Supervitri device validation in the mouse model. Part II (oocyte tests)
Experimental protocol

**Oocyte survival tests**

Mouse oocytes were collected from F1 hybrid females (B6/CBA) and washed thoroughly. Oocytes were vitrified following a standardized protocol and using commercial vitrification solutions. Briefly, samples were exposed to buffer solution and equilibration solution gradually for 15 min and transferred to vitrification solution for 1 min. Afterwards, oocytes were loaded onto the surface strip of the Supervitri device and directly plunged into liquid nitrogen (LN2). Subsequently, the Supervitri device containing the loaded samples was capped under the LN2. A maximum of 5 oocytes were loaded at a time on each Supervitri device.

For the warming, the cap was first removed from the Supervitri under LN2, and then the Supervitri strip was transferred from the LN2 into the first warming solution (4ml, at 37°C) solution for 1 min. Then, oocytes were gradually moved to dilution solution for 3 min, to washing solution 1 for 5 min and finally to washing solution 2 for an additional 1 min (at room temperature). After warming, samples were extensively washed and kept in culture medium. After 1-2h, oocytes were evaluated as detailed below (see paragraphs 1, 2 and 3).

Control oocytes were evaluated following the same set-up and conditions, without exposure to vitrification/warming media nor LN2. A Fisher’s exact test was used to analyse statistical differences between the test and control oocytes. A p value < 0.05 was considered statistically significant.

1. **Morphological evaluation of vitrified oocytes at different time-points after warming**
   
   Oocyte survival and vacuolization rates were assessed 1-2h after warming under the inverted microscope.

2. **Meiotic spindle visualization after oocyte warming using a polarized light microscope**
   
   A glass bottom dish was prepared with microdroplets of manipulation medium covered with mineral oil. Between one and two hours after warming, metaphase II (MII) mouse oocytes were placed in the glass bottom dish maintained at 37.3°C. The birefringence signals of the meiotic spindles were analysed using polarAIDETM technology (Vitrolife).

3. **Oocyte fixation and processing for evaluation of the meiotic spindle structure and chromosome distribution by immunofluorescence**
   
   By 2h post-warming, oocytes were fixed and processed for the analysis of the meiotic spindle morphology and chromosome distribution. Triple-labelling protocol was used for the detection of microtubules, microfilaments, and chromatin. Labelled oocytes were examined using an epifluorescence microscope and digital images were acquired. Oocytes that were not subjected to vitrification were processed in parallel following the same protocol and used as controls. Meiotic spindles with a bipolar barrel shape and chromosomes aligned in the metaphase II plate were considered as morphologically normal. Meiotic spindles with an abnormal (elongated bipolar or multipolar) shape and dispersed chromosomes were considered abnormal.
Results

Oocyte survival tests

1. **Morphological evaluation of vitrified oocytes at different time-points after warming**

A total of 32 oocytes were vitrified/warmed following the previously described protocol. During the warming, 30/32 oocytes were retrieved (93.8%). The survival rate of oocytes vitrified with the Supervitri device was 96.7% (Table 1). After warming, major vacuolization did not occur and differences in minor vacuolization rates were not statistically significant (Fisher’s exact test, p = 0.56) (Table 2). Examples of oocytes that lysed or presented minor vacuolization after the warming with the Supervitri are shown in Figure 1.

<table>
<thead>
<tr>
<th>Survival</th>
<th>Processed oocytes n</th>
<th>Retrieval n (%)</th>
<th>Survival n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supervitri</td>
<td>32</td>
<td>30 (93.8)</td>
<td>29 (96.7)</td>
</tr>
</tbody>
</table>

**Table 1.** Retrieval and survival rates after warming with the Supervitri device.

<table>
<thead>
<tr>
<th>Vacuolization</th>
<th>Processed oocytes n</th>
<th>Major n (%)</th>
<th>Minor n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Supervitri</td>
<td>29</td>
<td>0 (0)</td>
<td>4 (13.8)</td>
</tr>
</tbody>
</table>

**Table 2.** Oocyte vacuolization rates observed in the fresh control group and after the warming with the Supervitri device.

**Figure 1.** A) The only oocyte that did not survive after the warming with the Supervitri showing the lysed cytoplasm. B) Example of an oocyte showing minor vacuolization (indicated by red circles) after the warming with the Supervitri device.
Results

2. **Meiotic spindle visualization after oocyte warming using a polarized light microscope**

No differences in spindle birefringence signal were found between the control and Supervitri groups (Fisher’s exact test, p > 0.5) (Table 3). Examples of oocytes with a positive spindle birefringence signal for both control and Supervitri groups are shown in Figure 2.

<table>
<thead>
<tr>
<th>Spindle birefringence</th>
<th>Processed oocytes n</th>
<th>Positive signal n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Supervitri</td>
<td>29</td>
<td>28 (96.6)</td>
</tr>
</tbody>
</table>

**Table 3.** Analysis of the meiotic spindle birefringence in control oocytes and oocytes vitrified with the Supervitri device.

![Figure 2](image-url) Spindle birefringence images of control oocytes (A-B) and Supervitri warmed oocytes (C-D).
Results

3. **Oocyte fixation and processing for evaluation of the meiotic spindle structure and chromosome distribution by immunofluorescence**

A 100% of normal meiotic spindle morphology and chromosome distribution was observed in both groups (Table 4). Examples of normal meiotic spindle morphology and chromosome distribution are shown in Figure 3.

<table>
<thead>
<tr>
<th>Meiotic spindle morphology and chromosome distribution</th>
<th>Processed oocytes n</th>
<th>Meiotic spindle morphology</th>
<th>Chromosome distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal n (%)</td>
<td>Abnormal n (%)</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>10 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Supervitri</td>
<td>29</td>
<td>29 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Table 4. Analysis of the meiotic spindle morphology and chromosome distribution in control oocytes and oocytes vitrified with the Supervitri device.

Figure 3. Immunofluorescence analysis of the morphology of the meiotic spindle and the chromosome distribution in metaphase II mouse oocytes. Control non-vitrified oocytes with a normal barrel shape spindle and the chromosomes aligned in the MII-plate (A-D). Oocytes vitrified with the Supervitri device with a morphologically normal spindle and the chromosomes correctly aligned (E-H). In the merged images of oocytes, microfilaments, microtubules and chromosomes are displayed in red, green and blue, respectively. The raw images of the chromosomes (A’-H’) and meiotic spindle (A”-H”) are displayed on the right side of its complementary image.
Conclusions

Conclusions:
• Oocytes vitrified with the Supervitri device showed very high survival rates.
• Oocyte spindle morphology and chromosome distribution was shown to be normal after vitrification with the Supervitri device.
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BPB MEDICA™ was founded in 1999 by the Bellini family, boasting thirty year's experience in the biomedical sector. The founder, Carlo Bellini Sr., started the business in 1968 and has passed down to his heirs ethics, integrity and spirit of sacrifice. Today BPB MEDICA™ has leveraged its 50 years experience to develop new innovative product lines, growing the company on international level.

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Carlo Bellini Sr's daughter founded Biopsybell srl: "Biopsy" as the initial core business and "Bell" as Bellini family name.

**THE EARTHQUAKE:**
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**THIRD GENERATION OF THE FAMILY:**
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**SURGICAL MASKS:**
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