Vitrification can modify embryo cleavage stage after warming. Should we change endometrial preparation?

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Abstract

Purpose Studies have shown that embryo metabolism and cell cleavage after warming vitrified embryos is faster than after thawing frozen embryos. We study vitrified embryo transfer (VET) results depending on the developmental stage of warmed embryos and the duration of progesterone treatment before embryo transfer.

Methods We designed a prospective study, patients were randomized in two groups, starting progesterone three (D+3) or four days (D+4) before embryo transfer. We recruited 88 patients with embryos vitrified on day 3.

Results We didn't find statistical differences in pregnancy rate when we transferred embryos in D+3 vs D+4 (38.2 % vs 40.5 % $p \geq 0.05$). The day after warming, 54.6 % of embryos had developed to morula or early blastocyst, 32.4 % to cleavage stage and 13 % didn't cleave. Transfers were with morula/blastocysts stage embryos (52.1 %; n:37), cleavage stage embryos (18.3 %; n:13) or mixed (29.6 %; n:21). Implantation rate was significantly higher in morula/blastocyst stage than in cleavage stage or mixed transfers (44 %, 22 % and 16.3 %; $p=0.011$). Pregnancy and implantation rates were significantly higher in morula/blastocyst transfers on D+4 than on D+3 (68.7 % and 64.7 % vs 33.3 %, and 33.3 %, $p=0.033$ and $p=0.034$).
Conclusions Our findings suggest that a majority of embryos will develop to morula/blastocyst stage after warming. VET results with morula/blastocysts, and after four days of

progesterone supplementation, are better than with cleavage stage embryos.

Keywords Vitrification · Progesterone · Endometrium · Blastocyst · Embryo cleavage · Implantation window

Introduction

The start of vitrification in embryos from mammals took place in 1985 by Rall and Fahy [15], and was later applied to human embryos, with a first term pregnancy reported in 1990 [5]. Compared with slow freezing, vitrification is not a method with osmotic equilibrium but a sudden entrance of cryoprotectant into cells that does not allow the formation of ice crystals; cooling is extremely fast and a higher concentration of cryoprotectant is needed [1,20]. Vitrification does not require a freezer and is extremely efficient, taking less than 15 min to vitrify or warm as compared with the lengthier programmed slow freezing. In a metanalysis by AbdelHafez in 2010 on cryopreservation techniques, four studies, out of 2700 publications, performed a direct comparison between vitrification and slow freezing, suggesting better results with the first technique. Embryo survival rate was significantly higher with vitrification than with slow freezing, and implantation and pregnancy rate were also higher in two of the studies [1,16,20].

Some authors have suggested that the selection of embryos for frozen/thawed embryo transfers could be decided not only on their morphological appearance after thawing, but also on their cleavage capacity after 24 h in culture. Van der Elst et al. [21] and Ziebe et al. [22] improved the delivery rate per transfer and the implantation and birth rates per transferred embryo following this strategy. Some authors have observed a higher rhythm of cell division in

Capsule Should we change endometrial preparation after warming D+3 embryos?

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vitrified/warmed embryos compared with frozen/thawed embryos. Desai et al. [3] found that 78 % of vitrified/warmed embryos showed signs of compaction and/or blastulation after 48 h in culture; and later suggested that this event could be an excellent indicator for pregnancy [4]. Balaban et al. [2] published that vitrified/warmed embryos had significantly higher pyruvate consumption compared with frozen/thawed embryos indicating a higher developmental rate of the former.

The observed higher rhythm of cell division might entail the need to change endometrial preparation before embryo transfer in order to obtain a better synchrony between embryos and endometrium. In *in vitro* fertilization cycles the high levels of oestradiol will accelerate endometrial development and the appearance of pynopodes one or two days compared to natural cycles, although this may vary depending on hormonal treatment and individuals [6,11,19]. In egg donation treatment with oestradiol /progesterone supplemented cycles, some authors have observed that pynopode formation in these cycles is similar to natural cycles [7], while others suggest that the preparation of the endometrium is 2 or 3 days slower than in natural cycles [10,12,14]. In frozen embryo transfer cycles the optimal progesterone supplementation before transfer is still debated. Embryos frozen in day two or three are generally transferred after three days of progesterone treatment, and embryos frozen in day 5 are transferred after 5 days of progesterone treatment [9].

The present study has two objectives. We want to study the influence of the developmental stage of warmed embryos in the pregnancy and implantation rates of the vitrified embryo transfers (VET); and, we want to assess whether there is a difference in pregnancy and implantation rates between transferring warmed embryos after 3 or 4 days of progesterone treatment.

Materials and methods

This is a prospective and randomized study performed between May 2008 and May 2011. We included in the study all VET cycles performed with embryos vitrified on day three of development that reached our quality cryopreservation criteria: embryos with 6 or more cells and with 20 % fragmentation or less. All patients had written consent form for VET.

Patients followed an artificial VET protocol, and they were divided randomly (following a computer generated randomization table) in two groups, starting progesterone three days before embryo transfer (D+3), or four days before embryo transfer (D+4). Following the clinic protocol a maximum of two embryos were transferred.

Vitrification

The vitrification procedure was carried out using the Vitri-fication Kit from Irvine Scientific (Santa Ana, CA, USA).

Following instructions of the commercial laboratory, the embryos are vitrified in two stages and the whole process is performed at room temperature, including the media used. For the first step embryos are placed in a 20 µl droplet of ES medium (Equilibration Solution) until they regain 80 % of their original volume (between 5 and 15 min). Afterwards, embryos are deposited in four 20 µl droplets of VS medium (Vitrification Solution): five seconds in the first and second droplet, 10 s in the third and finally, once they are in the fourth droplet, they are introduced in the Cryotip, which is a closed storage support. In less than 90 s cryotips must be sealed with a thermic sealer and submerged in liquid nitrogen.

Warming

We used Vitrification Thaw Kit Irvine Scientific (Santa Ana, CA, USA) and the whole process is performed at room temperature, including the media used. First the cryotip is submerged in a distilled water bath at 37 °C for three seconds; the cryotip content is deposited in a sterile petri dish and mixed 1:1 with TS medium (Thawing Solution). After one minute the embryos are changed to a 20 µl droplet of TS for another minute. Then they go through two 20 µl droplets of DS (Dilution Solution) two minutes each and three 20 µl droplets of WS (Washing solution) for 3 min each. Finally, embryos are deposited in a culture dish with G2-plus medium (Serie G5 Vitrolife, Sweden) and observed under the inverted microscope to confirm survival and to assess their morphology. Embryos that presented one or more viable cells were incubated at 37 °C, 5 % O₂ and 7.3 % CO₂ (to obtain a pH between 7.20–7.34 following instructions of culture medium). After 24 h in culture and previous to VET, embryos were observed and classified in three developmental stages: embryos with no cell division (the number of cells was the same as 24 h before), cleavage cell stage embryos (embryos with more cells after 24 h in culture) and embryos that had developed into morula or early blastocyst stage.

EmbryoGlue media (Vitrolife, Sweden) was used for embryo transfer. Transfer was guided with an abdominal scan and a Wallace catheter was used (Smith Medical Internacional Ltd.UK).

Endometrial preparation

Endometrium was prepared with a hormone replacement treatment. Ovarian down regulation is achieved using a GnRH agonist (Ginecrin depot, im, Abbott Laboratories, Madrid, Spain) in the luteal phase. With menstruation, patients start treatment with 2 oestradiol patches/2 days (Estradot® 75 mg; Novartis, Barcelona, Spain). After 8 to 10 days a transvaginal scan and a serum oestradiol measurement are performed. Endometrium is considered to be

Table 1 Demographic characteristics depending on embryo development

	Morula/blastocysts	Cleavage stage	Mixed	P-value
Medical diagnosis				NS
Male factor	21.6 % (8/37)	15.4 % (2/13)	23.8 % (5/21)	
Female factor	32.4 % (12/37)	30.6 % (4/13)	28.6 % (6/21)	
Mixed factor	46 % (17/37)	53.8 % (7/13)	47.6 % (10/21)	
Age in vitrification	38.2±5.3	35.4±3.8	33.7±5.7	NS
Duration of vitrification (months)	13.3±15.7	16.0±25.8	8.0±10.8	NS
Endometrial width (mm)	9.0±1.5	8.6±1.7	9.2±1.9	NS
No. embryos transferred	1.3±0.4	1.3±0.5	2.0±0.2	0.000

Values are mean±SD; Values are %(number / total); NS=not statistically significant ($p>0.05$)

prepared if its width is over 7 mm and serum oestradiol is above 100 pg/ml. From that moment 200 mg/8 h progesterone can be started (Utrogestan®, Seid, Barcelona, Spain or Progeffik®, Effik, Madrid, Spain) and embryo transfer is programmed. Both oestradiol patches and progesterone are continued until pregnancy test 15 days after commencement of progesterone. If the pregnancy test is positive hormonal treatment is continued until week 12 of gestation.

Clinical pregnancy is defined by the observation with ultrasound of an intrauterine sac four weeks after embryo transfer.

Statistical analysis

Categorical variables were compared with the χ^2 test, using Fisher when necessary.

Continuous variables for analysis of groups D+3 vs D+4 were compared with T-student test and continuous variables for analysis of the transfer with different embryo development were compared with ANOVA test. A P-value <0.05 was considered as being significant. All analyses were performed with the commercial software SPSS version 13.0.

The study was designed to obtain an increase of 5 % in implantation rate of VET. We considered a statistical power of 80 %. As a result we needed a sample size of 986 VET cycles in both groups.

Results

Eighty eight VET cycles were performed between May 2008 and May 2011. A total of 145 embryos were warmed with a survival rate of 96.5 %. After 24 h in culture, we observed that 13 % of embryos had viable cells but had not changed their cell number; 32.4 % of embryos showed some cleavage activity as the number of cells had increased; and 54.6 % of embryos showed not only an increased number of cells but had also developed into morula or early blastocyst stage.

We included in the study 71 VET cycles. We transferred embryos in morula or early blastocyst stage in 37 patients (52.1 %); cleavage stage embryos in 13 (18.3 %); and a combination of the two in 21 (29.6 %; called mixed transfers). In the morula/early blastocyst stage transfers, 78.4 % of the embryos were morulas and 21.5 % were early blastocysts. We excluded from the analysis cycles in which a non divided embryo was transferred ($n=17$; although these embryos have a very low implantation rate they might influence our results).

We compared the three groups (morula/early blastocyst stage transfers, cleavage stage embryo transfers, and mixed transfers) and observed that they were statistically homogeneous for clinical diagnosis: male factor (21.6 %, 15.4 % and 23.8 %), female factor (32.4 %, 30.6 % and 28.6 %) and mixed factor (46 %, 53.8 % and 47.6 %), the age of the patients at the time of vitrification (38.2, 35.4 and 33.7 years old), the duration of vitrification (13.3, 16 and 8 months) and the endometrial width (9, 8.6 and 9.2 mm) at the time of

Table 2 VET results depending on embryo development

	Morula/blastocysts	Cleavage stage	Mixed	P-value
Number	37/71 (52.1 %)	13/71 (18.3 %)	21/71 (29.6 %)	
Pregnancy rate	48.6 % (18/37)	23 % (3/13)	33 % (7/21)	NS
Implantation rate	44 % (22/50)	22 % (4/18)	16.3 % (7/43)	$P=0.011$
Miscarriage rate	22.2 % (4/18)	0 % (0/3)	43 % (3/7)	NS

Values are % (number / total); NS=not statistically significant ($p>0.05$)

Table 3 Demographic characteristics depending on days of progesterone supplementation

	D+3	D+4	P-valor
Medical diagnosis			NS
Male factor	20.5 % (7/34)	21.6 % (8/37)	
Female factor	32.3 % (11/34)	29.7 % (11/37)	
Mixed factor	47 % (16/34)	48.6 % (18/37)	
Age in vitrification	33.3±5.8	34.7±4.5	NS
Duration of vitrification (months)	11.5±15.2	13.0±18.4	NS
Endometrial width (mm)	9.0±1.8	9.0±1.6	NS
No. embryos transferred	1.6±0.5	1.5±0.5	NS

Values are mean±SD; Values are %(number / total); NS=not statistically significant ($p>0.05$)

starting progesterone (Table 1). However, groups were not homogenous for the number of embryos transferred (1.3, 1.3 and 2 embryos). In mixed transfers we always used 2 embryos, while in the other groups, transfers with one or two embryos were included. Clinical pregnancy rates (48.6 % in morula/early blastocyst, 23 % in cleavage stage, and 33 % in mixed transfers) and miscarriage rates (22.2 % in morula/early blastocyst, 0 % in cleavage stage, and 43 % in mixed transfers) were not significantly different between the three groups. However, implantation rates were significantly higher in the morula/early blastocyst transfers (44 %) than in the cleavage stage transfers (22 %) and in mixed transfers (16.3 %) ($p=0.011$) (Table 2).

Secondly we compared results between VET cycles in which progesterone supplementation had started three (D+3) or four (D+4) days before embryo transfer. We observed that both groups were statistically homogenous for clinical diagnosis: male factor (20.5 % and 21.6 %), female factor (32.3 % and 29.7 %) and mixed factor (47 % and 48.6 %), the age of the patients at the time of vitrification (33.3 and 34.7 years old), the duration of vitrification (11.5 and 13 months), the endometrial width (9 and 9 mm), and the number of embryos transferred (1.6 and 1.5 embryos) (Table 3). When including in the analysis all transfers irrespective of the degree of embryo cell division, clinical pregnancy rates, implantation rates and miscarriage rates

Table 4 VET results depending on days of progesterone supplementation (with all embryos, irrespective of the degree of embryo cell division)

	D+3	D+4	P-value
Number	34	37	
Pregnancy rate	38.2 % (13/34)	40.5 % (15/37)	NS
Implantation rate	30 % (17/56)	29 % (16/55)	NS
Miscarriage rate	15.4 % (2/13)	33.3 % (5/15)	NS

Values are % (number / total); NS=not statistically significant ($p>0.05$)

Table 5 VET results depending on embryo development and days of progesterone supplementation

	D+3	D+4	P-value
Pregnancy rate			
Morula/Blastocyst	33.3 % (7/21)	68.7 % (11/16)	$P=0.033$
Cleavage stage	33.3 % (1/3)	20 % (2/20)	NS
Mixed	50 % (5/10)	18 % (2/11)	NS
Implantation rate			
Morula/Blastocyst	33.3 % (11/33)	64.7 % (11/17)	$P=0.034$
Cleavage stage	33.3 % (1/3)	20 % (3/15)	NS
Mixed	25 % (5/20)	8.7 % (2/23)	NS
Miscarriage rate			
Morula/Blastocyst	14.3 % (1/7)	27.3 % (3/11)	NS
Cleavage stage	0 % (0/1)	0 % (0/2)	NS
Mixed	20 % (5/10)	100 % (2/11)	NS

Values are % (number / total); NS not statistically significant ($p>0.05$)

between the two groups were not significantly different (38.2 % vs 40.5 %; 30 % vs 29 %; 15.4 % vs 33.3 %; $p\geq 0.05$) (Table 4). However, results were different if we took into account differences in embryo development (Table 5). Transfers of morula/early blastocyst embryos produced higher pregnancy and implantation rates after 4 days of progesterone compared with 3 days of progesterone (68.7 % vs 33.3 %; $p=0.033$ and 64.7 % vs 33.3 %; $p=0.034$). Moreover, cleavage stage embryos and mixed transfers produced similar pregnancy and implantation rates after 3 or 4 days of progesterone treatment. Miscarriage rates were similar for all groups after 3 or 4 days of progesterone.

Conclusion

Embryo implantation can be seen as a successful end to two separate processes, embryo development and endometrial preparation, where synchrony between the two is important. The whole process is regulated by several endocrine, paracrine and autocrine factors acting both at endometrial and embryological levels [17].

In in vitro fertilization treatments, transfer of embryos in morula or blastocyst stage have been associated with higher implantation rates, compared to cleavage stage embryo transfers. Identifying optimal embryos for transfer is likely to be the cause for this improvement, although some have argued that the intrauterine transfer of embryos at the time when embryo and endometrium normally meet in vivo might also benefit the process [8,13,18,19,22].

In our clinic, we take into account cell division to make a prognosis of the implantation potential of warmed embryos [21]. Since cell cycle can vary between 15 and 24 h, embryos are observed immediately after warming and 24 h later. This

has allowed us to observe that day 3 vitrified embryos, warmed and cultured for 24 h, present a rhythm of cell division higher than frozen/thawed embryos (unpublished data). In this study 54.6 % of embryos (vitrified on day 3) divided to morula/compacting stage or early blastocyst after 24 h in culture. The implantation rate of these embryos was significantly higher (44 %) than rates obtained with cleavage stage embryo transfers (22 %) or mixed transfers (16.3 %). Our findings are similar to those of Desai et al. [3], as they obtained higher pregnancy rates with more advanced embryos after vitrification and warming. They had a pregnancy rate of 58 % (28/48) with warmed embryos in blastocyst stage (after 48 h in culture), 26 % (6/23) with morula/compacting stage embryos and 0 % (0/6) with embryos with no compaction signs. However, no statistical analysis was mentioned in their study.

In our study day 3 vitrified/warmed embryos, which after 24 h in culture had divided into cleavage stage, were transferred after 3 or 4 days of progesterone supplementation and no difference was observed in pregnancy or implantation rate (33.3 % vs 20 %). This finding would suggest that implantation of warmed embryos at cleavage stage do not benefit from a longer preparation with progesterone, as it had been defended in artificial cycles in egg donation [10,12,14]; although using frozen instead of fresh embryos may account for differences observed.

Day 3 warmed embryos that had developed into morula or early blastocyst produced better pregnancy and implantation rates if transferred after 4 days instead of 3 days of progesterone supplementation (64.7 % vs 33.3 %). This would suggest that VET cycles benefit from taking into account the higher developmental rate of vitrified/warmed embryos and preparing the endometrium similar to natural cycles, that is transferring morula/early blastocyst embryos (day 4 of development) on day 4 of progesterone supplementation.

Given the significant improvement in implantation rate of warmed embryos transferred after 4 days of progesterone, we decided to stop our study after 71 VET cycles. However, results can only be given then with 60 % of statistical power.

In summary, implantation rate of morula/early blastocyst embryos is significantly higher than cleavage stage embryos, and our results suggest that 4 days of progesterone supplementation before embryo transfer benefits implantation and pregnancy rates.

Declaration The authors report no financial or commercial conflicts of interest.

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