Techniques for Ovarian Tissue, Whole Ovary, Oocyte and Embryo Cryopreservation

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Abstract

In recent years, preservation of fertility in women has been of great importance, especially in patients exposed to deleterious conditions on fertility. Thus, cryopreservation of human gametes, embryos and ovarian tissue has become an essential part of assisted reproduction. This approach limits the number of embryos transferred, while supernumerary oocytes and/ or embryos can be used in subsequent treatment cycles. Furthermore, cryopreservation reduces the potential risk of hyperstimulation syndrome. Cryopreservation is carried out by two techniques; the slow freezing method, and the more recent rapid procedure called vitrification technology. Recently due the success and simplicity of vitrification, the balance between those two methods has been changed in advantage of vitrification. The use of slow freezing method has become controversial due to its difficulties, expense and respective low success rates in artificial reproduction. Therefore, vitrification seems to win the battle and will be the cryopreservation method of the future.

Keywords: Blastocyst, Cryopreservation, Embryo, Fertility preservation, Ovarian tissue, Ovary, PN Zygotes, Vitrification.

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Introduction

The cryopreservation methods of both human oocytes and embryos, and recently the ovarian tissue and the whole ovary have been acknowledged to be mandatory, in certain conditions, and beneficial tools in advanced techniques of artificial reproduction (1 - 3). It has been asserted that the routine use of a good and safe cryopreservation program could provide favourable outcomes in artificial reproduction regarding high cumulative rates of both clinical and ongoing pregnancy. In addition, the cost of a live birth could be potentially reduced and chances of a multiple pregnancy avoided as well when a single embryo is transferred.

As the efficacy of anticancer therapies has been increasing and quite efficient early diagnoses of the disease have been realized regarding gynaecological cancers, increased long-term survival of cancer patients and long-term complications of anticancer treatments are being encountered. In that point of view, fertility preservation is also being looked at as a viable option where cryopreservation of gametes in females is fundamental.

Preservation of gametes is not a novel idea and its history stretches back to about 200 years. In 1776, Spallanzani et al. recorded, for the first time, a successful cooling and re warming of spermatozoa in snow (4). Since then, great advances have been achieved, and two main methods, slow-rate freezing and vitrification, have been implemented in cryopreservation of human gametes and embryo (s). Unfortunately, initial applications of cryopreservation resulted in low success concerning low viability of the cells and poor clinical outcomes (1 - 3, 5 - 16). The poten-

tial cell damage that occurs during cryopreservation techniques and the toxic damage of cryoprotectants are the major limiting factors of clinical success (16, 17).

Notably, three potential cellular damages during cryopreservation have been defined previously. The first one is the chilling injury that occurs at higher temperatures such as between +15 to -5° C. This injury mainly damages the cytoplasmic lipid droplets and microtubules including the meiotic spindle (18). The next and most popular damage is the formation of intracellular ice crystals, which is the main source of fracture and damage of zona pellucida or cytoplasm which occurs between -50 to -150° C (13). The last one has been addressed under -150° C, which has been defined as the least dangerous one (16, 17).

Better and favourable clinical results could be achieved by modifications of techniques and use of intra and extra-cellular cryoprotectants combined. Subsequently, after overcoming the potential and toxic cell damages by laboratory experience and increased success with each cycle of cryopreservation, the battle of vitrification and slow-rate freezing will begin. Therefore, this study tries to compare and determine which cryopreservation technique will live on in the future.

Techniques of Cryopreservation of Human Gametes, Embryos, Ovarian Tissue and Ovary: The slow rate freezing method is the former number one, which is also known as equilibrium freezing due to the exchange of fluids between the extra and intra-cellular spaces of cells, allowing freezing without serious osmotic effects and deformation of cells (17 - 19). Thus, it was accepted to be a safe procedure due to unserious toxic and osmotic damages because of relatively low concentration of cryoprotectant solutions. In the past decades, slow cooling procedure was used for cryopreservation, but over the last few years, it has been suggested that the vitrification method may be a valuable alternative to this procedures (20 - 22). In comparing the principles, procedures and results of slow cooling and vitrification protocols, Kuleshova and Lopata (20) state that both methods have resulted in the successful cryopreservation of human oocytes and embryos, but the former has given much lower success rates. Substantially, the low concentration of cryoprotectants used in slow-rate freezing has been

commonly claimed to be insufficient in preventing the formation of ice crystals. These intra- and extracellular ice crystallizations are the main sources of fractures and damages of zona pellucida or cytoplasm resulting in decreased cell survival (17, 19). Therefore, in early 2005, opinion articles as well studies initiated to underline that vitrification might be a better alternative for cryopreserving human zygotes and embryos rather than the slow-rate method (9 - 15).

Vitrification was first clinically introduced in early 1980s. In 1985, Rall and Fahy reported the efficacy of vitrification in embryo cryopreservation (13). This method is a non-equilibrium one whereby cells are rapidly plunged into liquid nitrogen at -196° C after a short period of equilibrium and a subsequent glass-like solidification. Nevertheless, the method requires high cooling rate along with higher concentrations of cryoprotectants, which may exert toxic and osmotic effects on cells (17, 19). Thus, an increased probability of all other forms of cell injury except from the formation of ice crystals has been asserted. However, on the contrary to this hypothesis, vitrification offers a new prospective in attempts to develop an optimal cryopreservation, by producing a glass like solidification of cell, completely without intracellular ice crystallization during the cooling and warming processes (14).

Physically, there is a close link with the cooling rate and concentration of cryoprotectants as the higher cooling rate reduces the required concentration of cryoprotectants and vice versa (18, 19). Therefore, establishment of specific balances between a reliable highest cooling (and warming) rate and a safe concentration of cryoprotectants without any toxic effect are critically required for preventing the consequent cell damage in vitrification (19, 24 - 26). Therefore, vitrification bypasses the severe cytotoxicity resulting from the high cryoprotectant concentration required by the aforementioned techniques, by introducing cryoprotectants with higher membrane permeability and lower toxicity, together with an appropriate concentration of non-permeable cryoprotectants (20 - 27). Vitrification could be introduced without the use of expensive equipment and it could be completed by one embryologist within a few minutes, providing significant benefits for any

busy IVF program (13 - 15, 20 - 23, 25 - 27). Notably, groups working with vitrification have established their own unique procedures, by making alterations in the concentration of cryoprotectants, cooling rate and/ or carriers, and attempted to prove its superiority.

Aim and Advantages of Cryocarriers: Direct contact of cells for vitrification and the possibility of viral pathogens transmission make the vitrification procedure quite hard for a daily usage (28). Therefore, numerous carrier systems assumed to prevent both direct contact and requirement for a large volume of cryoprotectants have been introduced during this decade. The open pulled straw, flexipet-denuding pipettes (FDP), microdrops, electron-microscopic (EM) copper grids, traditional straws, hemistraw system, small nylon coils, and the minimum volume cooling by cryotops and recently the closed by cryotips are such examples (14, 29 - 32). However, the largest experiences were mostly with the use of cryotop, cryotip and cryoloop (14, 29 - 32).

In 2005, Kuwayama et al. reported improved vitrification success with the use of the former method in human oocytes (14).

Afterwards, the same group also published a comparison between open system, the CryoTop and a closed vitrification system, the Cryotip, in over 13,000 embryos at different stages (29). Considered the largest study to date concerning vitrification, the authors suggest that cryotop is an efficient and reliable way to freeze cleavage embryos, blastocysts and oocytes in daily practice. The idea of transmission of viral pathogens to vitrified embryos which are stored in contaminated nitrogen was raised by Bielanski et al (28). The possibility relies on the fact that many viruses and some bacteria, such as *Stenotrophomonas maltophilia* which is the most common*, cause of contamination may survive exposure to liquid nitrogen significantly suppress fertilization and embryonic development in vitro (32). Thus, a reliable coverage and cell isolation is required in all rapid cooling devices. CryoTip has been recently suggested to eliminate the danger of contamination of cells while maintaining the high efficacy of the procedure. However, more recently the same group indicated that cross-contamination of these agents and transmission between samples in open and closed media is not critically different

where a low rate of transmission has been observed as well in open systems (33).

Oocyte cryopreservation: It has been well introduced that cryopreservation of human gametes and embryos has been known to result in different success rates based on the developmental stages of cells (12). Mainly, the immature cells seem to be more sensitive than the latter stages, concerning clinical and laboratory applications or procedures. The methods of cryopreservation, especially vitrification, surely affect cells and cause damages due to harmful non-physiological conditions. The human oocyte ultra-structure is quite sensitive to changes of temperature and extracellular osmotic pressure. Thus, during freezing and thawing human oocyte can undergo several types of cellular damage such as cytoskeletal disorganization, chromosomal or DNA abnormalities, spindle disintegration, premature cortical granule exocytosis, related hardening of the zona pellucida and plasma membrane disintegration (34 - 36).

The majority of assisted reproduction laboratories are using slow-cooling methods for human embryos and oocytes based on the original work of Testard et al. (37). Porcu et al. described the use of intracytoplasmic sperm injection (ICSI) for fertilizing frozen-thawed human oocytes in 1997 (38), which led to the incorporation of oocyte cryopreservation in clinical practice (35). The technical difficulties of the method were highlighted in a series of reports published after the first successful attempts and were conducted as early as mid 80s. The difficulties include a low pregnancy rate and an increased percentage of aneuploidy after gamete exposure to cryoprotectants and the freezing-thawing process (2, 7, 27). On the other hand, studies showed no increase in the number of abnormal or stray chromosomes in previously cryopreserved oocytes (39 - 41). It is not a surprise that the rate of maturation, fertilization, and cleaving were found to be low in cryopreserved human oocytes compared to fresh ones (10). It has also been shown that the outcomes of human oocyte cryopreservation are rather unfavourable than the results of cryopreservation of human embryos (33 - 36, 41). This latter data completely supports the higher sensitivity of human oocytes to temperature and osmotic alterations rather than the human embryos,

leading to a significant decrease in survival rate (67% vs. 54%, respectively) as well as a reduction of pregnancy rate in to half (respectively, 14.2% vs. 28.2%) with cryopreservation of oocytes (33,36, 42,43). It has been previously mentioned that embryos and gametes of human can be possibly damaged in all stages of cryopreservation. The leading example for this occasion was the initial thoughts concerning meiotic spindle. The former experiments presumed that meiotic spindle could be lysed and damaged in cryopreservation procedures (36). However, more recently, was shown that meiotic spindle simply disassociates due to the decrease in temperature, and it can reform with normal function by the increase in temperature in 4 or 6 hours (41).

Recently, vitrification was suggested as a more suitable for cryopreservation of human oocyte rather than the slow freezing method by the application of higher concentration of cryoprotectants and a rapid cooling speed for preventing the formation of intracellular ice crystals (44). Kuleshova et al. reported a pregnancy from vitrified oocytes while Yoon et al, reported six deliveries (21, 23). More recently significantly increased success rates with the vitrification of MII oocytes by the use of cryotop method which was initially described by Kuwayama et al., were reported (14). Vitrification by the cryotop method, which is highly reproducible, is, therefore, superior to slow-rate freezing. In addition, at present, no other technique has consistently produced results as excellent as those obtained with this method. Notably, those results are no different than the ones obtained with fresh oocytes. (44, 45). Applying the cryotop method to human oocytes, Kuwayama et al, (14, 29) reported a 91% survival, 81% cleavage and 50% blastocyst rates, along with a 41% pregnancy rate per embryo transfer resulting in 11 live births. In another study (45), the concomitant outcome achieved by fresh and cryopreserved donor oocytes, vitrified by cryotop method, was compared and a 96.7% survival rate without any difference in fertilization (76.3% and 82.2%), day 2 cleavage (94% and 97.8%), day 3 cleavage (80.8% and 80.5%) and blastocyst formation (48.7% and 47.5%) rates were accomplished, in comparison to fresh oocytes.

Furthermore, pregnancy rate per embryo transfer was 65.2% for the vitrification group. Antinori et al., reported a 99.3% survival, 93.0% fertilization, 96.7% cleavage, 32.5% pregnancy and 13.2% implantation rate by applying cryotop method in oocyte vitrification. At present, cryotop is the most efficient vitrification method available and the best current alternative for the creation of reliable egg banks (44, 45).

Vitrification of immature human oocytes was also an interesting point upon the development of in vitro maturation programs (46). Initially, this idea was presumed to overcome the problem of damaging to meiotic spindles in frozen oocytes (39 - 41, 47). Presumably meiosis arrest tables place at prophase I and chromosomes are protected by the membrane of the germinal vesicle in the immature stage. As well, there are not any formed microtubular structures in this stage yet. Notably, cryopreservation of immature oocytes can be beneficial in IVM cycles, especially in patients with premature ovarian failure. Tucker et al. reported one birth after cryopreservation of immature oocytes collected in a stimulated cycle by traditional slow rate cooling and rapid thawing protocol (47). Furthermore, Cha et al. used vitrification for the cryopreservation of immature oocytes retrieved from unstimulated cycles in patients with polycyctic ovarian syndrome (48). However, they reported a cleavage rate of 90%; there was no successful implantation in this study (47, 48).

In fact, oocyte cryopreservation is useful for young women refraining use of a sperm donor, or who wish to retain a choice of, male partner or want to avoid custody battles if the relationship between the couples breaks up in the future. Further more, embryo cryopreservation asserted to be not feasible wherein spermatozoa and fertilization are required rather than oocyte cryopreservation. Therefore, in ethical point of view, oocyte cryopreservation seems to be favourable rather than further stage cryopreservation.

Embryo cryopreservation: 2PN (Zygotes) and cleavage-stage embryo cryopreservation: In assisted reproduction, the induction of ovulation techniques offers a large number of oocytes and generation of supernumerary embryos. Aiming to avoid wastage of supernumerary oocytes and embryos, decrease the risk of ovarian hyperstimulation syndrome, and limit the risk of multiple

pregnancies, cryopreservation of oocytes and generated embryos plays a fundamental role in assisted reproduction. Embryo cryopreservation is an established method offered by many fertility centres world wide. If embryos survive the freeze-thaw process and keep all their blastomeres intact, cryopreservation of embryos would have a comparable pregnancy rate with fresh IVF (49, 50). However, overall, the former cryopreservation technique slow-rate cooling leads to a 30 - 40% reduction in the implantation potential of embryos (49, 51).

The first trials of embryos freezing were in late 1970s. The first pregnancy and live birth from frozen-thawed embryos was reported in Australia in 1983 (1). Embryo freezing spread rapidly and became an essential part of the assisted reproduction techniques. Although many factors may limit the cryopreservation success, such as intra- or extra-cellular ice crystal formation during the freezing- and thawing which have a harmful effect on the cell membrane leading to the fracture of cytoplasm and decreases in cell survival rate (15). Toxic effects of permeable cryoprotectants may cause osmotic shock to the cells due to the replacement of the bound water molecules in and around proteins, particularly in high concentration (19).

Embryonic developmental stages, at which the process of cryopreservation will be performed, are important factors in cryopreservation success. Two-pronucleate (2PN)-stage embryos show no signs of their developmental competence when cleavage-stage embryos are damaged subsequent to thawing. The implantation of damaged blastomeres, which often coexist along with intact ones, is lowered, as expected, rather than the intact ones (49). Blastocyst-stage embryos are preimplantation embryos that have successfully passed the critical step of genomic activation and so have a high developmental potential (52). As blastocysts contain many cells, loss of some cells during freezing will cause lower harmful effect to the embryonic development. However, development of embryos with reduced viability will arrest after extended culture. Thus, they will not undergo cryopreservation (53).

In embryo cryopreservation, it is difficult to eliminate the ice crystals formation and its hazardous effects with slow freezing technique.

Critically, before the storage of embryos in liquid nitrogen, a long period of time for routine calibration of the controlled-rate freezing equipment and maintenance is obligatory for the cryopreservation success. Selection of the most appropriate cryoprotectants, the accurate duration, and the adequate temperature are important to improve the success of slow freezing cryopreservation (17 - 19). On the contrary, vitrification, as mentioned earlier is a time consuming method that is completed within 10 minutes by only one embryologist. Consequently, more attention has been focused on vitrification technology as an alternative to the slow freezing technique, especially after reporting the first embryo vitrification pregnancy by Yokota el al. (54).

In early 2000s, different survival rates were reported mainly because of variations in vitrification techniques (19). This difference might be due to the hardening of the zona pellucida after the cortical reaction that occurs with and after the process of fertilization, which gives the ooplasmic membrane more stability to cope with the low temperature and osmotic changes. Vitrification carries out significantly higher survival and pregnancy rates (20). Based on cryopreservation of over 16000 embryos, vitrification resulted in higher survival rates (84%) for all embryonic developmental stages and higher pregnancy rates when compared to slow-rate freezing technique (14, 29). However, the majority of studies which comprise vitrification of early stage embryos have reported as high survival rates as over 80%. The majority of studies have reported pregnancy rates in the range of 22 - 30%, which are completely in acceptable ranges and much higher than the rates of slow rate freezing procedures (29, 30, 55 - 63). More recently, even pregnancy rates as high as 35% have also been reported with vitrification of both cleavage embryos (60) and PN stage zygotes (61). These reported successful pregnancies and recent data suggest that vitrification of human zygotes and early stage embryos are perfect alternatives for slow freezing techniques. Especially in countries where cryopreservation of later-stage human embryos is not allowed by law or due to religious reasons, vitrification seems to be quite beneficial (61 - 63).

El-Danasouri et al. reported that the rate of survival tends to increase with the increasing

number of blastomeres and cell stage cleavage embryos (57, 58). As well, higher pregnancy and slightly higher survival rates have been attributed to the further stages of human embryos such as 8-cell (59, 60) and blastocyst stages (64, 65). However, other studies have shown equal (62, 63), or even higher survival rates (29, 61) by the vitrification of PN stage zygotes. As well, it should not be underestimated that the differences in pregnancy rates between further stage embryo vitrification might occur due to the effect of the transfer day.

Vitrification has an excellent survival and clinical success rates with a low rate of cellular cryo-damage (61). In a recent study published by the present group (66) using the slow freezing method for cryopreservation of human zygotes, the pregnancy rate per embryo transfer was reported to be 10.2%, while with the use of vitrification the pregnancy rate was found to be more than three times higher (61). Accordingly use of slow freezing method was stopped completely in the relevant centre and it has been replaced by a routine vitrification program after a long period of practicing the conventional slow freezing method. More recently, the present group reported a 100% survival rate of zygote PN-stage subsequent to vitrification along with high pregnancy (27 - 36.8%), and low abortion rates (17.42%) among vitrified 339 embryos (61). Desai et al. (67) performed a study of vitrification on the human embryos at 6-8 cell- stages. The post- warming survival rate, the clinical pregnancy and the implantation rates were 85%, 44.2% and 19.9% respectively.

Blastocyst Stage Cryopreservation: Blastocyst freezing has three major rationales: (1) the superiority of blastocyst-stage over earlier stage freezing in terms of implantation per thawed embryos which improves the overall expectations for cryopreservation programmes; (2) maximizing the cumulative pregnancy rates per oocyte retrieval; and (3) extended in-vitro culture of human embryos is becoming more common, encouraging the routine use of blastocyst transfer in IVF programs which is of reduced chances for multiple pregnancies (68).

Blastocysts and further stages of human embryos have different physiological requirements than early stage embryos. These requirements affect the survival rate of the organism exposed to harmful conditions like ultra rapid freezing (19). A major factor that affects the survival rate of blastocyst is the fluid-filled cavity called blastocoele. As expected, the formation of intracellular ice crystals is directly proportional to the volume of blastocoele. Vanderzwalmen et al. initially encountered low survival rates after vitrification of blastocysts (25). However, they were able to overcome the problem by reducing the size of the blastocoelic cavity through puncturing it with a special pipette before the procedure (69), by kewise, Mukaida et al. reported artificial shrinkage in blastocyst vitrification with increased success of the technique (64, 65). Nonetheless, nowadays, without artificial shrinkage or puncturing, blastocyst vitrification has tended to be extremely successful with increased clinical outcomes.

On the other hand, blastocyst stage embryo has also the advantage of possessing many cells, thus the loss of few blastomeres during the freezing and thawing processes might not compromise the integrity of the entire specimen. As well, no increase in chromosomal aneuploidy has been reported. On the contrary, an increased rate of DNA fragmentation was defined in frozen/thawed bovine blastocysts suggesting a possible damage by cryopreservation (70). Thus, a special attention still should be given on this issue; however, Takahashi et al. indicated a normal incidence of congenital defect and anomaly after blastocyst vitrification (71).

Until the year 2002, cryopreservation of blastocyst stage was performed mainly by the slow-rate method that let acceptable outcomes (72 - 78). However, with advances in vitrification, the method of choice for cryopreserving blastocyst stage embryo is vitrification rather than slow-rate technique. Moreover, after year 2002, the outcomes of vitrification in blastocyst stage were critically improved and reported to be as high as 97.5 - 100% survival and a 44 - 53% pregnancy rate (71). Furthermore; most of the recent reported data on survival and pregnancy rates were above 90% and 50%, respectively (79). Moreover, high implantation rates (25 - 29%) along with low abortion rates (17 - 22%) have been reported (72-79). These recent mainly suggested that vitrification seems to be the future of cryopreservation

with very high rates of pregnancy and survival outcome ever reported. A recent review by the present group, precisely indicated that vitrification is the best choice in further stage embryo cryo-preservation due to superior outcomes than slow-rate freezing which is quite similar to physio-logical rates (68).

Cryopreservation of Ovarian Tissue and the Whole Ovary: The first human live birth upon orthotopic transplantation of cryopreserved ovarian tissue was reported in 2004 (80), in spite of the introduction of the concept of ovarian transplanta-tion since 1906 (81). Ovarian tissue harvest can easily be performed using laparoscopy. There upon, ovarian cortex is sliced into small fragments after being cleaned from the medulla so that cryoprotectants can easily diffuse into cortical tissue.

There are two main methods to transplant frozen-thawed ovarian tissue. In the orthotopic ovarian transplantation, the frozen-thawed ovarian tissue is grafted into the pelvis (80, 82). In heterotopic ovarian transplantation, the ovarian tissue is grafted subcutaneously into the forearm or abdominal skin (83, 84). This method is much less invasive and tissue monitoring is simple for patients who have risk of cancer recurrence in the ovarian graft (85). The most important advantage of this method is prevention of cytotoxic effects of chemotherapy and/ or radiation therapy in patients who need further treatment. Although, heterotopic transplantation should require IVF to retain fertility, natural conception can occur in the case of orthotopic ovarian transplantation. The grafts starts producing hormone 3 - 4 months after trans-plantation (86) and the longest reported survival time for the graft is three years (84), which suggests that transplantation, should be performed close to the time when conception is desired.

Dspite its infancy to preserve fertility and lack of studies, ovarian tissue cryopreservation has several potential advantages, such as the presence of many primordial follicles with oocyte arrest in diplotene of prophase of the first meiotic division, primordial follicles being theoretically less cryosensitive than mature oocyes, and preserva-tion of the endocrinal function of the ovary. Therefore, this technique is useful for young women at risk of premature ovarian failure where spermatozoa or fertilization is not required and cryopreservation can be done independent of the menstrual cycle. However, it has the disadvantage of involving two operations for both harvesting and transplanting the tissue. The primordial follicles are unevenly distributed throughout the cortex with differences in the actual number of follicles in multiple samples from the same ovary. At least 60% of all follicles are expected to be lost during the initial ischemia after transplanting the ovarian tissue and ovarian grafts are also expected to have a limited lifespan (87, 88). It also bears the possibility of transmitting viruses including HIV (89, 90), hepatitis B and C (91). Transmis-sion of cancer cells is also possible and cancers have been known to recur in patients with remission after the replacement of autologous cryopreserved bone marrow (92).

Previously, ovarian tissue cryopreservation used to be done by slow freezing and rapid thawing with low follicular survival rates (5 - 50%) (87, 93). Presently ovarian tissue cryopreservation is done by modified vitrification with higher follicular survival rates (80.3%) (94). Isachenko et al. (95) reported that subsequent to thawing, culture of vitrified ovarian tissues in 30 millilitres of culture medium with agitation is associated with a higher number of none degenerated oocytes rather than culturing in two millilitres of medium or in 30 millilitres of medium without agitation. Till now, separation of primordial follicles from stromal tissue or cancer cells was not possible but it is still under trials, as polymerase chain reaction (PCR) has increased the sensitivity of tests to detect the presence of remnant cancer cells. It may be also possible to culture tissues in-vitro under conditions aimed at eliminating cancer cells, but the use of cytotoxic drugs has its deleterious effect on grafts.

To date, there have been two human live births after orthotopic transplantation of cryopreserved ovarian tissue (80, 82). Huang et al. (96) reported that immature oocytes can be retrieved successful-ly from the visible antral follicles of excised ovarian tissue, matured in vitro and cryopreserved by vitrification. Oocyte viability rate in ovarian tissue before and after cryopreservation by vitrification was studied, first in bovine and later in humans, and a 97% survival rate of oocytes has been reported for bovine ovarian tissue which was not different from the result of fresh bovine ovarian tissue (97).

Cryotissue method, which is similar to the cryotop, was performed for bovine ovarian tissue by vitrification as an animal model for human clinical trials. No ice crystal formation of ovarian tissue during cryotissue method has been documented and 88% survival rates have been obtained for cattle ovarian tissue vitrification where 100% of the ovarian tissues had been successfully autotransplanted in four cattle (98). In all of these cases, a prompt recovery of normal estrous cycle occurred two months after the transplantations. Subsequently, human ovarian tissue from donors was transplanted for a cancer patient. The transplanted tissue was vitrified by the cryotissue method and a similar post-thaw survival rate, as high as 89%, was obtained. This study critically showed the potential use of this technique in human ovarian tissue cryopreservation (98).

Whole ovary cryopreservation has been proved to be a feasible technique in fertility preservation and high follicular survival rates and successful histological appearances with viability have been demonstrated in several human and animal studies (99 - 101). Moreover, hormonal restoration of the ovary, and live births after cryopreservation of the whole or hemi-ovary and its contralateral orthotopic transplantation have been reported in some animal studies (93, 102) However, there are some difficulties in the cryopreservation of the whole ovary and its heterotopic or orthotopic transplantation indicated by other studies with extremely low follicular survival after transplantation (87, 103) These difficulties and low follicular survival rates have been tried to overcome by microvascular reanastomosis and/ or transplantation of the ovaries (93, 103). Both vitrification and slow-rate techniques have been studied and successfully implemented in whole ovary cryopreservation. Authors have asserted that cryopreservation of the whole ovary and transplantation will be the selected method which may replace the oocyte banking in the near future. Although the high survival and viability rates after cryopreservation have been defined, difficulties after overcome of the low follicular survival rates after ovarian transplantation.

Conclusion

Nowadays, vitrification seems to have replaced the former slow rate freezing protocols by improved survival and clinical outcomes. Although different stages of human gametes and embryos show different physiological needs and features that can affect the survival rate, especially, upon laboratory procedures. Undoubtedly, outcomes of vitrification of human embryos at different developmental stages are quite encouraging. Therefore, vitrification should be accepted as a real, viable and a more efficient alternative for cryopreservation of human embryos. Nevertheless, concerning cleavage and blastocyst stage vitrification, the vitrification in both embryonic developmental stages seem to be favorable and efficient in regards to increased survival and pregnancy rates. On the other hand, more advanced pregnancy rates have been reported by vitrification at blastocyst stage. However, pregnancy rates are not merely a fair parameter for comparison due to controversies about the differences of transfer day. On the contrary, we should benefit from the great advances both embryo culture and vitrification techniques offer. Acceptably high rates of pregnancy were also reported by vitrification at cleavage and PN stage. However, vitrification of cleavage and PN stage of human embryos are important and critical alternatives concerning similar survival rates, as well as high blastocyst formation rates. Vitrification at this stage will serve quite a lot in countries where further culturing is not allowed. The possibility of easy and safe application of early pre-implantation techniques and in vivo maturation procedure are also other advantages where professionals have benefited from vitrification of all embryonic stages. Therefore, in the current practice of vitrification, whether cleavage and PN or blastocyst stage, should be recommended instead of slow rate freezing which is costly and requires programmable freezers.

There are some unanswered questions; first, should we really expect less chromosomal damage in blastocyst stage due to increased inner cell number? And second, could other early selection assessments improve our embryo scoring and selection for vitrification during early embryonic stages? Third, is reduced number of vitrified embryos at blastocyst stage critical for success or does this give the chance of a better selection for vitrified embryos? Nevertheless, there is not a

quite reasonable point yet to select the blastocyst stage vitrification rather than the early stage one, where all the aforesaid advantages can be obtained by the early stage embryo vitrification along with extended embryo culturing.

Cryopreservation of both ovarian tissue and the whole organ has been proved a feasible technique in fertility preservation. Nevertheless transplantation of ovarian tissue or cortex is precisely working whereas some advances in improving follicular survival in the whole ovary transplantation is necessary. However, in the near future the cryopreservation of ovarian tissue and the whole ovary seems to replace oocyte banking for fertility preservation. On the other hand, oocyte banking should be the selected method in fertility preservation and artificial reproductive techniques where absolute follicular depletion or loss is not expected, especially in the ethical point of view. Nonetheless, great opportunities have been witnessed in cryopreservation and all developmental stages of gametes or generated embryos, as well as tissue, and the whole ovary could be cryopreserved with amazing success.

Acknowledgement

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