THE EFFECT OF REPEATED FREEZE-THAW CYCLES ON THE BIOMECHANICAL PROPERTIES OF CANINE CORTICAL BONE

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ABSTRACT

THE EFFECT OF REPEATED FREEZE-THAW CYCLES ON THE BIOMECHANICAL PROPERTIES OF CANINE CORTICAL BONE

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University of Guelph, 1999

As orthopedic investigations have become more intricate, bone specimens have sometimes undergone multiple freeze-thaw cycles prior to biomechanical testing. The purpose of this study was to determine if repeated freezing and thawing affected the mechanical properties of canine cortical bone. Six pairs of third-metacarpal bones were tested in three-point bending and six pairs of femurs were tested in torsion. One member of each pair was tested destructively at collection. The other member was tested nondestructively at collection and after each of five freeze-thaw cycles; followed by destructive testing after the fifth cycle. For destructive tests, the material properties (modulus, maximum stress, maximum strain and absorbed energy) of a specimen at collection were compared to those of the corresponding contralateral specimen which had undergone five freeze-thaw cycles. For repeated nondestructive tests, the modulus of a specimen at collection was compared to modulus of the same specimen at each of the five thaw intervals. During destructive testing, there was a significant ($p = 0.02$) decrease (20%) in maximum torsional strain. Other changes in bending and torsional destructive properties were not statistically significant. During repeated nondestructive testing, there were solitary significant ($p < 0.05$) increases (8% and 9%, respectively) in both bending

and torsional modulus. However, these isolated changes were not correlated to the number of freeze-thaw cycles. The pattern of alterations in destructive and nondestructive biomechanical properties was most consistent with varying specimen dehydration at each thaw interval. Despite using accepted methods to maintain specimen hydration, repeated freezing, thawing, handling and testing of cortical bone increased the risk of moisture loss. Unless stringent efforts are made to insure proper hydration, the mechanical properties of canine cortical bone will be altered by repeated freezing and thawing, affecting the results of studies utilizing this technique.

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DECLARATION OF WORK PERFORMED

I declare that all work reported in this thesis was performed by me, with the exception of some design, programming and interpretation associated with statistical analysis, which was performed by Ms. Anne Valliant.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 - INTRODUCTION

Biomechanical testing of bone is utilized extensively in orthopedic studies that evaluate metabolic diseases (McCalden, McGeough & Court-Brown 1997), new surgical procedures (Schulz, Waldron, Grant, *et al* 1996), or orthopedic implants (Trostle, Wilson, Dueland, *et al* 1995). Storage of bone specimens is required in nearly all of such studies, especially when access to biomechanical-testing equipment is limited. Effective storage techniques also reduce the complexity and cost of these investigations through batch-processing of samples at the researcher's convenience (Huss, Anderson, Wagner-Mann, *et al* 1995). Freezing has long been the most-accepted means of bone storage (Turner & Burr 1993), with the biomechanical effects of a single freeze and thaw well documented (Pelker, Friedlaender, Markham, *et al* 1984; Roe, Pijanowski & Johnson 1988). As orthopedic investigations have become more intricate, however, specimens have sometimes undergone multiple freeze-thaw cycles prior to biomechanical testing (Panjabi, Krag, Summers, *et al* 1985; Linde & Sorensen 1993; Kang, An & Friedman 1997). Unfortunately, the effects of repeated freezing and thawing on cortical bone have not been investigated.

1.1 - STATEMENT OF GOALS AND HYPOTHESES

The primary goal of this study was to determine the effect of repeated freezing on the mechanical properties of canine cortical-bone specimens. The mechanical effects from repeated nondestructive loading of these specimens were also assessed.

The primary hypothesis of this study was that repeated freezing of canine cortical-bone specimens did not affect their mechanical properties. It was also hypothesized that repeated nondestructive loading had no influence on the mechanical properties of these specimens.

1.2 - LITERATURE REVIEW

1.2.1 - Bone Structure

Bone exists in two basic forms, woven bone and lamellar bone. Woven bone, which makes up the immature skeleton, is characterized by the random organization of its coarse collagen-fiber matrix. As the skeleton develops, almost all woven bone is remodeled to form lamellar bone. In mature animals, woven bone only persists in a few places, such as the dental alveoli, osseous labyrinths (e.g., tympanic bullae), in regions of tendon-bone attachment, and at sites of initial fracture repair. Lamellar bone has a matrix composed of finer collagen fibers, which are arranged in sheets. Lamellar bone can form either a solid (compact cortical bone) or a spongy (trabecular cancellous bone) mass, both of which have highly organized infrastructures (Bailey 1984; Wheater, Burkitt & Daniels 1987).

Cortical (Compact) Bone - Cortical bone, which is found in the diaphysis of long bones, is organized into haversian systems (osteons). Haversian systems are arranged on the long axis of the appendicular skeleton, along lines of stress. Within each system, a haversian (central) canal is surrounded by concentric layers (lamellae) of bone. Connections between haversian canals are established by Volkmann's canals, which pierce through the columns of lamellae at right angles. Blood vessels, lymphatics and nerves course through the system of haversian and Volkmann's canals (Wheater *et al* 1987; Markel 1996a) (**Fig A**).

Each lamella is formed from bone laid down by osteoblasts. In doing so, the osteoblasts become entrapped within small cavities (lacunae) between the lamellae, and subsequently mature into osteocytes. Minute canaliculi connect adjacent lacunae and

ultimately extend to the haversian canal. These canaliculi contain cytoplasmic extensions of the osteocytes, allowing nutrients to reach the osteocyte cell bodies. Interstitial lamellae span regions between complete haversian systems. On the outer surface of the bone, periosteal osteoblasts form circumferential lamellae to surround the columns of haversian systems. On the inner surface, similar endosteal circumferential lamellae merge with cancellous bone in the medullary cavity (Wheater *et al* 1987; Markel 1996a) **(Fig A)**.

Surrounding each haversian system is a thin layer of cement-like ground substance, composed primarily of glycosaminoglycans. Collagen fibers span the lamellae within a haversian system, increasing the bone's resistance to mechanical stress. However, these fibers do not cross the ground-substance cement lines between haversian systems, possibly explaining why the cement line is the weakest portion of the bone's microstructure (Markel 1996a) **(Fig A)**.

Cancellous (Spongy, Trabecular) Bone - Cancellous bone is found in the medullary cavity and epiphyseal region of long bones, and in the axial skeleton. It is formed by a three-dimensional network of plates and columns called trabeculae (Carter & Spengler 1978; Alexander 1985; Markel 1996a). These thin trabeculae are composed of irregular lamellae, which contain osteocytes within lacunae (Wheater *et al* 1987). Osteoblasts and osteoclasts are also present in the endosteum which lines the trabeculae. Cancellous bone does not typically have haversian systems. Nutrients and cell metabolites are exchanged through canaliculi that communicate with blood sinusoids in the marrow (Wheater *et al* 1987).

Bone Composition - By weight, bone typically consists of 21% cellular and organic matrix, 71% inorganic material, and 8% water (Markel 1996a). Osteoblasts, osteocytes and osteoclasts make up the cellular components. These cells are responsible for bone development and for bone remodeling in response to stress or injury. They also maintain mineral homeostasis by regulating the flow of mineral ions between bone and extracellular fluid (Boskey 1985; Fetter 1985; Markel 1992). Osteoblasts, which reside on bone surfaces, are responsible for producing the organic matrix (osteoid). Approximately 10% of osteoblasts become enclosed within the matrix and are subsequently called osteocytes. Osteoclasts, which reside on or near bone surfaces, are responsible for the majority of bone resorption. The organic matrix is made up primarily of collagen (95%), with small amounts of proteoglycans and glycosaminoglycans (ground substance, 5%) (Alexander 1985; Markel 1992, 1996a). Collagen provides both tensile strength and a scaffold for the deposition of inorganic salts. The proteoglycans and glycosaminoglycans add resilience and flexibility to the matrix. The inorganic mineral component of bone is responsible for its mechanical strength and hardness (Carter & Spengler 1978; Markel 1996a). Bone mineral is comprised primarily of calcium and phosphorus (hydroxyapatite), plus small amounts of carbonate, magnesium, fluoride, sodium and citrate (Carter & Spengler 1978; Alexander 1985; Markel 1996a).

1.2.2 - Bone Mechanical Properties

As a rigid tissue, the primary functions of bone include structural support and the facilitation of movement. Consequently, bone is subjected to multiple forces: tension, compression, bending, shear and torsion, which often occur in combination (Markel

1996b). Tension applies equal and opposite loads away from the surface of the bone, causing the bone to become longer and more narrow. As ultimate tensile strain develops along a plane perpendicular to the applied load, the bone fails by separating haversian systems at the cement lines. Compression is applied in the opposite direction of tension, resulting in equal and opposite loads toward the bone surface. As the bone becomes shorter and wider, failure occurs by oblique fracture through the haversian systems. During bending, loads are applied that cause the bone to curve around an axis. The bone is subjected to tensile forces on the convex surface and compressive forces on the concave surface. In three-point bending, two equal moments are created by forces applied at three locations on the bone. The magnitude of these moments is determined by the force applied at two peripheral points and by their perpendicular distance from a third central point. Failure occurs at the central point by a fracture originating on the tensile surface. In four-point bending, two central and two peripheral forces also create two equal moments. Both central forces are applied in one direction, and both peripheral forces, of equal magnitude, are applied in the opposite direction. The bone fails at the weakest site between the two central points. When bone is subjected to shear force, a load is applied perpendicular to the bone's surface. As angular deformation increases, the bone fails by formation of a fracture parallel to the applied load. In torsion, the applied load produces twisting of the bone, usually along its long axis. Torsional loading causes shear forces to be distributed over the entire bone, perpendicular and parallel to the axis of rotation. Tensile and compressive forces also develop diagonal to the axis of rotation. The bone initially fails in shear by formation of a longitudinal fracture, which then propagates diagonally (spirals) along the plane of ultimate tensile stress (Markel 1996b).

Bone is said to be anisotropic, because its strength varies under different loading orientations. It tends to be stronger when loaded in orientations typical of those experienced *in vivo* (e.g., compressive strength is usually two times greater than tensile strength) (Pelker & Friedlaender 1987). Bone is also viscoelastic, behaving differently when subjected to different rates of loading. Under fast loading conditions, bone increases in strength and stiffness, sustaining greater loads and storing more energy prior to failure (Nordin & Frankel 1989).

A typical load-deformation curve for cortical bone is illustrated in **Figure B** (Nordin & Frankel 1989). Load, plotted on the ordinate, is the amount of externally applied force (load = mass x acceleration). Load forces are usually measured in newtons. One newton is equal to the force required to accelerate a 1-kg mass at 1 m/s^2 (1 N = 1 $kg \cdot m/s^2$) (Carter 1989). Loads are may also be expressed as kilogram force (kgf), with one kgf equal to the force required to give a 1-kg mass the acceleration of gravity (1 kgf = 9.8 $kg \cdot m/s^2$ = 9.8 N). Deformation, plotted on the abscissa, is the change in dimension that results from the applied load. Deformation is often measured in millimeters. From a load-deformation curve, the mechanical characteristics of a structure can be derived. The load-deformation curve has two phases. The initial portion of the curve is the elastic region. Loads applied within the elastic region will not cause a permanent change in shape of the structure (elastic deformation). However, once loads are applied that surpass the yield point, a permanent change in shape will persist after removal of the load (plastic deformation) (Turner & Burr 1993; Markel 1996b).

The overall strength of a structure is defined by the load sustained before failure, the deformation sustained before failure (ductility), and the amount of energy stored

before failure. The energy stored, or toughness, is defined by the area under the curve (e.g., N·mm) (Markel 1996b). The ultimate strength on a load-deformation curve refers to the ultimate load (or more commonly, ultimate stress) at the failure point. Stiffness of a structure is characterized by the slope of its load-deformation curve within the elastic region (e.g., N/mm) (Hayes 1991; Markel 1996b).

Load-deformation curves provide mechanical information on an entire structure, such as a bone specimen. However, they cannot characterize the material properties of that structure, independent of its geometry. By standardizing testing procedures to minimize geometrical effects, the material properties of a specimen can be determined through the use of stress-strain curves (Hayes 1991; Markel 1996b). Similar to load, which is defined by an applied force (N), stress is defined by an applied force per unit area. Pascals (Pa) or megapascals (MPa) are the commonly used units (1 Pa = 1 N/m^2, 1 MPa = 1 $N/mm^2 \approx 145$ psi) (Carter 1989; Turner & Burr 1993; Markel 1996b). Strain is defined as a localized change in dimension in response to an applied load. Linear strain is determined in relation to the original length of the specimen, and is therefore expressed as a percentage. Shear strain is a measure of angular deformation, and is expressed in radians (1 radian $\approx 57°$) (Carter 1989). Analogous to stiffness in a load-deformation curve, the slope of a stress-strain curve is defined as the modulus; an elastic (Young's) modulus when testing in tension, compression or bending, and a shear modulus when torsion or shear forces are applied. Units for these moduli (i.e., stress/strain) are the same as those for stress, because strain measurements have only relative values (Hayes 1991; Markel 1996b).

1.2.3 - Mechanical Differences Between Cortical and Cancellous Bone

Cortical and cancellous bone exhibit markedly different mechanical properties. Cortical bone is stronger, stiffer and substantially more brittle than cancellous bone. Cancellous bone is more ductile due to its porosity. Thus, it can sustain a higher strain (change in dimension) prior to failure. Cortical bone fails at approximately 2% strain, while cancellous bone fails at approximately 75% strain (Markel 1996b).

1.2.4 - Biomechanical Testing of Bone

Biomechanical testing has been utilized extensively in a multitude of orthopedic investigations. Studies evaluating metabolic and endocrine diseases, from cage-layer osteoporosis in chickens to age-related changes in humans, often employ biomechanical testing (Kasra & Grynpas 1994, 1995; McCoy, Reilly & Kilpatrick 1996; Kasra, Vanin, MacLusky, *et al* 1997; McCalden *et al* 1997). The establishment of new surgical procedures, ranging from pin-fixation techniques to decompressive spinal surgeries, may also rely heavily on biomechanical evaluation (Schulz *et al* 1996; Sukhiani & Holmberg 1997). In the development of novel orthopedic implants, such as locking intramedullary nails or external fixators, biomechanical testing again plays an integral role (Trostle *et al* 1995; Cervantes, Madison, Miller, *et al* 1996).

Destructive Versus Nondestructive Testing - Bone failure (destruction), in nature or during biomechanical testing, can occur after a single catastrophic loading event or after numerous smaller cyclic loading events (fatigue fracture) (Carter & Hayes 1976; Pelker & Friedlaender 1987; Markel 1996b). When cortical bone is repeatedly loaded

within its elastic region, both strength and stiffness can decrease over time. This degradation of mechanical properties (fatigue) is likely due to dispersion of strain energy through microcracking and slippage at cement lines (Turner & Burr 1993). Fortunately, this degradation is also extremely gradual, usually requiring hundreds to thousands of consecutive cycles to produce destruction/failure. When similar elastic-region loads occur singly or in limited cycles (e.g., three cycles at 35% of breaking strength), the biomechanical properties of cortical bone appear to be unaffected (Seldin & Hirsch 1966). Biomechanical testing in this manner is classified as nondestructive.

Bone Moisture and Temperature - Maintaining the water content of bone is a significant factor in preserving its biomechanical properties (Huss *et al* 1995). As bone dries, it becomes stiffer and more brittle. The stress required to cause failure may increase, but the associated strain (ultimate displacement) and the energy absorbed (toughness) both decrease (Turner & Burr 1993). In cortical bone, such changes develop after only 10 minutes of exposure to air, and become pronounced after 60 minutes (Seldin & Hirsch 1966). Therefore, saline solution is commonly applied to cortical-bone specimens during initial collection prior to storage, during pretesting procedures (e.g., placement of implants), and throughout biomechanical testing (Turner & Burr 1993). Such saline application is not recommended for cancellous-bone specimens. Bone preserved at -20°C also undergoes gradual loss of moisture due to evaporation (Stromberg & Dalen 1976b; Huss *et al* 1995). Effective methods of maintaining hydration during storage include placing samples in plastic containers (Tomford, Doppelt, Mankin, *et al* 1983; Panjabi *et al* 1985; Kang *et al* 1997), wrapping samples in saline-soaked towels (Kang *et al* 1997), and freezing samples in saline solution (Seldin &

Hirsch 1966; Griffon, Wallace & Bechtold 1995; Huss *et al* 1995). Griffon *et al* (1995) destructively tested paired cortical-bone specimens in torsion or four-point bending after 12 months of freezer storage. One member of each pair was frozen in saline solution, while the other was wrapped in polyethylene. Although no differences were found between groups evaluated in torsion, during four-point bending, specimens frozen in saline had up to 24% greater displacement and up to 30% greater absorbed energy. The results of this study, however, may have been affected by failure to keep the bone specimens moist after thawing.

Although not always practical, the mechanical properties of bone are most accurately evaluated at physiologic temperatures. Fortunately, errors associated with performing routine testing procedures at room temperature are negligible. Only the results of fatigue testing, involving numerous consecutive loading cycles, are significantly altered. Bone can usually withstand twice as many cycles before failure when tested at room temperature than when tested at 37°C (Turner & Burr 1993).

Sample Selection - The mechanical symmetry of bones has been verified in rabbits (White, Panjabi & Hardy 1974; Terjesen & Benum 1983; An, Kang & Friedman 1996), rats (Bak & Jensen 1992), dogs (Stromberg & Dalen 1976b) and humans (Mather 1967). Therefore, "bilateral" or "paired left-right" animal models are used extensively in orthopedic research (Griffon *et al* 1995; An *et al* 1996). Although significant biomechanical variations have been noted between paired bones within certain animals, such variations were equally distributed between left and right sides. These potential differences between paired bones from the same animal have also been substantially less than those routinely observed between bones from different animals (White *et al* 1974;

An *et al* 1996). Therefore, a higher level of statistical significance can be obtained with a given number of animals if paired samples are used (Steel & Torrie 1980a, 1980b).

Specimens should be selected that are well suited for the type of test desired. Bones with unusual geometry can induce errors. For example, curved or twisted bones, such as the humerus, can shift when tested in three-point bending. The resulting false measurement of displacement can cause overestimation of strain and underestimation of elastic modulus (Turner & Burr 1993). Straight relatively-symmetrical specimens best suit most testing procedures. In one study, the use of canine metacarpal/metatarsal bones not only accommodated both bending and torsion testing, but also maximized the number of paired long-bone specimens obtained per donor (Griffon *et al* 1995).

1.2.5 - Storage of Bone Specimens by Freezing

In orthopedic studies utilizing biomechanical testing, evaluation of freshly harvested bone specimens is impractical in most circumstances (Huss *et al* 1995). Freezing (cryopreservation), considered the best method for long-term preservation of bone (Turner & Burr 1993), has been the most frequently used technique for specimen storage (Pelker *et al* 1984; Griffon *et al* 1995; Huss *et al* 1995). Some investigations, however, have required samples to be frozen and thawed several times, due to the complexity of the experiment (Panjabi *et al* 1985; Linde & Sorensen 1993; Kang *et al* 1997) or due to unforeseen circumstances (Kang *et al* 1997). Thus, the potential for cryopreservation, especially with multiple freeze-thaw cycles, to alter the mechanical properties of bone has been questioned (Linde & Sorensen 1993; Kang *et al* 1997).

Single Freeze-Thaw Cycle - Bone frozen for subsequent biomechanical testing is routinely stored at -20°C, the temperature of conventional freezers. The mechanical properties of cortical samples properly stored at this temperature do not differ significantly from those of fresh samples (Seldin & Hirsch 1966; Pelker & Friedlaender 1987; Goh, Ang & Bose 1989), even after eight (Roe *et al* 1988) to 12 months (Tshamala, van Bree & Mattheeuws 1994). In 1984, Pelker *et al* evaluated the torsional characteristics of rat femurs. There were no significant differences between freshly harvested bone and bone stored at -20°C for two weeks. Although the lack of haversian systems in rat cortical bone initially limited extrapolation to other species, later investigations supported these results. After three to four weeks of similar freezer storage, feline humeral, feline femoral (Goh *et al* 1989) and human femoral (Seldin & Hirsch 1966) samples were tested in torsion, four-point bending and three-point bending, respectively. When compared to fresh control samples, no significant differences were noted. Several studies have evaluated the long-term effects of -20°C cryopreservation on canine cortical bone. When tested by compression, screw-pullout load and screw-stripping torque, femoral specimens frozen for four and eight months were similar to controls (Roe *et al* 1988). Even after 12 months of freezer storage, femoral samples (initially sterilized with ethylene oxide) showed no significant change in ultimate stress during compression, bending and torsion testing (Tshamala *et al* 1994).

Bone preserved at -20°C undergoes gradual loss of moisture due to evaporation (Stromberg & Dalen 1976b; Huss *et al* 1995). At temperatures warmer than -28°C, this evaporation may enlarge ice crystals within the bone, potentially causing structural damage through microcracking (Brown & Cruess 1982; Roe *et al* 1988). If ice-crystal

enlargement does occur at -20°C, there has been no evidence of any significant effect on biomechanical properties. In several investigations, however, trends toward increased stiffness and increased load at failure were observed in frozen cortical specimens (Seldin & Hirsch 1966; Roe *et al* 1988; Huss *et al* 1995). Such changes, along with decreased deformation and absorbed energy, are often associated with moisture loss (Seldin & Hirsch 1966; Turner & Burr 1993). To minimize the potential effects of evaporation, cryopreservation at -70°C is recommended if storage is anticipated to be prolonged (Roe *et al* 1988; Kerwin, Lewis & Elkins 1991; Kang *et al* 1997) Cortical bone frozen at -70°C shows no change in surface structure when evaluated with scanning electron microscopy (Vogenreiter, Ascherl, Blumel, *et al* 1994). However, the requirement of more-expensive specialized freezers for specimen preservation at this temperature makes its use less common among researchers.

The mechanical properties of bone may also be altered by cellular enzymes (collagenase and proteases). Although these enzymes, which break down the extracellular organic matrix, are most active when bone is thawed, their activity is not completely arrested at -20°C. As described above, storage temperatures of -70°C, or lower, are recommended to maximally inhibit enzyme effects (Pelker & Friedlaender 1987; Kerwin *et al* 1991; Kang *et al* 1997). Roe *et al* (1988) evaluated canine femurs sterilized with ethylene oxide and stored at room temperature for one, 16 and 32 weeks. The observed deterioration in mechanical properties (compression, screw-pullout load, and screw-stripping torque) of these specimens with increasing time was most likely due to enzymatic destruction of bone matrix.

Enzymatic degradation and ice-crystal formation have not been shown to significantly influence the mechanical characteristics of bone specimens undergoing a single -20°C freeze and subsequent thaw. With repeated freeze-thaw cycles, however, the potential for these processes to cause significant damage should increase.

Multiple Freeze-Thaw Cycles - In studies dealing with cancellous bone, specimens are usually frozen and thawed prior to any biomechanical testing (Linde & Sorensen 1993; Borchers, Gibson, Burchardt, *et al* 1995; Kang *et al* 1997; McCalden *et al* 1997). This freeze-thaw cycle is repeated if multiple tests are performed at intervals greater than one day (Linde & Sorensen 1993). Water expansion associated with each freeze was suspected to cause specimen damage. However, after subjecting bovine and human cancellous bone to five (Linde & Sorensen 1993; Kang *et al* 1997) and eight (Borchers *et al* 1995) freeze-thaw cycles, there were no significant changes during nondestructive (Linde & Sorensen 1993) or destructive (Borchers *et al* 1995; Kang *et al* 1997) compression testing.

The biomechanical effects of repeated freezing on cortical bone have not been evaluated. Extrapolating results from the above cancellous-bone studies would be unsound; since cancellous and cortical bone have markedly different mechanical properties, and since they may also differ in their response to techniques of storage, sterilization or decreasing antigenicity. For example, freeze-drying rat and human cortical bone for bone-graft banking caused microcracking, with subsequent decreased torsional strength, compressive stress and compressive elastic modulus. However, similar freeze-drying of a trabecular/cortical-bone construct (rat vertebrae) did not significantly alter its structural properties (Pelker *et al* 1984; Borchers *et al* 1995).

1.2.6 - Figures

Figure A - Microstructure of cortical bone.

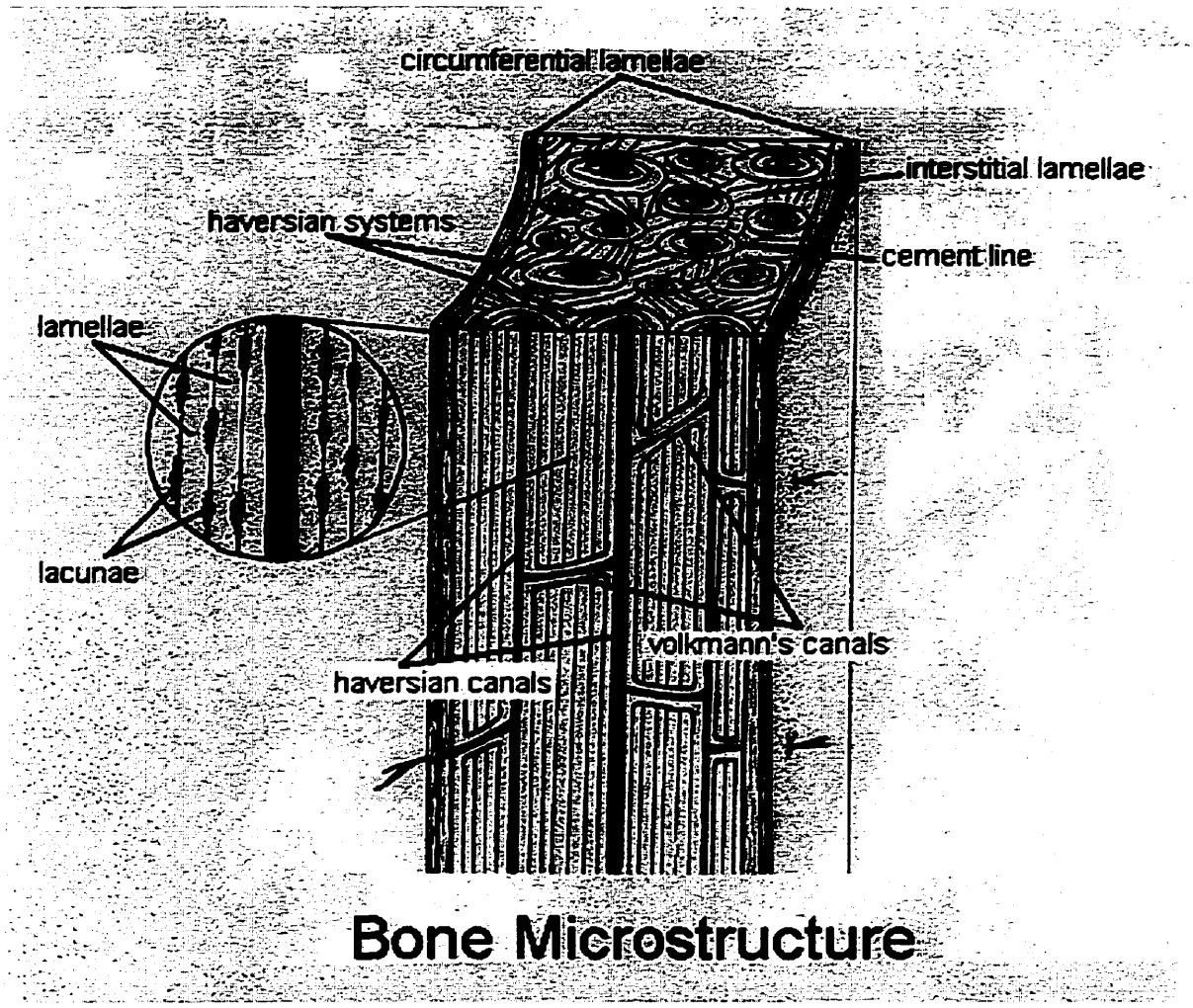
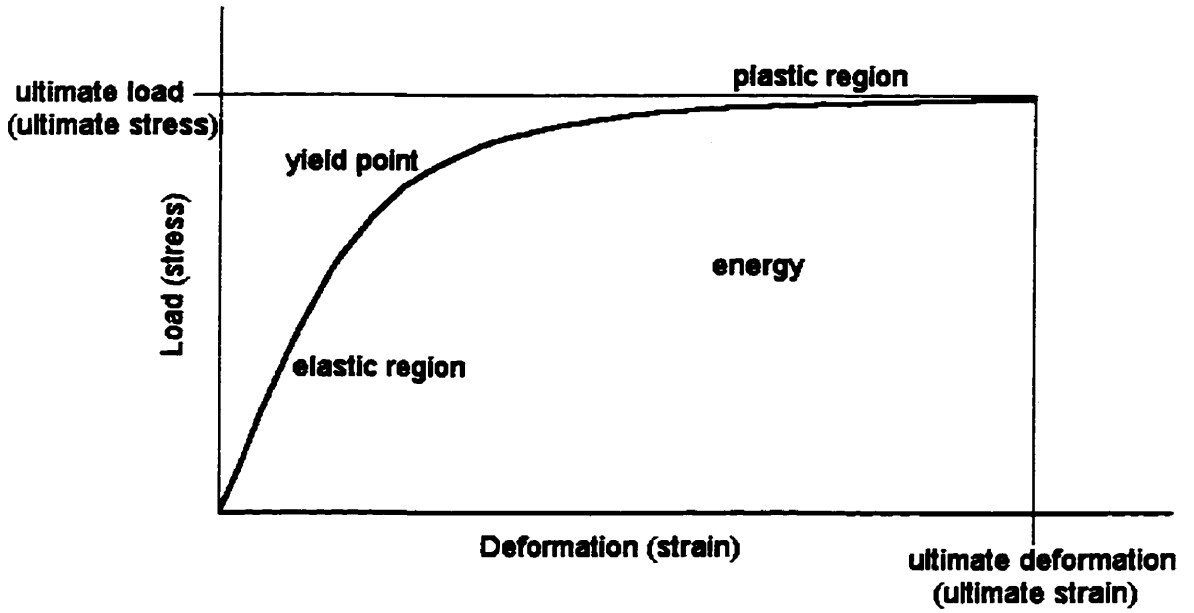


Figure B - Typical load-deformation curve for cortical bone.



2.0 - THE EFFECT OF REPEATED FREEZE-THAW CYCLES ON THE BIOMECHANICAL PROPERTIES OF CANINE CORTICAL BONE

(Submitted to Vet Comp Orthop Traumatol)

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2.1 - Side Abstract

The effect of five freeze-thaw cycles on paired canine cortical-bone specimens was evaluated using destructive and repeated nondestructive three-point bending and torsion tests. A significant decrease in destructive torsional strain and isolated significant increases in nondestructive bending and torsional modulus were most consistent with varying specimen dehydration at each thaw interval.

2.2 - Keywords and Summary

Biomechanical testing, cortical bone, repeated freezing, dog

Summary

As orthopedic investigations have become more intricate, bone specimens have sometimes undergone multiple freeze-thaw cycles prior to biomechanical testing. The purpose of this study was to determine if repeated freezing and thawing affected the mechanical properties of canine cortical bone. Six pairs of third-metacarpal bones were tested in three-point bending and six pairs of femurs were tested in torsion. One member of each pair was tested destructively at the time of collection. The other member was tested nondestructively at collection and after each of five freeze-thaw cycles; followed by destructive testing after the fifth cycle. For destructive tests, the material properties (modulus, maximum stress, maximum strain and absorbed energy) of a specimen at collection were compared to those of the corresponding contralateral specimen which had undergone five freeze-thaw cycles. For repeated nondestructive tests, the modulus of a specimen at collection was compared to modulus of the same specimen at each of the five thaw intervals. During destructive testing, there was a significant ($p = 0.02$) decrease (20%) in maximum torsional strain. Other changes in bending and torsional destructive properties were not statistically significant. During repeated nondestructive testing, there were solitary significant ($p < 0.05$) increases (8% and 9%, respectively) in both bending and torsional modulus. However, these isolated changes were not correlated to the number of freeze-thaw cycles. The pattern of alterations in destructive and nondestructive biomechanical properties was most consistent with varying specimen

dehydration at each thaw interval. Despite using accepted methods to maintain specimen hydration, repeated freezing, thawing, handling and testing of cortical bone increased the risk of moisture loss. Unless stringent efforts are made to ensure proper hydration, the mechanical properties of canine cortical bone will be altered by repeated freezing and thawing, affecting the results of studies utilizing this technique.

2.3 - Introduction

Biomechanical testing of bone is utilized extensively in orthopedic studies that evaluate metabolic diseases (13), new surgical procedures (18), or orthopedic implants (24). Storage of bone specimens is required in nearly all of such studies, especially when access to biomechanical-testing equipment is limited. Effective storage techniques also reduce the complexity and cost of these investigations through batch-processing of samples at the researcher's convenience.

Freezing has long been the most-accepted means of bone storage (26), with the biomechanical effects of a single freeze and thaw well documented. Without this technique, only the simplest biomechanical studies would be feasible. Factors that may alter mechanical properties, such as moisture loss, ice-crystal formation and enzymatic degradation, usually do not significantly influence bone subjected to one freeze-thaw cycle (5, 7, 16, 17, 19, 25). As orthopedic research has become more intricate, however, specimens have sometimes undergone multiple freeze-thaw cycles prior to biomechanical testing (9, 10, 15). In such studies, the potential for the above factors to cause significant damage increases, due to the elevated temperatures associated with each thaw interval (6, 8, 9, 17).

The biomechanical effects of repeated freezing on cancellous bone have already been investigated. After subjecting bovine cancellous specimens to five (9, 10) and eight (2) freeze-thaw cycles, no significant changes were noted during nondestructive (10) or destructive (2, 9) compression testing. However, there are no published reports of similar studies on cortical bone. Extrapolating results from cancellous samples would be unsound since: cancellous and cortical bone have markedly different mechanical

properties (11, 14), they may differ in their response to techniques of storage, sterilization or decreasing antigenicity (2, 16), and mechanical properties often differ between testing orientations (e.g., compression versus bending) (6, 11, 14). The objectives of this investigation were to evaluate the effect of repeated freezing on the biomechanical properties of canine cortical bone, and thus determine if this technique was suitable for orthopedic research.

2.4 - Materials and Methods

Six pairs of third-metacarpal bones and six pairs of femurs were collected from one-year-old clinically-normal beagles weighing 8.6 ± 0.4 kg (mean ± SEM). Specimens were collected immediately after euthanasia, which was performed for reasons unrelated to this study and for a project approved by the Animal Care Committee, University of Guelph, under guidelines from the Canadian Council on Animal Care. After removing all soft tissues, each sample was wrapped in paper towels saturated with isotonic saline solution and then placed in a sealed polyethylene bag[a]. Both ends of each femur were excised with a band saw at the metaphyses, resulting in a straight tubular segment of cortical bone. Specimens were refrigerated at 5°C until transportation, on ice, to the biomechanical-testing facility within 14 hours of collection.

Third-metacarpal bones were evaluated in three-point bending with a screw-driven mechanical testing machine[b]. Femurs were evaluated in torsion with a custom-made torsion testing machine[c]. Load and deformation data were directly recorded on a personal computer with commercial software[d]. One member (control) of each pair was randomly chosen for immediate testing to failure (destructive). Nondestructive

testing was performed on the second member of each pair, using 40% of the load applied within the elastic-deformation region (approximately 35% of failure load) of the control (19). After initial nondestructive testing (cycle 0), the second specimen was wrapped in saline-soaked paper towels, sealed within a polyethylene bag, and frozen at -20°C. At one-week intervals (cycles 1, 2, 3, 4 and 5), the specimen was thawed, tested nondestructively as described, and placed back into freezer storage. Third-metacarpal bones were thawed for eight hours at room temperature. The larger femoral specimens were thawed for 18 hours under refrigeration (5°C), followed by five hours at room temperature (22). After the fifth freeze-thaw cycle (cycle 5), each specimen was tested to failure. To confirm complete thawing, bone-marrow temperature was recorded immediately after this test. Throughout these procedures, specimens were kept moist by frequent application of isotonic saline solution or by wrapping specimens in saline-soaked paper towels.

During three-point bending tests, each third-metacarpal bone was positioned so that its dorsal surface contacted two end supports, which were 30 mm apart. To ensure identical placement for any repeated testing, the bone's contact points were marked with an indelible pen. Loading for both destructive and nondestructive tests was performed at a deformation rate of 2 mm/min, with the load point placed centrally on the specimen's palmar surface (**Addendum-Fig. A**). Immediately prior to destructive testing, the midshaft diameter of the third-metacarpal bone was determined by calculating the mean of dorsopalmar and lateromedial measurements. After failure, midshaft cortical thickness was determined by calculating the mean of dorsal, palmar, lateral and medial measurements.

During torsion tests, a square aluminum cup was initially attached to the chuck on each end of the testing machine. The proximal end of the femoral specimen was placed in the cup linked to the reaction torque cell, while the distal end was placed in the cup linked to the actuator. The femur was positioned to align its axis with the axis of rotation and to expose 58 ± 2 mm of diaphysis between the aluminum cups. After firmly seating the bone with four set screws, each cup cavity was filled with polymethylmethacrylate and allowed to cure. Thus, slippage between specimen and testing machine was prevented, and for any repeated testing, identical alignment between specimen and machine was ensured (**Addendum-Fig. B**). Loading for both destructive and nondestructive tests was performed at a rotation rate of 35°/min. Immediately prior to destructive testing, the mean diameter of the femoral diaphysis was determined by calculating the mean of craniocaudal and lateromedial measurements taken at the proximal, midshaft and distal aspects of the specimen. After failure, mean cortical thickness was determined by calculating the mean of cranial, caudal, lateral and medial measurements taken at these same three sites.

Load-deformation curves were created from recorded data. For specimens tested destructively, failure load and maximum deformation were noted. Absorbed energy was determined by the area under the entire curve (i.e., to the failure point)[c]. Stiffness was determined by the slope of the curve within its elastic region. These structural properties were then normalized, compensating for specimen geometry, to yield the analogous material properties of maximum stress, maximum strain, absorbed energy and modulus (either bending [elastic] or torsional [shear]). For specimens tested nondestructively, stiffness and modulus were calculated (**Addendum**).

To determine if significant differences were initially present between members of bone pairs, the bending or torsional modulus was compared between fresh control and contralateral fresh cycle-0 specimens, using a mixed-model analysis of variance[f]. To evaluate the results of repeated nondestructive testing, the bending or torsional modulus prior to freezing (cycle 0) was compared to that at each thaw interval (cycles 1, 2, 3, 4 and 5). This data was fitted to a mixed-model analysis of variance for repeated measures, with contrast statements between cycle 0 and each subsequent thaw interval[f]. A Bonferroni correction was applied to the contrast statements to protect against type-I error. For destructive testing, each bending or torsional material property (modulus, maximum stress, maximum strain and absorbed energy) was compared between control specimens and contralateral specimens which had undergone five freeze-thaw cycles (cycle-5 specimens), using a paired students t-test[g]. Significance was set at $p < 0.05$. To make graphic comparisons more meaningful, the initial differences between pair members (control versus cycle 0) and the results from repeated nondestructive tests (cycle 0 versus cycles 1-5) were expressed as percentage change from cycle-0 values. Results from destructive tests (control versus cycle 5) were expressed as percentage change from control values.

2.5 - Results

Prior to freezer storage, neither bending (third-metacarpal bones, $p = 0.37$) nor torsional (femurs, $p = 0.97$) modulus differed significantly between members of bone pairs. Mean differences in control specimens (bending, $-7.9 \pm 8.4\%$; torsional, $-0.8 \pm 14.5\%$) relative to contralateral cycle-0 specimens were small. However, the range of

initial differences between pair members, as indicated by each SEM, was relatively large compared to changes (means and SEMs) observed during subsequent repeated nondestructive testing of the member subjected to multiple freeze-thaw cycles (**Fig. 1**).

In nondestructive tests comparing cycle 0 against cycles 1-5, mild overall increases in both bending ($4.4 \pm 0.8\%$, $p = 0.11$) and torsional ($5.0 \pm 1.0\%$, $p = 0.04$) modulus were associated with repeated freezing. These effects, however, were primarily due to solitary significant ($p < 0.05$) increases in bending modulus ($7.5 \pm 2.9\%$) at cycle 3 and torsional modulus ($8.8 \pm 2.7\%$) at cycle 2. No temporal trends were observed (**Fig. 1**).

In destructive tests comparing controls against cycle-5 specimens, the only significant change was in maximum torsional strain, which decreased by $19.9 \pm 5.0\%$ ($p = 0.02$). A less prominent decrease in maximum bending strain ($8.7 \pm 4.8\%$, $p = 0.12$) also occurred. Although increases in modulus (bending, $12.2 \pm 11.7\%$; torsional, $27.4 \pm 27.5\%$) and maximum stress (bending, 9.8 ● 5.5%; torsional, 3.7 ● 10.2%) were observed, they did not differ significantly from control values. Minimal changes were associated with absorbed energy (bending, $-1.7 \pm 9.9\%$; torsional, $1.8 \pm 12.0\%$; **Fig. 2**). The bone-marrow temperature of cycle-5 specimens immediately after failure was 20 ± 1°C, thus confirming complete thawing of specimens at each testing interval.

2.6 - Discussion

Repeated freezing can significantly alter the biomechanical properties of canine cortical bone during both destructive and nondestructive testing. In destructive tests, which compared fresh samples to contralateral samples that had undergone five

freeze-thaw cycles, there was a significant ($p = 0.02$) mean decrease (20%) in maximum torsional strain. Other changes in bending and torsional destructive properties were not statistically significant (**Fig. 2**). In repeated nondestructive tests, which evaluated the same samples prior to freezing and at each of the five thaw intervals, there were solitary significant ($p < 0.05$) increases (8% and 9%, respectively) in both bending and torsional modulus. Since these isolated changes in modulus (bending, only cycle 3; torsional, only cycle 2) were not correlated to the number of freeze-thaw cycles, the results of biomechanical studies using fewer cycles could still be affected (**Fig. 1**).

To allow collection of multiple paired samples from each animal, different bone specimens were used for each biomechanical testing method (6). Metacarpal bones were chosen for three-point bending because they best fit the testing apparatus with minimal slippage. The straight tubular nature of femoral diaphyseal specimens was ideally suited for testing in torsion.

No significant differences in bending ($p = 0.37$) or torsional ($p = 0.97$) modulus were present between members of bone pairs prior to freezing. These results were consistent with those of other studies, which have evaluated mechanical symmetry in dog (23) and rabbit (1, 27) bone. Inadvertently during torsion tests, all left-femoral specimens were loaded in external rotation and all right-femoral specimens were loaded in internal rotation. However, members of each left-right pair were randomized between control and treatment (freezing) groups; and the straight tubular nature of femoral specimens should have helped minimize any variation associated with rotational direction. Mean differences in modulus among pair members prior to freezing were actually less during torsion (-1%) than during bending (-8%; **Fig. 1**). A previous investigation also showed

that the torsional properties of long bones do not differ significantly between internal and external rotation (27).

Despite the aforementioned symmetry among paired bone specimens, repeated nondestructive testing of the same specimen may more accurately and efficiently detect small yet significant changes induced by a treatment (e.g., freezing). If the testing method does not significantly alter mechanical properties, a smaller statistical error should occur when a specimen is compared to itself (4, 20, 21). The effect of repeated nondestructive testing on fresh cortical bone has been examined (19). When human femurs were tested in bending at 35% of their failure load for three cycles, no differences in modulus were observed among cycles. In our study, specimens were similarly loaded (approximately 35% of failure load) in bending or torsion for a total of six cycles, one prior to freezing and one at each of five thaw intervals. No consistent significant changes nor temporal trends in bending or torsional modulus were observed. Compared to the range of initial differences between members of bone pairs (bending SEM, 8.4%; torsional SEM, 14.5%), changes (means and SEMs) due to repeated freezing and nondestructive testing of the same specimen were relatively small (**Fig. 1**).

The limited cyclic loading associated with repeated nondestructive testing is also unlikely to alter a bone's failure properties during any subsequent destructive testing (26). To our knowledge, there have been no publications in which fresh specimens were tested destructively after multiple nondestructive tests. In our study, destructive tests were used to compare fresh specimens with those which had undergone five freeze-thaw cycles. Our results and the results of the aforementioned nondestructive study on fresh bone (19) do not eliminate the possibility that repeated nondestructive tests, alone, may alter

mechanical properties at failure. However, these results do indicate that modulus, maximum stress and absorbed energy are not significantly affected (**Figs. 1 and 2**).

Maintaining the water content of bone is a principal factor in preserving its biomechanical properties. As bone dehydrates, it becomes stiffer and more brittle (7). Although these changes are more pronounced at room temperature (8), gradual moisture loss through evaporation still occurs at -20°C, the temperature of conventional freezers (7, 17). At freezer temperatures above -28°C, ice crystals within the bone may also enlarge, potentially causing structural damage through microcracking (3, 17). No significant change in mechanical properties has been observed in cortical specimens which have undergone a single -20°C freeze and subsequent thaw (5, 7, 16, 17, 25). With multiple freeze-thaw cycles, however, the potential for damage should increase due to greater dehydration associated with each thaw interval and increased ice-crystal formation associated with each freeze.

Enzymatic degradation may also alter the mechanical properties of bone (6, 17). Although reduced, the activity of collagenase and proteases is not completely arrested at freezer temperatures of -20°C. In previous investigations, cortical specimens were stored at -20°C (17, 25) and at room temperature (12, 17) for periods longer than those respectively accumulated during the multiple freeze-thaw cycles in our study. No significant alterations in mechanical properties were observed. Therefore, it is unlikely that enzymatic degradation contributed substantially to any changes observed in our specimens.

When bone loses moisture, its modulus (stiffness) and the stress (load) required to cause failure may increase, but the strain (displacement) and energy absorbed at failure

usually decrease (6, 17, 19, 26). Similar trends in this study suggest that specimen dehydration was the primary cause of any altered biomechanical properties. During destructive bending and torsion tests, mean increases in modulus (bending, 12%; torsional, 27%) and maximum stress (bending, 10%; torsional 4%) were observed, along with mean decreases in maximum strain (bending, 9%; torsional, 20%) (**Fig. 2**). However, only the decrease in maximum torsional strain was statistically significant ($p = 0.02$). Absorbed energy remained essentially unchanged (bending, -2%; torsional, 2%). During repeated nondestructive testing, small overall increases in bending (4%, $p = 0.11$) and torsional (5%, $p = 0.04$) modulus were also noted (**Fig. 1**). These changes, however, were significant ($p < 0.05$) only at cycle 3 (8%) during bending and at cycle 2 (9%) during torsion. Perhaps specimen hydration was not maintained as well during these isolated cycles as during the other thaw intervals.

Despite our use of accepted methods to maintain bone hydration (9, 15, 17), repeated freezing, thawing, handling and testing of cortical samples increased the potential for moisture loss. Unless stringent efforts are made to ensure proper hydration, the mechanical properties of canine cortical bone will be altered by repeated freezing, affecting the results of studies utilizing this technique. Additional methods which could have been employed in our study include freezing samples in saline solution and maintaining thawed samples in a saline bath before and after testing procedures (6, 7, 19).

2.7 - Footnotes

[a] Whirl Pak, Nasco Plastics, New Hamburg, Ontario, Canada

[b] Model 1011, Instron Corp, Canton, MA, USA

[c] Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada

[d] Easyest LX, Asyst Software Technologies Inc, Rochester, NY, USA

[e] Sigmaplot 4, SPSS Inc, Chicago, IL, USA

[f] Proc MIXED in SAS, Statistical Analysis System, Release 6.12, SAS Institute Inc, Cary, NC, USA

[g] Proc MEANS in SAS, Statistical Analysis System, Release 6.12, SAS Institute Inc, Cary, NC, USA

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2.9 - Figures

Figure 1 - Percent change (mean ± SEM) in nondestructive bending and torsional modulus, relative to the treatment-limb value at the time of collection (cycle 0). Control limb = corresponding contralateral limb at the time of collection; cycles 1-5 = treatment limb after each of five freeze-thaw cycles; * = significant difference from cycle 0 ($p < 0.05$).

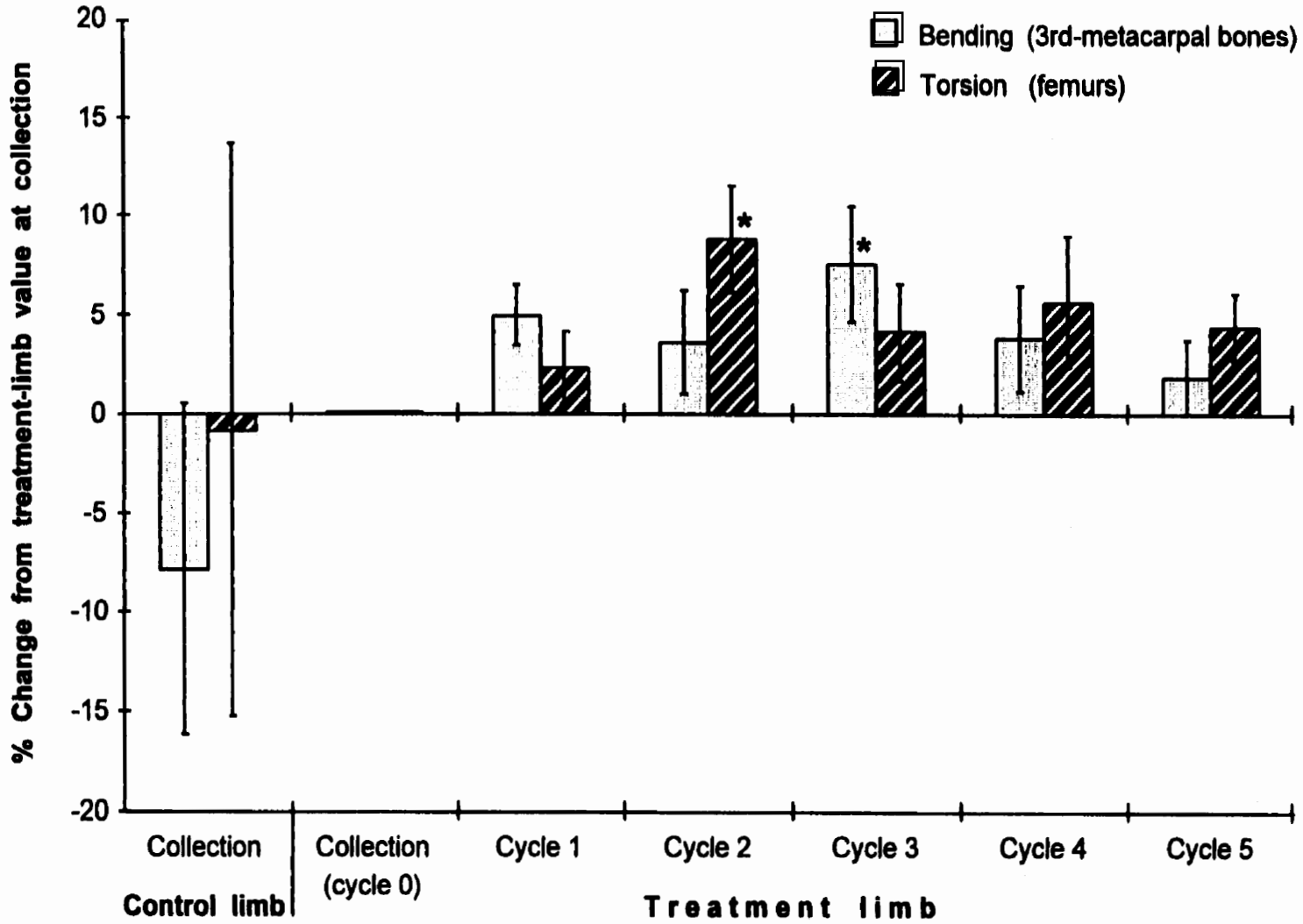
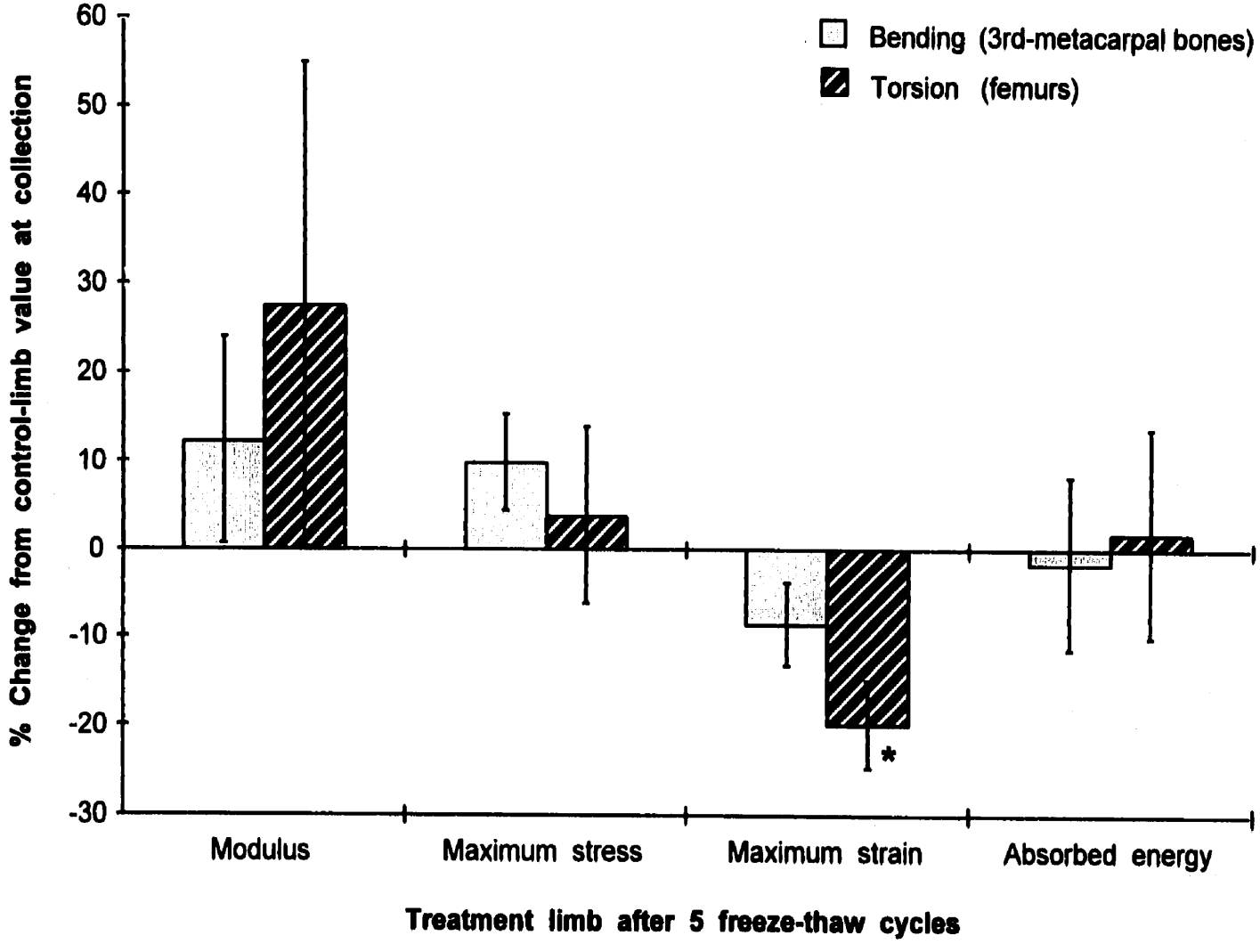

Bending (3rd-metacarpal bones)
Torsion (femurs)
% Change from treatment-limb value at collection
20
15
10
5
0
-5
-10
-15
-20
*
*
Collection
Collection
(cycle 0)
Cycle 1
Cycle 2
Cycle 3
Cycle 4
Cycle 5
Control limb
Treatment limb

Figure 2 - Percent change (mean ± SEM) in destructive bending and torsional modulus, maximum stress, maximum strain and absorbed energy of the treatment limb after five freeze-thaw cycles (cycle 5), relative to the contralateral control-limb value at the time of collection. * = significant difference from control limb ($p < 0.05$).


Bending (3rd-metacarpal bones)
Torsion (femurs)
% Change from control-limb value at collection
60
50
40
30
20
10
0
-10
-20
-30
*
Modulus
Maximum stress
Maximum strain
Absorbed energy
Treatment limb after 5 freeze-thaw cycles

2.10 - Addendum

Bending - Structural Properties *Units*

Length between supports (L) ..... mm

Midshaft diameter (d) ..... mm
(Mean of dorsopalmar & lateromedial measurements)

Midshaft cortical thickness (t) ..... mm
(Mean of dorsal, palmar, lateral & medial measurements)

Load at failure (F_{max}) ..... N

Displacement at failure (D_{max}) ..... mm

Stiffness (S) ..... N/mm

Absorbed energy (U) = Area under curve ..... J

Bending - Material Properties and Formulas *Units*

Midshaft cross-sectional area (A) = $\pi[d^2 - (d - 2t)^2]/4$ ..... mm^2

Moment of inertia (I) = $\pi[d^4 - (d - 2t)^4]/64$ ..... mm^4

Elastic modulus (E) = $SL^3/48I$ ..... MPa

Maximum bending stress (σ_{max}) = $F_{max}Ld/8I$ ..... MPa

Maximum bending strain (ε_{max}) = $D_{max}6d/L^2$ ..... %

Absorbed energy (U) = (Area under curve)/A ..... J/cm^2

Torsion - Structural Properties *Units*

Length of exposed specimen (L).....mm

Diameter: proximal, midshaft or distal (d).....mm
(Mean of craniocaudal & lateromedial measurements)

Mean of proximal, midshaft & distal sites (d_{av}).....mm

Cortical thickness: proximal, midshaft or distal (t).....mm
(Mean of cranial, caudal, lateral & medial measurements)

Mean of proximal, midshaft & distal sites (t_{av}).....mm

Torque load at failure (T_{max}).....N·mm

Angular displacement at failure (θ_{max}).....degrees

Stiffness (S).....N·mm/rad

Absorbed energy (U) = Area under curve.....J

Torsion - Material Properties and Formulas *Units*

Volume of exposed specimen (V) = $L\pi[d_{av}^2 - (d_{av} - 2t_{av})^2L]/4$.....$mm^3$

Polar moment of inertia:

Proximal, midshaft or distal (J) = $\pi[d^4 - (d - 2t)^4]/32$.....mm^4

Mean of proximal, midshaft & distal sites (J_{av}) = $\pi[d_{av}^4 - (d_{av} - 2t_{av})^4]/32$.....$mm^4$

Shear modulus (G) = SL/J_{av}.....MPa

Maximum shear stress (τ_{max}) = $T_{max}(d/2)/J$.....MPa
(Maximum of proximal, midshaft & distal sites)

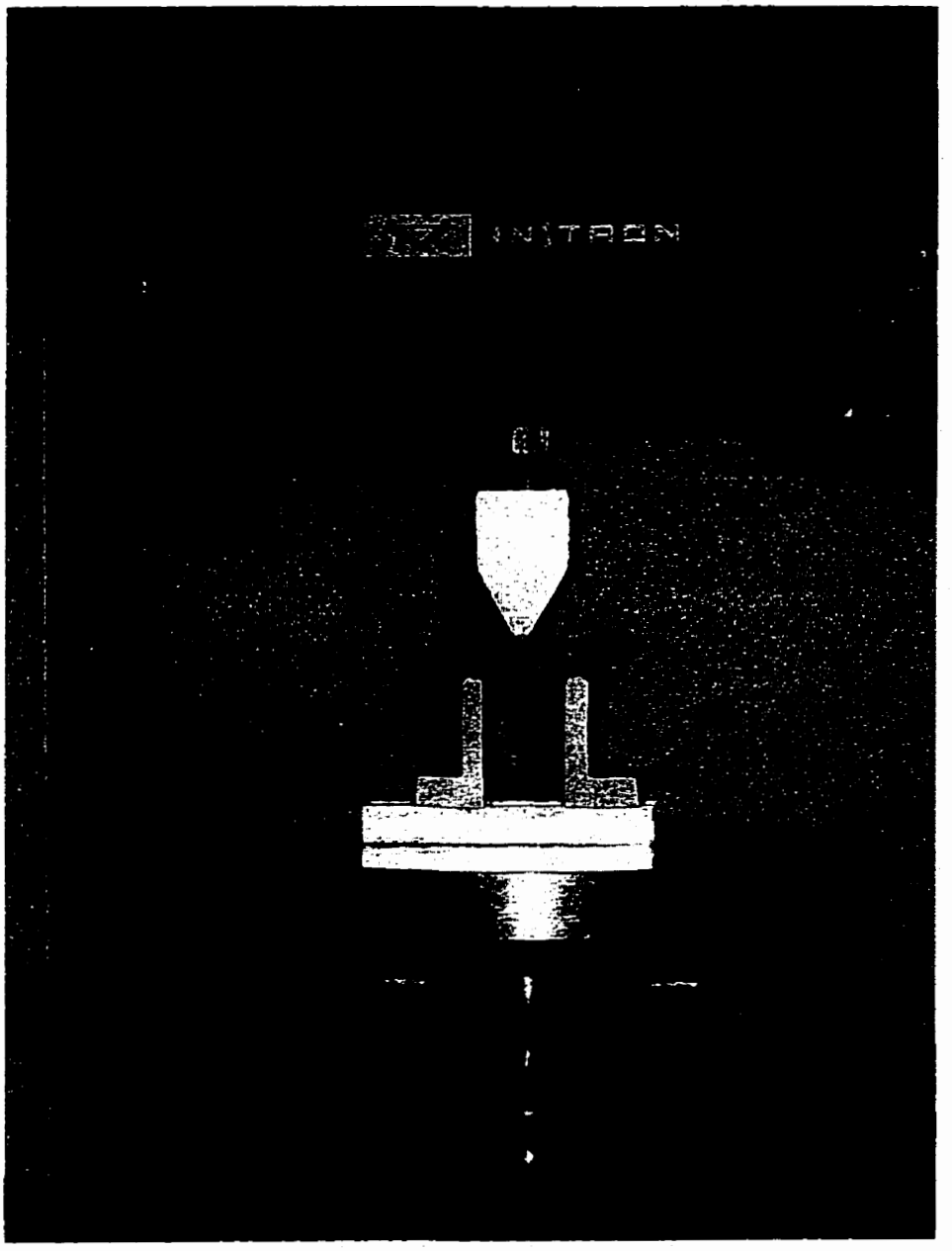
Maximum shear strain (γ_{max}) = $\theta_{max}\pi d_{av}/360L$.....%

Absorbed energy (U) = (Area under curve)/V.....J/cm^3

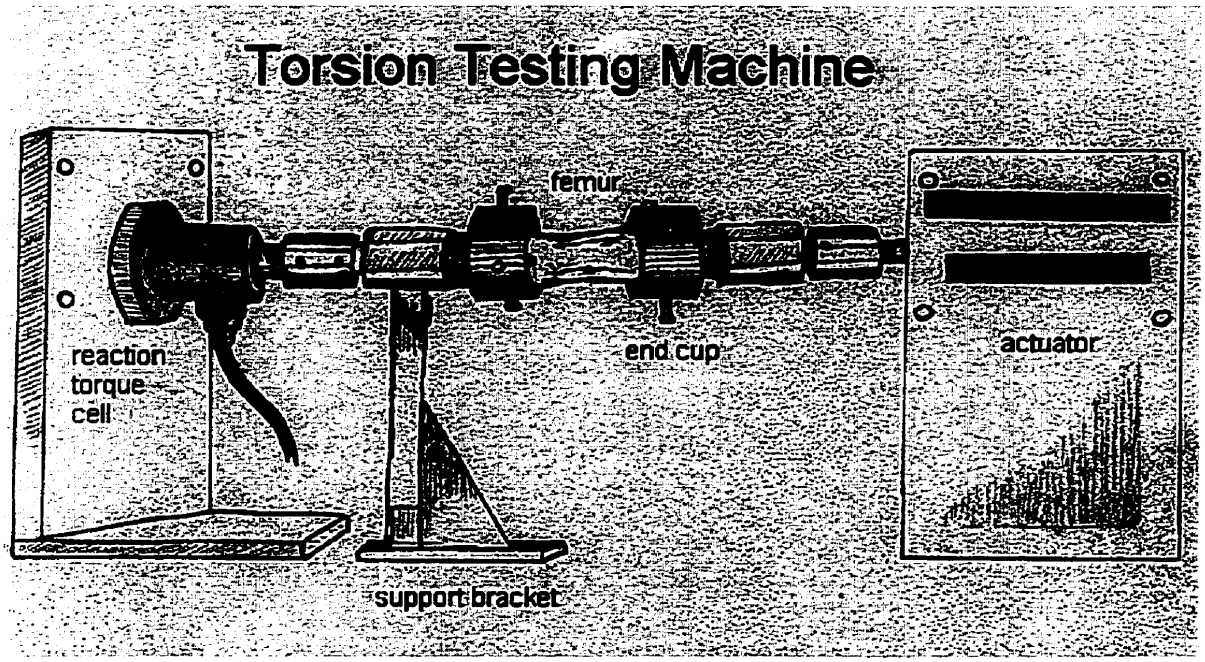
Addendum: Table A – Material properties (mean ± SEM) for nondestructive (modulus) and destructive (modulus, maximum stress, maximum strain and absorbed energy) testing in three-point bending and torsion. Control limbs were tested destructively at the time of collection. Contralateral treatment limbs were tested nondestructively at the time of collection (cycle 0) and after each of five freeze-thaw cycles (cycles 1-5), followed by destructive testing after the fifth cycle.

	Three-point bending		Torsion	
	Control limb	Treatment limb	Control limb	Treatment limb
Non-destructive testing				
Modulus (MPa): Cycle 0		7,535 ± 423		1,964 ± 77
Cycle 1		7,902 ± 446		2,011 ± 91
Cycle 2		7,793 ± 445		2,130 ± 65
Cycle 3		8,069 ± 381		2,041 ± 72
Cycle 4		7,805 ± 414		2,102 ± 80
Cycle 5		7,789 ± 518		1,999 ± 79
Destructive testing				
Modulus (MPa)	6,841 ± 482	7,789 ± 518	1,952 ± 274	2,134 ± 149
Maximum stress (MPa)	194 ± 7	212 ± 7	59 ± 5	59 ± 3
Maximum strain (%)	7.8 ± 0.5	7.1 ± 0.6	5.5 ± 0.4	4.4 ± 0.4
Absorbed energy (Bending = J/cm^2, Torsion = J/cm^3)	3.72 ± 0.28	3.64 ± 0.57	1.12 ± 0.10	0.95 ± 0.09

Addendum: Figure A - Mechanical testing machine configured for three-point bending[b].



Addendum: Figure B - Custom-made torsion testing machine[c].



3.0 - LIMITATIONS AND FUTURE AREAS OF STUDY

Despite using accepted methods of maintaining specimen hydration, repeated freezing, thawing, handling and testing of cortical bone resulted in altered mechanical properties due to moisture loss. Future studies should be aimed at determining if more stringent specimen storage and rehydration techniques would prevent this problem.

In destructive and nondestructive tests, moisture loss caused an increase in modulus of specimens loaded in bending and torsion. These changes, however, were not statistically significant during destructive tests, and were isolated, with no temporal trends, during repeated nondestructive tests. It would have been useful to test specimens destructively at each thaw interval, in order to evaluate all mechanical properties (modulus, maximum stress, maximum strain and absorbed energy). Thus, it may have been possible to determine the number of freeze-thaw cycles which elapsed before failure properties were significantly altered. However, testing in this fashion would have required a much larger sample size.

A progressive increase in moisture loss likely occurs with increased specimen freezing, thawing and handling. Changes in failure properties were more pronounced in femurs, which were tested in torsion, than in third-metacarpal bones, which were tested in three-point bending. Femurs were subjected to more involved handling and longer thawing intervals. Because bone contains only a small amount of water (8%) (Markel 1996a), shorter periods of thawing may be used than for tissues with higher water content. Only limited data is available on the duration of thawing needed for cortical bone specimens (Stromberg & Dalen 1976a). Studies to standardize thawing procedures and to evaluate thawing in a saline bath would be beneficial.

3.1 - GENERAL CONCLUSIONS

As orthopedic investigations have become more involved, repeated freezing as a storage technique has become necessary. The purpose of this study was to determine if repeated freezing altered the mechanical properties of canine cortical bone. Accepted methods of maintaining specimen hydration were used in this study. However, repeated freezing, thawing, handling and testing of cortical bone resulted in altered mechanical properties due to moisture loss. If repeated freezing and thawing of bone specimens is to be used by researchers, the minimum number of freeze-thaw cycles necessary should be utilized. Stringent methods of maintaining bone moisture, such as freezing in saline solution or storage in a saline bath during specimen handling, should be performed.

4.0 - MASTER REFERENCE LIST

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