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TITLE:

Fertility Preservation Through Oocyte Vitrification: Clinical and Laboratory Perspectives

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KEYWORDS:

Fertility preservation, ovarian stimulation, metaphase II oocyte, vitrification, warming, Key Performance Indicators (KPI)

SUMMARY:

Oocyte cryopreservation is recognized by several international scientific societies as the gold standard for fertility preservation in postpubertal women. Appropriate clinical and laboratory strategies ensure maximum efficacy, efficiency, and safety of fertility preservation treatments.

ABSTRACT:

Preserving female fertility is crucial in a multifunctional healthcare system that takes care of patients' future quality of life. Oocyte cryopreservation is recognized by several international scientific societies as the gold standard for fertility preservation in postpubertal women, for both medical and non-medical indications. The main medical indications are oncologic diseases, gynecologic diseases such as severe endometriosis, systemic diseases compromising the ovarian reserve, and genetic conditions involving premature menopause. This paper describes the whole clinical and laboratory work-up of a fertility preservation treatment by outlining recommendations for objective and evidence-based counseling. Furthermore, it focuses on the effectiveness of the procedure and describes the most appropriate strategies to fully exploit the ovarian reserve and maximize the number of oocytes retrieved in the shortest possible time. The evaluation of the ovarian reserve, the definition of an ideal stimulation protocol, as well as oocyte retrieval, denudation, and vitrification procedures have been detailed along with approaches to

maximize their efficacy, efficiency, and safety.

INTRODUCTION:

The development and implementation of an efficient cryopreservation program for human oocytes has been a significant breakthrough in reproductive medicine. According to recent evidence, vitrification is the most effective strategy to cryopreserve metaphase II (MII) oocytes, as it results in statistically higher survival rates compared to slow freezing, independently of the patient population (infertile patients or oocyte donation program)[1-3]. The remarkable achievements of oocyte vitrification led the Practice Committees of the American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART) to pronounce this technique to be the most effective for elective fertility preservation in postpubertal women, for both medical and non-medical indications[4,5,6]. Medical indications for fertility preservation include (i) cancer and autoimmune diseases that require therapies[7] such as radiotherapy, cytotoxic chemotherapy, and endocrine therapy (whose detrimental effect on the ovarian reserve is associated with maternal age as well as type and dose of the treatment); (ii) ovarian diseases requiring repeated or radical surgery (such as endometriosis)[8]; and (iii) genetic conditions (e.g., X-fragile) or premature ovarian failure. In addition, fertility preservation has become a valuable option for all women who have not accomplished their parental objective for non-medical reasons (also known as social freezing).

Regardless of the indication for fertility preservation and according to the major international guidelines on fertility preservation, all patients willing to vitrify their oocytes should receive appropriate counseling to be informed about their realistic chance of success, the costs, risks, and limitations of the procedure[9-13]. Most importantly, it should be clear that vitrifying a cohort of MII oocytes does not ensure a pregnancy, but that it offers a higher chance of success for future in vitro fertilization (IVF) treatment, if necessary[14]. In this regard, the woman's age at the time of oocyte vitrification is certainly the most important limiting factor[15] as advanced maternal age (AMA; >35 years) is the main cause of female infertility[16]. Besides a progressive reduction in the ovarian reserve, AMA is associated with an impairment of oocyte competence due to defective physiological pathways such as metabolism, epigenetic regulation, cell cycle checkpoints, and meiotic segregation[17]. Therefore, the reasonable number of eggs to vitrify mainly depends on maternal age. In women younger than 36 years, at least 8–10 MII oocytes[18] are required to maximize the chance of success. In general, the higher the number of vitrified oocytes, the higher is the likelihood of success. Therefore, tailoring ovarian stimulation according to ovarian reserve markers such as anti-Müllerian hormone (AMH) levels or antral follicle count (AFC) is crucial to fully exploit the ovarian reserve in the shortest possible time.

The safety of the whole procedure is the other key issue when enrolling patients for fertility preservation. Clinicians should employ the best strategies to minimize the risks and prevent (i) ovarian hyperstimulation syndrome (OHSS) by using safe approaches such as the gonadotrophin-releasing hormone (GnRH) antagonist protocol followed by a GnRH agonist trigger[19] and (ii) the remote, yet possible, risks of peritoneal bleeding, injury to the pelvic structures (ureter, bowel,

appendix, nerves), or pelvic infection during oocyte retrieval. Lastly, (iii) traditional regimens for stimulation are associated with supraphysiologic serum estradiol and therefore, are not recommended in estrogen-sensitive diseases such as breast cancer. Protocols involving aromatase inhibitors (such as letrozole or tamoxifen) are more suitable in these cases[20,21]. In the laboratory setting, the most widespread protocol for oocyte vitrification is still the one first described by Kuwayama and colleagues[2,23], which consists of a stepwise procedure involving the gradual addition of cryoprotectants (CPAs). In the first phase (equilibrium/dehydration), oocytes are exposed in a CPA solution containing 7.5% v/v ethylene glycol and 7.5% v/v dimethyl sulfoxide (DMSO), while in the second phase, oocytes are moved to a vitrification solution with 15% v/v ethylene glycol and 15% v/v DMSO, plus 0.5 mol/L sucrose. After a short incubation in the medium of vitrification, the oocytes can be placed in specifically designed, open cryodevices and finally plunged in liquid nitrogen at -196 °C to be stored until use.

Here, the whole clinical and laboratory work-up of a fertility preservation treatment has been described by (i) outlining recommendations for objective and evidence-based counseling, (ii) focusing on the cost-effectiveness of the procedure, and (iii) describing the most appropriate strategies to fully exploit the ovarian reserve and maximize the number of oocytes retrieved in the shortest possible time. The evaluation of the ovarian reserve, the definition of an ideal stimulation protocol, as well as oocyte retrieval, denudation, and vitrification procedures will be detailed along with approaches to maximize their efficacy, efficiency, and safety. As other protocols or adaptations of this protocol exist in the literature, the representative results and the discussion sections of this manuscript only apply to this procedure.

PROTOCOL:

1. Work-up and clinical counseling

NOTE: In case of patients requiring fertility preservation for oncologic reasons, ensure that there is no waiting list for scheduling consultation, and the appointment is provided as soon as possible.

1.1. Examine the medical history and previous documentation, and assess the patient's general health status.

1.2. Record all information (including the oncologist's approval to undergo ovarian stimulation in case of cancer patients) in a relational database.

1.3. Provide the patient with specific counseling about the feasibility of the procedure. Explain the steps of the procedure (ovarian stimulation, oocyte retrieval, oocyte vitrification), and inform her about the realistic chances of success (mainly dependent on maternal age and expected number of MII oocytes at the time of oocyte retrieval), as well as the cost and limitations of the procedure.

1.4. Perform transvaginal ultrasound to obtain information on the ovarian reserve (i.e., AFC)

and to assess the accessibility of the ovaries for egg collection.

1.5. Request blood tests to assess blood group and Rhesus factor, coagulation screening (blood count, prothrombin, thromboplastin, fibrinogen, Protein C, Protein S, anti-thrombin III, homocysteine), and infectious diseases (Hepatitis B, Hepatitis C, HIV, Venereal Disease Research Laboratory/Treponema pallidum Hemagglutination Assay).

NOTE: In case of patients accessing a fertility preservation program for non-urgent medical reasons, a more comprehensive assessment may include basal follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, AMH, breast examination, Papanicolaou test, genetic screening of coagulation (factor V of Leiden and prothrombin), and TORCH screening (toxoplasmosis, rubella, cytomegalovirus).

1.6. Request a cardiological evaluation (electrocardiogram).

1.7. Recommend psychological counseling.

2. Controlled ovarian stimulation protocols for fertility preservation

NOTE: When the time available before starting the cancer treatment is limited, the random-start protocol (i.e., starting ovarian stimulation at any time during the menstrual cycle) is recommended for the ovarian stimulation in oncologic patients who are candidates for fertility preservation. In a fertility preservation program for non-urgent medical reasons or social reasons, conventional stimulation starting in the early follicular phase is preferable, and ovarian stimulation is started based on the menstrual cycle. Controlled ovarian stimulation (COS) approaches should be performed according to the recent European Society of Human Reproduction and Embryology (ESHRE) guidelines[24].

2.1. Tailor COS according to the patient's characteristics and ovarian reserve markers, mainly maternal age, FSH, AMH, and AFC.

2.2. Start COS on day 5 of the menstrual cycle using recombinant or urinary FSH with a fixed dose of 150–300 IU/day (antagonist protocol).

NOTE: In a specific patient population with LH deficiency or poor-suboptimal response, additional LH 75/150 IU/day may be administered according to the ovarian response, LH levels, and the gynecologist's judgement.

2.3. In patients with estrogen-sensitive diseases, include gonadotropins associated with aromatase inhibitors (letrozole) from day 1 of stimulation until day 7 after oocyte retrieval.

2.4. Administer a fixed dose of gonadotropins for 4 days.

2.5. Monitor follicular growth on day 5 and then every 2–3 days; eventually, adjust the

gonadotropin dosage.

2.6. Once at least 3 follicles reach 17–18 mm in diameter, administer the trigger for final oocyte maturation with a single subcutaneous bolus of GnRH agonist at the dose of 0.5 mL.

3. Oocyte retrieval

3.1. Preparing for oocyte retrieval

3.1.1. For materials required, refer to the **Table of Materials**, and keep ready laboratory footwear and outfit, surgical facemask, hair cover, surgical gloves, a permanent non-toxic marker, tweezers, sterile small gauzes, disposable or reusable speculum, vaginal surgery equipment and surface disinfectant. Ensure the availability of resuscitation equipment, anesthetic drugs for reversal, a kit prepared for the treatment of anaphylactic shock, and oxygen in the operating room.

3.1.2. Perform the oocyte retrieval procedure according to the recommendations of the ESHRE Working Group on Ultrasound in assisted reproduction technologies (ART)[25].

3.1.2.1. Administer sedation or general anesthesia, and antibiotics for prophylaxis.

NOTE: In this protocol, deep sedation was achieved by administrating propofol (whose dosage is adjusted according to the patient's weight) and 50–100 µg of fentanyl, 1000 mg of paracetamol, and assisted mask ventilation with oxygen.

3.1.2.2. Perform oocyte retrieval using an aspiration unit composed of a vacuum pump, a collection tube connected to a 17 G single-lumen needle, and an oocyte-collecting tube. During the collection, do not exceed a pressure of ~120 mmHg to avoid the risk of damage to the oocytes such as stripping off the cumulus cells or fracturing the zona pellucida.

3.1.2.3. Calibrate working surface temperature to ensure 37 °C in the culture media. During the whole procedure, minimize oocyte exposure to even transient temperature that may affect their developmental competence.

3.1.2.4. At the end of oocyte retrieval, observe the patient for 3–4 h before discharge.

3.2. Operation theater

3.2.1. Before entering the operating room, identify the patient and confirm the time of ovulation trigger.

3.2.2. Have the patient lie down on the operating table in a gynecological position.

3.2.3. Cleanse the vagina/cervix prior to oocyte retrieval to minimize bacterial contamination.

3.2.4. Perform a preliminary transvaginal ultrasound to assess the position of the ovaries and the anatomical relationships with the various organs and blood vessels.

3.2.5. Under ultrasound guidance, insert a single-lumen needle through the vaginal wall and into an ovarian follicle, taking care not to injure the organs or blood vessels located between the vaginal wall and the ovary.

3.2.6. Start aspiration from the closest follicle and moving on to the most distal ones.

3.2.7. Puncture all follicles of a diameter larger than 11–12 mm, performing "twisting movements" of the needle to aspirate the whole follicular fluid, which is then released into a sterile tube (round bottom 14 mL) preheated in the block heater of the operating room.

3.2.8. Immediately after retrieval, seal and label the tube with details of the patient's identity.

3.2.9. Ensure that the nurse brings the tube to the laboratory, where it is immediately screened for the presence of cumulus–oocyte complexes (COCs).

3.2.10. Instruct the embryologists to inform the clinician of the total number of COCs retrieved.

3.2.11. Once the procedure is complete for the first ovary, flush the needle with clean medium, and proceed with the second ovary using the same procedure.

3.2.12. After oocyte retrieval, evaluate any bleeding from the ovaries or blood vessels of the parametrium and free fluid in the pouch of Douglas.

NOTE: To automate and improve the effectiveness of gamete and embryo traceability at the clinical level, an electronic witnessing system (EWS) was implemented in the center[26]. Nevertheless, this protocol does not mention the EWS, to ensure reproducibility of the protocol in any IVF laboratory. Still, consider that all steps of the procedure require a second operator (i.e. a witness) to ensure gamete and embryo traceability.

4. IVF laboratory

4.1. The day before the oocyte retrieval procedure

4.1.1. Prepare oocyte collecting tubes (refer to the **Table of Materials**).

4.1.1.1. Dispense 1 mL of IVF medium (refer to the **Table of Materials**) in each oocyte collection tube (round bottom, 5 mL), and cover with 0.2 mL of mineral oil (refer to the **Table of Materials**) for embryo culture.

NOTE: The number of tubes will be defined according to the number of follicles expected to be

retrieved. Each tube might contain up to 4 COCs.

4.1.2. Seal the tubes with the cap (first snap). Label the tubes with details of the type of medium and date of preparation. Incubate the tubes overnight at 37 °C in a controlled atmosphere (6% CO_2, 5% 0_2).

4.2. On the day of the oocyte retrieval

4.2.1. Prewarm the plastic supplies at 37 °C (sterile culture dishes and Pasteur pipettes).

4.2.2. Ask the patients to confirm their identity (full name and date of birth) and the time of ovulation trigger. Annotate on the laboratory sheet that the identification (ID) procedure has been accomplished, and that the time of ovulation trigger has been confirmed.

4.2.3. Take the oocyte collection tubes off the incubator (right before the procedure begins), and push down the cap to ensure tight closure. Label the oocyte collection tubes with the patient's information. Place the oocyte collection tubes in the block heater at 37 °C.

4.2.4. Examine the follicular fluid in the prewarmed sterile culture dishes, and identify COCs. Once one or more COCs are identified, rinse them twice in two drops of medium to remove the follicular fluid and blood contamination.

4.2.5. Transfer the COCs to the oocyte collection tubes and annotate the number of COCs on the tube. Loosen the caps of the medium tubes, and promptly incubate them at 37 °C in an a atmosphere of 6% CO_2, 5% O_2.

4.2.6. Repeat steps 7 to 9 according to the number of oocytes retrieved. Update the laboratory sheet.

NOTE: Ensure that a witness checks that all the tube-warming blocks (including the ones in the operating theater) are empty and signs the closing of the procedure on the laboratory sheet.

4.2.7. Wipe down the working stage after completion of the procedure.

5. **Oocyte denudation**

5.1.1. Prewarm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered medium and hyaluronidase (refer to the **Table of Materials**) at 37 °C at least 1 h before starting.

5.1.2. Prepare a 4-well IVF plate (refer to the **Table of Materials**) with 0.6 mL/well of prewarmed HEPES-buffered medium (supplemented with 5% human serum albumin [HSA]) covered with 0.3 mL of mineral oil for embryo culture, and warm at 37 °C for at least 30 min.

5.1.3. Label the oocyte denudation plate with details of the patient's identity.

5.1.4. Immediately before starting the procedure, add hyaluronidase to the first well to obtain a final concentration of about 20 IU/mL.

5.1.5. Place a limited number of COCs (up to 6) in the first well containing the enzyme to disperse the cumulus cells.

5.1.6. To enhance enzymatic removal of the cumulus and corona cells, perform the stripping of cumulus cells by pipetting the oocytes repeatedly through a Pasteur pipette with an inner diameter of approximately 250 µm for up to 30 s. After initial cell dissociation is observed, transfer the oocytes to the second well containing only HEPES-buffered medium, taking care to carry over a minimum amount of the enzyme.

5.1.7. Perform further denudation to remove the corona cells by using denuding pipettes with decreasing inner diameters (170–145 µm). Use lower diameters (135µm) only if strictly necessary.

5.1.8. Carefully wash the denuded oocytes to wash out the enzyme.

5.1.9. After denudation, examine the oocytes under an inverted microscope to assess their integrity and stage of maturation. Separate MII oocytes from the immature ones (germinal vesicle and metaphase-I).

5.1.10. Move the MII oocytes to the vitrification area to immediately perform cryopreservation. Update the laboratory sheet.

6. Oocyte vitrification

NOTE: Perform oocyte vitrification preferably within 38 h of oocyte retrieval and immediately after denudation. The vitrification procedure described here has to be accomplished at room temperature (RT) and by using a stripper pipette with an inner diameter of 170 µm so as not to damage oocytes during manipulation.

6.1. Approximately 30 min before the procedure, bring the equilibration solution (ES) and vitrification solution (VS) to RT (25–27 °C).

6.2. Properly label the cryodevices with the patient's name and ID, treatment ID, date of freezing, number of oocytes, and cryobarcode.

6.3. Fill a disposable cooling rack up to the top with fresh liquid nitrogen, and start the sterilization process.

6.4. Label the vitrification plate (refer to the **Table of Materials**) with the patient's name and ID. Ask a witness to check the correct ID of the cryodevices.

6.5. Carefully invert each vial twice to mix its contents before use, and prepare the lid of a 6 mm Petri dish with one drop of 30 µL of HEPES-buffered medium (with 5% HSA) and one adjacent drop of 30 µL of ES.

NOTE: Place drops just before use to limit medium evaporation.

6.6. Using a 170 µm diameter stripper pipette, place the oocytes (up to 9) in the first drop with the smallest possible volume of medium. Using the stripper pipette, create a bridge of medium between the drop n.1 and n.2 to obtain a gradual increase in concentration of the CPAs (**Figure 1A**).

6.7. Incubate the oocytes in the first drop for 3 minutes. Add a third drop of 30 µL of ES (n.3). After 3 min, transfer the oocytes into the second drop of ES, and create a medium bridge between drops n.3 and n.2 (**Figure 1B**). Incubate the oocytes in drop n.3 for 3 min.

6.8. During the incubation, add one 30 µL drop of ES for each cryodevice that will be used (if 9 oocytes are to be cryopreserved, place 3 drops of ES with 3 oocytes in each drop of ES). Move the oocytes into pure ES solution, and leave them for 6–9 min (until they recover their initial size after shrinkage) (**Figure 1C**).

6.9. Prepare a central well dish (60 x 15 mm) with 1 mL of VS. At the end of the first 6 min, transfer the oocytes to be cryopreserved into the VS solution, releasing as little medium as possible. Leave the oocytes in VS for 1 min, and wash them carefully by moving them from the bottom to the top of the dish to remove the excess of ES.

6.10. Approximately 10 s before the end of the minute of incubation, place the cryodevice under the microscope, and adjust the focus on the black mark (i.e., the tip of the cryodevice). Place the oocytes on the cryodevice beside the black mark with the minimum amount of VS (**Figure 2A**).

6.11. Move the stripper pipette away from the oocytes, and remove the excess of VS medium (**Figure 2B**) so that the oocytes remain covered by a thin layer of medium (**Figure 2C**).

6.12. Quickly plunge the cryodevice in liquid nitrogen, rapidly shaking it to remove air bubbles from its surface. Hold the protective cap of the cryodevice with tweezers, and fill it with liquid nitrogen; then insert the cryodevice into it while keeping the propylene strips in liquid nitrogen.

6.13. Store the cryodevice in a visiotube previously labeled with the patient's information. Update the laboratory sheet.

7. Oocyte warming

7.1. Approximately 30 min before the procedure, warm the thawing solution (TS), dilution

solution (DS), and washing solution (WS) to RT (25–27 °C). Carefully invert each vial twice to mix its contents. Place 1 mL of TS in a central well Petri dish, and warm it at 37 °C for at least 1 h before starting the procedure.

7.2. Label all plastic supplies with the patient's name and ID and the type of solution. Ask a witness to confirm the patient's information on the cryodevice.

8. For each cryodevice to be warmed, prepare a 6-well plate with 200 µL of DS in the first well and an equal amount of WS in the second and third ones (named WS1 and WS2, respectively). Add phosphate buffered saline (PBS) or sterile water in the area outside the wells to prevent evaporation.

7.4. Take the TS dish out from the incubator, and place it under the microscope. Adjust the focus of the microscope to the center of the Petri dish.

7.5. Carefully twist and remove the protective cap of the cryodevice, while keeping the propylene strips in liquid nitrogen. Transfer the cryodevice from liquid nitrogen into the TS as quickly as possible to avoid the risk of devitrification and initiate the countdown (1 min).

7.6. Localize the oocytes by focusing on the tip of the cryodevice (i.e., the black mark). Using a stripper pipette, release the oocytes from the cryodevice.

NOTE: Try not to aspirate the oocytes from the cryodevice; gently release some media on them until they move into the TS.

7.7. Using a 170 µm diameter stripper pipette, transfer the oocytes to DS with a little amount of TS (to create a gradient), and leave them in the DS for 3 min. Move the oocytes to WS1 likewise, and leave them for 5 min. Finally, transfer the oocytes to WS2 for 1 min.

7.8. Transfer the oocytes into an appropriate preequilibrated IVF culture medium, and incubate them for 1 h before proceeding with intracytoplasmic sperm injection (ICSI). Update the laboratory sheet.

REPRESENTATIVE RESULTS:

Overview of the fertility preservation program at the center

Over a 12-year period (2008–2020), 285 women underwent at least one oocyte retrieval entailing the vitrification of the whole cohort of mature eggs collected. Most of these women (n=250) underwent a single retrieval, and 35 underwent multiple retrievals. The reasons for undergoing oocyte retrieval for egg vitrification are summarized into 4 categories: medical (except for cancer), cancer, non-medical, and others. Among the 250 women undergoing a single oocyte retrieval for egg vitrification, 8% had medical reasons other than cancer (10 endometriosis, 3

myoma, 4 ovarian cysts, 1 hydrosalpinx), 16% had cancer (31 breast cancer, 3 ovarian cancer, 2 colorectal cancer, 2 Hodgkin's lymphoma, 1 vulvar cancer e 1 cervical cancer), 53% had non-medical reasons, and 23% had other reasons (43 absence of sperm retrieved, 10 risk for OHSS, 4 infections, and 1 fever). This distribution was different among patients undergoing multiple oocyte retrievals for egg vitrification. Specifically, 9% had medical reasons other than cancer (1 endometriosis, 2 reduced ovarian reserve), 6% had cancer (2 breast cancer), 80% had non-medical reasons, and 3% had other reasons (1 absence of sperm retrieved). None of the patients undergoing multiple oocyte retrievals for egg vitrification warmed those eggs, while 78 of the 250 women undergoing a single egg vitrification cycle returned to use those oocytes (**Figure 3**).

Table 1 summarizes the data of the 250 women undergoing a single oocyte vitrification cycle clustered according to the related reasons. The patients with a medical reason for egg vitrification and the patients undergoing fertility preservation because of cancer were younger (mean maternal age < 35 years) and showed a better ovarian reserve (higher AFCs) than the patients with non-medical or other reasons. However, the mean maturation rates (number of MII oocytes/number of COCs retrieved) were slightly lower (72–73% versus 77–79%), so that the number of oocytes vitrified on average was similar in the 4 groups (9–10 oocytes). Importantly, 9 out of 40 oncologic patients (22.5%) underwent a random-start ovarian stimulation protocol because they had limited time before starting chemo- or radiotherapy. It is interesting that approximately half of the patients with medical reasons other than cancer (53%) and the majority of patients with other reasons for egg vitrification (76%) actually returned for warming. Conversely, very few patients who underwent fertility preservation for cancer (17.5%) or non-medical reasons (13%) used their vitrified oocytes for IVF. Also remarkable is the time elapsed between vitrification and warming among the patients who returned: on average, 283 days in patients with medical reasons other than cancer, 132 days in patients with other reasons, 1264 days in oncologic patients, and 1547 days in patients with non-medical reasons. Regardless of all these relevant differences, the survival rate was similar (83–88%; on an average, 8–11 oocytes were warmed, and 7–9 oocytes survived) between the patients in the 4 groups, thereby confirming the efficacy and safety of oocyte vitrification and warming protocols. Moreover, the survival rate is independent of vitrification and the warming operator's experience (**Figure 4A,B**). **Table 1** shows the fertilization rates in the 4 groups, which are ~70%, except for the patients with non-medical reasons for oocyte vitrification (~80%). However, these data are not comparable owing to a small sample size and the bias of the sperm factor on the fertilization outcome[27].

FIGURE AND TABLE LEGENDS:

Figure 1: Medium droplet configuration for oocyte cryopreservation. To gradually perform equilibration, oocytes are first placed in a (**A**) drop of BS and mixed with (**B**) a drop of ES**.** After 3 min of incubation, (**C**) a third drop of ES solution is mixed**,** and oocytes are incubated for 6–9 min. Abbreviations: HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BS = HEPES-buffered medium; ES = equilibration solution.

Figure 2: Oocyte loading on cryopreservation device. The oocytes are placed on the cryodevice

in (**A**) a single small drop of VS. (**B**) The stripper pipette is shifted away from the oocytes**,** and (**C**) the excess of VS is re-aspirated to leave just a thin layer around each oocyte. Abbreviation: VS = vitrification solution.

Figure 3: Oocyte vitrification cycles performed at the GENERA center for reproductive medicine of Rome (years 2008–2020). Over the 12-year period, 250 patients underwent a single oocyte retrieval for oocyte vitrification, while 35 underwent multiple oocyte retrieval cycles. The inherent reasons for oocyte vitrification are shown in the figure. The patients returning for warming (n=78) belong only to the group of women who underwent a single oocyte vitrification cycle.

Figure 4: Mean survival rate per cohort of warmed oocytes. (**A**) Vitrification and (**B**) warming operators' experience. Each patient is included only for the first warming cycle. Statistically significant differences were assessed using Mann-Whitney *U*-test.

Figure 5: Oocyte morphology at the beginning and the end of the equilibration procedure. To determine the outcome of the equilibration procedure, it may be useful to annotate (**A**) oocyte morphology before starting the procedure. (**B**) A sharp shrinkage of the oocyte is observed after first exposure to the cryoprotectant solution. The equilibration procedure can be considered complete when (**C**) the oocyte has recovered its initial volume. Scale bars = 25 µm.

Table 1: Patients undergoing a single cycle of oocyte vitrification. Abbreviations: FSH = follicle stimulating hormone; AMH = anti-Müllerian hormone; BMI = body mass index; COC = cumulus oocyte complexes; MII = metaphase-II; N = normozoospermic; MMF = moderate male factor (1–2 sperm defects); OAT = oligoasthenoteratozoospermic; NOA = non-obstructive azoospermia (sperm were collected through testicular sperm extraction).

DISCUSSION:

Clinical considerations

Although emerging strategies, such as ovarian tissue cryopreservation and in vitro maturation, have been explored, oocyte vitrification after COS is the gold standard technique for fertility preservation. In this scenario, the number of oocytes retrieved and cryopreserved should be maximized in the shortest possible time as most cancer patients might benefit solely from one ovarian cycle before they have to commence their cancer treatment(s). Thus, a proper ovarian stimulation protocol is crucial to fully exploit the ovarian reserve and increase the cumulative chance of a future live birth[28], an outcome strongly dependent on maternal age at oocyte retrieval. In this regard, an ideal age for oocyte vitrification for fertility preservation purpose has not been proposed yet[29], and a consensus is required between either 35 years[18] or 37 years[30,31]. However, fertility preservation should be accessible for all patients, including women older than 37 years, provided that they are given proper counselling on the success rates based on their age.

The protocol and starting dose of medications should be outlined according to the gynecologist's judgment and most importantly, based on the time available[32,33]. Starting conventional stimulation in the early follicular phase is recommended when time is not an issue. However, if required, the random-start approach is feasible to minimize delays in commencing urgent cancer/medical treatments[34,35]. Follicular development has been reported to be an extremely dynamic process as multiple waves of follicle recruitment have been described throughout a single ovarian cycle. Although the biological mechanisms behind this phenomenon still need to be unveiled, competent oocytes can be retrieved and cryopreserved independently of the phase of the menstrual cycle in which COS is started[36].

In this center, ovarian stimulation is typically performed using the GnRH antagonist protocol and 150–300 IU/day of recombinant-FSH or human menopausal gonadotropin. In specific populations of patients older than 35 years, with LH deficiency, or showing suboptimal response after standard COS, LH might be added during COS to increase the recruitment and growth of follicles through a synergic action with insulin-like growth factor 1[16,37,38]. Moreover, the GnRH antagonist protocol followed by a GnRH agonist trigger has been reported to be a short, safe, and highly convenient stimulation protocol to maximize ovarian response and minimize the risk for OHSS[39]. In patients with estrogen-sensitive diseases such as breast cancer, gonadotropins associated with aromatase inhibitors, such as letrozole, are administered[20].

In fact, a recent review showed that letrozole involves similar ovarian response as conventional stimulation with no complications in terms of congenital defects, malignancy recurrence, and increased mortality[40]. Similarly, tamoxifen might be used during COS in case of estrogen-sensitive tumors, which, like letrozole, showed no impact on oocyte competence[41,42]. However, this needs to be confirmed in larger studies. In this center, letrozole is administered from day 1 of stimulation up to day 7 after oocyte retrieval in case of estradiol-sensitive tumors. The risk for OHSS, which is the most important complication of COS that would also further delay anticancer treatments, should be considered, particularly in young patients with high AFCs. In these cases, triggering ovulation with a GnRH agonist instead of human chorionic gonadotropin (hCG) has proven to be beneficial. Other issues that must be prevented are (i) thrombo-embolism, which requires the administration of low-molecular weight heparin during COS, and (ii) a reduced ovarian response after COS[43]. To compensate for the latter, two consecutive stimulations in a single ovarian cycle might be performed. This novel unconventional COS protocol, known as DuoStim, entails follicular and luteal phase stimulations and two oocyte retrievals in a short timeframe (~15 days) and should be investigated for fertility preservation purpose in the future[44-46].

Other possible strategies for fertility preservation in women are: (i) ovarian tissue cryopreservation, which is the only option available for prepubertal females. This possibility is promising for restoring reproductive and endocrine activity and avoiding delay in starting cancer treatment as no hormonal stimulation is needed. However, it is still experimental and requires laparoscopic surgery with later transplantation, and there is the risk for orthotopic cancer retransmission. (ii) Embryo cryopreservation, which involves a higher survival rate after warming, but delays cancer treatment and requires a male partner or donor to be involved, thereby also

limiting a woman's future reproductive autonomy[47]. Moreover, it might be subject to legal limitations in some countries. Considering these limitations, oocyte vitrification is considered the most established and ethically acceptable approach. Ideally, all major hospitals, where women affected from cancer in their reproductive age are treated, should provide programs for fertility preservation, and the whole process should be free of charge for these patients to ensure equal access to this program. Still, oocyte cryopreservation must be performed solely in centers with enough expertise not to affect oocyte competence in the process[48].

Critical steps in the vitrification protocol and troubleshooting

Vitrification is a pseudo second-order phase transition (IUPAC Compendium of Chemical Terminology) that induces a glass-like solidification inside the cells preventing ice crystal nucleation and growth, the main causative factor of cell injury. To achieve proper vitrification, a combination of at least two CPAs is required (typically ethylene glycol and DMSO as permeating agents and sucrose as the non-permeating agent) along with an extremely high cooling rate (>20,000 °C/min) achieved either by minimizing loading volumes or by direct exposure of the samples to liquid nitrogen (open devices). As oocytes are the largest cells of the body, they contain the largest amount of water. Therefore, they are more sensitive to freezing injuries than embryos. During oocyte cryopreservation, damage to intracellular organelles (e.g., the cytoskeleton or meiotic spindle), alteration of membrane permeability, zona pellucida hardening, oocyte activation, alteration of the biochemical pathways, and possibly cell death can occur[49]. For this reason, a delicate balance between multiple factors must be ensured for successful preservation of oocyte viability and developmental competence. To achieve consistency in the survival rate after vitrification and reach the benchmark levels, all the crucial steps of the procedure must be strictly controlled.

CPAs, cell osmolality, and cooling rates

To increase the probability of vitrification, the viscosity of the medium (and therefore CPA concentration) must be maximized. However, the toxicity of the CPAs should always be kept under control[50]. To this end, it is crucial to minimize both the time of cell exposure to the CPAs and the loading volumes, and always work at room temperature (25–27 °C)[51]. Other protocols involve different combinations of CPAs and cryodevices and are carried out at 37 °C; however, they have not been described here. Thus, the notes, representative results, and discussion sections of this manuscript only apply to the vitrification protocol detailed here.

During cryopreservation, the oocytes are exposed to CPA solutions of increased osmolyte concentration to promote cell dehydration and cytoplasmic shrinkage. During warming, they are exposed to solutions with decreased osmolyte concentration to restore the cytoplasmic volume. During vitrification, the oocytes are dehydrated, and unintentional fluctuations in cell volume can cause severe osmotic shock, thereby compromising survival and developmental potential after warming[52]. Finding the optimal cooling rate is a key aspect for preserving cell viability as it affects the osmolality[53] and cell developmental potential[54]. For instance, when a cell is exposed to

cooling rates slower than the optimal rates, it may be extensively exposed to hypertonic conditions leading to cell death.

To achieve the optimal cooling rate, the loading volumes should be minimized, RT should be controlled so as not to affect the speed of the equilibration process, and samples should be directly exposed to liquid nitrogen. One way to assess when equilibration has been accomplished is to annotate the morphology of the oocyte (in particular, the width of the perivitelline space and the thickness of the zona pellucida) before the beginning of the procedure (**Figure 5A**). After an initial phase of cell volume reduction (**Figure 5B**), the oocyte is expected to recover its initial volume (**Figure 5C**). Although correct pH is maintained during oocyte handling under air atmosphere because of the zwitterions buffering the vitrification-warming solutions, medium osmolality is instead particularly dependent on temperature[53]. Therefore, the method of dish preparation is critical to reduce any possible detrimental effect[55].

Oil overlay is certainly pivotal to prevent evaporation and avoid any increase in osmolality in the IVF media[56]. However, it cannot be used while performing the vitrification and warming procedures. Therefore, some tips are important for operators: (i) prepare the droplets as quickly as possible and immediately before use; (ii) consider larger volumes of droplets to minimize shifts in osmolarity (<30 µL is not recommended); (iii) when environmental conditions cannot be standardized, a 6-well plate can be used, and sterile water or PBS can be added to the adjacent reservoir to limit evaporation; (iv) pay attention to the date of the first opening of the vial of medium as osmolality can change if the bottle is repeatedly opened.

Oocyte warming and oocyte devitrification

The most crucial step that may affect the consistency in the survival rate is definitely the warming process[57]. During this step, CPAs are gradually removed from the oocyte and diluted to prevent any potential cytotoxic effect. Devitrification, namely the formation of ice nuclei or ice crystals during the warming of a vitrified solution or accidentally during audit, transport, or storage in vapor, is one of the main risks of vitrification[58-60]. Therefore, to prevent devitrification and injury during warming, the difference in temperature between the first and last steps of the process must be maximized. As shown by Seki and Mazur[57] in murine oocytes subject to vitrification and warming at different rates, the faster the warming, the higher the survival. Volume and temperature of the TS are the main factors to control: TS should be properly warmed to 37 °C (at least 1 h before the procedure), and the liquid nitrogen rack should be filled to the edge of its capacity and placed as close as possible to the stereomicroscope. The operator should be as fast as possible while transferring the cryopreservation carrier from the liquid nitrogen to the TS. Because of the high efficiency of vitrification, a universal warming protocol can be used irrespective of the freezing protocol involved, making the management of all the warming cycles easier even when oocytes are imported from a different IVF center[61].

Open devices and risk of contamination

The employment of open systems and direct exposure of the samples to liquid nitrogen are

required to achieve the extremely high cooling and warming rates that support the effectiveness of this protocol. Although vitrification is a procedure that poses low risk for cross-contamination, because it involves very small volumes and is carried out after several sample washings that dilute any putative viral load, it is essential to adopt all precautionary measures to increase its safety. Based on current evidence, closed systems do not offer a competitive alternative to open systems at least for oocyte vitrification[62,63]. To maintain the effectiveness of open vitrification systems while minimizing the risks associated with direct contact with liquid nitrogen, the latter might be sterilized by ultraviolet irradiation[64,65]. Alternatively, vapor storage tanks might be used, which are known to pose a lower risk of contamination than conventional ones, but have proven to be effective in preserving oocyte viability[66,67].

Importance of constant monitoring of key performance indicators for oocyte vitrification programs

In an IVF laboratory, the monitoring of key performance indicators (KPIs) is essential for monitoring and constantly improving results[68]. In general, when defining KPIs for monitoring processes and procedures, three main areas should be considered: structure, process, and outcome. Structural KPIs measure the quality of the IVF laboratory by outlining the characteristics of physical and human resources. An example of a structural KPI related to the facility in a cryopreservation program might be the number of cryotanks relative to the total number of ART procedures requiring cryopreservation conducted in a given time period or the number of cryo-tanks relative to the total square meters of the cryoroom. However, human resources and in particular, operator skills are of utmost importance when dealing with a delicate procedure such as vitrification. In fact, although extremely effective, the vitrification technique is a challenging procedure involving several procedural phases with stringent timings that must be strictly controlled: a very small amount of a significantly viscous medium should be managed over a very short period.

No freezing machine with specific cooling parameters setting is involved in the procedure; therefore, standardization of protocol details and training are essential. The manual process has strict skill requirements that must be fulfilled to obtain consistent and highly reproducible cell survival rates. Specific training should be provide to each novice operator to make them proficient in controlling all the critical points of the procedure, in particular, the exposure time of the samples to CPAs and the handling of the cells in a highly viscous medium. However, according to Dessolle and coauthors,[69] the learning curve for the vitrification procedure should not be so long even for junior embryologists as the achievement of proficiency is mainly limited by manipulation challenges. Once expertise in vitrification has been achieved, the performance of each individual operator must be accurately and regularly verified through the use of KPIs to regularly assess the maintenance of competency values established by consensus papers[70]. Moreover, operator confidence/competence must be comparable so as to not affect the outcomes.

Process KPIs measure how well the IVF laboratory works. Vitrification and warming protocols and procedures should be conducted in a timely manner, and fluctuations in culture conditions

should be minimized by paying particular attention to maintaining adequate osmolarity and temperature for the preservation of not only oocyte developmental competence, but also of operator safety while handling liquid nitrogen. Examples of process KPIs are the percentage of laboratory staff injuries while handling liquid nitrogen per number of IVF procedures per year, the percentage of gametes/embryos lost/damaged during vitrification/warming procedures, and audits per number of procedures per year.

Finally, outcome KPIs measure the effectiveness of the IVF laboratory and generally refer to postwarming oocyte survival defined as the proportion of morphologically intact oocytes at the time of ICSI (in case of oocyte vitrification, the competency value should be >50% and the benchmark value is 75%)[70]. Additionally, the rates of fertilization (<10% lower than the fresh oocytes inseminated at the center from a comparable patient population), embryo development (the same as a comparable patient population using fresh oocytes), and implantation (<10–30% lower than a comparable population of fresh embryos at the center) are applicable as outcome KPIs for vitrified oocytes[70]. However, clinical outcomes are more subject to couple-specific characteristics than to faulty clinical procedures[71,72].

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Figure 1

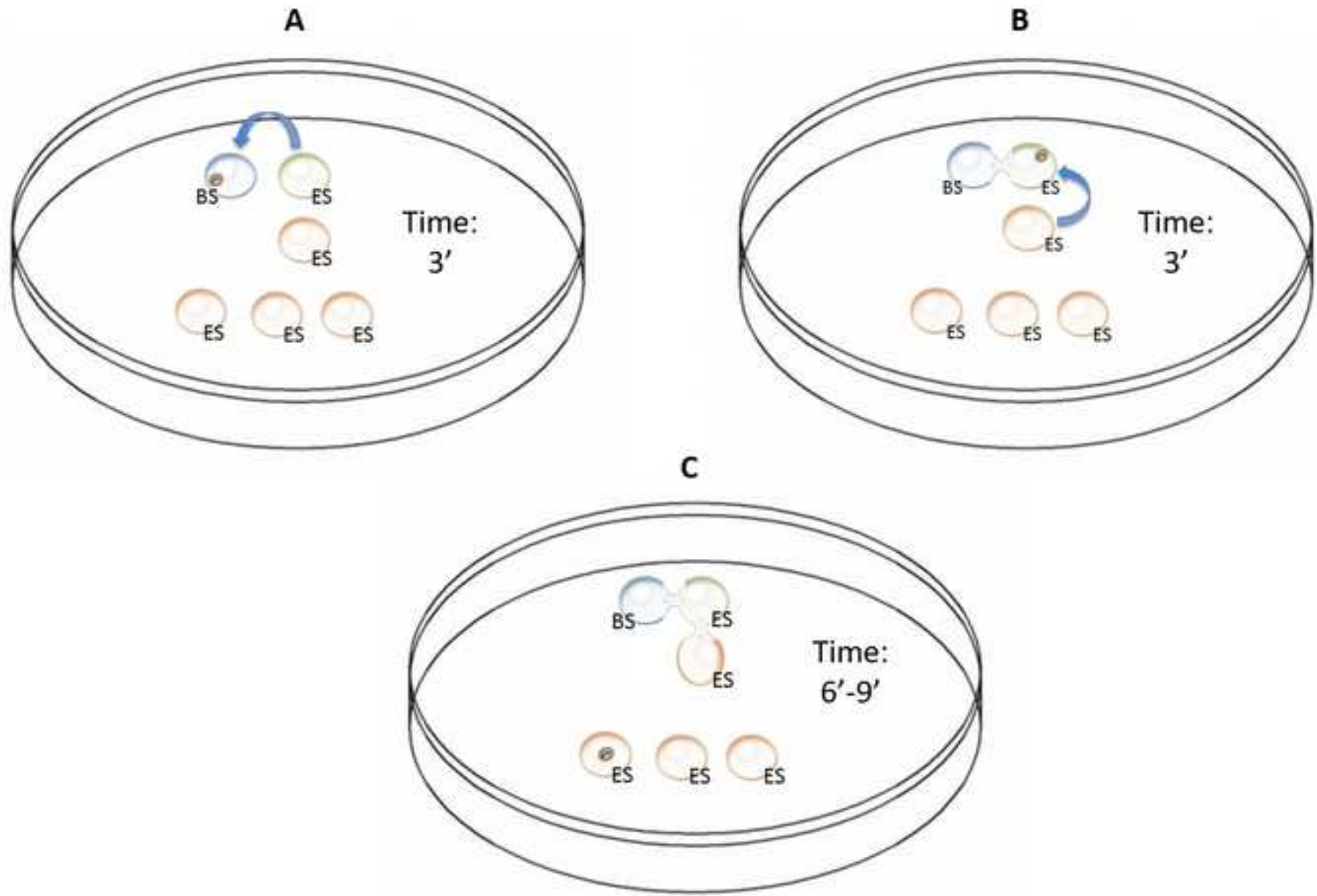


Figure 2

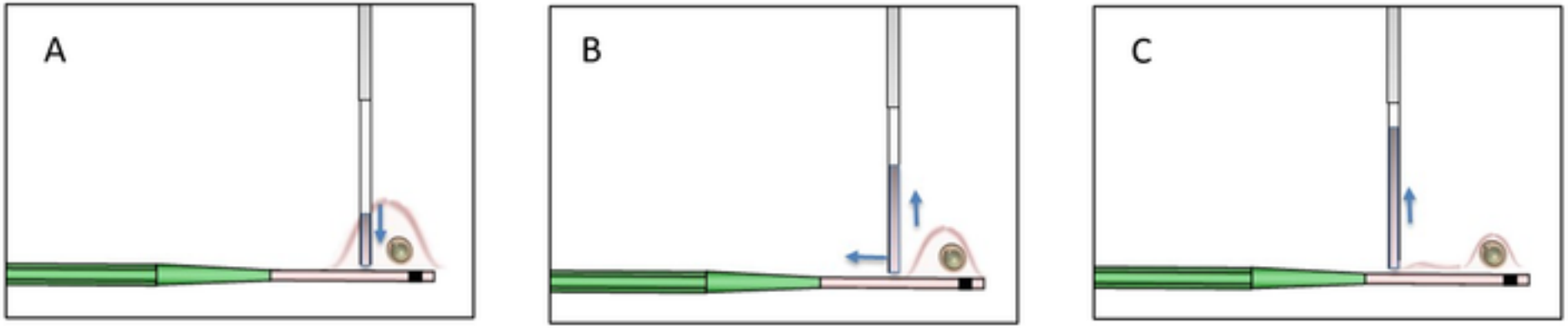


Figure 3

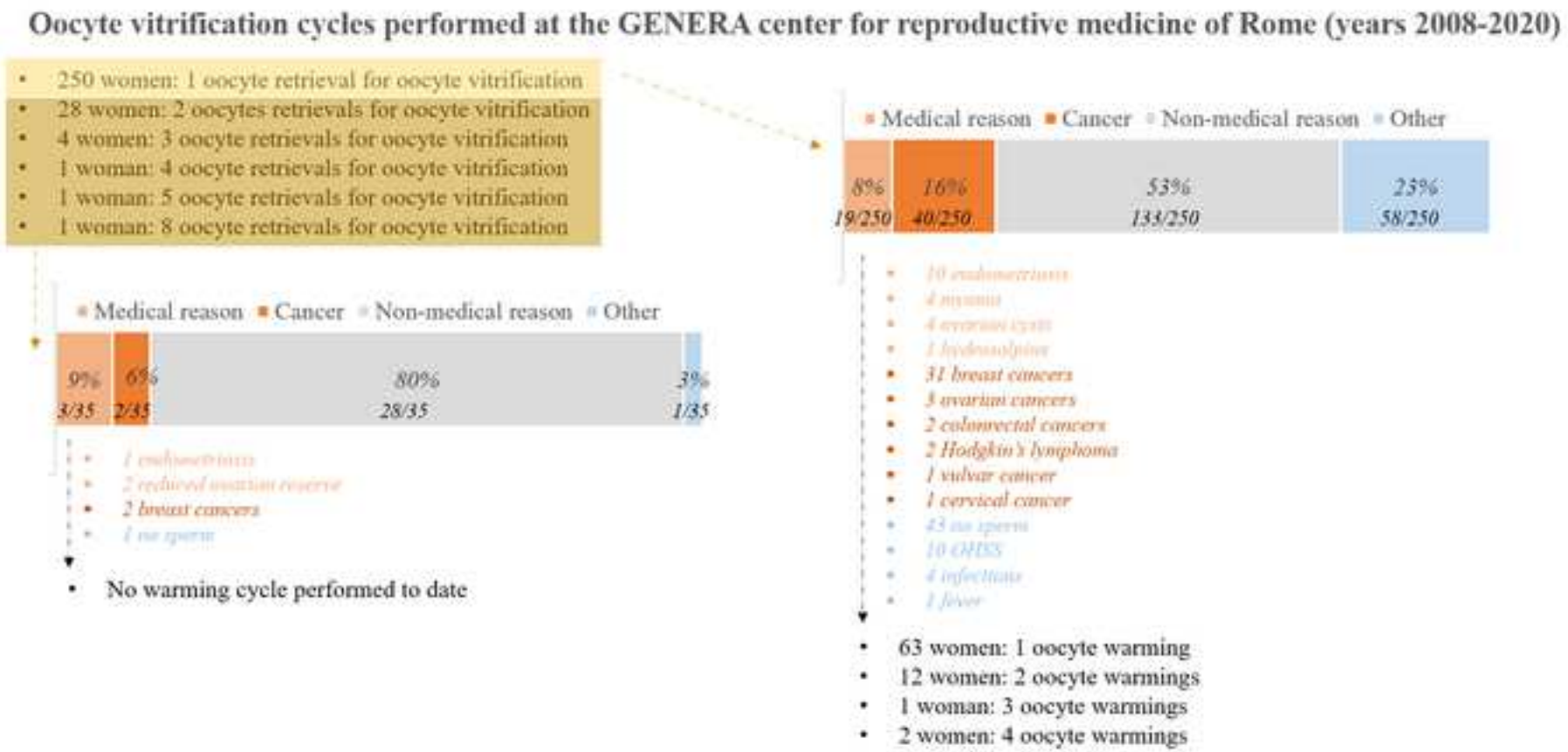


Figure 4

Click here to access/download;Figure;Figure 4.tif

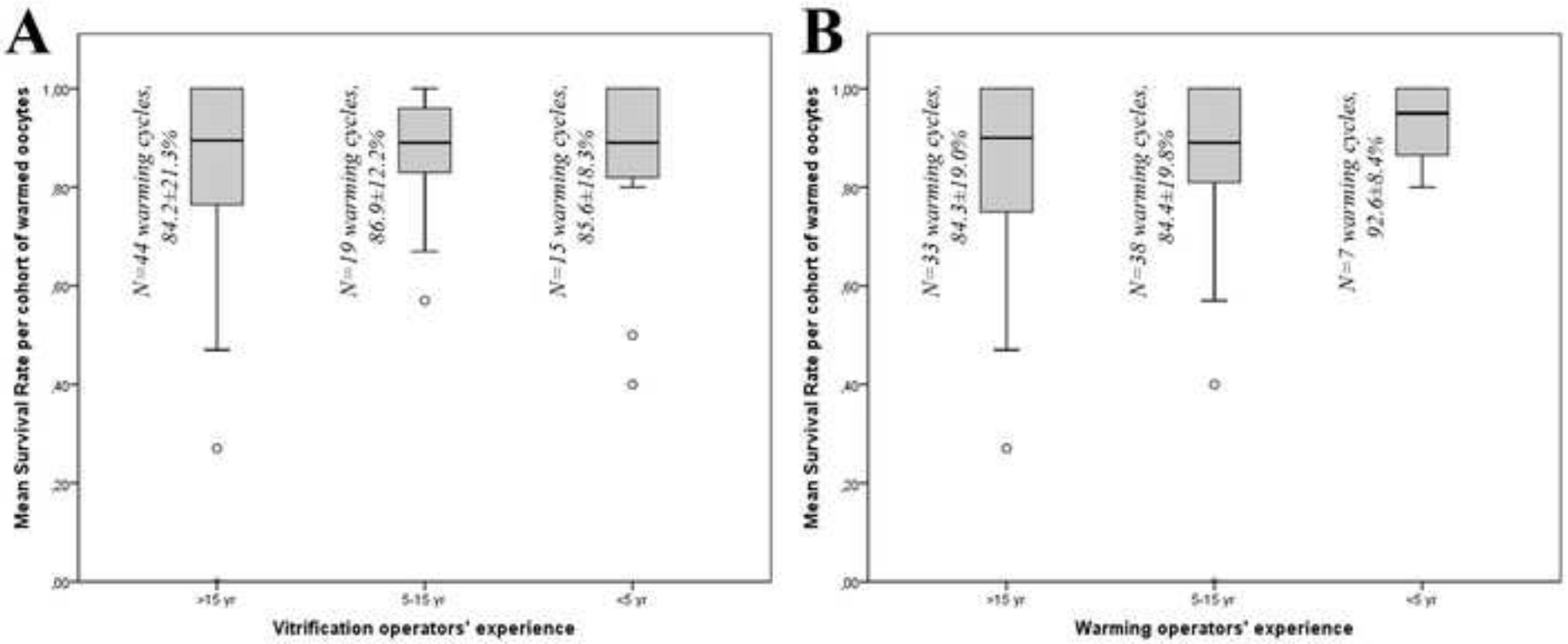
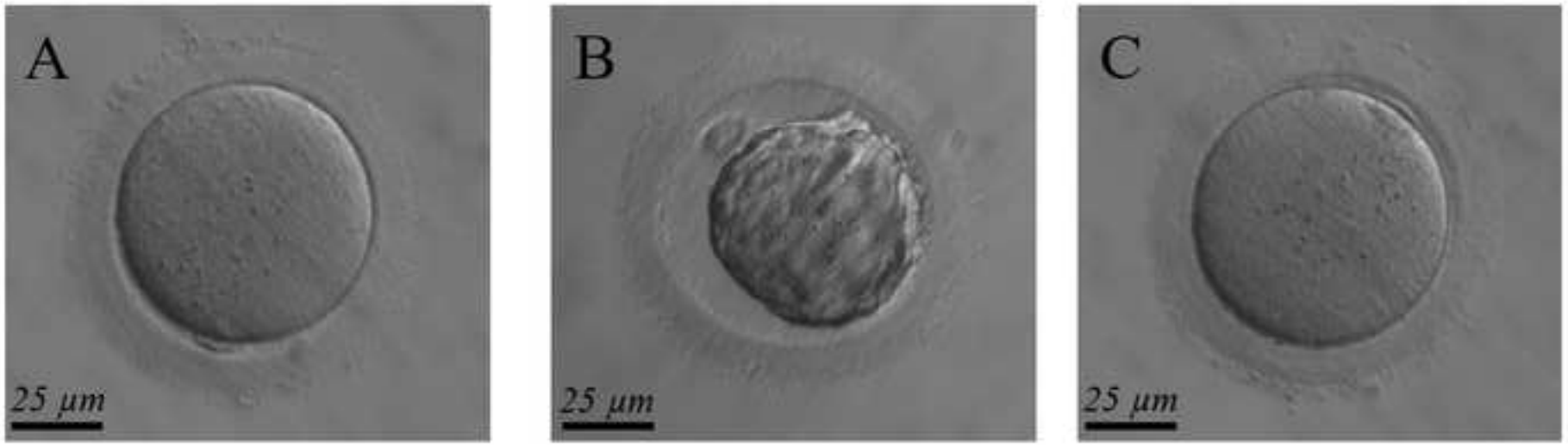


Figure 5



Reason for oocyte vitrification
Maternal age at oocyte retrieval, mean±SD
Basal FSH, mean±SD
AMH, mean±SD
AFC, mean±SD
BMI, mean±SD
Duration of stimulation, mean±SD
COCs, total, mean±SD
MII oocytes, total, mean±SD
Maturation rate, mean±SD
Patients returning for warming, total % of patients who underwent vitrification
Days between vitrification and first warming, mean±SD
Warmed MII oocytes, total mean±SD
Survived MII oocytes, total mean±SD
Survival rate, mean±SD
Sperm Factor
N, n, %
MMF, n, %
OAT, n, %
NOA, n, %
Fertilization rate, mean±SD

Medical other than cancer (n=19)	Cancer (n=40)	Non-medical (n=133)	Other (n=58)
33.6±6.4 years	34.6±5.9 years	37.0±3.4 years	38.2±4.3 years
7.5±4.2 IU/L	7.6±1.7 IU/L	7.8±4.1 IU/L	8.8±5.2 IU/L
1.8±1.7 ng/mL	1.9±0.2 ng/mL	2.2±2.4 ng/mL	3.0±2.5 ng/mL
12.7±5.0	13.3±7.0	11.6±6.1	10.7±6.7
20.1±1.5	21.8±1.6	20.2±3.1	22.9±3.6
10.0±1.5	9.6±1.7 *9/40, 22.5%* *Random start*	9.9±2.1	10.3±2.1
239	551	1493	592
12.6±5.3	13.8±9.1	11.2±7.1	10.2±8.5
167	400	1161	462
8.8±3.3	10.0±7.2	8.7±5.7	8.0±6.7
72.4±13.6%	72.1±19.6%	79.5±17.5%	77.4±22.2%
10/19, 52.6%	**7/40, 17.5%**	**17/133, 12.8%**	**44/58, 75.9%**
283±225	1264±764	1547±709	132±203
82	66	191	409
8.2±2.8	9.4±4.8	11.2±5.8	9.3±7.2
73	55	160	345
7.3±3.3	7.9±4.0	9.4±4.8	7.8±6.9
87.6±18.1%	83.2±1.7%	85.4%±14.5%	84.7±21.9%
5/10, 50%	4/7, 57%	13/17, 76%	13/44, 29%
3/10, 30%	3/7, 43%	3/17, 18%	17/44, 39%
2/10, 20%	-	1/17, 6%	11/44, 25%
-	-	-	3/44, 7%
70.0±14.4%	70.0±17.0%	78.5±20.6%	71.0±24.3%

Name of Material/ Equipment	Company	Catalog Number
Collection		
Equipment		
Hot plate	IVF TECH	
Lab Markers	Sigma Aldrich	
Laminar Flow Hood	IVF TECH	
Stereomicroscope	Leica	Leica M80
Thermometer		
Test tube Warmer		
Tri-gas incubator	Panasonic	MCO-5M-PE
Vacuum Pump	Cook	K-MAR-5200
Consumables		
CSCM (Continuos single culture complete) medium	Fujifilm Irvine Scientific	90165
Mineral Oil for embryo culture	Fujifilm Irvine Scientific	9305
Ovum Aspiration Needle (Single lumen)	Cook	K-OSN-1730-B-60
Primaria Dish	Corning	353803
Round- bottom tubes	Falcon	352001
Round- bottom tubes	Falcon	352003
Rubber Bulb	Sigma Aldrich	Z111589-12EA
Sterile glass Pasteur pipettes	Hunter Scientific	PPB150-100PL
Denudation		
Equipment		
CO2 incubator	Eppendorf	Galaxy 14S
Flexipet adjustable handle set	Cook	G18674
Gilson Pipetman	Gilson	66003
k-System Incubator	Coopersurgical	G210Invicell
Lab Markers	Sigma Aldrich	
Laminar Flow Hood	IVF TECH	
Stereomicroscope	Leica	Leica M80
Consumables		
Biopur epTIPS Rack	Eppendorf	30075331
Human Serum Albumin	thermoFisher Scietific	9988
Hyaluronidase	Fujifilm Irvine Scientific	90101
IVF culture dish (60 x 15mm)	Corning	353802
IVF dish 4-well plate with sliding lid	ThermoFisher Scietific	176740
IVF One well dish	Falcon	353653
Mineral Oil for embryo culture	Fujifilm Irvine Scientific	9305
Modified HTF Medium	Fujifilm Irvine Scientific	90126
Rubber Bulb	Sigma Aldrich	Z111589-12EA
Sterile glass Pasteur pipettes	Hunter Scientific	PPB150-100PL
stripping pipette tips (140 µm)	Cook	K-FPIP-1140-10BS

stripping pipette tips (130 µm)	Cook	K-FPIP-1130-10BS
stripping pipette tips (170 µm)	Cook	K-FPIP-1170-10BS

Vitrification

Equipment

Electronic Timer		
Flexipet adjustable handle set	Cook	G18674
Gilson Pipetman	Gilson	F123601
Lab Markers	Sigma Aldrich	
Laminar Flow Hood	IVF TECH	
Stainless Container for Cooling Rack	Kitazato	
Stereomicroscope	Leica	Leica M80

Consumables

Biopur epTIPS Rack	Eppendorf	30075331
Human Serum Albumin	Fujifilm Irvine Scientific	9988
IVF culture dish (60 x 15 mm)	Corning	353802
IVF dish 6-well	Oosafe	OOPW-SW02
Modified HTF Medium	Fujifilm Irvine Scientific	90126
stripping pipette tips (170 µm)	Cook	K-FPIP-1170-10BS
Vitrification Freeze kit	Fujifilm Irvine Scientific	90133-so
Vitrifit	Coopersurgical Origio	42782001A

Warming

Equipment

Electronic Timer		
Flexipet adjustable handle set	Cook	G18674
Gilson Pipetman	Gilson	F123601
k-System Incubator	Coopersurgical	G210Invicell
Lab Markers	Sigma Aldrich	
Laminar Flow Hood	IVF TECH	
Stainless Container for Cooling Rack	Kitazato	
Stereomicroscope	Leica	Leica M80

Consumables

Biopur epTIPS Rack	Eppendorf	30075331
CSCM (Continuos single culture complete) medium	Fujifilm Irvine Scientific	90165
IVF culture dish (60 x 15 mm)	Corning	353802
IVF dish 4-well plate with sliding lid	ThermoFisher Scietific	176740
IVF dish 6-well	Oosafe	OOPW-SW02
Mineral Oil for embryo culture	Fujifilm Irvine Scientific	9305
SAtripping pipette tips (300µm)	Cook	K-FPIP-1300-10BS
Vitrification Thaw kit	Fujifilm Irvine Scientific	90137-so

Comments/Description

Grade A air flow

02/CO2

IVF culture medium supplemented with HSA

Corning Primaria Dish 100x20 mm style standard cell culture dish
Falcon 14ml Round Bottom Polystyrene Test tube with snap cap
Oocyte collection tubes/ Falcon 5ml 12x75 Round Bottom Polipropilene Test tube with snap cap

Pipette Pasteur Cotonate, 150mm, MEA e CE

Stripper holder
p20

Grade A air flow

Micropipettes epTIPS Biopur 2-200 µl

80 IU/mL of hyaluronidase enzyme in HEPES-buffered HTF
Corning Primaria Falcon Dish 60X15mm TC Primaria standard cell culture dish
Multidishes 4 wells (Nunc)
Falcon 60 x 15 mm TC treated center-well IVF

HEPES-Buffered medium
1 mL for pasteur pipettes
Pipette Pasteur Cotonate, 150 mm, MEA e CE
PIPETTE FLEXIPETS PER DENUDING

PIPETTE FLEXIPETS PER DENUDING
PIPETTE FLEXIPETS PER DENUDING

Stripper holder
p200

Grade A air flow
Liquid nitrogen container for vitrification

Micropipettes epTIPS Biopur 2-200 µL

Corning Primaria Falcon Dish 60 x 15 mm TC Primaria standard cell culture dish
OOSAFE 6 WELL DISH WITH STRAW HOLDER
HEPES-Buffered medium
PIPETTE FLEXIPETS PER DENUDING
2 Vials of ES (Equilibration Solution, 2 x 1 mL) and 2 Vials of VS (Vitrification Solution, 2 x 1 mL)
VitriFit Box

Stripper holder
p200

Grade A air flow
Liquid nitrogen container for vitrification

Micropipettes epTIPS Biopur 2-200 µL
IVF culture medium supplemented with HSA
Corning Primaria Falcon Dish 60X15mm TC Primaria standard cell culture dish
Multidishes 4 wells (Nunc)
OOSAFE® 6 WELL DISH WITH STRAW HOLDER

PIPETTE FLEXIPETS PER DENUDING
4 Vials of TS (Thawing Solution, 4 x 2 mL) + 1 Vial of DS (Dilution Solution, 1 x 2 mL) +1 Vial of WS (Washing Solution, 1 x 2 mL)

Authors: We want to thank the reviewers for their interest in our manuscript and their time. Their observations significantly improved its quality. We have revised it accordingly and we hope our revision fulfils your expectations. Please see hereafter our point by point responses.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Authors: Done accordingly.

2. Please make the title concise; consider “Fertility Preservation Through Oocyte Vitrification: Clinical and Laboratory Perspectives”.

Authors: Changed accordingly.

3. Please provide an email address for each author.

Authors: Added.

4. Is oocyte retrieval done under anesthesia? If so, please specify the method of anesthesia.

Authors: Yes. Details have been added to the manuscript. Briefly, oocyte retrieval is performed under deep sedation achieved by administrating propofol (whose dosage is adjusted according to patient’s weight) and fentanyl 50-100 µg, paracetamol 1000 mg, and assisted mask ventilation with oxygen. An antibiotic prophylaxis is also adopted routinely. At the end of oocyte retrieval, the patient is observed for 3-4 hr and then discharged. The patient is clearly given also an emergency contact number.

5. Line 230: which medium is to be used?

Authors: Hepes Buffered medium.

6. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Authors: Done.

7. Lines 387-388: what do you mean by “At our center, ovarian stimulation is conventionally performed using GnRH antagonist protocol and rec-FSH or hMG with a dosage between 15-300 IU die”?

Authors: According to our policy, we adopt an antagonist protocol for all woman undergoing fertility preservation so to improve the safety, as suggested also by the ESHRE guidelines. GnRH antagonist protocol is in fact associated with several advantages over the GnRH agonist one, including shorter duration, lower dose of gonadotrophins, reduced risk for OHSS, possibility to use GnRH agonist to trigger ovulation, absence of perimenopausal symptoms due to pituitary desensitization, no risk for ovarian cyst formation, reduction of psychological burdens.

8. Please move Figure legends to appear after Representative Results.

Authors: Done.

9. Please do not abbreviate journal names in the reference list.

Authors: Done.

10. Please add scale bars to the micrographs in Figure 5 (and in the legend).

Authors: Done.

11. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. The title and a description should appear after the Representative Results of the manuscript text.

Authors: Done.

12. Please sort the Materials Table alphabetically by the name of the material.

Authors: Changed accordingly

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript answers the call for a methodological paper covering both clinical and laboratory aspects of human oocyte vitrification. It will provide valuable guidance to IVF clinics introducing the procedure to the practice. It must be appreciated that besides pointing to critical steps of the protocol (temperature, osmolarity, sufficient training, etc.), the authors emphasize the need for sufficient consulting and distinguish between oocyte freezing for medical reasons and so-called "social freezing". Nevertheless, a few points should be clarified before publication.

Major Concerns:
1) The manuscript should state in the abstract or introduction that Sage protocol and open vitrification system is used.

Authors: Done.

2) The existence of alternative protocols (e.g. Vitrolife and most widely used Kitazato) should be mentioned. For instance, temperature requirements are different in Vitrolife protocol. Thus, it should be stated that a necessity to work at RT (line 443) applies to this particular protocol (Sage)

Authors: thanks for the suggestion. We have better specified in both the introduction and discussion that the method described here refers to a specific vitrification protocol, therefore the results and the discussion cannot be applied to all vitrification protocols.

3) The vitrification (and ICSI) is performed immediately after denudation, 36-38 hours after hCG (lines 227 and 256). Could it be excluded that oocyte displaying PB is not in the anaphase/telophase/interkinesis stage? Was the assessment of egg maturity (MII spindle imaging) considered before vitrification? Leaving oocytes 2-3 hours before ICSI/vitrification would provide time for completion of maturation….Also, the timing of ICSI (1 hour after thawing, line 326) differs from what is recommended for other systems (2 hours). Is 1 hour indeed sufficient according to the author´s experience?

Authors: Thanks for the crucial observation. According to our protocol, oocyte vitrification is performed right after denudation and within 38 hours post hCG administration. All the oocytes displaying PB are vitrified and no spindle imaging is performed before vitrification to avoid the extra exposure of the oocytes to environmental conditions. After warming, the oocyte are pre-incubated for at least 1 hour and then inseminated. According to both our personal observations and the data retrieved from the literature (Chen et al., 2004, Sereni et al., 2009) the oocytes display spindle birefringence within 1 hr of thawing and no significant progresses might be observed by extending the culture to 2 hr. In other terms, early spindle recovery allows early insemination of the oocytes, thereby avoiding in vitro aging and allowing to optimize the fertilization rate after insemination.

4) Besides survival (line 363), fertilization rates should be reported in the reports session. Some systems provide the survival rate (2 hours after thawing) higher than 90%, yet fertilization and utilization are low.

Authors: Thanks for the observation. We provided also the fertilization rates and underlined that they are nor representative of the common practice of an IVF lab due to small numbers and variable sperm factors in the 4 groups.

Minor Concerns:
Introduction:
line 44-49 - complex, difficult-to-read sentence, consider rephrasing

Authors: ok, we have rephrased it.

Protocol:
To avoid confusion, the terms "oocyte collection tubes", "IVF culture medium", "Hepes-bufered medium" should be stated throughout the protocol and noted in the table of materials

Authors: Done.

Line 145 - how many follicles must reach 17-18 mm in diameter? Please state minimum number.

Authors: At least 3. Added.

Line 203 - "place all materials" ..please specify

Authors: Done.

Line 214 - COC rinsing in IVF medium - this medium is CO2 dependent.. In ambient conditions the buffered medium would be preferable to avoid changes in pH.

Authors: Correct. However we prefer avoiding the exposure to buffered medium during oocyte retrieval. To preserve the pH, the IVF medium is pre-equilibrated, covered by oil and maintained in closed tubes during the procedure.

Line 231-233 - "supplemented with 5% albumin" - please add "human"

Authors: Done.

Is medium supplemented with albumin in-house, or the albumin-containing medium is used? I many countries addition of substances to media is forbidden, and only certified media can be used.

Authors: Albumin is certified by Fujifilm Irvine Scientific and simply added to the medium before use.

Line 211-264 "Labelling with ID"…Some clinics use automated witness system. Please mention the option of identity validation via unique codes scanning.

Authors: Thank you for the observation. Also in our clinic we use an electronic witnessing system (EWS) and barcodes scanning to guarantee the traceability throughout the procedure. We mentioned this possibility. Yet, we preferred describing a protocol entailing the minimum requirements for correct gamete identification, so that the protocol can be adopted in every IVF center. In the future, we might consider describing the EWS, but this will require a dedicated manuscript.

Line 275 "move the oocyte" - please specify whether the oocyte is transferred (aspirated and released from micropipette) or pushed on the dish bottom toward the next droplet.

Authors: Done. Thanks for the observation.

Line 283 - is 1ml of VS to be shared by all 9 oocytes (in 3 steps), the transferred ES would gradually dilute VS, which might have and an adverse effect on 2nd and 3rd subset of oocytes.

Authors: We use a large volume of VS (1 ml) and we always take a small amount of VS in the micropipette so the volume of ES that is transferred is negligible and would not affect the concentration. We have specified this now.

311 - "Add PBS or sterile water…" please make it clear that PBS/water is not supposed to dilute DS/WS solution but fill the area around the wells

Authors: Done, Thanks for the observation.

321 - "spoon movement" please specify the type of movement

Authors: Done.

Discussion:
Line 397-399 - difficult-to-read sentence, consider restructuring

Authors: Revised. We hope it is clearer now.

Line 464- "consider larger volumes in the droplets to minimize shifts in osmolarity".
The evaporation of small (30ul) droplets is the primary concern of this vitrification system. Have the authors researched how increasing volume affects vitrification efficiency? Do bigger volumes work better?

Authors: It is indeed true that droplets' evaporation is a critical point of the protocol. In fact, we have increased the droplets volume to 30 μl (10 μl more than the 20 μl recommended by the manufacturer) and we have also provided some tips here to minimize evaporation during the procedure. Still, we have now specified that in non-standardized environmental conditions or in case of unexperienced operators, the use of a Reploplate as plastic support might be considered. In the Reproplates the volumes can be increased gradually and PBS (or water) might be added to the dish so to prevent evaporation and changes in osmolarity.

Figures:
Figure 3 and Figure 4 appear blurred in the pdf version of the manuscript (both printed and the screen-displayed).

Authors: The quality has been improved to 600 dpi.

Reviewer #2:
Manuscript Summary:
Well written. Details are more than adequate. Covered all areas of oocyte vitrification.

Major Concerns:
Verification of patient identity on labeling for vitrification and warming by a second lab staff is never mentioned. It is critical to include this step.

Authors: Thanks for the observation, we have specified the need of a second witness.

Under section Oocyte Retrieval, steps 8 and 9 should mention to loosen caps of the tubes when they are kept in an environment of 6%CO2 and 5% O2.

Authors: Thanks for the observation, we have specified it.

Minor Concerns:
Is not cancer a medical condition? Maybe the medical condition should be decided as cancer and non cancer for indications to vitrify oocytes.

Authors: With "medical" we mean conditions other than cancer for which fertility preservation is advisable. Better specified now.

Under section Oocyte warming, step 7 describes to add PBS but does not describe where to add as there are 6 wells in the plate.

Authors: Thanks for the observation, we have better described it.

Reviewer #3:
Manuscript Summary:
The authors describe the whole clinical and laboratory work-up of a fertility preservation treatment by (i) outlining recommendations for an objective and evidence-based
counseling, (ii) focusing on the cost-effectiveness of the procedure, (iii) describing the most appropriate strategies to fully-exploit the ovarian reserve and maximize the number of oocytes retrieved in the shortest possible time.

Excellent manuscript with a comprehensive review of this topic.

Authors: Thanks.