HYDROGEL ENCAPSULATION AS A HANDLING AND VITRIFICATION TOOL FOR ZEBRAFISH OVARIAN TISSUE

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Abstract

Zebrafish is an important animal model, thousands lines have been developed, thus having a great need for their preservation. However, the cryopreservation of fish oocytes is still limited and needs improvement. The sodium alginate hydrogel, in addition to providing support for the cells, has been shown to be a potential cryoprotectant. Therefore, the aim of this study was to evaluate the sodium alginate hydrogel encapsulation technique efficiency during zebrafish ovarian tissue vitrification. The encapsulation methodology was standardized in the first experiment. In Experiment 2, we evaluated four vitrified groups: standard protocol without encapsulation (VS); encapsulated with cryoprotectants (VS1-A); encapsulated with half the cryoprotectants concentration (VS2-A); encapsulated without cryoprotectants (VA). VS treatment (54.6 ± 12.3%; 23.7 ± 9.9%; 12.6 ± 5.0%) did not differ from the VS1-A and VA showed a lower membrane integrity percentage (1.2 ± 1.4%; 0.3 ± 0.6%; 0.5 ± 1.5%). Mitochondrial activity was significantly greater in non-encapsulated treatment (VS) when compared to the encapsulated treatments. VS1-A and VS obtained the lowest lipid peroxidation (39.4 ± 4.4 and 40.5 ± 3.3 nmol MDA/mg respectively) in which VS was not significantly different from the VS2-A treatment (63.6 ± 3.1 nmol MDA/mg), unlike, VA obtained the highest lipid peroxidation level (124.7 ± 7.9 nmol MDA/mg). The results obtained in this study demonstrate that the sodium alginate hydrogel encapsulation technique did not have a cryoprotective action, but maintained the membrane integrity when used the standard concentration of cryoprotectants. However, halving the cryoprotectant concentration of fragments encapsulated in alginate hydrogel did not cause an increase in lipid peroxidation. In addition, it provided support and prevented the oocytes from loosening from the

tissue during the vitrification process, being an interesting alternative for later *in vitro* maturation.

Keywords: Sodium alginate, *Danio rerio,* fish oocyte, cryopreservation, cell support.

1. Introduction

Thousands of zebrafish lines, including wild-type, mutant, and transgenic strains are available in laboratories around the world for research [1]. The preservation of these valuable genotypes is particularly important and presents significant and urgent challenges. To meet this demand, gamete cryopreservation has proven to be an efficient tool. Sperm cryopreservation protocols have been widely studied for many different fish species, making the cryopreservation of male gametes well established [2]. However, sperm cryopreservation only preserves the paternal genome, which is not enough to maintain genetic diversity [3]. Cryopreservation of embryos and mature oocytes is very challenging due to their complex structure, large size and amounts of yolk material, high chilling sensitivity, and poor membrane permeability to solutes [4]. Recently, in a vitrification study of zebrafish embryos, it was possible to obtain some viable larvae that grew to adulthood and spawned [5]. However, it is a technique that requires the use of sophisticated equipment and gold nanorods, and is not very accessible. Thus, more research is needed on the preservation of female genetic material.

Previous studies have shown that the cryopreservation of ovarian tissue fragments is a promising alternative for the conservation of fish immature oocytes [6–8] and oogonia [9-12], allowing female genetic preservation. The main advantage for ovarian tissue cryopreservation to an embryo and mature oocytes, is

the greater permeability of the immature oocyte membrane to cryoprotectants [4]. However, to fertilize early-stage follicles, *in vitro* or *in vivo* maturation would be required. *In vitro* maturation is a very interesting and promising alternative, as it is capable of generating offspring without the need for a live animal. Studies with zebrafish vitellogenic oocytes maturation *in vitro* had the ability of to develop into hatching embryos [13] and live larvae [14]. Thus, cryopreservation of oocytes in primary growth, cortical alveolar and mainly vitellogenic stages instead of oogonia is a better alternative for further maturation *in vitro,* as there is no protocol for oogonia *in vitro* maturation.

In order to avoid cell damage on zebrafish ovarian tissue, the vitrification proved to be more efficient than slow freezing, especially in preventing mitochondrial damage and oxidative stress [8]. However, the vitrification method involves ultra-fast cooling, which prevents ice crystals formation, and the use of high concentrations of cryoprotective agents (CPAs) [15]. The use of high concentrations of cryoprotectants can be a problem, as cryoprotectants can be cytotoxic, depending on the concentration, and may cause disruption or damage to cell membranes, reduced mitochondrial function, damage to proteins or other macromolecules, among other toxic effects [16].

To promote greater protection in relation to the damages caused by the cryopreservation procedures to cells and tissues, cell encapsulation in hydrogel biomaterials has been shown to be able to reduce the usage of high CPAs concentration without compromising the vitrification outcomes [17]. Sodium alginate is an anionic polysaccharide produced by brown seaweed, which in contact with calcium ions it crosslinked, forming the alginate hydrogel. The main advantages of alginate hydrogel are biodegradability, biocompatibility, non-

toxicity and capacity of absorbing large amount of water [18], in addition to present antioxidant properties [19]. Alginate ionic crosslinking has advantages, such as the low amount of salt required to form the gel and the control of crosslinking by the concentration of alginate [20]. The gel-like structure within the alginate hydrogel produces in the encapsulated cells creates an environment similar to an extracellular matrix (ECM) [21]. Alginate hydrogel encapsulation, maintains cell structure *in vitro* that morphologically resembles *in vivo* ambient, with improved porosity, cell proliferation and mechanical strength [22]. Alginate hydrogel is usually used in tissue engineering [23], cell culture structure [24] and cell/tissue transplantation [25].

Studies using alginate hydrogel for cryopreservation of mammalian cells, have demonstrated promising results. A study with vitrification of encapsulated murine embryonic stem cells and human adipose-derived stem cells in alginate hydrogel showed 80% membrane integrity using 1.5 M 1,2-propanediol (PROH) and 0.5 M trehalose (approximately 4-times lower concentration than normal concentrations used for vitrification) and encapsulated cells had a major impact on inhibiting intracellular ice formation during warming [26]. The slow freezing of human fibroblasts encapsulated in alginate hydrogel in the absence of CPAs showed a metabolic activity similar to that of cryopreserved fibroblasts with Me_2SO and trehalose CPAs [27]. These results evidence that cell encapsulation in alginate hydrogel may be an alternative to replace or reduce the CPAs in cryopreservation process. However, there has been no study using the crosslinked alginate encapsulation in cryopreserved fish cells.

Therefore, the aim of this study was to evaluate the sodium alginate hydrogel encapsulation technique efficiency on vitrification of zebrafish ovarian tissue fragments.

2. Materials and methods

The study was conducted in accordance with the National Council for Control and Animal Experimentation (CONCEA) and approved by the Ethics Committee of the Federal University of Rio Grande do Sul. Project number: 38864

2.1. Fish care and sample collection

Female wild-type zebrafish age 1 - 2 years old, average weight 1.49 ± 0.39 g, were maintained in filtered and aerated 40 L aquariums in stock density of 7 fish / L, at 27 °C ± 2 °C, under a photoperiod 14:10 h (light/dark), pH between 7.2± 2, zero levels of toxic ammonia and nitrite (Alcon Labcon® Toxic Ammonia and Nitrite test Kit). These animals were not in breeding routine. Fish were fed with TetraMin® dry flake fish food (Tetra, Germany) three times a day until apparent satiety.

After acclimatation, 28 females were euthanized with a lethal dose of buffered tricaine methane sulfonate solution (0.6 mg/mL, pH 7.4, 22 ºC) [28], followed by decapitation. A ventro longitudinal section in the animals' abdomen was performed to access and collect their ovaries. After collection, the ovaries were weighted, 0.249 ± 0.103 g on average. Fragmentation was performed in a petri dish with markings on its base in a grid format containing 3x3 mm squares (Fig 1a), where the tissue was sized and cut with the aid of a needle and bistoury, so that all fragments were the same size (3x3 mm) (Fig 1b). The fragments were placed in

90% Leibovitz L-15 medium diluted in distilled water (pH 9.0) and randomly distributed among treatments.

2.2. Experimental design

This study was divided in two experiments. The Experiment 1 aimed to define the best encapsulation technique for the zebrafish ovarian tissue in alginate hydrogel. Parameters investigated were type of hydrogel encapsulation, time of CPA exposure, and warming temperature. For the type of encapsulation two groups were tested, in group 1 the fragments were encapsulated in 30 µL of sodium alginate hydrogel (Fig 2a) and in group 2 the fragments were encapsulated by immersion in alginate (Fig 2b). The time of exposure to cryoprotectants was tested before or after encapsulation, and different warming temperatures investigated were 28, 37 and 50 °C (Fig 3). The definition of each warming temperature was based on previous protocols. The warming temperature of 28 °C was according to vitrification protocol of zebrafish ovarian tissue [8]; 37 °C warming temperature was according to cryopreservation protocols with alginate hydrogel encapsulated mammalian cells [29,30]; and 50 °C warming temperature, in a previous test, was the temperature that defrosted the 30µl alginate bead in 60s.

Ovarian tissue fragments from nine females were randomly distributed into one fresh control and 12 vitrified treatment groups (VA1-VA12) combining the parameters described above (Fig 3). Eight replicates were performed for each group (vitrified treatments and control/fresh ovarian tissue) and the membrane integrity analyzed by trypan blue staining was the main parameter evaluated. The group that presented oocytes with the highest percentage of membrane integrity was used for the next experiment.

The aim of Experiment 2 was to evaluate the alginate hydrogel as a cryoprotectant or as auxiliary cryoprotectant along with traditional cryoprotectants. Ovarian tissue fragments from 19 females were randomly distributed into one fresh control and 4 vitrified treatment groups: VS: Standard protocol without encapsulating the fragments (1.5M Methanol + 5.5M Me_2SO + 0.5M sucrose); VS1-A: Fragments encapsulated in alginate and exposed to the same concentration of cryoprotectants as the standard protocol (1.5M Methanol + 5.5M Me_2SO + 0.5M sucrose – encapsulated in alginate); VS2-A: Fragments encapsulated in alginate and exposed to half the concentration of cryoprotectants than the standard protocol (0.75M Methanol + 2.75M Me_2SO + 0.25M sucrose – encapsulated in alginate); VA: Fragments encapsulated in alginate and vitrified without any other cryoprotectant (Fig 3). Membrane integrity were analyzed by SYBR-14 and propidium iodide (SYBR-14/PI), morphology (membrane, cytoplasm and nucleus) by histology with hematoxylin and eosin stain, lipid peroxidation by thiobarbituric acid reactive substances (TBARS) and mitochondrial activity by MTT assay, that was analyzed immediately after warming and 120 min after in 90% Leibovitz L-15 medium. All the parameters evaluated were validated for fish oocytes. Eight replicates were performed for each analysis.

2.3. Ovarian tissue encapsulation in alginate hydrogel

The sodium alginate solution was prepared at 2% concentration in 90% Leibovitz L-15 medium (pH 9), the solutions were kept under agitation until complete solubilization at room temperature (22 °C). For encapsulation in 30 µL of sodium alginate (VA1 to VA6 groups) the tissue fragments were placed in a 1/8 plastic teaspoon, and 30 µL of alginate solution was added then, for the crosslinking

199 the spoon was immersed in 4% calcium chloride solution for 60 s (22 °C), forming
200 a bead (Fig 4a). For encapsulation by immersion in alginate (VA7 to VA12 groups),
201 the fragments were immersed in sodium alginate solution with the aid of a 1ml
202 syringe with curved needle and then immersed in 4% calcium chloride solution (22
203 °C), forming a thin film of sodium alginate hydrogel around the ovarian tissue
204 fragment (Fig 4b).

205 *2.4. Vitrification and warming procedure*

206 Ovarian tissue fragments were carefully transferred with the aid of a curved
207 needle into a cryotube containing 300 µL of equilibrium solution (ES1: 1.5 M
208 methanol + 2.75 M Me_2SO - vitrified treatments of Experiment 1 and VS, VS1-A
209 of Experiment 2; or ES2: 0.75 M methanol + 1.375 M Me_2SO - VS2-A treatment)
210 for 7 min (4°C). Then, the samples were exposed to 300 µL of vitrification solution
211 for 90 s (1.5 M methanol + 5.5 M Me_2SO - vitrified treatments of Experiment 1 and
212 VS, VS1-A of Experiment 2; or 0.75 M methanol + 2.75 M Me_2SO - VS2-A
213 treatment). After that time, the vitrification solution was removed, the samples with
214 alginate were encapsulated and the cryotubes were directly plunged at the liquid
215 nitrogen. The protocol and cryoprotectants used on the first experiment, VS and
216 VS1-A treatments were according to Marques et al. [8], with modifications. After
217 7 days, the cryotubes were warmed in a water bath for 60 s at 28, 37 or 50 °C on
218 the first experiment and for 60 s at 28 °C in Experiment 2, then were exposed to the
219 to the first warming solution containing 1 M sucrose, to a second solution
220 containing 0.5 M sucrose for 3 min, and finally to a third solution of 0.25 M sucrose
221 for 5 min. The samples were washed three times in 90% L-15 medium (pH 9.0, 22
222 °C) and then primary growth (PG), cortical alveolar (CA) and primary vitellogenic

(Vtg1) oocytes - according to Brown-Peterson et al. [31] classification - were analyzed using the following methodologies. Results of oocytes at more advanced stages than Vtg1 were not demonstrated because they were all with damaged membranes.

2.5. Membrane integrity assays

Trypan blue is a dye exclusion method based on the principle that cells with damaged membranes are dead, in which they are stained in blue. The oocytes were gently separated using syringe needles in 90% Leibovitz L-15 medium, were incubated in 0.4% trypan blue solution for 3 min and then washed three times in 90% L-15 medium. The fragments were observed under light microscope (Nikon Eclipse E200, Tokyo, Japan 40× objective lens).

SYBR-14/PI staining is a fluorometric method to assess membrane integrity, which SYBR-14 is an inclusion dye and PI is an exclusion dye. SYBR-14 passes through plasma membranes and loses the acyl group and binds to the double stranded DNA of living cells, and emits green fluorescent light. In contrast, PI pass through cell damage membrane. Thus, intact membrane cells are bright green stained with SYBR-14 and membrane damage cells are bright red stained with PI . The fragments were incubated with 100 µL of PBS with 4 µL of SYBR-14 (0.02mM) for 4 min and 1.5 µL of PI (4.8mM) for 1 min in the dark at room temperature (22 °C) and analyzed under fluorescence microscope (Carl Zeiss, AxioVert, Germany, 20× objective lens). To analyze, a picture of the same field was taken with two different excitation filters, ex 490 nm for SYBR-14 and ex 545 nm for PI. Cells stained with SYBR-14, but which were also stained with propidium iodide, were considered to have ruptured membrane and cells stained

with SYBR-14 and which were not stained with PI, were considered to have intact membrane.

The ovarian tissue pieces were flattened and stretched until all oocytes were clearly visible, before stain (Tripan blue or SYBR-14/PI). Then, percentage of membrane integrity of all oocytes of each phase (PG, CA and Vtg1) contained in whole fragment was calculated as follows: (Membrane integrity (%) = [Number membrane damage oocytes/Total number of oocytes] × 100). To classify the stages of the oocytes after staining, the imageJ software was used to measure the diameter of the cells. In which were classified into PG (7–140 μm diameter), CA (140–340 μm diameter) and Vtg1 (340–690 μm diameter) [32]

2.6. Histological analysis

Ovarian tissue fragments were fixed in Karnovsky's solution (2% paraformaldehyde + 2.5% glutaraldehyde + phosphate buffer solution, pH 7.5 – 7.8) for 24 h, then were dehydrated in an increasing series of alcohol concentration (70, 80 and 95%) embedded in Leica historesin (methacrylate glycol) and sectioned at 3 μm on a Leica RM2245 microtome with glass knives and stained with Hematoxylin and Eosin (HE). Morphological integrity of oocytes (phase PG, CA, Vtg1, Vtg2 and Vtg3) were evaluated with a light microscope (Nikon Eclipse E200, Tokyo, Japan 10x and 40× objective lens). All oocytes visible on the histological slides were morphologically evaluated (membranes, cytoplasm and nucleus). As the fragments were small, it was not possible to evaluate quantitatively, as few cells were visible. Therefore, evaluation of the fresh/control group and the vitrified ovarian tissue was descriptive.

2.7. Mitochondrial activity

Mitochondrial activity was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay [33], based on the action of the mitochondrial enzyme succinyl dehydrogenase, that promotes the reduction of MTT to formazan crystals, which is active only in living cells. MTT was evaluated immediately after ovarian tissue fragments warming and 120 min later - incubated in 90% L-15 medium pH 9.0 at 28 °C. The ovarian tissue fragments were incubated in 400 µL of MTT (5mg/mL) for 120 min at 28 °C. The supernatant was carefully removed and then, dimethyl sulfoxide (400µL) was added to solubilize the formazan crystals, resulting in a purple solution. Next, 100µL of the colored solution was transferred in 96 well microplate and the absorbance were read at 570nm on a SpectraMax® 250 Microplate Spectrophotometer. Each sample was analyzed in triplicate.

2.8. Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) levels are indicative of lipid peroxidation, which occurs in the presence of reactive oxygen species (ROS). The ovarian tissue fragment was homogenized in cold (4 °C) 150µL of phosphate buffered saline solution (PBS), pH 7.4 and centrifuged for 10 min at 3000 x g [34]. The supernatant was collected and used to performed the assays. First the protein content was quantified according to the Coomassie blue method and the sample volume that corresponds 50 µg of proteins was calculated [35]. Then, the samples were incubated with trichloroacetic acid (TCA) 20% + thiobarbituric acid (TBA) 0.5% (150 µL) at 100 ºC for 30 minutes. The species reactive to thiobarbituric acid (TBARS) were determined by spectrophotometry at 532 nm as described by Sachett et al. [36]. MDA (2 mM) was used as the standard. Lipid peroxidation was expressed by nmol MDA / mg of protein.

2.9. Statistical analysis

All data are presented as mean values ± standard error (mean ± SD). Homogeneity (Levene's test) and normality (Shapiro-Wilk, Kolmogorov-Smirnov, and D'Agostino & Pearson) tests were performed. When necessary, the data were transformed (LOG) and the outliers were excluded. For Experiment 1, in the evaluation of membrane integrity, the data were analyzed using three-way analysis of variance (Three-Way ANOVA), considering the effect of the alginate encapsulation, the moment of exposure to CPAs, the heating temperature of the samples, and the interaction between these effects. When a significant effect was observed for one of the factors, the groups were compared using the Tukey or Bonferroni test. For Experiment 2, the membrane integrity data (SYBR-14/PI) and TBARS, were analyzed using Kruskal-Wallis analysis, followed by Dunn's test. The MTT test was analyzed by means of two-way analysis of variance (Two-Way ANOVA), considering the experimental groups (cryopreservation protocols), the timing of the MTT test (immediately and 120 minutes later), and the interaction between these factors. When a significant effect was observed for one of the factors, the groups were compared using the Tukey or Bonferroni test. The data analyzed by ANOVA are presented in bar graphs (mean and standard deviation) and the data analyzed by Kruskal-Wallis analysis are presented in Box and Whiskers type graphs (Median, maximum, and minimum). Analyzes were performed using Statistical Analysis System v.9.4 (SAS) software and GraphPad Prism 7.0.

3. Results

3.1. Experiment 1 - Standardization of ovarian tissue encapsulation

All parameters evaluated, encapsulation form, moment of exposure to CPA, and warming temperature had a significant effect on PG, CA and Vtg oocytes membrane integrity after warming for determination of the best protocol (Fig. 5a-d). The ovarian tissue encapsulated in 30 μL bead and exposed to the CPA after encapsulation resulted in greater damage to PG, CA, and Vtg1 oocytes membranes. The fragments encapsulated before or after cryoprotectant exposure, showed higher PG, CA and Vtg1 oocyte membrane integrity when warmed at 28 °C. Vtg1 oocyte membrane integrity was best preserved by immersion encapsulation combined to the CPA exposition before the encapsulation and warmed at 28 °C (VA7: 18.6 ± 4.7%) and PG (VA7: 37.7 ± 3.7%) and CA (VA7: 29.9 ± 4.2%). Therefore, the encapsulation form by immersion in alginate, the exposure to CPA before encapsulation and the 28 °C warming temperature (VA7) were chosen as the best protocol, and it was used for the following experiment.

3.2. Experiment 2

The membrane integrity evaluation by SYBR-14/PI stain for PG and CA oocytes was significantly higher in the VS experimental group (PG: 54.6 ± 12.3%; CA: 23.7 ± 9.9%), compared to the VS2-A (PG: 16.3 ± 5.5 %; CA: 5.4 ± 4.2%) and VA groups (PG: 1.2 ± 1.4%; CA: 0.3 ± 0.6%) (Fig. 6a-e). The VS1-A experimental group (PG: 33.0 ± 5.5%; CA: 17.7 ± 8.3%) differed only from the VA group. For oocytes in Vtg1 stage, the highest membrane integrity were observed in the VS (12.6 ± 5.1%) and VS1-A groups (12.8 ± 7.9%), which differed statistically from the other groups.

The histological analysis showed in all vitrified treatments (VS, VS1-A, VS2-A and VA) that CA and vitellogenics oocytes (Vtg1, Vtg2 and Vtg3) presented

cytoplasmic alterations, with apparent rupture of the cortical alveoli and nucleus degeneration (Fig. 7 e, h, k, n). However, no membrane damage was observed in vitellogenics oocytes. On the other hand, in PG vitrified oocytes membrane damage, nucleus fragmentation and the formation of vacuoles in the cytoplasm were observed (Fig. 7 f, i, l, o).

Mitochondrial activity by MTT assay showed no effect alteration for fragments analyzed immediately or 120 min after warming ($p = 0.2262$) (Fig. 8a). Samples analyzed immediately after warming, showed greater mitochondrial activity in the VA (43.4 ± 17.9 AU/g) and VS groups (36.4 ± 15.5 AU/g), which differed statistically from the VS1-A group (14.3 ± 5.1 AU/g). For the analyzed samples 120 min after warming, we observed greater mitochondrial activity in the VS groups (62.2 ± 28.6 AU/g), which differed from the other groups.

Lipid peroxidation by TBARS assay levels was higher in VA group (124.7 ± 7.9 nmol MDA/mg) and did not differ from VS2-A group (63.6 ± 3.1 nmol MDA/mg). The lowest lipid peroxidation level was in VS1-A group (39.4 ± 4.4 nmol MDA/mg) that did not differ from VS group (40.5 ± 3.3 nmol MDA/mg) (Fig. 8b).

4. Discussion

The preservation of maternal genetic material in fish has several limitations, since it is not possible to cryopreserve mature oocytes. Thus, cryopreservation of fish ovarian tissue has been shown to be a promising alternative, as it preserves immature oocytes. In this study, we evaluated the effect of encapsulating zebrafish ovarian tissue in alginate hydrogel on vitrification, providing the first information on this technique for fish ovarian tissue.

The initial challenge to propose a protocol using sodium alginate hydrogel was to build an enclosure that would maintain the best viability of the biological material (ovarian tissue), to be vitrified with cryoprotective solutions in a second step. In the literature, we did not find references regarding the encapsulation of a tissue (set of cells), but only suspension of isolated cells [27,37]. Thus, we started from numerous tests and developed two forms of encapsulation of ovarian tissue, in 30 µL alginate bead or by direct immersion in sodium alginate hydrogel. Two other important aspects that we had to take into account were the moment of exposure of the samples to CPA eg before or after of sodium alginate hydrogel encapsulation and the optimum temperature for warming the samples. From this, the treatment of ovarian tissue exposed to CPA prior to direct immersion in sodium alginate hydrogel and heated to 28 °C (VS7), demonstrated greater membrane integrity in the three stages of development. We have therefore concluded that the combination of the three parameters considered to compose the VS7 treatment, produced the best protocol in the use of sodium alginate hydrogel to cryopreserve the fish ovarian tissue. Therefore, the hypothesis of delayed penetration of CPA into the cell, due to the greater thickness of the walls of the envelope when the samples were encapsulated in 30 µL alginate hydrogel bead, seemed to us to be the most plausible. According to Rall [38], insufficient permeation of cryoprotectants can affect the survival of the oocyte which can also affect a set of cells (ovarian tissue), was demonstrated in our experiments. In addition, previous studies have reported that alginate hydrogel granules with a larger diameter, resulted in minor membrane integrity of isolated mouse Leydig cells [37]. According to these authors, larger granules have a small proportion between surface and volume and difficult temperature transfer with liquid nitrogen [37].

Finally, the temperature around 28 °C considered ideal for zebrafish maintenance [39] and it is the recommended temperature for warming fragments of zebrafish ovarian tissue following vitrification [8], which is in fact, the most appropriate for the proposed protocol. When the 30 µL alginate bead was warmed, we noticed that the bead took time to thaw, so we tested higher temperatures (37 and 50 °C), however, these warming temperatures did not produce good results in ovarian tissue encapsulated by immersion in alginate hydrogel. In addition, in the present study, when the ovarian tissue was exposed to cryoprotectants before being encapsulated by immersion in alginate hydrogel and warmed to 28 °C (VA7), the membrane damage of the Vtg1 and CA oocytes was significantly lower when compared to the treatment exposed to CPA after encapsulation, at the same encapsulation form and warming temperature (VA10). On the other hand, the integrity of the PG oocyte membrane did not differ in both moments of exposure to cryoprotectants. During maturation, fish oocytes become less permeable [40], which was demonstrated as the percentage of membrane integrity of the most advanced stages of ovarian tissue decreased (and the exposition to cryoprotectant after encapsulation may affect more CPA penetration in CA and Vtg1 oocytes).

The expectation, in Experiment 2 was that encapsulation with sodium alginate hydrogel could present efficiency at least equal to the traditional vitrification protocol, was realized. At all stages of cell development analyzed, PG, CA and Vtg1, membrane integrity, by SYBR-14/PI stain, the results did not differ between treatments VS and VS1-A. In the literature, the results regarding the protective efficiency of sodium alginate hydrogel encapsulation for membrane integrity, varies depending on the species and cell group studied. Bian et al. [41] observed that human preantral follicles, demonstrated no difference in membrane

integrity among follicles encapsulated in alginate hydrogel and vitrified with cryoprotectants and fresh follicles. The opposite was observed for bovine preantral follicles, that had a significant loss of quality after vitrification, when sodium alginate hydrogel was used [42].

In our study, sodium alginate did not function as a single cryoprotective element of zebrafish oocyte tissue, as observed in the almost null percentage of membrane integrity in the VA treatment. Notably, half of the concentration of CPAs (0.75M Methanol + 2.75M Me2SO + 0.25M sucrose) in VS2-A did not support the vitrification process compared to VS1-A. However, Mohanty et al. [27], observed that human fibroblasts encapsulated in 2% alginate hydrogel concentration, eliminates the need of CPAs of the encapsulated cells during slow freezing. Besides that, it has already been demonstrated that the alginate bead structure may not prevent the encapsulated cells from cryopreservation damages at non-optimal conditions [43]. Thus, the results obtained in our study demonstrate that the protocol for the vitrification of encapsulated ovarian tissue in alginate hydrogel needs to be adjusted, regarding the concentration of sodium alginate. Furthermore, the size of the fragments may have interfered negatively, considering that, most of the studies with cells encapsulated in sodium alginate hydrogel and cryopreserved, were carried out using a cell suspension, where the entire cell was involved within the hydrogel. Thus, it would be interesting to use smaller fragments of ovarian tissue.

In the present study, the histology analysis, showed nucleus fragmentation in all developmental stages of vitrified ovarians, indicating the occurrence of cellular apoptosis [44]. In vitrified PG oocytes vacuoles in cytoplasm were possible of, which may represent the dissolution of the organelles and autophagic cell death

441 [45]. Follicular membrane rupture of vitrified PG oocyte was observed That is one
442 of the main damages caused by the cryopreservation process [46]. However, no
443 membrane damage was observed on vitellogenic oocytes. Mature female zebrafish
444 have oocytes in an asynchronous development into ovaries, containing follicles of
445 all stages of maturation. Histology sections of small size ovarian tissue, it was not
446 possible to observe large number of each developmental oocyte stage. Then, some
447 characteristics may not have been possible to observe.

448 Membrane integrity alone is not as informative as the number of cells that
449 have survived the cryopreservation and warming procedures, followed by a
450 functional test, which evaluates metabolic activity [43]. This study was evaluated
451 the mitochondrial activity immediately and after, 120 min in L-15 culture medium
452 (28ºC). The results showed no effect for moment of the analysis, suggesting that
453 there are no changes in mitochondrial activity after 120 min on culture medium.
454 However, the encapsulated ovarian tissue in alginate hydrogel treatments (VS1-A,
455 VS2-A and VA) analyzed 120 min after warming, showed lowest mitochondrial
456 activity when compared to non-encapsulated and vitrified ovarian tissue (VS). The
457 concentration of alginate used in this study may have affected the passage of
458 nutrients from the culture medium. High alginate concentration reduces the pore
459 size and porosity, and can prevent the transport of oxygen and nutrients to the cells
460 and, consequently, contributed to reduced metabolic activity of cells [28].

461 Oxidative stress is caused when the physiological balance between reactive
462 oxygen species (ROS) production and antioxidant defenses is not efficient [47], it
463 can cause damage to DNA and induce lipid peroxidation which adversely affects
464 membrane structure, fluidity and function [48]. Cryopreservation and freeze–thaw
465 stress, can damage the antioxidant enzymes that protect against lipid peroxidation

466 [49]. Sodium alginate has antioxidant properties [19] and it has recently been
467 reported to prevent lipid peroxidation in cryopreserved buffalo sperm by slow
468 freezing [50]. The vitrified ovarian tissue encapsulated in alginate hydrogel without
469 CPA (VA) showed the highest levels of lipid peroxidation and membrane damage
470 (SYBR-14/PI), while the ovarian tissue encapsulated with CPA (VS1-A) had the
471 lowest levels and membrane integrity similar to non-encapsulated vitrified tissue
472 (VS). The freeze-thaw process is known to promote lipid peroxidation of the
473 membrane so that cell membranes lose their permeability barriers at a faster rate
474 than cell membranes not treated with CPA, thus shortening cell life [51]. In this
475 sense, the addition of cryoprotectants has a protective action against lipid
476 peroxidation. In our study, one of the cryoprotectants used was Me_2SO which is
477 described as being able to reduce the rigidity of the membrane, contributing to the
478 reduction of mechanical and osmotic stress during cell swelling and shrinkage [52].
479 Therefore, although CPAs have toxic effects, they have important benefits during
480 the cryopreservation process. The lipid peroxidation of non-encapsulated vitrified
481 ovarian tissue, but containing CPA (VS), did not differ from the encapsulated
482 vitrified ovarian tissue with half the CPA concentration (VS2-A). This information
483 suggests that the decrease in CPA in encapsulated and vitrified samples did not
484 result in an increase in lipid peroxidation. During the cryopreservation process, the
485 production of free radicals, as well the high content of lipids of samples, tends to
486 increase lipid peroxidation, which leads to cell death [48]. Considering the high
487 lipid content of CA and vitellogenic oocytes, it is particularly important that there
488 is no increase in the levels of lipid peroxidation.

489 The zebrafish ovarian tissue vitrification presented in this study, preserves
490 primary vetellogenic, cortical alveolar e mainly primary growth oocytes, in which

491 are immature oocytes. *In vitro* maturation of these oocytes would be necessary for
492 further fertilization. Fish oocytes *in vitro* maturation is still in the experimental
493 phase, but it is already possible to obtain live larvae from vitellogenic oocytes that
494 have not gone through the cryopreservation process [14]. However, a study with
495 *in vitro* maturation of cryopreserved early stage oocytes (primary growth and
496 cortical alveolar) did not develop [53]. Therefore, to continue the cryopreservation
497 process of immature oocytes, the next step will be to develop an efficient *in vitro*
498 maturation protocol.

499 In the present study, the sodium alginate hydrogel encapsulation (VS1-A)
500 maintained membrane integrity when compared to the traditional protocol and
501 provided greater antioxidant protection against lipid peroxidation, even with the
502 reduction in the concentration of permeable CPA. An important observation made
503 in the study was that the use of the alginate hydrogel encapsulation technique
504 reduced the loss of oocytes during the manipulation of fragments of ovarian tissue.
505 zebrafish ovaries have a morphology like bunches of grapes in which the oocytes
506 are easily detached from the tissue. Therefore, alginate hydrogel promoted greater
507 support for oocytes, consequently, encapsulation in alginate hydrogel limited the
508 consequences of mechanical stress. Given these results, an interesting alternative
509 for encapsulation of zebrafish ovarian tissue fragments in alginate hydrogel, would
510 be the oocyte maturation *in vitro*, promoting oocytes support.

511

512 **Acknowledgments**

513 We would like to thank the Coordination for the Improvement of Higher
514 Education Personnel (CAPES/PROEX) (N◦ _88887.354528/2019-00), National Council
515 for Scientific and Technological Development CNPq (N◦ _141423/2021-8). The authors

warmly thank UFRGS Reproduction Biotechnics Laboratory and the members of the AQUAM Laboratory in UFRGS.

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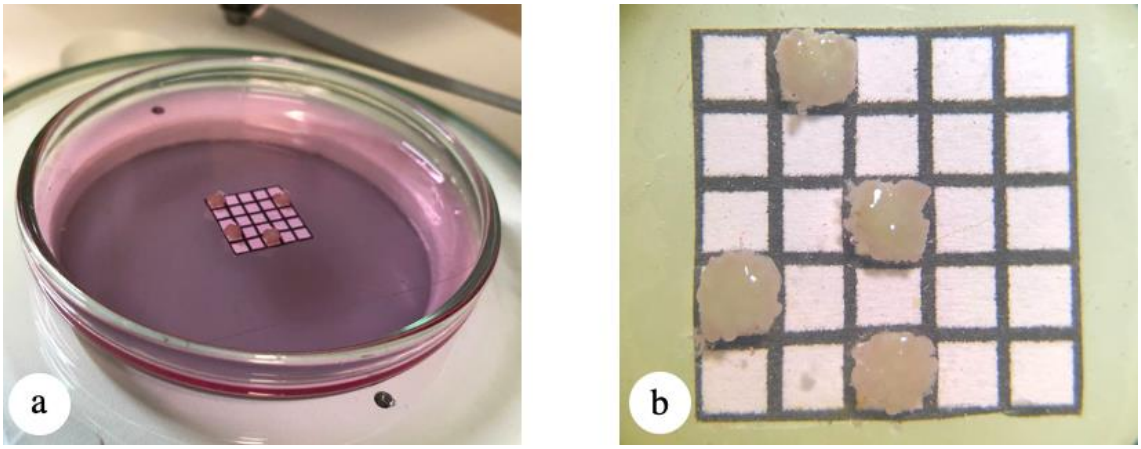
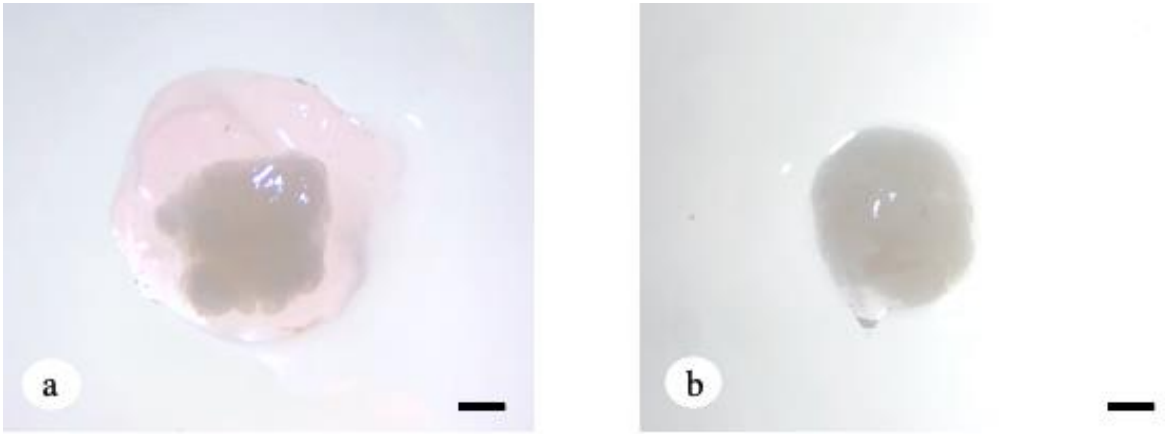


765

766 **Figure 1. Fragmentation of zebrafish ovarian tissue.** (a) Petri dish with markings
767 on its base in a grid format containing 3x3 mm squares for dimensioning the
768 fragments. (b) Ovarian tissue fragments (3x3 mm).

769

770

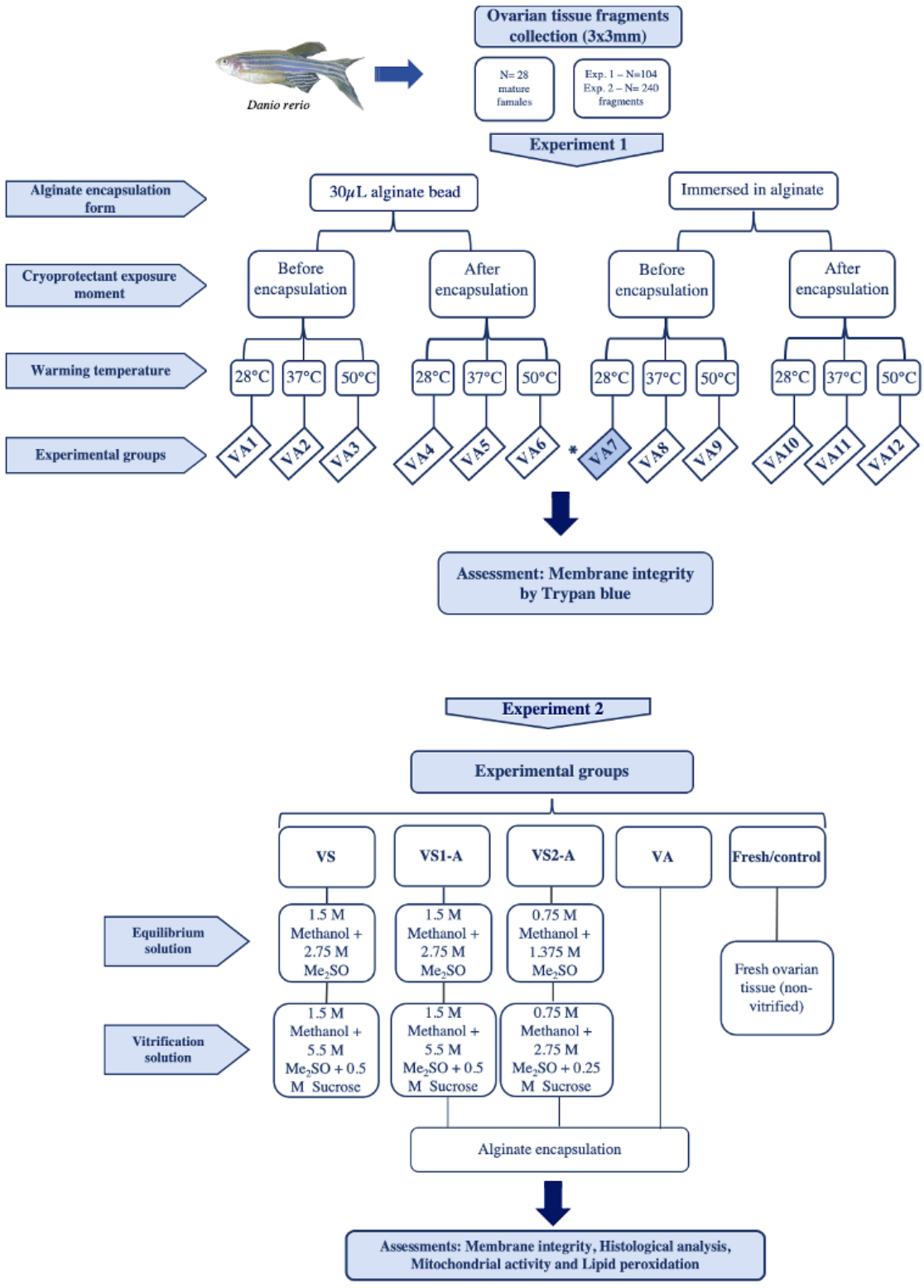


771

772 **Figure 2. Sodium Alginate encapsulation form.** (a) Zebrafish ovarian tissue
773 fragment encapsulated in 30 µL of sodium alginate hydrogel (b) Zebrafish ovarian
774 tissue fragment encapsulated by immersion in sodium alginate hydrogel. Scale
775 Bars: 1 mm

776

777



778

779 **Figure 3. Experimental design illustrating the two experiments conducted with**

780 **the zebrafish (*Danio rerio*) experimental model**. Cryoprotectant used in

Experiment 1: 1.5 M Methanol + 5.5 M Me_2SO + 0.5 M Sucrose. * Treatment chosen for experiment 2.

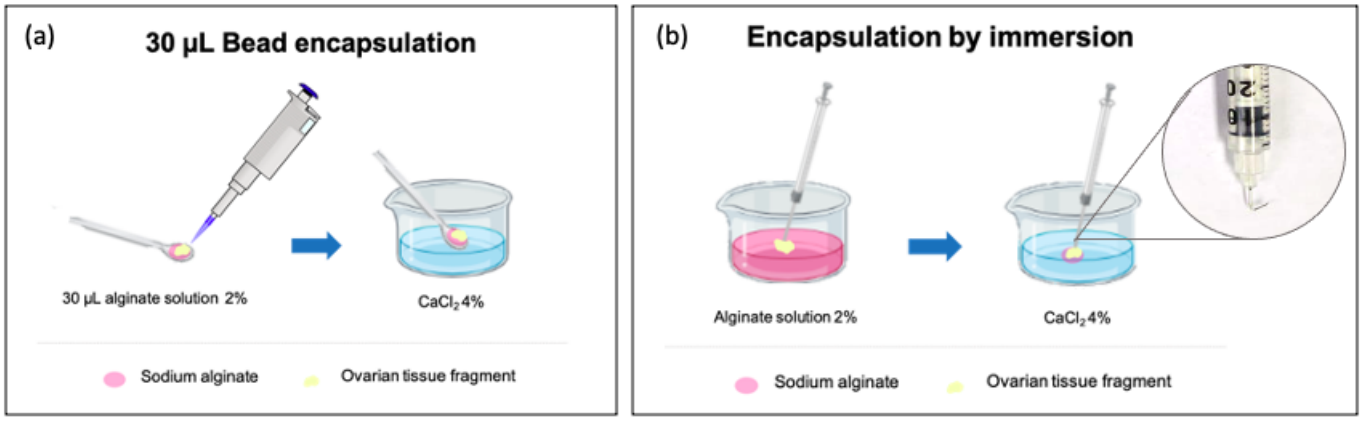
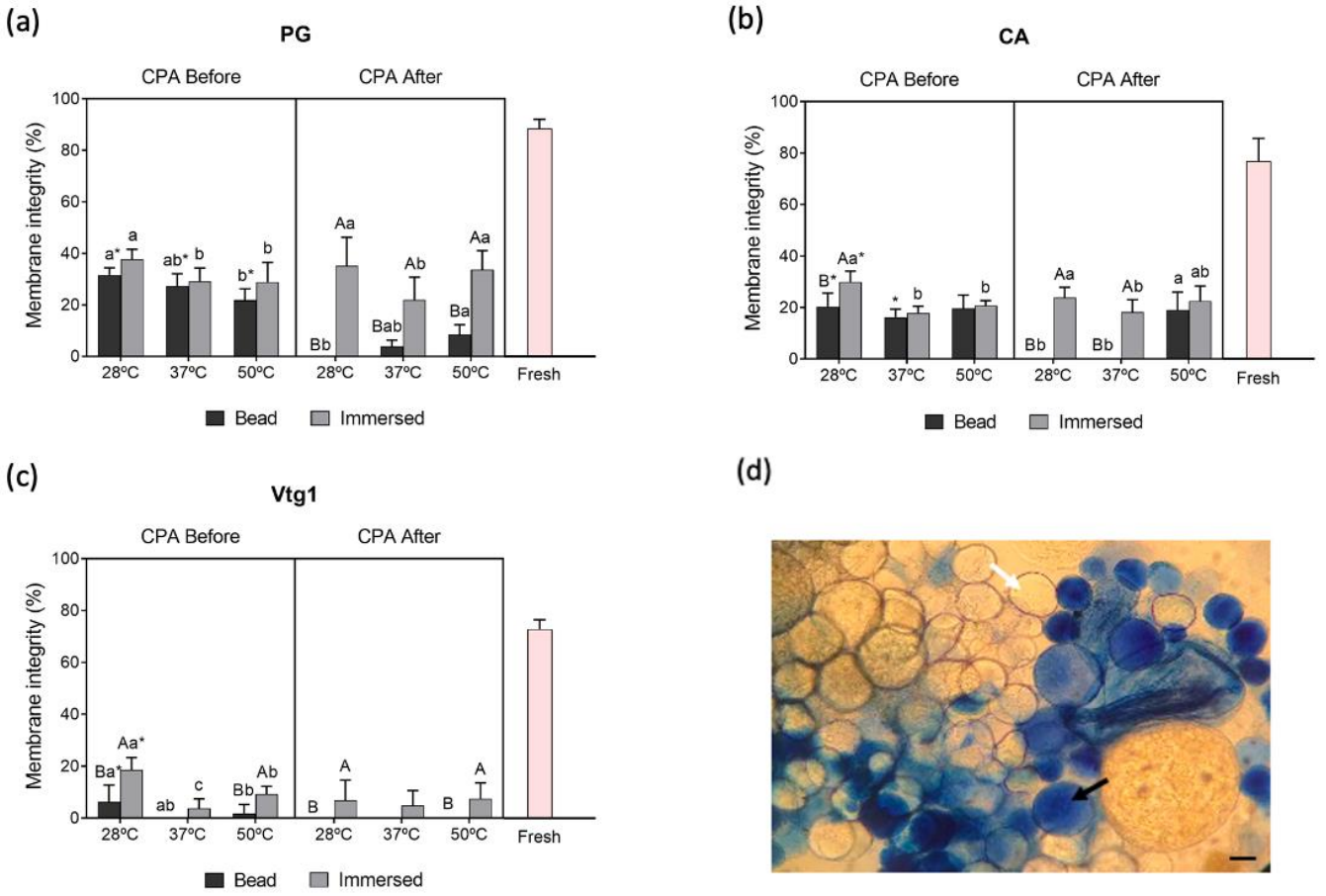
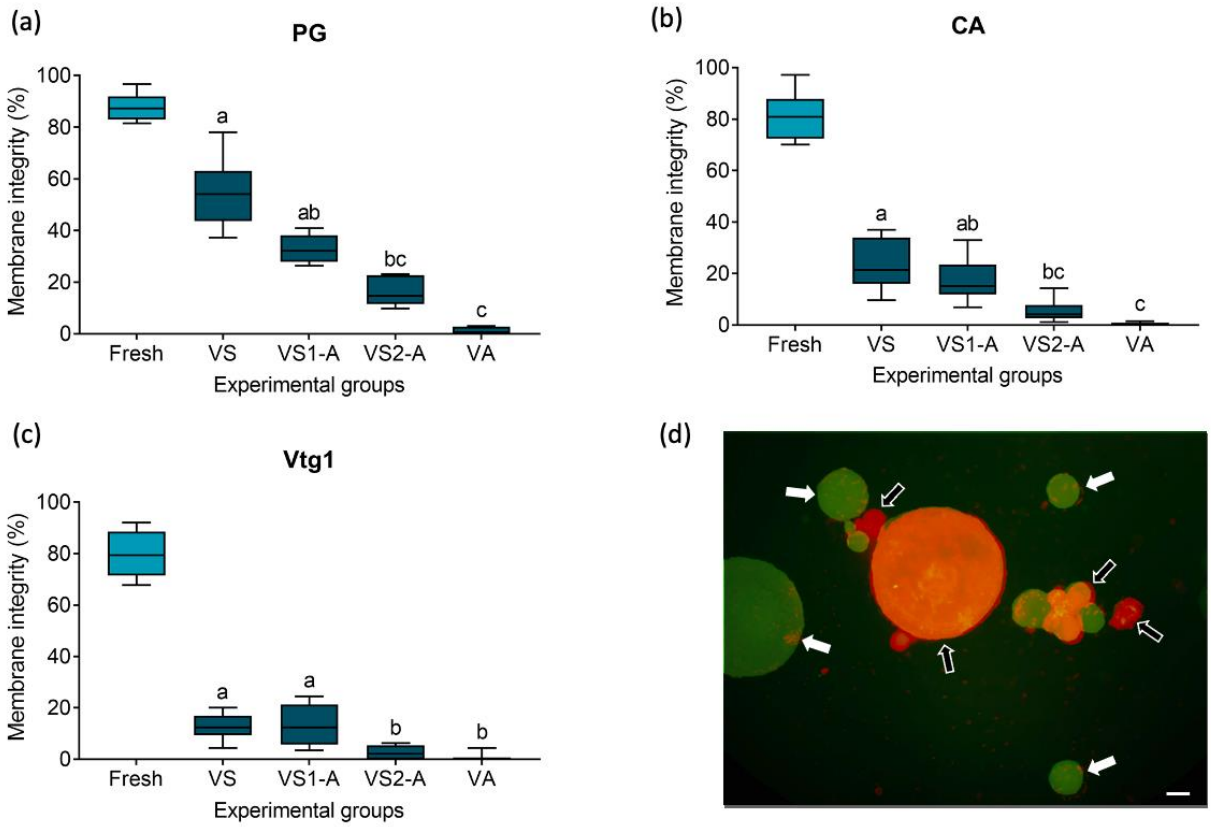


Figure 4. Ovarian tissue encapsulation in sodium alginate hydrogel. (a) Ovarian tissue encapsulation in 30 µL alginate bead; (b) Ovarian tissue encapsulation by immersion in alginate, with image of the curved needle.



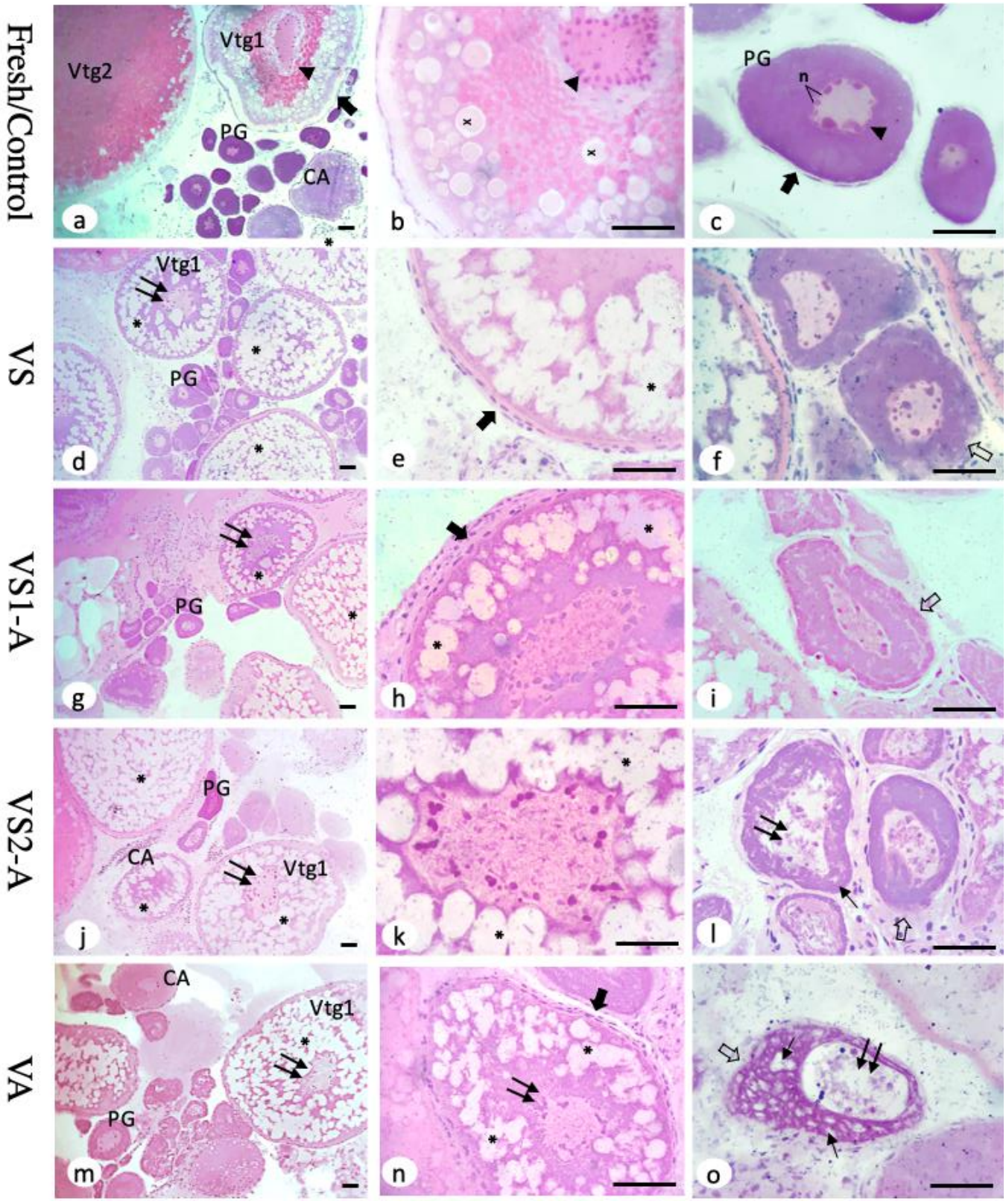
794

795 **Figure 5. Membrane integrity by trypan blue.** (a) Primary Growth oocytes; (b)
796 Cortical Alveolar oocytes; (c) Primary Vilellogenic oocytes. Different capital
797 letters indicate a difference in the form of encapsulation (bead and immersed),
798 within the same warming temperature and the form of exposure to CPA. Lower
799 case letters indicate difference between warming temperatures (28, 37 and 50 ºC),
800 within the same form of encapsulation and exposure to CPA. Asterisk (*) indicates
801 when there is a difference between the moment of exposure to CPA (before or
802 after), within the same warming temperature and in the same encapsulation form.
803 Data presented as mean ± SD. (d) Trypan blue oocyte stain. (white arrow) oocyte
804 with intact membrane, unstained; (black arrow) oocyte with ruptured membrane,
805 stained blue. Scale Bar: 30 µm.



806

807 **Figure 6. Membrane integrity after vitrification by SYBR-14 / PI.** (a) PG
808 oocyte; (b) CA oocyte; (c) Vtg1 oocyte. VS (1.5 M Methanol + 5.5 M Me_2SO + 0.5
809 M sucrose); VS1-A (1.5 M Methanol + 5.5 M Me_2SO + 0.5 M sucrose –
810 encapsulated in alginate); VS2-A (0.75 M Methanol + 2.75 M Me_2SO + 0.25 M
811 sucrose – encapsulated in alginate); VA (encapsulated in alginate); Fresh = fresh
812 ovarian tissue fragments. Data presented as mean ± SD. Different letters indicate a
813 difference between the experimental groups. (d) Merged images of the two
814 excitation filters used: Oocytes with damaged membrane stained by propidium
815 iodide (black arrow) and Oocytes with intact membrane stained by SYBR-14 (white
816 arrow). Scale Bar: 30 µm.



817

818 **Figure 7. Oocytes morphology after vitrification**. a-c) Fresh/control group; d-f)

819 VS group; g-i) VS1-A group; j-l) VS2-A group; m-o) VA group. Primary growth

820 stage (PG). Cortical alveolus stage (CA). Primary vitellogenic stage (Vtg1).

Secondary vitellogenic stage (Vtg2). Nucleoli (n). Rupture of the cortical alveoli (asterisks). Intact cortical alveoli (x symbol). Nucleus fragmentation (double arrow). Intact nucleus (arrowhead). Cytoplasm with vacuole formation (black arrow). Follicular membrane rupture (hollow arrow) intact follicular membrane layer (thick arrow). Scale Bars: 30 µm; Stain: H.E.

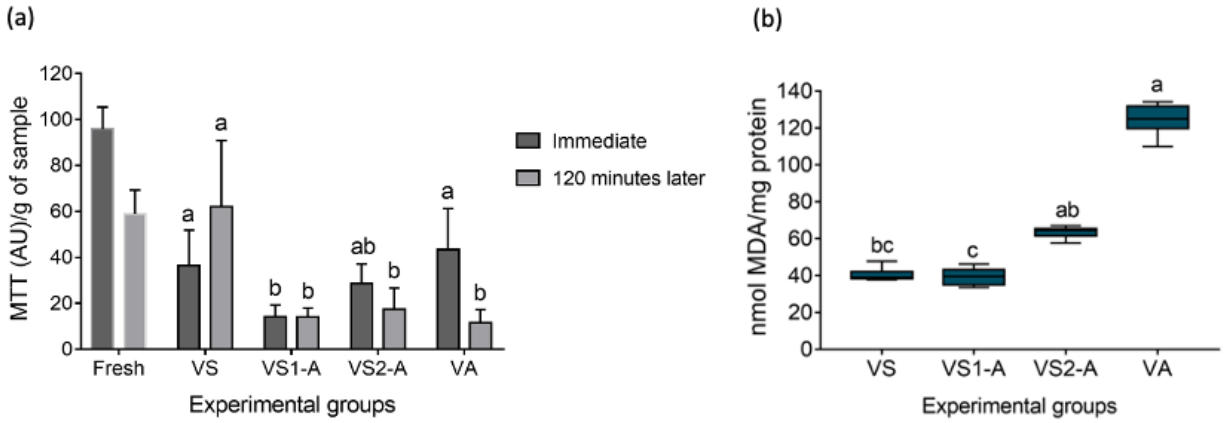


Figure 8. Mitochondrial activity and oxidative stress. (a) Mitochondrial activity (AU / g) by thiazolyl blue tetrazolium bromide (MTT). (b) Thiobarbituric acid reactive substances (TBARS) analyzed after vitrification. VS (1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose); VS1-A (1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose; encapsulated in alginate hydrogel); VS2-A (0.75 M methanol + 2.75 M Me_2SO + 0.5 M sucrose; encapsulated in alginate hydrogel); VA (Encapsulated in alginate hydrogel); Fresh = fresh ovarian tissue fragments. Different letters indicate difference between treatments. Data presented as mean ± SD.