

**AN EVALUATION OF STORAGE, HYDRATION AND REPORTING PROTOCOLS
FOR BIOMECHANICAL TESTING OF THE RAT FEMORAL NECK**

A Thesis

**Submitted to the Graduate Faculty of the Louisiana State University
and Agricultural and Mechanical College
in partial fulfillment of the requirements for the degree of
Master of Science**

in

The School of Human Ecology

**by
Michael Wayne Reeves
B.S., Louisiana State University, 1996
May 2002**

ACKNOWLEDGEMENTS

The author wishes to express his deep appreciation to his major professors, Dr. Michael Keenan and Dr. Maren Hegsted, for their continued guidance, support, and understanding throughout this project. Special thanks goes to Dr. Yan Chen for serving on the committee and sharing his knowledge of engineering with the author and other committee members. The author is also grateful to Ms. Gabie Church for her patient direction with the statistical analysis portion of this project.

Much thanks is expressed to Karen Jones, who acquired the test animals needed for this study and kept the lab organized and running smoothly. Dr. Nena Cross, who encouraged the author to pursue graduate studies and inspired confidence when times got tough, is given his special gratitude.

The author expresses his deepest love and respect to his wife, children, and parents for their patience, support, encouragement, and sacrifices, without which the completion of his graduate studies would not have been possible. Finally, thanks is given to God for giving the author the strength and comfort to persevere to see this project to completion.

TABLE OF CONTENTS

Acknowledgements	ii
Table of Contents	iii
List of Tables	v
List of Figures	vi
Definition of Terms	vii
Abstract	ix
Introduction	1
Statement of Problem	1
Objective	3
Research Questions	4
Review of Literature	5
Osteoporosis in Humans	5
Rat Model of Post-menopausal Osteoporosis	11
Biomechanical Testing of Bone	14
Biomechanical Studies of the Rat Femoral Neck	20
Materials and Methods	24
Study Design	24
Hydration Under Vacuum	24
Density Measurement	25
Femoral Neck Mechanical Testing	25
Design of Experiments	28
Data Collection	30
Statistical Analysis	30
Results	31
Density	31
Breaking Measurements	32
Discussion	41
Breaking Measurements	41
Terminology and Units of Measurement	46
Frozen Storage of Bones	48
Bone Hydration	54

Study Limitations	65
Recommendations For Future Studies	66
Summary and Conclusions	68
Summary	68
Conclusions	69
References	71
Appendix A: Mechanical Testing Procedure	76
Appendix B: Photos of Mechanical Testing Equipment	77
Vita	84

LIST OF TABLES

1.	Post-treatment Density Change (intra-sample) for 15-day Frozen Storage	35
2A.	Post-treatment Density Change Between Fresh & Post-freeze Groups for 34-day Frozen Storage	35
2B.	Post-treatment Density Change Within Post-freeze Group for 34-day Frozen Storage	35
3A.	Post-treatment Density Change Within Fresh, Post-freeze & Vac3 for 122-day Frozen Storage	36
3B.	Post-Treatment Density Change Within Post-freeze Group for 122-day Frozen Storage	36
3C.	Post-Treatment Density Change Within Vac3 Group for 122-day Frozen Storage	36
4A.	Post-Treatment Density Change Between Vac1 & Vac3 for 831-day Frozen Storage	37
4B.	Post-treatment Density Change Within Vac1 Group for 831-day Frozen Storage	37
4C.	Post-treatment Density Change Within Vac3 Group for 831-day Frozen Storage	37
5.	Breaking Measurements for 34-day Frozen Storage	38
6.	Breaking Measurements for 122-day Frozen Storage	39
7.	Breaking Measurements for 831-day Frozen Storage	40

LIST OF FIGURES

1.	Load-Deformation Curve	44
2.	Stress-Strain Curve	45
3.	Effect of Freezing on Breaking Values	60
4.	Effect of Freezing on Plastic Strain	61
5.	Effect of Hydration on Breaking Values – Fresh vs. Hydrated (122-Day Storage)	62
6.	Effect of Hydration on Breaking Values – Frozen vs. Hydrated (122-Day Storage)	63
7.	Effect of Hydration on Plastic Strain	64

DEFINITION OF TERMS

Breaking Load or Strength

Maximum load a material can withstand before it fails

Breaking Stress or Strength

Maximum stress a material can withstand before it fails

Density

Relationship of the mass of a material to its volume, defined as mass/unit volume [g/cm^3]

Ductility

Extent to which a material can sustain plastic deformation without failure

Elastic Limit

Greatest stress that can be applied to a material without causing permanent deformation.

Elastic Modulus

Rate of change of strain as a function of stress. The slope of the straight line portion of a stress-strain diagram. It also is called stress-strain ratio or Young's modulus.

Elasticity

Ability of a material to return to its original shape when load causing deformation is removed.

Gauge Length

Distance from the top of the femoral head to the the point where the femoral shaft is embedded in the resin. Used by the Instron testing machine to calculate non-vertical deflection of the specimens during axial loading

Load-Deformation Curve

Graph of applied load as a function of deformation, not normalized for size, shape, or structure of the material being tested. Provides a measure of the structural or extrinsic properties of a substance.

Plastic Strain

Deformation that remains after the load causing it is removed. It is the permanent part of the deformation beyond the elastic limit of a material.

Plasticity

Tendency of a material to remain deformed after the stress causing it is removed.

Stiffness

Measure of resistance of plastics to bending.

Strain

Change in length of a material relative to its original length, usually expressed % Strain.

Stress

Force applied to a material divided by the cross-sectional area through which it acts. Normalizes for material size and shape.

Stress-Strain Diagram

Graph of applied stress as a function of strain, normalized for size, shape, or structure of the material being tested. Provides a measure of the intrinsic properties of a material.

Toughness

Total area under the stress-strain curve, combining the elastic and plastic regions, that represents the energy absorbing capacity of the material.

Yield Strain at Failure

Maximum deformation a material can withstand before it fails under stress

Yield Stress at Failure

Maximum stress (applied force) a material can withstand before it experiences permanent deformation. Also called the elastic limit.

Yield Point

Point on the stress-strain curve where any additional stress (applied force) causes permanent deformation even after the stress is removed.

Yield Strength

Maximum stress (applied force) a material can withstand without experiencing permanent deformation after the stress is removed.

Yield Point Energy

The energy or work a material can absorb and still return to its original shape once the stress is removed.

Young's Modulus

Slope of the stress-strain curve that represents intrinsic stiffness of a material normalized for size and shape.

ABSTRACT

The current study evaluated the effect of frozen storage and hydration under vacuum on density and breaking strength of the rat femoral neck. Femurs were frozen in saline for periods of 15, 34, 122, or 831 days.

No significant effect on bone density was detected for freezing periods of 15, 34, and 122 days, indicating that frozen storage of specimens in saline is moisture-preserving for periods up to four months. Freezing periods of 34 and 122 days were used to examine the effect of frozen storage on bone biomechanical behavior. Plastic strain increased for the 34-day storage period ($p=0.0453$) and decreased for the 122-day storage period ($p<0.0001$). Strain to failure ($p<0.0001$) and yield strain ($p<0.0001$) decreased and Young's modulus ($p=0.0018$) increased after 122 days of frozen storage.

Hydration for one hour after the 15-day storage period significantly decreased density compared to fresh ($p=0.0407$) and frozen-stored ($p=0.0008$) specimens. In the 122-day storage experiment, hydration for three hours significantly decreased density compared to the frozen-stored bones, both between ($p=0.0059$) and within samples ($p=0.0270$). Hydration did not significantly alter the density of bones frozen for 831 days.

Hydration of bones frozen for 122-days decreased yield strain ($p=0.0100$) and strain at failure ($p=0.0214$) compared to fresh bones. Plastic strain ($p=0.0474$) and strain at failure ($p=0.00116$) both increased and Young's modulus decreased for hydrated bones compared to frozen-stored bones. Bones frozen for 831 days and hydrated for either one hour or three hours showed an increase in plastic strain ($p=0.0469$) with the longer hydration time.

These results indicate frozen storage for up to 122 days does not affect bone density, but does alter the biomechanical behavior of the rat femoral neck for storage periods as short as 34 days. Hydration decreases density in bones frozen for up to 122 days, but extending the frozen storage period to 831 days prevents additional dehydration of stored bones. The biomechanical behavior of the rat femoral neck is affected by hydration for bones frozen for both 122 and 831 days.

INTRODUCTION

Statement of Problem

According to the National Institutes of Health (NIH), osteoporosis is a major health concern for over 28 million Americans. Both men and women with osteoporosis are susceptible to bone fractures following minimal trauma. By age 50, one of every two women and one of every eight men in America will experience a bone fracture related to osteoporosis. Incidents of fracture total over 1.5 million annually, with approximately 300,000 being fractures of the hip.¹ With an aging American population, the number of hip fractures is expected to increase to 650,000 annually by the year 2050.² Approximately fifty percent of women² and seventeen percent of men experience a hip fracture by the age of ninety.^{3,4} Almost twenty-four percent of the Americans who experience hip fractures after the age of fifty have a survival rate of less than one year.^{1,2,6} Although only twenty to thirty percent of all hip fractures occur in men^{4,5} the one-year mortality rate for men is nearly twice that of women.^{1,7}

In order to study the effects of environmental and remedial agents on bone properties, the rat has been used extensively as a model of osteoporosis. Although the rat has certain limitations when modeling human disease, the NIH considers it to be one of the most appropriate experimental models, offering “unique advantages” and “important strengths.”⁸ Over 500,000 publications during the last thirty years have reported on studies involving the rat as an experimental model, according to the NIH.⁸

A wide variety of techniques are utilized as researchers attempt to gain an understanding of bone status in osteoporosis studies. These include dual energy x-ray absorptiometry and density measurement (mineral content), ultrasound (mass), markers found in blood and urine

(formation and resorption), biopsies (architecture and remodeling), and mechanical testing (biomechanical behavior).

Ex-vivo biomechanical testing is commonly used in rat studies to examine the effect of environmental and remedial agents on bone behavior. Subjecting the femoral neck to breaking tests has gained popularity in studies aimed at investigating the causes and possible prevention of hip fractures in conjunction with osteoporosis. Biomechanical testing of the femoral neck provides a direct measure of how much stress is actually needed to cause a hip fracture.

Although

other indicators provide useful information about bone status in relation to the development and treatment of osteoporosis, they are not true indicators of actual bone strength.

Even though biomechanical testing of the femoral neck has gained popularity, no standardization has been adopted by researchers for designing and reporting the results of these types of studies. Protocols for specimen preparation, storage, hydration, and mechanical testing vary widely between studies. Bones may be tested immediately after removal from the animal or kept in frozen storage until later testing. Storage methods vary, with bones being stored either wet or dry, with or without musculature, and at different temperatures. Specimens may or may not be rehydrated prior to testing, and rehydration methods and times also differ. Such variability in testing protocols introduces a degree of uncertainty when attempting to compare study results.

Another disparity between femoral neck breaking studies is the use of different methods to report test results. Researchers report a wide variety of measured variables in an attempt to present a comprehensive picture of bone biomechanics. This problem is compounded when

mechanical breaking values are reported with non-standard terminology and units of measurement. Results may be reported as extrinsic values, dependent on individual bone characteristics, or as intrinsic values, normalized to be independent of bone size and shape. This variability between reporting techniques makes cross-correlation of study results difficult, and often produces data which are meaningless for their intended purpose.

Objective

The overall purpose of this study is to construct a basic model of the biomechanical behavior for the rat femoral neck. This will be accomplished by identifying storage and hydration effects, determining which measured values are pertinent, and reporting these values in a standardized format.

The first objective is to determine which measured values should be used to report femoral neck breaking values. This will eliminate presenting values which are inaccurate, irrelevant, or repetitive. By limiting reported values to those determined to be pertinent, a more accurate representation of femoral neck biomechanical behavior can be provided to future researchers.

The second objective is to identify the appropriate terminology and units of measurement for reporting femoral neck breaking values. Reporting values in a standardized format will allow researchers to utilize the data from this study when conducting future studies involving rat femoral neck breaking tests.

The final objective is to examine the impact of storage and hydration methods on density and femoral neck breaking values. Four experiments will be performed to provide baseline data

on density and bone mechanical breaking values in response to frozen storage and hydration under vacuum. Femoral neck density and mechanical test results will be evaluated statistically to identify correlations between these values and the storage and hydration techniques used prior to testing. Both between-group and within-group examinations will be conducted to detect significant interactions.

Research Questions

This study will attempt to provide a clearer understanding of how density and breaking measurements of the rat femoral neck are affected by storage and hydration methods prior to testing, and how they combine to give an overview of the biomechanical behavior of the rat femoral neck. Specifically, this study seeks to answer the following questions regarding the rat femoral neck:

- 1) What terminology and units of measurement are most appropriate to report test results for biomechanical values?
- 2) What combination of measured values gives the best overall representation of femoral neck biomechanical behavior?
- 3) What effects do storage and hydration protocols have on individual biomechanical breaking and density values?
- 4) Do pre-test storage and hydration protocols, individually or combined, alter the overall representation of biomechanical behavior?

REVIEW OF LITERATURE

Osteoporosis in Humans

Osteoporosis Overview

Bone fractures associated with osteoporosis pose a major health risk throughout the world, with the elderly being especially vulnerable to fractures of the hip. As the world population ages because of decreased birth rates and increased life span, hip fractures related to osteoporosis will become a global health problem.⁹ Worldwide cases of osteoporosis-related hip fractures are estimated to increase from 1.66 million to 6.25 million each year by the year 2050.¹⁰ Fractures related to osteoporosis total over 1.5 million incidents annually in the United States, with approximately 300,000 being fractures of the hip.¹ By the year 2050, the number of hip fractures in the United States is estimated to increase to 650,000 annually. During the same time span, the yearly incidence of hip fracture in Europe is forecasted to increase from 414,000 to 972,000.¹¹ While most hip fractures presently occur in North America and Europe, worldwide demographic shifts in age and population will most likely alter this trend. The World Health Organization predicts that by the year 2050 the developing countries in Africa, Asia, and South America will account for nearly 75% of all hip fractures.⁹

In the United States, approximately fifty percent of women and seventeen percent of men will experience a hip fracture by the age of ninety.^{2,3,4} Figures for European countries indicate approximately thirty percent of women and eleven percent of men will suffer a hip fracture by the age of eighty.¹¹ Complications from hip fracture often have a fatal outcome, with twenty-four percent of Americans who experience a hip fracture after the age of fifty dying in less than a year.^{1,2,6} While men account for about twenty to thirty percent of the total incidence of hip

fracture,^{4,5} they have nearly double the one-year mortality rate of women.^{1,7} Less than sixty percent of those who survive can walk unaided a year after a hip fracture, and as many as one-fourth will spend the rest of their lives in a long-term care facility.^{1,7}

Etiology of Osteoporosis

The rate of bone resorption in humans generally exceeds the rate of formation after the age of forty, resulting in a gradual decline in bone density throughout the remaining years of life. The bone loss associated with osteoporosis was considered a consequence of aging until 1947 when Fuller Albright proposed the existence of a second type of osteoporosis associated with the onset of menopause.¹² Albright's theory failed to gain appreciable acceptance because the scientific community was unable to verify his suggestion of a hormonal deficiency as the causal agent in postmenopausal osteoporosis. Over two decades later, Nordin (1971) again proposed the existence of two distinct forms of osteoporosis to explain the accelerated bone loss which could not be accounted for by advancing age.¹³

Albright's (1947) findings received little acceptance until more than three decades later when a study of bone loss in postmenopausal women was conducted by Riggs et al. using dual-photon absorptiometry.¹⁴ Changes in bone density were examined in 123 healthy women ranging in age from 40 to 80+ years. Differing patterns of bone loss at various ages and between bone types were noted, with vertebral fractures (mainly trabecular bone) increasing sharply between the ages of 51 and 65, while hip fractures (trabecular and cortical bone) rose drastically at 75+ years of age. Riggs et al. concluded this accelerated loss of trabecular bone was associated with the postmenopausal cessation of estrogen production. The superposition of this more rapid phase of

trabecular bone loss upon the slower age-related loss of both bone types was suggested as the mechanism behind the sudden increase in hip fractures in advanced age.

In 1983, two physicians from the Mayo Clinic, Melton and Riggs, found the number of hip fractures in women to be twice that of men after age 75, which supported the theory of a postmenopausal mechanism of osteoporosis.¹⁵ Their research provided evidence for a dual etiology of osteoporosis which correlated closely to Albright's original theory.¹⁶

In 1990 Nordin et al. examined 87 pairs of women matched according to their years since menopause to determine how bone loss is affected by age and years since menopause. They found that by the age of seventy the amount of bone loss associated with menopause was eleven percent, while age accounted for eighteen percent. These results provide evidence that menopause has a significant effect on bone loss that is independent of the normal age-related loss.¹⁷

Rate of Bone Loss

Accelerated bone loss following menopause appears to compound the effects of the slower age-related bone loss seen almost universally in the elderly. This superposition of an earlier phase of rapid bone loss on the slower age-related phase may account for the fact that elderly women experience nearly twice as many hip fractures as their male counterparts.¹⁸ Riggs et al. (1998) proposed the theory that both the accelerated bone loss associated with the onset of menopause and the slower age-related bone loss are the result of estrogen deficiency.¹⁹

Study results regarding an accelerated phase of bone loss following menopause were sometimes conflicting. For example, Riggs et al. (1986), using dual-photon absorptiometry,

found both premenopausal and postmenopausal bone loss to be similar, with a mean loss rate of 1.2% per year.²⁰ In contrast, using computed tomography, Genant et al. (1982) found a 12% loss rate within two years after ovariectomy.²¹ A possible explanation of these varying results could lie in the fact that surgically-induced menopause is abrupt, while the hormonal changes associated with natural menopause are gradual. Differences in measurement techniques may also partially explain these conflicting results.

A sixteen-year study by Ahlborg et al. measured bone density in the forearm using single-photon absorptiometry to determine how menopause affects bone loss. Their results showed an accelerated period of bone loss following menopause during the first five years, followed by a slower phase over the next six years.²² Chittachoen et al. used dual energy X-ray absorptiometry to examine bone loss in the distal radius, midradius, femoral neck, lumbar spine and total body in two groups of women with surgically-induced and natural menopause. Their findings indicated a higher bone loss in the lumbar spine and distal radius during the first nine years after oophorectomy, typical with that seen in natural menopause. After nine years of menopause, bone loss was greater at the femoral neck, comparable to the slower phase of age-related bone loss.²³

Age of Menopause Onset

Studies investigating the effects of age at onset of menopause have produced conflicting results. Two large studies examined the correlation between a woman's age at the onset of menopause and her risk of hip fracture. Kiel et al. conducted a study using a cohort of the Framingham Heart Study and found no correlation between menopause onset age and hip

fracture risk.²⁴ Paganini-Hill et al. conducted a cohort study of 8600 postmenopausal women in California and also found no relationship between the risk of hip fracture and the age of menopause onset.²⁵

In contrast, Vega et al. (1996) compared 102 women with osteoporosis who had experienced a hip fracture to a control group without a history of osteoporosis or hip fracture. They found that the osteoporotic group had a significantly lower age at onset of menopause than the control group, indicating a positive correlation between early menopause and the incidence of hip fracture and osteoporosis.²⁶

In another study Vega et al. (1994) examined the age at onset of menopause on three different skeletal sites in 1050 osteoporotic women who had experienced a vertebral fracture. The subjects were categorized according to early, normal, and late menopause, with 45 and 52 years of age being the boundary points. Their results showed that women who experience early menopause have a significantly greater risk of hip fracture.²⁷

Conflicting results have also been reported in studies examining the effect of menopause onset age on bone mineral density. Soda et al. (1993) studied 166 postmenopausal Japanese women, divided into groups according to whether their menopause had occurred before or after the age of fifty. They determined that the women who experienced menopause before the age of fifty were significantly more likely to have a lower bone mineral density than those who had a later menopause.²⁸ The above-mentioned study by Vega et al. (1994) also determined that the early menopause group had a lower bone mineral density in the spine, femur, and radius, indicating a lower overall skeletal bone mineral density for women who experience earlier menopause.²⁷

Pouilles et al. (1994) conducted a study investigating the effect of age at menopause on vertebral bone mineral density. They found that women with early menopause had a lower vertebral bone mineral density than those who had undergone a normal menopause, and were therefore more prone to developing osteoporosis.²⁹

Ohta et al. (1996) examined bone mineral density in the second to fourth vertebrae to determine if early menopause increased the risk of osteoporosis. Menopause before the age of forty-three was considered early, with menopause after the age of forty-three considered normal. Their results showed a significantly lower bone mineral density in the women who had experienced an early menopause.³⁰

Some studies have produced results which conflict with the findings cited above. Seeman et al. (1988) and Seeman and Allen (1989) also conducted studies designed to determine what effect age at menopause had on bone loss. Both of these studies concluded that bone loss was not significantly different between the early and later menopause groups.^{31,32}

Natural vs. Surgically-Induced Menopause

Richelson et al. (1984) conducted a study to compare the bone loss of surgically-induced menopause in young women and to that experienced by older women who had undergone a natural menopause. The bone mineral density for both groups of menopausal women were significantly lower than controls, indicating a menopausal contribution to bone loss. The two menopausal groups did not differ from each other significantly in bone mineral density. Their results indicated that women who undergo oophorectomy have comparable bone mass to those women who experience a natural menopause.³³

Seeman and Cooper (1987) compared women with early natural menopause to those with surgically induced menopause, with both groups matched by age. They also found no significant difference in bone mineral density between the two groups.³⁴

Ohta et al. (1994) studied whether oophorectomy or natural menopause were more likely to induce the hormonal changes that lead to osteoporosis. They found no significant difference in bone mineral density of the spinal lumbar bone following surgically-induced or natural menopause, and concluded that both oophorectomy and natural menopause have the same negative effect on bone mineral density which leads to osteoporosis.³⁵ Ohta et al. (1992) concluded that oophorectomy and natural menopause produce the same degree of bone loss after three years, at which time they claim the hormonal effects on bone are well pronounced.³⁶

Rat Model of Postmenopausal Osteoporosis

Suitability of Ovariectomized Rat as Osteoporosis Model

Although the rat has certain limitations when modeling human disease, the NIH considers it to be one of the most appropriate experimental models, offering “unique advantages” and “important strengths.” Over 250 rat disease models have been developed, with approximately twenty-eight percent of the laboratory animals used in research being rats. Over 500,000 publications (PubMed) during the last thirty years have reported on studies involving the rat as an experimental model, according to the NIH.⁸

Using animals as models for postmenopausal bone loss offers researchers the opportunity to study osteoporotic development without the ethical limitations or compounding factors associated with human subjects. According to the Food and Drug Administration (FDA), there

is no experimental animal model for postmenopausal osteoporosis that perfectly emulates the disease in humans. The FDA characterizes ovariectomized animals as the preferred animal model in osteoporosis studies, since estrogen deficiency is considered the overriding risk factor in osteoporosis development. In their guideline paper for pre-clinical and clinical studies investigating agents for the prevention and treatment of osteoporosis, the FDA mandates that one of the animal models used must be the ovariectomized rat.³⁷

The rat model has some characteristics which make it particularly suitable as a model for postmenopausal osteoporosis. The castrated male rat and post-ovariectomized female rat both serve as examples of accelerated bone loss. These surgically altered animals are both bone modeling species which are used to represent bone growth and shaping in humans, and are considered a proper animal model for changes in cancellous bone in human.³⁷ The rat femoral neck contains both cortical and trabecular bone, both of which are lost in human menopausal and age-related osteoporosis. According to Bagi et al., the concentration of cortical bone in the rat femoral neck is 72.5% and is evenly distributed, compared to 12.5% in humans which is unevenly distributed.³⁸ Although the concentration of cortical bone in the rat femoral neck is higher than in humans, the FDA considers the ovariectomized rat an appropriate model for the accelerated trabecular bone loss associated with menopause in human females.^{37,38,39}

Appropriate Age of Rats for Osteoporosis Model

The age of rats used in osteoporosis studies varies. Two commonly used models are the mature rat (three months old) and the aged rat (6-24 months old).⁴⁰ In a review of related literature, Mattila claimed the mature rat model is used more often as a rat model because they

are inexpensive and show the effects of ovariectomy in a “reasonable time.”⁴⁰ No references for this claim were provided, and the term “reasonable time” was not defined.

Kalu et al. (1989) conducted a definitive study using related experiments to address the issue of appropriate age for an estrogen-deficient osteoporosis rat model. In one experiment, the femoral characteristics of female Wistar rats of various ages (1, 3, 6, 12, 18, and 24 months) were examined. Density, calcium content, length, and weight of the femur were measured for each age group. All parameters increased significantly between the ages of one and three months. By six months of age femur density and calcium content had stabilized, and length and weight of the femur were stable by twelve months of age. Their results provided evidence that no significant loss or gain of bone occurs between the ages of twelve and twenty-four months of age. By removing the complicating factor of changes in bone associated with a growing skeleton, this long-term bone stability in the aged rat model provides a foundation for examining the effects of estrogen deficiency.⁴¹

A study by Nnakwe (1995) examined the effect of normal aging on breaking strength, ash weight, phosphorus and calcium content, and phosphorus to calcium ratio on the bones of female *Fischer 344* rats. These parameters are commonly used as markers for osteoporosis-related bone loss. Although the rats ranged in age from 5 to 29 months old, no evidence of bone loss was detected with aging. This supported the earlier findings of Kalu et al. (1989) that the aged rat provides a stable model for estrogen-deficient postmenopausal bone loss.⁴²

Bagi et al. (1996) conducted a study designed to test the effects of ovariectomy on the rat femoral neck. Fourteen-week-old rats were ovariectomized and sham-operated, with baseline control animals sacrificed at the time of surgery. As expected, the ovariectomized rats

experienced a significant decrease in femoral neck strength compared to the sham-operated animals at twelve weeks, post-surgery. The sham rats had a significant increase in femoral neck strength compared to the baseline controls, indicating continued bone growth during the twelve-week experimental period.⁴³ A similar study by Bagi et al. (1997) also found a significant increase in femoral neck strength at four and eight weeks post-surgery, but not at twelve weeks.³⁹ These findings support the previously cited studies showing that rats continue to experience skeletal growth during the first six months of life.^{41,42}

Biomechanical Testing of Bone

Suitability of Femoral Neck as Testing Site

Based on their previous findings of bone stability in aged rats, Kalu et al. (1989) conducted another experiment using twelve-month-old female Wistar rats to examine the role of estrogen deficiency in bone loss. Sham and ovariectomized rats were studied for six months to examine changes in bone density and calcium content for three types of bone: femur, ilium, and vertebrae. The highest density values for both parameters were observed in the femur, with the lowest values in the vertebrae. Ovariectomy caused a significant decrease in all three bone types in density and calcium content. The decrease in density and calcium content was more pronounced in the vertebrae compared to the femur. This corresponds with the greater loss of trabecular bone seen in human females after early menopause. Kalu et al. also found increased bone resorption and decreased intestinal calcium absorption, which are similar to changes found in postmenopausal women.⁴¹

Peng et al. (1994) studied the effect of ovariectomy and orchidectomy on the mechanical behavior of the rat femoral neck and the tibia. Ovariectomized rats were twelve weeks old and orchidectomized rats were ten weeks old at the time of surgery, with testing carried out six and four weeks post-surgery, respectively. The femoral neck was tested in compression and the tibia was tested with three-point bending. Both treatments produced a significant decrease in the maximal load of the femoral neck compared to sham-operated controls. For ovariectomized animals, the maximum load of the tibia decreased only about one-third of that of the femoral neck (8.7% and 15.8% respectively). For orchidectomized animals, the maximum load decreased 10% in the tibia and 23.9% in the femoral neck. Based on their results Peng et al. concluded that the rat femoral neck is “a far more sensitive indicator” than the tibia for detecting bone loss.⁴⁴

Bagi et al. (1996) examined the mechanical behavior of the rat femoral neck in response to ovariectomy-induced estrogen deficiency. Fourteen-week-old rats were ovariectomized and tested against sham-operated controls after a period of twelve weeks. Control animals were sacrificed at the time of surgery for baseline comparison. Maximum load decreased significantly in the ovariectomized animals compared to the sham-operated rats, indicating an estrogen-deficiency effect. Sham-operated rats had femoral neck maximum loads that were significantly higher than controls, indicating the ability of femoral neck mechanical testing to reveal age-related changes in bone. Specific figures for the mechanical testing were not reported. Bagi et al. concluded from their data that the femoral neck offers a suitable site for mechanical testing in estrogen-deficient rats.⁴³

All of the rats used in the aforementioned studies were ten to twelve weeks old, which means they were still experiencing rapid skeletal growth.^{41,42} The detection of decreased bone strength in the femoral neck compared to baseline controls shows that ovariectomy has the ability to suppress the rapid bone growth seen in these young rats, and that the femoral neck site is sensitive enough to detect these changes.

Another study by Bagi et al. (1997) also examined the effect of ovariectomy on the mechanical behavior of the rat femoral neck. Fourteen-week-old rats were ovariectomized or sham-operated, and baseline controls were sacrificed at the time of surgery. Ovariectomized and sham-operated rats were sacrificed at eighteen, twenty-two, and twenty-six weeks of age for femoral neck mechanical testing. Maximum load was significantly higher for the ovariectomized and sham-operated rats compared to the baseline controls, indicating continued skeletal growth with age which can be detected at the femoral neck site. Maximum load significantly decreased in the ovariectomized compared to the sham-operated rats at fourteen and twenty-two weeks of age. No significant change was detected at the twenty-six-week period as was seen in their previously mentioned study, and no explanation was offered. Bagi et al. again concluded that their results confirmed the rat femoral neck site as good mechanical test site for ovariectomy-induced bone loss.³⁹

Guidelines for Testing Procedures

Database searches of Cambridge Scientific Abstracts, InfoSeek, Medline/PubMed, OldMedline, MedlinePlus, in addition to online searches utilizing Copernic 2001 and the U.S.

National Library of Medicine gateway, revealed no articles addressing the proper protocol for mechanical testing of the rat femoral neck.

Previous studies that have examined the effects of storage and hydration on the mechanical behavior of bone have varied greatly with regards to animal type, bone location, storage technique, and testing protocol. A well-written paper by Turner and Burr (1993) provides a comprehensive review regarding the biomechanical testing of bone specimens.⁴⁵ The U.S. Food and Drug Administration recommends the Turner/Burr paper as a reference for those interested in performing biomechanical testing of bone in their research.³⁷ The following is a brief summary of the recommendations made by Turner and Burr in their tutorial on biomechanical testing of bone.⁴⁵

Testing Parameters:

Load is the force that is actually applied to a bone specimen, and deformation is the amount of deflection the bone experiences in response to the load. A load applied to a bone will produce a corresponding deformation in the bone, which can be plotted as a load-deformation curve. The load-deformation curve is divided into an elastic region where the bone will return to its original shape once the load is released, and a plastic region where permanent deformation occurs.

Biological researchers commonly report mechanical test results in units of force, which represent the extrinsic properties that are dependent on bone size and shape. For meaningful results when testing bone specimens, it is necessary to convert the load-deformation curve to a

stress-strain curve. Stress is the load per unit area, and strain is the change in length compared to its original length. By reporting test results in units of stress, researchers can describe the intrinsic mechanical properties of bone specimens normalized for size and shape. This serves to standardize test results, facilitate comparisons between studies, and remove variability between specimens. Mechanical properties associated with the stress-strain curve include Young's modulus, brittleness, toughness, yield strength, and breaking strength.(see Definitions)

Specimen Storage:

Ethanol, ethanol/saline mixture, or saline can be used to store bones, with ethanol being the least effective at bone preservation and producing the greatest decrease in Young's modulus (2.5-4%; human femur). Storing specimens at -20EC in saline-soaked gauze is the preferred method of long-term storage, with the smallest decrease in Young's modulus (<2%; canine femur). Freezing at -20EC or at -70EC showed no change in stiffness (rat femur), but a significant increase in compressive strength and elastic modulus (rat vertebrae).

Specimen Hydration:

Bone should be kept hydrated in saline or saline-soaked gauze to prevent drying during mechanical testing. Young's modulus (17%; human femur) and tensile strength (31%; human femur) will increase (17% and 31%; human femur) during drying, and toughness will increase (55%; human femur).

Testing Temperature:

Bone should be mechanically tested at 37EC, although this temperature would be impractical for researchers. Testing at 23EC would produce an increase in Young's modulus (2-4%), which could be avoided by testing the specimen in a 37EC saline water bath.

Testing Methods:

Loading of bones can be accomplished through tensile, compression, or shear stresses. Even simple loading schemes involve a combination of these stresses acting on the bone specimen.

Tensile testing pulls the bone apart, and is one of the most accurate methods for the mechanical testing of bones. It is limited in usefulness with cortical bone since accurate machining of bone specimens is required, and should be reserved for larger bones.

Bending testing is appropriate for whole long bones of small animals, and can be accomplished with either a three-point or four-point loading system. Three-point loading is the more simple testing technique, but high shear stresses are formed near the middle of the bone. These shear stresses are eliminated with the four-point bending test.

Compression testing is well-suited to cancellous bone, such as vertebrae, but is considered less accurate than bending testing. Advantages of compression testing are the ability to use small bones and to more closely represent the actual loading forces seen in live animals. Disadvantages of compression testing are the need for precisely machined bone samples to provide accurate loading of the specimen.

Torsion testing can be used to examine machined bone specimens or whole bones (used often with rodents). Shear testing tends to be more accurate than torsion testing, but requires precisely machined specimens taken from larger bones.

Fatigue testing utilizes repeated loading cycles using tensile, bending, compression, or torsion techniques. Temperature must be carefully controlled during fatigue testing, since twice as many loading cycles can occur at 23EC compared to 37EC. Fatigue testing is expensive and time-consuming, but is important when validating osteoporotic models.

Strain Rate:

When load is applied to a dried bone, it behaves like a spring during mechanical testing. A wet or hydrated bone contains water that causes it to act like a shock absorber, thereby dissipating some energy. The rate of strain should be between one and eight percent per second to simulate the conditions that occur in live animals and prevent alteration of mechanical properties. No information was provided on how to obtain the desired rate of strain.

Biomechanical Studies of the Rat Femoral Neck

The following information was gained from a review of twelve studies chosen to be representative of current biomechanical testing of the rat femoral neck.^{39,43-53} Since the results and specific design of the studies was not the intended goal of this review, the information was summarized by category. Details were provided only when unusual or noteworthy information was noted in a particular study.

Age of Rats

The age of the rats used by researchers ranged from three to fourteen weeks of age in all but one of the twelve studies. The young rats used in these studies ranged in age from three weeks to 14 weeks of age. Sato et al. (1997) chose nine-month-old rats which were old enough to fit the stable “aged rat” model for estrogen deficiency described by Kalu et al. (1989) and Nnakwe (1995).^{24,25,53}

Specimen Storage

Minus 20EC was reported as the storage temperature for ten studies, with no storage temperature reported by the other two studies. Six studies reported specimens stored without musculature, three with musculature, and three did not report this information. Bones were stored in tubes in two studies and were stored in plastic wrap in two other studies. The remaining eight studies did not report this information.

None of the twelve studies indicated whether or not the specimens were stored in saline, although one did report that the specimens were stored wet. Also, none of the studies indicated what length of time the specimens were stored before testing.

Thawing

Five studies reported thawing specimens at room temperature, and five provided no information on thawing procedures. Specimens in one study were thawed in Ringer’s solution for an unspecified period of time, and overnight at 7EC in a refrigerator for another study. None

of the studies reported whether or not the thawing took place in water or air, or what length of time was allowed for thawing.

Specimen Hydration

Eight of the twelve studies reported no information on specimen hydration or rehydration before mechanical testing was performed. Specimens were soaked for one-hour in Ringer's solution in two studies, and for an unspecified period of time in another study. A one-hour soak in a phosphate buffer solution was reported by one group of researchers.

Testing Temperature

Ten of the twelve studies reported no information on temperature of the testing environment. One group of researchers utilized a 37°C water bath for the specimen during the testing procedure. Another study stated only that testing took place at room temperature.

Strain Rate

Strain rates were reported in a wide variety of units. These included mm/min, mm/sec, cm/min, and radians/sec. Converting all strain rates to mm/min (except for radians/sec) showed a reporting range of 2 mm/min to 254 mm/min. The most commonly reported value for strain rate was 2 mm/min (in five studies).

Testing Parameters

All twelve studies reported their mechanical test results in units of force derived from the load-deformation curve. This type of data reporting did not normalize the mechanical test results to provide information on the intrinsic properties of the bones independent of size and shape. There was also a wide variation in the mechanical values tested and the terminology used to denote these variables.

MATERIALS AND METHODS

Study Design

This study consisted of four experiments designed to obtain baseline data for bone density and mechanical breaking measurements of the rat femoral neck in response to frozen storage and hydration under vacuum. The experiments involved storing cleaned femurs for periods of 15, 34, 122, or 831 days. Specimens in three experiments were subjected to hydration under vacuum for times ranging from one to three hours, depending on the experimental design. Mechanical testing of the femoral neck was performed in three experiments, and density measurements were made in all experiments.

Hydration Under Vacuum

Femurs were tagged to maintain proper identification during the hydration process. Strips were cut from manila folders, since they remain impervious to the effects of submersion in water. Pencil was used to mark the tags to avoid contaminating the femurs with dyes or inks that might leach out during the rehydration process. The tags were attached to each femur using cotton string looped loosely over the mid-shaft of the femur. Femurs were hydrated using a vacuum pump connected to a Kimax flask (#27060) by 3/16-inch I.D. rubber tubing at the side tap. (Appendix B) The flask was filled with 4000 ml of 0.9% saline at room temperature. After the tagged femurs were placed inside, the top of the flask was sealed with a rubber stopper. The vacuum pump was set to hold a negative pressure of 360 mm Hg throughout the procedure.

Density Measurement

Density measurements were based on the Archimedean Principle, which equates the weight of a solid to the weight of liquid it displaces when submerged. A Mettler AE160 balance with a ME-33340 Density Determination Kit was used to obtain density values. (Appendix B) Ultra-purified water was used as the submersion liquid, with 0.13 ml of nonane added per 200 ml of water to prevent air bubbles from adhering to bones. Femurs were first weighed in air and weighed again while submerged in water.

Density was calculated using the following formula:

$$D_s = A / (A - B) \times D_w$$

D_s = density of solid

A = air weight of solid

B = submerged weight of solid

D_w = water density @ given temp

Femoral Neck Mechanical Testing

Specimen Description

The femur is the leg bone which attaches at the distal end to the knee and at the proximal end to the hip. The femoral neck is a smaller shaft of bone which extends at an angle from the proximal end of the femur. The ball-shaped femoral head is located at the end of the neck shaft and fits into a rounded socket in the pelvis called the acetabulum. The surfaces of the femoral head and acetabulum are covered with a smooth articular cartilage that cushions the ends of the bones during movement and impact.

Specimen Cleaning

Femurs were manually cleaned as soon as practical after removal of the leg from the animal. Gloves were worn to prevent the transfer of skin oils or contaminants to the femurs during the cleaning procedure. Musculature and connective tissue were removed using dissecting scissors and scalpels. Femurs were separated from the tibias by inserting the tip of a narrow-pointed scalpel into the joint and slowly working the bones apart. All remaining organic material was removed by gentle scraping with a scalpel equipped with a new blade, followed by wiping with Kimwipes[®] tissues.

To avoid a cushioning or “shock absorber” effect during compression testing, the articular cartilage covering the femoral head was removed. If the cartilage cap was loose, it was pried off using a narrow-pointed scalpel inserted under the edge of the cap. A tight-fitting articular cartilage was removed by making a shallow incision through the cartilage and then inserting a scalpel to separate the two halves.

Specimen Handling and Preparation

Prior to testing, femurs were imbedded in 1-inch lengths of 1/2-inch I.D. copper pipe using H.B. Fuller hot-melt wood glue. (Appendix B) The distal end of the femurs were inserted into the liquified glue up to approximately the greater trochanter, and immediately submerged in an ice-water bath to minimize heat transfer to the bone. After removal from the water bath, gauge lengths (see Definitions) and average femoral neck diameters were measured for each specimen using a Mitutoyo #2005 dial caliper. Gauge length is determined using the depth rod extension on the caliper, while femoral neck diameters are obtained from the gauge dial after the

specimen is placed between the moveable jaws. Specimens were wrapped in saline-moistened Kimwipes[®] tissues and placed in the refrigerator on a tray covered with Saran Wrap until mechanical testing could be performed.

Testing Equipment and Environment

Femoral neck breaking values were obtained using an Instron 4301 automated materials testing machine controlled by an IBM PC300PL computer with Merlin IX software. (Appendix B) A specimen holding device and actuator were fabricated to attach to the Instron testing machine. A four-way positioning system in the holding device allowed for precise alignment of the femoral head with the actuator. (Appendix B)

Testing was performed in a controlled environment regulated for temperature (18-21EC) and humidity (63-68%). All specimens remained wrapped in saline-moistened Kimwipes[®] tissues and covered with Saran Wrap until time for testing. During actual testing, specimens were sprayed with 0.9% saline solution to prevent drying.

Breaking Procedure

The specimen holding device was mounted in the vise-grip jaws attached to the Instron testing platform, and the actuator was attached to the loading arm. Each section of copper pipe containing an imbedded femur was inserted into the holding device. The top of the femoral head was positioned directly under the bottom of the actuator by adjusting the four alignment screws contained in the holding device. (Appendix B) To ensure accurate load deformation readings, a minimal load was applied to each specimen at the beginning of the testing procedure. This was

accomplished by manually bringing the actuator into contact with the femoral head until a load value of 0.006 lb-ft or less registered on the Instron machine. Control was then transferred to the computer for actual mechanical testing and recording of the results. (Appendix A)

Design of Experiments

15-Day Frozen Storage

Femurs were harvested from five male albino Sprague-Dawley rats immediately after sacrifice. These animals were subjected to spinal cord transection four days prior to sacrifice for a separate study, but this period of immobility was considered too short in duration to alter the biomechanical behavior of the femurs. Specimens were kept wrapped in saline-moistened Kimwipes[®] tissues and refrigerated before and after cleaning. Cleaned specimens were frozen at -16EC for fifteen days in glass vials filled with 0.9% saline. After thawing in the storage vials for ten minutes in a 25EC water bath, the sample group (n = 10) was rehydrated under vacuum in 0.9% saline for two 1-hour periods. Density testing was performed after each stage of treatment to examine the effects of freezing and hydration. No femoral neck breaking measurements were performed on this group of bones.

34-Day Frozen Storage

Femurs were harvested from seven Sprague-Dawley rats obtained from the LSU Department of Psychology immediately after sacrifice. As part of a separate study, these animals were subjected to microinjections of a pharmacological agent, but any effect on the biomechanical behavior of the femurs was considered unlikely. Specimens were stored in the

refrigerator overnight wrapped in saline-moistened Kimwipes[®] tissues on trays covered with Saran Wrap to prevent drying. Femurs were kept wrapped in saline-moistened Kimwipes[®] tissues and refrigerated before and after cleaning the following day. The left and right legs from each animal were randomly assigned to two treatment groups. Density and femoral neck breaking measurements were taken to examine the effects of freezing. One group (n = 7) was measured in the fresh state immediately after cleaning. The second group (n = 7) was frozen at -16EC in saline-filled glass vials for thirty-four days, and thawed in the storage vials for ten minutes in a 25EC water bath prior to testing.

122-Day Frozen Storage

Femurs were harvested from sixteen Sprague-Dawley rats obtained from the LSU Department of Psychology immediately after sacrifice. As part of a separate study, these animals were subjected to microinjections of a pharmacological agent, but any effect on the biomechanical behavior of the femurs was considered unlikely. Specimens were kept wrapped in saline-moistened Kimwipes[®] tissues and refrigerated before and after cleaning. Femurs were randomly assigned to three treatment groups. Density and femoral neck breaking measurements were made on all three sample groups. One group (n = 10) was tested immediately after cleaning, and the other two groups were frozen at -16EC in saline-filled glass vials for 122 days. Frozen specimens were thawed in the storage vials for ten minutes in a 25EC water bath. After thawing, one group (n = 11) was tested without rehydration. The remaining group (n = 11) of thawed femurs was rehydrated under vacuum in 0.9% saline for three hours and then tested.

831-Day Frozen Storage

Femurs harvested from fourteen Sprague-Dawley rats (6 male, 5 female, 3 unknown) were stored frozen in saline for 831 days. Specimens were frozen with the musculature on until they could be cleaned over a three-week period, at which time they were thawed in the refrigerator overnight. The femurs were kept wrapped in saline-moistened Kimwipes® tissues and refrigerated before and after cleaning. Cleaned femurs were frozen at -16EC in glass vials filled with 0.9% saline for 831 days. Bone pairs were randomly assigned to two treatment groups. After thawing in the storage vials for ten minutes in a 25EC water bath, each sample group (n = 14) was rehydrated under vacuum in 0.9% saline for either one or three hours. Density and femoral neck breaking measurements were taken immediately following rehydration to evaluate the effects of vacuum rehydration time.

Data Collection

Pre-defined breaking parameters were programmed into the Merlin IX software for all femoral neck breaking tests: stress at failure, strain at failure, Young's modulus, toughness, yield stress, yield strain, plastic strain to failure, and yield point energy (see Definitions). Gauge length and average diameter values were entered into the testing software immediately prior to testing each specimen. Breaking measurements and calculations were handled by the Merlin IX software.

Statistical Analysis

Results were analyzed for statistical significance using SAS-8E software. Independent and paired t-tests were used to analyze test results, with a 95% confidence interval.

RESULTS

Density

15-Day Frozen Storage

Density values for the fresh, post-freeze, one-hour vacuum, and two-hour vacuum sample groups are listed in Table 1. A freezing period of fifteen days resulted in a non-significant increase in density ($p=0.5902$) compared to the values taken before the bones were frozen. Density was significantly lower after a one-hour period of vacuum hydration compared to both the fresh ($p=0.0407$) and post-freeze measurements ($p=0.0008$). Another vacuum hydration period of one-hour also showed a significantly lower density compared to fresh ($p=0.0140$) and post-freeze ($p=0.0003$) values. No significant difference in density was detected between the one-hour and three-hour post-hydration measurements ($p=0.4188$).

34-Day Frozen Storage

A freezing period of thirty-four days did not produce any significant difference in density values between the fresh and post-freeze sample groups ($p=0.9157$). Within the post-freeze sample group, density measurements taken before and after freezing also showed no significant change ($p=0.1216$). Density values for the fresh and post-freeze groups are contained in Table 2.

122-Day Frozen Storage

Density values between the fresh, post-freeze, and three-hour vacuum hydration sample groups are shown in Table 3-A. No significant difference in density was detected between the fresh and post-freeze bones ($p=0.3700$). The density of the rehydrated bones was significantly

lower than the post-freeze bones ($p=0.0059$), and was closely approaching significance compared to the fresh bones ($p=0.0548$).

Measurements of density were also analyzed within both the post-freeze and three-hour vacuum hydration sample groups. Within the post-freeze sample group, no significant change was detected between the pre-freeze and post-freeze density values ($p=0.3030$), as shown in Table 3-B. Within the three-hour hydration group, as seen in Table 3-C, density values were compared for pre-freeze, post-freeze, and post-hydration conditions. A significant decrease was detected between the pre-freeze density values and both the post-freeze ($p=0.0419$) and the post-hydration measurements ($p=0.0002$). Post-hydration density significantly also decreased compared to the post-freeze density ($p=0.0270$).

831-Day Frozen Storage

Table 4 shows density values for the one-hour and three-hour vacuum hydration samples. No significant difference in density was detected between the one-hour and three-hour periods ($p=0.4256$). Density measurements were also analyzed within each vacuum hydration group, before and after hydration. No significant change in density values was detected within either the one-hour ($p=0.1858$) or the three-hour ($p=0.4534$) sample group.

Breaking Measurements

34-Day Frozen Storage

Measurements for Stress, Strain, Yield Stress, Yield Strain, Yield Point Energy, Toughness, and Young's Modulus showed no significant difference between the fresh and post-

freeze groups. A significantly lower value for Plastic Strain was detected for the fresh bones compared to those that were frozen ($p=0.0453$). Measured breaking values are listed in Table 5.

122-Day Frozen Storage

Measurements for Stress, Yield Stress, Yield Point Energy, Toughness showed no significant difference between the fresh, post-freeze, and three-hour vacuum sample groups.

Plastic Strain for the fresh bones was significantly higher compared to the post-freeze bones ($p<0.0001$), but was not significantly different compared to the rehydrated bones ($p=0.8612$). Post-freeze bones had a significantly lower Plastic Strain compared to the rehydrated bones ($p=0.0474$).

Young's Modulus was significantly lower for the fresh bones compared to the post-freeze bones ($p=0.0018$), and was significantly higher for the post-freeze bones compared to the rehydrated bones ($p=0.0291$). No significant difference in Young's Modulus was detected between the fresh bones and the rehydrated bones ($p=0.1896$).

Strain values for the fresh bones were significantly higher than both the post-freeze bones ($p<0.0001$) and the rehydrated bones ($p=0.0214$). A significantly lower Strain value was detected for the post-freeze bones compared to the rehydrated bones ($p=0.0116$).

Yield Strain for the fresh bones was significantly higher than both the post-freeze bones ($p<0.0001$) and the rehydrated bones ($p=0.0100$). Yield Strain was lower for the post-freeze bones compared to the rehydrated bones, and approached significance ($p=0.0538$).

831-Day Frozen Storage

Measured values for Stress, Strain, Yield Stress, Yield Strain, Yield Point Energy, Toughness, and Young's Modulus showed no significant difference between the one-hour and three-hour hydration periods. Yield Stress and Yield Strain values were lower in the one-hour hydration sample group, with both values approaching significance (0.0645 and 0.0539 respectively). Values for Plastic Strain were significantly lower in the one-hour hydration group compared to the three-hour hydration group ($p=0.0469$). Measured breaking values are listed in Table 7.

Table 1

**Post-treatment Density Change (intra-sample)
for 15-day Frozen Storage**

Group	Fresh	Post-freeze	Vac1 Hydration	Vac2 Hydration
Density^a	1.5404 ± 0.0086	1.5420 ± 0.0231	1.5304 ± 0.0071 ^{b,c}	1.5332 ± 0.0080 ^{d,e}

^a Mean ± SEM (g/cm³) — independent t-test

^b p<0.05 compared to Fresh

^c p<0.01 compared to Post-freeze

^d p<0.05 compared to Fresh

^e p<0.01 compared to Post-freeze

**Table 2-A**

**Post-treatment Density Change
Between Fresh & Post-freeze Groups
for 34-day Frozen Storage**

Group	Fresh	Post-Freeze
Density^a	1.5330 ± 0.0099	1.5316 ± 0.0081

^a Mean ± SEM (g/cm³) — independent t-test

Table 2-B

**Post-treatment Density Change
Within Post-freeze Group
for 34-day Frozen Storage**

Group	Fresh	Post-Freeze
Density^a	1.5398 ± 0.0085	1.5316 ± 0.0081

^a Mean ± SEM (g/cm³) — paired t-test

Table 3-A

**Post-treatment Density Change
Between Fresh, Post-freeze & Vac3 Groups
for 122-day Frozen Storage**

Group	Fresh	Post-Freeze	Vac3 Hydration
Density^a	1.5666 ± 0.0041	1.5709 ± 0.0025	1.5525 ± 0.0054 ^b

^a Mean ± SEM (g/cm³) — independent t-test

^b p<0.01 compared to Post-freeze

Table 3-B

**Post-treatment Density Change
Within Post-freeze Group
for 122-day Frozen Storage**

Group	Fresh	Post-Freeze
Density^a	1.5691 ± 0.0029	1.5709 ± 0.0025

^a Mean ± SEM (g/cm³) — paired t-test

Table 3-C

**Post-treatment Density Change
Within Vac3 Group
for 122-day Frozen Storage**

Group	Fresh	Post-Freeze	Vac3 Hydration
Density^a	1.5734 ± 0.0033	1.5606 ± 0.0072 ^b	1.5525 ± 0.0054 ^{c,d}

^a Mean ± SEM (g/cm³) — paired t-test

^b p<0.05 compared to Fresh

^c p<0.01 compared to Fresh

^d p<0.05 compared to Post-freeze

Table 4-A

**Post-treatment Density Change
Between Vac1 & Vac3 Groups
for 831-Day Frozen Storage**

Group	Vac1 Hydration	Vac3 Hydration
Density^a	1.1577 ± 0.0158	1.1767 ± 0.0174

^a Mean ± SEM (g/cm³) — independent t-test

Table 4-B

**Post-treatment Density Change
Within Vac1 Group
for 831-Day Frozen Storage**

Group	Vac1 Hydration	Vac3 Hydration
Density^a	1.1433 ± 0.0157	1.1577 ± 0.0158

^a Mean ± SEM (g/cm³) — paired t-test

Table 4-C

**Post-treatment Density Change
Within Vac3 Group
for 831-Day Frozen Storage**

Group	Vac1 Hydration	Vac3 Hydration
Density^a	1.1685 ± 0.0215	1.1767 ± 0.0174

^a Mean ± SEM (g/cm³) — paired t-test

Table 5**Post-treatment Breaking Measurements
for 34-day Frozen Storage**

	Stress (psi)	Strain (in/in)	Yield Stress (psi)	Yield Strain (in/in)	Yield Point Energy (lbf-in)	Plastic Strain (in/in)	Toughness (psi)	Young's Modulus (psi)
Fresh	2332.13 ± 261.35	0.0934 ± 0.0057	2300.83 ± 255.71	0.0909 ± 0.0049	0.5909 ± 0.0812	0.1020 ± 0.0042	70.0196 ± 10.2610	25694.4 ± 3516.8
Post- Freeze	2836.07 ± 126.12	0.0908 ± 0.0043	2665.00 ± 237.68	0.0993 ± 0.0087	0.4749 ± 0.0803	0.1196 ± 0.0067 ^b	75.7804 ± 6.3085	31464.6 ± 1349.9

^a Mean ± SEM (g/cm³) — independent t-test^b p<0.05 compared to Fresh

Table 6

**Post-treatment Breaking Measurements
for 122-day Frozen Storage**

	Stress (psi)	Strain (in/in)	Yield Stress (psi)	Yield Strain (in/in)	Yield Point Energy (lbf-in)	Plastic Strain (in/in)	Toughness (psi)	Young's Modulus (psi)
Fresh	3085.16 ± 126.65	0.1226 ± 0.0052	34189.10 ± 31279.00	0.1155 ± 0.0044	0.5570 ± 0.0451	0.1270 ± 0.0047	87.1370 ± 5.5468	25662.0 ± 1721.5
Post- Freeze	2819.22 ± 167.52	0.0810 ^c ± 0.0041	2719.46 ± 207.28	0.0753 ^c ± 0.0061	0.5241 ± 0.0598	0.0861 ^c ± 0.0049	82.4259 ± 7.1122	35059.0 ^c ± 1915.1
Vac3	2866.03 ± 116.67	0.1019 ^{b,d} ± 0.0063	2729.09 ± 145.16	0.0933 ^b ± 0.0063	0.6428 ± 0.0645	0.1310 ^d ± 0.0206	95.3361 ± 6.8148	28991.0 ^d ± 1730.2

^a Mean ± SEM (g/cm³) — independent t-test

^b p<0.05 compared to Fresh

^c p<0.01 compared to Fresh

^d p<0.05 compared to Post-freeze

Table 7**Post-treatment Breaking Measurements
for 831-day Frozen Storage**

	Stress (psi)	Strain (in/in)	Yield Stress (psi)	Yield Strain (in/in)	Yield Point Energy (lbf-in)	Plastic Strain (in/in)	Toughness (psi)	Young's Modulus (psi)
Vac1	1947.78 ± 124.96	0.0954 ± 0.0054	1568.96 ± 217.71	0.0844 ± 0.0090	0.2154 ± 0.0195	0.1060 ± 0.0091	49.4975 ± 4.4685	21736.1 ± 2320.1
Vac3	2102.71 ± 132.31	0.1033 ± 0.0049	2067.43 ± 138.78	0.1058 ± 0.0055	0.2351 ± 0.0111	0.1377 ^b ± 0.0122	49.0139 ± 3.2341	20876.3 ± 1542.0

^a Mean ± SEM (g/cm³) — independent t-test^b p<0.05 compared to Vac1

DISCUSSION

The overall objective of this study was to construct a basic model of biomechanical behavior of the rat femoral neck. Three objectives were defined to accomplish our goal: (1) determine which breaking measurements provide the most accurate representation of femoral neck biomechanical behavior; (2) determine what terminology and units of measurement are most appropriate for reporting breaking values; (3) identify the effects of storage and hydration protocols on the biomechanical behavior of the rat femoral neck.

The tutorial paper by Turner and Burr (1993) served as our primary guide throughout the course of this study.⁵⁴ Their paper addressed the biomechanical measurement of bone in general, and was not specific to animal type, bone location, or goals of research. The U.S. Food and Drug Administration recommends the Turner/Burr paper to researchers for guidelines on the mechanical testing of bone in osteoporosis studies, so we felt confident in adopting this paper as our primary information source.³⁷ References to “the tutorial” or “Turner and Burr” henceforth in this discussion refer to this publication.

Breaking Measurements

Load-Deformation Curve

Biomechanical testing of bone consists of measuring two components: the force applied (load) and the displacement in response to the load (deformation). The relationship between these two variables is expressed as an XY-coordinate graph called a load-deformation curve. (Figure 1) The curve can be divided into an elastic deformation region and a plastic deformation region. Loading forces at which the bone will return to its original shape once the load is

removed are represented by the elastic deformation region of the curve. Applied loads at which the bone specimen experiences permanent deformation are represented by the plastic deformation region.

The point at which the deformation changes from temporary to permanent is known as the yield point, but this is usually ill-defined with bone specimens. The slope of the curve within the elastic deformation region represents the rigidity of the specimen, which is a measure of the extrinsic stiffness. The area under the load-deformation curve, a combination of the elastic and plastic deformation regions, represents the total breaking energy of the specimen. Breaking load is the force at which the bone specimen actually fractures.

Stress-Strain Curve

By the application of engineering formulae, which can be obtained from the Turner and Burr tutorial, the load and deformation can be converted to stress and strain, respectively. Load is converted to stress, which is defined as the force per unit area. Deformation is converted to strain, which is defined as the change in specimen length relative to its original length. A stress-strain curve expresses the relationship between stress and strain on an XY-coordinate graph. (Figure 2) The stress-strain curve is also divided into two sections, the elastic strain and the plastic strain regions, which represent the areas of temporary and permanent strain, respectively.

The slope of the elastic strain region is referred to as the elastic modulus or Young's modulus, which represents the intrinsic stiffness of the specimen. The amount of strain between the yield point and the point of fracture is the ductility of the specimen. Brittleness is the term used for a material that can sustain little post-yield strain before it fractures under load. The

combined area under the curve comprised of the elastic strain and plastic strain regions represents the energy absorption capacity, or toughness, of the material. Breaking strength is defined as the maximum stress a material can withstand before experiencing fracture.

Appropriate Measurements for Biomechanical Testing

According to Turner and Burr, bone breaking measurements reported by researchers normally are determined directly from the load-deformation curve. Our review of the related literature confirmed this finding, with ten of the twelve studies we selected for review reporting only measurements derived from the load-deformation curve. When measurements are reported in this format, the data is not normalized for specimen size and shape, which makes data comparisons between different studies difficult or useless.

Following the suggestions of Turner and Burr, we presented the measurements from our study in the normalized format derived from the stress-strain curve. The Merlin IX software connected to the Instron materials testing machine automatically converted the measurements derived from the load-deformation curve. By reporting our data in this format we have removed the effects of structural variability between specimens, which was especially important considering that our study involved data collected from four independent experiments.

In order to provide a complete and accurate representation of biomechanical behavior, we chose eight measurements for reporting: stress at failure, strain at failure, Young's modulus, toughness, yield stress, yield strain, plastic strain to failure, and yield point energy. While all of these measurements may not be of interest to every researcher, our goal was to provide the most complete representation possible of the biomechanical behavior observed in our tests.

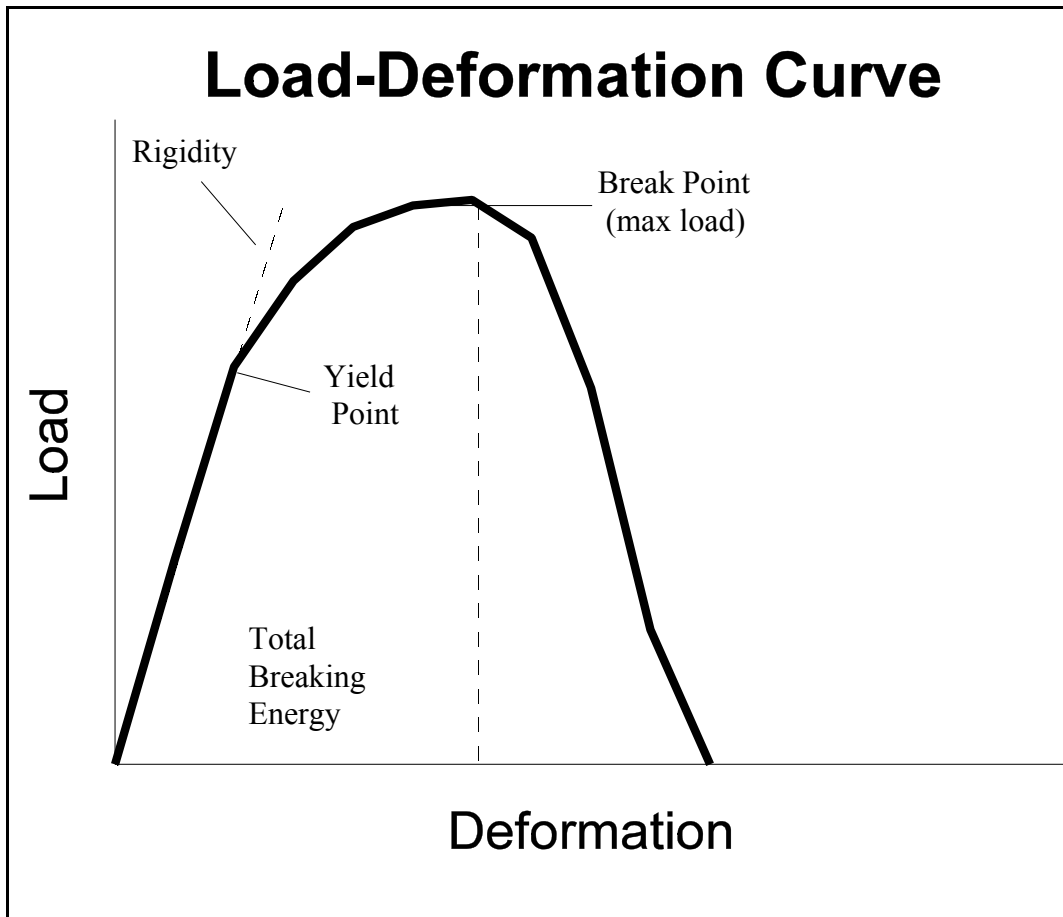


Figure 1: Load-Deformation Curve

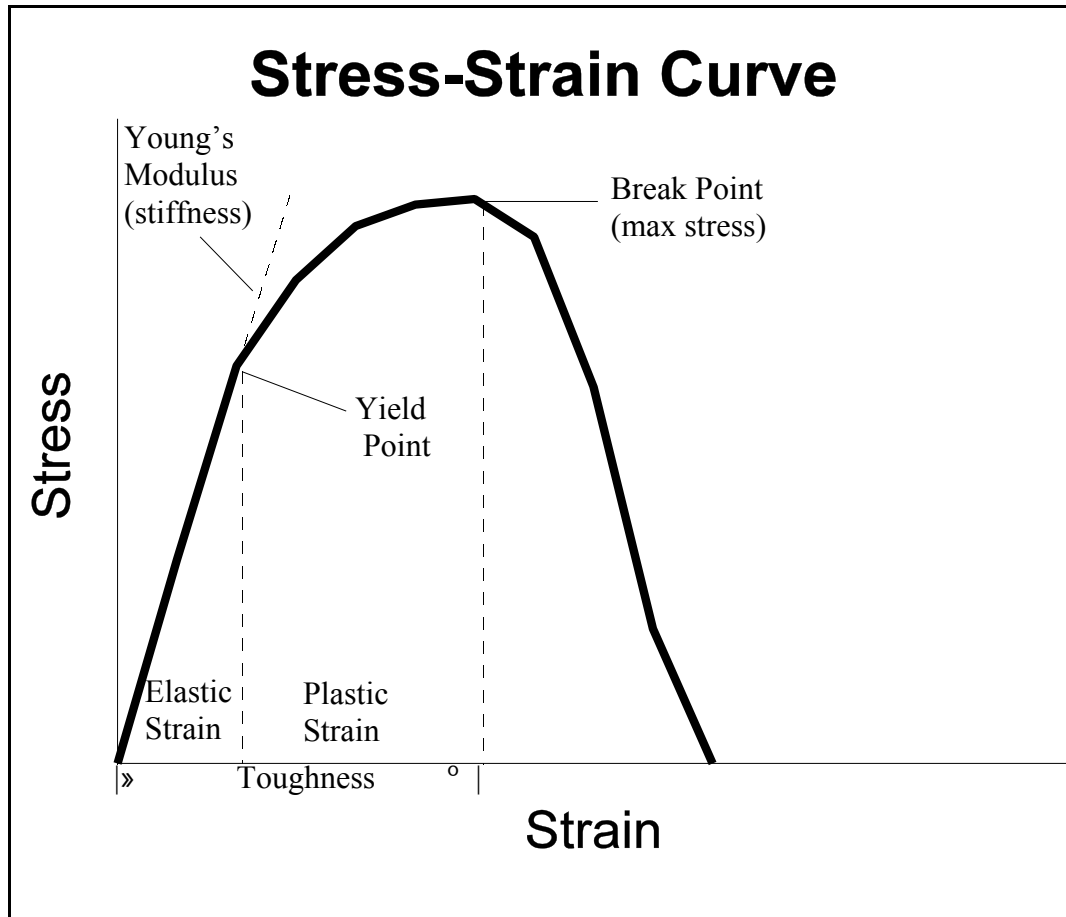


Figure 2: Stress-Strain Curve

Terminology and Units of Measure

General Terminology of Biomechanical Measurements

Turner and Barr did not address the issue of terminology in their tutorial, however we found a wide variation in terminology between published studies. The terminology used to describe the biomechanical measurements of bones as a whole is inconsistent between studies. One noticeable problem in the published literature is the use of the term *bone strength* to refer to the entire group of mechanical measurements taken in a study. Turner and Burr point out that *strength* refers to either the load or stress at which the bone actually breaks. We found references in five of our twelve selected studies where the term *strength* was used in this broader context. This more general use by researchers of the term to refer to all biomechanical measurements leads to confusion.

The term used most often in our sample studies to refer to the overall group of mechanical measurements was *biomechanical properties*. Although this term is much more appropriate than *strength*, we still considered it inadequate for its intended description. A *property* of a material carries with it the connotation of relevance to all members of the group to which it refers. For instance, referring to the *biomechanical properties* of the rat femoral neck conveys the meaning that those characteristics are common to all bones of this type. However, as many decades of research have proven, bones respond differently when subjected to various stimuli and conditions. This individualized response is what makes it possible for researchers to study bones for the cause and treatment of diseases such as osteoporosis.

We chose the term *biomechanical behavior* to represent the overall group of measurements gained from biomechanical testing in our studies. The term *behavior* by

definition is an action or reaction under a specified set of circumstances. We believe this term most accurately describes the biomechanical response of bones to test environments, and suggest its adoption as a standard way of referring to biomechanical test measurements in general.

Specific Terminology of Biomechanical Measurements

One of the more confusing points in the published literature for bone studies is the terminology used to refer to a specific type of measurement. For example, *stiffness* was used in seven out of ten of our sample studies reporting that measurement. The term *rigidity* was used in the other three sample studies. In general, the term *stiffness* describes the ability of a material to resist deformation under an applied load. However, bones have both extrinsic and intrinsic stiffness, depending on whether or not the measurements have been normalized for size as previously described. Rigidity refers to the non-normalized measure of stiffness, and Young's (elastic) modulus refers to the intrinsic or normalized stiffness of a bone. It is easy for readers of the three studies reporting rigidity as a measurement to know exactly what value was being referred to, whereas use of the term stiffness leaves readers uncertain.

Similar problems of unclear terminology in the published literature for measured values occur with breaking strength, energy to failure, and deformation. Without proper distinction, the only way for readers to define which measurements the researcher is reporting is to observe the units assigned to those values. Since some readers may be uninformed regarding which units of measurement are used for specific measured values, we feel it is important for researchers to use terminology which is unambiguous. By doing so, researchers can facilitate a reader's ability to understand, apply, and compare the results of biomechanical studies.

Units of Measure

The issue of proper units of measure was not addressed in the tutorial by Turner and Burr. We did not find the reporting of units of measurement to be a major problem within our sample studies. However, some measurements such as toughness, stress, and energy can be correctly reported with more than one unit of measurement. As pointed out earlier, units of measurement are sometimes necessary for the reader to determine which values are being reported. If a mechanical value is commonly associated with more than one unit of measurement, we suggest the researcher make note of this as another method of presenting data in a meaningful manner.

Frozen Storage of Bones

Suggested Guidelines for Frozen Storage of Bones

Turner and Burr point out that biomechanical data is always relative unless the specimens are tested immediately after removal from the animal. We have found that both manpower and time restrictions make testing at the time of animal sacrifice impractical, thereby necessitating frozen storage of bone specimens. Turner and Burr stated that freezing bone specimens at -20°C in saline-soaked gauze is “unquestionably the best method of long-term preservation prior to testing.” While we respect the acknowledged expertise of Turner and Burr in the area of bone biomechanics, we recognize several shortcomings with the above-mentioned statement in general, and specifically in relation to the rat femoral neck.

A general concern is that Turner and Burr did not define *long-term preservation*. Studies cited to substantiate their statement were based on freezing periods of fourteen, twenty, and

thirty days. In comparison, our research group has bone specimens from nutritional intervention studies that have been stored for periods greater than a year. Instead of disposing of these bones, we kept them based on the possibility of conducting future research when appropriate.

Also, Turner and Burr did not provide any information as to how specimens wrapped in saline-soaked gauze should be stored. Should specimens be stored in vials, plastic wrap, freezer bags, or uncovered? For storage periods as short as a week, we suspect uncovered specimens would experience sublimation of the saline and thereby expose the bones to drying. The opportunity for sublimation would be enhanced if specimens were stored in a forced-air freezing environment as occurs with some refrigerator/freezer units.

Finally, the findings cited by Turner and Burr were based on results from a variety of bone sources, testing techniques, and reported measurements and may not be applicable to studies of the rat femoral neck. Specimens in the cited studies included human, canine, and rat femurs, and rat vertebrae. Testing techniques included bending (human femur/bending properties), torsion (rat femur/strength & stiffness), and compression (rat vertebrae/strength & elastic modulus). The testing technique used with the canine femur (Young's modulus) was not stated.

Although these cited study results may be accurate, we feel more comprehensive testing is needed before researchers "unquestionably" adopt the use of saline-soaked gauze as the most appropriate method of long-term preservation for bones in general, and specifically for the rat femoral neck.

Specimens used in our current study were submerged in 0.9% saline in glass vials and frozen at -16EC. Storage in saline guards against moisture loss, as shown by our results

described below. Also, the ice surrounding the specimen provides short-term protection against temperature rise in the event of freezer malfunction or power failure.

Effect of Frozen Storage on the Rat Femoral Neck - Previous Studies

Two studies involving breaking of the rat femoral neck have included preliminary tests to examine the effect of freezing and subsequent thawing on biomechanical behavior. Peng et al. used eighteen-week-old male and female rats, testing one femoral neck at the time of sacrifice and the other after seven days of -20°C frozen storage. They determined that the freeze/thaw process did not significantly alter the biomechanical behavior of the femoral neck.⁴⁴ Bagi et al. also reached the same conclusion in their preliminary study.³⁹

While both of these studies specifically address the issue of frozen storage on the rat femoral neck, we still have some concerns in applying their results on a broad scale. The study by Bagi et al. did not report age of the rats, storage temperature for the femurs, or length of storage time. Therefore, we are unable to consider their study results as evidence for the stability of femoral neck biomechanical behavior following frozen storage. Peng et al. stored their test femurs with the musculature on, which may or may not correlate to specimens stored after being cleaned of soft tissue. Also, the bones in their study were only frozen for seven days, which is half the frozen-storage period used in the studies cited by Turner and Burr.

Additional testing needs to be conducted to determine if frozen storage of bones in a cleaned state affects their biomechanical behavior. Also, longer periods of frozen storage, both with and without musculature, need to be investigated to ensure density and behavioral characteristics remain unchanged in response to frozen storage. Our current study examines the

effects of varied periods of frozen storage on the biomechanical behavior of bones stored in a cleaned state.

Effect of Frozen Storage on the Rat Femoral Neck - Current Study

One goal of our current study was to examine the effects of frozen storage on the density of the rat femur and the biomechanical behavior of the rat femoral neck. We included density measurements in our study to determine if the moisture content is affected by frozen storage. Three different experiments comparing bones which were tested immediately after excision and bones frozen for periods of time 15, 34, or 122 days. The two shorter frozen-storage periods correlated well with the storage times cited by Turner and Burr in studies examining the effects of freezing on bone mechanical behavior. Our longest frozen storage period was four times the longest period cited by Turner and Burr, which provided information about the effects of extended frozen-storage times on the rat femoral neck not found in the published literature.

Effect of Frozen Storage on Density

Our results between the fresh and frozen groups showed that femoral density was not significantly affected by frozen storage in all three experiments. As a matter of redundancy in our testing, we ran intra-sample analyses comparing density before and after a group of femurs was frozen. Two of three intra-sample comparisons showed no significant change in density. We are unable to explain why the third intra-sample analysis detected a significant change in density. However, since five of the six analyses detected no significance, we felt confident concluding that frozen storage for up to four months does not significantly affect density of the

rat femur as a whole, and therefore should not alter femoral neck density. This indicates that freezing specimens in 0.9% saline serves to protect against moisture loss for at least four months of frozen storage. (Tables 1; 2-A,B; 3-A,B,C)

Effect of Frozen Storage on Biomechanical Behavior

In two experiments we also examined the effect of freezing (34 & 122 days) on the biomechanical behavior of the femoral neck. The portions of each experiment that compared fresh and frozen-stored bones were identical in design, except for the length of the frozen storage period. We measured the following variables for all specimens in both experiments: breaking stress, breaking strain, Young's modulus, toughness, yield stress, yield strain, yield point energy, and plastic strain to break.

Breaking strength (stress at failure) between the fresh and frozen-stored samples was not significant in either of these experiments. We cannot correlate these findings with the results of the studies by Peng et al. and Bagi et al. mentioned above, because they measured breaking load. According to Turner and Burr, breaking stress and breaking load can show different trends within the same study. Since Peng et al. conducted some of the limited work done specifically with the effect of freezing on the rat femoral neck, we would be interested in seeing their data size-normalized for comparison with our current results.

The only measured behavior that showed significance between fresh and frozen-stored specimens in both experiments was plastic strain to failure. (Tables 5 & 6) However, the results were significant in opposite directions, showing a significant increase in plastic strain for the 34-day storage period and a significant decrease for the 122-day storage period. (Figure 4) The only

difference between the two experimental designs was the length of the frozen-storage period. We concluded that the longer 122-day frozen-storage period negated and reversed the increase in plastic strain caused by the shorter 34-day freezing period. If this conclusion is correct, then additional testing could possibly identify a frozen storage period that produces a non-significant change for plastic strain. Identification of such a breakeven point could prove useful to researchers interested in measuring plastic strain when designing future intervention experiments.

Other than plastic strain, no other measurements in the 34-day frozen storage experiment showed a significant change in biomechanical behavior between fresh and frozen-stored specimens. The experiment with the 122-day frozen storage period showed significant behavioral differences between the fresh and frozen-stored specimens in several measurements. Strain and yield strain values decreased and Young's modulus increased significantly in this experiment. Since the design for the fresh/frozen-storage portions of these experiments was identical, we concluded that the extended storage time was responsible for these biomechanical behavioral changes. (Figure 3; Table 6)

Compared to fresh specimens, the 122-day frozen-storage period decreased the amount of strain to failure the femoral neck could tolerate once it reached the yield point, thereby reducing its plastic behavior. The intrinsic stiffness of the femoral neck increased, and the deformation occurring at the yield and failure points both decreased.

Bone Hydration

Suggested Guidelines for Preserving Hydration of Bones

Turner and Burr stated that bone specimens should be tested in a hydrated condition to ensure accurate biomechanical test results, although they did not define the term *hydrated condition*. Common sense dictates defining a bone in a hydrated condition as one which does not significantly differ in moisture content from its in-vivo state. However, once a bone is cleaned of soft tissue for testing purposes, the researcher must become concerned about dehydration of the specimen. With long-term storage of bones, the problem of dehydration becomes more of a concern, since more opportunity exists for moisture loss associated with extended handling and storage times.

As a way to ensure bone specimens remain hydrated, Turner and Burr suggested submergence in saline or wrapping with saline-soaked gauze during testing as ways to ensure the specimens remain hydrated. Hou et al. (1991) tested the rat femoral neck with specimens submerged in a circulating buffer solution.⁵⁵ However, such testing requires fabrication of equipment which may be beyond the ability or budget of some researchers. Since the biomechanical measurements of the rat femoral neck are small, we question if a forced-circulation system might apply sufficient pressure to the specimen to alter test results.

In our current study, we kept specimens hydrated by spraying them with 0.9% saline during cleaning, preparation, measuring, and testing procedures. Specimens were wrapped in saline-soaked Kimwipes[®] tissues when in temporary storage or between handling steps.

Effect of Hydration on the Rat Femoral Neck - Previous Studies

In their discussion of bone hydration, Turner and Burr did not address the issue of rehydrating a bone that may have become moisture deficient. We found two studies in the published literature that investigated the relationship between bone moisture content (as measured by density) and rehydration procedures. Broz et al. (1993) provided evidence that mouse bones (femur) air-dried for forty-eight hours could be rehydrated to 88% of their original water content with a three-hour immersion in saline.⁵⁶ Keenan et al. (1992) showed that additional water can be drawn into a whole rat bone (tibia and femur) through hydration under vacuum at 380 mm Hg for one hour, but additional vacuum time was ineffective.⁵⁷ We found no published information dealing with hydration of the rat femoral neck.

Effect of Hydration on the Rat Femoral Neck - Current Study

The second goal of our current study was to examine the effects of hydration under vacuum on the density and biomechanical behavior of the rat femoral neck. This part of our study included three different experiments comparing the effect of hydration under vacuum on previously frozen bones with fresh and frozen-stored bones that had not been rehydrated. Frozen-storage periods for the three experiments consisted of 15, 122 or 831 days. For the purposes of our study, we considered a hydrated bone as one that does not significantly differ in moisture content from its immediate post-excision state. Vacuum periods ranged from one to three hours, depending on the experimental design.

Effect of Hydration on Density

Results for the 15-day frozen storage experiment showed a significant decrease in density for a single group of frozen-stored bones after one hour of hydration under vacuum compared to the non-hydrated fresh and frozen-stored densities for the same group. An additional hour of hydration produced no significant change in density compared to the one-hour hydration period. (Table 1)

The 122-day frozen-storage experiment showed a significant increase in density of bones rehydrated under vacuum for three hours compared to the frozen-stored bones. For redundancy, we also tested within the three-hour vacuum group. A significant decrease was detected in the rehydrated bones compared to the frozen-stored bones. (Table 3-A,C)

Results for the 831-day experiment showed no significant difference in density between one-hour and three-hour vacuum times for separate groups of frozen-stored bones. Redundant testing within each group hydration group also showed no significant change in density after hydration under vacuum. (Table 4-A,B,C)

As previously stated, our study has shown that freezing has no significant effect on density for up to four months of frozen storage in 0.9% saline, indicating that the frozen-stored bones did not need rehydrating compared to their fresh state. However, the one-hour and three-hour rehydration periods significantly decreased the density compared to frozen-stored bones, both between and within sample groups, in the 15-day and 122-day experiments. This decrease resulted in a density that was significantly lower than the the fresh bone density in two comparisons, and closely approached significance in another. This indicates that even one hour of vacuum rehydration can significantly lower the density of bones stored frozen in saline for up

to four months compared to their fresh density. Since the density is lowered compared to fresh bones, we speculate that the vacuum hydration is removing either water or organic materials from the bones.

The failure of either the one-hour or three-hour hydration under vacuum to significantly alter density after 831 days of frozen storage, either between or within sample groups, does not correspond to the results of the other two experiments. Hydration under vacuum decreased density of specimens stored frozen in saline for 15 and 122 days , but increased density of bones stored for a period of 831 days. The design was identical for this portion of all three experiments, except for the length of the storage period. Therefore, we feel confident concluding that the extended period of frozen storage has somehow altered the ability of rehydration under vacuum to lower bone density by removing either water or organic material from the bones. Any attempt on our part to explain the mechanics of this effect is beyond our expertise and will be left to others for explanation.

Effect of Hydration on Biomechanical Behavior

The 122-day and 831-day frozen-storage experiments were used to test for effects of hydration on biomechanical behavior of the rat femoral neck. We measured the same biomechanical variables as previously stated.

Results from the 122-day storage experiment showed significant biomechanical changes for the bones hydrated under vacuum for three hours compared to the non-hydrated fresh and frozen-stored bones. (Table 6) Comparing the fresh and hydrated bones, we detected a significant decrease in strain at failure and yield strain at failure. (Figure 5) As previously

stated, freezing for 122 days had the effect of decreasing the values for strain at failure and yield strain compared to fresh bones as was seen with hydration under vacuum. Since the hydrated bones were also frozen at one time, we speculate a freezing effect (within the hydrated sample group) may be superimposed on the effect of hydration for these two variables. When compared to fresh bone values, the rehydrated bones (previously frozen) had a smaller decrease in both strain to failure and yield strain than the non-hydrated frozen bones. This indicates that hydration under vacuum was able to partially reverse the effect of freezing on these two variables by moving these two variable in the positive direction. Additional evidence to support this conclusion is provided by comparing the breaking values for the non-hydrated frozen bones to the vacuum-hydrated bones. Hydration moves both strain to failure and yield strain in a positive direction, although only the strain to failure value is statistically significant. This indicates that hydration under vacuum for three hours is able to reverse the depression of strain to failure and yield strain caused by freezing for a period of 122 days.

A significant increase in strain at failure and plastic strain to failure, and a significant decrease in Young's modulus was detected when comparing the frozen-stored and hydrated bones after the 122-day frozen storage period. (Figure 6) Yield strain at failure was approaching significance ($p=0.0538$). As previously stated, freezing was shown to move these two variables in the opposite direction when compared to the fresh bones. Since the rehydrated bones were also frozen, we conclude that there is an effect of freezing superimposed on the hydrated bones which three hours of vacuum hydration was able to reverse.

Biomechanical behavior in the 831-day frozen-storage experiment also showed some significant differences, but not to the degree seen in the 122-day experiment. (Table 7; Figure 7)

Comparing the one-hour and three-hour hydration periods, plastic strain to failure increased significantly with the longer hydration time. Yield stress at failure and yield strain at failure both increased with hydration time, but were only approaching significance ($p=0.0645$; $p=0.0539$). Again, we must rely on others with more biomechanical expertise to interpret these behavioral changes.

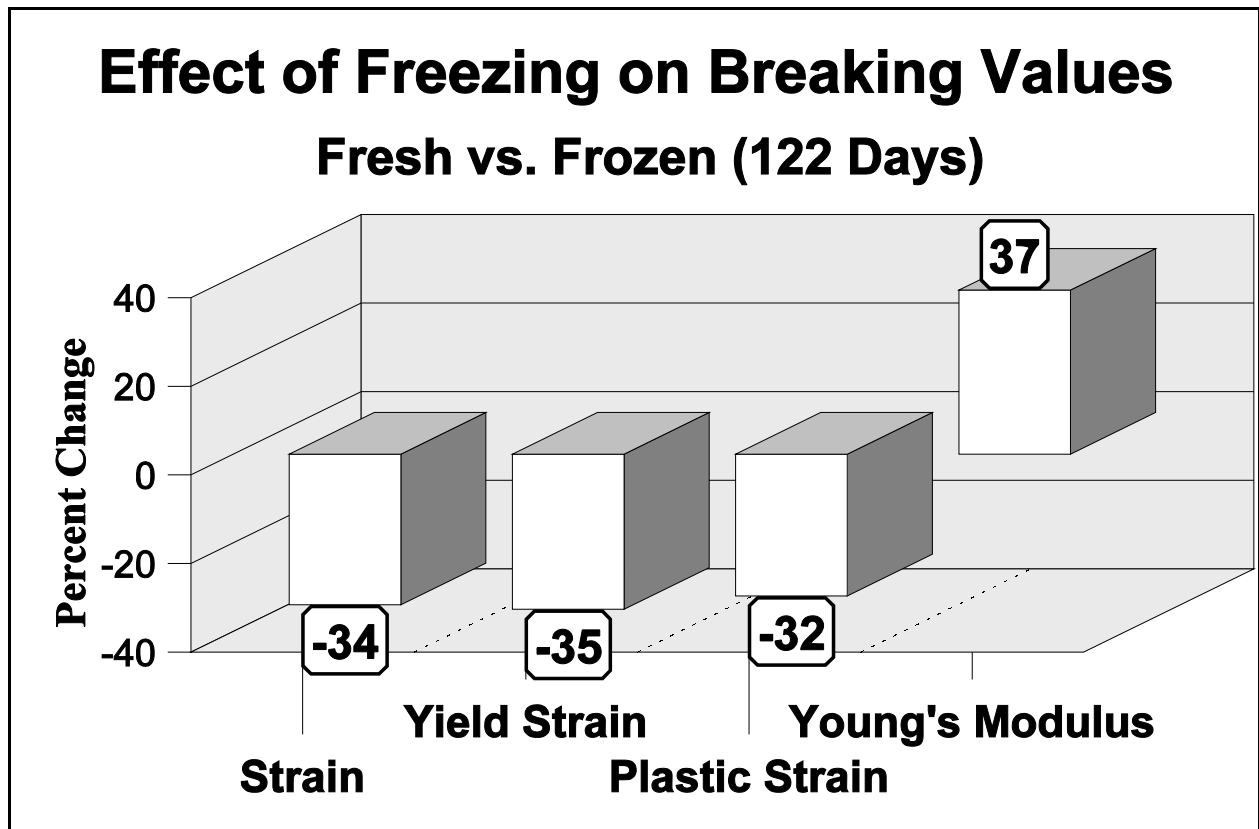


Figure 3: Effect of Freezing on Breaking Values – Fresh vs. Frozen (122-day storage)

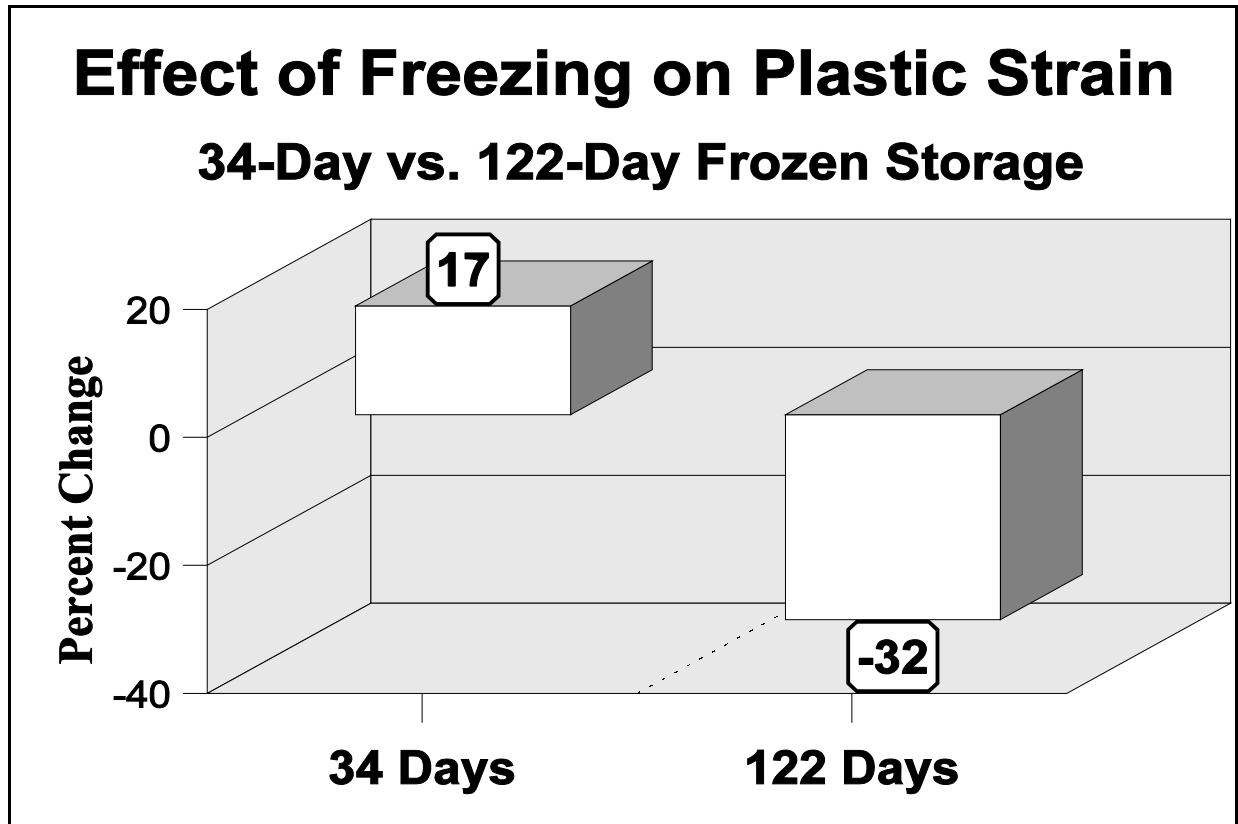


Figure 4: Effect of Freezing on Plastic Strain – 34-day vs. 122-day Frozen Storage

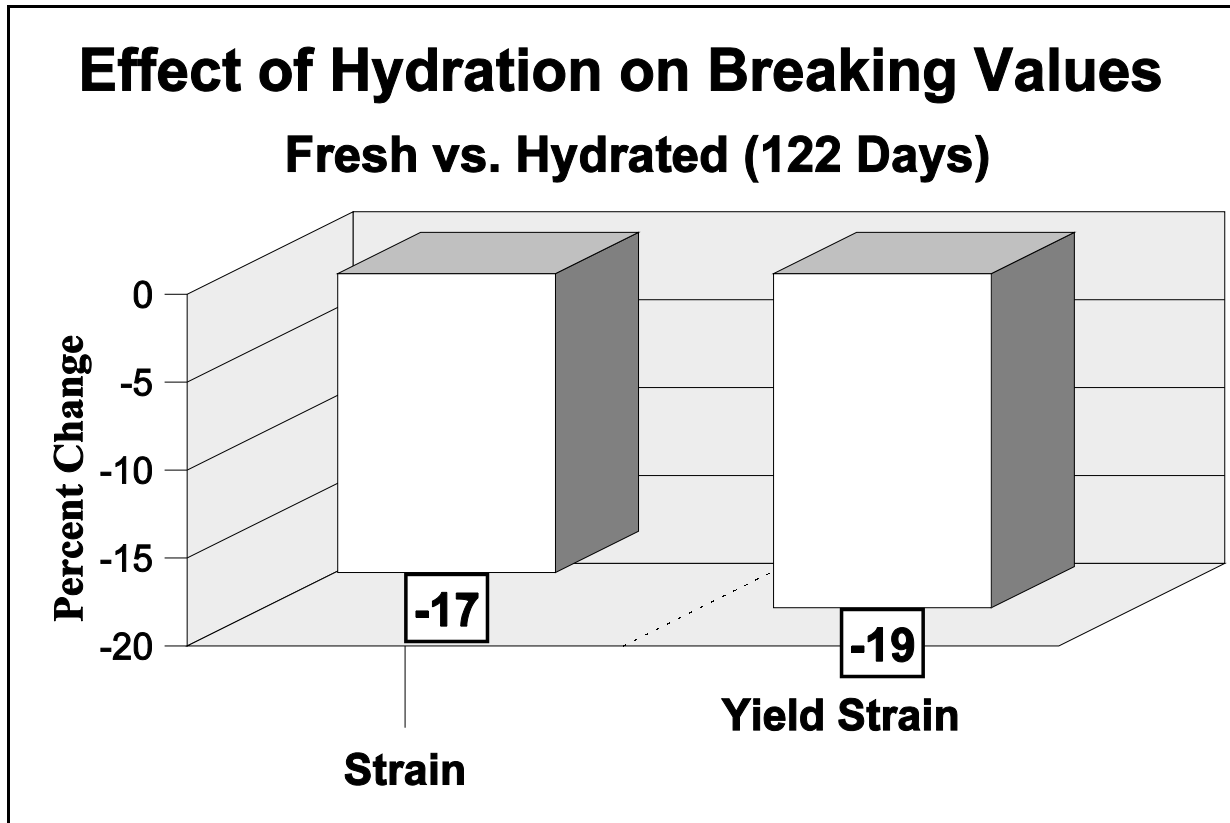


Figure 5: Effect of Hydration on Breaking Values – Fresh vs. Hydrated (122-day storage)

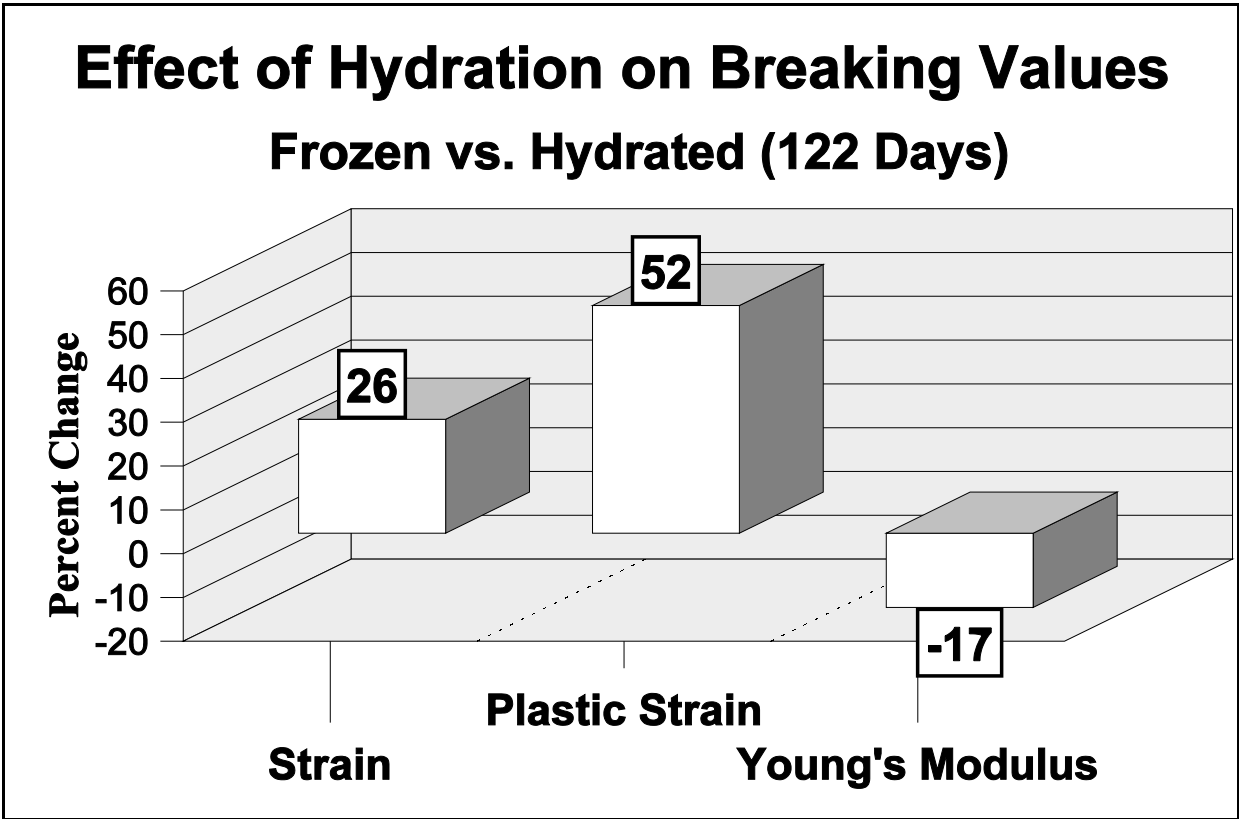


Figure 6: Effect of Hydration on Breaking Values – Frozen vs. Hydrated (122-day storage)

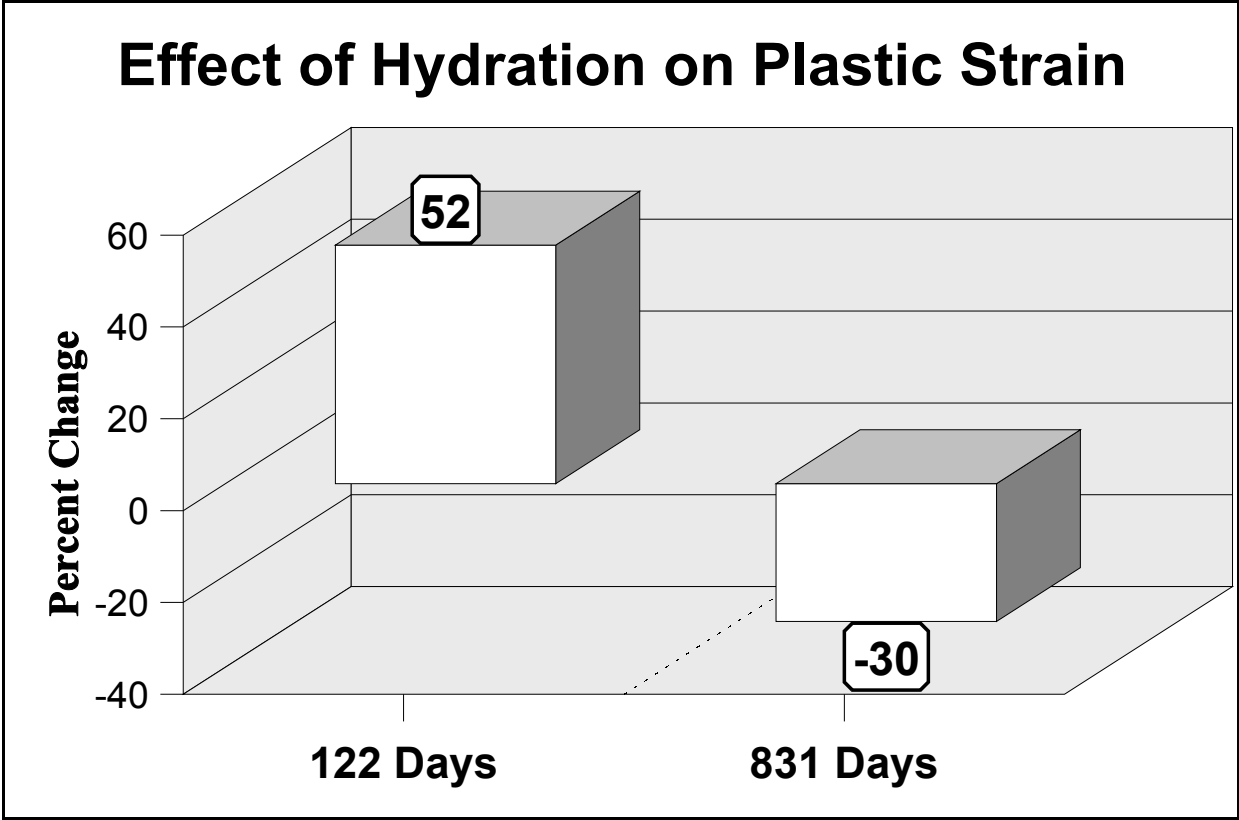


Figure 7: Effect of Hydration on Plastic Strain

Study Limitations

Variability and Sample Size

Since our current study was unfunded, we were required to obtain rats for our experiments as they became available from outside sources. Our reliance on donated rats resulted in a lack of control over the age, size, gender, and breeding history of the rats used in each experiment. The variability among the animals used in our study was relatively broad for age, size, and gender, and was unknown for breeding history. All of the rats in our experiments were the Sprague-Dawley strain, so breed was not a variable that could affect our results.

Another factor we were unable to control was the sample size for each of our experiments. The smaller sample size probably magnified the effect of the uncontrolled variability, thereby contributing to an overall lower power for our statistical analysis. We feel our sample size was sufficiently large to ensure statistical validity, but would have preferred a larger sample size to provide more statistical power to our study. A more powerful analysis might have detected additional statistical differences in our current study. Adequate funding of future studies will provide appropriate sample size and control of experimental variability to ensure higher statistical power.

Testing Protocol

Our testing protocol has the proven ability to detect statistical differences in the mechanical properties of the rat femoral neck caused by freezing and hydration. However, the ultimate goal in developing this protocol was its application in intervention studies involving the treatment and prevention of osteoporosis. To be useful in osteoporosis studies, a testing protocol needs to be able to detect statistical difference in mechanical properties between ovariectomized and sham-

operated rats. Before using this protocol in an actual study situation, additional testing should be conducted to verify its suitability for use in osteoporosis studies.

Fixation of Femurs

Our method of fixating the femurs for mechanical testing involved using a quick-setting hot glue product that was immediately submerged in an ice-water bath to minimize the transfer of heat from the glue to the femur. We do not know if enough heat was transferred to the femur to have an effect on the biomechanical behavior of the femoral neck. Additional testing should be conducted to determine if the heat released during the fixation process alters femoral neck mechanical properties.

Recommendations For Future Studies

Based on the results of our current study, we feel confident making the following recommendations to researchers who plan to use the rat femoral neck as a test site for biomechanical measurements:

- femurs can be safely frozen in 0.9% saline for up to four months without affecting density (based on the assumption that the density of the whole femur is representative of the femoral neck)
- femurs frozen for up to four months in 0.9% saline should not be hydrated before testing
- hydrating femurs for as little as one hour under vacuum can decrease bone density below their fresh-state density, which could adversely affect mechanical measurements
- the effect of freezing and hydration protocols on measurements of strain, yield strain, plastic strain, and Young's modulus should be considered when designing experiments

- the term “biomechanical behavior” should be adopted as a general description of the mechanical properties of bones under various test conditions
- use specific and appropriate terminology to describe the actual mechanical properties of bones that are being tested
- biomechanical measurements should be reported as normalized data derived from the stress-strain curve to allow correlation of results between studies, using units of measurement appropriate for the stress-strain relationship

SUMMARY AND CONCLUSIONS

Summary

In designing our current study, we attempted to provide baseline data on how frozen storage and hydration protocols affect density and biomechanical values. We selected a set of breaking measurements that we felt gave the best overall representation of the biomechanical behavior of the rat femoral neck. Our data was reported as size-normalized results which can be correlated with other studies without concern for variations in specimen size and shape between studies. The terminology we used to report our results accurately describes the data it was intended to identify.

We have provided evidence that frozen storage of specimens in saline for periods up to four months will not significantly alter density of the whole rat femur. Although we have not proven that this stability in density applies to the femoral neck specifically, we believe it is reasonable to conclude that the density of the whole femur is also representative of the femoral neck density. Our results also showed that hydration under vacuum can decrease bone density of femurs frozen in 0.9% saline for up to four months compared to the density of fresh specimens. We have shown that extending the frozen-storage period to twenty-seven months somehow interferes with the ability of hydration under vacuum to reduce bone density. Even though not statistically significant, hydration under vacuum for as little as one hour increases bone density of femurs frozen for this extended period of time. Finally, we presented evidence that both frozen-storage and hydration under vacuum can alter the biomechanical behavior of the femoral neck.

Our review and summary of the published literature provided evidence of the need for standardization of testing and reporting procedures in femoral neck breaking studies. Hopefully

this will highlight the problem and lead others toward adopting a more standardized approach to their testing and reporting techniques.

Conclusions

Hip fracture associated with osteoporosis is a major health concern for the aging world population. The aged ovariectomized rat has proven itself to be an appropriate model for studies involving osteoporosis induced by estrogen-deficiency. Testing of the rat femoral neck is gaining popularity in osteoporosis studies to examine changes in the mechanical behavior of bone in response to intervention treatments.

Although this testing site is being used more frequently, very little baseline literature has been published regarding the biomechanical behavior of the rat femoral neck. Reporting techniques are non-standardized, with results often reported in non-normalized measurements that make correlation of data between studies difficult and inaccurate. No consistency exists between studies for behavioral parameters that are measured or for the terminology used to report this data.

The current study had three goals: (1) determine which breaking measurements provide the most accurate representation of femoral neck biomechanical behavior; (2) determine what terminology and units of measurement are most appropriate for reporting breaking values; (3) identify the effects of storage and hydration protocols on density and biomechanical behavior of the rat femoral neck.

The information gained from the current study can provide the research community with a starting point for better understanding the biomechanical behavior of the rat femoral neck. Our baseline data supplies lacking information regarding the effects of frozen storage and hydration

on whole femur density and femoral neck mechanical behavior. By following the guidelines set forth by Turner and Burr in their tutorial on biomechanical testing of bone, we have offered an example of appropriate testing and reporting protocols. Presenting our data in this format will allow a smooth and accurate transfer of information for future researchers interested in using the femoral neck as a testing site.

REFERENCES

- 1) National Osteoporosis Foundation. (2001). Fast facts on osteoporosis. [WWW page]. URL <http://www.nof.org/osteoporosis/stats.htm>
- 2) American Association of Orthopaedic Surgeons. (2001). Falls and hip fractures. [WWW page]. URL http://orthoinfo.aaos.org/fact/thr_report.cfm?Thread_ID=77&topcategory=Hip
- 3) National Institutes of Health. (1988). Type II osteoporosis. [WWW page]. URL <http://www.niams.nih.gov/rtac/funding/grants/pa/pa88-10.htm>
- 4) Orwoll, E., MD. (1999). Strategies for osteoporosis. [WWW page]. URL http://www.nof.org/men/strategies_men.htm
- 5) Seeman, E. (1995). The dilemma of osteoporosis in men. American Journal of Medicine, 98 (2A), 76S-88S.
- 6) National Osteoporosis Foundation. (2001). Men and osteoporosis. [WWW page]. URL <http://www.nof.org/men/>
- 7) National Osteoporosis Foundation. (2001). Backgrounder. [WWW page]. URL http://www.nof.org/news/pressreleases/background_hipfracture.htm
- 8) National Institutes of Health. (1999). Report of the rat model priority meeting. [WWW page]. URL <http://www.nhlbi.nih.gov/resources/docs/ratmtg.htm>
- 9) World Health Organization. (1999). Osteoporosis: Both health organizations and individuals must act now to avoid an impending epidemic. [WWW page]. URL <http://www.who.int/inf-pr-1999/en/pr99-58.html>
- 10) Cooper, C., Campion, G., & Melton, L. J. (1992). Hip fractures in the elderly: A world-wide projection. Osteoporosis International, 2, 285-289.
- 11) European Communities/European Foundation for Osteoporosis. (1998). Report on osteoporosis in the european community: Action for prevention. [WWW page]. URL <http://www.connect.ie/effo/summary/uk/ukintro.htm>
- 12) Albright, F. (1947). Osteoporosis. Annals of Internal Medicine, 27 (6), 861-868.
- 13) Nordin, B. E. C. (1971). Clinical significance and pathogenesis of osteoporosis. British Medical Journal, 1, 571-576.

- 14) Riggs, B. L., Wahner, H. W., Seeman, E., Offord, K. P., Dunn, W. L., Mazess, R. B., Johnson, K. A., & Melton, L. J. (1982). Changes in bone mineral density of the proximal femur and spine with aging: Differences between the postmenopausal and senile osteoporosis syndromes. Journal of Clinical Investigation, *70*, 716-23.
- 15) Melton III, L. J., & Riggs, B. L. (1983). Epidemiology of age-related fractures. In L. V. Avioli, The Osteoporotic Syndrome (pp. 45-72). New York: Grune & Stratton.
- 16) Riggs, B. L., & Melton III, L. J. (1983). Evidence for two distinct syndromes of involutional osteoporosis. American Journal of Medicine, *75*, 899-901.
- 17) Nordin, B. E. C., Need, A. G., Chatterton, B. E., Horowitz, M., & Morris, H. A. (1990). The relative contributions of age and years since menopause to postmenopausal bone loss. Journal of Endocrinology & Metabolism, *70*, 83-88.
- 18) Riggs, B. L., & Melton III, L. J. (1986). Involutional osteoporosis. New England Journal of Medicine, *314* (26), 1676-86.
- 19) Riggs, B. L., Khosla, S., & Melton, L. J. (1998). A unitary model for involutional osteoporosis: Estrogen deficiency causes both type I and type ii osteoporosis in postmenopausal women and contributes to bone loss in aging men. Journal of Bone and Mineral Research, *13* (5), 763-773.
- 20) Riggs, B. L., Wahner, H. W., Melton III, L. J., Richelson, L. S., Judd, H. L., & Offord, K. P. (1986). Rates of bone loss in the appendicular and axial skeletons of women: Evidence of substantial vertebral bone loss prior to menopause. Journal of Clinical Investigation, *77*, 1487-1491.
- 21) Genant, H. K., Cann, C. E., Ettinger, B., & Gordon, G. S. (1982). Quantitative computed tomography of vertebral spongiosa: A sensitive method for detecting early bone loss after oophorectomy. Annals of Internal Medicine, *97*, 699-705.
- 22) Ahlborg, H. G., Johnell, O., Nilsson, B. E., Jeppsson, S., Rannevik, G., & Karlsson, M. K. (2001). Bone loss in relation to menopause: A prospective study during 16 years. Bone, *28* (3), 327-331.
- 23) Chittachoen, A., Theppisai, U., Sirisriro, R., & Thanantaseth, C. (1997). Pattern of Bone loss in surgical menopause: A preliminary report. Journal of American Medical Association, *80* (11), 731-737.
- 24) Kiel, D. P., Felson, D. T., Anderson, J. J., Wilson, P. W. F., & Moskowitz, M. A. (1987). Hip fracture and the use of estrogens in postmenopausal women: The Framingham Study. New England Journal of Medicine, *317*, 1169-74.

- 25) Paganini-Hill, A., Chao, A., Ross, R. K., & Henderson, B. E. (1991). Exercise and other factors in the prevention of hip fracture: The Leisure World Study. Epidemiology, *2*, 16–25.
- 26) Vega, E. M., Mautalen, C. A., Carrilero, P., Wittich, A., Otano Sahores, A., & Silberman, F. S. (1996). Proximal femoral fractures: Risk factors, bone mineral density, body composition and biochemical changes in age-matched patients and controls. Medicina (Buenos Aires), *56* (4), 353-62.
- 27) Vega, E. M., Egea, M. A., & Mautalen, C. A. (1994). Influence of the menopausal age on the severity of osteoporosis in women with vertebral fractures. Maturitas, *19*, (2), 117-24.
- 28) Soda, M. Y., Mizunuma, H., Honjo, S., Okano, H., Ibuki, Y., & Igarashi, M. (1993). Pre- and postmenopausal bone mineral density of the spine and proximal femur in Japanese women assessed by dual-energy x-ray absorptiometry: A cross-sectional study. Journal of Bone and Mineral Research, *8* (2), 183-189.
- 29) Pouilles, J. M., Tremollieres, F., Bonneu, M., & Ribot, C. (1994). Influence of early age at menopause on vertebral bone mass. Journal of Bone and Mineral Research, *9* (3), 311-315.
- 30) Ohta, H., Sugimoto, I., Masuda, A., Komukai, S., Suda, Y., Makita, K., Takamatsu, K., Horiguchi, F., & Nozawa, S. (1996). Decreased bone mineral density associated with early menopause progresses for at least ten years: Cross-sectional comparisons between early and normal menopausal women. Bone, *18* (3), 227-231.
- 31) Seeman, E., Cooper, M. E., Hopper, J. L., Parkinson, E., McKay, J., & Jerums, G. (1988). Effect of early menopause on bone mass in normal women and patients with osteoporosis. American Journal of Medicine, *85*, 213–216.
- 32) Seeman, E., & Allen, T. (1989). Risk factors for osteoporosis. Australian and New Zealand Journal of Medicine, *19* (1), 69–75.
- 33) Richelson, L. S., Wahner, H. W., Melton III, L. J. III, & Riggs, B. L. (1984). Relative contributions of aging and estrogen deficiency to postmenopausal bone loss. New England Journal of Medicine, *311* (20), 1273–1275.
- 34) Seeman, E., & Cooper, M. (1987). The effect of early and surgically induced menopause on bone mass. In C. Christiansen, J. S. Johansen, & B. J. Riis, Osteoporosis, (Vol.1, pp. 85-86). Denmark: Osteopress ApS.
- 35) Ohta, H., Masuda, A., Komukai, S., & Nozawa, S. (1994). Osteoporosis after menopause and oophorectomy. Nippon Rinsho, *52* (9), 2382-2394.

- 36) Ohta, H., Masuzawa, T., Ikeda, T., Suda, Y., Makita, K., & Nozawa, S. (1992). Which is more osteoporosis-inducing: Menopause or oophorectomy? Journal of Bone and Mineral Research, *19* (3), 273-85.
- 37) Food and Drug Administration. (1994). Guidelines for preclinical and clinical evaluation of agents used in the prevention or treatment of postmenopausal osteoporosis. [WWW page]. URL <http://www.fda.gov/cder/guidance/osteo.pdf>
- 38) Bagi, C. M., Wilkie, D., Georgelos, K., Williams, D., & Bertolini, D. (1997). Morphological and structural characteristics of the proximal femur in human and rat. Bone, *21* (3), 261-267.
- 39) Bagi, C. M., Ammann, P., Rizzoli, R., & Miller, S. C. (1997) Effect of estrogen deficiency on cancellous and cortical bone structure and strength of the femoral neck in rats. Calcified Tissue International, *61*, 336-344.
- 40) Mattila, P. (1999). Dietary xylitol in the prevention of experimental osteoporosis: Beneficial effects on bone resorption, structure and biomechanics. [WWW page]. URL <http://herkules.oulu.fi/isbn951425158X/>
- 41) Kalu, D., Liu, C., Hardin, R., & Hollis, B. (1989). The aged rat model of ovarian hormone deficiency bone loss. Endocrinology, *1*, 7-16.
- 42) Nnakwe, N. (1995). The effect of aging on bone compositions of female fischer 344 rats. Mechanisms of Ageing and Development, *85*, 125-131.
- 43) Bagi, C. M., DeLeon, E., Ammann, P., Rizzoli, R., & Miller, S. C. (1996). Histo-anatomy of the Proximal femur in rats: Impact of ovariectomy on bone mass, structure, and stiffness. The Anatomical Record, *245* (4), 633-44.
- 44) Peng, Z., Tuukkanen, J., & Zhang, H. (1994). The mechanical strength of bone in different rat models of experimental osteoporosis. Bone, *15*, 523-532.
- 45) Nordsletten, L., Kaastad, T. S., Madsen, J. E., Reikeras, O., Ovstebo, R., Stromme, J. H., & Falch, J. (1994). The development of femoral osteopenia in ovariectomized rats is not reduced by high intensity treadmill training: A mechanical and densitometric study. Calcified Tissue International, *55*, 436-442.
- 46) Suponitzky, I., & Weinreb, M. (1998). Differential effects of systemic PGE2 on bone mass in rat long bones and calvaria. Journal of Endocrinology, *156*, 51-57.
- 47) Tuukkanen, J., Peng, Z., & Väänänen, H. K. (1994). Effect of running exercise on the bone loss induced by orchidectomy in the rat. Calcified Tissue International, *55*, 33-37.

- 48) Hou, J. C-H., Zernicke, R. F., & Barnard, R. J. (1991). Experimental diabetes, insulin treatment, and femoral neck morphology and biomechanics in rats. Clinical Orthopaedics, 264, 278-285.
- 49) Toolan, B.C., Shea, M., Myers, E. R., Borchers, R. E., Seedor, J. G., Quartuccio, H., Rodan, G., & Hayes, W. C. (1992). Effects of 4-amino-1-hydroxybutylidene bisphosphonate on bone biomechanics in rats. Journal of Bone and Mineral Research, 7, 1399-1406.
- 50) Sogaard, C. H., Danielsen, C. C., Thorling, E. B., & Mosekilde, L. (1994). Long-term exercise of young and adult female rats: Effect on femoral neck biomechanical competence and bone structure. Journal of Bone and Mineral Research, 9 (3), 409-416.
- 51) Sogaard, C. H., Wronski, T. J., McOsker, J. E., & Mosekilde, L. (1994). The positive effect of parathyroid hormone on femoral neck bone strength in ovariectomized rats is more pronounced than that of estrogen or bisphosphonates. Endocrinology, 134, 650-657.
- 52) Peng, Z., Tuukkanen, J., & Väänänen, H. K. (1997). Ovariectomy-induced bone loss can be affected by different intensities of treadmill running exercise in rats. Calcified Tissue International, 60, 441-448.
- 53) Sato, M., Zeng, G. Q., & Turner, C. H. (1997). Biosynthetic human parathyroid hormone (1-34) effects on bone quality in aged ovariectomized rats. Endocrinology, 138, 4330-4337.
- 54) Turner, C. H., & Burr, D. B. (1993). Basic biomechanical measurements of bone: A tutorial. Bone, 14, 595-608.
- 55) Shen, Y., Li, M., & Wronski, T. J. (1997). Calcitonin Provides complete protection against cancellous bone loss in the femoral neck of ovariectomized rats. Calcified Tissue International, 60, 457-461.
- 56) Broz, J. J., Simske, S. J., Greenberg, A. R., & Lettges, M. W. (1993). Effects of rehydration on the flexural properties of whole mouse bones. Journal of Biomechanical Engineering, 115, 447-449.
- 57) Keenan, M. J., Hegsted, M., Reisenauer, A. M., Ward, T. L., & Southern, L. L. (1992). Effect of increased hydration and removal of bone fat on a method for determination of bone density. Journal of Bone and Mineral Research, 7, 247-248.

APPENDIX A

Mechanical Testing Procedure Using the Instron 4301 Materials Testing Machine

- 1) place specimen holding device into the grip jaws on the loading stage and secure loosely
- 2) attach actuator to the loading arm
- 3) power-up Instron 4301 and align actuator with center of holding device and tighten grip jaws securely
- 4) place copper pipe with embedded specimen (preferably a test specimen) into the holding device and tighten loosely
- 5) power up computer and start Merlin IX control software
- 6) select desired testing protocol (compression) from the menu
- 7) place a specimen in the holder and lower actuator until it rests about 1/4" above the femoral head
- 8) use adjustment screws in holding device to center the femoral head directly under the actuator and tighten securely
- 9) lower actuator until a minimal load reading appears on the digital display
- 10) zero *load* and *displacement* readings on Instron control panel
- 11) swap Instron to "IEEE" mode
- 12) start test using Merlin software
- 13) enter specimen *diameter* and *gauge length* when requested
- 14) end test using Merlin software
- 15) reset Instron display panel to the non-IEEE mode to repeat procedure for the next specimen

APPENDIX B

Photos of Mechanical Testing Equipment

Photo 1: Pump setup for hydration under vacuum

Photo 2: Mettler balance for density testing

Photo 3: Femur mounted for mechanical testing

Photo 4: Instron testing machine and computer

Photo 5: Femoral head breaking apparatus

Photo 6: Close-up of femoral head breaking setup



Photo 1: Pump setup for hydration under vacuum

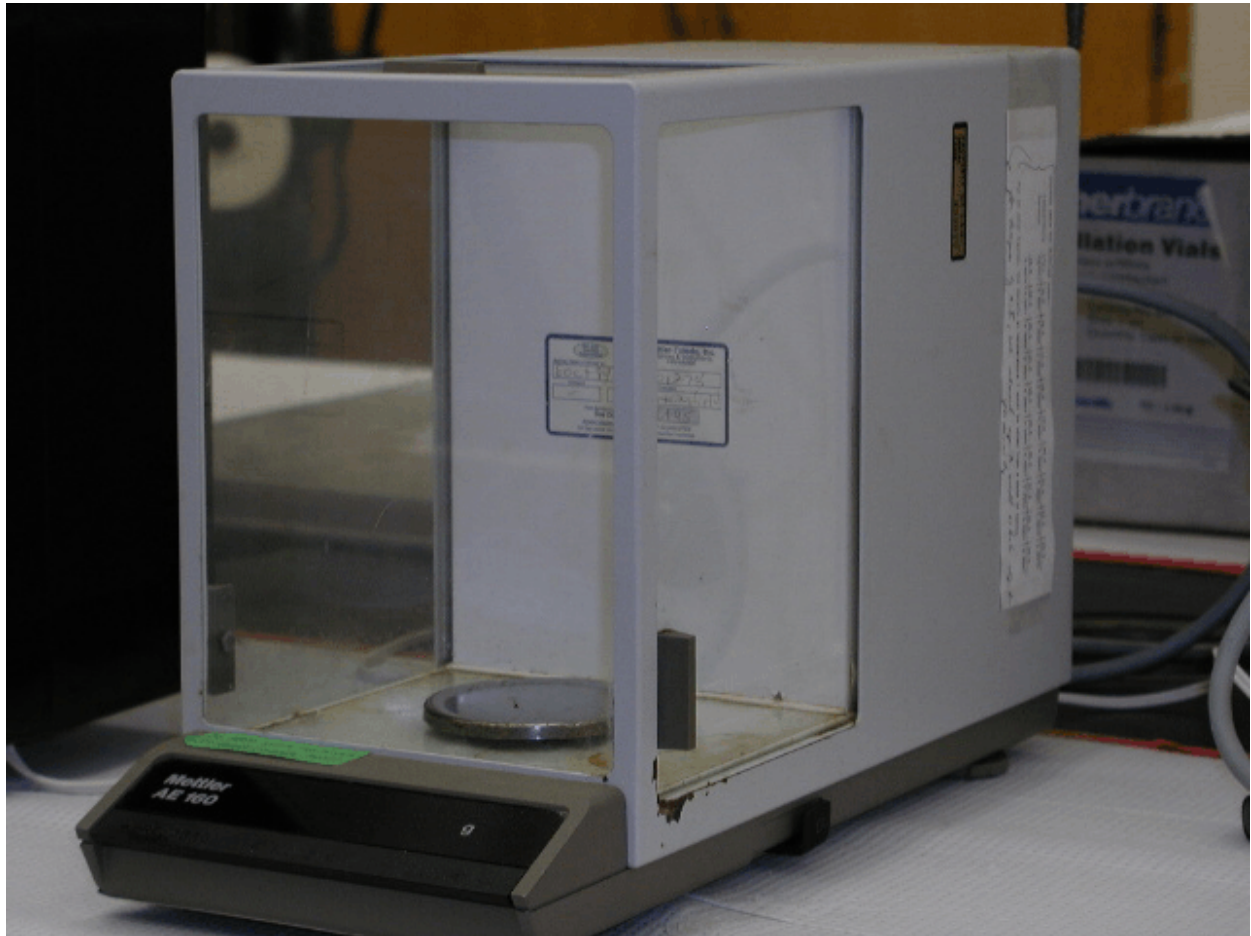


Photo 2: Mettler balance for density testing



Photo 3: Femur mounted for mechanical testing



Photo 4: Instron testing machine and computer

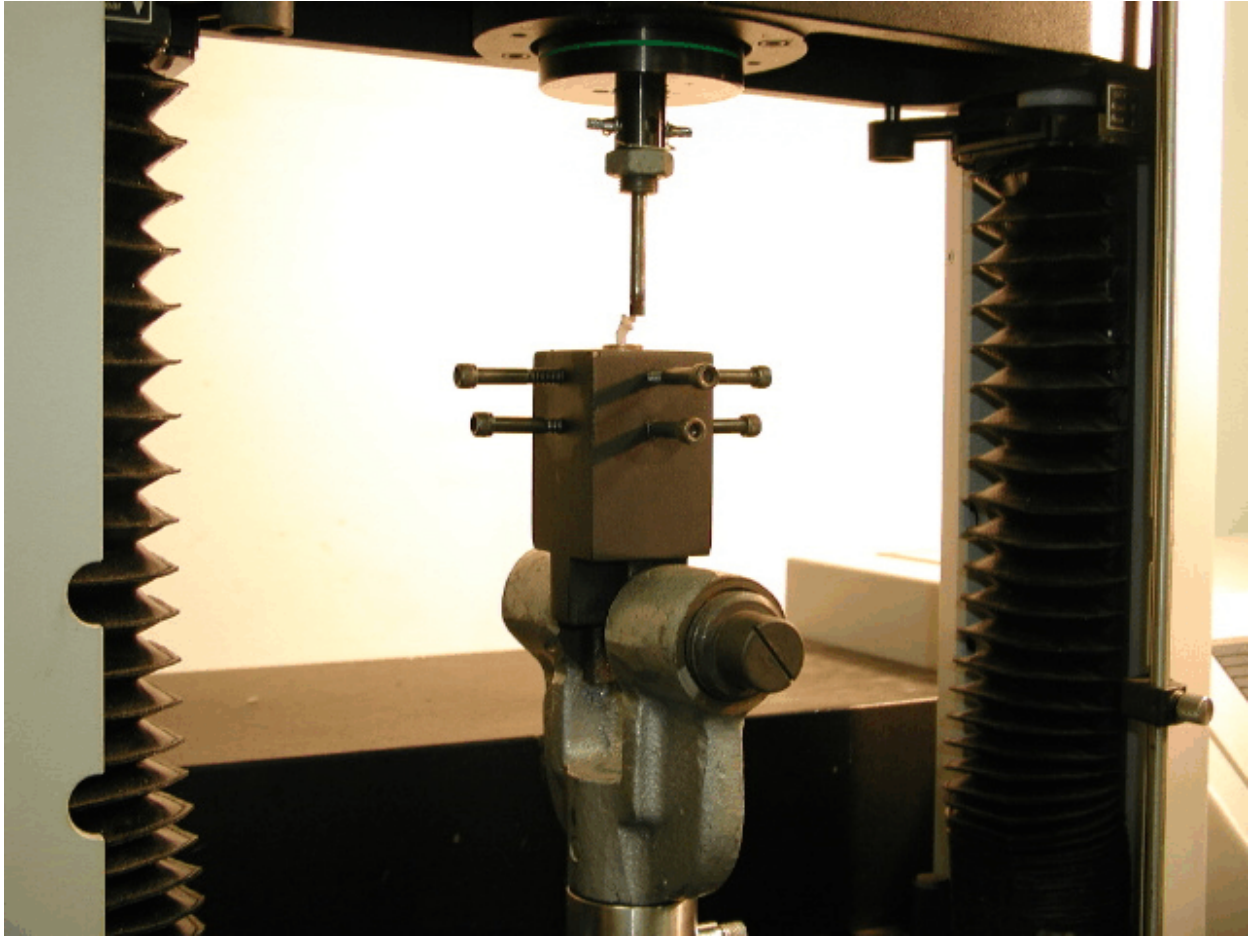


Photo 5: Femoral head breaking apparatus

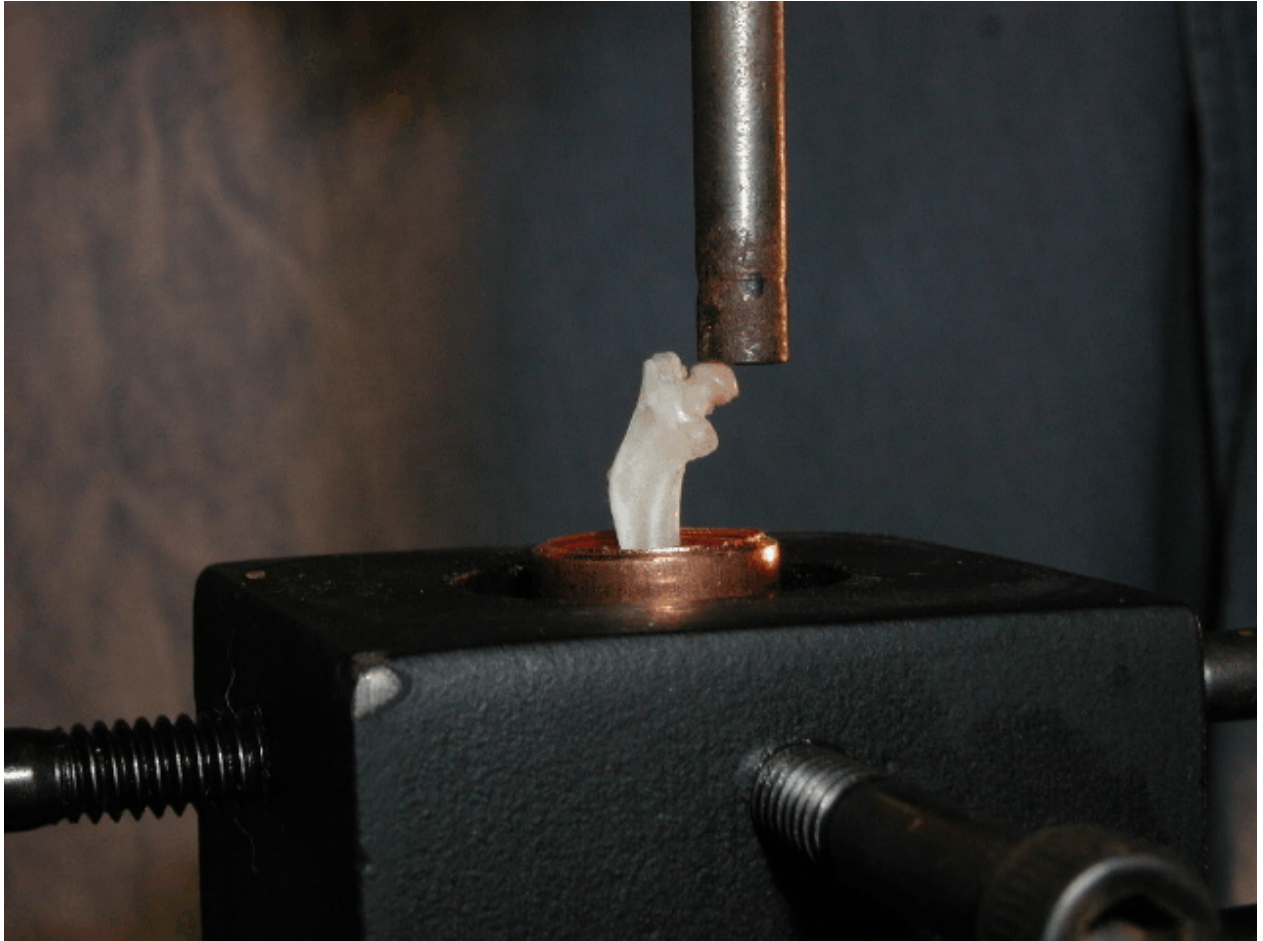


Photo 6: Close-up of femoral head breaking setup

VITA

The author was born in McComb, Mississippi on May 17, 1954 to parents Mary and Buford Reeves, who moved to Baton Rouge, Louisiana in 1959. After finishing high school, he received four semesters of credit in a professional geology curriculum at Louisiana State University. He worked for 13 years as a power plant equipment operator, during which time he married Karen Lynn Brittingham and had three children, Brandon, Kala, and Krista. After a disabling industrial accident, the author returned to his university studies in September, 1992.

In May of 1996 the author received his Bachelor of Science in Human Ecology, majoring in Dietetics. He began his graduate studies in September, 1996 working toward a Master's degree in Human Nutrition and Food in preparation for entering the dietetic internship sponsored by Louisiana State University. The author plans are to begin his dietetic internship in September, 2002.