Optimization of permeabilized fibers preparation for mitochondrial respiration measurements using Design of Experiments methodology.

Fabienne Cortade, Virginie Gaillard, Béatrice Chabi

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Aim of the study

To optimize the permeabilized fibers (pf) preparation from mouse *Tibialis anterior* in our lab, we used the Design of Experiments (DoE) methodology that evaluates the impact of 6 experimental conditions or factors, on the pf respiration parameters (Pyruvate Malate Succinate respiration (PMS leak) and respiratory control ratio (RCR<sub>PMS</sub>)), to provide a maximum of information using a limited number of experiments and animals.

Materials and Methods

**Test system**

- **Animals**: C57BL/6 mice, 25 week-old, male and female (n=18)
- **Muscle**: *Tibialis anterior*, n=2 per mouse
- **Device**: High-resolution Oxigraph-2k (OROBOROS Instruments)
- **DoE software**: NemrodW®, version 2015, NemrodW SAS, Marseille, France

**Fixed experimental conditions**

- Resting rate (PMS leak): 5 mM pyruvate, 5mM malate and 10 mM succinate
- ADP-stimulated rate (PMS<sub>s</sub>): addition of 5 mM ADP
- Respiratory Control Ratio (RCR<sub>PMS</sub>) set as the ratio of oxygen consumption at PMS leak (PMS<sub>s</sub>) over oxygen consumption at PMS<sub>s</sub>, Y<sub>i</sub>: PMS<sub>5</sub> level to be maximized (at least 40 pmol O<sub>2</sub>/s*mg fibers)
- Y<sub>2</sub>: variability of RCR<sub>PMS</sub> estimated by coefficient of variation of 4 repeated experiments to be minimized

**Responses studied**

- The influence of 6 factors on Y<sub>1</sub> and Y<sub>2</sub> responses has been evaluated using a Hadamard matrix with 8 experiments (instead of 64 experiments if all combinations had been tested with a « One-Factor-At-A-Time » (OFAT) method), see below. To evaluate experimental variance for Y<sub>i</sub> response, each experiment has been replicated 4 times. To evaluate experimental variance for Y<sub>1</sub> response, one experiment (n=6) has been replicated 4 additional times. In total, 36 experiments have been performed.

**Experimental domain**

<table>
<thead>
<tr>
<th>Factors evaluated</th>
<th>Level -1</th>
<th>Level +1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber Types</td>
<td>Red</td>
<td>White</td>
</tr>
<tr>
<td>Manual Teasing</td>
<td>Gentle</td>
<td>Rough</td>
</tr>
<tr>
<td>Saponin content</td>
<td>8-25%</td>
<td>20-35%</td>
</tr>
<tr>
<td>PMS concentration</td>
<td>25 µg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Permeabilization time</td>
<td>0 hour</td>
<td>6 hours</td>
</tr>
</tbody>
</table>

**Design of Experiments**

The influence of 6 factors on Y<sub>1</sub> and Y<sub>2</sub> responses has been evaluated using a Hadamard matrix with 8 experiments (instead of 64 experiments if all combinations had been tested with a « One-Factor-At-A-Time » (OFAT) method), see below. To evaluate experimental variance for Y<sub>i</sub> response, each experiment has been replicated 4 times. To evaluate experimental variance for Y<sub>1</sub> response, one experiment (n=6) has been replicated 4 additional times. In total, 36 experiments have been performed.

**Experimental matrix and results**

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Fiber types</th>
<th>Manual teasing</th>
<th>Saponin concentration (µg/ml)</th>
<th>Permeabilization time (min)</th>
<th>Resting period (h)</th>
<th>Y&lt;sub&gt;1&lt;/sub&gt;: PMS&lt;sub&gt;5&lt;/sub&gt; level (pmol O&lt;sub&gt;2&lt;/sub&gt;/s*mg )</th>
<th>Y&lt;sub&gt;2&lt;/sub&gt;: RCR&lt;sub&gt;PMS&lt;/sub&gt; variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>N'1</td>
<td>White</td>
<td>Gentle</td>
<td>S1</td>
<td>25</td>
<td>30</td>
<td>30.7</td>
<td>0.196</td>
</tr>
<tr>
<td>N'2</td>
<td>Red</td>
<td>Gentle</td>
<td>S1</td>
<td>50</td>
<td>10</td>
<td>80.1</td>
<td>0.194</td>
</tr>
<tr>
<td>N'3</td>
<td>Red</td>
<td>Rough</td>
<td>S1</td>
<td>50</td>
<td>30</td>
<td>56.4</td>
<td>0.084</td>
</tr>
<tr>
<td>N'4</td>
<td>White</td>
<td>Rough</td>
<td>S2</td>
<td>50</td>
<td>30</td>
<td>31.3</td>
<td>0.204</td>
</tr>
<tr>
<td>N'5</td>
<td>Red</td>
<td>Gentle</td>
<td>S2</td>
<td>25</td>
<td>30</td>
<td>51.2</td>
<td>0.096</td>
</tr>
<tr>
<td>N'6</td>
<td>White</td>
<td>Rough</td>
<td>S1</td>
<td>25</td>
<td>10</td>
<td>32.8</td>
<td>0.099</td>
</tr>
<tr>
<td>N'7</td>
<td>White</td>
<td>Gentle</td>
<td>S2</td>
<td>50</td>
<td>10</td>
<td>31.8</td>
<td>0.120</td>
</tr>
<tr>
<td>N'8</td>
<td>Red</td>
<td>Rough</td>
<td>S2</td>
<td>25</td>
<td>10</td>
<td>62.4</td>
<td>0.202</td>
</tr>
</tbody>
</table>

Best experimental conditions to maximize PMS<sub>5</sub> level are red fibers, rough manual teasing and 20-35% saponin content.

To be noticed:

- Six hours resting period had no deleterious impact on PMS<sub>5</sub> level, allowing a more convenient organization of the protocol schedule.
- Objective level of 40 pmol O<sub>2</sub>/s*mg fibers is reached with both saponin content allowing to choose between the more practical/less toxic mode of preparation.

**Influencing factors**

- Fiber types
- Manual teasing
- Saponin content
- Permeabilization time
- Resting period

**Non influencing factors**

- Saponin concentration

Evaluation of experimental variance with only one replicate of one experiment over 8 was not accurate enough to discriminate with confidence which of the 6 tested factors are really influencing RCR<sub>PMS</sub> variability. Nevertheless, it seems that levels of influencing factors that maximize PMS<sub>5</sub> level were not deleterious in minimizing RCR<sub>PMS</sub> variability.

**Conclusion**

Using a DoE analysis, we were able to optimize pf assay conditions with a reduced number of experiments and animals, and rapidly obtain valuable data in accordance with ethical recommendations (3Rs). The optimization of pf preparation by DoE will be pursued with two objectives (i) studying the possible interactions existing between the 3 factors related to saponin (saponin content, saponin concentration and incubation time), (ii) calculating the optimal sample size (n) needed to observe statistically significant differences between two animal groups.