Dimethylsulfoxide vs. 1, 2 Propandiol as Cryoprotective Additives during Vitrification in Cryopreservation of Human Embryos

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Abstract

Objective: Assessment of the survival rate of human embryos after vitrification/thaw process using two different freezing (vitrification) media with two different solvents; Dimethylsulfoxide (DMSO) based medium and 1,2Propandiol (PROH) based medium. These two solvents act as cryoprotective additives (CPA) that provide a protective effect for human embryos vitrification.

Subject & Methods: Day2 or day3 divided human embryos were exposed to DMSO or PROH based media before plunging into Liquid Nitrogen (LN2) storage. Later on, during replacement cycles, the embryos were thawed and their survival rate was evaluated.

Results: Two hundreds and twelve embryos in vitrification process were into two groups. Group A (114 embryos) were treated with sequential vitrification medium based on DMSO and Group B (98 embryos) were treated with PROH based medium. Thawed embryos were transferred to physiological media designed for cleavage stage embryos and kept in the CO2 incubator for 2 to 8 hours. After vitrification/thawing procedure, the survival rates of group A (DMSO) and group B (PROH) were 85 survived embryos of 114 (74.6%) and 52 survived embryos of 98 (52%) respectively (P value<0.05).

Conclusion: DMSO based medium used for vitrification/thaw process of day2 or day 3 divided embryos demonstrated a significant higher embryo survival rate than that of PROH based vitrification medium.

Keywords: Embryo cryopreservation; Dimethylsulfoxide; DMSO; 1, 2 Propandiol; PROH; Vitrification.

Introduction

The use of controlled ovarian stimulation protocols during ART programs led to the production of large numbers of human oocytes and consequently embryos. In a routine IVF practice, 60 % of stimulated IVF cycles may yield surplus embryos suitable for storage by freezing (Cryopreservation) [1]. Cryopreservation now plays a pivotal role in clinical assisted reproduction and has a profound impact on treatment strategy [2]. The first successful pregnancy after transfer of a frozen-thawed embryo was achieved in 1983 by [3] using a slow freezing protocol with dimethylsulfoxide (DMSO). Since then, thousands of other babies have been born as a result of the transfer of frozen embryos.

Cryopreservation is the technique of freezing cells and tissues at very low temperatures (-196°C) at which the biological material remains genetically stable and metabolically inert, while minimizing ice crystal formation. In general, when a tissue is subjected to low temperatures, ice crystals will eventually form. These crystals may disrupt the cell membrane leading to the death of the cell. The goal of cryopreservation is to replace some of the water with other compounds that will not form large crystals when frozen. These compounds are known as the cryoprotective additives (CPA) [4]. The most common methods of human embryos cryopreservation are the Slow Freezing and the Vitrification:

1.Slow Freezing

Slow-freezing protocol means a slow, controlled rate of cooling, after exposing the embryos stepwise to low concentrations of CPA. During slow-cooling, cells dehydrate, shrink and the concentration of solutes increases as water freezes in the medium. Slow freezing method is consuming more time up to 3 hours and requires a special freezing programming machine to provide the controlled freezing procedure [5].

2.Vitrification

Vitrification is a process which, by combining the use of concentrated solutions with rapid cooling, avoids the formation of ice. Samples reach low temperatures in a glassy state, which has the molecular structure of a viscous liquid and is not crystalline. This method has the potential advantages of being rapid to carry out and does not require controlled rate cooling apparatus. Good survival of human embryos has been demonstrated by vitrification in a number of laboratories [6]. It has been suggested

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that vitrification imparts less trauma to cells and is, therefore, a more effective means of cryopreservation of the human embryo than conventional slow freezing [7],

The general properties of cryopreservation media (CPAs) are they are mostly chemical solvents of a low molecular weight, non-toxic and can permeate cells [4]. Nowadays dimethylsulfoxide (DMSO) and propandiol (PROH) are the most successfully used, FDA approved CPAs. At the same time, they are the most widely studied CPAs for decades. In general, it is widely accepted that PROH is best used for pronucleate stage (day 1) - based cryopreservation programs, while DMSO is superior in dividing embryo stage (day 2 or 3) –based cryopreservation programs [8-10].

However, these studies were widely based on slow freezing protocols and as previously mentioned; the vitrification technique is now gaining wide popularity and is suggested to be superior for cleavage-stage embryos [11]. We hypothesized that the rule of superiority of DMSO based slow freezing program applies to DMSO based vitrification program. However, lack of comparative studies supporting this hypothesis for human embryos has led us to design this study to investigate this point.

Subjects and Methods

The study was conducted during the period from March 2009 till January 2011. The study was carried out in a private setting in Cairo, Egypt. Study protocol was approved by ethical committee of Suez Canal University. All eligible women who had undergone IVF/ICSI cycles with supernumerary good quality embryos during this period were included in the study after signing initial relevant consents. All women had long protocol IVF or ICSI cycles. They received GnRH a in the form of 0.1 mg triptorelin acetate as daily subcutaneous injection starting on day 21 to 23 of the menstrual cycle. Triptorelin acetate administration was continued until loss of follicular activity by transvaginal ultrasonography. At this stage exogenous gonadotropins was initiated and triptorelin acetate was decreased to half. When the largest 3 follicles reached 18 mm diameter, a single 10,000 IU intramuscular dose of human chorionic gonadotropins (hCG) were administered. Transvaginal follicular aspiration took place 35-36 hours later. The fertilization procedure was applied either by IVF or ICSI. Embryo transfer was performed two or three days later by replacement of morphologically best three embryos.

Supernumerary good quality embryos (class A or B [10]) were subjected to cryopreservation under the local ruling conditions. Cryopreservation procedure was applied on day 2 or 3 at 4-8 cell stage embryos. Patient-based randomization allocated frozen embryos into two groups; group A; embryos were cryopreserved with ready-to-use commercially available DMSO vitrification media & group B; embryos were cryopreserved with ready-to-use commercially available PROH vitrification media using the following protocol:

1. After exposure to equilibrium solution for 5 to 15 min embryos in group A and B were exposed to ready to use commercially available DMSO or PROH vitrification media for 60-90 seconds based on simple randomization table.
2. Embryos were then loaded in HSV Straws with two parts; inner one where the embryos were loaded and external cover to protect the inner part.
3. After loading the embryos in HSV straws, the external part of the straws were sealed by heating sealer and then immediately stored in LN2 tanks where they were completely covered with LN2 to be stored at -196 ˚C.
4. Later on, during the replacement cycle, thawing procedure was applied to the embryos of each group using ready-to-use commercially available thawing solutions. Thawing solutions are applied in 3 steps with different sucrose concentrations; 1M, 0.5M, 0M. Embryos were exposed to the media at each step for 2-3 minutes. At the last step, the embryos were exposed to physiological sucrose free solution as a final wash step. These steps allow the withdrawing of the intracellular CPA gradually and not in a sudden manner to minimize the cell shock and maintain the cell viability.
5. Embryos were then incubated in CO2 incubators, cultured in physiological cleavage media for 2 to 8 hours. Then examination for survival was performed.

The main outcome measure was embryo survival rate during thawing in replacement cycles. Survival was indicated by complete return of all embryo blastomeres to the cytoplasmic and morphological conditions existing prior to vitrification. SPSS 19 package (SPSS, Chicago, IL, USA) was used for statistical analysis. Data was expressed as means ± SD. Student t, chi square and factorial ANOVA tests were used when appropriate. Significant values were set at $p<0.05$ level.

Results

Overall, 66 women were eligible for the study and had undergone thawing of embryos in 66 replacement cycles. All participants were undergoing a replacement cycle after a failed first fresh cycle. A total of 212 embryos were included with 114 embryos in group A and 98 embryos in group B.

Table 1 shows a comparison between the two groups as regards to female age, number of oocytes retrieved, oocyte maturity, the fertilization rate and the division rate. The only significant difference was shown in fertilization rate (56.2% vs. 47.6% for group A and group B respectively, p=0.04).

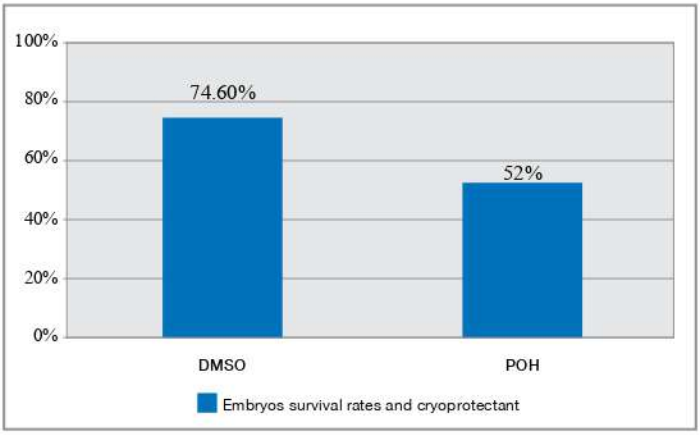
Table 1: Cycle characteristics of the two groups

	DMSO(n=36)	PROH(n=30)	P
Age	28.9 ± 4.6	29.67±5.2	0.51
Retrieved oocytes	16.77±4.3	18.55±3.6	0.40
Mature oocytes	14.43±2.3	14.08±3.1	0.83
Fertilized oocytes	10.9±1.8 (65.2%)	8.86 ±2.3 (47.6%)	0.04 (S)
Divided embryos	10.7±2.4 (64%)	8.83±1.4 (47.5%)	0.05

S: Significant at 0.05

After thawing survival rate was examined in the two groups. 85 out of 114 embryos (74.6%) survived group A, in contrast to 51 out of 98 embryos (52%) for group B, the difference was highly significant, $p < 0.005$. Figure 1

Figure 1: Post-thawing survival rate in the two groups.



To avoid bias, the following factors: age, number of retrieved oocytes, number of mature oocytes, number of fertilized oocytes and number of divided oocytes that were retrieved at fresh cycles, were tested in relation to number of survived embryos in replacement cycles via factorial ANOVA test. None of the mentioned factors significantly affected the number of survived embryos (table 2).

Table 2: ANOVA test relating different influencing factors to number of survived embryos in the two groups

	Group A		Group B	
	F	P	F	P
Age	0.549	0.892	1.128	0.413
No. retrieved oocytes	0.791	0.69	0.889	0.609
No. mature oocytes	0.439	0.952	1.46	0.243
No. fertilized oocytes	1.54	0184	2.043	0.087
No. divided oocytes	1.626	0.157	2.007	0.092

We investigated embryo survival per stage of embryo development. For 4-cell stage , 29 out of 43 embryos (67.4%) survived in group A, in contrast to 25 out of 41 survivor embryos in group B, (60.9%), p=0.13. For 6-cell stage, 34 out of 44 embryos (77.2%) survived in group A, in contrast to 19 out of 35 survivor embryos in group B (54.2%), p=0.02. For 8-cell stage, 22 out of 27 embryos (81.4%) survived in group A, in contrast to 7 out of 22 survivor embryos in group B (31.8%), p=0.01. Table 3

Table 3.Survival rate per stage of embryo development

	Group A	Group B	p
4-cell stage	67.4%	60.9%	0.13
6-cell stage	77.2%	54.2%	0.02 S
8-cell stage	81.4%	31.8%	0.01 S
Fertilized oocytes	10.9±1.8 (65.2%)	8.86 ±2.3 (47.6%)	0.04 (S)

S: Significant at 0.05

Disscussion

The role of cryopreservation in assisted reproduction has long been a milestone in IVF structure, with 60% of cycles having extra embryos suitable for freezing and up to 18% of total IVF cycles in USA are non-donor frozen cycles [12]. IVF centers usually adopt one of two strategies for embryo replacement upon having a good number of fertilized oocytes. The choice is principally based on local expertise and preference of individual centers. The 1st strategy is based on 1st day post oocyte collection (pronucleate stage) embryo freezing. It usually entails keeping five 2 PN embryos for fresh replacement and freezing the extra embryos for future replacement. The 2nd strategy is based on divided embryo (day 2, 3 or 5) freezing. It usually entails choosing the best 1-3 embryos for fresh embryo transfer and freezing the extra good quality dividing embryos for further replacement cycles. This strategy is more widely accepted by most of the centers. Traditionally, with the use of slow freezing protocols, the two most widely used cryoprotective agents were 1,2 propandiol (PROH) and Dimethylsulfoxide (DMSO), with plethora of evidence suggesting superiority of PROH for pronucleate stage freezing and DMSO for dividing stage embryo freezing programs [8-10]. Van der Elst et al [10]in 1995 have conducted a randomized controlled trial on different protocols of slow freezing, two of them were using each of the two cryoprotective additives solely and the third was a combination of the two. The study proved superiority of Dimethylsulfoxide based freezing protocol on the other two protocols as regards to embryo survival as well as pregnancy per transfer rates. Their suggested explanation was a change of membrane permeability and surface to volume ratio in multicellular as opposed to unicellular embryos, in addition to difference in freezing temperature favor Dimethylsulfoxide for freezing of divided embryos. Other explanations were suggested by Brian Wowk [13] that unnecessary formation of hydrogen bond between POH molecules and proteins less active sites causing the risk of a complete lethal dehydration and over consumption of POH but DMSO is leaving these sites for some water molecules preventing the lethal complete dehydration. Also, crowding occurs due to over-reaction between 1,2 propandiol and protein molecules weakens the hydrogen bonds that in return minimizes the activity of 1,2 propandiol as a cryoprotective agent , thus, allowing the protein molecules to regain its chemical activity leading to a lethal effect..

Vitrification, as a method of cryopreservation, has been gaining wide acceptance during the last decade. It entails rapid cooling after emersion with concentrated cryoprotective agents, avoiding the formation of ice and possible cell trauma related to over dehydration or possible rehydration. Good evidence has demonstrated survival of human or animal embryos comparable or even better to conventional slow freezing method [6, 7, 14].

As the technique entails use of high concentration of cryoprotective agent, it is expected that type of cryoprotectant agent is of utmost importance to achieve this tissue equilibrium. Several agents, including dimethylsulfoxide, acetamide, and 1,2propandiol propylene glycol have been used without evidence of superiority of one agent on the others [7, 15]. To our knowledge, this is the first study comparing two cryoprotective agents during vitrification. As a pragmatic study, our results concentrate on embryo survival, rather than pregnancy rate in order to avoid the need to control for many confounding factors that could affect pregnancy, especially as we adopt a strategy of dividing embryo freezing with all patients enrolled in this study having failed their fresh cycles. Our results show a significantly higher survival rate in embryos subjected to dimethylsulphoxide as a cryoprotective agent in comparison to those subjected to 1,2 propandiol (74.6% vs.

52% respectively). Interestingly, the difference was more apparent with advancement of stage of embryo development. It is logic to think that the superior protective effect of dimethylsulphoxide over 1,2 propandiol on dividing cells using slow freezing protocol applies also with the higher concentration used for vitrification. This appears to be more evident with higher stages of embryo development. However, this point needs further validation.

As it was unpractical to randomize embryos, we opted to patient-based randomization at the outset of the study. This has led to inability to control for all possible influencing factors from the start. As a result, we ended with slightly unequal arms of the study (114 vs. 98 embryos for dimethylsulphoxide and 1, 2 propandiol groups) and slightly different fertilization rate for the two groups. However, it is our policy to freeze only good quality embryos. When all confounding factors, including patient age and fertilization rate were tested for effect on embryo survival, none had shown any significant effect.

In conclusion: Dimethylsulphoxide based medium used for vitrification/thaw process of day2 or day 3 divided embryos demonstrated a significant higher embryo survival rate than that of 1,2propandiol based vitrification medium.

References

1. Camus M. Human embryo cryopreservation: review of clinical issues related to the success rate", Proceedings of Symposium on "Cryobiology and Cryopreservation on Human Gametes and Embryos" ESHRE Campus 2004. Brussels, Belgium, 12th to 13th March; 24–26.
2. Edgar DH, Archer J and Bourne H. The application and impact of cryopreservation of early cleavage stage embryos in assisted reproduction. Hum Fertil, 2004; 8 (4):225–230
3. Trouson A and Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. Nature, 1983; 305 (5936): 707-9.
4. Mazur P, Rall WF and Leibo SP. Kinetics of water loss and the likelihood of intracellular freezing in mouse ova. Influence of the method of calculating the temperature dependence of water permeability. Cell Biophys, 984; 6 (3):197–213.
5. Ashwood-Smith MJ, Morris GW, Fowler R, Appleton TC, Ashorn R. Physical factors are involved in the destruction of embryos and oocytes during freezing and thawing procedures. Hum Reprod, 1988; 3 (6): 795-802.
6. Elder K and Brian D. Cryopreservation In: In vitro fertilization", 2nd edition. Cambridge University Press; 2000: 192-228.
7. Balaban B, Urman B, Ata B, Isiklar A, Larman MG, Hamilton R, Gardner DK. A Randomized controlled study of human Day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. Hum Reprod, 2008; 23 (9): 1976–1982.
8. Quinn P. Success of oocyte and embryo freezing and its effect on outcome with in-vitro fertilization. Semin Reprod Endocrinol, 1990; 8:272-80.
9. Veek LL, Amundson CH, Brotman LJ, DeScisciolo C, Maloney MK, Muasher SJ, Jones HW Jr. Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes. Fertil Steril, 1993; 59:1202-7.
10. Van der Elst J. Camus M, Van den Abbeel E, Maes R, Devroey P, Van Steirteghem AC. Prospective randomized study on the cryopreservation of human embryos with dimethylsulfoxide or 1, 2-propanediol protocols. Fertil Steril, 1995; Jan 63(1):92-100
11. Lin TK, Su JT, Lee FK, Lin YR, Lo HC. Cryotop vitrification as compared to conventional slow freezing for human embryos at the cleavage stage: survival and outcomes. Taiwan J Obstet Gynecol, 2010; 49(3):272–278
12. Center for Disease Control. Assisted Reproductive Technology national summary. Fertility Clinic Report, 2009; P 16.
13. Wowk, B. "Revolutionary breakthroughs in the cryobiology", 21st century of medicine seminar, CRYOCARE UPDATE, Alcor's CRYONICS, 1999 issue of the Cryonics Institute's THE IMMORTALIST; Jan-Feb
14. Gautam SK, Verma V, Palta P, Chauhan MS, Manik RS. Effect of type of cryoprotectant on morphology and developmental competence of in vitro-matured buffalo (Bubalus bubalis) oocytes subjected to slow freezing or vitrification. Reprod Fertil Dev, 2008; 20(4):490-6.
15. Kasai M and Mukaida T. Cryopreservation of animal and human embryos by vitrification. Reprod Biomed Online, 2004; Aug: 9(2):164-70.