Role of oocyte vitrification for fertility preservation programs

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Summary

Advances in oncology and cancer treatments have allowed to drastically improve the patient recovery prognosis. However, the therapies used to battle the disease often imply an irreversible damage to their reproductive potential due to which an increase in demand for fertility preservation has gradually emerged in the society. Cryopreservation has been historically used as a first line approach in fertility preservation for women, but initially this option was limited to embryos. Major advancements achieved in the past few years in the protocols for oocyte cryopreservation have opened a window of hope to women who wish to preserve their fertility potential either for social or oncologic reasons. The aim of this review is to provide current knowledge on oocyte cryopreservation being focused on vitrification as a standardized, simple, reproducible, and efficient option.

KEY WORDS: fertility preservation, cancer, social freezing, slow cooling, vitrification.

Introduction

The knowledge about cancer and hence the design of more efficient therapies has significantly evolved during the last decades. This has led clinicians to go a step further exploring approaches to offer a better quality of life to survivors beyond the cure of the disease. In this context, Fertility Preservation (FP) aims to give individuals at risk of losing their reproductive ability the chance to conceive and have their own genetic offspring, especially in young women without descendant (1).

In a completely different scenario, the percentage of women who decide to delay the motherhood due to personal reasons have gradually increased during the last years (2, 3).

The two realities presented above may help to understand why the interest in FP methods has recently grown so markedly. The aim of this review is to appraise the validity of oocyte vitrification within the framework of FP either for oncological or nononcological reasons.

Oocyte cryopreservation

Shifting the pendulum from cell death to immortality at low temperatures is a far from negligible goal that cryobiology has eagerly pursued (4). The incorporation of cryopreservation in the field of Assisted Reproduction Techniques (ART) has represented a before and an after in the management of *In Vitro* Fertilization (IVF) cycles. Embryo cryopreservation protocols have been established and efficiently applied in the IVF laboratories for more than three decades. As proof of this, the first report of a pregnancy after a transfer of cryopreserved embryos date from 1983 (5).

In this review we focus on the analysis of oocyte cryopreservation as a strategy for FP, due to which studies considering male gamete or embryo cryopreservation at different stages of preimplantation development will be omitted.

Besides the application of oocyte cryopreservation in FP for either oncological (6, 7) or nononcological reasons (8), the aforementioned approach has been previously used for egg

banking in ovum donation programs (9-11), for pooling oocytes in poor responders as a strategy to optimize the IVF cycle (12) and for storing surplus oocytes in normal responders when embryo cryopreservation is not feasible either for moral/religious objections of the couple or for legal impediments in some countries (13, 14).
Despite its present widespread use, the search for methods to efficiently cryopreserve the female gamete has posed quite a challenge and still establishing the most optimal protocol is a topic of discussion (15).
Crystallization, defined as irreversible damage after exposing cells to low temperatures (+15C to -5 C) before the nucleation of ice (16), and hyper concentration of solutes have been identified as the two main sources of chilling cellular injury. Cryobiology has tried to bypass ice crystal formation through the use of solutions containing Cryoprotectant Agent/s (CPA/s) resulting in an effective expulsion of intracellular water. Slow freezing and vitrification protocols have proposed different combinations and concentrations of both non-permeating (e.g. sucrose) and permeating CPA/s (e.g. propanediol PROH, dimethyl sulphoxide DMSO or ethylene glycol EG). The first provides a continuous solutes gradient across the oolemma while the second participates actively displacing intracellular water in an osmotic interchange process causing an extreme shrinkage (4). Unfortunately, the toxicity of the CPA/s, which depends on the particular composition and concentration, is an irrefutable fact (17) that compromise the success of cryopreservation methods.
On the other hand, the survival of any sample to freezing is determined by the structure, size and shape of the cells. Human oocytes are one of the most vulnerable cells to cryopreservation in nature. This is due to its large size and the high water content in the cytoplasm that increase drastically the probability of intracellular ice formation (crystallization) and osmotic damage during the process (18-20). Furthermore, the permeability of the cell membrane to water, the principal factor involved in dehydration may be different to later stages of development (21-23) and also very variable between individual human oocytes (23, 24). And if this weren't enough, the sensitivity of the meiotic spindle to temperature variation or cell dehydration/rehydration (25-31) may alter the subsequent chromosomal distribution during the meiotic segregation.
While sharing common principles, the protocols for the two main strategies proposed in cryobiology to date differ in the way in which the CPAs are used.

Slow freezing

Slow freezing protocols present an initial phase of gradual cell dehydration in the presence of one or more solutions containing permeating (≤1.5 M) and non-permeating CPA/s (≤0.3M) for periods up to 10 min before loading into the straw. Thereupon, the temperature is slowly reduced (at -0.38°C/min) to -30°C and then rapidly (at -50°C/min) to -150°C before immersing into liquid nitrogen (32).
The exposure of the oocytes to low temperatures (+15C to -5C) for an excessive time may lead to an irreversible damage in the cytoskeleton and cell membranes due to the chilling injury (16, 33, 34). Because of this, the cooling rate achieved is crucial for the success of the technique allowing for adequate expulsion of water and decreasing the time of exposure to stressful osmotic conditions. Also, the use of higher concentrations of non-permeating CPA/s may result in improved oocyte (29, 35) and embryo (36) survival rates.
Nevertheless the application of slow freezing for oocyte cryopreservation has got limited success to date (37) suggesting that there has been reached a plateau at 70-80% in the oocyte survival rate (Gook and Edgar 2007) (36) with currently available methodology. It has been stated a clear improvable clinical pregnancy rate (CPR) per transfer and implantation rate (IR) of 20.6 and 10.1%, respectively, on average (38).
The fact that the modifications made to the protocols described in early '70s by Whittingham et al. (39) are gathering encouraging results on slow cooling of oocytes, suggests that the optimal protocol is yet to be established.

Vitrification

The term vitrification refers to the conversion of a superviscous and supercooled liquid into a glassy state when it is cooled below its glass transition temperature (40, 41).
The vitrification process is directly proportional to the solution viscosity and the reduction in temperature and inversely to the sample volume (42). Considering the proposed equation, the current vitrification protocols have opted for: a) using more efficient combination of multiple

CPA/s to obtain extreme cell shrinkage and a highly viscous solution reducing the exposure times significantly (43-45), b) loading the samples in a very small volume of vitrification solution (0.1–2 µl) facilitating a faster cooling (42) and c) direct contact of the oocyte/embryo with liquid nitrogen generating very high cooling rates (>10000°C/min) (45).
Being the main purpose of this review, the outcome of oocyte vitrification will be discussed in detail within the following sections.

Comparative studies of outcomes
Most of the RCT studies regarding oocyte cryopreservation have been focus in the analysis of outcomes comparing fresh *versus* vitrified/slow-cooled metaphase II oocytes (10, 46, 47).
Very few studies have been published comparing the outcomes and relative efficiency of slow freezing and vitrification till date. Two of them have shown comparable survival rates, but a possible decrease in embryo development in the vitrification group using a close device (6, 48). Another two studies using the more widely used open system protocol suggested an increased survival, fertilization and implantation rates in the vitrification (49, 50). The only prospective RCT in these series of studies (51) threw an increased survival, fertilization, development and clinical pregnancy rates in the vitrification group. All these studies have considered the PROH with 0.3 M sucrose method for slow cooling which might be suboptimal.
In light of published data it can be stated that vitrification is the most efficient approach to cryopreserve human metaphase II oocytes. However the aforementioned assertion presents two major weak points. Strictly speaking, any study that aims to weight the outcomes and relative efficiency of both approaches should use identical material and setups (4). Secondly, such trials should compare the clinical outcomes obtained with optimal protocols in both of the techniques including live birth rates and long term follow-ups in the offspring.

Oocyte vitrification in assisted reproduction laboratories

Due to the limited amount of existing data about FP patients who have decided to attempt pregnancy with their own vitrified oocytes, the clinical efficiency of the procedure has to be based on the outcome obtained with vitrified oocytes from ovum donation and autologous IVF cycles.

Ovum Donation cycles
In many countries ovum donation is a well-established ART option offered to patients who for different reasons cannot use their own oocytes (52-55). Being possessor of the highest outcomes that can be reached for any assisted reproductive method (56), the main drawbacks of ovum donation are related to logistic limitations. Constraints in the availability of a suitable donor and the lack of synchronization between donor and recipient leads to long and undesirable waiting lists.
Oocyte vitrification for donor egg banking can overcome the aforementioned logistic downsides. The first live birth reported from a donated vitrified human oocyte was in late 90's (57). Thenceforth, many groups have related their experience with egg banking showing promising blastocyst and/or pregnancy rates with vitrified oocytes and, wherever it has been analyzed, equivalent outcomes to those obtained with fresh oocytes (10, 11, 46, 58-61). With an overall survival rates of 92.5% and 43.7% OPR after oocyte vitrification and storage, the only one large prospective randomized controlled trial published to date has demonstrated the clinical usefulness of cryo-banking in oocyte donation programs (10).
However, today oocyte banking is not a very widespread service in infertility clinics due to the lack of standardization in the methodology used to cryopreserve human oocytes and the consequent uncertainty to reproduce the results.

Autologous IVF cycles
The results obtained from autologous vitrified oocytes from infertile IVF patients have been also studied in detail. Different studies, including a prospective randomized study, have shown an equivalence in the fertilization, embryo development, implantation and ongoing pregnancy rates (IR and OPR, respectively) between vitrified and fresh oocytes (47, 48). Interestingly, the analysis of the cumulative rates from the same group revealed a negative association between age and the clinical outcomes (62).
The reproducibility of oocyte vitrification was analyzed in a multicenter prospective longitudi-

nal cohort study from Rienzi et al. which achieved a satisfactory overall outcomes without significant differences between centers attributable to the technique (63). The patient age and number of vitrified oocytes were found to be predictive parameters of success concluding that each year of maternal age decreases by 7% the delivery rate. This is consistent with other study from Kim et al. obtaining significantly better outcomes when using vitrification in young fertile women (64) confirming the already known negative association of age with ovarian reserve and oocyte quality. In this sense, the results obtained from two different studies seem to indicate that 7 would be the turning point in the number of oocytes that should be vitrified to reach acceptable outcomes (63, 65).

Finally, a systematic review and meta-analysis of 17 studies on oocyte vitrification from Potdar et al. was recently published including randomized and non-randomized, controlled and prospective cohort studies and both donor and non-donor oocytes (66). The study shows an overall 0.88 survival rate, 0.74 fertilization rate, 0.87 cleavage rate and 0.07 of both clinical and ongoing pregnancy rates (CPR and OPR, respectively) per warmed oocyte. As expected, these rates were higher in donor *versus* non-donor oocytes but no significant differences were observed between vitrified and fresh oocytes, except for the OPR being lower in the vitrified group. The review concludes that oocyte vitrification is an effective and safe cryopreservation method without losing sight of the impact of the age and the reason for cryopreservation on the results.

Oocyte vitrification for fertility preservation

As a summary of the previous section, introduction of vitrification has allowed to achieve similar results between cryopreserved and fresh oocytes. The extrapolation of these results to the cancer patient population might seem automatic but it needs to be made with caution. The validity of oocyte vitrification for FP should be determined considering exclusively data extracted from that population.

At this point, it should be pointed the difficulty of establishing a consensus on effectiveness due to the heterogeneity of the protocols (cryoprotectant types/concentrations) and devices described in the literature. This may be the underlying reason for the discrepancies observed between different groups regarding some technique-related parameters such as the oocyte survival rate (67). Furthermore, age, oocyte quality or indication for vitrification are some patient-dependent factors that have been already mentioned as confounding factors (64). Studies about oocyte vitrification have considered predominantly good prognosis cases (good responders or oocyte donors) so the results could be bias. It becomes necessary to conduct more large-scale prospective randomized controlled trials in typically infertile patients to strengthen the conclusions drawn. However, the aforementioned evidences could be of great help for counseling case-to-case to FP patients with real data about the success expectations (68).

Age-related fertility decline (social) and nononcological patients

Intuition leads to think that FP concerns only cancer patients but other medical conditions such us endometriosis or an imminent adnexectomy that may compromise fertility can represent also an indication for FP (68).

On the other hand, modern societies are facing two realities that are directly opposed: the trend towards the postponement of the first pregnancy for various economic, educational, and social reasons and the gradual and unstoppable loss of fertility potential in women after the age of 30 (69, 70). This tendency is placing the total fertility rate of most developed countries below the replacement level becoming a demographic concern (71).

The term ‘‘social’’ refers to women who electively decide to collect and preserve their gametes for a future use (68). Leaving aside the ethical, political and religious connotations that this approach entails (72, 73), the life style related issues represent a challenge for the future and welfare of the society that reproductive medicine has to address. Although ART cannot manipulate the impact of time on reproductive ageing, an appropriate counseling and planning about oocyte vitrification can safeguard the future chances of healthy women who decide to attempt pregnancy at a more advanced age (71).

For about 10 years, in parallel with the technical improvement of oocyte cryopreservation,

the possibility of egg storing for nonmedical purposes is more extensively discussed and more commonly accepted by the general population and expert committees in the USA and Europe (74, 75). One example of the increasing acceptance is the inclusion of FP programs in most of the reproductive medicine centers of the United States during the last years (76).

Clinical outcome in nononcological patients

Data about outcomes in nononcological patients is scarce, mainly because their gametes have not yet been used. Different circumstances could explain that low rate of use including absence of partner, disapproval of the oncologist or hesitancy indecision on the wish of motherhood itself (77).

Garcia-Velasco et al. evaluated the outcome achieved in their FP program including 560 nononcological patients (78). The predominantly reason to delay the childbearing was for social reasons (90.6%) being the remaining percentage covered by other medical indications such as endometriosis or imminent adnexectomy. 26 women came back to attempt pregnancy with a mean oocyte survival rate obtained per patient of 84.8% and an OPR and cumulative OPR of 30.7 and 70.9%, respectively, resulting in the birth of five healthy babies.

Oocyte vitrification has been also described as a FP option for medical reasons (nononcological) in patients with Turner syndrome and impending premature menopause (79, 80).

Oncological patients

Progress in cancer treatments and the consequent increase in the proportion of patients who overcome the disease has improved noticeably, especially in prepubescent and breast cancer patients (81-84). From the FP perspective, the challenge now is to design effective strategies to give opportunities for survivors to conceive children with their own oocytes despite the devastating impact that the radiation or chemotherapy has on the fertility (85).

Strategies for safeguarding fertility in oncological patients should be based on patient age, cancer type, type of treatment, presence of a male partner, time available before the cancer therapy and the likelihood of ovarian metastasis. Oocytes, embryos and ovarian tissue delineate the variety of cryopreservation approaches explored to date (7, 38, 83, 85-91).

The effectiveness of oocyte vitrification based on the results with typically infertile patients have turned oocyte vitrification into the treatment of choice for FP (74, 75). In any case, the oocyte storage for oncological reasons has got a better social acceptance than FP for nonmedical reasons (92). Nevertheless, oocyte cryopreservation eliminates the ethical and legal concerns that arise with vitrified embryos in case of not overcoming the cancer or further separation from the partner who generated the embryos (93).

Keeping to the collection of oocytes, as summarized in De Vos et al. the reproductive maturity of the patient (pre or postpubertal) will determine the FP approach ranging from: i) Controlled Ovarian Stimulation (COS) and cryopreservation of mature or *in vitro* mature oocytes or ii) ovarian cortex cryopreservation with or without *ex vivo* retrieval of immature oocytes and either cryopreservation of immature oocytes or cryopreservation of *in vitro* matured oocytes to iii) transvaginal retrieval of immature oocytes and either cryopreservation of immature oocytes or cryopreservation of *in vitro* matured oocytes (94). It has to be mention that there has not been reported a single live birth from vitrified *in vitro* matured oocytes in an oncological patient so far.

Regardless of the adopted preservation strategy, the success expectations of oocyte cryopreservation will ultimately depend on both the quantity and quality of the starting material. In oncology, the time interval between primary surgery and the start of chemotherapy is based on the assumption that chemotherapy should be started without any delay. It has been found that the overall survival decreased by 15% in breast cancer patients for every 4-week delay in initiation of chemotherapy (95). Hence, many oncologists still consider that the time required for COS which initiation has to be synchronized with the menstrual cycle and the associated increase in circulating estradiol (E_2) concentrations (96) are consistent reasons to dissuade patients for this FP approach.

A strategy to avoid the unwanted rise in E_2 is to collect oocytes from unstimulated cycles although the efficiency and success expectations with this approach are inevitably limited. By contrast, protocols for COS using aromatase inhibitors (letrozole) have demonstrated to be efficient in patients with estrogen receptor-posi-

tive breast cancer by maintaining physiological E_2 levels (6, 97, 98). Beside this advantage, and while waiting for long-term follow-up studies, letrozole does not seem to increase the risk of breast cancer recurrence (99). Nonetheless it has been shown that hormone-dependent cancer patients stimulated with letrozole have an inferior response to COS resulting in a reduction in the number of mature oocytes retrieved (100).

Regarding the interference with the start of chemotherapy, a recent finding has found a way to dissociate the onset of COS and the phase of the menstrual cycle (101). The fact that multiple batches of antral follicles can be developed irrespective of the stage of the menstrual cycle, has been translated into random-onset COS protocols. This strategy allows obtaining an acceptable number of oocytes with a drastic reduction in the time required (102, 103) providing the option of two consecutive stimulations before the chemotherapy in some cases (104).

Clinical outcomes in oncological patients

Similarly to what has been previously described in nononcological patients, the information about oocyte vitrification outcomes in nononcological patients is very lacking because the limited experience with this technique in this subgroup of patients. Obtaining a large enough database from cancer survivors who have decided to attempt pregnancy with their vitrified oocytes still will take a few years.

Before vitrification appeared on the scene, several publications reported the birth of a commendable number of babies conceived after oocyte slow freezing in cancer survivors (105-107).

Up to the present time, there are very few papers showing clinical outcomes with oocyte vitrification in oncological patients. In 2009, Sánchez-Serrano et al. supplied documentary evidence about the birth of twins in a breast cancer patient by using a combined approach of ovarian tissue cryopreservation and oocyte vitrification after grafting (108). Subsequently, Kim et al. reported the birth of a baby after oocyte vitrification in a patient with chronic myeloid leukemia (109). Finally, Garcia-Velasco et al. reported the second birth of a baby in a study summarizing the outcome achieved in their FP program for oncological patients (78). A total of 340 patients underwent COS for oocyte vitrification being breast cancer the most frequent cancer type (67%). Most of the patients could be stimulated only once with an average number of mature oocytes vitrified per patient of 8.5 ± 6.4. Out of the four patients who attempted to use their cryopreserved oocytes two pregnancies were achieved. One finished in a miscarriage and the second one, in a patient with non-Hodgkin lymphoma, resulted in the birth of a healthy boy.

Subsequently, the same group published an update of the FP program focused in the obstetric outcome including 7 patients more who returned to thaw their oocytes (110). The overall oocyte survival rate reported in the study was 92.3%. Out of the 11 embryo transfers, 7 (63.6%) had positive pregnancy test resulting in 3 more babies, which make a total of 4 in these series. None of these 4 pregnancies had additional obstetric complications and neonatal intensive care unit was not required for any of the babies.

Discussion

Currently there are enough consistent data to conclude that vitrification is an effective, simple, safe, and efficient technique to cryopreserve oocytes. This makes it a promising option of FP for medical and nonmedical reasons. However, the uncertainty that the bias in the selection of specific populations or the heterogeneity in the protocols used in those studies may generate should be properly analyzed before taking its validity in FP patients for granted. Likewise, clinical settings offering those techniques must be equipped with the necessary infrastructure and possess appropriate training ensuring good practice with the highest guarantees of success.

Certainly, this experience obtained in infertile patients can be used for counseling purposes in FP. The opportunities must be exposed with objectivity and accuracy stressing the importance of having a minimum number of oocytes to be stored. In order to avoid false hopes, it must be clearly transmitted to the patient that oocytes can be preserved but not fertility. Fertility refers to the childbearing capability and here many more factors come into play.

As reflected in this review, preliminary data about oocyte vitrification in FP patients are now available. Despite the promising outcome

achieved, the limited sample of this series becomes necessary to be cautious and wait for more results to definitively confirm the efficiency of oocyte vitrification in this group of patients. Similarly, long-term follow-up studies in babies born after oocyte vitrification are mandatory to definitively consolidate the safety of the technique.

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