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Vitrification vs. slow cooling protocol using embryos cryopreserved in the 5th or 6th day after oocyte retrieval and IVF outcomes

Paweł Kuć[1,2], Agnieszka Kuczyńska[2,3], Bożena Stankiewicz[2], Piotr Sieczyński[2], Joanna Matysiak[4], Waldemar Kuczyński[2,5]

[1]Department of Perinatology, Medical University of Białystok, Poland
[2]Centre for Reproductive Medicine KRIOBANK, Białystok, Poland
[3]Department of Endocrinological Gynecology, Medical University of Białystok, Poland
[4]Warsaw University of Technology, Faculty of Mathematics and Information Science, Warsaw, Poland
[5]Department of Gynecology, Medical University of Białystok, Poland

Abstract: Modifying cryopreservation protocols may be seen as a way to simplify cryobanking procedure and increase satisfying outcomes. The aim of the study was to evaluate the influence of slow cooling protocol and vitrification on IVF outcomes using embryos preserved in the 5th or 6th day after oocyte retrieval. The study compared 2 groups of human embryos underwent slow cooling protocol (n=189) and vitrification (n=58). All embryos were cryopreserved in the 5th or 6th day after oocyte retrieval. Pre- and postfreezing embryo evaluation was performed in 2 or 3 steps scale, respectively. The study evaluates the effectiveness of two freezing methods and influence of the freezing day, pre- and postfreezing embryo grading on clinical pregnancy rate. Study showed higher pregnancy rate after vitrification (50.4%) than slow cooling protocol (25.9%). Significantly higher pregnancy rate was observed, when embryo preserved in the 5th day after oocyte retrieval (50.3%) than in the 6th day (22.7%). Postfreezing embryos evaluation showed that high quality blastocysts gave nearly four times better pregnancy outcomes than the ones evaluated as poor quality, and three times better than the ones evaluated as moderate. Prospective trials are needed to evaluate pregnancy and neonatal outcomes after vitrification. The number of controlled studies concerning vitrification has not been large enough, yet.

Key words: human embryo cryopreservation, vitrification, slow cooling protocol, IVF/ICSI, clinical pregnancy rate

Introduction

According to science development, there is a need to use quicker, simpler and safer technique for embryo cryostorage. The slow cooling protocols are a routine use in infertility clinics nowadays. However there are documented limitations to the current method. Damage the zona pellucida may results from the biological changes and has been correlated with poor outcomes. Modifying cryopreservation protocols – freezing and thawing using polymers and change the length of cooling methods may be seen as a way to simplify and speed up cryobanking procedure and correlated with more satisfying outcomes. Despite of many problems connected with vitrification technique, it has been a challenge for reproductive medicine, nowadays.

The first reproducible method for cryopreservation of mammalian embryo was reported in 1972 for the mouse embryos using DMSO as the cryoprotectant [1]. This slow-freezing method was proved effective also for humans. The first pregnancy from frozen-thawed human embryo was reported in 1983 [2]. Since then, this method has been widely used for human embryo at early cleavage state. Twenty two years ago a new alternative method for human cryopreservation – vitrification was reported by Rall and Fahy [3]. Cryprotectant in high concentration, in very small volume is used in this method to induce a glass-like state to rapid embryo cryopreservation, avoiding the formation of intracellular ice. The high osmolarity rapidly

Correspondence: P. Kuć, Dept. of Perinatology, Medical University of Białystok, Sklodowskiej-Curie Str. 24A, 15-276 Bialystok, Poland; tel.: (+4885) 7468352, e-mail: kucp@poczta.fm

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Table 1. Prefreezing blastocyst evaluation according to modified Gardner's scale.

Grade	Stage status and blastocyst development	Inner cell mass quality (ICM)	Trophectoderm quality (TQ)
1– high quality	1. An early blastocyst, blastocel being less than half volume of that of the embryo or 2. A blastocyast with a blastocel whose volume is half of, or greater than half of that of embryo or 3. A blastocyst with a blastocel completely filling the embryo or 4. An expanded blastocyst with a blastocel volume larger than that of the early embryo, with a thinning zona	A. Tightly packed, many cells or B. Loosely grouped, several cells	A. Many cells forming a cohesive epithelium or B. Few cells forming a loose epithelium
0 – low quality		B. Loosely grouped, several cells or C. Very few cells	C. Very few large cells

Table 2. Postfreezing blastocyst evaluation scale.

Quality grade	Thawed blastocyst status	Inner cell mass quality (ICM)	Trophectoderm quality (TQ)
2 – high	An expanded blastocyst with the same blastocels volume or larger than that of the before freezing	ICM is the same size or larger than before freezing. Tightly packed, many cells	At least 90% of healthy appearing cells
1– moderate	An expanded blastocyst with the same blastocels volume	The same size of ICM than before freezing. Loosely grouped, several cells	Between 70% - 90% of healthy appearing cells
0 – poor	An expanded blastocyst with the same blastocels volume	Smaller ICM than before freezing. Loosely grouped, very few cells	Below 70% of healthy appearing cells

dehydratates the embryo cells and submersion into liquid nitrogen rapidly solidifies cells without damaging by ice crystals.

The aim of the study was to compare two cryopreservation techniques efficiency in patients undergoing controlled ovarian stimulation in GnRH agonist "long protocol". The study evaluates also an influence of the freezing day, prefreezing and postfreezing embryo grading on pregnancy rate.

Material and methods

The retrospective study compared 2 groups of embryos underwent slow cooling protocol (83 patients – 189 embryos) or vitrification (38 patients – 58 embryos). All human embryos observed in the study were retrieved from patients undergoing controlled ovarian stimulation in GnRH agonist "long protocol" with the use of follitropin alpha and menotropine. Oocytes were collected under ultrasound guidance by standard means and were fertilized in all cases using ICSI procedure. Only embryos remained after fresh embryo transfer were involved in. All embryos development was observed to blastocyst stage and blastocysts were cryopreserved in 5th or 6th day after oocyte retrieval. Vitrification was performed using Freeze & Thaw (IRVINE Scientific) kits according to producer protocol. Quinn's Advantage Blastocyst Freeze Kit (Sage Media) was used in blastocyst slow cooling protocol according to protocol. Single or double embryo transfer was performed according to blastocyst survival rate in both groups of patients. Prefreezing and postfreezing embryo evaluation was performed in 2 steps or 3 steps scale, respectively. Prefreezing embryo evaluation was assessed according to modified Gardner's scale [4]. (Table 1) Postfreezing embryo evaluation was expressed in own "inner centre" embryologic scale: high quality thawed blastocysts (grade 2), moderate (grade 1) and poor quality (grade 0). (Table 2) Logistic regression for binary data was used to compare effectiveness of two protocols and influence of single or double embryo transfer, day of freezing, prefreezing and postfreezing embryo evaluation on pregnancy rate. Pregnancy rate was present as positive βhCG pregnancy test performed 14 days after embryo transfer and clinical pregnancy confirmed by ultrasound in 4-5 week after embryo transfer.

Results

Our study showed higher effectiveness of vitrification (50.4%) than slow cooling protocol (25.9%) in ongoing pregnancy rate (p=0.0371) (Fig. 1). Analysis of day of embryo freezing presented significantly higher pregnancy rate, when embryo was preserved in the 5th day after oocyte retrieval (50.3%) than in the 6th day (22.7%) (p-=0.0017) (Fig. 2). The embryo survival rate after thawing process was 84% and 82% for vitrification as well as 83% and 79%, for the 5th and 6th day of freezing respectively. Prefreezing embryo development evaluation in 2 steps scale was not statistically significant in pregnancy outcomes. Postfreezing 3 steps embryos evaluation showed that blastocyst estimated as high quality (grade 2 in the scale) gave near-

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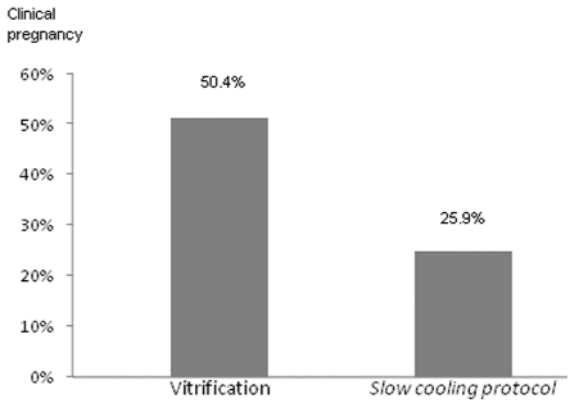

Fig. 1. Comparison of clinical pregnancy rate after vitrification vs. slow cooling protocol (p-value 0.0371).

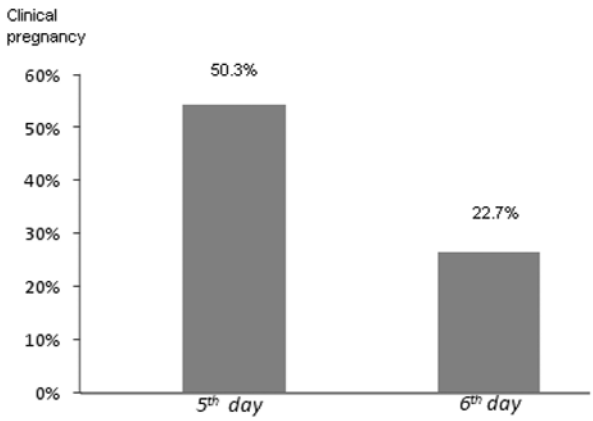

Fig. 2. Comparison of clinical pregnancy rate of blastocyst preserved in the 5th and in the 6th day after oocyte retrieval (p-value 0.0017).

ly four times better pregnancy outcomes than the ones evaluated as poor quality (grade 0), and three times better than the ones evaluated as moderate (grade 1) (Table 3).

The logistic regression model for binary data was applied, together with the backward elimination analysis. In this analysis we used a significance level of 0.05 to retain variables in the model, and it turned out that significant ones are the following: the day of preservation of the embryo (5th or 6th – with the p=0.0017), the cryopreservation technique used (slow cooling protocol or vitrification – with the p<0.002), postfreezing embryo evaluation (in three steps scale – with the p<0.01). The results of performing logistic regression analysis are shown in Table 2.

The pregnancy rate after vitrification was more than twice higher than the pregnancy rate under the slow cooling protocol. Similarly, it turns out that the pregnancy rate was nearly three times higher when blastocyst had been preserved in the 5th day after oocyte retrieval than in the 6th day. The analysis showed that the embryos evaluated as 2 grade in the

Table 3. Logistic regression analysis

	Odds ratio	95% confidence interval
Vitrification vs. Slow protocol	2.127	1.046-4.325
5th day vs. 6th day freezing	2.825	1.475-5.411
Postfreezing embryo eval.: 0 vs. 2	0.223	0.114-0.434
Postfreezing embryo eval.: 1 vs. 2	0.330	0.145-0.751

3 steps scale gave the best pregnancy outcomes – nearly four times better than the ones evaluated as grade 0, and three times better than the ones evaluated at 1 grade. The statistical computations were performed in the SAS System (PROC LOGISTIC from the SAS/STAT application).

Discussion

Cryopreservation and cryostorage have several potential advantages in human *in vitro* fertilization. The aim of cryopreservation procedures is to reach the highest survival rate and viability of human embryos after thawing. Commonly used slow cooling protocols have been used to cryopreserve all stages of human embryos, but clinically satisfactory outcome have not been obtained, yet. Slow cooling protocols require more expensive equipment and are more time-consuming. Various mechanisms which could damage embryos structure as intra and extracellular ice formation, cryoprotectant toxicity, osmoting swelling shrinkage should be circumvented to obtain high embryo survival rate. Vitrification is a new method which allows abbreviating time of procedure using combinations of a high concentration of cryoprotectant. To prevent the loss of embryos during vitrification they must be preserved at temperature below the glass transition temperature of cytoplasm, which is around -130°C. In practice liquid nitrogen -196°C is used for maintaining the temperature. The most important advantage of vitrification is producing any ice crystals during cooling and warming. At the other side the most important limitations of this method are toxic effects caused by high concentration of cryoprotectants and liquid nitrogen-mediated contamination. In contrast, in slow cooling protocols the effect of cryoprotectant toxicity is rather small, supported relatively constant results. Many research studies from last decade showed that vitrification method could be better than slow-cooling protocols in fertility treatment [5-8]. In last few years this technique has received increasing interest among embryologist leads to significant development. It is used to freeze animal and

human embryos at any stage of their development as well as germinal cells [7,9-17].

Blastocyst is the structure formed of numerous small cells. In contrast to slow freezing, during vitrification less number of cells is lost, increasing chances to further regular embryo development. A reliable development for vitrification is needed to preserve the supernumerary blastocysts because only few numbers of blastocysts are usually available to cryopreservation after fresh transfer. Some infertility clinics tried to preserve blastocyst with the slow-freezing method, but clinically satisfactory results have not been obtained [18]. The survival rate of blastocysts following the slow freezing method is about 60%, and pregnancy rate is generally less than 30% [19,20]. Vitrification process could become an alternative to slow freezing protocol, nowadays. Many research studies have been focused on vitrification due to significantly higher survival and pregnancy rates observed. The pregnancy rate and survival rate are reported as 37-48% and 70-95%, respectively [7,21-24].

Liebermann *et al*. [6] observed almost similar pregnancy rates in vitrified and slow-frozen blastocyst transfers 46% and 43% respectively. Authors presented also similar survival rate 96% and 92%, respectively. They also did observe any congenital defects in the newborns delivered after slow cooling and vitrification protocol. Congenital malformation rate in infants delivered after vitrification protocol is rather low and is similar to observed after fresh embryo transfer [24]. Other authors showed higher pregnancy rate after vitrification preservation. Stehlik *et al*. [25] reported that the pregnancy rate was 16% and survival rate 83% in slow cooling protocol in comparison to vitrified blastocysts which gave 100% survival and 50% pregnancy rate. The pregnancy rate following vitrification observed by Huang *et al*. [26] showed 53.8% and 77% survival rate. The largest study published till now comparing slow cooling protocol versus vitrification was performed on over than 6000 blastocysts by Kuwayama *et al*. [27]. Authors observed 53% pregnancy rate after vitrification and 51% pregnancy rates following slow cooling. Authors compared the blastocysts survival rate from slow-freezing and vitrification protocols obtained a post-thaw survival rate between 57 and 91% after slow freezing, and 90-100% after vitrification, similarly to Stehlik *et al.* studies. The randomized controlled trials by Kolibianakis *et al*. [19] compared effectiveness of vitrification and slow cooling protocol. The study showed no significant difference in pregnancy rates between two preservation methods. Authors showed that vitrification technique seemed to be better in postthawing survival rates. Postthawing blastocyst development of embryos cryopreserved in the cleavage state was significantly higher after vitrification than slow freezing. Authors maintained that vitrification does not appear to be associated with an increased chance of pregnancy, but has a significant advantage in postthawing survival rates in the cleavage as well as blastocyst stages.

Analysis of the day of blastocyst freezing, showed adverse data regarding pregnancy rates. Studies by Behr *et al*. and Shapiro *et al*. [28,29] did observed any differences between the 5th day and 6th day blastocyst freezing in pregnancy rate as well as survival rate. Marek *et al*. study [30] confirmed hypothesis for fresh transfers that embryos reaching blastocyst stage faster (the 5th day) could have better quality than slower ones (the 6th day) and could have better pregnancy rate for frozen blastocyst. Authors maintained that pregnancy rate for 5th day frozen/thawed blastocyst were significantly higher than for 6th day, and presented 50% and 29% respectively. Survival post-thaw rates were similar in both studied groups.

From the other site, analyzing cleavage-stage embryos and a traditional slow-freezing cryopreservation, survival rates of 76-80% are reported [31]. Most studies on vitrification of cleavage-stage embryos reported high survival rates of over 80%, and pregnancy rates in the range of 22-35%, which were much higher than the rates of slow-freezing procedures [7,27]. Besides Balaban *et al*. [5] presented pregnancy rates about 49% using vitrification of cleavage-stage embryos. Balaban *et al*. [5] compared survival rate of human 3-day embryo preserved by slow freezing and vitrification in the randomized, controlled study. Significantly higher embryos survival was observed after vitrification in 94.8% than after slow freezing protocol 88.7%. The development to the blastocyst stage was also higher following vitrification (60.3%) than following slow freezing (49.5%). Authors obtained 30% implantation rate in case of vitrification. Kuwayama *et al*. [27] presented higher survival rate in 4 cell human embryos after vitrification 98% than after slow cooling protocol 91% as well as higher pregnancy rate 53% in vitrification, and were 51% in slow cooling protocol, respectively. Rama Raju *et al*. [7] study showed that preservation of 8-cell human embryos by vitrification is more efficient than in slow cooling protocol. The survival rate after vitrification was 95%, as well as the pregnancy rates 35%, and it was significantly higher than after slow freezing – 60% and 17.4% respectively.

Conclusions

Vitrification is an alternative to slow freezing protocol and is associated with the higher pregnancy rate and higher embryo survival. The prospective trials are needed to confirm this hypothesis and to evaluate pregnancy and neonatal outcomes. There is still not enough number of childbirths and controlled studies concerned vitrification technique. Furthermore, vitrifi-

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cation is suitable in infertility clinics, where a small number of embryos are cryopreserved in a short period by a simple method. Multiple pregnancies risk connected with freezing lots number of embryos using slow cooling protocols could be limited using vitrification technique, on condition that it present significantly higher efficacy. It could also reduce the ovarian hyperstimulation syndrome [32].

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