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# Alternative Methods for Long Term Storage of Skeletal Muscle Tissue

Byron Hempel

# Alternative Methods for Long Term Storage of Skeletal Muscle Tissue

Student: Byron Hempel
Faculty Mentor: Kenneth Campbell
Other Contributors: Ben Lawson

## Introduction

I have been doing research in Dr. Kenneth Campbell's laboratory in the Department of Physiology here at the University of Kentucky. Dr. Campbell is also the director of the Muscle Mechanics Core for the Center for Muscle Biology. Researchers from laboratories outside of UK can send in various skeletal muscle samples for functional measurements at the Muscle Mechanics Core. In order to do this, tissue must be stored for shipment.

Storing the samples using traditional methods can be complicated and allows for little shipping time. Therefore, cryopreservation, or freezing tissue, presents the best alternative method for effective long term storage. However, there are many obstacles that arise when freezing muscle tissue. Relatively large ice crystals form inside the cell when freezing, and as the ice expands, the cell is torn apart. The introduction of a cryopreservant solution should help preserve cells by displacing water[1]. The rate of temperature change for both the freezing and thawing process may also affect cell viability. Tissue can either be flash frozen in liquid nitrogen or be frozen very slowly. A final parameter, thaw-rigor, damages cells by inducing uncontrolled contraction[2], which may be mitigated by fixing the tissue length with a capillary tube.

**This project entails finding the most cost effective, efficient, and least damaging means of preserving skeletal muscles for biophysical experiments months after storage.** The effectiveness of storing the tissue will be determined by both visual and mechanical studies of the frozen tissues. Solution type, tissue support, and rate of freezing are controlled in an attempt to meet these criteria. Therefore, this research will focus primarily on the effects generated by the freezing and thawing process.

## Methods and Materials

### Tissue Collection

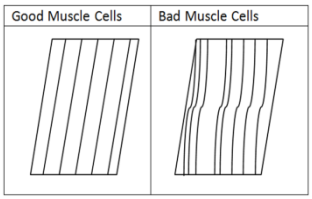
*Sprague-Dawley* rats were sacrificed and the soleus and psoas muscle were dissected. After each dissection, the muscles were placed in a cryopreserving solution (or dry control) to begin the freezing process. These muscles were chosen for two main of reasons: they are fairly easy to dissect and they are different functionally and structurally.

Tissues were then either fixed to a capillary tube or left untied and placed into a cryogenic vial containing the experimental solution. The rate of freezing was controlled by either immediately placing the excised tissue into liquid nitrogen (flash freezing), or placing the tissue in an insulated cryogenic vial into ice for 30 minutes, then switching over into dry ice temperatures (slowly freezing). The types of cryopreservants were obtained from background papers and previous studies, including a dry sample, varying levels of glycerol and relax (a universal lab solution which allows muscle cells to maintain their integrity while in *vitro*), and 2M sucrose[3].

**Visual Inspection**

The viability of the storage method was determined by looking at the muscle tissue and observing structural damage from freezing. In general, any deviation from parallel muscle strands will be deemed damaged tissue (see figure 1). Also, the cells were stretched with forceps and the resistivity to ripping was noted. For control, muscle was observed and studied under the dissecting microscope immediately after dissection. Muscle groups deemed effectively stored were used as an outline and more tissue was dissected and stored in a freezer for two months before beginning functional assays.

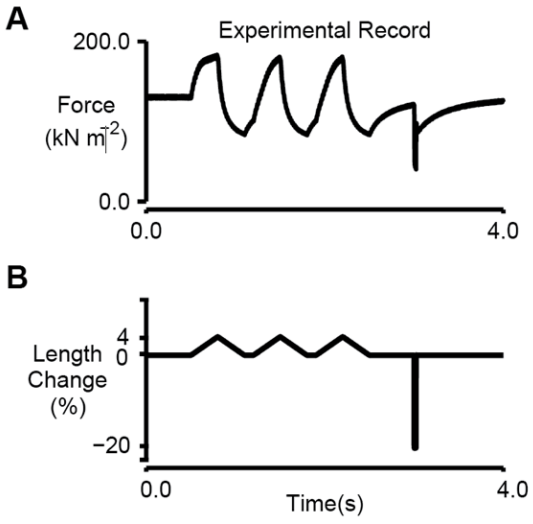
Figure 1



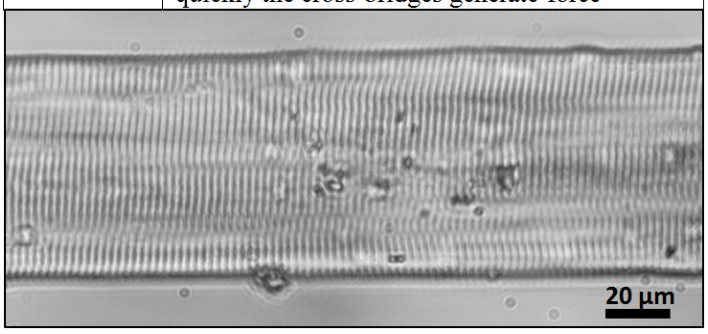

**Mechanical Experiments**

Quantitative analysis was performed on the muscles that were previously frozen in different solutions to test the effectiveness of the storage method. The frozen muscle tissue was thawed and separated into bundles (groups of 10-20 cells about 3mm in length), then chemically permeabilized. Control tissues were separated and permeabilized immediately after dissection. An individual muscle fiber was then removed from the muscle bundle and tied between a force transducer and a motor. Functional parameters were measured by performing mechanical assays to determine many variables, most importantly the isometric tension ($P_0$), the rate of tension recovery ($k_{tr}$), and the $pCa_{50}$. All experiments were completed within two weeks after permeabilization. Below is a representative trace from a mechanical experiment and the parameters being measured:

**Figure 2**




| Parameter | This parameter indicates |
|---|---|
| $P_0$ | Maximum isometric force normalized to the fibers cross-sectional area |
| $pCa_{50}$ | Calcium sensitivity of the contractile apparatus |
| $k_{tr}$ | Rate of tension recovery – a measure of how quickly the cross-bridges generate force |




The fiber length change (B) was caused by the motor, while the force transducer reported the force transients (A). When a cell is placed in solutions containing different calcium concentrations, it will produce different isometric tensions. A soleus muscle fiber placed in pCa 4.5, which has the highest $Ca^{2+}$ concentration, generally generates the highest isometric tension,

while placing the fiber in pCa 9.0, which has minimal concentrations of calcium, generates low isometric tension. The parameter indicated by $pCa_{50}$ is the calcium concentration where a fiber generates half the maximum isometric tension recorded. The rate of tension recovery is the rate at which the cell's cross bridges, or "miniature motors", cycle.

The results from previously frozen muscle fibers will be compared to muscle fibers that were freshly excised from the rat. The sarcomeres were observed and photographed under a high power microscope.

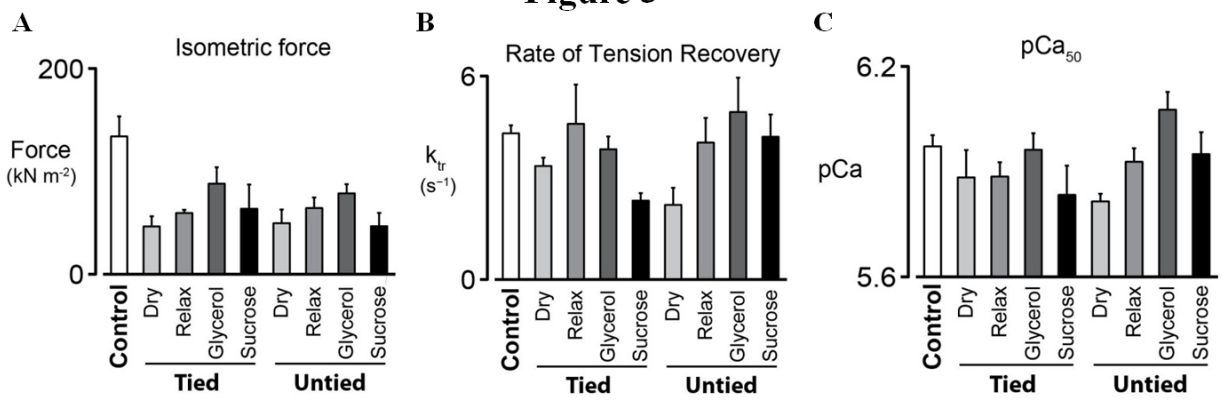
**Results**

Several observations were made during the visual inspection of frozen tissue. None of the flash frozen tissues were considered healthy. All of the tissues frozen without solution were also badly damaged. Although none of the frozen tissues equaled the quality of fresh, tissues stored in solution seemed much more viable, with the quality improving as the cryopreservant concentration increased. The tied muscles also appeared less damaged than those left untied. The following groups were determined effectively stored qualitatively from the visual inspection:

| Group | Cell type | Cryopreservant | Tied/Untied |
|---|---|---|---|
| 1 | Soleus | 100% Relax | Untied |
| 2 | Soleus | 100% Relax | Tied |
| 3 | Soleus | 50% glycerol | Untied |
| 4 | Soleus | 50% glycerol | Tied |
| 5 | Soleus | 2M sucrose | Untied |
| 6 | Soleus | 2M sucrose | Tied |
| 7 | Soleus | 100% glycerol | Untied |
| 8 | Soleus | 100% glycerol | Tied |
| 9 | Psoas | 100% Relax | Untied |
| 10 | Psoas | 100% Relax | Tied |
| 11 | Psoas | 50% glycerol | Untied |
| 12 | Psoas | 50% glycerol | Tied |
| 13 | Psoas | 2M sucrose | Untied |
| 14 | Psoas | 2M sucrose | Tied |
| 15 | Psoas | 100% glycerol | Untied |
| 16 | Psoas | 100% glycerol | Tied |

The following graphs compare the maximum isometric tension, the rate of tension recovery, and the $pCa_{50}$. Note that not enough time was available to study every group, so only groups 1-6 were studied, along with 2 dry stored groups of tied/untied and the fresh control tissue.

**Figure 3**




In figure 3, note that the "Glycerol" indicates 50% glycerol in relax solution. Looking at the isometric force, none of the samples were as high as the freshly excised tissue. However, as concentration of cryopreservants increases, the mechanical function nears the control, with the exception being in 2M sucrose. It also appears that tied tissue offers a slight improvement over the untied groups.

**Discussion**

Having two different muscle types was necessary for this experiment. The soleus contains mostly slow twitch muscle fibers, while the psoas exhibits fast twitch (IIB) fibers[4]. The soleus displays pennation[5] and attaches to connective tissue, while the psoas does not contain much connective tissue. The soleus resists damage much better than the psoas. Therefore, the different subsets of skeletal muscle may require different solutions to be effectively stored, such as the soleus being less susceptible to freezing damage by being a hardier cell. Continued research performing mechanical assays on the unstudied psoas tissue and the other groups that have not yet been studied will be done.

The visual inspection portion of the experiment could not determine an absolute for which method stored the cells well. However, the groups were narrowed down effectively into a much smaller group size, and the mechanical assays will determine which method works best.

From the mechanical assay results so far, there has not been a consistent storage method which maintains the integrity of the cells. However, looking at the trends on the graphs, as glycerol levels rise, it is predicted that the 100% glycerol will be the most effective way to preserve the tissue thus far. With each storage method, there were still cells that were effectively stored (it produced the same properties of a fresh cell: an isometric tension around $9x10^4\ N\ m^{-2}$, had a $k_{tr}$ around 4 $s^{-1}$, and pCa around 6), but the storage was more of a hit and miss. More time is needed to study the rest of the groups, and to see if other groups were stored effectively. Also, different levels of sucrose could be tested. The 2M sucrose could be too high of a concentration and be toxic to the cells.

Looking at photographs of the sarcomeres and the output of data, force output of a cell cannot be wholly predicted by the way the sarcomeres look after freezing. Many of the frozen cells looked healthy (contained parallel, uniform sarcomeres), yet the force output was drastically lower than fresh tissue. For fresh muscle cells, the cell's mechanical properties could generally be predicted by the way the sarcomere's lined up: the more parallel and uniform, the higher the force output would be. This leads one to believe that the freezing process and/or the

increased concentration of cryopreservants are damaging cells at a protein or sarcomere level. More intensive study and lab equipment beyond the scope of this experiment is needed to determine the cause of the results, such as using electron microscopy to look further into the protein structure.

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