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Original Research

Effect of Shorter Pretreatment Time on Clinical and Neonatal Outcomes in Human Blastocysts Vitrification after Artificial Shrinkage

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Abstract

Background: Prolonged pretreatment time may be harmful to frozen embryo's developmental potential. This study was conducted to evaluate the effect of different equilibration times on the clinical and neonatal outcomes of frozen-warmed blastocyst transfer. **Methods**: This is a retrospective study based on data collected from our medical records from March 2018 to March 2022 and including a total of 763 expanded blastocysts from 538 warming blastocyst cycles. These cycles were divided into two groups according to the equilibration time: (A) 6–7 minutes, and (B) 9–10 minutes. The survival rate, clinical, and neonatal outcomes were investigated. **Results**: The survival, implantation, and clinical pregnancy rates of vitrified-warmed shrinkage blastocyst were not different between the two groups. Other variables analyzed including live birth, multiple gestation, and neonatal outcomes were similar between the two groups. **Conclusions**: The results of this study illustrated that vitrification of artificially collapsed blastocysts with a shorter equilibration time (6–7 minutes) and pre-vitrification is able to lead to similar clinical and neonatal outcomes in patients undergoing assisted reproductive technology (ART).

Keywords: blastocyst vitrification; artificial collapse; exposure to equilibration solution; pregnancy and implantation rates; spontaneous abortion; neonatal outcomes

1. Introduction

Since the birth of Louise Joy Brown in 1978, assisted reproductive technology (ART) has been constantly increasing, and have permitted millions of infertile couples to conceive. Currently, more than 3.5 million cycles are annually performed, with over 500,000 deliveries worldwide. It has been estimated that about 9 million children have been conceived globally following ART [1]. The advancements in ART have been achieved thanks to several improvements, including ovarian stimulation procedures, fertilization, embryo transfer methods, and importantly to embryo cryopreservation. Vitrification has become a highly important step of ART for a variety of reasons: to store supernumerary embryos for future use, for preimplantation genetic testing, or freeze-all cycle, once the patient is exposed at high risk to develop ovarian hyperstimulation syndrome (OHSS) and the fresh transfer cannot be performed [2,3]. Cryopreservation of oocytes and embryos by "slow freezing" was first applied in the 1980s [4], which was subsequently replaced by the "vitrification" procedures [5]. This practice has been considered a real breakthrough in ART, especially with oocyte cryopreservation, allowing embryologists to obtain a higher survival rate at the warming, fertilization rates, and embryonic development compared to the slow freezing procedure [6,7]. Practically, cryopreservation enables long-term preservation of cells (gametes/embryos) at ultra-low temperatures in a state of suspended animation. To obtain that state, it is fundamental to avoid ice crystals formation, which will damage irreversibly the cell and induce death. This can be achieved through vitrification, using a high concentration of cryoprotective agents (CPAs) to increase viscosity, inhibit the growth and formation of ice crystals, and finally induce the solution to enter a "glassy state" [8]. The ability to cryopreserve human embryos has also improved significantly in the last decade [6,9,10]. There is some sufficient evidence showing results from vitrification are superior to those achieved with the slow freezing protocols [11–16]. However, the methods utilized to cryopreserve human embryos still have some weak points that might be improved. Several approaches have been applied and tried to optimize the vitrification procedure. These procedures employed are not always designed to specifically take account of the osmotic tolerance response of the cells according to the temperature, time, and exposure to CPAs [17]. Thus, a critical aspect is represented by the high concentration of CPAs. One of the most used for cryopreserved gametes and embryos is dimethyl sulfoxide (DMSO), an amphipathic chemical compound. Exposure to this molecule might cause unexpected changes in cell fates, probably af-

fecting epigenetic regulation, especially when used at high concentrations [18,19]. Further, CPA might impact negatively cellular metabolism and function, enzyme activities, cell growth, and apoptosis [20], and might be correlated with increased levels of reactive oxygen species (ROS) and apoptotic events [21]. Several publications reported that human embryos should be kept in the vitrification solution for a maximum of 1 minute [12–14], while the equilibration times normally ranged from 5 to 15 minutes [15–18]. The exposure time of embryos to CPAs represents an important concern for the success of vitrification. Longer exposure to equilibration solutions may be detrimental to further embryo development, while a briefer time may not be enough for the penetration of CPAs into the cells. Contrasting results have been found in the literature, with studies reporting a fixed equilibration time of 5 minutes and others increasing the exposure time up to 15 minutes [22–28]. These variations imply that an agreement is missing on the equilibration time for human embryos vitrification and, therefore, the need to improve our vitrification protocol. We retrospectively analyzed our results to investigate the effects of different equilibration times on embryo survival, clinical, and neonatal outcomes.

2. Materials and Methods

2.1 Experimental Data

This was a retrospective cohort study performed at Center for Reproductive Medicine of Mary Hospital, from March 2018 to March 2022, and included 538 frozen embryo replacements with women aged <35 years. Women who had endometrial thickness <7 mm or uterine anomaly were excluded. Patients whose partner had severe male factors and infected by COVID-19 were also not included. Following the fresh embryo transfer, supernumerary good quality blastocysts ≥ 2, according to Gardner's score [29], excluding those with a grade of CC, BC, or CB, were frozen on day 5 after fertilization. The study contained a total of 758 expanded blastocysts, which were divided into two groups according to the equilibration time: group (A) 6–7 minutes (n: 361); and group (B) 9–10 minutes (n: 402). The primary outcome was live birth and abortion rates. The secondary outcomes included survival rate at warming, clinical pregnancy, and implantation rates. The Ethics Committee of Haikou Mary Hospital approved this study (Reference no. 2021-10-02).

2.2 Ovarian Stimulation

Ovarian stimulation and blastocyst preparations were achieved using the protocol as described in our previously reported method [11–13]. Patients were treated with either a long gonadotropin-releasing hormone (GnRH) agonist (triptorelin, Diphereline, Ipsen, France) or a GnRH antagonist (cetrorelix, Cetrotide, Merck Serono, Switzerland) protocol. Ovulation trigger was achieved with recombinant human chorionic gonadotropin (rhCG, Ovidrel, MerckSerono) as soon as 50% of the follicles of >10 mm reached a diameter of ≥ 18 mm. After 36 h, cumulus–oocyte complexes (COC) were retrieved transvaginally under ultrasound guidance using a 17-gauge single lumen needle (K-OPS-7035-RWH-ET, Cook, Queensland, Australia) under general anaesthesia [16].

2.3 Oocyte Retrieval and Embryo Development

COC were isolated from follicular fluid, rinsed, and transferred to 0.6 mL of Universal *in vitro* fertilization (IVF) Medium (CooperSurgical Fertility Solutions, Malov, Denmark), covered with oil for culture tissue (CooperSurgical Fertility Solutions, Malov, Denmark) in four-well dishes (Nunc™, ThermoFisher Scientific, Mexico city, Mexico), and returned to the incubator (Astec Co., Ltd, Fukuoka, Japan) equilibrated at 37 °C, 6% CO_2, 5% O_2 and 89% N_2. All media used were covered with 0.35 mL of oil and incubated overnight. Sperm used for either *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) was collected by masturbation [30]. All oocytes were inseminated by conventional *in vitro* fertilization or intracytoplasmic sperm injection and cultured in IVF Medium as illustrated by Zhang and colleagues [31]. Fertilization was assessed 17–19 h after insemination for the appearance of two distinct pronuclei and two polar bodies. At this stage, normally fertilized oocytes were cultured individually in a 25 µL preequilibrated droplet of Quinn's Advantage Cleavage medium (CooperSurgical Fertility Solutions, Malov, Denmark) under oil in a standard incubator (Astec Co., Ltd, Fukuoka, Japan) equilibrated at 37 °C (6% CO_2, 5% O_2, 89% N_2). On the morning of day 3, 66–68 hours post-insemination, embryos were moved from cleavage medium to a 25 µL droplet of blastocyst medium (CooperSurgical Fertility Solutions, Malov, Denmark) and were cultured in groups of two or three embryos.

The morphologic features of the blastocysts were assessed on day 5 according to Gardner's score [29]. The best quality blastocyst was replaced in embryo transfer medium; any remaining good quality blastocysts were cryopreserved. Some patients had no fresh embryo replacement, and all the blastocysts were vitrified for future use. Most of the blastocysts were cryopreserved on day 5 (93.1%), the remaining on day 6.

2.4 Artificial Shrinkage (AS) of Blastocyst

Hatching and expanded blastocysts (grade 3 or more) were artificially shrunk by applying one or two laser pulses (Hamilton Thorn Bioscience Inc, Beverly, MA, USA) before vitrification. The blastocyst was positioned to provide a safe distance between the inner cell mass (ICM) and the focus of the laser beam before being exposed to a minimum setting (200 ms) laser pulse to produce a small hole at the junction of two trophectoderm cells, resulting in the discharge of fluid from the blastocoel cavity (Fig. 1A). Normally, a blastocyst shrinkage occurred within 1 or 2 min-

utes. Rarely a second laser pulse was applied, and for some blastocysts responding slowly, it took up to 5–8 minutes to observe the shrinkage and disappearance of the blastocoel (Fig. 1B). Subsequently, the embryo was rapidly vitrified.

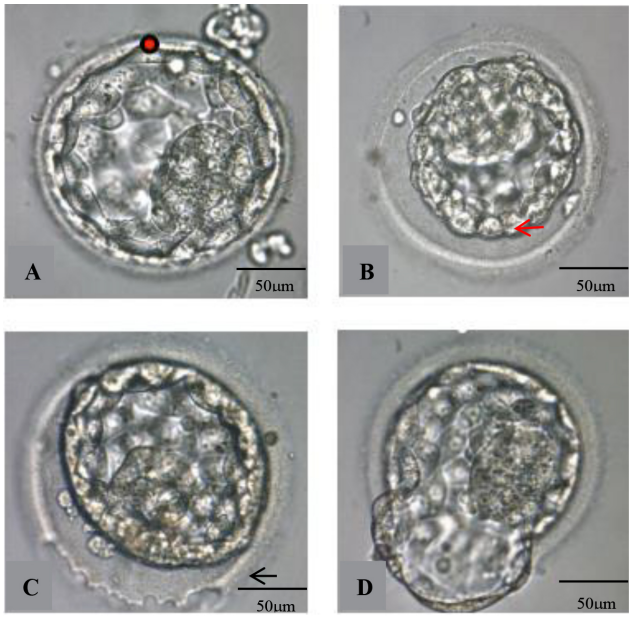

Fig. 1. Photographs of AS and laser-assisted hatching of blastocysts. (A) Laser drilling at the cellular junction of the trophectoderm (red arrow) before vitrification (red dot). (B) Blastocyst after AS. (C) A continuous laser beam was emitted tracing the zona pellucida (ZP) (black arrow) to drill a hole in about one fourth of the ZP surface. (D) Blastocyst partly hatched from ZP after about two hours' culture. AS, artificial shrinkage; ZP, zona pellucida. Magnification is 400×.

2.5 Blastocyst Vitrification

The Cryotop® method (Kitazato Cryotop®, Kitazato Corporation, Shizuoka, Japan) initially described by Kuwayama and colleagues [5,32,33] was applied for blastocyst vitrification. The procedure comprised two different steps: equilibration and vitrification, which are both carried out at room temperature (22–25 °C). Following the shrinkage, the blastocyst was transferred into the equilibration solution (ES) for 6–7 minutes (group A) or 9–10 minutes (group B). In the second phase, the embryo was exposed to a vitrification solution (VS) containing dimethyl sulfoxide (DMSO) in combination with ethylene glycol, trehalose, which functions as an osmotic agent, gentamicin, and hydroxypropyl cellulose for 45–60 seconds. Blastocysts were immediately placed on the Cryotop® device, using a narrow and sterile micropipette (Kitazato Biopharma Co. Ltd., Fuji, Shizuoka, Japan), with the smallest possible amount of VS, and were quickly immersed into liquid nitrogen (LN2). A single blastocyst was always vitrified on each Cryotop® device.

2.6 Blastocyst Warming and Laser-Assisted Hatching

The Cryotop® Thawing Media Kit (Kitazato Cryotop®, Kitazato Corporation, Shizuoka, Japan) was used for warming. In a Nunc 35 × 10 mm culture dish (Nunc™, ThermoFisher Scientific, Mexico city, Mecico), 1 ml of Thawing Solution (TS) was heated at 37 °C for 30–60 minutes, and then positioned on the stage warmer. The Cryotop® device containing the embryo was removed from the LN2 and placed as quickly as possible into the dish containing the TS preheated. The blastocysts instantly fell from the device and could be easily detected in the media under the microscope. After 1 minute, blastocysts were transferred to the Diluent Solution (DS) for 3 min at room temperature 22–25 °C. The last step was for 5 minutes, twice, in the Washing Solution (WS), after which the embryo returned to culture in blastocyst medium (CooperSurgical Fertility Solutions, Malov, Denmark) equilibrated at 37 °C, 6% CO_2, 5% O_2 and 89% N_2. At final step of warming, laser-assisted hatching (Hamilton Thorn Bioscience Inc, Beverly, MA, USA) was completed. Approximately a quarter of the zona pellucida (ZP) was removed, using up to six laser pulses at a minimum setting (200 ms). The laser pulses were orientated on the ZP where the largest perivitelline space was seen, distant from the cells to avoid any damage (Fig. 1C). However, the size of the laser hole would never exceed the thickness of the ZP, which is usually 5–10 um in diameter.

After two hours of culture, the embryo was reassessed and often the re-expansion of the blastocoel was observed (Fig. 1D). Embryo transfer was normally performed within 2 to 3 hours. Patients received one to a maximum of two blastocysts, based on quality. In case the embryo did not survive, another available embryo was warmed; otherwise, the transfer was cancelled. All frozen-warmed embryo cycles were transferred in day 5 to the endometrium.

2.7 Programmed Warmed Embryo Transfer

Common modalities for blastocyst transfer were natural cycles or hormonal replacement cycles for endometrial preparation. In women with regular menstrual cycles, warmed blastocysts were transferred into the uterus during natural cycles that were monitored with ultrasound and in which ovulation was confirmed based on urine luteinizing hormone (LH) tests (Tianjin Recare Co., Ltd., Tianjin, China). For artificial preparation of endometrium, the administration of progesterone (50 mg in oil, daily) (cetrorelix, Cetrotide, Merck Serono, Switzerland) was initiated when endometrial thickness exceeded 8 mm. On day 5 after the initiation of progesterone treatment, the blastocysts were warmed and the surviving blastocysts were transferred into the patient's uterus using embryo transfer catheters (Cook Incorporated, Bloomington, IN, USA) under ultrasound guidance (Kaixin, Xuzhou, Jiangsu, China) [31,34]. For luteal supplementation, progesterone pessary was utilized, which was continued daily for at least 2 weeks after embryo transfer. β-human chorionic gonadotropin (β-

hCG) serum was measured 14 days after embryo replacement. Clinical pregnancy was determined by a fetal heartbeat on ultrasound screening after 35 days [31].

2.8 Clinical Outcome Definitions

Clinical outcomes in this study included: implantation rate (IR), clinical pregnancy rate (CPR), live birth rate (LBR), miscarriage rate, and multiple pregnancy rate (MPR). IR was confirmed when a gestational sac was visualized via an ultrasound examination. CPR was defined as the detection of a foetal heartbeat. LBR was calculated by dividing the number of live birth deliveries by the number of transfers performed. The loss of a foetus with a gestational age of $<$12 weeks was considered a miscarriage. The evaluated neonatal outcomes were as follows: sex; gestational age; birth weight; preterm birth, defined as a baby born before 37 weeks of gestation; low birth weight, defined as birth weight less than 2500 g at any gestational week; macrosomia, defined as birth weight greater than 4000 g at any gestational week; the delivery method and presence of malformations.

2.9 Statistical Analysis

Data were presented as mean $\pm$ standard deviation (SD) beside numbers and percentage. Statistical analysis was performed with either Student's *t*-test for comparison of mean values or a chi-square test to compare percentages using the Stata 13 program (StataCorp LLC, College Station, TX, USA). A *p*-value of $<$0.05 was considered statistically significant.

3. Results

A total of 763 vitrified-warmed blastocysts were analyzed in this study, of which 758 survived at the warming step (99.3%; 758/763). All surviving blastocysts were replaced in 538 women. The patient's characteristics are depicted in Table 1. No significant differences were observed regarding the mean age of patients (30.17 $\pm$ 3.48 *vs* 29.50 $\pm$ 4.09 years), the average number of blastocysts transferred (1.50 $\pm$ 0.55 *vs* 1.33 $\pm$ 0.52), the basal follicle-stimulating hormone (FSH) (7.33 $\pm$ 1.51 *vs* 7.00 $\pm$ 1.79 mIU/mL), body mass index (BMI) (22.67 $\pm$ 2.16 *vs* 21.17 $\pm$ 2.14 kg/m^2), infertility duration (4.17 $\pm$ 1.47 *vs* 4.67 $\pm$ 1.75 years), primary infertility (26% *vs* 21.5%), endometrial thickness (8.83 $\pm$ 1.47 *vs* 9.0 $\pm$ 1.10 mm), and artificial cycle (86% *vs* 80.6%).

Regarding clinical outcomes (Table 2), results show a similar survival rate after warming for group A (99.4%) and group B (99.3%), as well as the same implantation rate (A: 59.1% *vs* B: 61.2%), and clinical pregnancy rate (A: 70.4% *vs* B: 68.4%) for both groups. Further, the live birth (A: 64% *vs* B: 57.3%), multiple gestation rates (A: 21.0% *vs* B: 24.4%), and spontaneous miscarriage rate (A: 9.1% *vs* B: 14.2%) were comparable in the two groups.

Table 1. Characteristics of patients following 6–7 (group A) and 9–10 (group B) minutes equilibration vitrification protocols.

	Group A	Group B	*p*-value
Transfer cycles	250	288	
Maternal age (years)	30.17 $\pm$ 3.48	29.50 $\pm$ 4.09	0.767
Transferred blastocyst (n)	1.50 $\pm$ 0.55	1.33 $\pm$ 0.52	0.599
Basal FSH (mIU/mL)	7.33 $\pm$ 1.51	7.00 $\pm$ 1.79	0.734
Maternal BMI	22.67 $\pm$ 2.16	21.17 $\pm$ 2.14	0.254
Infertility duration (years)	4.17 $\pm$ 1.47	4.67 $\pm$ 1.75	0.604
Primary infertility (%)	65 (26.00)	62 (21.5)	0.223
Endometrial thickness (mm)	8.83 $\pm$ 1.47	9.0 $\pm$ 1.10	0.828
Artificial cycle, n (%)	215 (86.00)	232 (80.6)	0.093
Natural cycle, n (%)	35 (14.0)	56 (19.4)	

Data are presented as mean $\pm$ SD and proportion (%).
FSH, follicle-stimulating hormone; BMI, body mass index; SD, standard deviation.

Table 2. Comparison of clinical outcomes between group A and group B.

	Group A	Group B	*p*-value
Survived blastocysts (%)	99.4 (359/361)	99.3 (399/402)	1.000
Implantation (%)	59.1 (212/359)	61.2 (244/399)	0.555
Clinical pregnancy (%)	70.4 (176/250)	68.4 (197/288)	0.616
Live birth (%)	64 (160/250)	57.3 (165/288)	0.113
Spontaneous miscarriage (%)	9.1 (16/176)	14.2 (28/197)	0.126
Multiple gestation (%)	21.0 (37/176)	24.4 (48/197)	0.442

Data are presented as proportion (%).

Table 3 depicts the neonatal outcome of patients who completed the vitrified-warming program. In group A, a total of 180 children were born, *vs* group B, 199 children were born. There were no differences between the two groups concerning the prevalence of male babies (A: 54.4% *vs* B: 54.3%), average gestational age (A: 38.67 $\pm$ 1.37 *vs* B: 38.33 $\pm$ 1.21), preterm birth rate (A: 20% *vs* B: 20.6%), birth weight (A: 2.95 $\pm$ 0.58 *vs* B: 3.05 $\pm$ 0.63 kg), and low-birth-weight (A: 21.1% *vs* B: 25.1%). Additionally, the rates of macrosomia (A: 3.3% *vs* B: 2.0%), caesarean section (A: 65.6% *vs* B: 68.5%), and incidence of congenital abnormalities (A: 0.6% *vs* B: 0%) were comparable in the two groups.

4. Discussion

The objective of this study was to investigate the effects of different equilibration times on clinical and neonatal outcomes of human blastocysts vitrified following AS with the laser pulse. Results demonstrate that a shorter equilibration time of 6–7 minutes resulted in optimal survival, clinical pregnancy, and live birth rates, suggesting that the extension of ES to 9–10 minutes does not bring any further benefits to the vitrification process. In ART, a two-step protocol is commonly applied to vitrify human embryos, and

Table 3. Comparison of neonatal outcomes between group A and group B.

	Group A	Group B	*p*-value
Male babies (%)	54.4 (98/180)	54.3 (108/199)	0.973
Gestational age (weeks)	38.67 ± 1.37	38.33 ± 1.21	0.664
Preterm birth (%)	20 (36/180)	20.6 (41/199)	0.884
Birth weight (kg)	2.95 ± 0.58	3.05 ± 0.63	0.780
Low birth weight (%)	21.1 (38/180)	25.1 (50/199)	0.355
Macrosomia (%)	3.3 (6/180)	2.0 (4/199)	0.630
Cesarean section (%)	65.6 (105/160)	68.5 (113/165)	0.583
Congenital abnormalities (%)	1	0	

Data are presented as mean ± SD and proportion (%).

in China, this protocol is very popular and adopted by several IVF units. Embryos in the ES are in contact with a lower concentration of CPAs and rapidly start losing water. Lately, in the VS, embryos are exposed to a higher percentage of CPAs, which induces a profound volumetric change and osmotic imbalance of the embryos. Exposure to high concentrations of CPAs is thought to be very critical for the efficiency of vitrification [35], considering that high CPAs might cause cytotoxicity, osmotic stress, and epigenome alterations [36,37]. Thus, extended exposure to ES might be detrimental and impair future embryo development, while a shorter exposure may not be enough for the penetration of CPAs into the cells, therefore the balance between CPAs concentration and time of embryo exposure is decisive for vitrification success [17,19,38]. It is worth mentioning that temperature also plays an important role during the vitrification, regulating the flow of CPAs into the cells [39]. Indeed, in this study, vitrification was performed at room temperature, using ethylene glycol and DMSO as CPAs. Kitazato's protocol suggests maintaining blastocysts in VS within 1 minute (45–60 seconds), while the time in ES generally fluctuated between 5 and 15 minutes, which agrees with several published articles [5,6,10,11,40,41]. Animal studies have reported contrasting results on this topic. Kader and colleagues [22] evaluated the impact of equilibration time on the DNA integrity of vitrified-warmed mouse blastocysts. They recommended an equilibration time of 8 minutes at room temperature to improve mouse blastocyst DNA integrity [22]. Conversely, Bagis and collaborators [23] found that vitrification with a 15 minutes equilibration resulted in a higher hatched blastocyst rate compared to that seen at 5 or 10 minutes. Recently, Berteli and co-authors [38] analyzed about 1000 vitrified mouse oocytes, aiming to define the effect of the exposure time to ES on lipid characterizations and future embryonic development. They found that a longer equilibration time (10 minutes) produced lower oocyte survival and blastocyst rates compared to 7 minutes of exposure. As such, these authors concluded that a longer equilibration phase pre-vitrification can impair embryo development and cause modification in oocyte lipid composition, associated with membrane integrity [39]. Divergent results have also been found in humans, where some reports adopted a fixed equilibration time of 5 minutes [24,25], while others increased the equilibration phase pre-vitrification to 10 [26] or up to 15 minutes [27,28]. Xiong and colleagues [42] analyzed the topic in 517 frozen-warmed human embryos. They split the cycles into four groups according to the equilibration time: 5–6 min, 6–7 min, 9–10 min, and 11–12 min, and found no differences in terms of survival rate between the groups. But implantation and live birth rates were lower in the 5–6 minutes exposure group compared with the three other groups. However, the mentioned study was performed on cleavage-stage embryos and our study is performed on blastocysts, which responded differently to the permeation of CPAs [42]. Mitsuhata and collaborators [43] reviewed 80 non-expanded and 112 expanded blastocysts and applied two equilibration times pre-vitrification: 8–11 and 12–15 minutes. They found no difference between the two groups in survival, implantation, and live birth rates, which agrees with our results. However, the authors reported a significantly improved outcome for the expanded blastocysts in the 12–15 minutes group compared to the 8–11 minutes group [43]. However, in the cited study, expanded blastocysts were defined when the blastocoels occupied greater than half of the embryo volume with a diameter $>$150 µm, while our study adopted a laser pulse to induce blastocyst shrinkage. This is an important difference compared to our study, where all blastocysts were completely collapsed before being placed into ES. AS impacts the flowing of CPAs into the embryo, and thus reducing the ES to 6–7 minutes would be adequate to obtain an efficient vitrification process [44–47]. Expanding and fully expanded blastocysts enclose a considerable quantity of fluid and water in the blastocoel, which may increase the risk for ice crystal production during vitrification. On the other hand, when AS is applied, those expanded blastocysts collapsed in a few minutes, and are rapidly converted into a morula-like stage without any fluid-filled cavity. Laser technology is a simple, accurate, and effective microsurgery tool that currently has been applied in many medical fields, including ART [48]. In the current study, AS was obtained using a laser pulse at a minimal setting, orientated at the junction between the two trophectoderm cells, at a safe distance from the ICM. This is concordant with several studies reporting significant improvements in survival rate, clinical pregnancy, and implantation rates applying AS before vitrification [16,44–49]. Mukaida and collaborators [45] produced one of the first studies on that topic. The authors showed a significant improvement in survival and pregnancy rates in 502 blastocysts collapsed by laser pulse prior to vitrification compared to a retrospective control group.

In an animal model study, different times in ES influence the abortion rate. The possible mechanisms could be correlated with several reasons, such as DNA damage and fragmentation as demonstrated elsewhere [22]. Spin-

dle abnormalities were observed in vitrified blastocyst compared with fresh blastocyst [50], severe changes in temperature, and osmotic stress [17,35], as well as damage induced by exposure to high concentrations of CPAs [18,19]. Preliminary studies analyzing this concern have reported that cell exposure to high concentrations of DMSO might induce epigenomic alteration, as well as impair cellular metabolism, cell growth, and apoptosis [19–21,51–55]. In our study, the miscarriage rate was lower in the 6–7 minutes group than in the 9–10 minutes group, however, data displayed no significant difference in the two groups.

Additional studies, especially large-scale epidemiological reports are urgently needed to further understand the implications that cryopreservation and high concentration of CPA might have on the health of children conceived following ART, not only at the time of delivery but also during adult life. Particularly noteworthy is the increase in macrosomal and large for gestational age (LGA) newborns, in addition to a decrease in low birth weight (LBW) and small for gestational age (SGA) newborns [56].

However, no significant differences have been found between group A and group B in terms of neonatal outcomes. The current study carries the limitation of its retrospective design. This is not a randomized controlled trial, it is a retrospective observational cohort study aiming to calculate the effect of different ES time pre-vitrification of artificially collapses blastocyst on the survival and pregnancy rates, as well as neonatal outcomes. In addition, the presence of potential confounding factors due to the heterogeneous nature of the samples investigated may impair the efficacy of our conclusions. However, the comparison of confounders such as female age, maternal BMI, basal FSH, the number of blastocysts transferred, years of infertility, and endometrial thickness were not statistically different between the two groups. Furthermore, it is worth mentioning that the manipulation skills of each embryologist may influence the overall vitrification process, but in this study, vitrification and warming were performed by only two experienced embryologists and their performance were similar each year. Thus, we do believe that variations in technique between operators presumably did not influence the results. In our study, open vitrification system was used to allow direct contact of biological samples with LN2. Gullo *et al*. [57] showed that there was no statistically significant difference between closed and open vitrification with regards to survival, implantation, clinical pregnancy, and live birth rates.

As far as COVID-19 is concerned, the pandemic has seriously affected the lives of the global population. While the novel coronavirus has broad health implications across the globe, being overlooked in response and policy debates is the impact on women's reproductive health. In 2021, Owens *et al*. [58] reported that the COVID-19 pandemic has significantly impacted the reproductive health of women. However, women undergoing ART after the COVID-19 pandemic exhibited no significant difference in the clinical pregnancy rate, miscarriage rate, embryo cryopreservation rate, and other clinical outcomes [59]. Furthermore, patients who were infected by COVID-19 were not included in the study.

5. Conclusions

To conclude, our findings demonstrate that laser collapse of blastocysts before vitrification and shorter equilibration time of 6–7 minutes leads to similar clinical and neonatal outcomes. However, our results still require further investigations and prospective studies to confirm the benefit of shorter ES time as a routine protocol to improve the efficacy of the vitrification process.

Availability of Data and Materials

The original data of individual participants underlying this article will be shared on reasonable request to the corresponding author.

Author Contributions

LGZ conceived the idea, designed the study, conducted the analysis, and wrote the manuscript. YHL, NL designed the figures and tables. RS designed the figures and tables and revised the manuscript. All authors participated in the discussion of analysis and interpretation of data in this article. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All participants provided written informed consent. Furthermore, the study was approved by the Ethics Committee of Assisted Reproductive Medicine at the Haikou Mary Hospital (Ethics approval number: 2021-10-02).

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Conflict of Interest

The authors declare no conflict of interest.

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