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

Fabienne Cortade 1, Virginie Gaillard 2 and Béatrice Chabi 1

1 INRA, UMR DMeM, UNIVMONTPELLIER, FRANCE; 2 ITG, INSTITUT DU TEMPS GÈRE, PARIS, FRANCE

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 (RCRpfM)., 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 mice
Device: High-resolution Oxygraph-2k (OROBOROS Instruments)
DoE software: NemrodW®, version 2015, NewrodW SAS, Marseille, France

Fixed experimental conditions

Resting rate (PMS leak): 5 mM pyruvate, 5mM malate and 10 mM succinate
ADP-stimulated rate (PMSp): addition of 5 mM ADP
Respiratory Control Ratio (RCRpfM) set as the ratio of oxygen consumption at PMS leak (PMS) over oxygen consumption at PMSp, Y1: PMS level to be maximized (at least 40 pmol O2/s*mg fibers)
Y2: variability of RCRpfM estimated by coefficient of variation of 4 repeated experiments to be minimized

Responses studied

The influence of 6 factors on Y1 and Y2 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 Y1 response, each experiment has been replicated 4 times. To evaluate experimental variance for Y2 response, one experiment (n=6) has been replicated 4 additional times. In total, 36 experiments have been performed.

Design of Experiments

Factors evaluated

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level 1</th>
<th>Level 2</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>50 µg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Permeabilization time (min)</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Resting period before permeabilization</td>
<td>0 h</td>
<td>6 hours</td>
</tr>
</tbody>
</table>

Experimental domain

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>Y1: PMS level (pmol O2/s*mg)</th>
<th>Y2: RCRpfM variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>White</td>
<td>Gentle</td>
<td>S1</td>
<td>25</td>
<td>30</td>
<td>30.7</td>
<td>0.196</td>
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<tr>
<td>N2</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>N3</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>N4</td>
<td>White</td>
<td>Rough</td>
<td>S2</td>
<td>50</td>
<td>30</td>
<td>31.3</td>
<td>0.204</td>
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<tr>
<td>N5</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>N6</td>
<td>White</td>
<td>Rough</td>
<td>S1</td>
<td>25</td>
<td>10</td>
<td>32.8</td>
<td>0.094</td>
</tr>
<tr>
<td>N7</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>N8</td>
<td>Red</td>
<td>Rough</td>
<td>S2</td>
<td>50</td>
<td>10</td>
<td>62.4</td>
<td>0.202</td>
</tr>
</tbody>
</table>

Best experimental conditions to maximize PMS 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 level, allowing a more convenient organization of the protocol schedule.
- Objective level of 40 pmol O2/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 -14.9%
Manual teasing +17%
Saponin content +4.63%
Non influencing factors
Saponin concentration -2.2%
Permeabilization time -3.96%
Resting period

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 RCRpfM variability. Nevertheless, it seems that levels of influencing factors that maximize PMS level were not deleterious in minimizing RCRpfM variability.

Results / Interpretations

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.