Current aspects of blastocyst culture, biopsy and vitrification

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Summary

Despite the considerable improvements achieved in the field of human reproduction since the first baby was born in 1978, the IVF success rates reported worldwide are still unsatisfactory. A possible explanation is the high proportion of aneuploidies observed in human embryos, which lead to a high percentage of implantation failure. Recently, with the enhancement of culture conditions and the development of accurate technologies for whole genome amplification, it has become possible to screen the genetic status of the blastocysts in order to select those with higher potential to implant. These applications, together with highly successful vitrification procedures for embryo cryopreservation are leading to significantly increased clinical outcomes. In this review, we present the current state of art of some technical procedures and applications regarding blastocyst culture, vitrification and biopsy.

KEY WORDS: blastocyst culture, blastocyst vitrification, trophectoderm biopsy, PGS.

Introduction

The first report of a human baby born thanks to in vitro fertilization techniques dates from 1978. Thereafter, more than 5 million babies have been born worldwide due to assisted reproductive technologies (1). Since then, considerable advances have been made in clinical and embryological procedures, leading to highly performing stimulation protocols and to improved methods for embryo selection and quality assessment. Nevertheless, the clinical results achieved to this day are still quite unsatisfactory and insufficient especially for certain patient categories. In Europe the average clinical pregnancy rates reported for the overall population are around 32% with fresh and of 22% with frozen-thawed embryos (1). The high prevalence of aneuploidy in human embryos could be at the origin of these poor results. However, the success of assisted reproductive techniques depends on both embryo and endometrium quality. In 2/3 of the cases the primary cause for implantation failure seems to be endometrial receptivity impairment, while the embryo itself is responsible for only 1/3 of the failures (2). The highest pregnancy rates are obtained in fresh oocyte donation cycles, where the embryos are transferred to an unstimulated endometrium that has not suffered the effects of the supra-physiologic hormonal levels occurring during ovarian stimulation. Frozen embryo transfer has similar conditions. A recent systematic review showed that the use of frozen instead of fresh embryos for transfer significantly improved clinical outcomes, suggesting that it may be advantageous to cryopreserve all viable embryos and replace them in a subsequent natural cycle (3). Recently, the development of sophisticated technology for preimplantation genetic screening, combined with improved blastocyst culture protocols and with more efficient vitrification procedures, seems to be promising for the achievement of consistently increased clinical results. In this review we present a state of art overview of some technical issues regarding blastocyst culture, vitrification and biopsy.

Blastocyst culture and cryopreservation

After the introduction of improved culture media
combined with low-oxygen tension culture system, the extension of embryo culture to the blastocyst stage has become more successful (4). It is now generally accepted that blastocyst culture is an effective strategy for selecting the most viable embryos within a cohort, leading to a decrease in the total number of embryos transferred per patient and also to a reduction of multiple pregnancies. It seems that performing transfer at the blastocyst stage, the uterine receptivity is increased and the embryo is more capable to implant. Higher implantation, pregnancy and live birth rates are widely reported with blastocyst transfer compared to cleavage stage transfer (5). Recently, a review analyzing 50 randomized trials reported a higher live births rate in blastocyst transfer compared to cleavage transfer (32 vs 42%). Anyway the pregnancy and miscarriage rates were similar in the two groups. In addition, a higher cumulative clinical pregnancy rate was found in cleavage transfer, probably due to the higher percentage of frozen embryos transferred and lower failure to transfer per couples achieved in this group (6). On the other hand, this approach faces the risk that some or even all embryos may not reach the blastocyst stage. Anyway, it is impossible to known if a cleavage stage embryo who is unable to reach the blastocyst stage in vivo, would be able to develop and implant in vitro if transferred in uterus (7). The first report on a human baby born from frozen-thawed embryo was in 1983. At the moment, cryopreservation plays an important role on the success of IVF programs, reducing multiple pregnancies while achieving very good cumulative pregnancy rates. For a long time, slow freezing has been the procedure commonly applied for human oocyte and embryo cryopreservation. Over the last decade, the optimization of vitrification procedures has translated into excellent survival rates (8). A recent systematic review and meta-analysis analyzes the literature regarding the main embryo cryopreservation procedures for different embryonic developmental stages. The survival rate after thawing is higher after vitrification compared to slow freezing, both at cleavage and blastocyst stages (9). Vitrification seems to be superior to slow freezing in terms of clinical pregnancy and implantation rates. Edgar and Gook in a recent review of 220 studies published about cryopreservation, found that slow freezing of metaphase II oocytes lead to a lower survival rate and to a compromised development in respect to vitrification. On the contrary, cleavage embryos can be cryopreserved successfully with both procedures. Finally, blastocysts may be cryopreserved better with vitrification than slow freezing, even if optimal slow cooling can produce similar results (10). However, wide differences in cryoprotectants, protocols and type of supports employed are reported and randomized controlled trials comparing different vitrification methodologies are necessary. The key performance indicators should not limit to the survival after warming but also to the resumption of cleavage, blastocyst formation, implantation and even more importantly, the live birth rate. Ideally, a universal cryopreservation protocol for each developmental stage should be developed. However, nowadays it would not be realistic due to the variety of cryobiological and technical factors involved in the process (11).

In the last years, the literature has shown promising results. It has been reported an increased number of frozen-thawed embryo transfers with a success rate almost equal to that obtained with fresh embryos (12). A retrospective matched-cohort comparison of single embryo transfer showed higher pregnancy rates with frozen-thawed day-6 blastocysts compared to fresh day-6 blastocysts, although this difference did not apply to day-5 blastocysts (13). Another retrospective study pointed out statistically significant higher implantation and pregnancy rates in vitrified-warmed blastocyst transfer compared to fresh blastocyst cycles (14). A recent retrospective cohort study highlighted that after at least one blastocyst implantation failure episode, patients have a significant higher probability of live birth with a freeze-all strategy, followed by a subsequent frozen-thawed transfer on a natural cycle, instead of repeating a new fresh cycle (15). Anyway, additional prospective randomized trials are necessary to confirm these encouraging results, analyzing also the cumulative successes rates across all subsequent transfer using any frozen supernumerary blastocysts.

**Preimplantation genetic screening**

Preimplantation genetic diagnosis (PGD) allows to test the genetic status of the embryos before transfer; PGD has been conceived as an alternative to fetal prenatal diagnosis for those couples at risk of transmitting genetically inheritable disorders to their offspring. Lately, preimplantation genetic screening (PGS) has also been introduced to exam-
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ine the genetic status of the embryos of infertile couples undergoing IVF. Clinical indications for PGS include repeated implantation failure, recurrent miscarriages, severe male factor infertility and advanced maternal age (16). For a long time, embryo biopsy at cleavage stage has been the preferred procedure. More recently, trophectoderm biopsy with comprehensive chromosome screening (CCS) has been rapidly introduced into routine clinical practice; CCS allows the analysis of all 23 of chromosomes pairs, facilitating embryo selection according to the evaluation of their full karyotype. However, depending on the diagnostic methodology employed, the time for obtaining the genetic results for embryo selection could be highly variable (from 4 to 72 hours) (17). This may imply the need for vitrification because embryo transfer cannot be postponed indefinitely. There are some reasons why it seems to be convenient to perform trophectoderm compared to blastomere biopsy. First of all, embryos biopsied at the blastocyst stage have been found to have a higher euploidy rate compared to cleavage stage embryos. Performing trophectoderm biopsy, only those embryos with the best chance to be chromosomally normal and developmentally competent are analyzed (18). Secondly, blastocyst biopsy has a lower impact on developmental and implantation potential. An explanation for this evidence could be that a smaller proportion of cells are removed at the blastocyst stage. In addition, only extra-embryonic cells are biopsied leaving untouched the cells that will generate the fetus (19). Thirdly, performing trophectoderm biopsy, a large number of cells (between 3 and 10) are removed allowing a much more accurate diagnosis. This is particularly important to identify mosaic embryos. Lastly, based on the hypothesis of a self-correction of the cleavage embryos, performing a biopsy too early could lead to a loss of potentially competent embryos (20). However, the major limitation when performing blastocyst biopsy is that this developmental stage can be reached on day-6 or even on day-7 allowing no extra time to perform a fresh transfer. The vitrification procedure becomes necessary and the transfer has to be delayed to a subsequent natural cycle.

Technical issues

Extended embryo culture increases the efficacy of embryo selection but does not provide any benefit to the embryos themselves. It is generally accepted that blastocyst culture is not an appropriate option for all patients and there is not a general consensus on patient selection criteria. It has been proposed that a minimum number of good quality day-3 embryos could be one of the parameters important to make the decision to proceed with extended culture or not (21). Very recently, with the introduction of time lapse technology, a more accurate embryo morphological evaluation combined with embryo developmental kinetic analysis can be performed. However, very conflicting data are reported in the literature. There is still a considerable disagreement regarding which parameters are useful to predict blastocyst formation, implantation potential and ploidy status of the embryos (22). For this reason, each laboratory should set its own parameters to identify for each couple the number of expected viable embryos, based on the specific culture conditions employed (atmospheric versus physiologic oxygen concentration, single step versus sequential media, culture volume, separate versus grouped culture, and so on). The final goal should be the achievement of a number of blastocyst adequate to guarantee the performance of the genetic screening, followed by vitrification and/or transfer, avoiding cycle cancellation. When the number of available embryos is expected to be low, for example in poor responder patients or in severe male infertility, it is possible to apply an oocyte accumulation strategy. It has been demonstrated that the accumulation by vitrification of oocytes obtained both in natural (23) and in stimulated cycles (24) can lead to a lower drop-out and embryo transfer cancellation rates and also to better clinical outcomes in low responder patients. Ideally, the same accumulation strategy should be applied also to cleavage stage embryos, although it could not be applicable in some countries due to ethical and legal limitations. As mentioned before, the blastocyst stage can be reached on days 5, 6 or, rarely, 7 after insemination. When the expanded blastocyst is obtained early on day-5, the biopsy could be performed on day-5 followed by a fresh embryo transfer on day-6, if the genetic screening is carried out on site. When the expanded blastocyst stage is reached later on day-5, or on day-6 or -7, blastocyst vitrification is necessary immediately after the biopsy and the transfer has to be delayed to a subsequent natural cycle.

There are some evidences showing that expanded blastocysts frozen on day-5 or on day-6 have the same viability and implantation potential. The clinical outcomes achieved with day-7 frozen blas-
tocysts are significantly lower compared to day-6, but however much higher than those obtained in fresh day-7 blastocyst transfers. Probably the timing of endometrial receptivity may contribute to the lower clinical results achieved in fresh transfer and therefore cryopreservation of day-7 blastocysts seems to be the best choice (11). It has been reported that the risk of aneuploidy is 10% higher in embryos that do not blastulate until day-6. Anyway, even the slower blastocysts can be diagnosed as euploid leading to good clinical outcomes and contributing to increasing the cumulative pregnancy rate (25). However, more data on vitrified biopsied day-7 blastocysts transfer to determine their survival, implantation and live birth rates are necessary.

In case of absence of the genetic result after the biopsy, there is the possibility to perform a second biopsy on the same blastocyst, followed by a re-cryopreservation. There are some reports supporting the efficacy of the double vitrification procedure at the same (26) or a different developmental stage (27). On the contrary, there are still very few data on the outcome of a double trophectoderm biopsy. Taylor et al. reported a lower survival rate of three blastocyst twice biopsied and twice cryopreserved compared to blastocyst once biopsied and twice cryopreserved. In addition, two of the twice biopsied blastocysts were transferred but they did not implant (28). The authors hypothesized that the removal of too many trophoblast cells could impair the blastocyst viability. These results are not confirmed by our experience: in our fertility center eight single frozen transfer of twice biopsied blastocyst were performed achieving six clinical pregnancies and four live birth (unpublished results). Anyway, the number of analyzed blastocysts is too small yet and other studies taking in account also the perinatal outcome of the newborn are necessary.

Assisted hatching (AH) is the artificial disruption of the zona pellucida and can be created mechanically, chemically or by laser energy. To perform the blastocyst biopsy, AH can be made on day-3 at cleavage stage with the aim of promoting hatching when the blastocysts stage is reached. However, in this way it is not possible to predict where the inner cell mass will form, leading to some possible technical difficulties during the trophectoderm removal. An alternative method is represented by performing AH at the blastocyst stage on the opposite site of the inner cell mass, on the same day of the biopsy. Anyway, it seems that a natural hatching site usually develops in close proximity to the inner cell mass of human blastocysts. Recently, a comparison on the effect of the hatching site of 32 blastocysts has been reported. A higher performance of complete hatching was found when AH was performed near the inner cell mass than on the opposite side, although no data on implantation are reported (29). Even the optimal size of the holes in the zona pellucida have been investigated. Small holes were found to cause trapping or hatching delay of the blastocysts, while large holes resulted in higher pregnancy rates. Two recent publications, performed on 197,327 and 6,223 pregnancies respectively, obtained from assisted reproductive cycles showed an higher incidence of monozygotic twins associated with AH and with blastocyst transfer. In vivo, the zona pellucida remains intact until implantation. It was hypothesized that by drilling the zona pellucida the embryo may split resulting in monozygotic twins (30, 31). Another meta-analysis suggests that AH is related to increased clinical outcomes in repeated failure and frozen-thawed cycles (32).

Very recently, amplifiable DNA fragments were found in blastocoele fluids of around 90% of examined blastocysts. The amount of DNA was estimated to be equivalent to one embryonic cell. The samples are aspirated without performing a cell biopsy. The employment of whole genome amplification technologies to these fluid samples were shown to be feasible (33). Despite this new non-invasive technique seems to be really promising, the potential clinical use needs to be evaluated. A following trophectoderm biopsy should be performed to confirm that the DNA extracted from the blastocoele is representative of the blastocyst itself (34).

Conclusions

Following the scientific progress in assisted reproductive technologies, involving whole genome sequencing, extended embryo culture and vitrification protocols, PGS with trophectoderm biopsy has been rapidly introduced into routine clinical practice. As a consequence, more frozen-thawed embryo transfers are generally performed. Postponing embryo replacement to another natural unstimulated cycle is associated with many positive aspects. First of all, it permits to have more time for counselling and data analysis of the genetic results. Another benefit is the prevention of the risk of ovarian hyperstimulation syndrome. Furthermore, there are some evidences showing increased birth defects and adverse perinatal and obstetric outcomes.
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In fresh versus frozen-thawed embryo transfer, when both were compared with spontaneously conceived babies (35, 36). A large retrospective study on women who achieved pregnancy after both fresh and frozen embryo transfer demonstrated a significant increase of babies with low birth weight following fresh embryo transfer. Importantly, this reduction in birth weight was not observed in donor egg cycle, eliminating the possibility that the freeze-thaw procedure was responsible for it (37). Implantation is also dependent on endometrial receptivity. The implantation window in a natural cycle has an average duration of four days, after which the endometrium becomes a hostile environment for the blastocyst. It is known that the hormonal environment during a stimulated cycle is significantly impaired, due to exogenous hormones, leading to an altered or even absent receptivity. Current efforts are directed towards defining markers of endometrial receptivity during the putative window of implantation (38). Recently, it has been shown that a mechanical manipulation of the endometrium (scratching) could potentially improve endometrial receptivity. By performing this technique, it has been possible to obtain a live birth rate approximately twice as high to the one obtained in a control group, particularly in women with previous implantation failure (39, 40).

The recent data on morphokinetic evaluations are controversial and randomized controlled trials are necessary. Even if there seems to be a relationship between the ploidy status, blastocyst morphology and development dynamics, at this moment morphological and morphokinetics evaluation cannot replace PGS. However, these non-invasive methods can be used in combination with PGS to choose, within a cohort, the blastocysts to analyze or, when there are several euploid blastocysts, to select one for the transfer. Time lapse could be more appropriate than PGS for those patients whose embryos are of insufficient quality to recommend further extension of the culture up to the blastocyst stage. Morphokinetics could also represent a valid alternative to genetic analysis when PGS is not applicable for legal or personal reasons (41, 42).

In conclusion to date, combining extended culture, trophectoderm biopsy with whole genome amplification and blastocyst vitrification seems to be a highly efficient strategy to achieve excellent clinical results, even if there is still a great heterogeneity among different reproductive centers and there is no consensus on the superiority of any specific procedure regarding the optimal timing of blastocyst biopsy and vitrification or transfer. For these reasons, further high quality randomized controlled trials are needed to establish the optimal strategy to perform blastocyst biopsy, vitrification and transfer.

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