J Assist Reprod Genet (2014) 31:1605–1610
DOI 10.1007/s10815-014-0343-8

ASSISTED REPRODUCTION TECHNOLOGIES

Perinatal outcomes for transfer of blastocysts vitrified and warmed in defined solutions with recombinant human albumin: 374 babies born after 898 embryo transfers

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Received: 24 July 2014 / Accepted: 9 September 2014 / Published online: 19 October 2014

Abstract
Purpose To assess the efficacy of a novel, defined vitrification procedure using recombinant human albumin (rHA) for cryopreservation of human blastocysts. Design: Retrospective study. Setting: Private IVF clinic. Patients: 1,496 patients received vitrified/warmed embryo transfer (ET).
Methods Surplus blastocysts, and blastocysts from patients undergoing elective embryo cryopreservation, were vitrified/warmed using Cryotop carriers in homemade solutions containing either human serum albumin (HSA) or rHA. Main Outcome Measures: Clinical and neonatal outcomes regarding the vitrified/warmed ET procedures.
Results The HSA and rHA groups had a total of 1,163 and 898 vitrified/warmed cycles, respectively. Embryo survival rates (98.7 % vs. 98.9 %, respectively) and the number of embryos transferred (1.08±0.01 vs. 1.06±0.01, respectively) were similar in the HSA and rHA groups. Clinical pregnancy rates/ET were higher ($P<0.05$) in the rHA group (56.0 %) than in the HSA group (51.5 %). The HSA and rHA groups had similar live delivery rates/pregnancy (72.2 % vs. 72.3 %, respectively) and perinatal outcomes, including birth weight (2,988±28 vs. 3,046±26 g, respectively). Birth defects occurred in 0.9 % and 1.6 % of neonates in the HSA and rHA groups, respectively.
Conclusions rHA effectively replaced HSA for human embryo vitrification procedures, and yielded high rates of pregnancy and live births after vitrified/warmed ET. This new approach will support the development of defined ART systems, which will eliminate the variation and risks associated with the use of blood-derived products.

Keywords Blastocyst · Human · Vitrification · Defined · Recombinant human albumin

Capsule High rates of pregnancy and live births were obtained after transfer of human blastocysts that had been vitrified/warmed in chemically defined solutions containing recombinant human albumin.

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Introduction

Vitrification is a viable alternative to the conventional slow-freezing method used in assisted reproduction technology (ART), since it is associated with high embryo survival rates and successful outcomes [1–4]. Vitrification/warming solutions (VS/WS) typically contain high concentrations of protein, most frequently human serum albumin (HSA), to confer their cryoprotective effects. The presence of albumin, which is abundant in the female reproductive tract, improves both embryo development and handling. However, HSA can also contribute to biological variation, the introduction of toxic residue, and potentially disease transmission.

Seeking to develop defined embryo vitrification procedures, previous animal studies have described the feasibility of VS/WS containing synthetic polymers, including polyvinyl alcohol (PVA) [5]. However, the potentially toxic effects of these synthetic polymers on human embryos have not yet been thoroughly investigated in the clinical settings, and none can be considered a physiological alternative to albumin.

In contrast, both human and animal studies have shown the effectiveness of recombinant human albumin (rHA) in ART protocols, including in vitro fertilization (IVF) and embryo culture [6–9]. The use of rHA as the sole protein source provides superior batch-to-batch consistency and biological stability, and minimizes viral and prion contamination. rHA is considered a highly desirable substitute for HSA in ART

protocols, including embryo cryopreservation [10], but limited literature is available on rHA use. Unfortunately, the high cost of rHA currently limits its widespread use. Therefore, establishing efficient protocols, with low rHA concentrations, may lead to more practical and cost-effective procedures.

A previous pilot study, using in vitro-produced bovine blastocysts, found that the HSA (50 mg/mL) added to conventional VS/WS could be replaced with much lower concentrations of rHA (0.5, 1.0, and 2.5 mg/mL), which yielded high embryo viability after vitrification, similar to that of fresh embryos [11]. Based on these findings, a clinical trial was conducted to evaluate human blastocysts vitrified/warmed in defined solutions containing 2.5 mg/mL rHA, and high embryo survival and pregnancy rates were observed [12]. Consequently, the chemically defined VS/WS with rHA for blastocyst vitrification was implemented.

In the present study, we retrospectively analyzed these clinical data regarding human blastocyst vitrification, including the perinatal outcomes of children born after embryo transfer (ET), and compared the results obtained with the conventional (HSA) solutions and the novel, defined rHA solutions. These results provide a new step towards developing completely defined media for all ART protocols.

Materials and methods

Patients

This study included patients who received IVF or intracytoplasmic sperm injection (ICSI) treatment cycles at a private IVF clinic between January 2006 and June 2013. The study population consisted of patients who failed to conceive after fresh ET and subsequently received ET with the surplus vitrified blastocysts, as well as patients who underwent elective cryopreservation of all embryos and received ET with the vitrified blastocysts.

Between March 2006 and July 2013, 831 patients received 1,163 cycles of ET (IVF=487; ICSI=676), using blastocysts vitrified/warmed in the conventional solutions containing HSA (HSA group). Between November 2010 and July 2013, 665 patients received 898 cycles of ET (IVF=343; ICSI=555), using blastocysts vitrified/warmed in the defined solutions containing rHA (rHA group). In the clinic where this study was conducted, the mean age of women receiving IVF/ET treatment has slightly increased year-over-year. Consequently, patient age was younger ($P<0.05$) in the HSA group compared to the rHA group (Table 1). Informed consent had previously been obtained from the patients before the current study was conducted, and the study was approved by the Institutional Review Board.

IVF/ICSI and embryo culture

All patients used a long protocol, or a GnRH antagonist protocol, for controlled ovarian hyperstimulation. Oocytes were retrieved under transvaginal ultrasound guidance, 34 to 35 h after hCG injection, and were subjected to conventional IVF/ICSI procedures (day 0). After insemination, oocytes were cultured individually in 20-μL droplets of G-1 PLUS (Vitrolife, Göteborg, Sweden) or EmbryoAssist medium (Origio, Jyllinge, Denmark), at 37 °C in a humidified atmosphere of 6 % CO_2, 5 % O_2, and 89 % N_2. On day 3, good-quality embryos (6- to 10-cell embryos with ≤25 % fragmentation) were selected for either fresh ET, vitrification with Cryotop carriers (Kitazato, Fuji, Japan), or extended culture in G-2 PLUS (Vitrolife) or BlastAssist medium (Origio) until blastocyst formation.

For IVF/ICSI treatment cycles where the patients had only one good-quality embryo on day 3, the embryos were subjected to fresh ET, or vitrification in the commercial solutions (Kitazato) on day 3. When the patients had two or more good-quality embryos on day 3, one embryo was vitrified in the commercial solutions and the culture of the remaining embryos was extended. From the cultured embryos, good-quality blastocysts (blastocysts with Gardner's scores ≥2, excluding CC grades of the inner cell mass and trophectoderm) were selected on days 5 and 6 for fresh ET or vitrification in homemade solutions as described below.

Vitrification/warming of blastocysts

The base solution used for both vitrification and warming was Dulbecco's phosphate-buffered saline (DPBS; Irvine, Santa Ana, CA), supplemented with 50 mg/mL HSA (Buminate 25 %; Baxter, Tokyo, Japan) (HSA group) or 2.5 mg/mL rHA (G-MM; Vitrolife) (rHA group). The rHA concentration was chosen based on a previous study using bovine models [11], and vitrification was performed according to a previously described method [13]. In summary, blastocysts were first equilibrated in 10 % ethylene glycol (EG) at 37 °C for 12 to 15 min. In the solution, the expanded blastocysts in the HSA group were shrunk by manual pipetting, which has been proposed to improve the survival rate [14]. Conversely, this method was not used in the rHA group, as the blastocyst viability was already high without inducing shrinkage (unpublished data). After equilibration, the blastocysts were placed in a solution of 15 % dimethyl sulfoxide (DMSO), 15 % EG, and 0.5 mol/L sucrose for 1 min. During this exposure, the blastocysts were placed on the Cryotop carrier strip, and the Cryotop was quickly plunged into LN_2 (one embryo per carrier).

In this study, one or two vitrified blastocysts were warmed per cycle. For warming, the cryopreserved Cryotop was quickly placed in 0.5 mol/L sucrose. After 1 min, the blastocysts

Table 1 Patient characteristics and clinical outcomes for vitrified/warmed embryo transfers

	HSA	rHA	*P*-value
No. of patients	831	665	
Patient age (range)	34.8±0.1 (22–44)	35.4±0.2 (23–45)	.005
BMI (range)	20.6±0.1 (14.4–32.5)	20.8±0.1 (15.1–36.7)	.109
Patients undergoing their first or second IVF/ICSI treatment cycle (%)	735 (88.5)	604 (90.8)	.149
Cause of infertility			
Male factor	326 (39.2)	264 (39.7)	.873
Female factor	142 (17.1)	102 (15.3)	.398
Combined and other factor	363 (43.7)	299 (45.0)	.638
No. of warmed cycles	1,163	898	
No. of embryos warmed	1,274	963	
No. of surviving embryos (%)	1,258 (98.7)	952 (98.9)	.848
No. of ETs	1,159	897	
No. of embryos transferred	1,250	951	
Mean no. of embryos transferred	1.08±0.01	1.06±0.01	.108
No. of implantations (%)	621 (49.7)	521 (54.8)	.018
No. of clinical pregnancies (%/ET)	597 (51.5)	502 (56.0)	.045
No. of live deliveries (%/pregnancy)	431 (72.2)	363 (72.3)	>.999
No. of miscarriages (%/pregnancy)	158 (26.5)	131 (26.1)	.945
No. of stillbirths (%/pregnancy)	2 (0.3)	2 (0.4)	>.999
No. of patients lost to follow up (%/pregnancy)	6 (1.0)	6 (1.2)	.779

Values are expressed as mean±SEM. The vitrification/warming solutions contained either HSA (HSA group) or rHA (rHA group)

were washed at 37 °C with the following stepwise dilution: 2.5 min in 0.25 mol/L sucrose, 5 to 10 min in 0.1 mol/L sucrose, and 5 to 10 min in the base solution alone. Warmed blastocysts were cultured for at least three hours, and assessed for survival based on the re-expansion of the blastocoel.

ET of the surviving blastocysts (one or two per cycle) was performed with hormonal replacement cycles, and mechanically assisted hatching, with the use of micromanipulation, was carried out before ET. Implantation rate was defined as the number of gestational sacs per embryos transferred. Clinical pregnancy referred to the identification of a gestational sac in the uterus.

Statistical analysis

Data were compared using Student's *t*-test or χ^2 analysis and Fisher's exact probability test. Differences with a probability value (P) of <0.05 were considered significant.

Results

Table 1 shows the clinical outcomes for vitrified/warmed ET. Patient characteristics did not differ significantly between the HSA and rHA groups, with the exception of mean age. Most blastocysts survived warming in both groups, and ET could be performed in 1,159/1,163 (99.7 %) and 897/898 (99.9 %) of the warmed cycles in the HSA and rHA groups, respectively. The mean number of embryos transferred was slightly higher in the HSA group than in the rHA group, though this did not reach statistical significance. In this study, single ET of the vitrified/warmed blastocysts was performed in 1,068/1,159 (92.1 %) and 843/897 (94.0 %) of the ET cycles in the HSA and rHA groups, respectively. Implantation rates and clinical pregnancy rates per ET were significantly higher ($P<0.05$) in the rHA group compared to the HSA group. The percentages of live delivery, stillbirth, and miscarriage per pregnancy were similar in both groups. The percentages of patients lost to follow-up, due to either no response or changed address, were also similar in both groups.

Table 2 shows the perinatal outcomes of children born after vitrified/warmed ET. Fewer males were born in the HSA group than in the rHA group ($P<0.05$). The HSA group had slightly lower mean gestational age and birth weight, and higher rates of preterm delivery and very-low-birth-weight (<1,500 g), compared to the rHA group. However, these differences did not reach statistical significance. The percentages of multiple pregnancies were similar in the groups. Preterm birth complications, including respiratory distress, septicemia, and subependymal hemorrhage, were reported for three singleton babies in the HSA group.

Table 2 Perinatal outcomes of vitrified/warmed embryo transfers

	HSA	rHA	P-value
Live-born	447	374	
M (%)	207 (46.3)	203 (54.3)	.025
F (%)	240 (53.7)	171 (45.7)	.025
Vaginal delivery (%)	261 (60.6)	218 (60.1)	.942
Cesarean section (%)	170 (39.4)	145 (39.9)	.942
Mean gestational age (wk)	38.7±0.1	38.9±0.1	.158
Preterm (<37 wk) (%)	39 (9.0)	22 (6.1)	.141
Mean birth weight (g)	2,988±28	3,046±26	.133
<1,500 g (%)	13 (2.9)	5 (1.3)	.154
1,500–2,499 g (%)	53 (11.9)	41 (11.0)	.742
>2,500 g (%)	381 (85.2)	328 (87.7)	.358
Twins (%)	16 (3.7)	9 (2.5)	.416
Triplets (%)	0	1 (0.3)	-
Preterm birth complications (%)	3 (0.7)	0	-

Values are expressed as mean±SEM. The vitrification/warming solutions contained either HSA (HSA group) or rHA (rHA group)

Table 3 shows the incidence of congenital birth defects in neonates. There was no significant difference between the groups, and no reports of neonatal mortality in either group.

Discussion

Our results indicate that rHA effectively replaces HSA in the base solutions for vitrification/warming of human blastocysts, and yields high rates of pregnancy and live births after ET. To our knowledge, this is the first report of successful deliveries achieved after chemically defined embryo vitrification procedures with rHA.

Table 3 Incidence of birth defects in neonates born after vitrified/warmed embryo transfers

	HSA	rHA
Birth defects	1 (9 trisomy and double outlet right ventricle)	2 (ventricular septal defect)
	1 (patent ductus arteriosus)	1 (multicystic dysplastic kidney [left], hydronephrosis [right], and hypertrophic pyloric stenosis)
	1 (cleft lip and palate)	1 (imperforate anus)
	1 (sebaceous nevus)	1 (polydactyly)
		1 (polysyndactyly)
Total (%)	4 (0.9)	6 (1.6)

The vitrification/warming solutions contained either HSA (HSA group) or rHA (rHA group)

In the present study, human blastocysts retained high viability after vitrification/warming with a low concentration of rHA (2.5 mg/mL), which should facilitate the introduction of chemically defined and cost-effective procedures. Typically, large amounts of HSA are added to the VS/WS, since the high levels of protein may promote vitrification [15, 16]. In these cases, the toxicity of the solution can be reduced by decreasing the amount of intracellular cryoprotectants (CPs) used for vitrification [17]. Albumin, as a CP, is thought to protect cell membranes during the cryopreservation process and reduce the amount of visible ice in the solution [18, 19]. In addition, the presence of albumin in the vitrification solution may protect embryos from physical damage to the zona pellucida [20]. On the other hand, the Cryotop vitrification used in this study is a method developed to increase the cooling/warming rates by decreasing the sample volume [21]. This method can reduce the likelihood of chilling injury and ice crystal formation, and thereby promotes vitrification without the need for high concentrations of CP [22–24]. Rapid cooling minimizes chilling injury, and may help maintain membrane stability [25], while decreasing the vitrification volume can also minimize the probability of glass fracture [26]. Furthermore, combining two permeating CPs in the vitrification solution (e.g., EG and DMSO in this study) enables the use of a lower concentration of the each additive, thereby reducing CP toxicity [27]. Therefore, based on our results, high amounts of HSA are no longer essential, and can be replaced with lower amounts of rHA in VS/WS to achieve successful embryo vitrification.

Likewise, a previous study confirmed that low amounts of PVA were effectively substituted for serum in VS/WS for the ultra-rapid vitrification of porcine embryos [5]. Furthermore, macromolecule-free VS/WS was feasible, and produced results similar to those obtained with VS/WS containing serum or PVA. However, the use of VS/WS containing few macromolecules with surfactant properties can increase risks, such as embryos adhering to plastic surfaces [28]. In our previous study, bovine embryos vitrified/warmed in solutions with a very low concentration of rHA (0.5 mg/mL) tended to adhere tightly to the Cryotop strip surfaces, even though the post-warm hatching rate was high. We found that when vitrified Cryotop strips with embryos were warmed, removal of the embryos was more difficult in VS/WS containing 0.5 mg/mL rHA, compared to VS/WS containing 2.5 mg/mL rHA (unpublished data). Therefore, in the present study, defined solutions with 2.5 mg/mL rHA were used for human blastocyst vitrification to reduce the risk of adherence during the procedure.

In the present study, implantation and clinical pregnancy rates were lower in the HSA group, although the HSA group also had slightly lower gestational age and birth weight. The exact reason(s) for these results is unknown, though the toxic effects of high albumin concentration (e.g., high osmolality,

low pH, biological instability, toxic residues/components, and/or unknown interaction[s] with CPs) may be partly responsible [29]. In contrast, a previous study, examining bovine embryo vitrification with 0.25-mL straws, found that the addition of the macromolecule polyvinylpyrrolidone (PVP) and trehalose to vitrification solutions containing EG had a beneficial effect on post-warming hatching rates [30]. However, the addition of PVP and trehalose to the CP mixture of DMSO and EG did significantly reduce the hatching rates. The authors suggest that supplementation of the CP mixture with PVP and trehalose may have altered CP permeation before and after vitrification, resulting in irreversible damage to the cells during warming. Therefore, although their vitrification procedure differed greatly from that in the present study, it is noteworthy that when formulating VS, the addition of specific macromolecules or sugars to specific CP mixtures can alter the permeation characteristics of the CPs [31].

Blastocyst culture, as performed in this study, may favor the selection of male embryos for transfer, since they are thought to have greater preimplantation developmental rates [32]. Accordingly, blastocyst transfer has been associated with a sex ratio skewed in favor of male offspring [33]. Our results also revealed a slightly higher male:female ratio in the rHA group, though the ratio was lower in the HSA group. The reason for this difference remains unclear. However, in the HSA group, blastocysts that remained expanded before vitrification were artificially shrunk as described above. The shrunk, vitrified blastocysts were preferred for warming and ET, whereas fully expanded blastocysts did not easily shrink using this method. Therefore, it can be speculated that these expanded blastocysts were more likely to be male, and were less likely to be selected for ET. As the method was not used in our rHA group, further studies are warranted to evaluate whether the artificial shrinkage before blastocyst vitrification could be a cause of the gender bias observed in this study.

Results from this study, along with those from previous studies, indicate that Cryotop vitrification of human embryos is satisfactory and relevant to clinical settings [2–4, 21]. However, the major drawback of this procedure is the direct exposure of the biological samples to LN_2, which carries a potential risk of cross-contamination. Therefore, we are currently refining a technique for using the defined rHA vitrification/warming procedure in a closed loading system that can achieve adequate cooling/warming rates [34]. Studies involving the use of the defined vitrification procedure for human oocytes and early cleavage stage embryos are also being performed. We hope these efforts will ultimately lead to the establishment of efficient and hygienic embryo vitrification systems.

In conclusion, the results of this study clearly demonstrated the efficacy of the chemically defined vitrification procedure with rHA for human blastocysts, given the rates of embryo viability and successful pregnancies after ET. Although long-term follow-up studies with more participants are required to validate the safety of the procedure, this new approach will aid in the development of standardized and defined ART systems. By using rHA in place of HSA, these systems may one day eliminate the variation and risks associated with the use of blood-derived products.

Acknowledgments The authors thank Dr. David K. Gardner (Department of Zoology, The University of Melbourne) for his critical review of the manuscript.

References

1. Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. Fertil Steril. 2005;84(1):88–92.
2. Shi W, Xue X, Zhang S, Zhao W, Liu S, Zhou H, et al. Perinatal and neonatal outcomes of 494 babies delivered from 972 vitrified embryo transfers. Fertil Steril. 2012;97(6):1338–42.
3. Liu SY, Teng B, Fu J, Li X, Zheng Y, Sun XX. Obstetric and neonatal outcomes after transfer of vitrified early cleavage embryos. Hum Reprod. 2013;28(8):2093–100.
4. Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. Fertil Steril. 2014;101(5):1294–301.
5. Sanchez-Osorio J, Cuello C, Gil MA, Parrilla I, Maside C, Alminana C, et al. Vitrification and warming of in vivo-derived porcine embryos in a chemically defined medium. Theriogenology. 2010;73(3):300–8.
6. Gardner DK, Lane M. Recombinant human serum albumin and hyaluronan can replace blood-derived albumin in embryo culture media. Fertil Steril. 2000;74 Suppl 1:S31–S2.
7. Bungum M, Humaidan P, Bungum L. Recombinant human albumin as protein source in culture media used for IVF: a prospective randomized study. Reprod Biomed Online. 2002;4(3):233–6.
8. Bavister BD, Kinsey DL, Lane M, Gardner DK. Recombinant human albumin supports hamster in-vitro fertilization. Hum Reprod. 2003;18(1):113–6.
9. Lane M, Maybach JM, Hooper K, Hasler JF, Gardner DK. Cryosurvival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. Mol Reprod Dev. 2003;64(1):70–8.
10. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Changing the start temperature and cooling rate in a slow-freezing protocol increases human blastocyst viability. Fertil Steril. 2003;79(2):407–10.
11. Murakami M, Egashira A, Araki Y, Kuramoto T. Recombinant human albumin can replace blood-derived albumin for the vitrification and warming of in vitro-produced bovine blastocysts. Hum Reprod. 2011;26(1):i45–i6.
12. Murakami M, Egashira A, Nagafuchi E, Tanaka K, Mine C, Araki Y, et al. Use of a chemically defined vitrification procedure with recombinant human albumin for human blastocysts. Hum Reprod. 2012;27 Suppl 1:i71–i2.
13. Murakami M, Egashira A, Murakami K, Araki Y, Kuramoto T. Perinatal outcome of twice-frozen-thawed embryo transfers: a clinical follow-up study. Fertil Steril. 2011;95(8):2648–50.
14. Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. Hum Reprod. 2004;19(12):2884–8.

15. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology. 1984;21(4):407–26.
16. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196° C by vitrification. Nature. 1985;313(6003):573–5.
17. Kasai M, Mukaida T. Cryopreservation of animal and human embryos by vitrification. Reprod Biomed Online. 2004;9(2):164–70.
18. Rall WF. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology. 1987;24(5):387–402.
19. Leibo SP, Oda K. High survival of mouse zygotes and embryos cooled rapidly or slowly in ethylene glycol plus polyvinylpyrrolidone. Cryo-Letters. 1993;14:133–44.
20. Titterington JL, Robinson J, Killick SR, Hay DM. Synthetic and biological macromolecules: protection of mouse embryos during cryopreservation by vitrification. Hum Reprod. 1995;10(3):649–53.
21. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online. 2005;11(3):300–8.
22. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod. 1996;54(5):1059–69.
23. Vajta G, Holm P, Greve T, Callesen H. Vitrification of porcine embryos using the open pulled straw (OPS) method. Acta Vet Scand. 1997;38(4):349–52.
24. Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. Nat Biotechnol. 1999;17(12):1234–6.
25. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. Theriogenology. 2007;67(1):81–9.
26. Arav A, Yavin S, Zeron Y, Natan D, Dekel I, Gacitua H. New trends in gamete's cryopreservation. Mol Cell Endocrinol. 2002;187(1–2):77–81.
27. Ishimori H, Takahashi Y, Kanagawa H. Factors affecting survival of mouse blastocysts vitrified by a mixture of ethylene glycol and dimethyl sulfoxide. Theriogenology. 1992;38(6):1175–85.
28. Blake D, Svalander P, Jin M, Silversand C, Hamberger L. Protein supplementation of human IVF culture media. J Assist Reprod Genet. 2002;19(3):137–43.
29. Checura CM, Seidel Jr GE. Effect of macromolecules in solutions for vitrification of mature bovine oocytes. Theriogenology. 2007;67(5):919–30.
30. Pugh PA, Tervit HR, Niemann H. Effects of vitrification medium composition on the survival of bovine in vitro produced embryos, following in straw-dilution, in vitro and in vivo following transfer. Anim Reprod Sci. 2000;58(1–2):9–22.
31. Gutierrez A, Garde J, Artiga CG, Munoz I, Pintado B. In vitro survival of murine morulae after quick freezing in the presence of chemically defined macromolecules and different cryoprotectants. Theriogenology. 1993;39(5):1111–20.
32. Maalouf WE, Mincheva MN, Campbell BK, Hardy IC. Effects of assisted reproductive technologies on human sex ratio at birth. Fertil Steril. 2014;101(5):1321–5.
33. Chang HJ, Lee JR, Jee BC, Suh CS, Kim SH. Impact of blastocyst transfer on offspring sex ratio and the monozygotic twinning rate: a systematic review and meta-analysis. Fertil Steril. 2009;91(6):2381–90.
34. Larman MG, Gardner DK. Vitrification of mouse embryos with super-cooled air. Fertil Steril. 2011;95(4):1462–6.