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Effects of Cryopreservation on the Mechanical Properties of Bone

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

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Abstract

Effects of freezing bone at -20°C were tested using the Reference Point Indentation instrument on swine, foul, bovine femur, and human tibia. The effect of freeze-thaw cycles varied depending on the anatomy of the bone, but in all cases, the effect on the mechanical properties was smaller than the natural variation of those properties across a sample. Degradation on key mechanical tests was always found to be 15% or less. Subsequent freeze thaw cycles had no effect on further degration of the bone samples. Duration of freezing was negligible compared to phase-change and too small to measure on the time scale of 35 days. Furthermore, significant evidence was found supporting the theory that freezing degrades the organic portion of the extra-cellular matrix.

Acknowledgements

I would like to thank Paul Hansma. He helped me obtain funding for this project and mentored me with great wisdom, while at the same time giving me plenty of space to learn on my own. I would also like to thank Connor Randall, the lab manager, for helping me with so many different aspects of research that I do not have the space to list them on this page. I would also like to thank James Weaver for helping me understand the biology of bone, extensive graphic design help, SEM images, and helping me create some of the figures in the paper. I had spent so many hours reading literature, but I did not develop an intuitive feeling of bone until I started collaborating with James. I would like to thank Daniel Walsh for spending hours upon hours going through PSTAT textbooks with me as well as lending me his outside perspective on my research. I would like to thank Dan Bridges for contributing a figure and for general help in the lab. Finally, I would like to thank my dad, Steven Kaye, for teaching me how to navigate the world of Medicine and Biology.

I would like to thank URCA for funding this research project (URCA #37), UCI Willed Body Program for human tissue samples, and NIH (NIH R01 GM065354) for funding the Hansma Lab

This thesis is in memory of my loving Uncle Mike
1953-2009

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Introduction

As people age, they become much more susceptible to bone fracture. It is estimated that ten million Americans have osteoporosis[1]. Because of this, diagnosing and treating fractures has become a large and growing medical sector. In the U.S. alone, over $15 billion is spent on osteoporotic treatment each year. In addition, over 300,000 Americans break their hips each year, and have a 30% chance of dying within a year after hip replacement surgery[2]. Because of these facts, increasing and diagnosing bone strength has become a major field of study.

In the clinical setting, bone fracturability is currently determined *indirectly* by measuring its Bone Mineral Density (BMD) using low level radiation[3]. Although this technique is good, it is far from perfect. BMD reveals the density of mineral in the bone, but does not reveal the architecture of the bone. The density of mineral is only one factor in determining fracturability. In the lab, this principal is seen when dealing with thermally degraded bone, which is much more fracturable than untreated bone, but has the same BMD index. In the real world, this point is best exemplified with the disease *Osteogenesis Imperfecta (*aka Brittle Bone Disease). An X-Ray from a patient suffering from this disease is shown in Figure 1. This disease is caused by a patient's inability to produce an adequate amount of Type 1 collagen[4], an integral part of the bone organic extracellular matrix. These patients therefore have an incredibly high fracturability, which is due to a weakened organic matrix, and not necessarily a low bone mineral density[5].Therefore BMD would not be able to diagnose the fracture risk of many of these patients. In addition, recent studies support the theory that BMD alone is not always an accurate predictor of fracture risk due to the significant contributing effects of the extracellular organic matrix. Therefore, direct mechanical testing of the bone could be greatly advantageous.

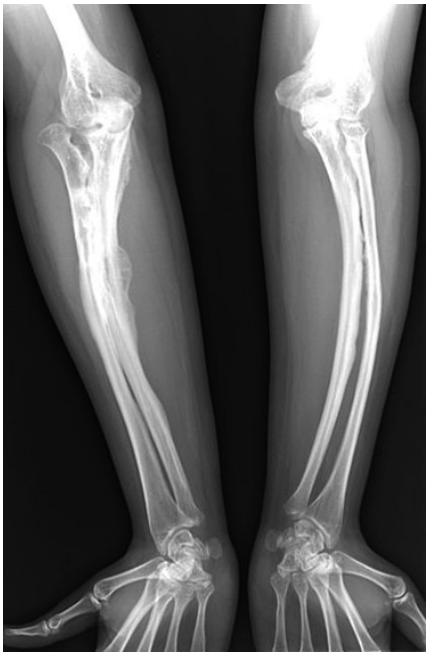

Figure 1: X-Ray photograph of a patient diagnosed with Osteogenesis Imperfecta, clearly showing the deformed (and highly fracturable) skeletal structure and high Bone Mineral Density.

Introduction to the Reference Point Indentation Instrument

Currently available methods for directly testing bone mechanical properties require invasive bone sampling, making routine use in clinical settings unfeasible. For this reason, there is a critical need to better quantify bone mechanical properties at the tissue level *in vivo.* In particular, the ability of bone to resist the growth of micro-cracks that can ultimately result in bone fracture is a property of great significance.

In a concerted effort to develop a reliable method to directly quantify fracture risk in living patients in a noninvasive manner, we have developed a new diagnostic instrument, the Reference Point Indentation Instrument[6] (RPI) (aka. Tissue Diagnostic Instrument[7], Bone Diagnostic Instrument[8-10], and Osteoprobe[11]) that uses a controlled indentation protocol to investigate the micro-mechanical response of the tibia to a defined loading regime. The procedure requires only a local anesthetic and because of the small size of the indents (375 microns across, on order of 1/1000 mm^3), it does not alter the macro-mechanical properties of the bone or cause injury to the patient.

Fundamental research has revealed that bone fractures begin when the organic matrix of the bone, or "glue" holding mineralized collagen fibrils together, fails causing crack propagation[12]. Preliminary results with the Reference Point Indentation instrument have shown that an individual's susceptibility to this fundamental failure mode can be measured by indentation tests in which bone is forced, on a microscopic scale, into the same types of failure - separation of mineralized collagen fibrils - that is the root event of bone fractures.

The RPI uses a probe assembly that can be inserted through the skin of a living patient and pressed up against the medial section of the tibia. The RPI can then measure the mechanical properties of bone by creating microscopic fractures in an indentation on the surface of the bone. The RPI obtains the distance of the indenting probe as a function of the force applied and uses this information to calculate key parameters which are highly correlated with fracturability.

Cryopreservation

Frozen bone is a crucial aspect of forthcoming bone research. Freezing is the preferred method of preservation of bone used for transplantation[13]. In addition, fresh bone is very difficult to obtain. Because of this, it is critical to understand how this method of preservation affects the original bone strength. Low temperature preservation is not just utilized on bones, but on many biological samples because at these temperatures, biological and biochemical processes slow down or stop completely[14]. In fact, most enzymes of normothermic animals show a 1.5 to 2 fold decrease in metabolic rate for every 10°C decrease in temperature[14]. Semen and embryos have been stored at cryogenic temperatures for years, then used successfully after thawing[15, 16]. Generally speaking, cryogenic temperatures are used for preserving cells, whereas hypothermic temperatures are used for organs[14]. Cowin suggests that -20°C to be an optimal temperature for bone preservation [13].

As for the effect of freezing, previous experiments have found no difference after freezing[17, 18], while others have found significant differences[19, 20]. These experiments incorporated a variety of mechanical tests, which include hardness, bending and compression tests. However, this is the first report on cryopreservation effects using the Reference Point Indentation[6] (RPI) instrument.

The extracellular matrix is approximately 20% water by weight[21]. My hypothesis is that this water crystallizes, creates nano-fractures in the cortical bone’s extracellular matrix and decreases bone strength. This research is designed to quantify the mechanical effects of the

duration of freezing, as well as to quantify the effects of the phase change. Furthermore, we aim to determine if freezing affects the organic part of the ECM differently than the inorganic part.

The Main Obstacle

The main problem is that the RPI's testing technique (specifically micro-indentation) is a destructive technique, as is shown in figure 2. *This means we cannot test the exact same location twice*. If we test different locations on the bone, (even locations which are very close to each other) the different locations can yield different mechanical properties. This is problematic because it is not possible to measure a specific location, and then treat the bone, and then test the same location after treatment and then compare measurements. However, if a very large number of randomly selected locations on many samples are measured, followed by cryopreservation of the samples, and then another very large number of measurements were made on the samples, this would dilute out the effect of variation in the bone, and the difference in the means should represent the effect of freezing.

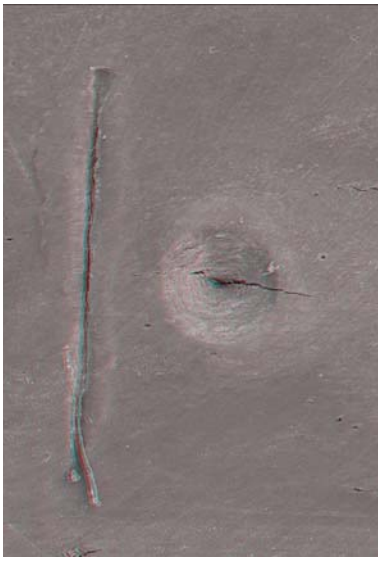

Figure 2: SEM image of a micro-indentation on Bovine Femur generated with the RPI. The indentation at the center is the product of the conical indentation probe probe while the line to the left is the product of the reference probe. Note the micro crack at the center of the indent induced by the indentation process.

Methods

Loading Regime and Key Measurements

The RPI uses a custom hypodermic needle as a reference probe that rests on the surface of the bone. The test probe cycles in and out of the reference probe as it indents the surface of the bone. The loading on the test probe increases linearly from 0 to 11 N in 1/3 of the indenting cycle. The load stays at 11 N for 1/3 of the cycle. Then the load decreases linearly from 11 to 0 N for the last 1/3 of the cycle. 20 indentation cycles are used per measurement at 2 Hz.

Indentation Distance Increase (IDI) and Total Indentation Distance (TID) are two measurements that highly correlate with fracturability from clinical trials[6]. IDI is defined as the distance between the indenter's displacement at maximum force on the 1st cycle to the displacement at maximum force on the 20th cycle, as is shown in Figure 3. We hypothesize that this is a measure of how well the bone can resist the growth of a fracture. TID is defined by the total distance the test-probe indented the bone, as is shown by Figure 3. This may represent how well a bone can resist the start of a fracture as well as resist the growth of that fracture. High values of IDI and TID represent high fracture risk.

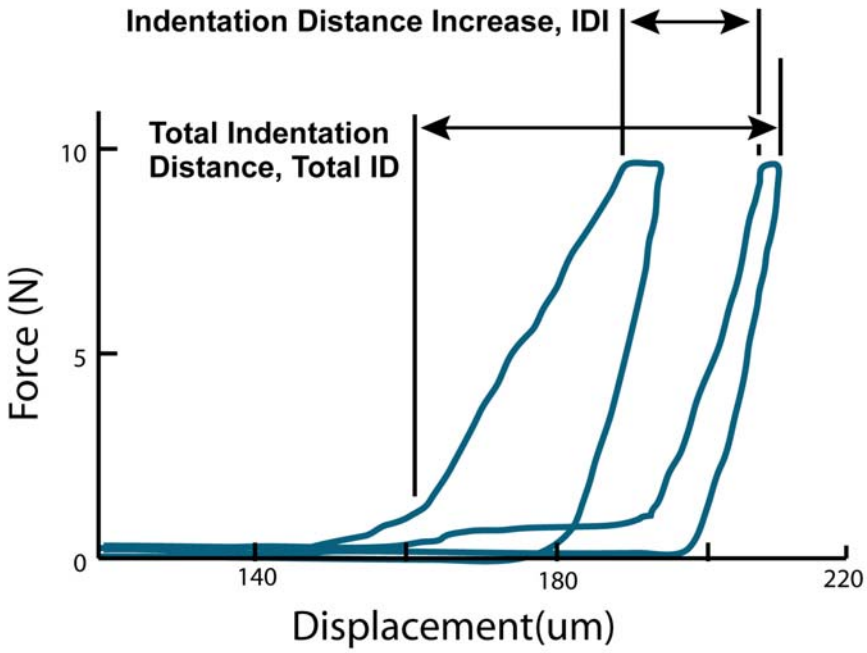

Figure 3: IDI and TID shown on a force vs. distance curve made by the RPI. IDI is the distance between the first cycle and last cycle. TID is total distance from traversed by the test probe. High values of IDI and TID are correlated with fracturability.

Testing Methods Used in All Three Experiments

There were three main experiments. In all experiments, the samples were frozen at -20°C. Samples were always hydrated in Hank's balanced salt solution (HBSS)[22] to ensure no degradation due to dehydration and to simulate *in vivo* conditions. Samples were tested in a small holding vice that was placed in a plastic container filled with Hank's solution. The samples were placed lengthwise into the vice so that the flat (cut) ends were in contact with the vice plates.

Measuring Changes in Apparatus

The same probe assembly was used to eliminate any possible systemic errors that are associated with changing probe assemblies in the middle of an experiment. The test probe was put under the microscope after each day of testing and inspected for any wear, as is shown in figure 4. The radius of the probe tip was always smaller than 12.5 microns. In addition to monitoring the tip radius, effects of degradation were measured by watching for changes in key parameters of the force vs. distance graph and for changes in IDI and TID values of PMMA over the course of the experiment.

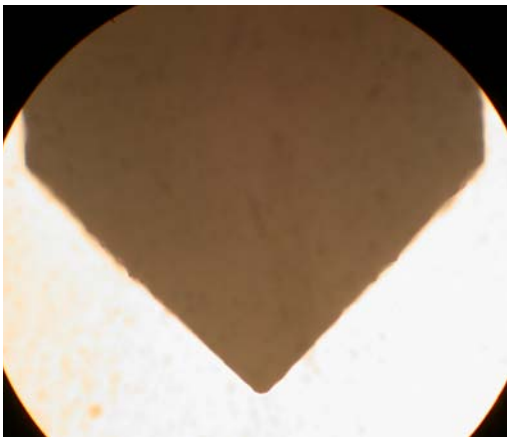

Figure 4: Test probe viewed with a 40x optical microscope. Note the sharp tip which was measured to have radius less than 12.5 microns.

Multi-Animal Experiment Method

The purpose of the first experiment was to obtain a rough idea of the effect of cryopreservation. For this reason we used multiple animal models, all with distinctly different cortical bone anatomy.

All the samples had their flesh and periosteum removed. They were stored individually in zip-locked bags with Hank's solution. The samples were refrigerated from 3 to 7 days. Before all testing sessions, PMMA measurements were made to ensure that the test equipment was functioning properly. In addition, the internal friction between the test and reference probe, which was always under .2N, was measured with this procedure.

All post-freeze samples were defrosted before testing. This was done by placing the sample in Hank's solution and then letting the sample defrost at room temperature for a minimum of two hours. In future experiments, this time was reduced to about 10 minutes by pouring room temperature Hank's solution into the sample bag.

A row of 20 measurements was made before the bone was frozen. The sample was then frozen for a minimum of five days and a maximum of 20 days. The sample was defrosted (as described above) and another row of 20 measurements was taken.

Bovine Femur Experiment Method

Bovine was chosen because its cortical bone is of the fibrolamellar type[13]. We hypothesize that this type of bone should exhibit greater changes due to freezing because of its woven bone layers, while still maintaining quintessential mechanical properties of laminar cortical bone due to its laminar bone layers.

Five bovine femur diaphyses were cut into 3x2 (approximate) cm pieces. The pieces were mixed together and approximately half of the pieces were selected for heating at 150°C for two hours (thermally degraded). Control and thermally degraded pieces were chosen randomly for this experiment. The cortical sides of the samples were then sanded flat and polished. This was done to minimize the error due to surface deformities abundant in bovine bone, and thus reduce the standard deviation.

The time from the beginning of preparation to the first freezing was less than two days. Samples were thawed in 20°C Hank's balanced salt solution (HBSS)[22] buffer for ten minutes before testing. IDI and TDI were calculated by averaging the values across a row of ten measurements.

Four control samples were used. Each sample had a control test, and five freeze-thaw cycles. For a given test (for example, Freeze-Thaw Cycle 3) all the samples were always tested together. Each test was four days apart. Because of the minimal time between tests, we can assume minimal effects due to duration of freezing.

Three thermally degraded samples were used. They went through three freeze-thaw cycles. There were four-day intervals between tests. Because of the minimal time between tests, we can assume minimal effects due to the duration of freezing. As is discussed below, the combined effect of phase change and duration of freezing were undetectable on thermally degraded bone.

To help quantify the "minimal effects" from the duration of freezing of control samples, a separate sample (sample #5) was used. Instead of waiting four days between control testing and the first freeze-thaw cycle, this sample was frozen for 20 days (the total duration of freezing for the other samples). Assuming duration of freezing increases IDI, then Sample 5 should have more of an increase in IDI between control and the first freeze-thaw cycle than the other samples. By calculating the how much more Sample 5 increased versus the other samples, we can gain a quantitative idea of the "minimal effect" of duration.

The same methodology of estimating the "minimal effects" from the duration of freezing of the control samples was used to estimate the "minimal effects" from the duration of freezing of the thermally degraded samples (with the exception that the 'separate sample' was thermally degraded).

Mechanical mapping of a control sample was done because of the possibility that two different rows on the same sample would have statistically different IDI or TID due to variation in IDI or TDI across the surface of the bone. When the sample was mapped, all four rows had no

statistical difference in either IDI or TID (P>.2), and no patterns due to spatial variation could be found.

Human Tibia Experiment Method

Unfrozen human tibia from a 71 and a 83 year old female were obtained from the University of California, Irvine (UCI) Willed Body Program. Although the exact date and time of death of the donors are unknown to Hansma lab researchers, the tissue had been preserved at hypothermic temperatures for an estimated two weeks before arriving at Hansma lab. Samples were kept in hypothermic preservation (with no chemical fixation) until they were measured with RPI. This experiment was composed of 3 stages, and thus the hypothermic preservation time of the bone samples vary. Preliminary Stage, Stage 1, and Stage 2 started at approximately three days, ten days, and twenty-one days, respectively, after tissue arrival at UCSB.

The medial section of the diaphysis region of the tibia was cut into sections approximately 2cm by 2cm. Because the medial section is tested *in vivo* clinically, only medial sections were tested in this experiment.

The sections were sanded flat and polished. The legitimacy of polishing was examined by measuring unpolished samples (periosteum removed), then polishing the samples and re-measuring the sample. It was found that measurements on the polished sample had approximately the same mean, but half the standard deviation. This helped the study gain more statistical power with the same sample size.

On each sample there were three rows of measurements made before the freeze-thaw cycle and three rows of measurements made after the freeze-thaw cycle. There were ten measurements per row. Rows extended lengthwise down the long axis of the bone. Control and Post-Freeze-Thaw rows were staggered in an effort to offset the effect of variation in the bone mechanical properties across the sample. This is illustrated in figure 5. PMMA was tested throughout the experiment to document the effect of probe assembly wear or damage and other factors which affect the IDI and TID values of the samples.

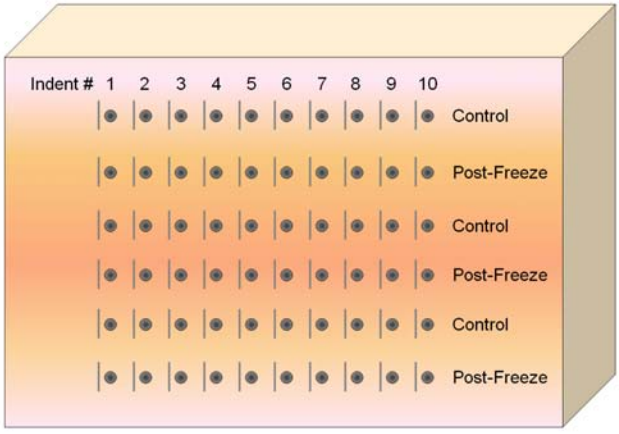

Figure 5: Illustration of staggered row testing method. Three control rows are measured before treatment. After a freeze-thaw cycle, three rows of post freeze measurements are staggered between control measurements to offset the effect of any patterns in the variation of mechanical properties across the bone.

Measurements were made in a preliminary stage followed by Stages 1 and 2. The Preliminary Stage was used to obtain an order of magnitude estimation of the effects of freezing. In Stage 1, four samples were tested. Samples were measured separately at different times to reduce the influence of systematic errors on the difference of control and post-freeze-thaw values. In Stage 2, six samples were tested.

In addition, two samples from the posterior section of the tibia were de-mineralized in 5% and 10% acetic acid respectively for seven hours on a shaker table with no exchange of acetic acid.

Results

Cryopreservation has two main parameters of particular interest. First is the degradation due to the water in the sample freezing and turning into ice (referred to as “phase change” in this paper). The second parameter is “Duration,” which examines the degradation of a frozen sample simply due to the passage of time.

Duration

We are interested in separating the degradation due to phase change from the degradation due to duration of freezing. All samples must go through at least one phase change in order to have been frozen for any duration. Therefore, the effect of duration can only be measured by keeping phase change constant and varying duration. By comparing the difference *in degradation* (degradation is the difference in IDI or TID from control measurements to post-freeze-thaw measurements) from two samples with different durations but the same number of phase changes, we can estimate the effect of duration.

Sample 5 had an increase of 16.3% from Control to Post-Freeze. Since the range of increases of the other samples is 9-22% (average is 15.1%), we can assume the degradation due to duration of freezing to be minimal and small compared to the degradation due to phase-change on the time scale of 35 days. This is seen in Figure 6. This agrees with theory, since biological processes slow-down exponentially with temperature.

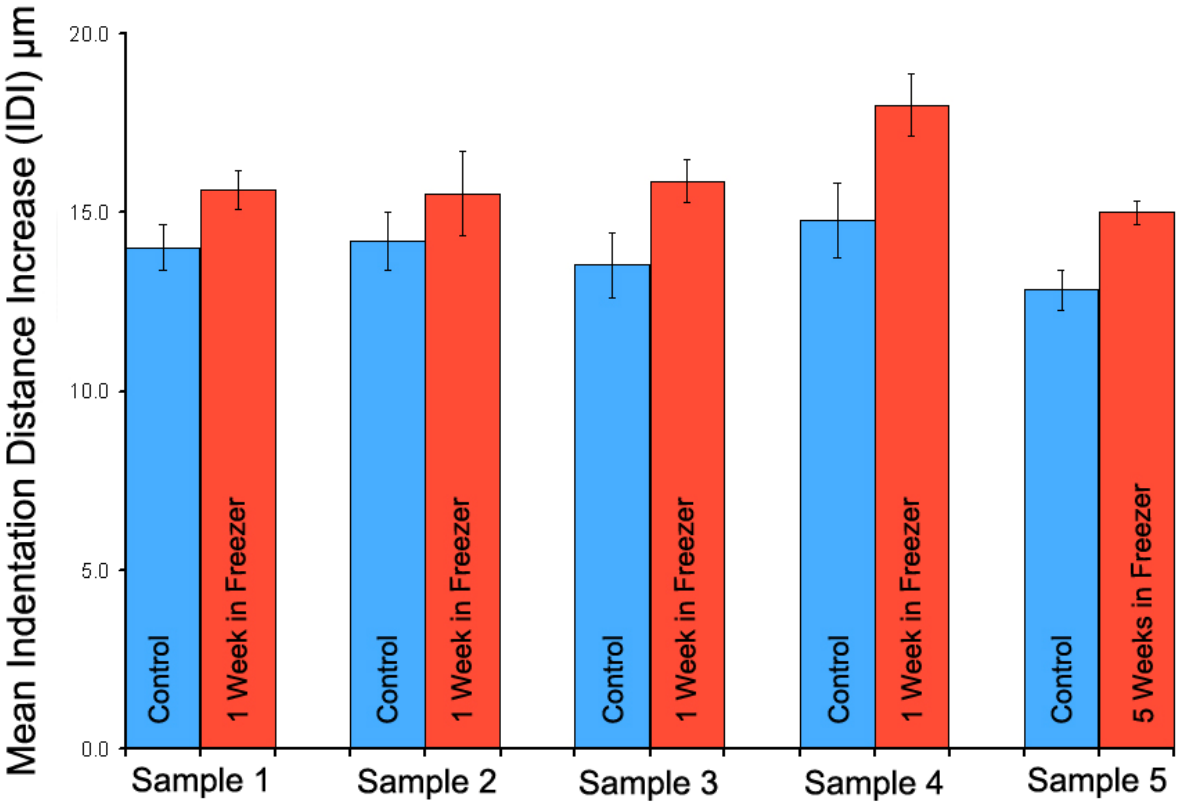

Figure 6: Degradation of bone mechanical properties due to duration of cryopreservation. Bone samples frozen for five weeks at -20 °C exhibited no significant difference in mechanical properties (as measured by IDI) compared to those frozen for 1 week (N=4) at the same temperature. Error bars represent standard error.

Phase Change

Multi-Animal Experiment

Since the results show the effects of duration to be negligible, we can assume (with good approximation) any degradation from control to post-freeze measurements to be due to phase-change. Figure 7 shows a graph of the results from experiment 1. Because micro-indentation is a destructive technique, and a different location on the bone must be tested after a freeze-thaw cycle, the natural variation of IDI and TID from location to location is of great importance. The natural variation in mean IDI between rows is substantially larger than the effect of a freeze-thaw cycle. This can be seen by simply comparing the standard deviation (a measure of the deviation of IDI from measurement to measurement) to the difference in mean IDI betweens Control and Post-Freeze measurements.

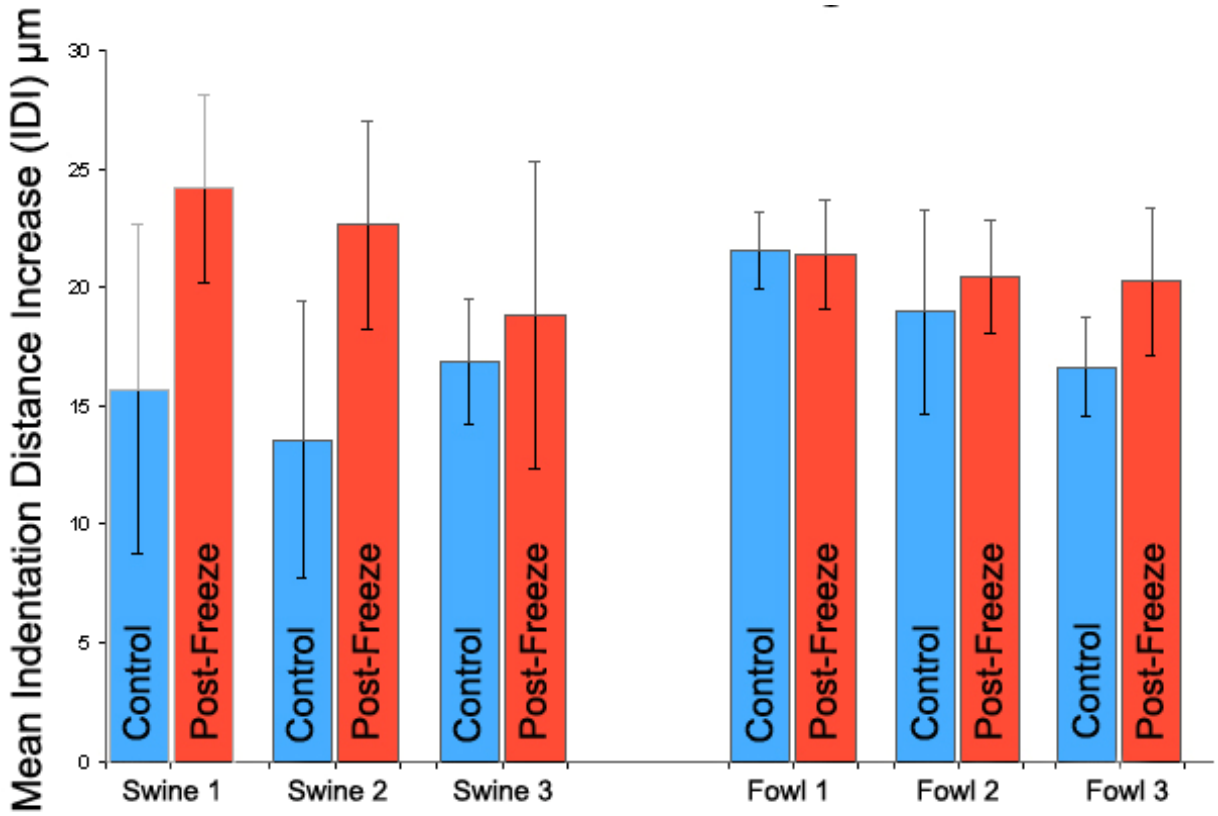

Figure 7: The effect of phase change on IDI. For both swine and fowl bone samples, the effects of a single freeze thaw cycle had no significant effect on their mechanical properties (as measured by IDI). Error bars represent +/- one standard deviation.

Figure 7 indicates that there is an increase in mean IDI due to phase-change. Five out of six of the samples have an increase in mean IDI. The magnitude of the increase seen in five samples is much larger than the magnitude of the slight decrease in one sample (Fowl 1). This implies that there is an increase in mean IDI with freezing, but this increase is less than the variation in IDI from location to location.

In the Bovine Femur Experiment, we had many more rows of measurements per sample, and more parameters held constant between samples. This helped us quantify the results, something which was not possible in Multi-Animal Experiment.

Bovine Femur

Control

When the data from the four different samples are grouped together, there was a 15.1% increase in IDI from before freezing to after the first freeze-thaw cycle (t-test .0004). In addition, four out of four samples showed an increase in IDI after one freeze-thaw cycle, and in three out of four samples, the increase was statistically significant ($P<.05$). All four subsequent freeze-

thaw cycles were not statistically different from the first. Figure 8 shows the mean IDI of control measurements and the first three freeze-thaw cycles.

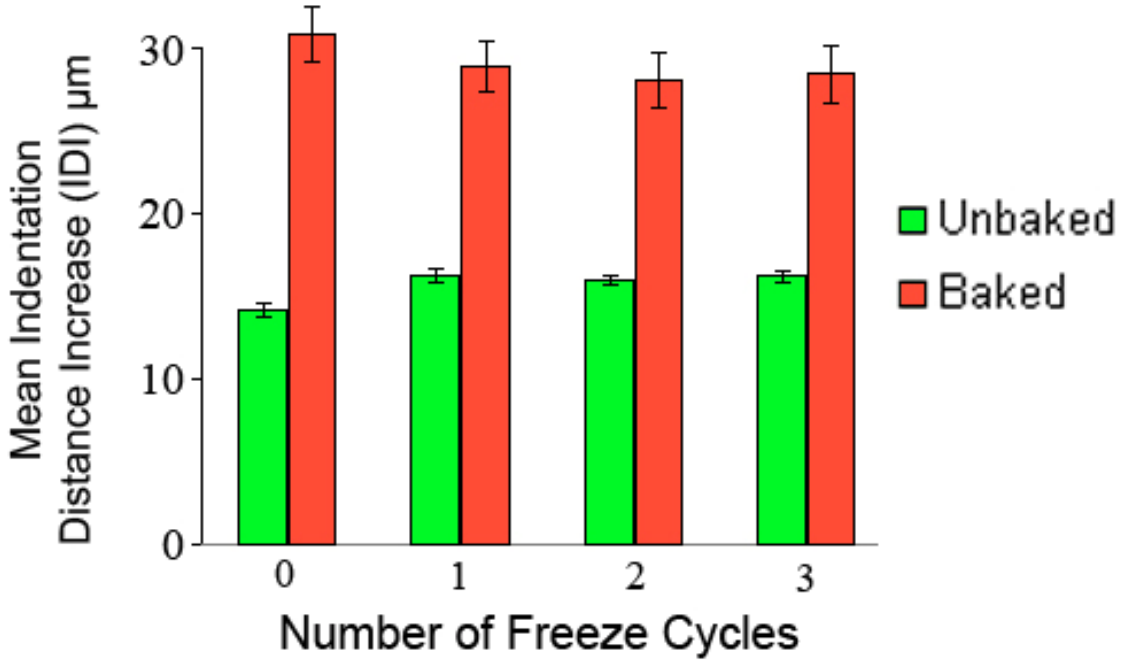

Figure 8: Degradation of mechanical properties (measured by IDI) of thermally degraded bone due to multiple phase-changes. Standard error is shown. Thermally degraded bone has no statistically significant change in IDI with multiple freeze-thaw cycles. Control bone shows a minor degradation after the first freeze-thaw cycle. Subsequent freeze-thaw cycles show no statically significant degradation.

It appears that freezing did not affect TID. Freeze-Thaw Cycles 1, 2, 3 and 5 all returned p values of .05 or greater with respect to control. There was no clear pattern of increasing or decreasing IDI with subsequent freeze-thaw cycles as is shown in figure 9.

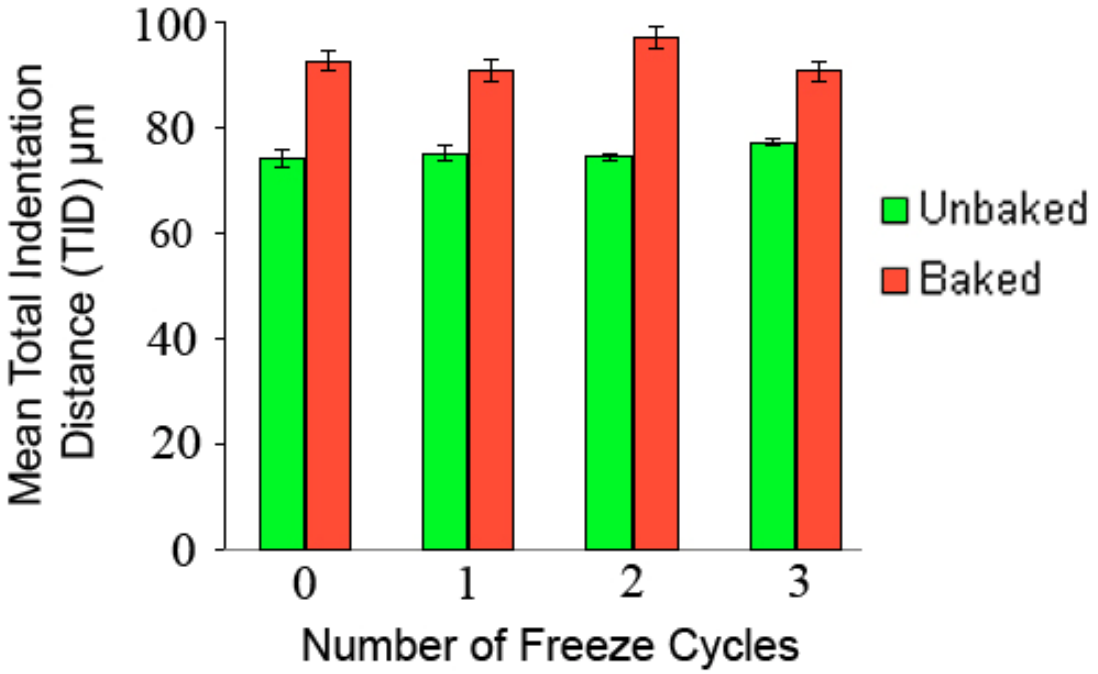

Figure 9: Degradation of mechanical properties (measured by TID) of thermally degraded bone due to multiple phase-changes. Standard error is shown. Phase-change has no measureable effect on Total Indentation Distance.

Thermally Degraded

When the samples are grouped together, there was no statistical difference in IDI and TID between control and freeze-thaw cycle 1, 2 or 3 (all t-test values above .2). This is shown figure 8.

When looking at each sample individually, we see no statistical difference between control, and six out of seven of the post-freeze-thaw cycle tests.

Three out of three bones showed no difference in TID between control and Post-Freeze tests. When all the samples are grouped together, there still is no difference between Control and Post-Freeze-Thaw tests (figure 9).

Results: Human

Human results show no statistically significant change in IDI or TID with phase change. Stage 1 and Stage 2 show an average increase of 1.9% (from 18.1 to 18.4 µm) and 3.1% (from 19.4 to 20.0) in IDI from Control measurements to Post-Freeze-Thaw measurements. Both of these increases were not statistically significant with p value of 0.17 and 0.32 respectively (from a 1 tailed t-test). This increase is shown in figure 10.

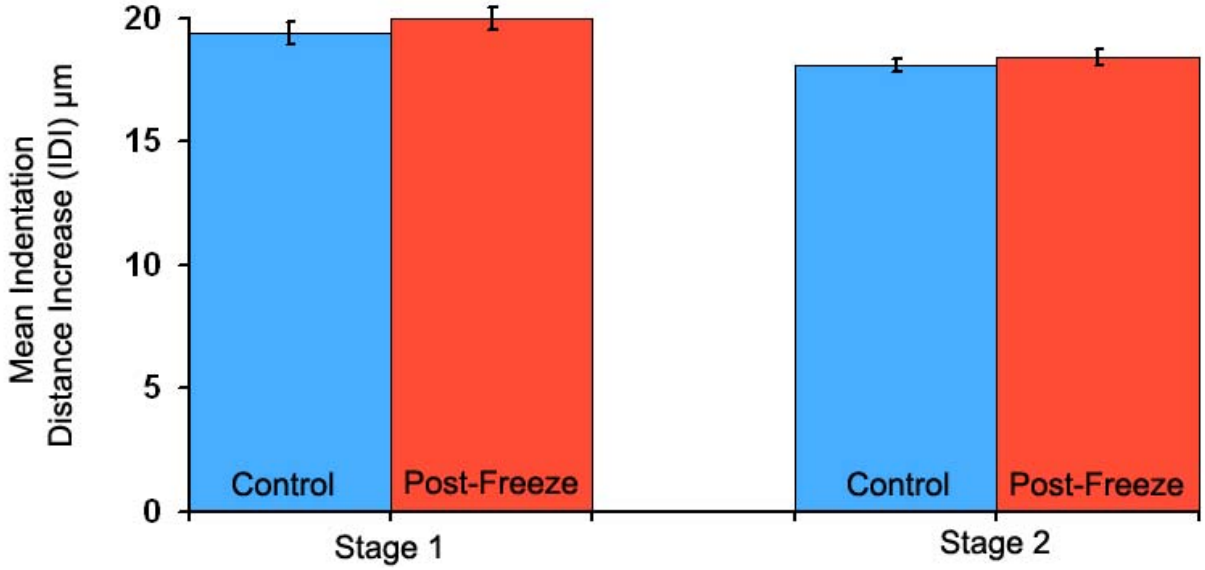

Figure 10: Two stage experiment showing the degradation due to cryopreservation at -20°C on the mechanical properties of human tibia (as measured by IDI). The degradation is a minimal and not statistically significant. Note that standard error bars overlap.

Stage 1 and Stage 2 show an average *decrease* of 1.6% (from 86.2 to 84.8 µm) and 3.2% (from 83.6 to 80.9) in IDI from Control measurements to Post-Freeze-Thaw measurements. Both of these increases were not statistically significant (Figure 11) with p value of 0.21 and 0.16 respectively (from a 1 tailed t-test).

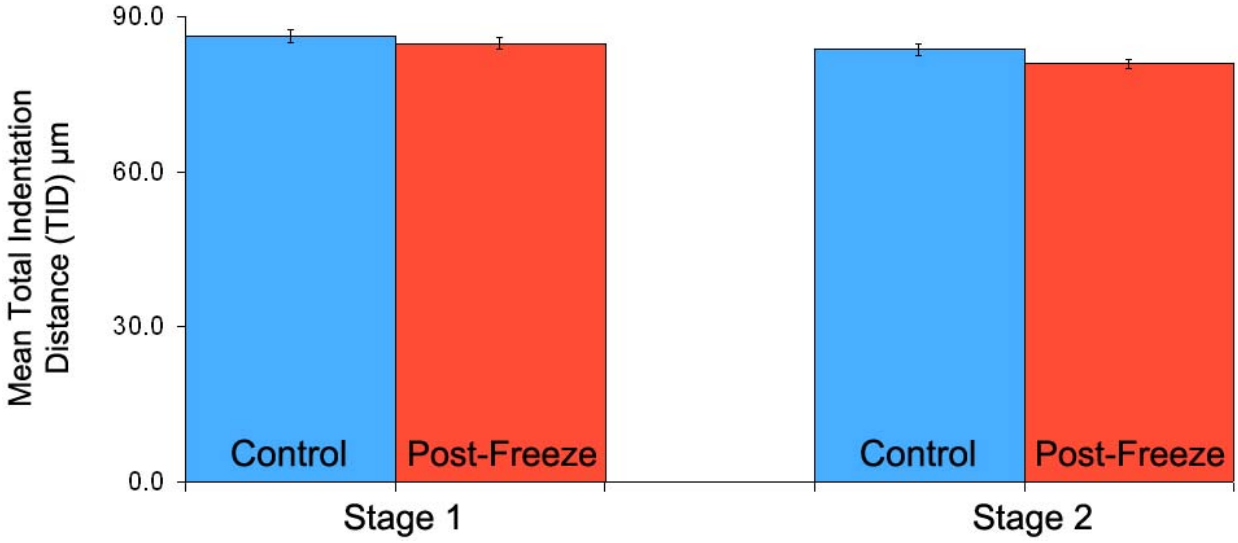

Figure 11: The minimal and not statistically significant increase of Total Indentation Distance due to phase-change on human tibia of a two stage experiment. Stand error is shown, and error bars overlap. Both stages exhibit similar results.

Demineralized bone also showed no statistically significant changes in IDI and TID after freezing ($p > 0.13$).

Discussion

Untreated Bone

There is minimal degradation due to duration of freezing on the time scale of 35 days.

After the first freezing, the bone no longer becomes more fracturable with consequent freezing and thawing. Results from this study suggest the organic ECM reaches saturation degradation due to phase change after a single freeze-thaw cycle (at least on the order of 5 freeze-thaw cycles). Because bone collagen is only barely soluble[23], finding that crystallization damage only affects the organic portion of the ECM explains why the effect of cryopreservation is minimal.

It is interesting to note that TID remained constant but IDI increased after freezing. If we look at the force vs. distance curves, we see that the distance the bone compresses before the force saturates is smaller. However, the bone compresses more at maximum force than it did before freezing. If this pattern is seen in future experiments, it will most likely be useful in discovering the length scale at which the crystallization is breaking up the ECM.

Thermally Degraded and Demineralized Bone

It has been established that heating of bone to 150 ˚C significantly degrades the extracellular organic matrix[23]. When thermal degradation or freezing are applied to bone individually, the bone has an increased fracturability. However, since we found that freezing does not significantly alter the mechanical properties of thermally degraded bone, results from this study suggest the process of thermal degradation alters bone in the same way freezing does. It follows that freezing degrades the organic matrix.

Conclusion

Cryopreservation has very minimal effects on the mechanical properties of bone. This means that frozen samples can be tested without concern about degradation due to the preservation process (although the bone may have degraded before storage). In addition, researchers should strongly consider cryopreservation as an optimal preservation technique.

References

1. *Fast Facts on Osteoporosis*. [cited 2010 March 19]; Available from: http://www.nof.org/osteoporosis/diseasefacts.htm.
2. Moran, C.G., et al., *Early mortality after hip fracture: is delay before surgery important?* Journal of Bone and Joint Surgery-American Volume, 2005. **87**(3): p. 483-9.
3. Rivadeneira, F., et al., *Femoral neck BMD is a strong predictor of hip fracture susceptibility in elderly men and women because it detects cortical bone instability: the Rotterdam Study.* Journal of Bone and Mineral Research, 2007. **22**(11): p. 1781-90.
4. Rauch, F. and F.H. Glorieux, *Osteogenesis imperfecta.* Lancet, 2004. **363**(9418): p. 1377-85.
5. Rauch, F. (2007) *Bone Mineral Density: What it Means and How to Measure it.*
6. Diez-Perez, A., et al., *Microindentation for in vivo Measurement of Bone Tissue Mechanical Properties in Humans.* Journal of Bone and Mineral Research, In-Press.
7. Hansma, P.K., et al., *The tissue diagnostic instrument.* Review of Scientific Instruments, 2009. **80**(5): p. 054303.
8. Randall, C., et al., *The bone diagnostic instrument III: testing mouse femora.* Review of Scientific Instruments, 2009. **80**(6): p. 065108.
9. Paul Hansma, P.K., Barney Drake, Eugene Yurtsev, Alexander Proctor, et al., *The bone diagnostic instrument II: Indentation distance increase.* 2008.
10. Hansma, P.K., P.J. Turner, and G.E. Fantner, *Bone diagnostic instrument.* Review of Scientific Instruments, 2006. **77**(7): p. 075105.
11. Hansma, P.K., et al., *A new bone diagnostic instrument: the osteoprobe II.* Journal of Bone and Mineral Research, 2007. **22**: p. S226-S227.
12. Fantner, G.E., et al., *Hierarchical interconnections in the nano-composite material bone: Fibrillar cross-links resist fracture on several length scales.* Composites Science and Technology, 2006. **66**(9): p. 1205-1211.
13. Cowin, S.C., *Bone Mechanics Handbook.* CRC Press. 2001. 20.3.1.
14. Rubinsky, B., *Principles of Low Temperature Preservation.* Heart Failure, 2003(8): p. 227-284.
15. *Child born after 21 year semen storage using Planer controlled rate freezer*. 2004 [cited 2010 March 19]; Available from: http://www.planer.com/PlanerComWebsite.nsf/7dc62b5d9e44dd40802575460048fc4e/df90c37237a74df88025757d005b0057?OpenDocument.
16. *'Twins' born 16 years apart*. 2006 [cited 2010 March 19, 2010]; Available from: http://www.planer.com/PlanerComWebsite.nsf/7dc62b5d9e44dd40802575460048fc4e/76a07726303dac468025757d005b004d?OpenDocument.
17. Weaver, J.K., *The microscopic hardness of bone.* Journal of Bone and Joint Surgery-American Volume, 1966. **48**(2): p. 273-88.
18. Sedlin, E.D., *A rheologic model for cortical bone. A study of the physical properties of human femoral samples.* Acta Orthop Scand Suppl, 1965: p. Suppl 83:1-77.
19. Kang, J.S.a.K., N.H., *The biomechanical properties of deep freezing and freeze drying bones and their biomechanical changes after in vivo allograft.* Yonsei Medical Journey, 1995(36): p. 332.
20. Pelker, R.R., et al., *Effects of freezing and freeze-drying on the biomechanical properties of rat bone.* J Orthop Res, 1984. **1**(4): p. 405-11.

21. Gray, H., et al., *Gray's anatomy : the anatomical basis of clinical practice*. 39th ed. 2005, Edinburgh ; New York: Elsevier Churchill Livingstone. xx, 1627 p.
22. Habelitz, S., et al., *Nanoindentation and storage of teeth.* J Biomech, 2002. **35**(7): p. 995-8.
23. Danielsen, C.C., *Age-related thermal stability and susceptibility to proteolysis of rat bone collagen.* Biochemical Journal, 1990. **272**(3): p. 697-701.