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TECHNOLOGICAL INNOVATIONS

A combination of hydroxypropyl cellulose and trehalose as supplementation for vitrification of human oocytes: a retrospective cohort study

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Abstract
Purpose This study aimed to determine whether the new formulation of vitrification solutions containing a combination of hydroxypropyl cellulose (HPC) and trehalose does not affect outcomes in comparison with using conventional solutions made of serum substitute supplement (SSS) and sucrose.
Methods Ovum donation cycles were retrospectively compared regarding the solution used for vitrification and warming of human oocytes. The analysis included 218 cycles ($N=2532$ oocytes) in the study group (HPC + trehalose) and 214 cycles ($N=2353$ oocytes) in the control group (SSS + sucrose).
Results No statistical differences were found in ovarian stimulation parameters and baseline characteristics of donors and recipients. The survival rate was 91.3 % (95 % confidence interval (CI) = 89.8–92.9) in the HPC + trehalose group vs. 92.1 % (95 % CI = 90.4–93.7) in the SSS + sucrose group (NS). The implantation rate (42.8 %, 95 % CI = 37.7–47.9 vs. 41.2 %, 95 % CI = 36.0–46.4), clinical pregnancy rate (CPR) per transfer (60.7 %, 95 % CI = 53.9–67.5 vs. 56.4 %, 95 % CI = 49.3–63.5), and ongoing pregnancy rate (OPR) per transfer (48.5 %, 95 % CI = 41.5–55.5 vs. 46.3 %, 95 % CI = 39.2–53.4) were similar for patients who received either HPC + trehalose-vitrified oocytes or SSS + sucrose-vitrified oocytes. Statistical differences were found when analyzing blastocyst rate both per injected oocyte (30.2 %, 95 % CI = 28.3–32.1 vs. 24.1 %, 95 % CI = 22.3–25.9) and per fertilized oocyte (40.8 %, 95 %CI = 38.5–43.1 vs. 33.2 %, 95 % CI = 30.8–35.5) ($P<0.0001$). Delivery rate was comparable between groups (37.2 %, 95 % CI = 30.8–46.6 vs. 36.9 %, 95 % CI = 30.4–43.4; NS).
Conclusions Our data demonstrate that HPC and trehalose are suitable and safe substitutes for serum and sucrose. Therefore, the new commercial media can be used efficiently in the vitrification of human oocytes avoiding viral and endotoxin contamination risk.

Keywords Hydroxypropyl cellulose · Macromolecular supplement · Oocyte vitrification · Survival rate · Clinical outcome

Capsule Our data demonstrate that HPC and trehalose are suitable and safe substitutes for serum and sucrose.

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Introduction

Oocyte vitrification has become one of the most important achievements in assisted reproductive technology (ART), and in recent years, it has been established as a routine technique given its good efficiency and consistency. Moreover, embryo development and clinical outcomes are similar to those achieved with fresh oocytes [1, 2]. As a result, this strategy has proven to be a valuable tool for ovum donation programs as it allows easier, more flexible procedures [3].

Other clinical applications for oocytes vitrification include fertility preservation for both oncological [4, 5] or social reasons [6], oocyte accumulation in low-responder patients [7], storage in cases of sperm collection difficulties [8], and ethical concerns or legal restrictions associated with embryo cryopreservation [9]. In addition, oocyte vitrification has been proposed as a safe and efficient alternative to postpone embryo transfer in patients at high risk of ovarian hyperstimulation syndrome [10].

Vitrification is defined as the glass-like solidification of an aqueous solution at low temperature. Using a high concentration of cryoprotectant agents (CPAs), in combination with very high cooling rates, avoids ice formation. This approach also requires high warming rates to ensure that glass is not converted into ice during warming [11].

Since the first vitrification procedure was developed [12], different CPAs, exposure times, and devices have been studied. Nowadays, a mixture of penetrating CPAs, such as ethylene glycol (EG) and dimethylsulfoxide (DMSO) in combination with a nonpenetrating CPA, such as sucrose, is the most widely used approach. Vitrification solutions also include a protein supplement. Formerly, whole serum was routinely added to media as a protein source. However, in an attempt to use more highly defined media, human serum albumin (HSA) became a replacement for serum and is the most widely used protein today. Serum substitute supplement (SSS), which contains globulins and HSA, represents more complex protein supplementation than HSA alone and was specifically designed as a protein supplement for culture media in ART procedures [13].

The current trend of removing any component of human origin has led to the development of new formulations for vitrification protocols that are free of viral contamination risk and plasma derivatives. Given the physical properties of hydroxypropyl cellulose (HPC), a fully synthetic macromolecule, which includes the ability to form a viscous gel at low temperatures, it has been proposed as a substitute for HSA [14].

Sucrose, the commonest sugar used as a nonpenetrating cryoprotectant in the majority of cryopreservation protocols, acts as a partial dehydrant by creating osmotic imbalance, which helps prevent the formation of ice crystals. Disaccharide trehalose is employed by certain species to survive in extreme conditions [15–19]. Further benefits of using this sugar as an osmotic agent in some human and mouse cryopreservation protocols have been reported previously [20–23].

Due to the benefits of using HPC and trehalose, the combination of both has been included in the new formulation of vitrification solutions. The present study aimed to assess the outcome of ovum donation cycles conducted with vitrified oocytes with HPC and trehalose, as opposed to vitrified oocytes, with traditional available solutions containing SSS and sucrose.

Material and methods

Study design and participants

A retrospective cohort study was conducted at the Instituto Valenciano de Infertilidad (IVI—Valencia, Spain). The procedure and protocol were approved by an institutional review board, which regulates and approves database analysis and clinical IVF procedures for research at IVI. Both vitrification media used in the present study were from the same manufacturer (Kitazato-Dibimed). The new formulation, which contains HPC + trehalose, was introduced in our laboratory in January 2012 and replaced the traditional containing SSS + sucrose. In an attempt to check outcomes with the new commercial media, computerized data from recipients who received vitrified oocytes from our ovum donation program were analyzed. In the first phase of the study, from November 2011 to January 2012, vitrification procedures were performed with SSS + sucrose solution (control group), while in the second period from January 2012 to May 2012, oocytes were vitrified with HPC + trehalose solution (study group). The analysis included 218 cycles ($N = 2532$ oocytes) in the study group and 214 cycles ($N = 2353$ oocytes) in the control group. Donors' oocytes were assigned to each recipient following our routine procedure, which consists in matching and considering blood type, phenotypical characteristics, and special requirements like screening for specific disease, etc. Oocytes were warmed using the corresponding warming solution according to that used for donors' oocyte vitrification. Recipients with severe male factor (total motile sperm <1 million), clinical history of recurrent miscarriage, and preimplantation genetic diagnosis were excluded from the analysis.

Stimulation protocol for donors

The controlled ovarian stimulation (COS) protocols employed in our center for donors have been described elsewhere [3, 24, 25]. In the luteal phase, long agonist protocol donors were downregulated with daily doses of a GnRH agonist, which started in the luteal phase during the cycle before stimulation (Synarel®, nafarelin, intranasal; Pfizer, Barcelona, Spain). After menses, COS was initiated with 150 or 225 IU/day of recombinant FSH (Gonal-F®; Merck-Serono, Madrid, Spain; or Puregon®; MSD, Madrid, Spain) combined with 75 IU/day hMG (Menopur®; Ferring Pharmaceuticals, Madrid, Spain). Dose was adjusted to ovarian response. In the GnRH agonist protocols, triggering was performed with 250 μg of recombinant human chorionic gonadotrophin (rHCG) (Ovitrelle®; Serono, Madrid, Spain). Alternatively, the flexible GnRH antagonist protocol was used as follows: COS was initiated D2–D3 after bleeding with 150 or 225 IU/day of recombinant FSH (Gonal-F®; Merck-Serono, Madrid, Spain; or Puregon®; MSD, Madrid, Spain) combined with 75 IU/day hMG (Menopur®; Ferring Pharmaceuticals, Madrid, Spain). Dose was adjusted to ovarian response. Daily doses of 0.25 mg of a GnRH antagonist (ganirelix, Orgalutran®; MSD, Madrid, Spain, or cetrorelix, Cetrotide®; Merck-Serono, Madrid, Spain) were started when a follicle measured >14 mm. A single GnRH agonist dose (0.1 mg of triptorelin,

Decapeptyl®; Ipsen Pharma, Barcelona, Spain) was administered to trigger final oocyte maturation when at least three follicles measured more than 17.5 mm or when one follicle measured more than 20 mm. In some cases, triggering was performed with 250 μg of rHCG. Transvaginal oocyte retrieval was conducted 36 h later.

Endometrial preparation for oocyte recipients

The endometrial preparation protocol is described elsewhere [25]. Women with ovarian function were first downregulated in the luteal phase with a single dose of a GnRH agonist depot (Decapeptyl®, 3.75 mg, Ipsen Pharma, or Gonapeptyl® 3.75 mg, Ferring). After menses, all the subjects received oral estradiol valerate (EV) (Progynova®, 6 mg/day; Schering, Madrid, Spain). Approximately 10–15 days after EV initiation, serum E_2 levels and endometrial thickness were measured. Administration of micronized progesterone (P) (800 mg/day, vaginally; Progeffik, Effik Laboratories, Madrid, Spain) was initiated the day after oocyte donation. If pregnancy was achieved, EV and P administration was maintained until gestation week 12.

Oocyte handling

Donors' oocytes intended for vitrification were maintained in fertilization media (fertilization medium: Cook IVF) at 5.5 % CO_2 in air and 37 °C for 2 h after ovum pick-up and were then enzymatically denuded. Oocyte denudation was performed by mechanically pipetting 40 IU/ml of hyaluronidase in the same medium. Vitrification was carried out immediately after assessing nuclear maturity. Only metaphase II (MII) oocytes were vitrified using HPC + trehalose or SSS + sucrose solutions. Insemination was performed 2 h after warming by ICSI.

Oocyte vitrification/warming

Oocyte vitrification was performed with the Cryotop® method using HPC + trehalose or SSS + sucrose solutions. Oocytes were equilibrated at room temperature for 12 min in 7.5 % EG + 7.5 % DMSO in TCM199 medium containing HPC or SSS according to the vitrification group. Subsequently, they were then placed in vitrification solution that included 15 % EG + 15 % DMSO in TCM199 medium + 0.06 mg/ml HPC or 20 % SSS and 0.5 M trehalose or sucrose. After 50–60 s in this solution, oocytes were loaded on the Cryotop strip and immediately submerged in liquid nitrogen. No more than four oocytes per Cryotop were loaded. For warming purposes, the Cryotop was removed from liquid nitrogen and was directly placed in 1.0 M trehalose or sucrose in TCM199 medium + HPC or SSS at 37 °C. After 1 min, oocytes were placed in 0.5 M trehalose or sucrose in TCM199 medium that contained the corresponding macromolecule or serum supplement according to each group, at room temperature for 3 min. Finally, one 5-min wash, followed by one 1-min wash, was performed in the same solution at room temperature before incubating oocytes in culture media (cleavage medium: Cook IVF) for 2 h before ICSI.

Embryo score, culture conditions, and selection

All the embryos were incubated at 37 °C, 5.5 % CO_2, and atmospheric O_2 concentration and were cultured individually until day 3 (72 h after ICSI) in the cleavage medium (Cook IVF); in the extended culture cases from day 3 to day 5 or 6, CCM Medium was used (Vitrolife). Embryo transfer was performed on both day 3 and day 5 following each patient's clinical indication.

Embryo quality was assessed morphologically according to the criteria of the Asociación para el Estudio de la Biología de la Reproducción (ASEBIR), the Spanish embryology society [26], with slight modifications. A summary of the ASEBIR classification system can be found in the Istanbul Consensus Workshop document on embryo assessment, published by Alpha Scientists in Reproductive Medicine [27]. In brief, a type A embryo (with optimal quality and the best implantation potential) was defined as that with four cells on day 2 and seven to eight cells on day 3, with <11 % focal fragments, absence of vacuoles, no multinucleation, and no irregularities in the zona pellucida (ZP).

Day 3 type B embryos showed seven to eight cells (from a four-cell embryo on day 2 with 11–25 % of fragmentation) or nine to ten cells (from a four-cell embryo with <26 % fragmentation and a maximum of one multinucleated cell) and having the same parameters for vacuoles and ZP evaluation as those described above. The embryos assessed as type A or B on day 3 were selected for either transfer or vitrification, except for the cases of blastocyst stage transfer indications. Suboptimal embryos were maintained in extended culture and were vitrified only if they developed into good-quality blastocysts.

The ASEBIR blastocyst scoring is based on the evaluation of inner cell mass (ICM) and trophectoderm appearance, as proposed by Gardner et al. [28]. Type A ICM was well defined, oval and compacted, and consisted of many cells (1900–3800 mm^2 in diameter). Type B ICM presented the same size as type A, but with lesser compaction. Type A trophectoderm was homogeneous, well defined, and made up of many cells, whereas a type B trophectoderm presented an irregular epithelium. Type A blastocysts were defined as having both type A ICM and type A trophectoderm. Type B blastocysts were defined as those having a type A ICM and type B trophectoderm, a type B ICM and type A trophectoderm, or both type B ICM and type B

trophectoderm. Type A and B blastocysts were selected for either transfer or vitrification. Artificial blastocyst collapsing was occasionally performed in hatching embryos.

Surplus embryos, suitable for further cryopreservation, were vitrified using the corresponding vitrification solution according to that used for donors' oocyte vitrification.

Outcome measures and statistics

The primary end point of the present study was survival rate per donation cycle. The secondary end points were fertilization rate; embryo quality; implantation rate (defined as the number of gestational sacs detected by transvaginal ultrasound examination divided by the number of replaced embryos); clinical pregnancy rate (CPR, confirmed by the detection of an embryonic sac during a transvaginal scan at least 5 weeks after ET) [29]; and ongoing pregnancy rate (OPR, confirmed by the presence of a gestational sac with fetal heart beat observed during the aforementioned scan at ≥12 weeks) [30]. The miscarriage rate was defined as the percentage of pregnancies that terminated before gestation week 22. Delivery and live birth rate (LBR) were considered when the fetus was born alive beyond the 22 weeks of pregnancy.

The variables considered were presented as proportions or the mean together with a confidence interval of 95 % (CI 95 %). The values of the different variables we examined were compared for the two groups. Comparison of quantitative variables was done using Student's *t* test for independent samples when data were normally distributed (tested by Kolmogorov-Smirnov test). Chi-square test was performed to compare proportions among the groups. $P < 0.05$ was considered statistically significant.

To confirm and quantify the two crude group analyses, ongoing pregnancy was fitted to logistic regression with the following covariates: type of macromolecule supplement (HPC or SSS), class variable, two states; day of transfer (day 3 or blastocyst-stage embryo transfer), class variable, two states; number of MII oocytes microinjected, discrete variable; number of embryos transferred, discrete variable; recipient's BMI, continuous variable, measured in units; and donor's age at the time of donation, continuous variable, measured in years.

Results

No statistical differences were found when analyzing donor's baseline characteristics and ovarian stimulation parameters (Table 1). The number of MII oocytes vitrified/warmed per donation cycle was similar in both groups (Table 1). No differences were observed in recipient's baseline characteristics (Table 2). The survival rate was 91.3 % (95 % CI = 89.8–92.9) in the HPC + trehalose group vs. 92.1 % (95 % CI = 90.4–93.7) in the SSS + sucrose group (NS).

There were no differences in the number of injected oocytes (Table 2). The fertilization rate was comparable among groups (NS). Embryo morphological quality on day 3, calculated as per cleaved embryo and per injected oocyte basis, was also comparable between groups (NS) (Table 2). The blastocyst rate per embryo subjected to extended culture was similar between the HPC + trehalose (65.9 %, 95 % CI = 63.0–8.8) and SSS + sucrose (61.1 %, 95 % CI = 57.8–64.4) groups (NS). Moreover, the morphological appearance of the achieved blastocysts was similar between both groups. However, statistical differences were found when analyzing the blastocyst rate per injected oocyte (30.2 %, 95 % CI = 28.3–32.1 vs. 24.1 %, 95 % CI = 22.3–25.9) and per fertilized oocyte (40.8 %, 95 % CI = 38.5–43.1 vs. 33.2 %, 95 % CI = 30.8–35.5, respectively) ($P < 0.0001$).

Clinical outcomes are summarized in Table 3. Embryo transfer was performed in 89.9 % (95 % CI = 85.9–93.9) of the donation cycles conducted with HPC + trehalose-vitrified oocytes and in 87.8 % (95 % CI = 83.4–92.2) of cycles with SSS + sucrose-vitrified oocytes (NS). The proportion of day 3 and blastocyst embryo transfers was similar between groups (Table 3). The number of embryos replaced in the recipient's uterus was similar between the two analyzed groups. No differences were found in the implantation rate (42.8 %, 95 % CI = 37.7–47.9 vs. 41.2 %, 95 % CI = 36.0–46.4, NS). The patients who received HPC + trehalose-vitrified oocytes achieved a CPR of 60.7 % per embryo transfer (95 % CI = 53.9–67.5). No statistical differences were observed when compared to the SSS + sucrose group (56.4 %, 95 % CI = 49.3–63.5). The OPR per embryo transfer was also comparable between groups (48.5 %, 95 % CI = 41.5–55.5 vs. 46.3 %, 95 % CI = 39.2–53.4, NS). No statistical differences were found when these outcomes were analyzed per cycle (Table 3).

As for the miscarriage rate, no significant differences were seen among groups (17.6, 95 % CI = 10.8–24.4 vs. 17.9, 95 % CI = 10.6–25.2). Delivery rate was also similar between both groups (37.2 %, 95 % CI = 30.8–46.6 vs. 36.9 %, 95 % CI = 30.4–43.4, NS).

A logistic regression analysis was performed on the OPR to account for the effect of the other confounding factors addressed in the "Material and methods" section, i.e., the factors used in the logistic regression model were the macromolecule supplement type, day of transfer (day 3 or blastocyst-stage embryos), number of microinjected MII oocytes, number of embryos transferred, recipient's BMI, and donor's age at the time of donation. The final modeling results are provided in Table 4. The analysis was based on 432 patients who either presented an ongoing or failed pregnancy. Of the six covariates, none had a statistically significant effect on the model.

Table 1 Demographics, baseline characteristics, and ovarian stimulation of donors

	HPC	95 % CI	SSS	95 % CI
Number of donors and donation cycles	218		214	
Age (years)	24.7 ± 4.0	(24.2–25.2)	25.6 ± 3.9	(25.1–26.1)
BMI (kg/m^2)	22.5 ± 2.9	(22.1–22.9)	22.9 ± 3.1	(22.5–23.3)
Days of stimulation	10.3 ± 1.4	(10.1–10.5)	10.4 ± 1.3	(10.2–10.6)
rFSH dose (IU)	1612.9 ± 528.7	(1535.4–1690.4)	1593.6 ± 513.6	(1513.5–1673.7)
E_2 on day of hCG (pg/ml)	2606.9 ± 1369.1	(2415.8–2798.0)	2577.7 ± 1523.7	(2367.1–2788.3)
P_4 (ng/ml)	0.96 ± 0.59	(0.84–1.08)	0.83 ± 0.48	(0.75–0.91)
Total MII oocytes vitrified (mean per cycle)	2532 (11.6 ± 3.3)	(11.2–12.0)	2353 (11.0 ± 3.2)	(10.6–11.4)

Unless otherwise indicated, values are mean ± SD with 95 % confidence limits in brackets

BMI body mass index, *rFSH* recombinant FSH

Discussion

The present study, which included a large number of patients, shows that the use of HPC and trehalose to replace conventional solutions with protein supplement and sucrose does not affect the oocyte survival rate, embryo development, and clinical outcomes in an ovum donation program. This demonstrates the great efficiency achieved when using this new commercial media allowing safer, more harmless procedures.

Ever since ART emerged, both cryopreservation and culture media have been supplemented with a protein source [31]. Although it has been proven that fertilization, embryo cleavage, and even pregnancy can be achieved by transferring embryos that originate under protein-free culture conditions [32], better clinical outcomes may occur when a protein supplement is added to culture media [33, 34].

In the 1980s, the commonest protein source for media supplementation was human serum. Serum is a reservoir of different molecules, such as steroids, vitamins, fatty acids, and growth factors that promote cell proliferation and differentiation. Moreover, serum acts as a scavenger of embryotoxic components and protects cell membranes during the freezing process [35].

Traditionally, serum proteins have been obtained from either adult donors' pools or fetal cord serum [31]. Nonetheless, a high risk of transmitting different infectious diseases has been associated with serum as a protein source [36]. Some studies have reported that the use of whole serum can be deleterious upon embryogenesis in vitro [37], plus the inconvenience of inherent variability from batch to batch.

In an effort to overcome these drawbacks, the trend has been to replace the use of whole serum with more highly

Table 2 Recipients' baseline characteristics and embryo development according to the group

	HPC	95 % CI	SSS	95 % CI
Number of recipients	218		214	
Mean age (years)	41.2 ± 4.0	(40.7–41.7)	41.2 ± 4.3	(40.6–41.8)
BMI (kg/m^2)	23.1 ± 3.9	(21.6–24.6)	24.2 ± 4.1	(22.7–25.7)
Days of endometrial preparation	16.1 ± 5.2	(15.4–16.8)	16.5 ± 5.1	(15.8–17.2)
Number of MII oocytes injected (mean ± SD)	2288 (10.5 ± 2.9)	(10.1–10.9)	2131 (10.0 ± 2.6)	(9.6–10.3)
Fertilization rate	1693/2288 (74.0)	(72.2–75.8)	1545/2131 (72.5)	(70.6–74.4)
Top-quality day 3/cleaved embryo	816/1643 (49.7)	(47.3–52.1)	726/1469 (49.4)	(46.8–52.0)
Top-quality day 3/injected oocyte	816/2288 (35.7)	(33.7–37.7)	726/2131 (34.1)	(32.1–36.1)
Blastocyst rate/embryo subjected to extended culture	691/1049 (65.9)	(63.0–68.8)	513/839 (61.1)	(57.8–64.4)
Blastocyst rate/oocyte injected	691/2288 (30.2)	(28.3–32.1)	513/2131 (24.1)	(22.3–25.9)*
Blastocyst rate/oocyte fertilized	691/1693 (40.8)	(38.5–43.1)	513/1545 (33.2)	(30.8–35.5)*
Good-quality blastocyst rate	329/691 (45.6)	(41.9–49.3)	269/513 (52.4)	(48.1–56.7)

Unless otherwise indicated, values are mean ± SD or *n* (%) with 95 % confidence limits in brackets

BMI body mass index

*$P < 0.05$

Table 3 Clinical outcomes according to the group

	HPC	95 % CI	SSS	95 % CI
Number of embryo transfers	196 (89.9)	(85.9–93.9)	188 (87.8)	(83.1–92.2)
Number of transfers on day 3	108 (55.1)	(48.1–62.1)	123 (65.4)	(58.6–72.2)
Number of transfers on blastocyst stage	88 (44.9)	(37.9–51.9)	65 (34.6)	(27.8–41.4)
Number of embryos replaced (mean ± SD)	362 (1.8 ± 0.4)	(1.7–1.9)	345 (1.8 ± 0.4)	(1.7–1.8)
Number of cycles with embryo cryopreservation	171/218 (78.4)	(72.9–83.9)	160/214 (74.8)	(69.0–80.6)
Number of re-vitrified embryos (mean ± SD)	549 (3.2 ± 1.9)	(2.9–3.5)	471 (2.9 ± 1.8)	(2.6–3.2)
Implantation rate	155/362 (42.8)	(37.7–47.9)	142/345 (41.2)	(36.0–46.4)
Clinical pregnancy rate/cycle	119/218 (54.6)	(48.0–61.2)	106/214 (49.5)	(42.8–56.2)
Clinical pregnancy rate/embryo transfer	119/196 (60.7)	(53.9–67.5)	106/188 (56.4)	(49.3–63.5)
Ongoing pregnancy rate/cycle	95/218 (43.6)	(37.0–50.2)	87/214 (40.6)	(34.0–47.2)
Ongoing pregnancy rate/embryo transfer	95/196 (48.5)	(41.5–55.5)	87/188 (46.3)	(39.2–53.4)
Miscarriage rate	21/119 (17.6)	(10.8–24.4)	19/106 (17.9)	(10.6–25.2)
DR/cycle	81/218 (37.2)	(30.8–46.6)	79/214 (36.9)	(30.4–43.4)
LBR/cycle	96/218 (44)	(37.4–50.6)	97/214 (45.3)	(38.6–52)

Unless otherwise indicated, values are mean ± SD or *n* (%) with 95 % confidence limits in brackets

defined albumin sources. Menezo et al. first demonstrated that HSA can be used instead of serum; indeed, it is now the most widely used protein as a supplement in embryo culture [38]. Even though the simplification of culture media supplementation has been recommended, some studies have suggested improved clinical outcome after using some preparations made of HSA and human globulins [39, 40]. The beneficial effect of more complex protein supplementation led to the introduction of SSS that consisted in HSA supplemented with another serum fraction rich in α- and β-globulins [13]. In fact, SSS has been specifically designed as a protein supplement for culture media in ART procedures and has been found to give higher implantation and pregnancy rates when added to media [41]. In a randomized controlled trial, Meintjes et al. demonstrated that SSS added to commercial HSA-supplemented embryo culture media resulted in an overall increase in implantation and live birth rates [42].

SSS is also commonly employed as a protein supplement in cryopreservation solutions that contain different CPAs for both slow freezing and vitrification protocols. However, concerns about using plasma derivatives for the supplementation of both cryopreservation and culture media have led to alternative macromolecules being developed. Addition of low concentrations of high molecular weight compounds, such as ficoll [43], PVP [44], or sodium hyaluronate [45], has had different degrees of success. Recombinant albumin is also available for the supplementation of human embryo culture media, although it is more expensive than human-derived protein products and there are no works to report that it improves clinical outcomes [46].

HPC is a cellulose polymer with hydroxypropyl groups that confers high solubility in both water and organic solutions. It has been widely used in both the pharmaceutical and food industries. Among other properties, HPC includes thermoplasticity and surfactant properties, as well as other stabilizing factors. HPC solutions have extremely high viscosity, which permits transitions to a glassy state at low temperatures. All these properties, associated with this fully synthetic macromolecule, make HPC the perfect choice for replacing serums that contain human derivatives to prepare

Table 4 Logistic regression analysis of the vitrification protocol on ongoing pregnancy, including possible confounding factors

Model effect	Values	OR (95 % CI)	*P* value
Macromolecular supplement	SSS vs. HPC	0.790 (0.502–1.244)	NS
Day of transfer	Day 5 vs. day 3	1.053 (0.942–1.176)	NS
Number of MII oocytes microinjected	Per MII oocyte	1.079 (0.981–1.186)	NS
Number of ET	Per oocyte embryo	1.440 (0.778–2.664)	NS
Recipient BMI	Per unit	0.977 (0.907–1.052)	NS
Donor age	Per year	1.024 (0.969–1.082)	NS

BMI body mass index, *CI* confidence interval, *OR* odds ratio, *NS* not statistically significant

cryoprotective solutions. Given that oocytes and embryos remain in vitrification and warming solutions only for a short time, we speculate that they do not need the beneficial components of serum for these procedures. Therefore, the outcomes should not be adversely affected when this synthetic macromolecule is used as a serum replacement.

The potential use of HPC as a macromolecular supplement in oocyte and embryo vitrification solutions has been previously described [14, 47, 48]. Inoue et al. first evaluated the use of HPC as a substitute for animal-derived serum and proteins in human oocytes, finding no differences in survival rate when compared with using SSS [14]. This finding has been confirmed very recently in a study conducted with mouse and human blastocysts [48]. Nevertheless, none of these studies provide clinical outcomes; thus, the safety of this macromolecule has not yet been demonstrated.

We also analyzed the use of trehalose as an osmotic agent. In addition to the osmotic role, sugars have also attributed other protective properties due to the high ice transition temperature compared to other CPAs, such as EG, DMSO, and propanediol [21]. Trehalose is a disaccharide that is composed of two glucose molecules, where the glycoside bond involves the OH groups of the two anomeric carbons. This sugar is present in hemolymphs of insects and also in some microorganisms. Some of these species can remain in a latent state for years, and even centuries, as they conserve themselves in a glassy state by using sugars, such as glucose, sucrose, or trehalose [15–19]. Thus, several animals utilize these sugars to survive freezing and extreme dryness [20–23].

Despite the fact that the vast majority of commercial media still use sucrose, trehalose has been previously employed in cryobiology and applied to reproduction protocols [21]. A study in mouse embryos has demonstrated that addition of both extracellular and intracellular trehalose helps raise not only survival rates but also fertilization and embryo development rates [21]. This disaccharide has also been successfully used in combination with glycerol to cryopreserve various types of cells and tissues, e.g., hepatocytes, cardiac tissue, platelets, adipose tissue, umbilical cord blood, and human embryonic stem cells [49]. The powerful protective effect of trehalose has been associated not only with facilitating the glassy state but also with a stabilizing effect on lipid membranes and proteins as a result of their direct interaction with polar residues through hydrogen bonds. This effect is known as water replacement hypothesis [49]. Moreover, trehalose is free of endotoxins allowing safer and harmless procedures.

To our knowledge, this is the largest sample size study to have been carried out to date that compares survival, embryo development, and clinical outcome by employing HPC + trehalose vs. SSS + sucrose as a supplement in an oocyte vitrification protocol.

No differences were obtained with regard to the primary end point (i.e., survival rate). This result is of great interest since it demonstrates that using HPC as a macromolecular totally synthetic supplement allows efficient vitrification while avoiding the use of supplementation containing human derivatives. Additionally, we observed a similar fertilization rate, top-quality embryos on day 3, and blastocyst formation per embryo subjected to extended culture in the HPC + trehalose group and the SSS + sucrose group, respectively. This scenario advocates an unaltered embryo development, therefore suggesting no damage that could compromise embryo viability.

We also found that a higher blastocyst rate was obtained in the HPC + trehalose-vitrified oocytes when analyzed with both per injected oocyte and per fertilized oocyte basis. These data indicate that the procedure's biological efficiency could improve if HPC was employed as a macromolecule supplement. However, a larger sample size would probably be required to confirm these results.

In addition, the potential of embryos to implant and achieve viable pregnancies in rates is comparable to that obtained using traditional solutions. These results also show the efficiency and safety of HPC and trehalose contained in vitrification and warming solutions.

Furthermore, the current study does not reveal statistical between-group differences in the number of patients who vitrified surplus embryos and the number of cryopreserved embryos. However, we did not analyze the clinical outcomes after transferring these cryopreserved surplus embryos in this study. There was not enough time for patients who failed to achieve pregnancy to return for a new attempt, let alone for those who wished to try for a second child.

This study also shows similar delivery rate when comparing both groups. However, it should be pointed out that we only considered the births for which we had notification. Thus, the data of delivery rate per cycle are probably underestimated, since no assumptions were made about the final outcome of the recipients lost to follow-up (22 recipients).

According to the linear regression model, macromolecule supplement type, day of transfer, number of microinjected MII oocytes, number of embryos transferred, recipient's BMI, and donor's age at the time of donation had no impact on OPR. We used OPR for the analysis instead of our primary outcome because once the survival was demonstrated to be similar among groups, we wanted to analyze the effect of these covariates in the further development and clinical outcomes. Obviously, we did not use delivery rate because of the incomplete data recovery.

It is necessary to underline that the retrospective design of this study represents a main limitation. Therefore, prospective studies would be required to confirm our results. Additionally, we cannot know the individual contribution of HPC and trehalose in the survival rate and clinical outcomes, since the effectiveness of both components has been jointly evaluated.

However, in this case, our aim was not to evaluate each component separately but test the new formulation of the vitrification solutions. A secondary limitation could be the fact that the embryo transfer was performed both on day 3 and in the blastocyst stage, following each patient's clinical indication. Nonetheless, our logistic regression analysis showed a negative influence of the day of transfer on the final outcome.

It is widely accepted that the ideal alternative to human-derived products is a synthetic macromolecule to make vitrification media safe, pyrogen-free, standardized, and acceptable worldwide. In conclusion, our data demonstrate that using HPC as a macromolecule supplement in combination with trehalose as an osmotic agent in vitrification protocols impairs neither survival or embryo development nor clinical outcomes.

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