“Vitrified particulated articular cartilage for joint resurfacing: A swine model”

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APPENDIX

Methods for Mechanical Testing

A surgical blade was first used to remove the bone from the dowels and the remaining cartilage was submerged in PBS until mechanical testing. Before testing each sample, the thickness and diameter were measured at three different locations using a vernier caliper and the average values for cartilage thickness and diameter were recorded. Unconfined compression testing was performed on all samples using a Bose ElectroForce 3200. The samples were placed on a steel disc and immersed in PBS at room temperature. A steel disc connected to a shaft was used to compress the samples from the top. The loading protocol (shown below) comprised of three cycles of rapid loading and holding the displacement for stress relaxation after each step.

1) Contact was established by applying a pre-load of 10 N

2) Contact held for 120 s

3) Rapid loading at a strain rate of 15%/s to a strain of 10%

4) Displacement held for 900 s

5) Rapid loading at a strain rate of 15%/s to a strain of 20%

6) Displacement held for 900 s

7) Rapid loading at a strain rate of 15%/s to a strain of 30%

8) Displacement held for 1200 s
Different parameters were calculated and used to compare the mechanical properties of the samples. Instantaneous modulus was calculated by plotting the stress-strain curve and fitting a linear regression model to each rapid loading phase (Figure A1). The model was fit to the portion between 25% and 75% of the peak stress for each cycle. This was done to account for the nonlinear responses occurring in the initial toe region and final strain hardening region. The slope of the linear regression line was determined, and this represented the instantaneous modulus.

![Figure A1: Example plot of the stress-strain curve for the positive control sample of Pig 1.](image)

Equilibrium stresses were calculated by averaging the last 100 stress values in each stress relaxation phase. Equilibrium modulus was then determined for each cycle by dividing the change in equilibrium stress (equilibrium stress of current cycle minus equilibrium stress of previous cycle)
by the change in strain (10%). A 2-term Prony series model [1, 2] was used to find the relaxation time constants (τ₁ and τ₂) using the following formula:

\[ \sigma(t) = \sum_{i=1}^{2} \sigma_i e^{-t/\tau_i} + \sigma_\infty \]

where \( \sigma(t) \) is the compressive stress, \( t \) is the time, \( \sigma_i \) are the Maxwell spring constants, \( \tau_i \) are the relaxation time constants, and \( \sigma_\infty \) is the equilibrium stress.

After calculating the four parameters (instantaneous modulus, equilibrium modulus, and the two relaxation time constants), paired two-tailed t-tests were performed between the positive control and fresh groups, the positive control and vitrified groups, as well as the positive control and negative control groups.

**Results of Mechanical Testing**

The outliers in the data were removed before summarizing the results and conducting the paired t-tests by using a threshold of 1.5 times the interquartile range. Values greater than or less than this range were considered outliers and omitted from the analysis. In addition, the Bose ElectroForce 3200 experienced some technical issues during the tests which lead to inaccurate values for the relaxation time constants in the second and third loading phases for some of the samples. These results were also removed from the data.

A summary of the results is presented in Figure A2, Table A1, and
Table A2 as the average ± standard deviation of each parameter in each loading phase of all four experimental groups. The results indicate that the instantaneous modulus and the equilibrium modulus are each highest in the positive no-defect control group and are similar in the fresh and vitrified particulated cartilage transplant groups. The vitrified group had slightly higher instantaneous and equilibrium moduli, making it closer to the positive no-defect control group. The results are similar in the relaxation time constants where the positive no-defect control group has the highest values and the fresh and vitrified groups have similar values. The vitrified group, again, has relaxation time constants that are closer to the positive no-defect control group.

Statistical differences in obtained parameters between the experimental groups were examined by conducting paired two-tailed t-tests for all the parameters. The results are displayed in Table A3 and a p-value of 0.05 was used to determine statistically significant differences. T-tests between the positive no-defect control and fresh groups as well as between the positive no-defect control and vitrified groups indicated significant differences in all instantaneous and equilibrium moduli (highlighted in orange in the table). Some of the relaxation time constants were also significantly different in both comparisons. These results align with the bar graphs in Figure A2.
Figure A2: Bar charts showing the average and standard deviation of the instantaneous modulus, equilibrium modulus and relaxation time constants for all experimental groups in all three loading phases.
Table A1: Instantaneous modulus and equilibrium modulus of all experimental groups in the three loading phases (Average ± s.d.). The n represents the number of samples used to obtain each parameter.

|                          | Instantaneous Modulus (MPa) | Equilibrium Modulus (kPa) |
|--------------------------|----------------------------|----------------------------|
|                          | 10% strain | 20% strain | 30% strain | 10% strain | 20% strain | 30% strain |
| **Positive control** (no defect) |           |            |            |            |            |            |
| 10% strain               | 10.5 ± 5.80 n=19 | 22.3 ± 11.6 n=19 | 35.7 ± 18.1 n=19 | 286.8 ± 228.2 n=19 | 716.1 ± 365.0 n=19 | 1024.5 ± 499.1 n=19 |
| Fresh                    | 3.47 ± 1.22 n=7 | 9.27 ± 5.63 n=8 | 15.2 ± 7.52 n=7 | 23.9 ± 35.2 n=7 | 92.3 ± 71.3 n=7 | 186.4 ± 156.9 n=7 |
| Vitrified                | 4.71 ± 2.75 n=8 | 9.59 ± 5.54 n=8 | 19.1 ± 10.3 n=8 | 35.8 ± 38.0 n=7 | 135.3 ± 118.4 n=7 | 280.1 ± 215.4 n=7 |
| **Negative control** (untreated defect) |           |            |            |            |            |            |
| 10% strain               | 3.44 ± 1.43 n=3 | 6.36 ± 2.13 n=3 | 13.4 ± 6.02 n=3 | −13.9 ± 22.4 n=3 | 49.9 ± 27.6 n=3 | 99.6 ± 1.79 n=3 |

Note: The negative equilibrium modulus values for the negative control samples are a result of the samples having lower stresses at the end of the first loading phase when compared to the end of the contact phase. Since the equilibrium modulus at 10% strain is calculated as the equilibrium stress of the first loading phase minus the equilibrium stress of the contact phase divided by 10% strain, lower stresses in the first loading phase would lead to a negative equilibrium modulus. The contact phase had a relaxation time of 120 seconds, whereas the first loading phase had a relaxation time of 900 seconds, so these results suggest that these samples did not fully relax after contact.
Table A2: Relaxation time constants of all experimental groups in the three loading phases (Average ± s.d.). The n represents the number of samples used to obtain each parameter.

|                | τ₁ (s)     | τ₂ (s)     |
|----------------|------------|------------|
|                | 10% strain | 20% strain | 30% strain | 10% strain | 20% strain | 30% strain |
| **Positive control (no defect)** |            |            |            |            |            |            |
|                |            |            |            |            |            |            |
| Positive control (no defect)       | 6.49 ± 2.08 | 10.7 ± 4.90 | 16.4 ± 8.69 | 101.5 ± 29.1 | 112.9 ± 40.8 | 131.3 ± 73.5 |
|                | n=17       | n=13       | n=16       | n=19       | n=13       | n=18       |
| Fresh          | 2.77 ± 1.67 | 3.73 ± 0.40 | 4.89 ± 2.20 | 55.1 ± 30.0 | 57.3 ± 4.09 | 70.0 ± 25.7 |
|                | n=7        | n=4        | n=6        | n=7        | n=4        | n=6        |
| Vitrified      | 4.42 ± 3.52 | 6.36 ± 5.83 | 3.72 ± 2.08 | 81.9 ± 38.9 | 79.5 ± 32.5 | 62.8 ± 15.8 |
|                | n=8        | n=7        | n=4        | n=8        | n=7        | n=4        |
| Negative control (untreated defect) | 2.67 ± 1.46 | 2.69 ± 1.12 | 3.19 ± 0.78 | 101.0 ± 94.5 | 40.0 ± 17.9 | 43.8 ± 13.4 |
|                | n=3        | n=3        | n=3        | n=3        | n=3        | n=3        |

Table A3: Summary of paired two-tailed t-tests conducted between the experimental groups (p-value < 0.05 highlighted in orange).

|                                | Positive Control vs. Fresh | Positive Control vs. Vitrified | Positive Control vs. Negative Control |
|--------------------------------|-----------------------------|--------------------------------|---------------------------------------|
| Instantaneous Modulus at 10% strain | 0.008                       | 0.021                          | 0.338                                 |
| Instantaneous Modulus at 20% strain | 0.023                       | 0.008                          | 0.300                                 |
| Instantaneous Modulus at 30% strain | 0.006                       | 0.024                          | 0.474                                 |
| Equilibrium Modulus at 10% strain   | 0.006                       | 0.009                          | 0.113                                 |
| Equilibrium Modulus at 20% strain   | 0.000                       | 0.006                          | 0.006                                 |
| Equilibrium Modulus at 30% strain   | 0.000                       | 0.011                          | 0.003                                 |
| τ₁ at 10% strain                  | 0.018                       | 0.088                          | 0.547                                 |
| τ₁ at 20% strain                  | 0.202                       | 0.067                          | 0.352                                 |
| τ₁ at 30% strain                  | 0.112                       | 0.026                          | 0.464                                 |
| τ₂ at 10% strain                  | 0.083                       | 0.085                          | 0.652                                 |
| τ₂ at 20% strain                  | 0.197                       | 0.005                          | 0.212                                 |
| τ₂ at 30% strain                  | 0.664                       | 0.029                          | 0.385                                 |
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