

# The Effect of Different Vitrification Protocols on Cell Survival in Human Ovarian Tissue: A Pilot Study

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## Research

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# **Abstract**

## **Background**

Vitrification has superseded the slow freezing method for cryopreservation of oocytes, embryos, and sperm, but there are yet no standard protocols for its use in ovarian tissue cryopreservation (OTC). Published protocols diverse mainly in the supplementation of dimethyl sulfoxide (DMSO) to the vitrification medium and the use of an open or closed vitrification system.

We investigated the vitality of ovarian tissue of transgender patients by Fluorescence Activated Cells Sorting (FACS) and histomorphological analyses using a DMSO-containing (P1) and a DMSO-free protocol (P2) in an open or closed vitrification setting.

## **Results**

Twelve ovarian samples were donated from female-to-male transgender patients: 6 were vitrified according to protocol 1, the other 6 according to protocol 2. The amount of vital cells was 90.1% (P1) and 88.4% (P2) before vitrification. After vitrification and subsequent warming, vital cells were reduced to 82.9% (P1,  $p=0.093$ ) and 72.4% (P2,  $p=0.019$ ). When comparing the closed and the open systems, the decline in cell vitality from pre- to post-vitrification was significant only for the latter ( $p=0.037$ ). Histological examination reveals no significant differences with respect to degenerated follicles before or after vitrification.

## **Conclusion**

These results lend support to the hypothesis that a protocol containing DMSO results in a higher vitality of ovarian cells than a protocol that uses ethylene glycol as cryoprotective agent in vitrification. The use of an open vitrification system led to significant decline in the rate of vital cells.

Trial registration: NCT03649087, retrospectively registered 28.08.2018

# **Introduction**

Ovarian tissue cryopreservation (OTC) is reported to be a successful way to preserve the fertility in women undergoing sterilizing cancer therapy (1, 2) and the interest in its re-transplantation is rapidly growing, as more than 130 live births were reported to date (3, 4). Although the majority of these live births were achieved with the slow freezing method (5, 6), live births were also reported following vitrification of ovarian tissue, which represents an ultra-fast freezing procedure by direct immersion of the tissue in liquid nitrogen (1, 7). The literature, however, provides conflicting results with respect to the outcomes after cryopreservation of ovarian tissue with vitrification and the slow freezing method, which to date serves as the standard method (6, 8).

In OTC, it is vital to retain the complex nature of ovarian tissue with its variety of cell types in order to restore the ovarian function after re-transplantation. Studies evaluating the slow freezing method reported negative effects on ovarian tissues, postulating that different cell types in ovarian tissue require different factors to prevent damage from ice crystal formation (9). Moreover, Sheikhi and colleagues postulated that vitrification could be advantageous over the slow freezing method by not inducing apoptosis in mouse and human ovarian tissue after warming (10). Presupposed that the tissue is small enough to assure rapid cooling during vitrification, cell-damaging ice crystal formation can be avoided. Vitrification is routinely used in assisted reproduction for cryopreservation of oocytes, embryos and sperm - which are very small compared to an ovarian tissue segment. OTC and re-transplantation is still experimental and published vitrification-protocols diverse mainly in supplementation of dimethyl sulfoxide (DMSO) to the vitrification media on the one hand, and the use of an open or closed vitrification system on the other (1, 11). With a direct exposure of the ovarian tissue to liquid nitrogen, an open vitrification system bears the risk of a contamination with pathogens. Hence, open vitrification systems are viewed critically and some authors request a scientifically and successfully proven closed vitrification system (12, 13).

In general, the chemicals used in cryoprotective solutions are considered as toxic to various cells. The degree of toxicity is dependent on individual concentration and cell type. Therefore, the current difficulty is to identify cryoprotective agents that do not affect cell viability and can be used for vitrification in acceptable concentrations at feasible cooling and heating rates.

As there are yet no standard protocols for vitrification in OTC, we aimed to study the impact of vitrification on human ovarian tissue by comparing the effects of two solutions either in- or excluding DMSO in an open and closed vitrification system.

## Results

In total, 12 female-to-male transgender patients, treated with androgens for a minimum of one year, donated their ovaries for this study and 12 ovarian samples could be obtained. Six were vitrified according to the DMSO-containing protocol 1, the other six were vitrified according to the DMSO-free protocol 2. For both protocols, an open and closed vitrification was performed (study flow chart: Fig. 1). Median age of patients enrolled to protocol 1 and protocol 2 was 22.6 (IQR 19.1–32.1) and 21.9 (IQR 18.9–29.1) years, respectively ( $p = 0.72$ ).

### FACS analysis

As ovarian tissue consists of a multitude of different cells that are embedded in a cellular structured surrounding, the structures and the cell-cell connections in such a cell network may eventually be affected during vitrification. Therefore, individual cell types might be differently accessible for mechanical and/or enzymatic digestion. Figure 2 exemplarily shows that the cell populations before and after vitrification did not differ fundamentally according to our FACS analysis: their size and complexity seemed to be similar in fresh and vitrified ovarian tissue.

Representative picture of unvitrified (left) and vitrified (right) ovarian cells after enzymatic digestion and Fluorescence Activated Cells Sorting (FACS) analyses. Upper lane: Contour Plot of ovarian cells. Lower lane: Dot Plot of DAPI (4,6 Diamino-2-Phenylindole, Dihydrochloride) stained cells.

In all specimen, the amounts of dead and alive cells were determined directly after surgical removal of ovarian tissue before vitrification. In all 12 samples, regardless of the vitrification methods used, the amount of vital cells was 91.4% (IQR: 80.7–95.7) before vitrification and 80.1% (IQR: 68.8–92.1) after vitrification and subsequent warming ( $p = 0.050$ ). Concerning the specimen planned to undergo the DMSO-containing protocol 1 and the DMSO-free protocol 2, the amount of vital cells was 91.4% (IQR: 84.5–97.0) and 88.4% (IQR: 78.1–95.1) before vitrification, respectively ( $p = 0.528$ ; see Figs. 2 and 3, first bars). After vitrification and subsequent warming, the amount of all vital cells was 82.9% (IQR: 78.1–91.9) for protocol 1 and 72.4% (IQR: 53.9–92.4) for protocol 2 ( $p = 0.057$ ). This decline in cell vitality was significant only for the DMSO-free protocol 2 ( $p = 0.019$ ), but not for the DMSO-containing protocol 1, ( $p = 0.093$ ; Fig. 3).

In a next step, the open and the closed methods were compared to each other. After the vitrification and thawing process, there were no differences in cell vitality between the open (median 79.4%, IQR: 59.1–90.5) and the closed system (84.1%, IQR: 69.4–92.4;  $p = 0.459$ ). Within the subgroups of the open system, the decline in cell vitality from pre- to post-vitrification was significant ( $p = 0.037$ ), whereas this was not the case for the closed system ( $p = 0.139$ ).

## Histological analysis

For ovarian tissue re-transplantation and subsequent restoring of the woman's fertility, the amount of intact follicles in the tissue after freezing is a crucial parameter. We therefore determined the amount of defective follicles in histological examinations before and after vitrification with respect to the different protocols (Fig. 4). An example of normal and damaged follicles is given in Fig. 5. Although not statistically significant, this analysis revealed that the proportion of defective follicles was highest in the DMSO-free group, whereas we did not find differences in the group vitrified with DMSO (protocol 1: 11.53% before vs. 11.11% after vitrification,  $p = 0.9$ ; protocol 2: 6.42% before vs. 22.65% after vitrification,  $p = 0.12$ ).

Histological determination of the percentage of defective follicles after vitrification in relation to the percentage of defective follicles before vitrification. Values are given in percent. (b-V EG: Percentage of defective follicles before vitrification with ethylene glycol, b-V DMSO: Percentage of defective follicles before vitrification with dimethyl sulfoxide, V-EG: Percentage of defective follicles after vitrification with ethylene glycol, V-DMSO: Percentage of defective follicles after vitrification with dimethyl sulfoxide)

Histological sections of ovarian fragments after HE-staining showing morphologically normal (A) and degenerated (B) follicles (Magnification 400x)

## Discussion

The results of our study show that in general, the majority of ovarian cells were vital after vitrification and subsequent warming despite a significant decline during the process. Moreover, there were differences depending on the protocol used.

When considering the effects of vitrification on ovarian tissue, several factors must be considered, including size, cooling and warming rates as well as the type and concentration of cryoprotectants. Both, DMSO and ethyleneglycol were reported to have concentration-dependent toxicological effects: whereas DMSO has been shown to affect the methylation status of embryonic stem cells and bodies, ethyleneglycol was considered to be embryotoxic at high concentration in mice and rats (14, 15). Depending on its concentration, DMSO decreases the cell viability and increases apoptosis and necrosis in certain cell lines (16). Notably, at higher concentrations, it acts pro-oxidant, whereas at low concentration it acts as a radical scavenger exhibiting antioxidant activity (17). Our results show that vitrification using a protocol DMSO-free protocol resulted in a significant decline in the rate of vital cells after the vitrification and thawing process ( $p = 0.019$ ), whereas this was not the case when a DMSO-containing protocol was used ( $p = 0.093$ ). Of note, this result is in accordance with our previous findings where a DMSO-containing protocol resulted in a lower amount of dead granulosa cells compared to an ethylenglycol-containing protocol (18).

Primordial follicles – routinely used for determining the efficiency of cryopreservation – represent more than 90 percent of ovarian follicles (9, 19, 20). Even if they seem morphologically intact after warming, it is uncertain if their reproductive capacity might have been affected by cryopreservation (6). As a composite tissue it must be taken into account that ovarian tissue consists of a large number of different cell types requiring different parameters to avoid ice crystal formation during the freezing process (6). Cortical ovarian tissue contains different cell types such as stroma cells, follicles – formed by an oocyte and granulosa cells – and blood vessels. Of note, adequate preservation of stroma and vascular system is of fundamental importance due to their critical role in follicular development and restoration of gonadal function after re-transplantation. It appears therefore logically consistent, that the efficiency of a cryopreservation protocol should be evaluated not only by the analysis of primordial follicles but also of other cells contained in cortical ovarian tissue: with respect to our FACS analysis, the difference between non-vital cells before and after vitrification is rather small with the DMSO-containing protocol and higher with the EG based protocol.

An open vitrification system bears the risk of a transmission of infective agents, and infections following artificial reproductive technologies (ART) have already been described in animals and humans (13, 21–23). Although none of these reported infections was ascribed to the cryopreservation technique itself, infective contamination via cryopreservation has experimentally been shown (12, 13). In order to prevent possible contaminations, the use of a closed vitrification system or sterile liquid nitrogen was suggested. Sterilization of liquid nitrogen is complex and so far, hardly suitable for ART. Moreover, closed vitrification systems are considered controversial as they may prolong the cooling rate especially in larger tissue, which is critical in very sensitive samples. Hence, there is still a demand for a scientifically and successfully proven closed vitrification system in OTC (13). Interestingly, our data show that the use of an

open system was associated with a significant decline in the rate of vital cells after vitrification and thawing ( $p = 0.037$ ), which was not the case for the closed system ( $p = 0.139$ ). Although there was no difference in final survival rates between the systems, this can be seen as a hint that open systems might be more prone to increased cell death. However, our findings are in contrast to previous results of Iwahati and co-authors, who were able to show in a retrospective study of blastocysts that there were no significant differences between the use of closed or open vitrification systems in embryo development after implantation as well as no impairment of neonatal development (24).

Of course, our study has to be interpreted within its major limitation, namely the small amount of samples donated from female-to-male transgender patients. The specific effect of androgens on the vitality of ovarian tissue is unknown, but as long-term androgen treatment does not seem to reduce the primordial follicle pool, their ovaries represent an excellent source of tissue for research purposes (25, 26). We are aware, that the viability of ovarian tissue can only be determined after reimplantation – but using FACS analysis, we were able to demonstrate the vitality of different cell types, which we regard as a strength of our study. Evaluating ovarian tissue after vitrification by using morphological parameters and histologic analyses is the main focus of most studies, based on the assumption that morphological intact follicles are representative for the success of ovarian tissue re-transplantation. Of note, the variety of cell-types surrounding the follicle seems to play an essential role after re-transplantation of ovarian tissue as well (27, 28). Taking into account that individual cell types might react differently on mechanical and/or enzymatic digestion, we initially showed with our FACS analysis that size and complexity of cell populations before and after vitrification do not differ fundamentally. To the best of our knowledge, we are the first to report such results in OTC.

In conclusion, our results lend support to the hypothesis that a protocol containing DMSO results in a higher vitality of ovarian cells than a protocol that uses ethylene glycol as cryoprotective agent in vitrification. With the protocols used, the closed system revealed no significant decline in the rate of vital cells after vitrification and thawing, whereas the open system did. Larger studies and the consideration of an animal model (such as a SCID mouse) for reimplantation might prove beneficial to confirm our findings.

## **Materials And Methods:**

### **Study design and collection of donor ovaries**

Donor ovaries were collected from female-to-male transgender donors during their combined gender reassignment operation, which has been published previously and included total laparoscopic hysterectomy, bilateral salpingo-oophorectomy and bilateral mastectomy (29).

Patients were recruited between February 2017 and December 2018. Inclusion criteria were: (i) gender-completing operation; (ii) age 18–40 years; (iii) willingness to donate ovarian tissue for research purposes.

## *Ethical approval*

Oral and written informed consent was obtained from all participants. The study was approved by the ethics committee of the Medical University of Vienna (EK 2240/2016), was conducted in accordance with the Declaration of Helsinki and was retrospectively registered in the Current Controlled Trials Register (NCT03649087).

## *Surgical technique and tissue preparation*

Total laparoscopic hysterectomy and bilateral salpingo-oophorectomy was performed in all patients. To ensure short ischemia times, the ovarian perfusion through the infundibulo-pelvic (IP) ligament was obtained until dissection of the uterus was completed. After dissection of the IP ligament and opening of the vaginal cuff, uterus, fallopian tubes and ovaries were removed en block through the vagina. Dissection of ovarian tissue was performed on a side table. The remaining part of the ovary was sent for histo-pathologic examination and morphological evaluation of the follicles. After an incubation in PBS for a maximum of 10 minutes the ovarian tissue was split into pieces of 10mm x 5-10mm x 2mm for subsequent vitrification.

## *Vitrification and Warming*

Two different vitrification protocols were used and both were performed in an open and in a closed system. For the open system, the tubes with the tissue pieces (see below) were closed with a lid after immersion in liquid nitrogen, whereas for the closed system, this was done directly before the nitrogen step. The study flow chart is provided in Fig. 1.

The first protocol contained DMSO as cryoprotective agent and was previously described by *Silber et al.* (protocol 1) (1). The second contained ethylene glycol and propylene glycol (propane-1,2-diol) as cryoprotective agents and was purchased by ORIGIO (Origio, Måløv, Denmark (protocol 2).

Except otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St Louis, USA).

### *Protocol 1*

For vitrification according to the protocol previously published by *Silber* and colleagues (1) the washed pieces were equilibrated for 10 min. in equilibration solution (ES) containing 7,5% Dimethylsulfoxid (DMSO) and 10% fetal calf serum (FCS) in DMEM. Afterwards the pieces were transferred with a tweezer to the vitrification solution (VS) containing 20% ethylene glycol (EG), 20% DMSO, 0.5M Sucrose and 10%FCS in DMEM. After 10 to 15 minutes of incubation, the tissue pieces were removed from the VS and transferred to a 1.8 ml tube (Nunc, ThermoFisher Waltham, Massachusetts, US) with tweezers. The tubes were then quickly immersed in liquid nitrogen using a tube holder.

For warming, the samples were removed from liquid nitrogen and 1 ml of a 37°C pre-warmed warming solution containing 1 M sucrose and 20% FCS were added. After incubation for 1–3 min. in a water bath

(37°C) the pieces were transferred to 1 ml of a solution containing 0.5M sucrose and 20% FCS for 3 min. After a washing step with PBS the ovarian pieces were either used for formalin fixation and histological examination or for FACS analyses.

#### *Protocol 2*

For vitrification according to the DMSO-free protocol by *Origio*, we used the *MediCult Vitrification Cooling kit* for human oocytes, cleavage stage embryos and blastocyst and adapted the protocol for our purposes as follows: The equilibration and vitrification solutions were warmed to room temperature. The ovarian tissue pieces were equilibrated for 15 min. in equilibration solution and transferred afterwards with a tweezer to the vitrification solution (VS) for a maximum of 1 min. The tissue pieces were then removed from the VS and transferred to a 1.8 ml tube (Nunc, ThermoFisher Waltham, Massachusetts, US) with tweezers. The tubes were immersed in liquid nitrogen using a tube holder.

For warming we used the *MediCult Vitrification Warming kit* by *Origio* and adapted the protocol as follows: After removing the samples from liquid nitrogen 1 ml of a 37°C pre-warmed warming solution was added directly into the tube, the tube was incubated in a 37°C water bath for a maximum of 3 min. Afterwards the ovarian tissue pieces were transferred into dilution media 1 for 3 min. Then the tissue was transferred for another 3 min in dilution media 2. After washing steps with washing media and PBS the ovarian pieces were either used for formalin fixation and histological examination or for enzymatic digestion and subsequent FACS analyses.

#### *Enzymatic digestion with Liberase*

For the enzymatic digestion with Liberase, the tissue was sliced into small pieces with a scalpel and was transferred in 10 ml PBS. After addition of 215µl of Liberase DH (2,8 Wünsch Units) the suspension was incubated for 1 h at 37°C. The suspension was shaken every 15 min. with a pipette for additionally mechanically disruption. After the digestion step, the cell suspension was filtered through a 100µm filter and rinsed with PBS. The cells were collected by centrifugation (300rcf) and washed twice in PBS.

#### *Fluorescence Activated Cell Sorting (FACS) analysis*

The cells were suspended in 1 ml PBS and analyzed on a BD FACSVersa Flow cytometer. DAPI (4,6-Diamino-2-Phenylindole, Dihydrochloride) was added to the samples ten minutes before start of the analysis to distinguish vital and non-vital cells. A minimum of 10.000 events was collected. Data were analyzed using a BD FACSuite V1.06 and FLOWJO software ([www.flowjo.com](http://www.flowjo.com)).

#### *Histological analysis*

Fresh and thawed ovarian pieces were fixed in 4% buffered formaldehyde and embedded in paraffin blocks. The paraffin blocks were serially cut into 4 µm sections and stained with hematoxylin and eosin. Within these sections, the number of follicles present were recorded and classified as primordial (oocyte surrounded by a single flat layer of follicle epithelial cells/pre-granulosa cells), primary (single layer of

cuboidal granulosa cells), secondary (two or more layers of granulose cells, no antrum), or antral (presence of an antrum), similar to previously described methods (19, 30, 31). Morphologic evaluation of the follicles was based on examination of the integrity of the basement membrane, cellular density, presence or absence of pyknotic bodies, and integrity of the oocyte. Based on these criteria, follicles were classified as morphologically normal or abnormal.

### *Statistical analysis*

The statistical analyzes were performed using the SPSS 27.0 software. Data are provided as median and interquartile ranges (IQR). The data of the FACS analyzes were analyzed using ANOVA for independent samples followed by Tukey's HSD Test. Differences were considered statistically significant at  $p < 0.05$ .

## **Declarations**

**Ethics approval and consent to participate:** The study was approved by the ethics committee of the Medical University of Vienna (EK 2240/2016), was conducted in accordance with the Declaration of Helsinki and was retrospectively registered in the Current Controlled Trials Register (NCT03649087).

**Consent for publication:** All authors have seen and approved the content of the manuscript.

**Availability of data and Materials:** The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing Interest:** JO received remuneration for lecturing from Lenus Pharma GesmbH outside the submitted work. All other authors declare that they have no conflict of interest.

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### **Author Contributions:**

Conceptualization, Julian Marschalek and Detlef Pietrowski; Data curation, Julian Marschalek and Detlef Pietrowski; Formal analysis, Johannes Ott and Detlef Pietrowski; Funding acquisition, Julian Marschalek and Detlef Pietrowski; Investigation, Julian Marschalek, Sabine Dekan and Maria Frank; Methodology, Johannes Ott and Detlef Pietrowski; Project administration, Julian Marschalek, Christian Egarter and Detlef Pietrowski; Resources, Sabine Dekan, Maria Frank and Detlef Pietrowski; Software, Johannes Ott and Detlef Pietrowski; Supervision, Christian Egarter and Kazem Nouri; Validation, Julian Marschalek, Sabine Dekan, Johannes Ott and Detlef Pietrowski; Visualization, Kazem Nouri, Johannes Ott and Detlef Pietrowski; Writing – original draft, Julian Marschalek, Johannes Ott and Detlef Pietrowski; Writing – review & editing, Julian Marschalek, Christian Egarter and Kazem Nouri.

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## Figures

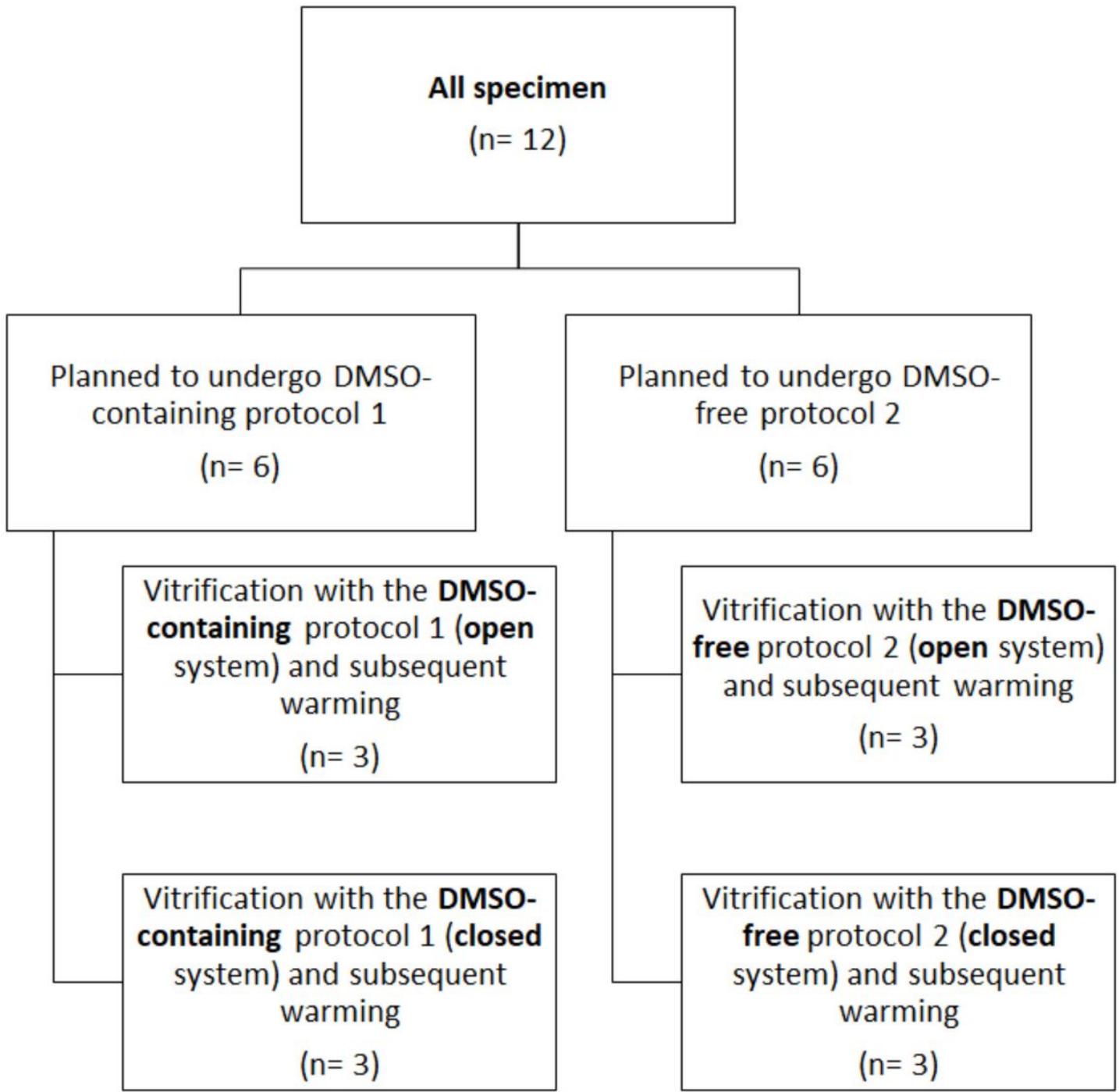
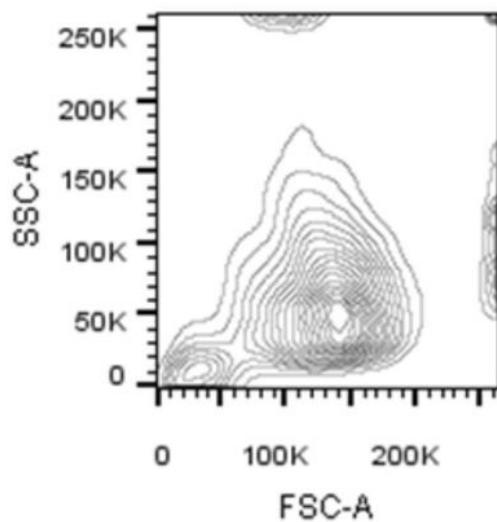
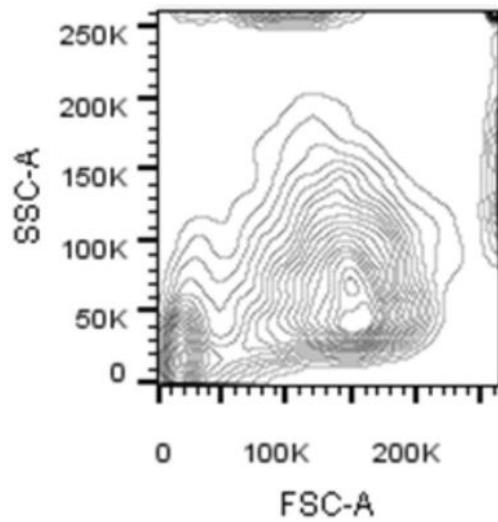


Figure 1

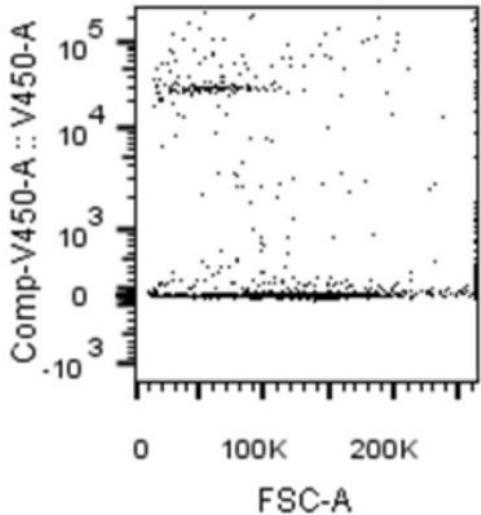
Study flow chart.



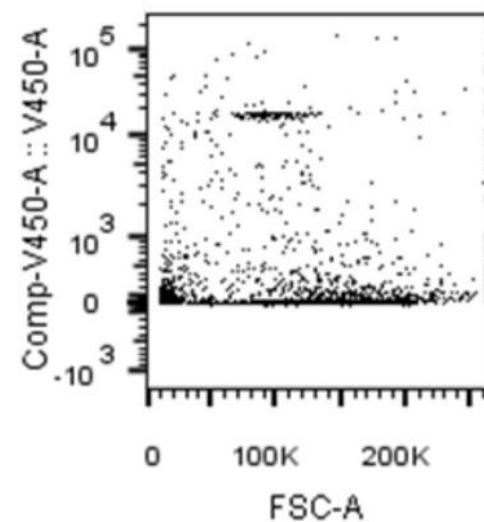
non vitrified ovarian tissue  
sorted by size and complexity



vitrified ovarian tissue  
sorted by size and complexity



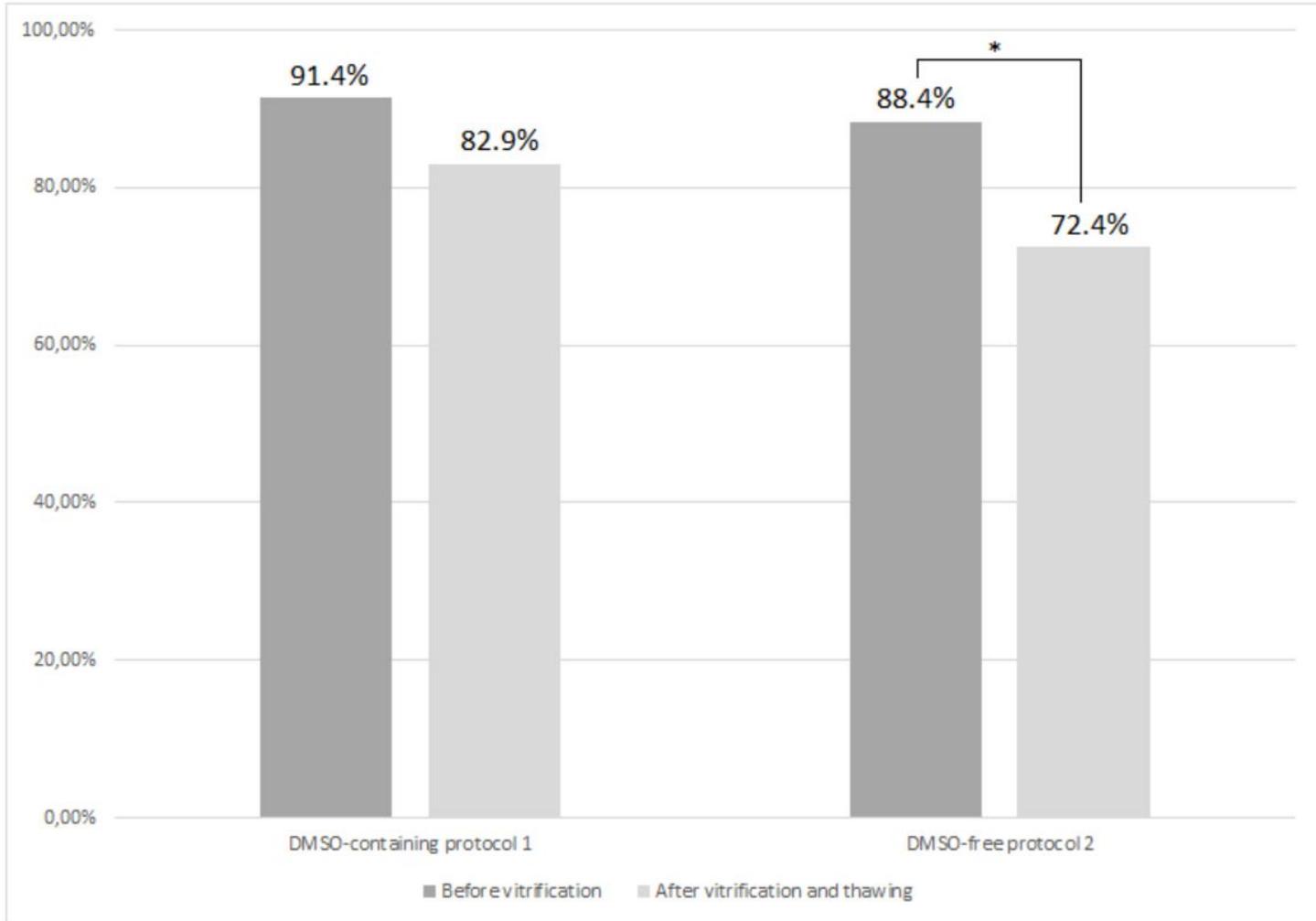
non vitrified ovarian tissue  
after DAPI staining



vitrified ovarian tissue a  
fter DAPI staining

**Figure 2**

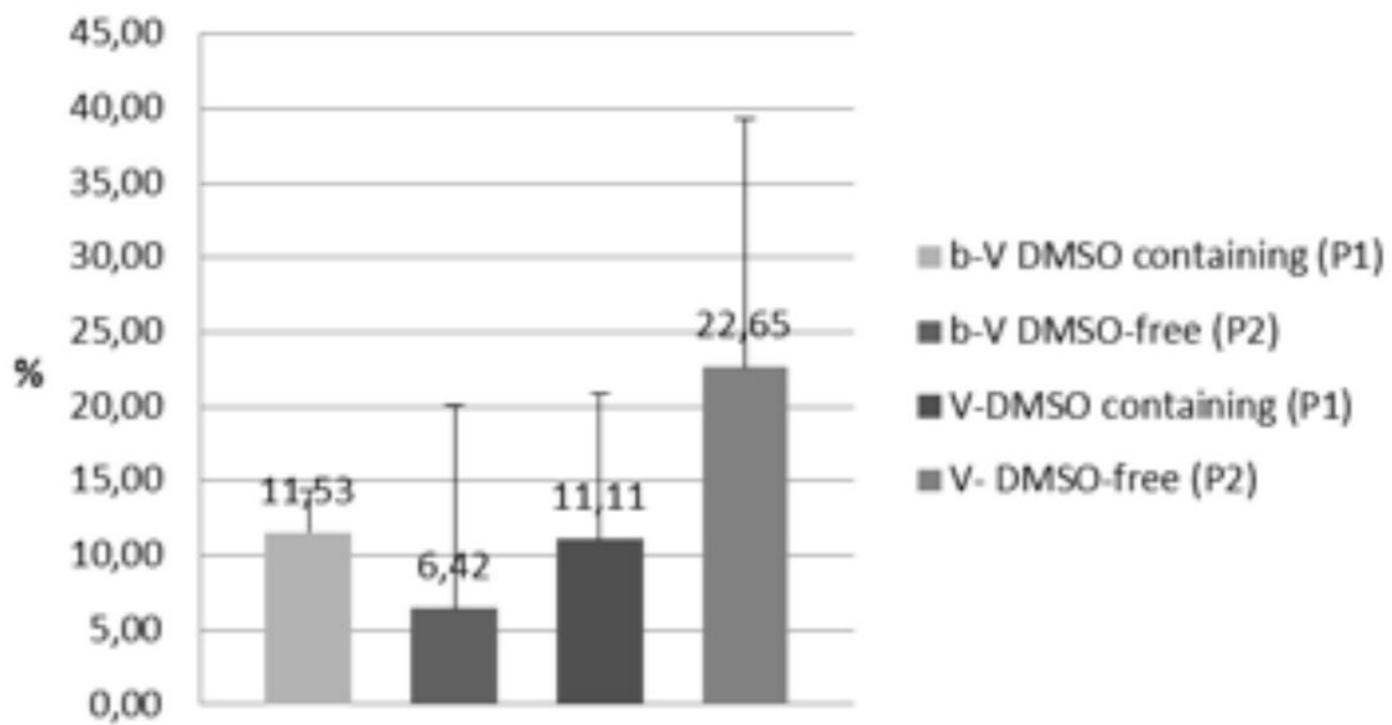
Representative picture of FACS analysis before and after vitrification.



**Figure 3**

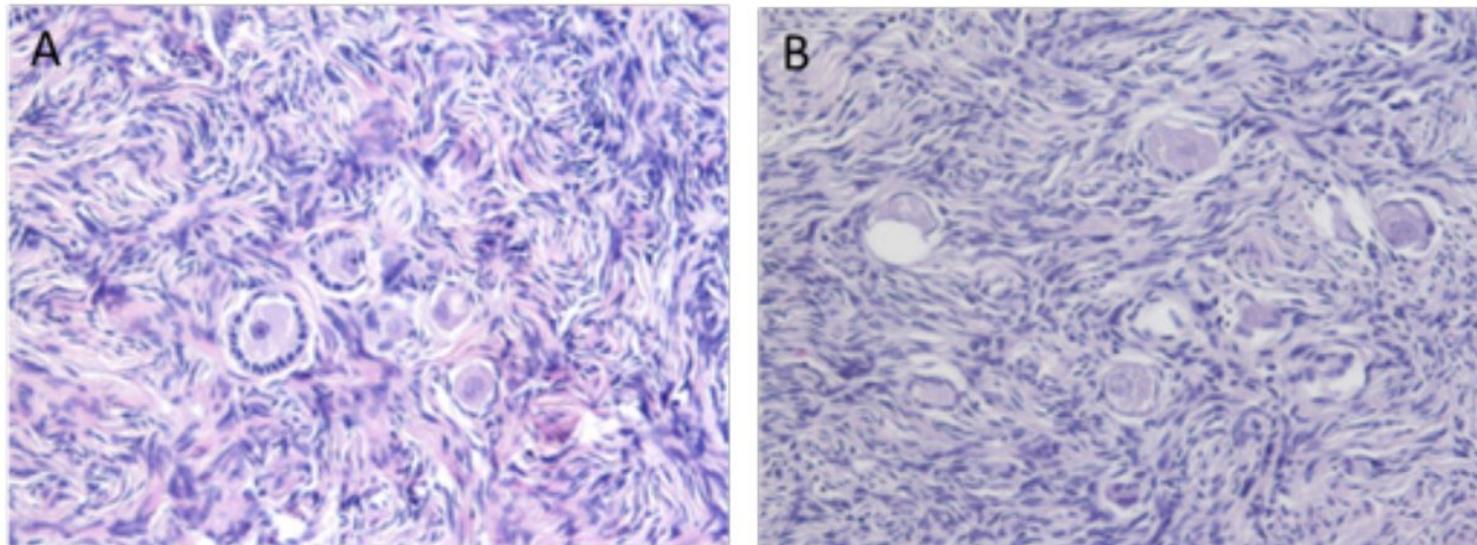
Rate of vital ovarian cells before and after vitrification/thawing using the DMSO-containing protocol 1 and the DMSO-free protocol 2. \* $p < 0.05$

## Percentage of defective follicles



**Figure 4**

Number of defective follicles with regard to different protocols.



**Figure 5**

Example of morphological normal and defective follicles.