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EMBRYO BIOLOGY

Artificial shrinkage of blastocysts prior to vitrification improves pregnancy outcome: analysis of 1028 consecutive warming cycles

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Abstract
Purpose This study aims to compare implantation, pregnancy, and delivery rates in frozen transfer cycles with blastocysts that were vitrified either with artificial shrinking (AS group) or without (NAS group).
Methods Retrospective comparative study of artificial shrinking of blastocysts prior to vitrification and frozen embryo transfer cycles in infertile patients undergoing frozen embryo transfer (FET) was done at the Humanitas Fertility Center between October 2009 and December 2013. Main outcome measure(s) were implantation (IR), pregnancy (PR), and delivery rates (DR) between the two groups.
Results A total of 1028 consecutive warming blastocyst transfer cycles were considered. In 580 cycles (total of 822 blastocysts), artificial shrinking was performed prior to vitrification (AS group), while in the remaining 448 cycles (total of 625 blastocysts), the artificial shrinking was not performed (NAS group). There were no differences in patient age (36.4 ± 3.7 vs. 36.3 ± 3.9) and number of embryos transferred (1.41 ± 0.49 vs. 1.38 ± 0.50) between groups. The IR, PR, and DR in the AS group were significantly higher ($p < 0.05$) than in the NAS group (29.9 vs. 23.0 %, 36.3 vs. 27.9 %, and 26.5 vs. 18.1 %, respectively).
Conclusions Performing AS of blastocysts prior to vitrification appears to improve implantation, pregnancy, and delivery rates probably related to a decreased risk of ultrastructural cryodamages, plausible when cryopreserving expanded blastocysts.

Keywords Blastocyst · Cryopreservation · Artificial shrinkage · Frozen embryo transfer

Capsule Performing artificial shrinkage of expanded blastocysts prior to vitrification appears to improve implantation, pregnancy, and delivery rates probably related to a decreased risk of ultrastructural cryodamages, plausible when cryopreserving expanded blastocysts.

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Introduction

In Italy, after the Constitutional Court removed most of the restrictions in the practice of ART [1, 2], embryo cryopreservation became permitted again. In our unit, only supernumerary embryos that reach the blastocyst stage are cryopreserved by vitrification [3–5]. During vitrification, the blastocysts are placed in a loading device surrounded by vitrification media [6–8]; however, despite high concentrations of cryoprotectants and reduced volume of solutions, expanded blastocysts are still prone to ice crystal formation due to the presence of large amounts of water-based fluid in the blastocoele cavity, which can cause ultrastructural damages and interfere with the exchange of cryoprotectants present in the vitrification media. Indeed, many articles have reported a negative correlation between blastocoele volume and the clinical outcome [9, 10]. The artificial shrinkage (AS) is a technique aimed at dehydrating and collapsing the blastocoele cavity prior to embryo vitrification. The blastocysts' trophectoderm undergo

physiological cycles of contraction and expansion, and it has been demonstrated that even a shrunken blastocoele still retain the potential to gradually recover its original dimensions and shape upon warming [11].

In literature, several AS methods have been described [12, 13]. In our study, we employed the mechanical technique using a holding pipette. Another AS method is with the laser whereby the inner cell mass (ICM) is positioned away from the targeted laser beam and the trophectoderm cells are exposed to a brief ultra-high temperature pulse that causes shrinkage in 1 to 2 min [12]. Some recent literature has demonstrated that after AS, the re-warmed blastocysts have an improved survival rate and subsequently a better clinical outcome [13, 14]. To confirm or negate these initial reports, we retrospectively compared pregnancy (PR) and implantation rates (IR) of frozen embryo transfer (FET) cycles using blastocysts vitrified either with or without AS. A secondary end point of analysis was the delivery rate and adverse pregnancy outcome.

Materials and methods

Patients and thawing cycle data

Data were collected on all patients who underwent FET treatment at Humanitas Research Hospital from October 2009 to December 2013. The causes of infertility were male factor, female factor (endometriosis, tubal), and/or unexplained infertility.

A total of 869 patients undergoing 1028 consecutive warming blastocyst transfer cycles were considered eligible for inclusion and divided into two groups: AS ($n=580$) and non-artificial shrinkage (NAS) ($n=448$). In the AS cycles, a total of 822 blastocysts were collapsed using the mechanical method of injection/holding micropipettes, while in the NAS cycles, a total of 625 blastocysts were cryopreserved intact. In this study, to stabilize the expanded blastocyst, we positioned the ICM at the 12 or 6 o'clock and using an injection pipette to pierce thru the trophectoderm cells and the zona pellucida causing a complete collapse of the blastocoele cavity. In this study, the expanded blastocysts were first stabilized with the ICM positioned at the 12 or 6 o'clock and then an injection pipette pierced thru the trophectoderm cells and the zona pellucida causing a complete collapse of the blastocoele cavity.

Implantation and pregnancy rates were considered the primary outcome while delivery rates and miscarriages were the secondary outcome of analysis.

The study was approved by our IRB on July 2009 as part of a multicenter trial on blastocoele fluid collection in order to test the safety of this procedure on pregnancy outcome [15]. Our center maintains an external audit anonymized electronic research queries system, exported from our certified only access web database, which at time of this analysis included 25,911 consecutive fresh non-donors IVF cycles and 4792 frozen embryo cycles. Patients who underwent these cycles had consented in writing that their medical records could be used for research purposes, as long as the patients' anonymity was protected and confidentiality of the medical record was assured.

Embryo transfer protocols

Two different protocols were used: natural cycle and hormonal replacement cycles. In natural cycles, patients had ultrasound examination on cycle day 8 and instructed to start urinary luteinizing hormone (LH) monitoring. Upon confirmation of the LH rise and in the presence of a follicle ≥17 mm and endometrial thickness of ≥7.5, the embryo transfer was scheduled 7 days later without any hormonal supplementation. If no LH rise was documented despite adequate follicular growth and endometrial thickness, the patients received 5000 IU of hCG subcutaneous and progesterone supplementation (400 mg intravaginally) was started 48 h later (modified natural cycle). In hormonal replacement cycles, estradiol (Progynova 2 mg Bayer, Italy) was started on cycle day 2 at 4 mg/day for 7 days and then increased to 6 mg/day until the day of ET. An ultrasound was performed 8–10 days from the estradiol start and when the endometrial thickness reached 8 mm, 600 mg of intravaginal progesterone was started and the ET was performed after 5 days.

In vitro culture of embryos

Zygotes were cultured in sequential medium (Cleavage and Blastocyst, Sage) organized in micro-drops covered with mineral oil with changeover performed on day 3. All embryos were incubated in a CO2/O2/N2 (5:5:90 %) environment. On day 5 of culture, the blastocysts were assessed for expansion and given a morphological grading according to the Istanbul Consensus criteria [16]. Blastocysts were vitrified either on day 5 or 6 depending on their developmental timing [17].

Embryo transfer and blastocyst cryopreservation

Blastocysts were cryopreserved using Cryotop in an open system and with Kitazato medium (Kitazato BioPharma Co., Shizuoka, Japan). In some patients (randomly selected), 10 min before vitrification, their expanded blastocysts were subjected to AS using injection/holding micropipettes (Cook Medical, ADD City and Country). After warming, the blastocysts were assessed for survival and observed for 2 h before embryo transfer in order to document their re-expansion.

No specific equilibration time according to the volume of blastocoelic was performed because the embryologists and the clinicians were blinded at warming regarding the pre-vitrification blastocyst grade of expansion or shrinkage.

Clinical and birth outcome

To confirm the establishment of a clinical pregnancy, an ultrasound was performed at 7 weeks to visualize a gestational sac and fetal cardiac activity. Pregnancies ≥24 weeks were considered deliveries, and pregnancies ending in spontaneous abortions were divided in miscarriages prior to 12 and >12 weeks. Ectopic pregnancies were also considered in the study. Birth outcomes were recorded within 28 days from the date of delivery by a structured questionnaire.

Statistical analysis

Data were described as number and percentage where appropriated. Differences between groups were explored by χ^2 test or Wilcoxon test where appropriate. Logistic regression multivariable analysis was used to determine the role of AS on pregnancy and delivery rate, corrected for female age and grade of expansion after removal of not surviving embryos and cases where only one blastocyst implanted when two embryos were transferred. All statistical analyses were carried out with the Stata 13 program. A p value of <0.05 was considered statistically significant.

Results

Overall, 1447 blastocysts were re-warmed and 1408 survived (survival rate (SR) of 97.3 %) (The details are summarized in Table 1). The two groups (NAS and AS) were comparable for patients' mean age (36.3 ± 3.9 vs. 36.4 ± 3.7) and average number of blastocysts transferred (1.4 ± 0.5 vs. 1.4 ± 0.5). In the NAS group (448 cycles), a total of 625 blastocysts were re-warmed, 604 survived (SR 96.3 %) and were transferred in 437 cycles. In 11 cases, embryo transfer was not performed (no blastocyst survival).

In the AS group (580 cycles), a total of 820 blastocysts were re-warmed, 804 survived (SR 97.6 %) and were transferred in 570 cycles. In ten cases, embryo transfer was not performed (no blastocyst survival). Concerning the types of replacement in the 1028 warming cycles, 238 (23.2 %) were natural or modified natural cycles and 790 (76.8 %) were hormonal replacement cycles. The implantation rate and pregnancy rate were significantly higher in the AS group than in the NAS group (29.9 vs. 23.2 % and 36.3 vs. 27.9 %, respectively $p < 0.005$).The delivery rate per transfer (Table 1) was significantly higher in the AS group than in the NAS group (26.7 vs. 18.1 %, $p = 0.001$). In Table 2, the pregnancy outcome is shown. In the NAS group, 79 live births (89 babies born) were observed, of which 69 were singleton and 10 were twins. In the AS group, 152 live births (174 babies born) were observed, of which 129 were singleton and 23 were twins. There were no differences in abortion rate and gestational length between the two groups.

The pregnancy outcome analysis confirmed no significant differences in the delivery, abortion, and ectopic rate as well as in the gestational age at delivery and mean singleton birth weight. A significant difference was found in the twin birth weight in favor of NAS. The abortion rate although not significantly different between groups showed an 8.4 % difference that represents a 27 % lower abortion rate in the AS group.

After removal of the non-surviving embryos and one only blastocyst implanted when two were transferred, a logistic analysis taking into account women's age, grade of expansion, pregnancy, and delivery rate was performed on 1280 blastocysts. The

Table 1 Overall results of frozen blastocyst embryo transfer in the non-artificial shrinkage (NAS), artificial shrinkage, and in the total

	NAS	AS	Total	*P*
Cycles (*n*°)	448	580	1028	
Mean age[a]	36.3 ± 3.9	36.4 ± 3.7	36.3 ± 3.8	0.909
Frozen Embryo Transfers (n°)	437	570	1007	
FET canceled (*n*°)	11	10	21	
Thawed blastocysts (*n*°)	625	820	1445	
Survived blastocysts (*n*°)	604	804	1408	
Blastocyst survival rate (%)	96.6	97.8	97.3	0.192
Transferred blastocysts (*n*°)	604	804	1408	
Mean transferred blastocyst[a]	1.38 ± 0.50	1.41 ± 0.49	1.40 ± 0.49	0.318
Implants (*n*°)	140	240	379	
Implantation rate (%)	23.2	29.9	27.0	0.005
Pregnancies (n°)	122	207	329	
Pregnancy rate (%)	27.9	36.3	32.7	0.005
Delivery (*n*°)	79	152	231	
Delivery rate (%)	18.1	26.7	22.9	0.001

[a] Mean ± SD

Table 2 Pregnancy and delivery outcome of frozen blastocyst embryo transfer in the non-artificial shrinkage (NAS), artificial shrinkage, and in the total

	NAS	AS	Total	*P*
Pregnancies (*n*°)	122	207	329	
Live birth rate (%)	79 (64.8 %)	152 (73.4 %)	231 (70.2 %)	0.106
Abortion rate (%)	39 (32.0 %)	48 (23.2 %)	87 (26.4 %)	0.093
Ectopic pregnancies (%)	4 (3.3 %)	7 (3.4 %)	11 (3.3 %)	1.000
Singleton (%)	103 (84.4 %)	170 (82.1)	273 (83.0 %)	0.959
Twin (%)	15 (12.3 %)	29 (14.0 %)	44 (13.4 %)	
Triplet (%)	0	1 (0.5 %)	1 (0.3 %)	
Births	79	152	231	
Singleton Birth (%)	69 (87.3 %)	129 (84.9 %)	198 (85.7 %)	0.695
Twin Birth (%)	10 (12.7 %)	23 (15.1 %)	33 (14.3 %)	
Triplet (%)	0	1 (0.5 %)	1 (0.3 %)	
Babies Born (n°)	89	175	264	
Gestational age (weeks)[a]	38.2 ± 2.4	38.1 ± 2.3	38.1 ± 2.3	0.410
Singleton gestational age (weeks)[a]	38.6 ± 2.2	38.7 ± 1.7	38.7 ± 1.9	0.544
Twin gestational age (weeks)[a]	35.5 ± 1.7	34.8 ± 2.0	35.0 ± 1.9	0.272
Singleton weight (g)+	3363 ± 578	3313 ± 509	3330 ± 533	0.449
Twin weight (g)[a]	2530 ± 393	2250 ± 483	2334 ± 473	0.019

[a] Mean ± SD

results for pregnancy rate were: number of obs = 1280. LR $\chi^2(3) = 26.11$. Prob > $\chi^2 = 0.0000$. Log likelihood = −751.15154. Pseudo $R^2 = 0.0171$. The AR odd ratio was 1.315605 with a standard error of 0.1700093, $p = 0.034$, 95 % CI 1.021242–1.694814. For delivery rate, the AR odd ratio was 1.438675 with a standard error of 0.2098151, $p = 0.013$, 95 % CI 1.080997–1.914702.

Discussion

The results of our analysis demonstrated an improvement in frozen embryo transfer outcome (better implantation, pregnancy, and delivery rates) when expanded blastocysts undergo AS prior to vitrification. Our data are in agreement with the recent work of other authors supporting the AS of expanded blastocysts to improve blastocyst viability upon re-warming and the implantation/pregnancy rate after embryo transfer [9, 11, 18].

Vanderzwalmen et al. [9] demonstrated that AS can enhance the implantation and pregnancy rate when used for expanded blastocysts. Yong Soo Hur et al. [11] utilized AS also in fresh blastocyst transfer cycles and proposed this technique as a useful approach to improve the clinical outcome in fresh cycles. Mukaida et al. [18] showed that AS was associated with higher embryo survival rate after vitrification/thawing.

Although several methods to perform AS have been described [13, 19, 20] such as micro-needle and holding pipette, chemical substances, laser pulse, and stripper pipette, we performed AS using the micropipette method being the least expensive and easy to carry out.

It is known that the trophectoderm forms a critical part of the placenta and umbilical cord. Sharen Cao et al. [21] in their article argued that AS could damage the blastocyst structure and have a negative impact on pregnancy outcome. In our study, we did not find any differences in terms of abortion rate and gestational duration between the two groups proving that AS is beneficial for frozen embryo transfer cycles of expanded blastocysts. A possible bias in our results can be a difference in the transfer regimen and his effect on implantation rate, although most of the cycles (76 %) were hormonal replacement cycles and this variable was not considered in our analysis. In AS 385 (66.4 %) were hormonal replacement cycles and 405 (90.4 %) in NAS. However, the type of hormonal replacement should not be influential on our results as demonstrated by a Cochrane meta-analysis [22] reporting no differences in IR and PR among hormonally prepared transfer cycles and other regimens. Another recent paper also confirmed that all of the current methods of endometrial preparation appear to be equally successful in terms of ongoing pregnancy rate [23].

The beneficial impact of artificially shrinking expanded blastocysts prior to vitrification can be attributed to a reduced likelihood of ice crystal formation once the volume of blastocoelic cavity is minimized. The same mechanism was suggested also by Vanderzwalmen et al. [9] who demonstrated an inverse relationship between volume of blastocoelic cavity and survival rate after thawing. Our work demonstrated that although there was no difference in survival rate between

expanded blastocysts that had AS prior to vitrification versus those who did not, the IR and the PR were higher in the group who received AS. It is therefore likely that expanded blastocysts due to their large volume of fluid-filled blastocoele cavity, may suffer ultrastructural damages not impairing their survival but impacting their subsequent potential for implantation. A positive aspect of our work although retrospective is the high number of cycles enrolled in the study with a complete follow-up.

Another important observation from our work is the demonstration that aspirating blastocoele fluid is not detrimental to the survival of the blastocyst thus reinforcing the concept of a potential role of this fluid for performing genetic and chromosomal analysis of the embryonic (and not trophoblast) compartment [24–27].

In conclusion, despite no differences in survival rates, our results confirm that AS before vitrification does not damage the blastocyst and appears to improve IR and PR after frozen blastocyst embryo transfer cycles. A prospective randomized trial is needed to confirm these initial observations.

Compliance with ethical standards

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