14 Food-grade chicken tibias were tested for the bending stress and strain, as well as the Young’s modulus using 4-point stress test. After the first testing, the tibias were divided into two groups, with one group being cryopreserved at -80°C for a week while the other group was kept in a 4°C refrigerator for a week. The tibias were then tested for the bending stress and strain again, using the 4-point stress test. Images were taken with each load test to measure the bending angle with ImageJ, which was required for the strain calculation. Afterwards, the all of the bones were broken using a 3-point stress test and the ultimate breaking stress was measured. The cross-sectional pictures were taken of the broken bones and the inner and outer radii were measured using ImageJ. There were significant differences (p<0.05) in the Young’s modulus between the cryopreserved bones and the non-cryopreserved bones. However, there were no significant differences (p>0.05) in the ultimate breaking stress between the cryopreserved bones and the non-cryopreserved bones. The results from this experiment may have implications on bone and tissue transplants.
Introduction

Bones make up an integral part of any vertebrate organism. It is associated with the integrity of the organism and for storage of various minerals and ions. Therefore, it is essential that the mechanical properties of the bone stay constant to a number of stimuli and changing conditions.

Cryopreservation refers to storing biological tissues below -70°C. It has many uses in the field of medicine and research but is primarily used in surgical settings as a means to store tissues or organs. The stored tissue or organ can be then used for transplants when it is needed.

The objectives of this experiment were to:

- Determine the mechanical properties of bone before and after cryopreservation by applying 4-point and 3-point bending stress
- Determine the correlation between cryopreserved bone and non-cryopreserved bone

It is hypothesized that the cryopreserved bones will have less elasticity than the control group. Based on the histology of mammalian bone, it is composed of approximately 20% water (Timmins and Wall, 1977). This may be accounted for from various cells inside the bones, such as osteoblasts, osteoclasts and marrow matter. Since cell cytoplasm is made up of mostly water, it is expected that the ice crystals will form inside the cytoplasm or on the outside of the cell in the extracellular space. These crystals may rupture the lipid bilayer membrane of the cell, causing the cell to lyse. This in turn will affect the mechanical property of the bone since the cells which contribute to water content have been destroyed.
Theory

From a biomechanical perspective, the bone is composed of collagen and hydroxyapatite. The hydroxyapatite in turn is composed of a matrix of calcium ions and osseous tissue, which stems from special cells called osteoblasts. There are also blood vessels that run vertically through the bone with latitudinal channels occurring occasionally. At the very middle of the bone is the bone marrow matter, which is a key component to an organism’s lymphatic system as well as the immune system (Fung, 1993). However, when the mechanical properties of bone are considered, the cortical part of the diaphysis (the middle part of the bone) is usually used for measurement. Hydroxyapatite is very rigid in nature and contains very little water. This leads to a very strong structure for any cortical bone (for a typical human, the ultimate tensile strength for the tibia is approximately 124MPa), as well as being very rigid.

Cryopreservation is the process of freezing tissues or organs at very low temperatures. Uses for cryopreservation are mainly in the medical field where an organ or tissue is frozen at very low temperatures for storage, then transplanted on another subject during a surgery. Usually, the tissues or organs are frozen at temperatures from -70°C to -196°C, which is the boiling point of liquid nitrogen. In theory, cryopreservation is supposed to provide ‘infinite life’ to cells since preserving in this method slows down the cellular clock to a point that the cell is neither in a living or dead state (G₀ point on the cellular cycle). However for cryopreservation to work properly, the cell must also regain its function after it is thawed (Chesné et al, 1993).

There are also many risks with cryopreserving cells. Since the cell is composed of mainly water (Lodish et al, 2007), it has serious consequences if there is ice crystal formation. There could be ice crystal formation on the inside of cells, which may lead to the cell bursting as it freezes. There could also be ice crystal formation on the outside of the cell, which can penetrate into the cell as the temperature is lowered. Although the cortical part of the bone does not contain much water due to the structure of
hydroxyapatite crystals, the inner part of the bone contains marrow and other vasculature which is composed of live cells. Thus, freezing of the bone can result in cell destruction due to ice crystals.

4-point bending test is a method of applying a load to the middle of a structure to determine the bending strain and stress without any large point shear stress applied. For the purposes of this experiment, the chicken tibia was approximated to be a hollow circular cylindrical object with uniform inner and outer radii throughout all of the testing area. With this approximation, a formula can be used to calculate the bending stress applied (Akay, 2006):

$$\sigma = \frac{PLr_o}{4I}$$

- $P$ is the applied load, in newtons
- $r_o$ is the outer radius of the bone, in meters
- $L$ is the distance between the support and load point, in meters
- $I$ is the cross sectional moment of inertia, in $m^4$

The cross sectional moment of inertia for a hollow cylinder is obtained by (Akay, 2006):

$$I = \frac{\pi(r_o^4 - r_i^4)}{64}$$

- $r_o$ is the outer radius of the bone, in meters (same as $a$ in the previous equation)
- $r_i$ is the inner radius of the bone, in meters

The bending distance can then be calculated by obtaining angles from the bones as the load is increased. If the bone is marked along three equidistant points from which the angle is measured, one can obtain the bending distance by using simple trigonometry using:
\[
\varepsilon = \frac{L\left(90 - \frac{\theta}{2}\right)}{2 \sin \left(\frac{\theta}{2}\right)}
\]

- \(L\) is the length of the bone diaphysis over the testing area, in meters
- \(\theta\) is the angle of the bone at the middle of the three points

Afterwards, the bending strain can be calculated by using the bending distance from above and subtracting that value from the original bending distance value, when the applied load is at 0.

The bending Young’s modulus can be obtained by dividing the stress by the strain.

Usually to test for bending elasticity of different material, the 3-point bending test is not used because the single point applied at the middle of the material produces a very large shear stress on the material which may throw off the results. However, for the purposes of this experiment, the 3-point bending test is used to test for the ultimate breaking stress (ultimate tension strength) of the bone. The same assumptions are made about this test as the 4-point bending test. The bending stress is calculated using (Akay, 2006):

\[
\sigma = \frac{PLr_o}{8I}
\]

- \(P\) is the applied load, in newtons
- \(L\) is the length of the bone diaphysis over the testing area, in meters
- \(r_o\) is the outer radius of the bone, in meters
- \(I\) is the cross sectional moment of inertia, in \(m^4\), also given in the formula above

For the duration of this experiment, a factor that had to be considered was measurement error. Since each of the angles have to be measured using ImageJ (Rasband, 2011), an imaging software, it is up to the experimenter to make very accurate measurements with the angle tool. To minimize this error,
an uncertainty value had to be calculated using the standard deviation from measurements made and plugging in this value into the equation for the bending distance, ultimately obtaining the uncertainty in bending strain (Halliday et al, 2006).

**Methods**

Samples of chicken bones were obtained by using food-grade chicken drumsticks and peeling the flesh, tendons and cartilages until the shaft of the tibia remained. The tibia samples were then fixed within two metal rectangular blocks using plaster. The length of the bone was measured along the testing area (defined as the distance between two metal blocks). The bone was then marked along 3 equidistant sections with a pen, effectively dividing the tibia section into four parts.

After the plaster had dried, the bending stress was applied in an inverted 4-point bending stress fashion, where the force is applied at the bottom of the tibia along two points (Fig. 1). These points are both 30cm from the middle of the tibia sample. Pieces of rectangular plywood of similar mass (135g for the plywood on left side and 141g for the plywood on the right side) were inserted to either end of the metal blocks. The 4-point bending apparatus was then created by attaching a double sided metal hooks at the equidistant points and running a metal rod under the tibia sample, connecting each end of the hooks on the bottom side. The combined weight of this apparatus (2 hooks and the metal rod) was measured to be 162g. Weights were then applied form the middle of the rod, which is also directly below the middle of the tibia. The bone with the attached 4-point bending stress was placed on
protruding rods affixed to the table.

Figure 1. The inverse 4-point bending apparatus used in the experiment. The bone is placed in the middle between the two plywood pieces and weights are hung at the middle of the rod hanging underneath.

A picture was taken of the bone before any of the 4-point bending stress apparatuses were attached to the metal blocks. A picture was taken with each increase in applied mass. The masses were applied in the form of a bucket and steel nails. The bucket used weighed 132g and each of the nails weighed 64.7g. The number of steel nails increased in increments of 5 until 20 nails, where the increment was changed to 10 nails until 100 nails were applied in total.

Before applying the stress, there were a total of 14 tibia samples. Bones broken at this stage were sawed at the middle and the inner and outer radii were measured with ImageJ. The remaining 11 bones were divided into two groups: the group for cryopreservation (‘Frozen’) and the group for the
cold room ('Cold'). The Frozen group was kept in a -80°C freezer for 7 days while the Cold group was kept in a 4°C cold room for 7 days as well.

A second 4-point stress testing was done at 7 days after the bones had been stored in the freezer or the cold room. The Frozen group was thawed out for 30 minutes before the stress testing since touching the cold metal blocks with bare hands could have resulted in an injury, as well as throw off results. This concept will be further explored in the discussion section. The inner and outer radii of bones broken in this stage were measured in the same fashion as the bones broken during the first stage of the 4-point stress testing. It is noteworthy to say that the apparatus and the weights applied were exactly the same as the first 4-point stress testing. The images were also taken as new increments of masses were applied.

With all the pictures taken for each increase in mass, an image analysis program called ImageJ was used to measure the angle of the tibia. As mentioned previously, the three equidistant points marked on the bone were used as the points of measurement. With the angle obtained, the strain was calculated.

After all the angles were measured for each load, the bones were then subjected to a 3-point bending stress to obtain the ultimate breaking stress. The bone was placed on the protruding rods in the same fashion as the 4-point bending stress, but with a longer metal rod running through the middle of the bone (Fig. 2). This created a lever system where the mass was applied at the end of the metal rod. This was done in part due to the lack of nails that could have been hung from the middle of the bone. The rod was 76cm in length, with a mass of 135g. This made any weight that was hung from at the end of the lever 16.9 times greater than its actual mass. The nails were applied in increments of 2 until the bone broke. The inner and outer radii of the bone were then also measured with ImageJ.
Figure 2. The 3-point bending apparatus, with the lever system shown. The metal rod protruding to the right is placed at the middle of the bone.

Using the strain and stress values obtained from various calculations, the values were plotted and the Young’s modulus for bending stress was calculated.

The measurement uncertainty value was also obtained by opening up one image on ImageJ and measuring the same angle 25 times and by applying the formula outlined beforehand.
Results

Figure 3. First load test for all 14 bones, with the stress plotted against the strain
Figure 3 shows the results from the first load test of all 14 tibia samples. As it is very messy, it has been converted into 14 lines of best fits onto Fig. 4. Using the slope values from the lines of best fit, the average Young’s modulus for the first load test was calculated to be 27.7MPa. During this stage of experimentation, 3 bones broke.
Figure 5. Second load test for ‘Frozen’ bones

Figure 6. Line of best fit for ‘Frozen’ bones from Fig. # (above)
Figure 5 shows the results from the first load test of 6 ‘Frozen’ tibia samples. It also has been converted into 6 lines of best fits onto Fig. 6. Using the slope values from the lines of best fit, the average Young’s modulus for the first load test was calculated to be 66.7MPa. During this stage of experimentation, 2 bones broke.

![Graph showing stress-strain relationship for different bones](image)

**Figure 7.** Second load test for ‘Cold’ bones
Figure 8. Line of best fit for ‘Cold’ bones from Fig. # (above)

Figure 7 shows the results from the first load test of 5 ‘Cold’ tibia samples. It has been converted into 5 lines of best fits onto Fig. 8. Using the slope values from the lines of best fit, the average Young’s modulus for the first load test was calculated to be 0.8 MPa.

The uncertainty in measurement was calculated to be $2.53 \times 10^{-8}$. This value is omitted from all figures since it is too insignificant to be seen on the graphs.
Figure 9. Ultimate breaking stress for all bones. Bones marked with a green star were frozen. Bones marked with a red star broke during the first test. Bones marked with a purple star were broken during the second test.

Paired t-tests with assumed equal variance were conducted on the obtained values for Young’s modulus between the ‘Frozen’ and the ‘Cold’ group after the second load test. This yielded in a p value of less than 0.05, indicating that there is a significant difference between the two groups.

Another paired t-test with assumed equal variance were conducted for the ultimate breaking stress between ‘Frozen’ and ‘Cold’ bones (Fig. 9). This yielded in a p value of more than 0.05, indicating that there is no significant difference between the two groups.
Discussion

The hypothesis proved to be correct. The results indicate that there is a significant difference (p<0.05) between the Young’s modulus of the ‘Frozen’ group and ‘Cold’ group. When the bone is observed cross sectionally, there is a noticeable difference between the cortical section of the bone and the inner section of the bone which contains the marrow. With the obtained inner and radii for a tibia sample (Bone 3), it was calculated that the chicken tibia is made up of approximately 62% cortical tissue and 38% inner parts. Even though the majority of the bone may be mechanically affected from the cortical tissue, it is hard to ignore the mechanical properties of the inner part of the bone. Since the inner part is composed of mostly marrow and vasculature, which in turn is composed of live cells containing water in the cytoplasm. Cryopreservation could have led to intracellular and extracellular ice crystal formation, leading to those cells to be destroyed. This would have decreased the elasticity of the ‘Frozen’ group significantly, as displayed in the results.

A point to be noted here is that the ultimate breaking stress between the ‘Frozen’ and ‘Cold’ groups are not significantly different (p>0.05). If the structure of the bone is considered, this is accounted for. As stated previously, the cortical section of the bone contributes to the majority of the cross-sectional area (62% for Bone 3). The cortical section of the bone is composed of mainly hydroxyapatite, which has a higher ultimate breaking stress than the interior section due to its rigidity. Since the bone must be broken all the way through, elasticity (Young’s modulus) will not have much of an effect on how much load the bone can take before breaking.

An interesting phenomenon arising from this experiment is that there is negative strain involved as the load is applied to the bone. The negative strain contributes to the graphs (Fig. #, # and #) by giving the appearance of squiggles and also gives a negative Young’s modulus value. In theory, the negative strain value makes sense since the point at which the strain is measured is subjective and is not absolute.
A possible explanation for this phenomenon is that the chicken tibia is so rigid that there were no actual changes in the angles or strain as the loads were increased, but the changes in strain resulted from inconsistent image capturing, which is explained in further detail below.

A problem with this experiment rises in the fact that cryoprotectants were not used to preserve the cells and tissue. Cryoprotectants serve to deter the formation of intracellular and extracellular ice crystals from forming. Some cryoprotectants, such as DMSO (dimethyl sulfoxide) function by intercalating between the water molecules and any salt molecules floating inside or outside the cell. Ultimately, this will decrease the amount of ice formed as the temperature drops and will prevent cell death to a certain degree (Pegg, 2007). If cryoprotectants were used, the outcome of this experiment could have been wholly different. An earlier study conducted indicates that if proper cryoprotectants are used for cryopreserving cells and tissue, the tissue after cryopreservation has no significant differences in elastiscity (Reuther et al, 2010).

A possible source of error in this experiment may stem from inconsistent capture of images as the load is increased. Although the camera was stabilized with a tripod, it was not affixed to the ground, which could have led to change in position of the camera every time a picture was taken. This may have led to inaccurate angle measurements which were crucial in calculating the bending strain. However, as mentioned in results, measurement uncertainty and error were calculated and were too insignificant to cause any doubts about the data.

This experiment could have been improved by increasing the sample size. Since only 14 bones were tested for bending stress and strain, it may not give a representative sample of the entire chicken tibia in the population. With more testing, the values can be refined. Also, the equipment used could have been improved by using an actual 4-point or 3-point test load devices. These devices use a system that places the testing material in the middle of the machine, while a specific load is applied with an
actuator and the strain is measured using lasers (Stanley and Chan, 1985). There is also another way the strain could have been calculated instead of using the angle and trigonometry method.

The formula for strain is given by (Akay, 2006):

\[
\varepsilon = \frac{6d r_0}{2c(3L - 4r_0)}
\]

- \(d\) is the displacement of the actuator, in meters
- \(r_0\) is the outer radius of the bone, in meters
- \(c\) is the distance between the support and the load point, in meters
- \(L\) is the distance between the supports

If this formula was to be used, it may have led to more accurate results as well as eliminate the negative strain phenomenon. However, access to a 4-point or 3-point test load devices were not possible for this experiment.

**Conclusion**

The bending stress and strain, along with the Young’s modulus were calculated based on the changes in angles from chicken tibia as loads were applied. The bones were then divided into a ‘Frozen’ group, where the bones were cryopreserved for a week and a ‘Cold’ group where the bones were put in a refrigerator for a week. Another load test was then applied to determine if there were significant differences in the Young’s modulus between the ‘Frozen’ and the ‘Cold’ group. The Young’s modulus for ‘Frozen’ bones was significantly higher than the ‘Cold’ group due to the structure of the bone, with its cortical and inner cellular parts.
References


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