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First successful vitrification of salmonid ovarian tissue

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ABSTRACT

Due to a lack of cryopreservation protocols for fish eggs and embryos, alternative techniques which will enable storage of female genetic resources are crucial for future development of reproduction management in conservation biology and aquaculture. Experiments were conducted to develop an optimal vitrification protocol for cryopreservation of brown trout *Salmo trutta* juvenile ovarian tissue. Needle immersed vitrification (NIV) method was used where ovaries were pinned on an acupuncture needle, passaged through equilibration and vitrification solutions containing different combinations and concentrations of methanol (MeOH), propylene glycol (PG) and dimethyl sulfoxide (Me_2SO) and subsequently plunged into liquid nitrogen. Vitrification solutions containing equal cryoprotectant concentrations (3M Me_2SO and 3M PG) yielded the highest oogonia survival rates (up to 40%) and qualitatively and quantitatively unaltered perinucleolar follicles. The method developed for brown trout could be applied to the conservation of female genetic resources of other salmonid species, including endangered and endemic species or populations.

Creation of viable cryobanks of gametes, pluripotent cells (primordial germ cells, spermatogonia, oogonia etc.) and gonadal or somatic tissues presents a valuable tool in medicine, biology (especially conservation biology) and agriculture for saving valuable genetic resources [10]. Ovarian tissue banking is a novel strategy for the preservation of female genetic material for its further use through transplantation or grafting procedures. In medical practice, usage of cryopreserved gonadal tissue often presents the only opportunity to sustain reproductive capability of patients with reproductive dysfunction [9]. From the point of view of conservation biology and aquaculture, it is a very promising and important method since an effective method for cryopreservation of fish oocytes or embryos still does not exist [10].

Slow-rate freezing is currently a standard procedure for the purpose of cryopreservation of ovarian tissue in medicine. However, due to several downsides of this technique, vitrification is starting to attract more attention in the recent years. During vitrification, liquid (or tissue) solidifies into an amorphous or glassy state without the formation of ice crystals [2]. This is achieved by using ultra-fast cooling and warming rates and by using high cryoprotectant concentrations. Successful vitrification of ovarian tissue has been evidenced in avian and mammalian species [5,9], thus opening possibilities for its application in fish, as well. To the best of our knowledge there is only one report on preservation of fish (*Danio rerio*) ovarian tissue using vitrification approach available [6], suggesting that ovarian tissue vitrification might be a promising technology to preserve the maternal genome in fish. The aim of this study was to develop a suitable protocol for vitrification of salmonid ovarian tissue which will result in viable cells after warming. As a model organism, brown trout *Salmo trutta* was used as a representative of the salmonid family. The developed protocol could then be applied to other endemic and endangered salmonid species for conservation efforts or aquaculture needs.

Brown trout (*Salmo trutta*) females were sampled at Bled fish farm (Slovenia) during September 2016. A total of ten fish at age of 1+ (SL: 245.1 ± 21.3 mm, W: 165.3 ± 40.7 g) were used. Fish were overdosed in the solution of 2-phenoxyethanol (Acros Organics) and dissected for gonadal sampling. Gonads were kept in Leibovitz L-15 media (Sigma-Aldrich) supplemented with 10% FBS (Sigma-

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Aldrich) on ice during the sampling procedure and transportation to the laboratory (maximum 1 h). Before the experiments, each gonad was checked under a stereomicroscope in order to verify sex, cleaned of large blood vessels and connective tissue and cut into several pieces (aiming to have pieces of ~30 mg). Tissue pieces were then pooled and three replicates with two additional pieces for histological examination were used for each test group.

The experiment included nine test groups with three equilibration (E1 – E3) and three vitrification solutions (V1 – V3). They were chosen according to Marques et al. [6] with minor modifications, and each of them containing different combinations and concentrations of methanol (MeOH), propylene glycol (PG) and dimethyl sulfoxide (Me_2SO) (Table 1). Extender consisted of L-15 media supplemented by 10% FBS, 25 mM HEPES and 0.5 M trehalose.

A needle immersed vitrification (NIV) method [9] was used where a total of five pieces of gonads per experimental group were pinned on an acupuncture needle and incubated in each equilibration solution for 15 min and in each vitrification solution for 90 s at 23 °C. After the last incubation, the remaining solution was absorbed from the tissue by a sterile paper towel and needles were plunged into liquid nitrogen (LN_2). After 10 min in LN_2, each needle was placed into a separate precooled cryotube (4.5 ml) and stored in a storage tank until further examination (at least 2 days). Each sample was warmed separately by plugging into three warming solutions (W1: L15 + 10% FBS + 0.3 M trehalose; W2: L15+ 10% FBS + 0.1 M trehalose; W3: L15 + 10% FBS) at 23 °C. Samples were serially transferred into W1 for 1 min, then into W2 for 3 min, and then into W3 for 5 min.

Each tissue sample was weighed before the dissociation process, placed in an Eppendorf tube filled with dissociating solution (L-15 media + 10% FBS + 2 mg/ml collagenase [Sigma-Aldrich] + 10 μg/ml DNase [Panreac AppliChem]), cut into small pieces and digested for 60 min on a shaking plate at 23 °C. Three fresh (control) pieces were also weighed and digested in the same manner. After incubation, cell suspensions were filtered through 50 μm filters and centrifuged at 200×*g* for 10 min at 10 °C. Pellets were resuspended in 20 μl of L-15 supplemented with 10% FBS and prepared for viability analysis. Among the female germ-lineage cells only oogonia had a diameter small enough to pass through the 50 μm mesh-size filters, therefore, only oogonia were analyzed by trypan blue live/dead staining. Prepared cell suspensions were mixed with 0.4% trypan blue (TB) in the ratio of 1:1 and incubated for 3 min at 23 °C. Each sample was analyzed in a Bürker-Türk type hemocytometer under the Nikon TE2000/U inverted microscope. The percentage of viable cells was calculated compared to the equivalent of control tissue weight of each sample as:

$$Viability\ (\%) = \left(N_{cryopreserved} \Big/ N_{fresh}\right) \times CF \times 100$$

where $N_{cryopreserved}$ is the number of TB- cells isolated from the cryopreserved tissue, N_{fresh} is the number of TB- cells isolated from the fresh tissue and *CF* is the correction factor calculated as:

$$CF = Weight_{fresh\ tissue} \Big/ Weight_{cryopreserved\ tissue}.$$

For histological analysis, fresh as well as vitrified/warmed ovarian fragments (two from each test group) were fixed in 8% formalin and stored at 4 °C until further work. Tissue pieces were processed in a series of ethanol and xylene and embedded into paraffin blocks. Each block was cut into 3 μm thick sections with a 70-μm distance between two sections. Slides were stained with the standard hematoxylin/eosin staining procedure. Sections were analyzed under a Nikon Eclipse 600 microscope and photographed using a QImaging Micro Publisher 3.0 digital camera. Follicles were determined according to Bromage and Cumaranatunga [1], and measurements of diameter and area of the follicles were taken by the open-source software ImageJ (https://imagej.nih.gov/ij/). For the sake of simplification of calculations, all follicles were assumed as spherical and their volume was calculated according to the following formula:

$$V = (4/3)r^3\pi$$

where *r* is the follicular radius. During qualitative histological analysis, two types of perinucleolar follicles were determined: early and late. Since we aimed to assess possible differences in follicle sizes between the tested vitrification groups, we employed an objective statistical analysis to firstly classify follicles from all experimental groups into early or late according to their size. Follicle measurements were ln-transformed and individual follicles were assigned to specific perinucleolar follicle subpopulation based on Euclidean distances and UPGMA. Follicles close to each other were assigned to the same subpopulation, whereas distant follicles were assigned to different subpopulations.

All values are presented as mean ± SD. Percentage data were arcsine-transformed prior to statistical analysis. One-way ANOVA followed by Tukey's HSD was used to determine the differences in viability data, morphological normality and stereological measurements between the tested vitrification groups. All statistical analyses were conducted in STATISTICA v12 software (StatSoft Inc., Tulsa, OK, USA).

Among the female germ-lineage only oogonia were present in the cells suspension, followed by a large number of erythrocytes (Fig. 1A). Vitrification process affected the ovarian tissue since after digestion erythrocytes were present in a much lower number (Fig. 1B), indicating they did not survive the process. The average number of oogonia isolated from 30 mg of fresh ovarian tissue was 93720 ± 8763. The vitrification process also had a significant effect on oogonia viability ($F_{(8,18)} = 7.17$, $p < 0.01$) and the highest number of viable cells was observed in the test groups E2V3 (40.34%) and E1V3 (37.98%) (Fig. 1C). This indicates that a 1:1 cryoprotectant ratio in VS is favorable, and that high concentrations of 4.5 and 5.5 M could be toxic for cells.

Approximately 2000 primordial follicles in the perinucleolar stage were histologically analyzed. The overall histological structure of ovarian tissue was normal in all test groups (Fig. 1D and E). Notable alterations in follicle structure observed after vitrification included slight segregation of the basal lamina or its indentations (Fig. 1E). The percentage of morphologically normal follicles was up to 88.32% in test group E2V1 (Fig. 1F). However, test groups E1V1, E1V2, E2V3, E3V1 and E3V2 had a significantly lower number of intact follicles compared to the control (one-way ANOVA, $F_{(9,87)} = 15.87$, $p < 0.01$; Fig. 1F). Similarly to the viability results for oogonia, groups with a cryoprotectant ratio of 1:1 had higher percentages of morphologically normal follicles (excluding group E2V3) indicating that similar protocols can yield favorable viability

Table 1
Test groups for the vitrification of brown trout ovaries with three equilibration (E1 – E3) and three vitrification solutions (V1 – V3) containing different combinations and concentrations of methanol (MeOH), propylene glycol (PG) and dimethyl sulfoxide (Me_2SO).

	Equilibration solution			Vitrification solution		
	E1	E2	E3	V1	V2	V3
MeOH	1.5 M	1.5 M	–	1.5 M	1.5 M	–
PG	1.5 M	–	1.5 M	4.5 M	–	3 M
Me_2SO	–	1.5 M	1.5 M	–	5.5 M	3 M