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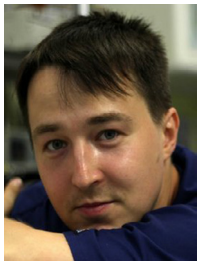
ARTICLE

Mechanical zona pellucida removal of vitrified-warmed human blastocysts does not affect the clinical outcome



BIOGRAPHY

In 2007, Konstantine Kirienko defended his PhD thesis entitled 'Development of cloned cattle and mice embryos *in vitro*, depending on conditions of their reconstruction and cultivation'. Since 2011, he has been working in Altravita IVF Clinic, Moscow, as Senior Embryologist. His research interests include mammalian cloning, nuclear transfer and transgenic research.

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KEY MESSAGE

Complete mechanical removal of the zona pellucida of vitrified–warmed human blastocysts did not modify clinical pregnancy, implantation and ongoing pregnancy rates.

ABSTRACT

Research Question: Does complete mechanical removal of the zona pellucida modify the outcome of transfer of vitrified–warmed human blastocysts?

Design: In a prospective randomized controlled study, 419 couples were allocated to either zona pellucida-free (n = 209) or zona intact (n = 210) vitrified–warmed embryo transfer. Main outcome measures included clinical pregnancy, implantation and ongoing pregnancy rates.

Results: Transfer of zona pellucida-free blastocysts resulted in clinical pregnancy, implantation and ongoing pregnancy rates (35,9%, 33,9% and 32,1%, respectively), similar to those achieved with zona intact control embryos (39%, 36,4% and 33,1%, respectively).

Conclusion: Total mechanical removal of the zona pellucida did not affect the tested parameters of clinical outcomes.

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KEYWORDS

Assisted hatching
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Human
Total zona pellucida removal
Vitrification

INTRODUCTION

Blastocyst hatching is an essential step for successful implantation and establishment of early pregnancy. Zona pellucida hardening has been considered a possible unfavourable consequence of in-vitro embryo culture and cryopreservation that could encumber hatching of blastocysts (*Larman et al., 2006; Fujiwara et al., 2010; Vajta et al., 2010*). Assisted hatching of cryopreserved embryos has been reported to improve clinical outcomes (*Li et al., 2016*). Previous studies reported that assisted hatching by zona pellucida thinning, or partial and complete zona pellucida removal, improved the clinical outcome of frozen–thawed (*Wong et al., 2003; Sifer et al., 2006; Valojerdi et al., 2008*) and vitrified–warmed (*Kinget et al., 2002; Hiraoka et al., 2007; Martins et al., 2011*) blastocyst transfer. It is possible, however, that blastocysts will not hatch even after zona pellucida thinning or partial zona pellucida removal. The ability of the trophectoderm to breach the inner zona pellucida and herniate through small gaps requires an energy-consuming effort, which may be incomplete and fail (*Vajta et al., 2010*). A preliminary study concluded that complete versus partial zona pellucida removal of vitrified–warmed blastocysts is associated with higher pregnancy, implantation and delivery rates (*Hiraoka et al., 2007*). Yet, a more recent report found impaired pregnancy rates after transfer of completely hatched euploid blastocysts compared with their expanded or hatching counterparts (*James et al., 2018*). The aim of our study was to investigate whether total mechanical removal of the zona pellucida will affect the outcome of vitrified blastocyst transfer.

MATERIALS AND METHODS

The study was conducted according to the approval of the Ethical Committee of Altravita IVF Clinic, Moscow, Russia on 21 November 2017. All involved patients signed informed consent.

In this prospective study, 463 high-quality blastocysts (≥3BB according to the grading scale proposed by *Gardner et al. 2000*; quality was accessed before vitrification) with intact zona pellucida from 419 patients who underwent autologous or donor/recipient vitrified embryo transfer cycles (maternal age 23–44 years; average 34.0 years;) were used. A random table generated by a computer programme (PyPI, randomization 1.0.0, Warehouse project, USA, 2016) was used to determine patients in the zona-free group with either zona intact group.

On day 5 or 6 after intracytoplasmic sperm injection or intracytoplasmic morphologically selected sperm injection, vitrification was carried out using Cryotech method (Vitrification Kit 101; Cryotech, Tokyo, Japan). Cryotech method (Warming Kit 102; Cryotech, Tokyo, Japan) was used for warming of the blastocysts on the day of the scheduled embryo transfer. In the zona-free group, mechanical zona removal was carried out while the blastocysts remained collapsed after warming, i.e. within 5–15 min of completing the warming procedure. Blastocysts were placed under mineral oil in 30-µl microdroplets of HTF w/HEPES (LifeGlobal, Guilford, USA) containing 5 mg/ml protein supplement (LifeGlobal) in a 60-mm culture dish. Partial zona pellucida opening (70–90% of circumference) was carried out mechanically by Partial Zona Dissection Pipette (Cook Medical, Brisbane, Australia) under an inverted phase-contrast microscope equipped with a heated stage at 37°C. After this procedure, the blastocyst was gently denuded of the zona pellucida by pipetting several times (FIGURE 1). The whole procedure was completed in less than 3 min.

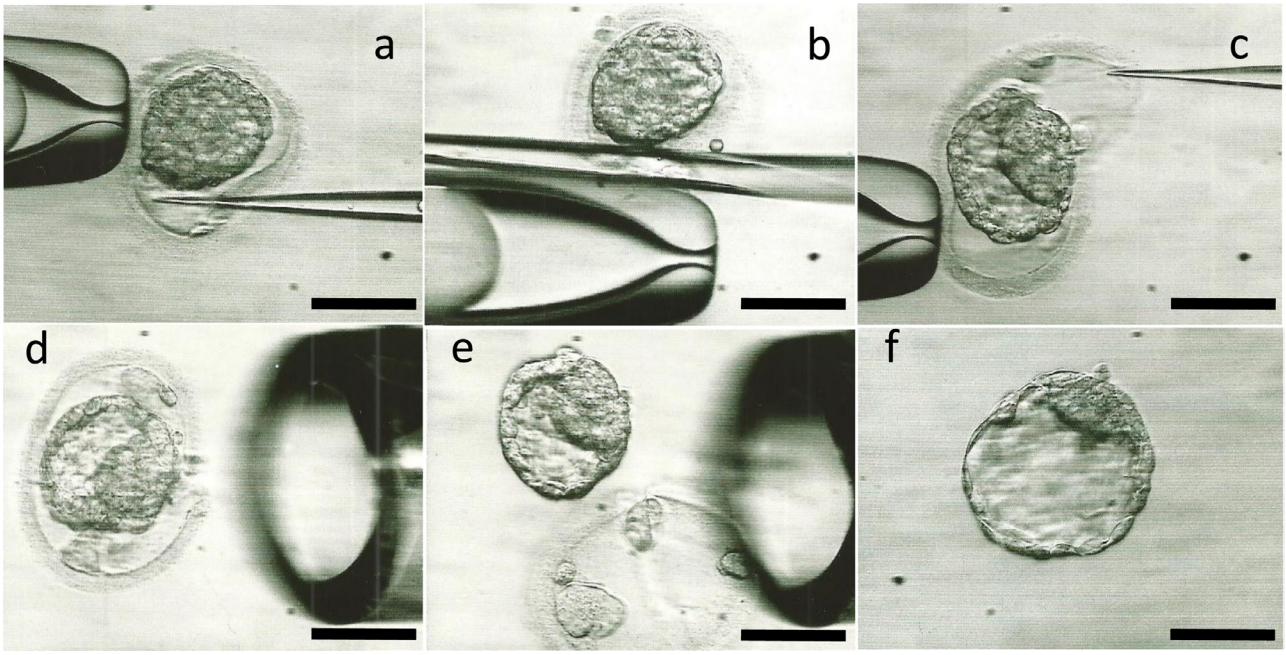

FIGURE 1 Total zona pellucida removal of vitrified–warmed blastocysts. (A–C) Mechanical partial removal of zona pellucida from blastocyst by partial zona dissection pipette; (D and E) complete zona pellucida removal by gentle pipetting; (F) blastocyst with zona pellucida completely removed. Scale bars represent 100 µm.

Subsequently, blastocysts were rinsed and cultured in HTF medium with 15 mg/ml protein supplement (LifeGlobal, USA) until transfer. Zona intact group blastocysts were cultured in HTF medium with 15 mg/ml protein supplement (LifeGlobal) after warming. Post-warming survival, i.e. re-expansion of blastocysts, was evaluated 1–3 h after warming.

In the natural frozen embryo transfer (FET) cycles, the development of the dominant follicle and endometrium were monitored from the 10th day of menstrual cycle by regular vaginal ultrasound. Once the leading follicle reached a mean diameter of 18 mm serum LH, oestradiol and progesterone were measured. If no LH surge was detected, 5000 IU HCG (Pregnyl®, Organon, Oss, Netherlands) was administered. Luteal support with 100 mg vaginal progesterone was given 12 h after LH surge or HCG injection. Supplementation with 200 mg, 300 mg and 600 mg progesterone were used, respectively, in the following 3 days.

In the hormone replacement treatment FET cycles, women received oestradiol valerate (4–6 mg daily from cycle day 1). Transvaginal ultrasound scan was conducted to assess endometrial thickness and ovulation from day 10–11 and oestradiol dosage was adjusted based on the endometrial thickness. When the endometrial thickness was 8 mm or wider, vaginal progesterone administration was started (400 mg for the first 2 days, then 600 mg daily from the third day).

Embryo transfer was scheduled after 4 or 5 days of progesterone administration in natural or hormone replacement cycles, respectively, regardless of the age of blastocysts at vitrification. Progesterone was continued until the 10th week of gestation if pregnancy occurred. The time from warming to transfer ranged from 1–3 h. Embryo number was one or two per transfer.

Biochemical pregnancy was defined by 10 IU/ml serum beta–HCG levels or more on day 10 and a fourfold or greater increase on day 14 after transfer, whereas clinical pregnancy was confirmed by the presence of a gestational sac (crown-rump length = 2–4 mm) and a fetal heartbeat 5 weeks after transfer. Implantation rate was calculated by dividing the number of gestational sacs observed by ultrasound 3 weeks after transfer by the total number of blastocysts transferred. The ongoing pregnancy rate was defined as the number of clinical pregnancies that progressed beyond 12 weeks divided by the number of embryo transfers. Spontaneous abortion rate was defined as the number of pregnancies lost before 20 weeks of gestation divided by the number of clinical pregnancies. Continuous data are expressed as mean ± SD. Student's t-test and chi-squared test were used for statistical analyses; $P < 0.05$ was considered statistically significant.

RESULTS

Both groups had similar maternal age, blastocyst rate (calculated by the number of high-quality blastocysts divided by the number of two-pronuclei stages), blastocyst morphology before cryopreservation and mean number of blastocysts per transfer (TABLE 1). The re-expansion rate of the zona-free group (98.1%) was comparable with that of the intact group (96.3%), suggesting that blastocyst viability was not affected by zona removal procedure. Clinical outcomes, including implantation rates, biochemical, clinical and ongoing pregnancies were not statistically different between the zona-free (33.9%, 43.5%, 35.9% and 32.1%) and zona-intact groups (36.4%, 47.6%, 39% and 33.1%) (FIGURE 2). No statistical difference was observed between zona-free and zona-intact groups concerning multiple pregnancy rates per clinical pregnancy (8.0% versus 3.6%), ectopic pregnancy rates per clinical pregnancy (1.3% versus 1.2%) and spontaneous abortion rates (9.3% versus 12.8%).

TABLE 1 CHARACTERISTICS OF EXPERIMENTAL GROUPS

Parameter	*Zona pellucida removal group*	*Control group*
Mean age of women (years) ± SD	34.2 ± 5.6	34.0 ± 5.2
Patients, *n*	209	210
Autologous cycles, *n*	168	174
Donor cycles, *n*	41	36
Embryo transfers, *n*	209	210
Natural cycles, *n*	112	119
Hormone replacement cycles, *n*	97	91
Blastocyst rate (%)	41.0	41.2
Blastocysts/two pronuclei, n	879/2143	893/2168
Transferred warmed blastocysts, *n*	230	233
Blastocysts according to quality[a], *n*		
2AA/2AB/2BA/2BB, *n*	2/2/0/4	0/3/0/10
3AA/3AB/3BA/3BB, *n*	16/13/13/53	9/11/15/57
4AA/4AB/4BA/4BB, *n*	19/21/11/76	20/10/21/77
Mean number of embryos per transfer ± SD	1.1 ± 0.3	1.1 ± 0.3

Blastocyst rate was calculated by the number of high-quality blastocysts divided by the number of 2PN stages.

[a] Quality according to Gardner *et al*., 2000.

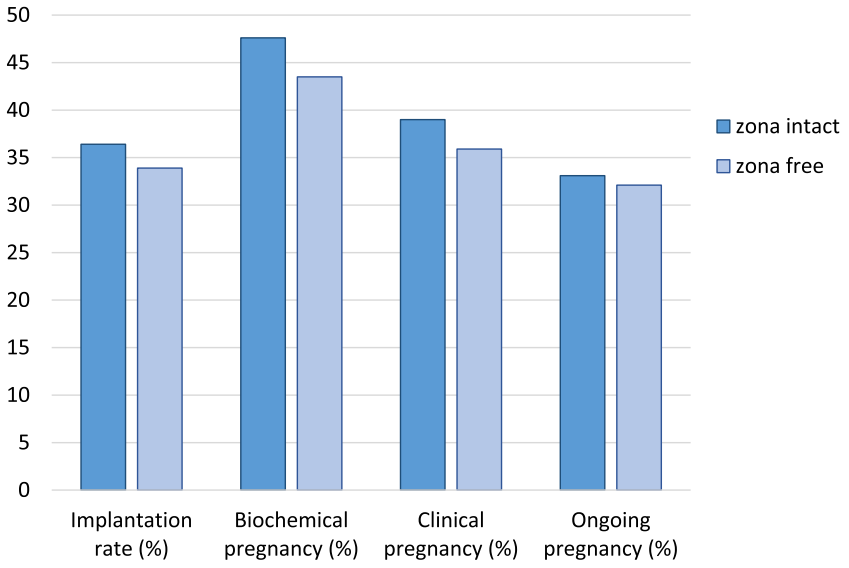

FIGURE 2 Results of transfers of zona-free and zona-intact blastocysts.
Biochemical pregnancy was defined by more than 10 IU/ml serum beta–HCG levels on day 10 and a more than fourfold increase on day 14 after transfer; clinical pregnancies were defined by ultrasonography confirming the presence of a gestational sac (crown–rump length = 2–4 mm) and a fetal heartbeat 5 weeks after transfer; implantation rate was defined as the number of gestational sacs detected by means of ultrasound 3 weeks after transfer divided by the number of embryos transferred; ongoing pregnancy rate was defined as the number of clinical pregnancies that progressed beyond 12 weeks divided by the number of embryo transfers. Student's t-test and chi-squared tests were used for statistical analyses; $P < 0.05$ was considered to be significant. No significant differences between corresponding rows of the two groups were observed ($P > 0.05$).

DISCUSSION

To the best of our knowledge, the present study is the first controlled randomized trial comparing transfer results after total mechanical zona pellucida removal with zona-intact blastocysts transfer. The potential of physical damage to the cytoplasm when the zona pellucida is completely removed by pipetting is a concern. We first determined that the blastocyst re-expansion rate after complete zona pellucida removal using hatching pipette and mechanical pipetting was comparable to that without zona removal. Previous studies have shown that the absence of the zona pellucida does not affect fertilization, pre-implantation development or subsequent full-term pregnancy (*Vajta et al., 2010; Ueno et al., 2014a*). We demonstrated no statistical difference between zona-free and zona-intact groups concerning multiple pregnancies, ectopic pregnancies, spontaneous abortions and clinical outcomes, indicating that mechanical zona removal and handling of completely zona-pellucida-removed blastocysts does not damage the blastocyst and does not seem to adversely affect the pregnancy outcomes after transfer.

A recent report showed that complete zona pellucida removal promoted blastocyst adhesion and outgrowth in fibronectin-coated dishes in the blastocyst outgrowth assay (*Ueno et al., 2016*). The blastocyst outgrowth assay mimics a part of the implantation process and is believed to reflect the potential for embryo differentiation and subsequent attachment and invasion into the endometrium (*Sherman, 1975; Glass et al., 1979; Wang and Armant, 2002*). Blastocyst outgrowth assays showed that the adhesion rate was improved, the time required for adhesion was shorter and the outgrowth area was larger in the complete-removal group than that in the partly-removal group. These results indicated that vitrified–warmed blastocysts with complete zona pellucida removal would have superior implantation potential compared with vitrified–warmed blastocysts with partial zona pellucida removal. It is possible, however, that results may vary between in-vivo and in-vitro conditions. The limitation of the study was the lack of clinical studies to explore the clinical efficacy of the complete zona pellucida removal as an assisted hatching method.

Hiraoka et al. (2007) showed that assisted hatching with total removal of the zona pellucida using a laser and mechanical pipetting significantly improves the implantation, pregnancy and delivery rates of vitrified blastocysts as compared with the partial opening of the zona pellucida using acid Tyrode's solution.

Both of these studies compared total zona removal with partial zona opening. It was observed *in vitro* that partial opening of the zona pellucida, in some cases, can also impede the hatching of vitrified human blastocysts, making some blastocysts unable to hatch and retain their integrity. The fact that partial opening may potentially be associated with entrapment of the blastocyst could explain why total zona removal considerably improved the clinical outcomes compared with partial zona removal.

Recently, it has been reported that the efficacy of spontaneous hatching of human blastocysts is not determined by the quality of the zona pellucida and gametes, but by the quality of the blastocysts themselves (*Syrkasheva et al., 2017*). Probably, the blastocyst can model its further development through its own genetic factors. Expression of *CTSV*, *GATA3* and *CGB* genes is lower in low-quality blastocysts and does not allow them to commit spontaneous hatching and to implant into the endometrium (*Syrkasheva et al., 2017*). From an evolutionary point of view, this factor may be a mechanism that prevents

the implantation of a defective embryo with retarded development or other developmental disorders. Therefore, hatching failure may be the indicator of developmental disorders, aneuploidy or low viability of the blastocyst and its inability to implant even after total zona pellucida removal.

In conclusion, the results of our observations suggest that total mechanical zona pellucida removal of vitrified-warmed human blastocysts had no significant detrimental or beneficial effect on the outcomes tested herein.

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