

Home-Made Vitrification and Thawing Media Did Not Differ Than a Commercially Available Brand on Blastocyst Survival, Expansion and Implantation

Original
Article

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ABSTRACT

Aim: This study was conducted to compare the cryo-survival and implantation rates of day 5/6 human blastocysts after using a home-made media versus a commercial freezing and thawing kit.

Materials and Methods: This is a prospective comparative study. Group 1 blastocysts were vitrified with home-made media (HM) (n = 48) according to shady grove protocol and thawed with gradual thawing technique using HM according to Kuwayama protocol. Group 2 (n = 30) blastocysts were vitrified and thawed with commercial media (CM). Blastocysts were subsequently cultured for 6-8 h, assessed for survival and expansion, then embryo transfer. The percentages of Positive hCG, clinical pregnancy, implantation, multiple pregnancy and abortion were recorded.

Results: Survival (following thawing and after 6-8 h culture), re-expansion, and implantation rate as well as the pregnancy rate were calculated for both media. Pregnancy rate for CM was 70% (n = 21/30) and 70.83% (n = 34/48; P = 0.8) for HM. Implantation rate for CM was 60% (n = 18/30) and 60.42% (n = 29/48; P = 0.9) for HM. There were no significant differences between the two groups. Both solutions provided high survival rate and of total 47 cases who recorded implantation, 45 went home with Healthy babies.

Conclusion: The two vitrification protocols, commercial kit and home-made solution, did not differ in blastocysts survival, re-expansion and implantation rates. So both methods could be used with equal success. HM it is providing an equal safety, and efficiency with lower cost in compared to CM.

Key Words: Blastocyst survival, home-made vitrification, thawing media, vitrification

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INTRODUCTION

The slow-freezing method was used in humans after success of cryopreservation of mouse embryos in 1972^[1]. The first successful frozen thawed human embryo pregnancy was reported in 1983^[2]. For each patient, only a small number of embryos can be cryopreserved. Then a more easier and rapid technique for cryopreservation was needed. This alternative new method for human cryopreservation and vitrification, was reported by Rall and Fahy^[3]. A small amount of medium harboring a high concentration of cryoprotectant was used in this method to induce a glass-like state for rapid embryo cryopreservation then submersion into liquid nitrogen, thus preventing ice crystals formation^[4].

The many documented papers on the success of vitrification along with less procedure time than the slow freezing and with the developing experience on the

procedure made it the procedure of choice by many IVF centers in storing both oocytes and embryos^[5-8].

As time goes by, frozen embryo transfer procedures have increased with more and more pregnancy rate as good as conventional fresh embryo transfer^[9,10] with publication showing that the frozen embryo transfer cases have lower preterm labour, birth weight and incidence of perinatal death than fresh cycles^[11,12].

Several vitrification kits are available in the market, but some of them result in inconsistent survival rates following thawing and others require extensive training^[13,14]. Different combined cryoprotectants media, as ethylene glycol (EG), dimethyl sulfoxide (DMSO) and propanediol (PrOH), or EG alone have been reported for vitrification of human embryos and oocytes^[15,16].

The most frequently used cryoprotectant: Dimethyl sulphoxide (DMSO), is due to its rapid transport through

cell membrane and it is highly efficient for oocyte, embryo and blastocyst vitrification. However, the possible toxicity of DMSO is of concern^[17].

There is also an evidence that a combined cryoprotectants might have better results than the media having a sole permeable cryoprotectant^[18]. In fact, the first pregnancy and birth from Vitrified/thawed blastocyst transfer were achieved using EG and DMSO as cryoprotectants^[19,20].

So, using a medium with EG, sucrose and DMSO as cryoprotectants for doing vitrification is both easy and competent for the cryopreservation of day 3, day 4 morulae or day 5/6 blastocysts^[21,22]. Other formulations are DMSO-free, which are volume independent. Larger straws are much simple to use and allow faster embryo exposure to vitrification solutions (e.g. Global Fast Freeze kit, LifeGlobal, Canada)^[23].

In this present study, we compared the two different vitrification and thawing media Home-made (HM) and Commercially-made (CM) in human blastocysts in intracytoplasmic sperm injection (ICSI) cycles.

We evaluated and compared survival, re-expansion, and percentage of live cells following vitrification and warming of Day 5 and Day 6 human blastocysts, vitrified and warmed with the Vit Kit Freeze/Thaw (Irvine Scientific, CA), or with two protocols using the Global Fast Freeze/Thaw Kits (LifeGlobal, Canada). Furthermore, cell survival of the vitrified/thawed embryos was compared with non-vitrified controls.

PATIENTS AND METHODS

Patients : This prospective study consisted of a total of 78 couples who had cryopreserved embryo transfer and vitrification of blastocysts after ICSI at a private fertility center, Banha city, Egypt between October 2015 and September 2018.

The mean age of all patients was 22-37 (28.6 ± 3.9). All patients included in this study were informed and gave a written consent for the cryopreservation procedure.

Experimental design : As shown in Table 1, a total of 154 blastocysts from 78 patients were randomly allocated into 2 treatment groups ; group 1 blastocysts were vitrified using the Global Fast Freeze Kit (Life Global; Commercial media) and thawed using Global Fast Freeze Thaw Kit (Life Global; Commercial media).

However, group 2 blastocysts were vitrified using Shady Grove Fertility RSC protocol in a Home-made

media and thawed as was described by Kuwayama in HM in a Home-made media^[24].

After blastocysts were vitrified/thawed according to the protocols described below, they were assessed for immediate survival, then cultured for a period of 6-8 hrs and reassessed for survival and expansion then prepared for embryo transfer.

Embryo grading and digital imaging: Assessment of blastocyst morphological quality and stage (days 5 or 6) was done according to the classification developed by Gardner and Schoolcraft^[25]. Digital images of each blastocyst were acquired before vitrification, immediately after thawing, and after 68- h culture, using a digital Watec camera connected to an inverted optical microscope with a thermal control microscope stage set on 37°C.

Table 1: Distribution of embryos by stage in both treatment groups:

Treatment Groups	Blastocyst stage						Total
	1	2	3	4	5	6	
1 Commercial media	7	5	8	18	21	2	61
2 Home-made media	4	9	13	25	37	5	93

**Vitrification-warming procedures:
Group 1 (CM)**

Vitrification procedure: Blastocysts were vitrified using the Global Fast Freeze Kit (LifeGlobal LLC, Guilford, CT, USA) which consists of 3 vitrification solutions, containing a combination of glycerol, ethylene glycol, human serum albumin (HSA), HEPES, and the base components of Global medium. Blastocysts were transferred to a drop of vitrification solution 1, and left for 5 min and subsequently to vitrification solution 2 where they were left for another 5 min, at room temperature. The blastocyst was then transferred and washed in several drops of vitrification solution 3 and immediately placed on the tip of the Cryotop (Kitazato, Japan) in a volume of <1µl a few millimeters back from the black tip. The straws were then plunged directly into liquid nitrogen (LN) and then were restored in specific labeled places inside the LN tanks. This loading technique was done the same for both groups.

Warming procedure: Blastocysts were thawed using the Global Fast Freeze Thaw Kit (LifeGlobal LLC, Guilford, CT, USA) which consists of 3 solutions, with

decreasing concentrations of sucrose. The protective cap of the straw was removed inside LN then transferred quickly and the blastocysts recovered into thawing solution 1. After 2 min, the blastocysts were transferred consecutively to thawing solution 2 (2 droplets) and were held for 2 minutes in each drop, then they were transferred to thawing solution 3 (3 droplets), where they were held for 3 min each. All procedures were done at room temperature, except for the last step where blastocysts were placed in the thawing solution 3, the dish was moved to a warm surface, at 37°C. After thawing, all blastocysts were transferred to a pre-equilibrated dish containing 20 µl culture drops of Global Total medium (LifeGlobal) under mineral oil and incubated 6-8 at 37°C in standard incubation conditions (6% CO₂ in air) in order to assess post-thawing survival and expansion.

Group 2 (HM)

Vitrification procedure: was performed according to the method that was described by Shady Grove Fertility Reproductive Science Center^[26]. In this protocol, 5 minutes prior to starting the vitrification process, expanded and hatching blastocysts were 'collapsed' artificially using one to two laser pulses directed at the trophectoderm at a site away from the inner cell mass.

Vitrification solutions

- A-36ml mHTF + 9ml SPS (mHTF + 20% SPS) [WS]
- B-2.25ml EG + 2.25ml DMSO + 10.5ml A (15% EG/DMSO + mHTF)
- C-7.5ml A + 7.5ml B (7.5% EG/DMSO + mHTF) [ES]
- D-7.5ml B + 1.2825g SUC (15% EG/DMSO + 0.5M SUC) [VS]

At room temperature blastocysts were put into drop 1(WS), then drop 1 (WS) connected with drop 2 (ES) for 2 minutes, then drop 2 (ES) was connected with drop 3 (ES) and waited 1min, drop 3 (ES) connected with drop 4 (ES) and waited 1min. Then Blastocysts were transferred to drop 5 (ES), and waited for re-expansion to 60-90% of the original volume. Then Blastocysts were moved into drops (VS) with minimal transfer of ES, and gently aspirated up and down to wash off the ES. Blastocysts were kept in VS for 45secs. Then blastocysts were loaded and stored as described previously with group 1.

Warming procedure: Thawing of blastocysts was done according to Kuwayama in HM [15] by placing the Cryotop in 1 ml of Thawing solution 1 (3.44 g sucrose/10 ml Global total hepes media) for 50-60 s at 37 °C and moved into a 0.5 ml of Thawing solution 2 (1.72 g sucrose/10 ml Global total hepes media) for 3 min, then to 0.5 ml Thawing solution 3 (0.86 g sucrose/10 ml Global total hepes media) for 5 minutes, then to 0.5 ml Thawing solution 4 (0.43 g sucrose/10 ml Global total hepes media) for 5 minutes.

All procedures were done at room temperature, except the last step where blastocysts were placed in the thawing solution 4, the dish was moved to a warm surface, at 37°C.

After thawing, all blastocysts were transferred to a pre-equilibrated dish containing 20 µl culture drops of Global Total medium (LifeGlobal) under mineral oil and incubated 6-8 at 37°C in standard incubation conditions (6% CO₂ in air) in order to assess post-thawing survival and expansion same like group 1.

Preparation of Patients' endometrium for embryo transfer:

Patient's endometrium was prepared initially by the administration of the white tab. (2mg estradiol valerate) (Cyclo-Progynova, Bayer Schering pharma, Germany) (2 mg three times- a day, initiated on the 2nd day of the menstrual cycle), then endometrial assessment with vaginal ultrasound is done. If the endometrial thickness reaches ≥ 8 mm and is triple in shape, we will start the progesterone if not then follow up every 2 days till the endometrial thickness is ≥ 8 mm.

Then, we give progesterone in the form of Pronotgest ampoule(progesterone 100mg/2ml, Produced by Nile Company for Pharmaceuticals, for: Marcyrl Co.) daily for 5 days then we do embryo transfer using soft transfer catheter (Labotect, Germany) under ultrasound guidance and continue with the treatment for 15 days and then a quantitative pregnancy test is done. If positive pregnancy test, assessment by ultrasound will be done after 10 days and the treatment (Cycloprogynova and Pronotgest) will be continued till at least the 7th week and if needed till the 14th week.

STATISTICAL ANALYSIS

Data are shown as mean values \pm SD, The effect of Vitrification protocol on the blastocyst immediate survival, survival and expansion at 6-8 hrs after thawing and other post transfer evaluations were evaluated using Pearson's Chi-square test.

RESULTS

The representative digital images of blastocysts before vitrification, immediately after thawing and 6-8 hrs later in both study groups [CM] and [HM] are shown in Fig. 1.

The differences between groups regarding age and years of infertility the mean age of the patients was 22-36 (28.42 \pm 3.79) for commercial media and 22-37 (28.71 \pm 4.16) for home-made media, respectively. The mean value of Duration of Infertility (years) Range for patients of CM was 1 – 6 (2.73 \pm 1.15) and 1-5 (2.7 \pm 0.94) for HM patient group (Table 2).

There were no significant differences between two vitrification-thawing protocols (Group 1: CM, Group 2: HM) on immediate survival. Using CM, vitrified/warmed Blastocysts had a survival rate of 98.3% (n = 60/61), while for HM, it was 98.9% (n=92/93; $P=0.27$), survival rate at 6-8 hrs with CM 96.7% (n=59/61), while for HM, it was 97.8% (n=91/93; $P=0.48$), or expansion for CM 93.4% (n=57/61), while for HM, it was 95.7% (n=92/93; $P=0.39$) after 6-8 h of culture following thawing (Fig. 2).

A total of 154 blastocysts from 78 patients were

vitrified during this study and these vitrified embryos were thawed to perform 79 transfer cycles. One patient had two transfer cycles because she didn't get pregnant in her first ET cycle. A positive serum hCG concentration was measured 14 days after embryo transfer in all patients. The chemical pregnancy rate for commercial kit was 73.3% (n=22/30) Vs 75% (n= 6/48; $P=0.74$) for home-made media. The clinical pregnancy rate for CM was 70% (n=21/30) Vs 70.8% (n=34/48; $P=0.84$) for HM. Implantation rate for CM was 60% (n=18/30) Vs 60.4% (n=29/48; $P=0.89$) was for HM. Of these 47 implantation cases, 45 went home with healthy babies (Table 3).

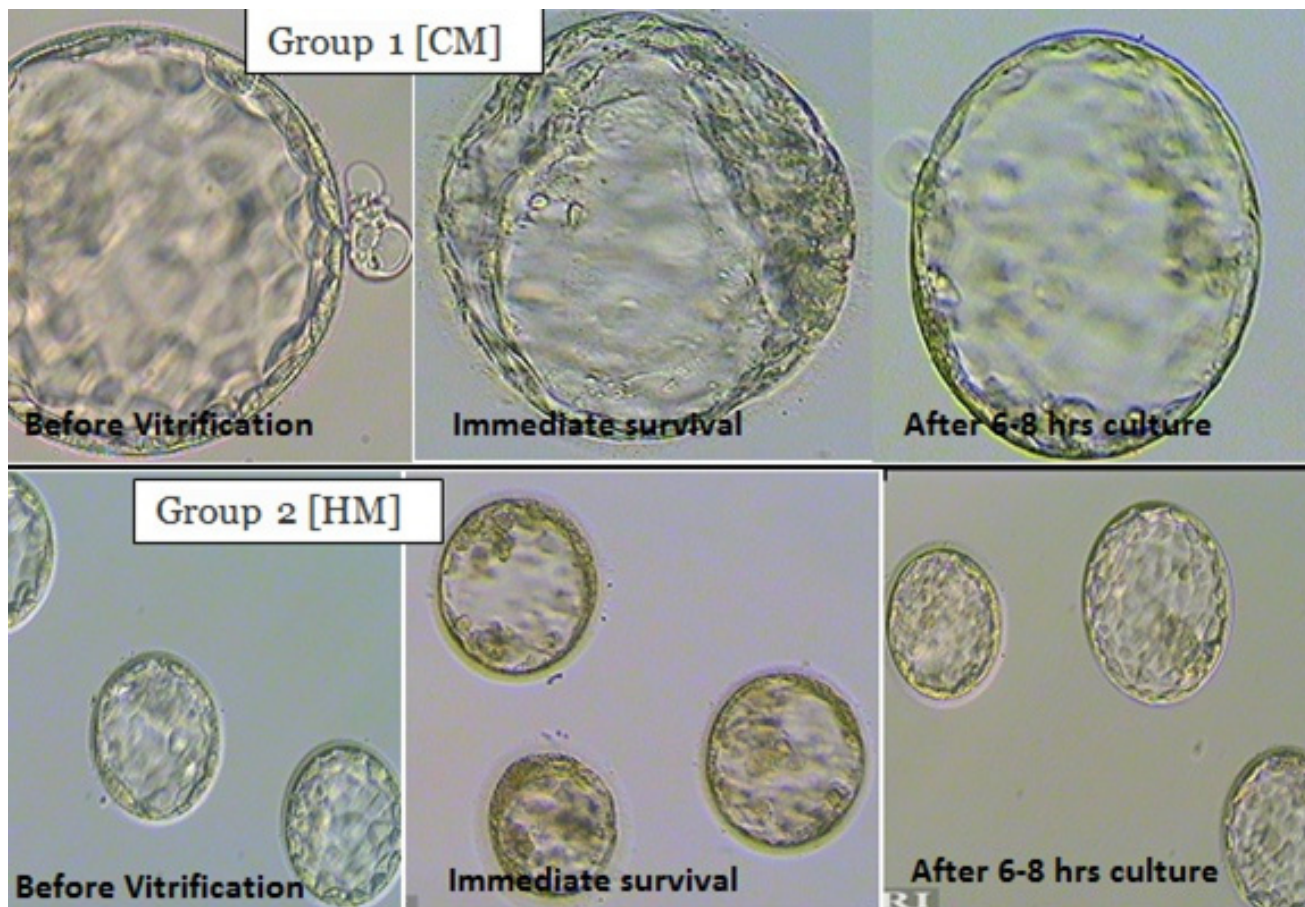


Fig. 1: Digital images of Blastocysts before vitrification and after thawing in both study groups.

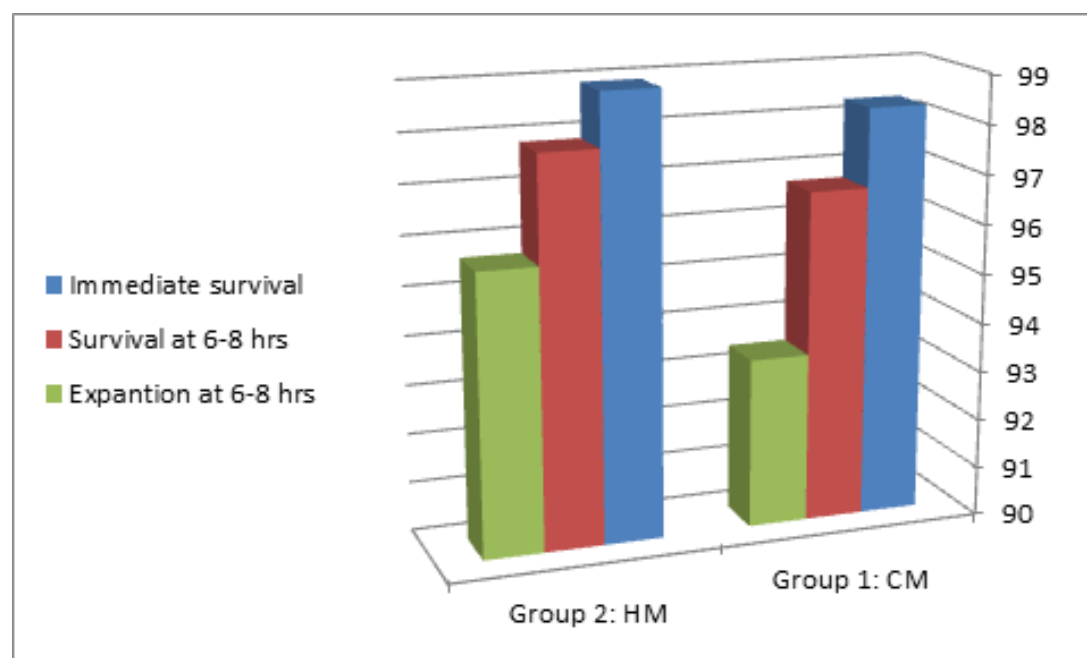


Fig. 2: Blastocyst survival and expansion following vitrification and thawing

Table 2: Difference between Groups regarding Initial Characteristics

	Group 1 [CM] (n=30)	Group 2 [HM] (n=48)	<i>P</i> *
Age (years)	22 – 36	22 – 37	0.731
Mean ± SD	28.42 ± 3.79	28.71 ± 4.16	NS
Age (years)	1 – 6	1 – 5	0.881
Mean ± SD	2.73 ± 1.15	2.7 ± 0.94	NS

Table 3: Main outcomes of the cycles of vitrified-thawed embryo transfer for both media

Parameter	CM (n=30)	HM (n=48)
No. of patients	30	48
No. of cycles of embryo transfer	30	49
No. of vitrified/warmed blastocysts	61	93
No. of Blastocysts survived	60	92
Survival rate (%)	98.3	98.92
No. of Blastocysts survived post 6-8 hrs	59	92
Survival rate post 6-8 hrs (%)	96.72	97.84
No. of Blastocysts expanded after 6-8 hrs	57	89
Expansion rate (%)	93.44	95.69
No. of chemical pregnancy (%)	22 (73.3%)	36 (75%)
No. of clinical pregnancies (%)	21 (70%)	34 (70.83%)
Implantation rate (%)	18(60%)	29(60.42%)
Incidence of twins	2	3
Incidence of triplet	1	3
No. of spontaneous abortions	3	4
Live birth (%)	15 (83.33%)	25(86.20%)

CM: Commercial Media

HM: Home made Media

DISCUSSION

The results found that vitrification did not affect blastocyst survival in any of the treatment groups. These findings confirm earlier results, which showed that thawed blastocysts which survived vitrification weren't different from fresh blastocysts, in terms of quality, DNA and chromosome integrity, ultra-structure, and developmental competence^[27-29].

Immediate survival, survival and re-expansion after 6-8 hrs, percentages of implantation and live birth rates were not significantly different for vitrified/thawed blastocysts between the two groups of HM and CM. This was observed despite the fact that the embryos were vitrified in different distinct developmental stages in the two groups, which may have negative effect on the development and impaired embryo health.

Neither survival, expansion nor percentage of implantation was significantly different between the two groups of the study. The results obtained with the Fast Freeze kit were in accordance with those observed in a preliminary study where a small sample of human blastocysts donated for research were vitrified with basis of the Global Fast Freeze media, and subsequently stained, leading to a survival rate following warming of 84 % and a cell survival rate of 87 %^[30]. Furthermore, the results of re-expansion rates, which is a positive prognostic marker associated with significantly increased implantation and clinical pregnancies,^[31] were similar.

It is well-established that Vitrification solutions that contain DMSO did not lead to cell membrane damage and death as quickly as the DMSO free vitrification solutions. However, those negative effects became apparent only after 10 min or longer exposure to the vitrification solution^[32]. With global fast freeze kit protocol the embryos are exposed to Vit. solution 1 for 5 minutes and to Vit. solution 2 for 5 minutes, maximum 10 minutes as documented.

Concerning to group 2 [HM]; the results confirmed that ethylene glycol combined with DMSO, with its low toxicity and high permeability^[33], diffuses into the embryos and leaves them very rapidly owing to its low molecular weight. Thus embryos may undergo less osmotic stress during vitrification and thawing. Glycerol has a higher molecular weight and moves across the plasma membrane through aquaporins 3 that predominantly facilitates diffusion^[34]. As earlier demonstrated by Stachecki and Cohen^[14], those 3 mixed materials are efficient cryoprotectants for blastocyst Vitrification.

The results also confirmed the previous findings that indicated that the homemade vitrification solution gave good results^[35].

CONCLUSION

The two vitrification protocols, commercial kit and home-made solution, did not differ in blastocysts survival, re-expansion and implantation rates. So, both methods could be used with equal success using simplified protocols and freezing straws which are cheaper and easy to load. HM it is providing an equal safety, and efficiency with lower cost in compared to CM.

CONFLICT OF INTEREST

There are no conflicts of interests.

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