Muscle tissue water content estimation in rat experimental intensive care unit model

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SUMMARY

The molecular mechanisms underlying acute quadriplegic myopathy (AQM) are not fully known. Mechanical stimulation may help slow the loss of muscle proteins, and elucidating the mechanisms of this effect could prove very valuable.

In this project, freeze-drying and desiccation methods were used to determine water content in muscles obtained from a novel experimental rat AQM-model. The focus was on evaluating the results and trying to refine the methods, which, in the future, may be used in combination with other methods to study muscle protein content.

I have shown that the freeze-drying method delivers reliable results, while the experimental room temperature desiccation method still needs to be refined.
INTRODUCTION

Skeletal muscle function and structure

Skeletal muscles have many important functions in our body. For example, they produce force and movement, help in maintaining posture, and support and protect bone and soft tissues. Skeletal muscle is also involved in thermoregulation, and is an important energy source during starvation and critical illness. (Martini et al. 2006)

Skeletal muscle cells, muscle fibers, are enormous multinucleated cells. Each fiber has hundreds of nuclei, which are necessary to sustain the large volume of cytoplasm (sarcoplasm). A muscle fiber contains cylindrical structures called myofibrils, which are made up of sarcomeres – the smallest functional unit of a muscle fiber. (Martini et al. 2006)

Muscle contractions depend on interaction between thin filaments (mainly composed of actin) and thick filaments (mainly myosin). Contraction is triggered by electrical signals from motor neurons, and depolarization of the cell membrane (sarcolemma) leads to intracellular Ca$^{2+}$ release and force generation by interactions between thin and thick filaments. (Martini et al. 2006)

Thick filaments consist of myosin and myosin binding proteins. Myosin is a motor protein that converts chemical energy from adenosine triphosphate (ATP) hydrolysis to mechanical energy to generate force and movement. (Norman 2006)

Thin filaments provide attachment sites to which myosin can bind. Actin, tropomyosin and troponin are the three major components that make up thin filament. Troponin is deactivated by an increase in calcium, and its suppressive effect on actin-myosin interaction is turned off. (Norman 2006)

During contraction, actin filaments and myosin filaments slide past each other, shortening the sarcomeres and thus the whole muscle. Myosin heads, which have bound exposed active sites on actin filament, change configuration when they release inorganic phosphate, producing movement. The myosin heads then detach and if ATP is available the cycle may be repeated. (Squire 2005)

![Figure 1. Sarcomere. Many sarcomeres are connected end-to-end, and when they shorten the muscle contracts. Actin filaments and myosin filaments are interdigitating, enabling cross bridge formation and force exertion. Adapted from Squire (2005)](image)

There are different types of skeletal muscle fibers. Three major types are present in the human body: fast, slow and intermediate fibers. This division is made with respect to contractile speed. The types use different substrates for ATP generation during contraction, have
different myosin isoforms, color, and fatigue resistance, to mention a few properties. (Martini et al. 2006)

Muscles not stimulated on a regular basis will undergo atrophy; lose size, tone and power. This effect can be caused even by a temporary reduction in muscle use. (Martini et al. 2006)

Skeletal muscles are essential in all voluntary movements, and any functional impairment can negatively affect a person’s quality of life. (Norman 2006)

**Acute Quadriplegic Myopathy**
A muscle paralysis frequently observed in mechanically ventilated intensive care unit (ICU) patients treated with non-depolarizing neuromuscular blocking agents and/or corticosteroids, acute quadriplegic myopathy (AQM) is also known as critical illness myopathy and thick filament myopathy. (Norman et al. 2006)

Decreased muscle membrane excitability, partial or complete loss of myosin, and dramatically impaired force-generating capacity are observed in AQM patients. Muscles innervated by proximal and distal spinal nerves are flaccid and weak. Craniofacial muscles, on the other hand, are typically spared or less affected. Cognitive and sensory functions are intact. (Norman et al. 2006)

Considered a consequence of modern treatment in anesthesiology and intensive care, AQM is found in up to 30% of ICU patients. Despite of the high prevalence, there is still poor understanding of basic mechanisms underlying the disease. This is in part due to the fact that patients are typically diagnosed 2-3 weeks after exposure to causative factors. (Larsson 2007)

There is accordingly a strong need for experimental animal models in mechanistic studies on AQM, as well as in specific intervention studies. Preliminary results from experimental animal studies indicate that the mechanical unloading may be an important factor and that the muscle wasting associated with AQM may be reduced by a loading intervention.

Larsson’s research group at Uppsala University combines clinical studies with experimental animal (rat and pig) models to study e.g. regulation of muscle contraction, muscle structure, muscle size, as well as the effects of different interventions. The effects of lengthening and shortening of limb muscles by mechanical loading is currently evaluated in the rat model, and this is where this project comes in.

Accumulation of water in the muscle tissue may be a confounding factor. It is therefore imperative to determine water content in muscle and how it is affected by loading. Also, tissue samples obtained from ICU patients are valuable and if water content estimation could be done with as little tissue as possible it would be good for all parts.

The aims of this project were to evaluate methods to determine water content in rat muscle, minimize waste of tissue in doing so, and then applying those methods to see if any trends could be observed in data from the experimental rat model.
RESULTS

Wet weight analysis
To investigate if the passive mechanical stimulation given in the rat ICU model has any effect on muscle weight, I compared the weight of muscles from left (stimulated) and right (internal control) hind legs. The weight of five muscles (Tibialis anterior, Extensor digitorum longus, Gastrocnemius, Soleus, and Plantaris) from each leg were pooled and compared.

Table 1. Mean weight difference with standard deviation of left and right hind leg musculature in mechanically stimulated rats.

<table>
<thead>
<tr>
<th>Time in model (h)</th>
<th>Mean difference (%)</th>
<th>Standard deviation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.8</td>
<td>1.7</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>1.1</td>
<td>1.5</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>1.2</td>
<td>7.4</td>
<td>5</td>
</tr>
<tr>
<td>48</td>
<td>4.1</td>
<td>4.6</td>
<td>2</td>
</tr>
<tr>
<td>72</td>
<td>8.6</td>
<td>5.6</td>
<td>3</td>
</tr>
</tbody>
</table>

*L/R-1, i.e. positive value means left is heavier than right.

Figure 2. Mean difference between left and right hind leg musculature. Positive Y-value means L > R. Standard deviation error bars. Linear regression line through origin ($R^2 = 0.936$ p<0.01).

A linear regression model indicates that there is a correlation between the time in model and the mean difference. A one-way ANOVA could not show a significant difference among any of the groups.

Water content estimation
An effect on actual protein content could be masked by edema; consequently it was desirable to find out the water content of the muscle tissue. To do this I used two methods, room temperature desiccation and freeze-drying. The room temperature desiccation method was experimental, the freeze-drying method more conventional.
Table 2. Mean water content with standard deviation in rat muscle tissue.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean water content (%)</th>
<th>Standard deviation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze-drying</td>
<td>72.7</td>
<td>1.7</td>
<td>20</td>
</tr>
<tr>
<td>Desiccation</td>
<td>78.4</td>
<td>1.5</td>
<td>20</td>
</tr>
</tbody>
</table>

Muscle tissue was collected from both legs of ten rats and water content measurements were done by freeze-drying and desiccation methods. A paired Student’s t-test found the difference in tissue water content obtained by the two methods to be significantly different.

A Pearson product moment correlation showed no correlation between the values obtained with the different methods.

**Dry weight analysis**

In order to compare the dry weight in mechanically stimulated and unstimulated muscle, I determined the water content in individual Plantaris muscles from ten model rats with the freeze-drying method. The water content in each individual muscle was subtracted from the muscle’s wet weight to obtain the dry weight.

Table 3. Mean dry weight (d/w) difference with standard deviation of Plantaris muscle from left and right hind legs in mechanically stimulated rats. Mean wet weight (w/w) difference of same muscles.

<table>
<thead>
<tr>
<th>Time in model (h)</th>
<th>D/w mean difference (%)</th>
<th>D/w standard deviation</th>
<th>W/w mean difference (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-3.3</td>
<td>12.5</td>
<td>-0.6</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>-2.3</td>
<td>13.9</td>
<td>-3.7</td>
<td>3</td>
</tr>
<tr>
<td>48</td>
<td>-0.4</td>
<td>N/A</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>72</td>
<td>5.4</td>
<td>21.8</td>
<td>3.1</td>
<td>3</td>
</tr>
</tbody>
</table>

* L/R-1, i.e. positive value means left is heavier than right.

![Figure 3](image.png)

**Figure 3.** Plantaris muscle dry weight difference (filled circles) in left and right leg in mechanically stimulated rats. Standard deviation error bars. Wet weight differences (empty circles) of same Plantaris muscles plotted without error bars. Positive Y-value means L > R. Linear regression (for w/w) line through origin ($R^2 = 0.863$, p=0.07).
A linear regression analysis failed to find a correlation between the time in model and mean dry weight difference. The power of the linear regression test is too low to draw any conclusions from, however. A one-way ANOVA could not show a significant difference among any of the groups.

Comparison of left-right difference at the different time points for wet and dry weight was done using a paired t-test, which failed to find any significant difference.
DISCUSSION

Water content determination by the freeze-drying method delivered satisfactory results; the mean result in this study (72.7%) is in good agreement with literature values for total body water (64-74%; Sahin et al. 2006).

The room temperature desiccation method, on the other hand, delivers a mean water content (78.4%) considerably higher than literature values. There are many conceivable sources of error; condensation on both tissue and the aluminum weighing pan, and the unfortunate subjectivity when reading out a maximum value because of the sensitive equipment used, to mention a few.

No conclusions can be drawn for the analyses of dry and wet weight in mechanically stimulated muscles, other than the fact that left leg muscles seems to be heavier than their right leg counterparts after some time in the ICU model.

There may be ways to go forward with the desiccation method. In a climate controlled laboratory environment, and using e.g. small sealed containers in which cryosections can be put to minimize evaporation and condensation, precision and reliability could improve.
MATERIALS AND METHODS

Animals
Female (4-5 months old) Sprague Dawley rats serve as an experimental intensive care unit model. They are pharmacologically paralyzed, isoflurane anesthetized, and mechanically ventilated. The left distal hindlimb muscles are mechanically loaded with a servomotor.

Figure 4 AQM rat model.

Tissue water content determination
Muscle cleaned of adipose and connective tissue was stored in liquid nitrogen freezers (approx. -170°C). Tissue samples were taken from stored muscles, and water content determined by room temperature desiccation and freeze-drying methods.

In the freeze-drying method, the frozen tissue samples were weighed and freeze-dried (-55°C) in vacuum for 48h, and then weighed again.

Figure 5 Microm HM 560 CryoStar.

In the room temperature desiccation method, the tissue samples were placed on Tissue-Tek OCT compound (Tissue-Tek®, Labonord, France) for cryostat sectioning (Microm HM 560 CryoStar, Thermo Fisher Scientific, U.S.A.).
The muscle tissue samples were oriented to obtain cross-sections with solely tissue. Ten micrometer-thick cryosections were cut and put on a 12 mm aluminium pan. Three to five cryosections were cut for each weighing. The pan was placed on a hangdown stirrup in a Cahn C-35 UltraMicro balance (Thermo Fisher Scientific, U.S.A.) for weighing. After stabilization, a maximum value was reached, after which evaporation pushed the value down towards a minimum value.

![Figure 6 Cahn C-35 UltraMicro balance.](image)

The minimum value in the room temperature desiccation method was corrected in order to compensate for the remaining water content, which was assumed to be equal to relative air humidity.

Water content was calculated by subtracting dry weight from wet weight and then dividing by wet weight (1).

\[
\text{Water content (\%)} = \frac{\text{Weight (wet)} - \text{Weight (dry)}}{\text{Weight (wet)}} \times 100 \tag{1}
\]

In order to achieve better precision in the room temperature desiccation method, five measurements were carried out, the highest and lowest values were left out and a mean was calculated from the three remaining values.
ACKNOWLEDGEMENTS

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REFERENCES


Norman, H. 2006. Cellular and Molecular Mechanisms Underlying Acute Quadriplegic Myopathy: Studies in Experimental Animal Models and Intensive Care Unit Patients *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 171: 79
