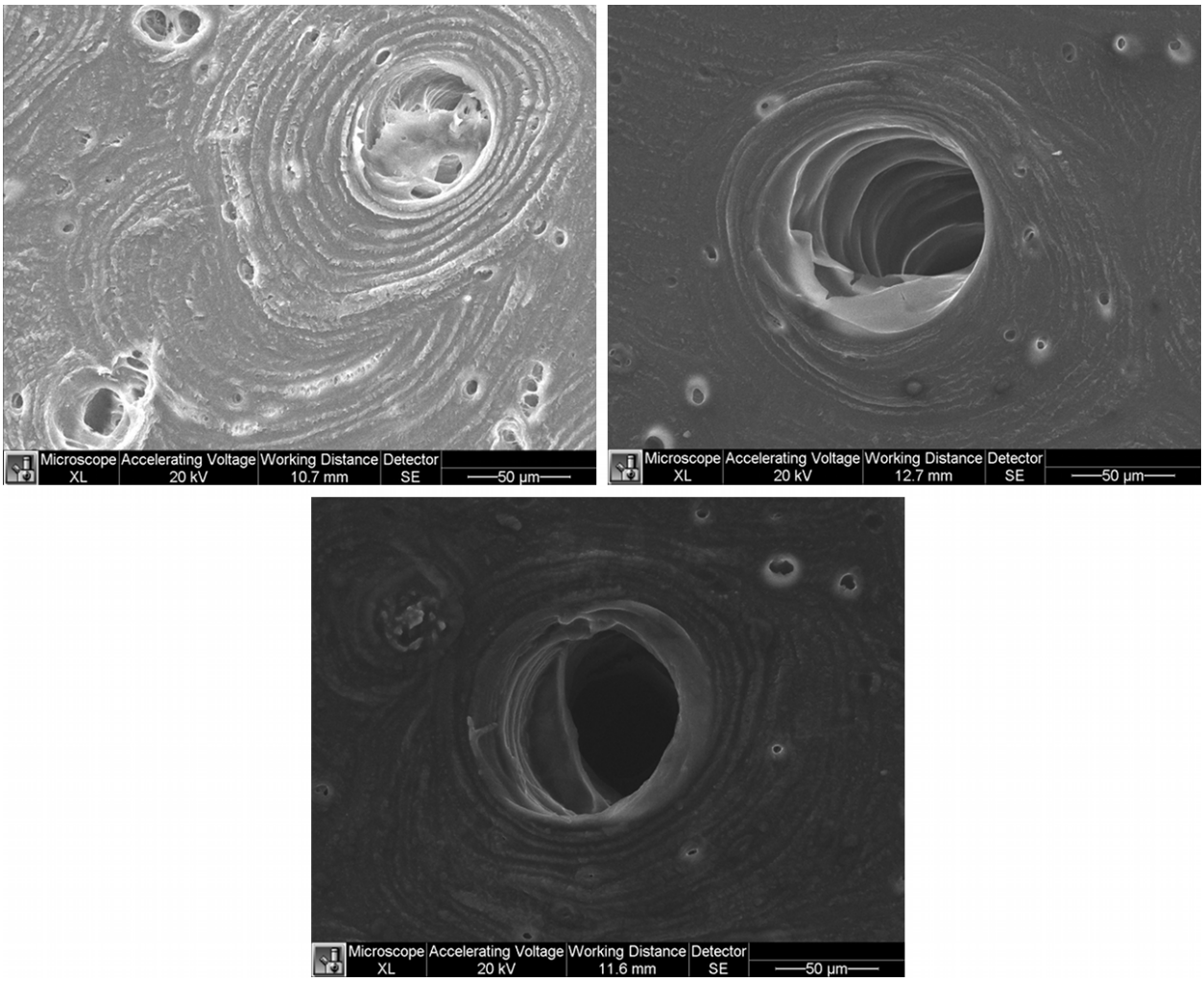

Figure 15: Examples of histology observed in three femora from Group 3 (specimens I(l), F(l), and J(r), all at 1000 times original magnification). Although these specimens were subjected to three freeze and thaw cycles, the histology was indistinguishable from the other previously frozen groups and the control group. Again, the Haversian canals presented were smooth and unbroken, and an examination of the surrounding cortical bone zones yielded no diagnostic markers.

CHAPTER V:

DISCUSSION

This study examined the effect of three freeze and thaw cycles on the microstructure of embalmed cadaveric human femora. Samples were investigated histologically with SEM, and each test group was compared with the control group and the other two test groups to establish any qualitative or quantifiable differences. No microstructural damage associated with freeze and thaw cycles was detected in the femoral sections analyzed and neither a novel diagnostic criterion or the criterion proposed by Tersigni (2002, 2007) was applicable to the differentiation of the three test groups from the control group. Considering the findings of Tersigni, an exploration of the inherent differences between this study and Tersigni's is warranted to examine any factors that might have precluded the formation of microstructural cracks or other diagnostic criteria caused by the application of cold temperature to the bone tissue.

Methodological differences were rejected as plausible reasons for the discrepancy between the findings of Tersigni's study and this study. Tersigni does not specify the regions of the bone examined during her study but does reference the well-documented problems pertaining to the histology of the *linea aspera*, so it is likely the entire cortical surface excluding the

posterior aspect of the bone was examined (2002: 16).[8] Similarly, this study excluded the *linea aspera* and looked at the entirety of the middle cortical zone, which follows the general tendency of histological research in biological anthropology (e.g. Alqvist and Damsten, 1969; Singh and Gunberg, 1970; Drusini, 1987; Drusini, 1996; Villa and Lynnerup, 2010). Magnification levels between the two studies are also comparable and were ruled out as a source of discrepancy.

The demography of the sample was considered as a factor that may have influenced the expression of microstructural cracks after the cold temperature treatment. This study used embalmed cadaveric femora from predominantly female donors. The ages of these individuals at time-of-death ranged from fifty-eight to ninety-five years. In contrast, Tersigni used unembalmed bone tissue and the age and sex of the samples were not disclosed in her study. In older females, such as those used in this study, the biological and chemical changes associated with menopause alter the normal deposition and resorption pattern of bone tissue. The hormonal changes associated with menopause reduce the number of osteoblasts in the BMUs (Frost, 1985). Fewer osteoblasts result in less deposition of bone tissue during secondary remodeling (i.e. the formation of Haversian systems), and the effect is Haversian canal diameters that are larger relative

[8] The *linea aspera* is a large bar of bone running longitudinally on the posterior aspect of the femur that serves as an origin and insertion site for numerous large lower body muscles. As a result, its histological organization is heavily influenced by an individual's activity levels. Its inclusion in histological studies can skew counts of secondary osteon population densities (Alqvist and Damsten, 1969), so it is generally avoided in anthropological research.

to other age groups (Wang and Ni, 2003). Techawiboonwong et al. (2008) found an increase in pore space does not result in an equal increase in the amount of vascular fluid. In the context of this study, this would mean more room for the expansion of cooling bodily fluids in the Haversian systems and, presumably, less intracanal pressure.[9]

The biomechanical properties of older bone tissue are altered in response to the biological and hormonal changes associated with old age. Bone tissue deposited later in an individual's life, particularly after menopause in women, contains a smaller ratio of mineral to organic components than older, earlier deposits of bone tissue (Simmons *et al.*, 1991). One role of the collagen matrix is to act as a cushion, permitting flexibility of the bone tissue when it's under mechanical stress. In older individuals, the shift to a higher ratio of mineral to organic components of bone tissue results in a higher incidence of bone failure (Macho *et al.*, 2005).[10] In the context of this study, the altered ratio of components in the bone tissue of older individuals would presumably induce a higher

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[9] The increased pore space found in older individuals was originally linked to the notion that the number of Haversian systems increased over time (Kerley, 1965). Histological studies have since shown that the number of secondary osteons in cortical bone remains relatively constant after the age of fifty (Walker et al., 1994: 666), and the increased pore space is largely due to the underlying biological and chemical changes.

[10] Conversely, Simmons *et al.* (1991) suggest the increased mineralization of bone, while reducing the overall tensile strength of the bone, may reinforce the cement line and the circumferential layers of bone, making the Haversian systems in older individuals more resistant to intracanal pressure. As biomechanical studies on the biomechanical strength of bone either look at whole bone strength or small cylindrical cores (Currey, 2009), there are currently insufficient data to support or refute this assertion.

incidence of microstructural cracking due to an increased tendency to weaken under stress.

The greatest contrast between Tersigni's study and this research is the latter's use of embalmed cadaveric bone. The cadavers used in this study were embalmed and formalin-fixed using an industry standard 6% formalin solution composed of formalin, phenol, glycerin, ethanol (EtOH), and water (Appendix C). Formalin is included in embalming fluids as it is a strong disinfectant and serves as a storage medium. As a result, a plethora of studies have sought to understand what impact the use of formalin has on the properties of bone tissue. The majority of these studies are concerned with the short-term effects of formalin on bone mineral density and its biomechanical properties. Despite ongoing research for the last five decades (Currey, 2009), this relationship is poorly understood, and contradictory findings on the effect of various formalin solutions on the stiffness (i.e. elasticity), compressive strength, and torsion strain resistance are found in the literature (e.g. McElhaney *et al*., 1964; Sedlin, 1965, Goh *et al*., 1989; Currey *et al*., 1995; Pöpperl *et al*., 1999; Van Haaren *et al*., 2008; Öhman *et al*., 2008). Studies have shown conclusively that formalin does not affect bone mineral density or otherwise interact with the mineral component of bone (Blanton and Biggs, 1968; Boskey *et al*., 1982; Edmondston *et al.*, 1994), but the aldehyde compound in formalin reacts with amines in the collagen matrix and forms an increased number of inter- and intra-fibrillar cross-links (Unger *et al*., 2010: 1048; Currey *et al*., 1995; Horan and An, 2006). The

prevailing theory (Currey *et al*., 1995) is that this interaction reduces bone flexibility and makes bone tissue more susceptible to failure under impact force. A review of the literature did not yield any research that addresses what effect, if any, formalin has on the biomechanical properties of Haversian canals.[11]

Studies on the effects of formalin storage on bone tissue for periods up to one year are sparse and the findings of these studies are inconclusive. Currey *et al*. (1995) examined the biomechanical properties of bovine cylindrical cores fixed in a 4% formalin solution for up to a year. Their results found that the necessary impact force required to cause bone failure was inversely correlated with the amount of time the bone was immersed in formalin. Similarly, Pöpperl *et al*. (1999) and Öhman *et al*. (2008) found bone tissue elasticity was significantly reduced in whole human calcanei fixed for six months and four millimeter cores of mid-shaft human femora fixed for eight weeks in a comparable 4% formalin solution, respectively. These findings are in contrast to Unger *et al*. (2010), who found that bovine femora and tibiae cores fixed in a 4% solution exhibited similar biomechanical results to the control bone, and Van Haaren *et al.* (2008), who tested the effect of a 10% formalin fixation of thirty mature goat femora and humeri for one year, found no statistically significant findings on biomechanical differences between fresh and formalin fixed bone tissue.

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[11] Goh et al. (1989: 467) explain that the reduced energy absorption capacity observed in their samples suggest that formalin changes the structural bond in osteons although no empirical data were found to directly support this assertion.

The summation of these observations on the properties of the bone tissue used suggest the bone tissues should have been conducive to microstructural damage; however, the bone samples exhibited no microstructural alteration consistent with prior observations of previously frozen bone. Therefore, these factors (i.e. age, sex, formalin effects on bone tissue's biomechanical properties) can be excluded as reasons for the observed differences between the previous studies and the current research.

A more plausible explanation for the observed discrepancy is the properties of the embalming fluid versus the bodily fluids naturally present in the pore spaces of the bone tissue. The process used to prepare the samples for the teaching and research collection involve pumping embalming fluid into the femoral artery (Appendix C). The natural bodily fluids are drained from the body, and are partially displaced by the embalming fluid. A variable and unknown amount of natural bodily fluids remains in the cadaver (Jason Papirny, March 23, 2011, personal communication). The presence of formalin as a precipitate in the HMDS prepared samples indicate that the embalming fluid is present to some degree in the middle cortical zone of the bone. Any natural bodily fluid still present in the bone tissue would be mixed with the embalming solution. The lack of any observed microstructural damage suggests that either not all of the mixture froze or the frozen mixture did not create enough intracanal pressure to damage the surrounding bone tissue.

Fractional freezing is the process where a solution with constituents that have different freezing points freeze partially or not at all. Although water is the major component of the embalming solution and of bodily fluid, the other constituents of the embalming solution have a much lower freezing point. It is possible that only a portion of the solution froze at the lowest temperature of the study or that none of the solution froze at all. If no freezing of the fluid occurred, then it naturally follows that no microstructural damage associated with the expansion of unbound water should be observed.

If the solution present in the pore spaces did freeze, the second factor could be that the unique properties of the mixture does not produce the same level of intracanal pressure exhibited by the natural expansion of bodily fluid. This is because the constituents other than water in the embalming fluid contract as they cool instead of expand (Petrenko and Whitworth, 2003). Therefore, the intracanal pressure produced by the expansion of the mixture would be insufficient to affect the bone tissue's integrity and result in observed freeze damage.

Although embalmed tissues are usually readily available for research, the use of embalmed tissues in cold temperature taphonomy studies of bone should be avoided. The mechanisms underlying histological freeze damage are not entirely clear, although the findings of this study could be interpreted to suggest the expansion of water in bodily fluid being cooled is the principal causative factor in the development of microstructural cracks

associated with cold temperature exposure. Because the embalmed fluid alters the natural state of the remains, any results garnered from the study of embalmed bone tissue are not applicable to the normal way in which a body would freeze.

To further research in this area, the caveats of this study should be addressed. One caveat of the study was not including unembalmed bone with the embalmed samples. This would have permitted a direct comparison between unembalmed and embalmed bone tissue to address whether the components of the embalming fluid account for the lack of microstructural cracks in the previously frozen embalmed femora. Since fresh pig femora were shown to be a suitable proxy in previous studies, the inclusion of fresh pig femora or fresh human bone in this study would have offered a basis of comparison for the effects of freezing on embalmed versus unembalmed tissue. A second caveat is the unknown freezing point of the embalming solution. It is presumed here that the embalming solution did not freeze at the coldest temperature in the study (-20^{o}c). In hindsight, a better approach would have been to determine the freezing point of the embalming solution and to determine if fractional freezing does or does not occur and at what temperature. Once these caveats are addressed, researchers can better understand the role of unbound water in the pore spaces of bone and the expression of freeze damage in previously frozen bone tissue.

CHAPTER VI:

CONCLUSION

Although this study did not identify histological indications of previous exposure to cold temperature in the embalmed cadaveric bone samples, it is constructive to treat the use of cadaveric bone as a test of assumptions (Kowalewski, 1999: 454). The results of this study suggest that embalmed bone tissue is not a suitable proxy for fresh human bone tissue in cold temperature taphonomy studies. This is presumably due to the nature of the embalming fluid and its interactions with natural bodily fluid present in the embalmed bone tissue.

The determination of whether bone tissue was previously frozen could be an important tool for forensic investigators in the investigation of a crime. The acceptance of any scientific approach in the forensic community is tied to its admissibility in legal proceedings after the implementation of the *Daubert standard* in the United States and the implications of the *R. v. Mohan* decision in Canada (Roger and Allard, 2004; Christensen, 2004). The consequences of these legal proceedings establish the necessity of methodologies to be testable for admissibility into court cases. The forensic study of previously frozen bone tissue is an area warranting further research to meet these standards of admissibility, but the findings of this study make clear that the results garnered by freezing embalmed bone are not applicable to understanding freeze damage in fresh

human bone. Though there might be ample embalmed cadaveric bone available to researchers, future cold temperature taphonomy studies should use fresh human bone, or human bone analogues such as a pig or deer bones (Marceau, 2007), to avoid the complications associated with the use of embalmed human bone tissue.

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Appendix A - Original SEM Methodology (HMDS)

The original SEM methodology followed the AMF's standard methodology for preparing biological tissue, which is based on Bray *et al*. (1993).

1. Specimens were fixed in ~15 ml of 2% glutaraldehyde/buffer fixative for 24 hours.
2. Two 15 ml 0.1 *M* phosphate buffered saline solution washes for 10 minutes.
3. Specimens were dehydrated using a series of ethanol (EtOH) and HMDS washes:
    a. 70% EtOH - 30 minutes
    b. 80% EtOH - 30 minutes
    c. 90% EtOH - 30 minutes
    d. 100% EtOH - 30 minutes
    e. 100% EtOH - 30 minutes
    f. EtOH:HMDS 75:25 - 30 minutes
    g. EtOH:HMDS 50:50 - 30 minutes
    h. EtOH:HMDS 25:75 - 30 minutes
    i. HMDS - 20 minutes
    j. HMDS - 20 minutes
4. HMDS was poured off and samples were air dried overnight in a fume hood.
5. Samples were sputter coated with gold/palladium (15 nm thickness).

Appendix B - Final SEM Methodology

This methodology was developed with the assistance of Arlene Oatway, AMF Technologist, and was used for the entirety of the study.

1. Specimens were fixed in ~15 ml of formalin for 24 hours.
2. 15 ml distilled H_2O wash for 3 minutes.
3. 30 ml acetone bath for 20 minutes.
4. 15 ml distilled H_2O wash for 3 minutes (two times).
5. 20 ml 10% HCl wash for 10 minutes.
6. 30 ml 0.1 *M* PBS wash for 20 minutes.
7. Air dried in fume hood overnight.
8. Sputter coated with gold/palladium (15 nm thickness).

Appendix C - Embalming Solution and Procedure

1. The femoral artery is opened up and 0.5 L of embalming fluid (see table below) is inserted up the leg and 0.5 L of embalming fluid is inserted down the leg.
2. After 24 hours, an aspirator and a trocar are used to inject between four to eight gallons of the embalming solution, at approximately 12 pounds per square inch, into the femoral artery. A variable amount of bodily fluid is recovered from the drainage of the femoral artery.
3. The cadaver is revisited after a few days to see how the fluid is settled. The radial artery is opened if the upper extremities require additional fluid.

Table 5: Embalming Solution

Ingredient (w/v %)	Amount	Freezing Point
Formalin (6%)	3.5 L	0°C
Phenol (100%)	4 L	42.0°C
Glycerine (10%)	6 L	-1.6°C
Ethanol (95%)	6 L	-114.1°C
H_2O (100%)	40.5 L	0°C

Appendix D - Temperature Log Data