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

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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 (RCRPMs)), 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 Oxigraph-2k (OROBOROS Instruments)
DoE software: NemrodW®, version 2015, NewroD W 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 (RCRPMs) set as the ratio of oxygen consumption at PMS leak (PMSL) over oxygen consumption at PMSp, Y2: PMSp level to be maximized (at least 40 pmol O2/s*mg fibers)
Y1: variability of RCRPMs estimated by coefficient of variation of 4 repeated experiments to be minimized

Design of Experiments
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 Y1 response, one experiment (n=6) has been replicated 4 additional times. In total, 36 experiments have been performed.

Experimental domain
Factors evaluated

<table>
<thead>
<tr>
<th>Level -1</th>
<th>Level +1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber Types</td>
<td>Red White</td>
</tr>
<tr>
<td>Manual Teasing</td>
<td>Gentle Rough</td>
</tr>
<tr>
<td>Saponin content</td>
<td>8-25% (S1) 20-35% (S2)</td>
</tr>
<tr>
<td>Saponin concentration for permeabilization</td>
<td>25 µg/ml 50 µg/ml</td>
</tr>
<tr>
<td>Permeabilization time</td>
<td>10 min 30 min</td>
</tr>
<tr>
<td>Resting period before permeabilization</td>
<td>0 hour 6 hours</td>
</tr>
</tbody>
</table>

Results / Interpretations

Experimental matrix and results

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Fiber types</th>
<th>Manual teasing</th>
<th>Saponin content</th>
<th>Saponin concentration (µg/ml)</th>
<th>Permeabilization time (min)</th>
<th>Resting period (h)</th>
<th>Y1: PMSp level (pmol O2/s*mg)</th>
<th>Y2: RCRPMs 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>0</td>
<td>30.7</td>
<td>24.6</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>6</td>
<td>80.1</td>
<td>56.1</td>
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<tr>
<td>N3</td>
<td>Red</td>
<td>Rough</td>
<td>S1</td>
<td>50</td>
<td>30</td>
<td>0</td>
<td>56.4</td>
<td>63.8</td>
</tr>
<tr>
<td>N4</td>
<td>White</td>
<td>Rough</td>
<td>S2</td>
<td>50</td>
<td>30</td>
<td>6</td>
<td>31.3</td>
<td>42.9</td>
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<td>Gentle</td>
<td>S2</td>
<td>25</td>
<td>30</td>
<td>6</td>
<td>51.2</td>
<td>53.4</td>
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<tr>
<td>N6</td>
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<td>Rough</td>
<td>S1</td>
<td>25</td>
<td>10</td>
<td>6</td>
<td>32.8</td>
<td>47.3</td>
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<tr>
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<td>S2</td>
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<td>10</td>
<td>0</td>
<td>31.8</td>
<td>24.3</td>
</tr>
<tr>
<td>N8</td>
<td>Red</td>
<td>Rough</td>
<td>S2</td>
<td>50</td>
<td>10</td>
<td>0</td>
<td>62.4</td>
<td>64.9</td>
</tr>
</tbody>
</table>

Y2: PMSp level

Influencing factors
- Fiber types
- Manual teasing
- Saponin content

Non influencing factors
- Saponin concentration
- Permeabilization time
- Resting period

Y2: RCRPMs variability

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