The Effects of 70% Ethanol Storage on Bone Mechanical Properties

A dissertation submitted in partial satisfaction of the requirements for the degree of Bachelors of Science in Physics by

Omar Ahmady

Committee:
Dr. Paul K. Hansma, Physics Department
Dr. Cyrus Safinya, Materials Department
Dr. James C. Weaver, Biology Department
The dissertation of Omar Ahmady is approved

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Paul K. Hansma

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Cyrus R. Safinya

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Deborah K. Fygenson
Abstract

The effects of storing bone in 70% ethanol for extended periods were tested using a novel micro-indentation device, the Reference Point Indentation (RPI) instrument. The initial experiment involved testing bovine bone samples as an animal model, while follow-up experimentation involved testing human cortical bone samples. These experiments showed no statistically significant changes in bone mechanical properties as measured by the RPI using its most reliable indentation parameter, total indentation distance. The duration of storage for the bovine animal model bone samples was 40 days, and the duration of storage for the human bone samples was 28 days. In both cases, no changes in bone mechanical properties occurred over the relevant storage periods, suggesting that ethanol storage has a negligible impact on the structural integrity of the extracellular matrix.
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This thesis is dedicated to my parents, who emphasized education above all else.
Introduction

The World Health Organization has argued that the global osteoporosis “time bomb” is ticking, with the projected global burden of osteoporotic hip fractures to exceed 6 million by 2050\textsuperscript{13}. In many countries, patients with hip fractures already occupy more hospital beds than patients with any other medical complication\textsuperscript{1}. Measures are urgently needed to identify individuals at risk of fracture that would benefit from pharmacological intervention. Currently, fracture risk is measured indirectly through noninvasive measurement of bone mineral density\textsuperscript{11}. The majority of studies have tried to relate fracture risk to bone densitometry criteria, but bone densitometry data has only been partially successful in explaining differences in fracture risk among individuals, as observed in vivo and in vitro\textsuperscript{2}. The reasons for the inability of bone densitometry data to accurately quantify fracture risk likely have to do with the biological determinants of bone mechanical properties.
Bone Biology

Bone is composed of approximately 70% mineral, 22% protein, and 8% water\textsuperscript{[2]}. The high rigidity, hardness, and toughness of bone relative to other tissue is a result of the complex that is formed by the inorganic salts that are fettered throughout the extracellular matrix, which are made up of collagen fibers, various non-collagenous proteins, and bone mineral\textsuperscript{[3]}. The bone mineral is made up of small crystals of hydroxyapatite, $\text{Ca}_10(\text{PO}_4)_6(\text{OH})_2$, which are located between the collagen fibrils\textsuperscript{[18]}. The organic matrix is made up of 90% collagen and 10% various other non-collagenous proteins\textsuperscript{[3]}. Type I collagen is the most abundant extracellular protein in bone, and is the most essential contributor to bone strength\textsuperscript{[2]}. This fact is exemplified by the disease Osteogenesis Imperfecta, which is caused by an inability to synthesize enough Type I collagen, resulting in brittle bones and increased fracturability.

The relationship between collagen and bone mineral deposition is demonstrated in vitro from electron micrographs in which the mineral crystals are aligned with the collagen fibril axis\textsuperscript{[17]}. Furthermore, the extent of cross-links between collagen molecules seem to be related to the degree of bone mineralization\textsuperscript{[3]}. Collagen forms a template for mineral deposition, and since the collagen-mineral complex is responsible for maintaining the structure and mechanical properties of bone\textsuperscript{[16]}, any hypothesis about the cause of bone mechanical degradation or lack thereof should include a mechanism accounting for the effects of the storage medium on the collagen-mineral complex in bone.

Recently, Landis, et. al., have demonstrated that the contribution of the mineral phase of bone to bone strength is also a function of the molecular structure and organization of the mineral crystals within the extracellular matrix\textsuperscript{[2]}. The hydroxyapatite crystals, as they occur in the extracellular matrix of bone, possess many discontinuities. These are formed within the structure of a single crystal as it grows, and the number of discontinuities increases with time\textsuperscript{[6]}. Impurities may also exist, causing a further number of discontinuities in the hydroxyapatite crystals\textsuperscript{[6]}.

Thus, differences in extracellular matrix structure and mineralization profiles both have a significant effect on the mechanical integrity of bone and its ability to resist the
growth of micron-level cracks into fractures\textsuperscript{[2]}. Other proposed mechanisms for differences in tissue mechanical properties of bone include changes in mineral crystal size, posttranslational changes in collagen, changes in collagen fibril orientation, and changes in non-collagenous proteins\textsuperscript{[15]}. With such a large number of potential variables, all of which are uncorrelated with Bone Mineral Density measurements, it is not surprising that researchers and clinicians have been unable to accurately quantify fracture risk with a large degree of precision.

Furthermore, it is not always necessary that an ultimate stress level be exceeded in order for a fracture to occur; repeated loading and unloading of bone can cause it to fracture even if loads are below the maximum stress threshold. This phenomenon is known as fatigue failure, and the fractures that result from this type of loading are known as stress fractures. Biomaterials such as bone have repair mechanisms for healing micron-level damage as it occurs. However, when the normal repair mechanisms are impaired (such as in osteoporosis), fractures are more likely to occur\textsuperscript{[2]}. Micro Indentation testing is one technique that may be able to quantify the likeliness of fracture regardless of cause because it directly measures bone mechanical properties.

Figure 1: Illustration of the micron-level cracking that is induced by the RPI. The length scale is small enough to avoid harming the patient, but large enough to fracture bone.
The Reference Point Indentation instrument (previously referred to as Osteoprobe\textsuperscript{[8]}, Bone Diagnostic Instrument\textsuperscript{[9-11]}, and Tissue Diagnostic Instrument\textsuperscript{[12]}) was developed to measure bone mechanical properties, particularly resistance to fracture at the tissue level\textsuperscript{[15]}. The instrument implements a cyclic indentation protocol to measure the ability of bone to resist growth in micron-level stress fractures, which are induced through repeated loading and unloading on the bone. The indentations caused by the instrument are small, on the order of 200 microns across, as illustrated in Figure 1. They are large enough, however, that the bone is fractured, as illustrated in Figure 2\textsuperscript{[15]}. Preclinical studies in human cadavers suggest that these indentations induce separation of mineralized collagen fibrils and initiation of cracks, likely the basic biological mechanism of bone fracture\textsuperscript{[15]}. Thus, the reference point indentation instrument directly measures the mechanical competence of bone tissue to resist fracture, integrating the myriad of variables that have been hypothesized to account for differences in bone fracturability.

Figure 2: Scanning Electron Microscope (SEM) image showing the separation of mineralized collagen fibrils that occurs during bone fracture. This separation of mineralized collagen fibrils also occurs during RPI indentation testing, suggesting that these tests do indeed simulate bone fracture at the micron level.
Chemical preservation methods have been developed by histopathologists to both prevent tissue decay as well as preserve cell structure for microscopic examination\textsuperscript{[3]}. Chemical fixation allows for good preservation of structures, with the possibility of cutting good quality thin sections for imaging\textsuperscript{[4]}. The fixatives are chosen for their ability to penetrate the specimen quickly and completely while extracting marrow fat and avoiding changes to the mineral content of the specimen\textsuperscript{[3]}.

The significance of ethanol (C\textsubscript{2}H\textsubscript{5}OH) in this study is that it is used by many laboratories\textsuperscript{[14]} and pharmaceutical companies\textsuperscript{[22]} for the purpose of preservation of bone prior to further investigation. Ethanol is the fixing agent usually employed in bone, because it allows for good preservation of its mineral phase\textsuperscript{[4]}. In order to avoid an acidic solution, which would cause complete or partial demineralization of samples, a pH neutral 70\% ethanol solution is generally used as the fixative and storage medium\textsuperscript{[4]}. This ethanol concentration causes no shrinkage or expansion of cells, which can still be recognized through imaging if necessary\textsuperscript{[4]}. The bone is stored in the 70\% ethanol solution at 5\textdegree Celsius\textsuperscript{[3]}.

In order to reliably test the effects that pharmacological treatments have on the mechanical properties of bone in vivo, it must first be determined whether long-term storage in ethanol has any effects on the mechanical properties of bone samples. Using the total indentation distance parameter that the Reference Point Indentation instrument measures, these experiments were conducted to measure the effect that long-term storage of bone in ethanol has on bone mechanical properties, using the newly developed RPI instrument. There is currently no study available that specifically addresses the effects of ethanol on the fracture mechanics characterization of bone\textsuperscript{[14]}.
Materials & Methods

Clinical trials have demonstrated that two of the three measured indentation parameters are significantly greater for patients with fractures than for control patients\textsuperscript{[15]}. These measures are the total indentation distance (the total distance that the test probe was able to indent the bone) and the indentation distance increase (the distance between the displacement after the linear loading on the 1\textsuperscript{st} indentation cycle to the displacement after the linear loading on the 20\textsuperscript{th} indentation cycle). Data results from clinical trials using the receiver operating characteristic (ROC) curve showed that the total indentation distance is a good discriminator between patients with and without fractures\textsuperscript{[15]}. This data showed more statistically significant results using total indentation distance than indentation distance increase, which provides the justification for using total indentation distance as the relevant variable of interest, rather than indentation distance increase.

Figure 3: Illustration of the derivation of the TID and IDI indentation parameters from the force vs. distance curves as measured by the RPI. My bovine animal model and human experiments utilized TID, as it was determined to be more statistically significant from the clinical trials.
The Reference Point Indentation (RPI) instrument uses a reference probe and test probe assembly in order to measure the key indentation parameters. The micro fractures are induced by the repeated loading and unloading of the test probe on the bone. The test probe cycles through the reference probe while the loading occurs, while the reference probe remains stationary. The test probe loading increases linearly from 0 to 11 Newtons for 1/3 of the cycle, stays at 11 Newtons for 1/3 of the cycle, and unloads linearly from 11 to 0 Newtons for 1/3 of the cycle. For each measurement, 20 indentation cycles are made at the rate of 2 Hz.

Figure 4: Scanning Electron Microscope (SEM) image of an indent made in human bone utilizing the novel Reference Point Indentation instrument. The central indent is made by the test probe, while the indent on the right is made by the reference probe. The central indent’s size is on the order of 200 microns across.

The first step of the experiments involved removing the periosteum from the bone samples, which was also done during clinical trials\textsuperscript{[15]}. The second step involved sanding down the bone to create a smooth, homogenous surface for testing. Previous testing has demonstrated that when the bone is sanded down, the variance of the TID and IDI measurements across a given bone sample at a given sampling time decreased by a large amount. After sanding the bone down on three different grades of sandpaper, the bone
was then polished on a rotary with deionized water. In order to prepare the 70% ethanol that was to be used for storage, 70 parts of 100% ethanol and 30 parts of deionized water were placed in a flask.

The procedure for testing the bone is as follows: first, the bone was placed in a clamp, to hold it firmly in place for the testing. In order to prevent the drying of the bone (which significantly changes the bone’s mechanical properties), the clamp/holding apparatus was filled with Hank’s solution, a buffer kept at a pH of 7.4 which ensures hydration\(^{[16]}\). It was then confirmed that the bone is fully immersed in the Hank’s solution buffer. If the aqueous environment that the bone is placed in were not properly buffered with respect to the bone mineral, the mineral matrix would deteriorate\(^{[3]}\).

Next, a new probe assembly was utilized for every new experiment in order to avoid any possible errors that could arise from variance in the measuring apparatus. I also photograph the test probe under a light microscope to ensure that it is sharp. If the test probe is bent or damaged, this can cause errors in the measured indentation parameters.

![Test Probe Image](image.png)

Figure 5: Picture taken of the test probe, utilizing a light microscope equipped with a digital camera. The radius of the test probe was measured as approximately 5 microns across.
After photographing the test probe, the reference probe and test probe assembly are then attached to the Reference Point Indentation instrument. The instrument’s controls were utilized to lower the test probe to the sample’s surface. A sample of PMMA was tested continually throughout the course of the experiment to ensure that the RPI was working accurately and consistently. When testing bone, the light was turned on to examine the parts of the bone that have been tested, and to find the new region of the bone that is to be tested (If the same region is tested over again, this also causes errors in the observed indentation values). For each bone sample, 10 IDI measurements were made for every round of testing, in order to minimize the statistical variance in bone mechanical properties across a given sample. After conducting the testing, the bone was then placed back in its respective storage medium.

However, one aspect of the mechanical testing must be handled with specific care. The mechanical properties of bone vary not only according to the nature of the force applied, but also to its direction and rate of application. Bone is thus an anistropic material, as it has different mechanical properties in different directions. For this reason, the protocol in the experiment (as with the protocol in the clinical trials) required that the test probe must be perpendicular to the bone’s surface, within a margin of 15 degrees or less.

**Bovine Bone Experiment**

For the bovine animal model research, two primary experiments were conducted. The first experiment involved testing different animal bone samples, such as swine, foul, and bovine, in order to determine which would serve as the best animal model. Because of the fact that bovine bone had the lowest standard deviation in indentation parameter measurements and the use of bovine as an animal model in previous bone mechanical experiments, bovine was ultimately chosen as the preferred animal model. The second experiment was conducted with 5 control bovine samples, and 5 thermally degraded bovine samples. The period of thermal degradation for the thermally degraded bovine bone samples was 2 hours at 150°C Celsius. The samples were thermally degraded to determine whether ethanol storage has a differing effect on baked bone, as well as to
ensure that the RPI does indeed give higher TID values for the baked bone, since it is known that thermal degradation weakens the extracellular matrix\textsuperscript{[19]}. The samples were cut by a band saw into 3 cm. by 3 cm. square pieces. The samples were first tested as a control pre-storage, and were subsequently tested post-ethanol storage after periods of 10, 20, 30, and 40 days. The main purpose of this experiment was to ascertain whether there is a statistically significant difference in mean TID between bovine bone that has and has not been previously thermally degraded, and whether ethanol storage has an effect on mean TID for control and thermally degraded bovine bone.

Human Bone Experiment

Human tibias from 71 and 83 year old females were obtained from the University of California at Irvine Willed Body Program. The human bone experiment involved 6 samples from the lateral section of the 71 year old female’s tibia, and 6 samples from the lateral section of the 83 year old female’s tibia. These samples were cut into 2 cm. by 2 cm. square blocks using a band saw. Of the 6 lateral samples from each individual, 3 were from the posterior region and 3 were from the dorsal region. As representative a sample as possible was obtained for each individual, but the samples obtained from one individual to the other are identical in terms of the particular bone region that was selected. This was to prevent the possibility of ethanol affecting two different bone regions differently, which would have skewed the end results.

For the human bone research, the initial stage involved control testing all 12 human bone samples. In order to log both the time at which the indent was made, as well as ensure that no two indents were made over the same space, 5 marks were made alongside the edge of the bone with a band saw, corresponding to control testing, 1 week of ethanol storage, 2 weeks of ethanol storage, etc. Beside each mark, a row of 10 indents was tested after each particular storage interval, for each bone sample. This allowed for keeping track of which indents corresponded to which storage intervals when the bone samples were SEM imaged. Since the samples were tested from right to left, this also allowed me to determine which indents correspond to which total indentation distance values.
Statistical Analysis

Each individual test was analyzed in order to calculate the indentation parameters such as total indentation distance\textsuperscript{[15]}. For the bovine animal model experiments, the control data and data for each subsequent period of storage time were calculated by taking the mean of all measurements taken on a particular day. For the human bone experiments, the mean was also calculated for each individual section of bone tested on a particular day. The justification for grouping the data in this manner came from a bone mapping experiment conducted in the lab, which discovered a large degree of variance in the indentation parameters that are measured across a single bone sample at a single point in time. For this reason, the most valid assessment of the effects of ethanol on bone mechanical properties involved assessing changes in a particular sample over time; however, the overall results of the human bone data have also been included, which take the mean of measurements made for control and 1, 2, 3, and 4 weeks of storage.
Results

Effects of Ethanol Storage on Unbaked vs. Baked Bovine Bone

The results of the bovine animal model experiment showed no statistically significant changes in total indentation distance over the storage period of 40 days. There was a modest increasing trend in TID values for the unbaked samples, but this did not reach the level of statistical significance. The baked bovine samples showed no trend and no statistically significant changes in TID values over the relevant storage period. Of the 5 control bovine samples and 5 thermally degraded bovine samples, none of the samples demonstrated statistically significant changes at the 1% level and only 1 sample demonstrated a statistically significant change at the 5% level. As Figure 4 illustrates however, in all cases the baked bovine samples had a higher total indentation distance than the unbaked samples, suggesting that thermally degrading bone causes a highly significant decrease in bone mechanical properties.

Figure 6: Chart depicting testing of unbaked and baked bovine bone stored in 70% ethanol at 5° Celsius for 40 days. After control testing, the bovine bone was tested every 10 days.
Effects of Ethanol Storage on the Mechanical Properties of Human Bone

The results of the human bone experiment replicated the bovine animal model experiment, as there was no statistically significant change in the TID values of the bones over the 4-week storage period in ethanol. Figure 7 shows the data trend for the six 2 cm. by 2 cm. samples from the 71 year old individual, and Figure 8 shows the data trend for the six 2 cm. by 2 cm. samples from the 83 year old individual. Each data point for each sample represents the mean of the 10 TID measurements made on a particular bone sample at a given moment in time.

Figure 7: Scatterplot depicting testing of 6 bone samples from 71 year old female stored in 70% ethanol at 5° Celsius for 4 weeks. After control testing, the human bone samples were tested on a weekly basis, and no statistically significant changes occurred.
Both individuals showed a slight negative trend in their measured TID values over the storage periods. However, these changes were largely statistically insignificant. Of the 6 bone samples tested from the 71 year old female, none of the samples showed a statistically significant change in TID values at the 1% or 5% significance levels. Of the 6 bone samples tested from the 83 year old female, only 1 sample showed a statistically significant change in TID values at the 5% significant level. Thus, most of the statistical evidence points to the conclusion that storage in 70% ethanol does not have a discernible impact on bone mechanical properties over the time scale of 4 weeks.

For consistency with the bovine experiments, the mean of all the TID values for bone samples from both the 71 year old and 83 year old female was also plotted. Though there was a slightly negative trend in the overall mean of TID values over the 4 week
storage period, this trend was only statistically significant for the control vs. week 1 t-test. However, the control vs. week 2 t-test (p-value = 0.06), control vs. week 3 t-test (p-value = 0.24), and control vs. week 4 t-tests (p-value = 0.07) were all statistically insignificant at the 1% level. Averaging all the storage periods and comparing it to the control data, we observe a statistically insignificant 4.88% decrease in TID values over the 4 week storage period. However, as noted earlier, since there is a large degree of variation in TID values across a given human bone sample, tests of statistical significance are likely more valid when conducted on individual bone samples across time, rather than the entire distributions across time.

![Effects of Ethanol Storage on Human Bone](chart.png)

Figure 9: Chart depicting testing of human bone samples stored in 70% ethanol at 5° Celsius for 4 weeks. The mean TID for control and each subsequent week was calculated as the mean of the TID values of the 6 bone samples from the 71 year old female and the 6 bone samples from the 83 year old female.
Discussion

These results demonstrate that no statistically significant changes occur in the bone mechanical properties of either bovine animal model samples or human bone samples stored in 70% ethanol for the relevant time periods, as tested by the Reference Point Indentation (RPI) instrument. The bovine animal model experiment showed no statistically significant change in bone mechanical properties; however, the baked samples had higher total indentation distance values than the unbaked samples over the entire storage period, by a statistically significant margin.

The reference probe indentation (RPI) instrument could potentially be used to determine the effects of novel pharmacological therapies on bone mechanical properties, following a storage period. This research was conducted in order to determine whether temporary storage in 70% ethanol has any significant effect on bone mechanical properties.

These results are consistent with earlier experiments that have demonstrated that thermally degrading bone causes degradation in the extracellular matrix\(^{19}\). It also reinforces the hypothesis that the primary determinant of bone structure is the integrity of the extracellular matrix, and that this variable is directly measured from total indentation distance values.

Similarly, the human bone experiments demonstrated no statistically significant changes in bone mechanical properties, particularly when the samples were sorted on an individual basis. The justification for this came from bone mapping tests conducted in the laboratory which determined that there is a large degree of variance in indentation parameter values across a given human bone sample.

In conclusion, storage of human bone in 70% ethanol has no significant effect on bone mechanical properties for at least 4 weeks of storage time. These results are consistent with previous research by Ashman\(^{20}\) and Linde and Sorenson\(^{21}\) which concluded that storage of bone in 70% ethanol does not lead to degradation in bone mechanical properties. For this reason, researchers should strongly consider post-mortem storage of bone in 70% ethanol before bone mechanical properties are investigated in the laboratory.
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