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Vitrification of male gametes — our experience

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Vitrification (ultrarapid freezing) is routinely used in clinics for assisted reproduction for cryopreservation of oocytes and embryos. In recent years, the possibility to vitrify male gametes is also gaining increasing attention and is widely investigated. Reports of the first babies born after the application of the method have already been published.

Spermatozoa vitrification has a number of advantages, compared to the conventional (slow) freezing. The duration of the procedure is significantly reduced. The use of penetrating cryoprotectants, which are toxic to the gametes, is avoided. The method allows the cryopreservation of semen with deteriorating parameters, as well as single spermatozoa, obtained after testicular puncture.

In the current investigation we have used human semen processed with microfluidic technology (ZyMot sperm preparation device) to select high motility spermatozoa. The effect of 2 different cryopreservation methods (conventional freezing and vitrification) on the viability and motility of the male gametes was studied.

The functional parameters of the spermatozoa have been assessed using the following methods:

- Motility and longevity (CASA)
- DNA Fragmentation (SCD — test)
- Apoptosis (Flow cytometry analysis with Annexin V Apoptosis Detection Kit)
- HBA — assay
- ICSI and embryo culture (including embryo quality assessment according to the ALPHA guidelines)

Our investigations show that after virtification the spermatozoa retain higher motility and lower level of apoptosis and DNA-fragmentation, compared to cryopreservation with conventional freezing methods (on the liquid nitrogen vapour). Good quality embryos were obtained after ICSI with vitrified spermatozoa and clinical pregnancies have been registered. The method is introduced into clinical practice in InVitro OG Medical Centre "Dimitrov".

The vitrification of human spermatozoa can be viewed as a reliable method for cryopreservation and can be introduced to routine use in clinical practice.

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Enhancing seed supply in mussel aquaculture: a cryopreservation study using two cooling systems

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The mediterranean mussel, *Mytilus galloprovincialis,* is one of the principal aquaculture species cultivated in Spain, with the majority of its production located in Galicia (NW-Spain). In this region, juvenile mussels (seed) are traditionally collected from natural sources. However, in recent years, seed availability has declined, likely due to increasing variability in the upwelling system, climate change and the introduction of invasive species. Additionally, the practice of juvenile harvesting has led to conflicts with other fisheries, such as the goose barnacle industry.

Cryopreservation of D-larvae presents a promising solution for stabilizing seed availability in mussel aquaculture, offering a consistent high seed production at convenient times for the sector. Despite cryopreservation protocols for mussel cryopreservation are very advanced, the volume that can be cryopreserved in a laboratory setting can not sustain industrial hatchery operations. Expanding cryopreservation capacity remains a challenge, and a logical first step involves comparing the performance of different freezers with larger capacity. The goal was to assess differences in cryopreservation efficiency and determine whether adjustments to the current protocol are necessary when scaling to larger novel freezers before even considering larger volumes.

The experimental protocol follows the methodology outlined by Heres *et al.* (2022). D-larvae at 72 hours post-fertilization were selected, concentrated, and mixed at a 1 : 1 ratio with a cryoprotectant solution containing 10% ethylene glycol and 0.4 M trehalose. After a one-hour equilibration period, the larvae were placed in straws and loaded into the respective freezers, where they were cooled at a controlled rate of –1 °C per minute. Upon reaching –35 °C, the straws were plunged into liquid nitrogen and stored until thawing. Thawing was performed by immersing the straws in a 35 °C water bath for six seconds.

The experiment was conducted five times to assess the success and interindividual variability and we have determined some variation in the fitness of the produced larvae based on the freezer used (affecting mostly the normal morphology of the surviving larvae $p < 0.05$), we suggest some protocol modifications moving forward. Post-thaw analyses included assessments of larval normality and larval length to evaluate the effectiveness of the cryopreservation process.

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