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Neonatal outcome and birth defects in 6623 singletons born following minimal ovarian stimulation and vitrified versus fresh single embryo transfer

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ABSTRACT

Objective: To compare neonatal outcome between children born after vitrified versus fresh single-embryo transfer (SET).
Study design: Retrospective, single-centre cohort study of 6623 delivered singletons following 29,944 single-embryo transfers. Patients underwent minimal ovarian stimulation/natural cycle IVF followed by SET of fresh or vitrified-warmed (using Cryotop, Kitazato) cleavage-stage embryos or blastocysts. Outcome measures were gestational age at delivery, birth weight, birth length, low birth weight (LBW), small for gestational age (SGA) and large for gestational age (LGA) infants, perinatal mortality and minor/major birth defects (evaluated by parent questionnaire).
Results: Gestational age (38.6 ± 2 versus 38.7 ± 1.9 weeks) and preterm delivery rate (6.9% versus 6.9%, aOR: 0.96 95%CI: 0.76–1.22) in singletons born after the transfer of vitrified embryos were comparable to those born after the transfer of fresh embryos. Children born after the transfer of vitrified embryos had a higher birth weight (3028 ± 465 versus 2943 ± 470 g, $p < 0.0001$) and lower LBW (8.5% versus 11.9%, aOR: 0.65 95%CI: 0.53–0.79) and SGA (3.6% versus 7.6% aOR: 0.43 95%CI: 0.33–0.56) rates. Total birth defect rates (including minor anomalies) (2.4% versus 1.9%, aOR: 1.41 95%CI: 0.96–2.10) and perinatal mortality rates (0.6% versus 0.5%, aOR: 1.02 95%CI: 0.21–4.85) were comparable between the vitrified and fresh groups.
Conclusions: Vitrification of embryos/blastocysts did not increase the incidence of adverse neonatal outcomes or birth defects following single embryo transfer.

1. Introduction

In Europe multiples account for approximately 21% of live births related to assisted reproduction techniques (ART) [1]. Because twins/triplets are associated with higher rates of adverse perinatal outcomes they are regarded as a serious complication following ART [2]. In recent years single-embryo transfer (SET) has been recognized as the most efficient way to reduce the risk of multiple pregnancies, which has also led to an increased use of cryopreservation of any surplus embryos [3]. To improve the efficiency of embryo freezing, vitrification was recently introduced into clinical practice. This new technique – where cryopreservation is achieved through ultra-rapid freezing in the tiniest amount of carrier media – yields high survival rates and consistent results not only with cleavage-stage embryos but also with blastocysts and oocytes, which are considered to be more difficult to freeze [4]. However, concerns have been raised due to potential risks associated with vitrification (related to higher concentrations of toxic cryoprotectants), highlighting the need for follow-up studies of children born after vitrification [5].

Neonatal outcome following frozen–thawed embryo transfer (FET) was found to be at least similar or even better compared to fresh embryo transfer, but this was based on the use conventional slow-freezing methods; and data on vitrification are still very limited [6]. To date only three recent papers [7–9] have been published on neonatal outcome of children born after embryo/blastocyst vitrification. Although those studies have found a comparable or better outcome when compared to fresh or slow-frozen embryo transfer cycles, they are based on data from only 89–147 vitrification children.

Since 1995 Kato Ladies Clinic (KLC) has pioneered the development of mild approaches in assisted reproduction and has established the world's largest IVF programme based on minimal ovarian stimulation, mandatory single-embryo transfer and the increased use of vitrification [10]. This unique policy has resulted in thousands of singletons born following embryo vitrification. Therefore the aim of the present retrospective study

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is to compare neonatal outcome and birth defects in a large number of children born after vitrified and fresh embryo transfer from a recent three-year period.

2. Materials and methods

All singleton deliveries that resulted from all consecutive single-embryo transfers performed between January 2006 and December 2008 in a private infertility centre (Kato Ladies Clinic, Tokyo, Japan) were evaluated retrospectively. Monozygotic twin deliveries (1.37%: 92/6715) were excluded because their outcome has already been presented in a recent paper [11]. A dataset – containing no identifying data – was extracted by the responsible person managing the centre's computerized database and who was not involved in the research project. The authors used only the above-mentioned dataset. The present research project was also evaluated by the centre's independent Institutional Review Board, which approved it and decided that no specific patient consent form was needed due to the retrospective examination of already existing data obtained during routine patient follow-up.

2.1. Minimal ovarian stimulation protocol, embryo/blastocyst culture

The details of the minimal stimulation protocol used have been described previously [10]. Ovarian stimulation was mostly (88%) carried out using clomiphene citrate (from cycle day 3 until the day before triggering) in combination with a small amount of hMG or rFSH. The final oocyte maturation was triggered exclusively with a nasal GnRH agonist and oocytes were retrieved 32–35 h later. In a smaller proportion of cases unstimulated natural-cycle IVF (9%), minimal stimulation with letrozole (1.4%) or (in some patients with hypophyseal dysfunction) gonadotropin-only ovarian stimulation (1.6%) was performed. Following insemination by conventional IVF or ICSI, normally fertilized 2PN zygotes were cultured individually in 20 μl of cleavage-stage medium (SAGE®, Pasadena, CA) until day 2 or 3. Blastocyst culture was performed until days 5–7 using commercially available media (Quinn's Advantage®, SAGE) enriched by 10% protein supplement (Serum Protein Substitute®, SAGE). All embryos were cultured at 37 °C under the gas phase of 5% O_2, 5% CO_2 and 90% N_2 with full humidity in water jacket small multi-gas incubators (Astec®, Fukuoka, Japan). In our centre blastocyst culture, elective vitrification and subsequent frozen–thawed single embryo transfer is routinely indicated in cases with previous failed cleavage-stage embryo transfer cycles or a history of tubal infertility/ectopic pregnancy. If these factors were absent fresh cleavage-stage embryo transfer was usually performed. This policy resulted in the majority of SETs in this series involving either a fresh cleavage-stage embryo (47%) or a vitrified-warmed blastocyst (43%).

2.2. Embryo/blastocyst vitrification/warming, transfer procedure and preparation for FET cycles

In FET cycles cleavage-stage embryos/blastocysts were cryopreserved using the Cryotop® vitrification method (Kitazato, Shizuoka, Japan) as described previously [12–14]. The detailed illustrated protocol (PDF and animation sequence) can be seen at www.kitazato-biopharma.com. The embryo transfer procedure was performed under vaginal ultrasound guidance using a specially designed soft silicone inner catheter (Kitazato) by placing a single embryo in a minimal volume to the upper part of the uterine cavity [15]. In FET cycles embryos were transferred during a natural cycle or after endometrial preparation with exogenous estrogens and progesterone. In natural cycles, cleavage-stage embryos and blastocysts were transferred on day 2 and 5 respectively after ovulation. In hormonal replacement cycles, transdermal estradiol patches were started from cycle day 2 and duphaston was added from cycle day 11, after which cleavage-stage embryos or blastocysts were transferred 2 or 7 days later, respectively.

2.3. Parent questionnaire, outcome measures and statistical analysis

Patients were routinely followed up by ultrasound scans performed at our centre until the end of the 1st trimester. Before being discharged to the further care of their treating gynaecologist they were given a two-part questionnaire (with prepaid envelopes) that they had to fill out and send back at the 20th pregnancy week and after delivery. Those patients who did not respond to this two-part questionnaire were reminded by a telephone call at each stage. The study's high response rate (99.6%) could be attributed to the effort of a highly motivated nurse staff as well as the patients' socio-cultural characteristics. In addition complete loss to follow-up is usually rare because patients who still have frozen gametes or embryos stay in regular contact with the centre. The questionnaire asked information about the date and mode of delivery, the gender, birth weight and length of the newborn(s) and the presence of any birth defect or other anomaly. Live birth was defined as delivery at $\geq$22 weeks of pregnancy. Preterm, very preterm and post-term delivery was defined as delivery occurring at $<$37, $<$32 and $\geq$42 weeks, respectively. Low birth weight, very low birth weight, small for gestational age (SGA) and large for gestational (LGA) infants were defined as those weighing $<$2500 g, $<$1500 g, and $<$10th or $>$90th percentile (using Japanese reference tables for singleton newborns stratified by infant sex and maternal parity), respectively. Perinatal mortality was defined as the sum of stillbirths ($\geq$22 pregnancy weeks) and early (within 7 days) neonatal deaths. Birth defects were classified using the Q-codes of the ICD-10 classification by reformatting the answers of the parent questionnaires [16]. Major (those causing functional impairment or requiring surgical intervention) and minor malformations were subdivided on the basis of a reference textbook by Aaese [17]. The association between vitrification and adverse neonatal outcomes (preterm delivery, LBW, SGA LGA infants, perinatal mortality and birth defects) adjusted for maternal age, BMI, parity, type of stimulation protocol, ICSI versus conventional IVF fertilization, blastocyst versus cleavage-stage embryo culture and infant sex was evaluated by a multiple logistic regression analysis. Backward stepwise regression was used to generate the most parsimonious model (a $p < 0.05$ value was used for inclusion and $p > 0.1$ for excluding variables). Statistical calculations were performed with SPSS 20.0 software.

3. Results

During a three-year study period 29,944 single-embryo transfers were performed in our centre. Out of the resulting 6623 singleton deliveries 4092 (62%) and 2531 (38%) were born after transfer of vitrified or fresh embryos/blastocysts, respectively. Basal characteristics and the neonatal outcome of the vitrified and fresh single-embryo transfer groups are summarized in Table 1. The age of the mothers in the vitrified SET group was significantly higher (although probably of little clinical significance) and they also had a slightly higher caesarean section rate. Mean gestational age and the rate of preterm, very preterm and post term deliveries were not significantly different between the vitrified and fresh groups. In contrast, birth weight, birth length and crude LGA rates were significantly higher whereas crude LBW and SGA were significantly lower in children born after vitrified embryo transfer. Perinatal mortality was not significantly different between groups. The overall rate of birth defects was comparable between the vitrified and fresh groups. No statistically significant

Table 1
Basal characteristics and neonatal outcome in the vitrified and fresh SET groups.

	Vitrified SET	Fresh SET
Total embryo transfers, *n*	14,456	15,488
Patient age, years (range)	38.1 ± 4.2 (22–52)	37.9 ± 4.7 (22–53)
Cleavage-stage embryo transfers, *n*	1642 (11.4)	14,856 (96.0)
Blastocyst embryo transfers, *n*	12,814 (88.6)	632 (4.0)
Deliveries[a]	4092 (28.3)	2531 (16.3)
Maternal age, years (range)	36.2 ± 3.7 (22–48)	35.1 ± 3.6 (22–47)
BMI (kg/m^2)	20.5 ± 2.5	20.6 ± 2.6
Nulliparous, *n* (%, 95%CI)	2988 (73.0, 71.6–74.4)	2177 (86.0, 84.6–87.3)
CS rate, *n* (%, 95%CI)	1466 (35.8, 34.4–37.3)	785 (31.0, 29.2–32.8)
Gestational age, weeks (range)	38.6 ± 2 (22–43)	38.7 ± 1.9 (25–44)
GA < 37 weeks, *n* (%, 95%CI)	283 (6.9, 6.2–7.7)	176 (6.9, 6.0–8.0)
GA < 32 weeks, *n* (%, 95%CI)	59 (1.4, 1.1–1.9)	36 (1.4, 1.0–2.0)
GA < 28 weeks, *n* (%, 95%CI)	26 (0.6, 0.4–0.9)	8 (0.3, 0.2–0.6)
GA ≥ 42 weeks, *n* (%, 95%CI)	23 (0.6, 0.4–0.8)	8 (0.3, 0.2–0.6)
Birth weight, g (range)	3028 ± 465 (367–4888)	2943 ± 470 (270–4726)
<2.500 g, *n* (%, 95%CI)	347 (8.5, 7.7–9.4)	302 (11.9, 10.7–13.2)
<1.500 g, *n* (%, 95%CI)	47 (1.1, 0.9–1.5)	38 (1.5, 1.1–2.0)
<1.000 g, *n* (%, 95%CI)	25 (0.6, 0.4–0.9)	16 (0.6, 0.4–1.0)
SGA, *n* (%, 95%CI)	146 (3.6, 3.0–4.2)	178 (7.0, 6.1–8.1)
LGA, *n* (%, 95%CI)	776 (19.0, 17.8–20.2)	339 (13.4, 12.1–14.8)
Birth length, cm (range)	48.9 ± 2.7 (21–62)	48.6 ± 2.8 (27–59)
Stillbirths, *n* (%, 95%CI)	15 (0.4, 0.2–0.6)	10 (0.4, 0.2–0.7)
Early neonatal mortality, *n* (%, 95%CI)	11 (0.3, 0.1–0.5)	2 (0.1, 0.0–0.3)
Perinatal mortality, *n* (%, 95%CI)	26 (0.6, 0.4–0.9)	12 (0.5, 0.3–0.8)
Birth defects total, *n* (‰, 95%CI)	100 (24.4, 20.0–30.0)	51 (20.2, 15.0–26.0)

[a] Per embryo transfer after excluding monozygotic twin deliveries.

difference was noted in any particular malformation type between the vitrified and fresh groups (Table 3.). The detailed breakdown of detected malformations is presented in Table 4. After adjusting for confounding factors (based on maternal age, BMI, parity, type of stimulation, ICSI fertilization, blastocyst culture and infant sex) in a multivariate analysis vitrified single embryo transfer was not statistically associated with preterm delivery, birth defects, perinatal mortality and LGA infants. In contrast it was associated with a lower risk of LBW and SGA infants. The results of the multivariate logistic regression analysis are shown in Table 2.

4. Discussion

Our large single-centre study, evaluating over six and half thousand singleton IVF children, suggests that neonatal outcome and birth defect rates after vitrification are not adversely affected when compared to fresh embryo transfer. Birth weight of infants after vitrified embryo transfer was even higher, which is consistent with many studies on FET outcome. Our findings, based on the largest number of children born after vitrification published to date, support the overall safety of this recently developed method of cryopreservation.

Since its first introduction several controlled studies have aimed to investigate the outcome of children born after embryo cryopreservation [18]. A systematic review performed in 2008 found that the outcome of children born after cryopreservation was similar or even better when compared to fresh embryo transfer [6]. In particular large registry-based studies from Scandinavia and Australia [19–22] demonstrated that birth weight of FET children was significantly higher and preterm birth rates were lower compared to infants conceived in a fresh cycle. Underlying causes for a better outcome might be related to a selection bias (of patients or embryos) and to optimized endometrial receptivity and improved early implantation and placental development in FET cycles [19].

Table 2
Unadjusted and adjusted odds ratios between vitrified versus fresh single embryo transfer for adverse neonatal outcomes.

Adverse neonatal outcomes	Unadjusted OR (95%CI)	Adjusted OR[a] (95%CI)
Preterm delivery	0.99 (0.82–1.21)	0.96 (0.76–1.22)
Perinatal mortality	1.34 (0.68–2.66)	1.02 (0.21–4.85)
Birth defects	1.22 (0.87–1.71)	1.41 (0.96–2.10)
LBW infant	0.68 (0.58–0.80)	0.65 (0.53–0.79)
SGA infant	0.49 (0.39–0.61)	0.43 (0.33–0.56)
LGA infant	1.51 (1.32–1.74)	1.23 (0.88–1.72)

[a] Adjusted for maternal age, BMI, parity, type of stimulation, ICSI fertilization, blastocyst culture and infant sex.

However, data on the neonatal outcome of children born after embryo or blastocyst vitrification are still very scarce; the above findings were mainly based on studies where early cleavage-stage embryos were cryopreserved with a conventional slow-freezing method. A Japanese group was the first to publish a study on the outcome of children born after vitrification: 147 such children were compared to a fresh blastocyst control group [8]. Although the preterm delivery rates in their study were relatively high due to a high proportion of multiple pregnancies, they found comparable neonatal outcome and birth defect rates between the groups. A more recent Scandinavian single-centre study [7] with a high proportion of single-embryo transfers (>90%) prospectively evaluated the outcome of the first 103 children born following the introduction of vitrification at the centre. Like our study, they found the outcome in the vitrification group was comparable to that of the fresh transfer group and noted a significantly higher birth weight in singletons of the vitrification group. Another recent study, where vitrification was uniquely applied to cleavage-stage day 3 embryos, also reported comparable outcome between the fresh and vitrified groups [9].

Interestingly, in our series a large proportion of infants qualified as LGA using Japanese reference tables for neonatal birth weight. This finding is in line with recently presented registry-based studies where an approximately 1.6–1.7-fold increase for being LGA was found in children born after FET compared to fresh embryo cycles [19,23,24]. The exact underlying mechanism and long-term consequences of this phenomenon are yet unknown and further follow-up studies are warranted until definite conclusions can be drawn.

Table 3
Birth defects in singletons born after vitrified versus fresh SET.

	Vitrified SET	Fresh SET
Deliveries	4092	2531
Chromosomal anomalies, *n* (‰, 95%CI)	12 (2.9, 1.7–5.1)	2 (0.8, 0.2–3.0)
Circulatory, *n* (‰, 95%CI)	29 (7.1, 4.9–10.2)	16 (6.3, 3.9–10.2)
Digestive system, *n* (‰, 95%CI)	11 (2.7, 1.5–4.8)	4 (1.6, 0.6–4.1)
Eye, ear, face, neck, *n* (‰, 95%CI)	9 (2.2, 1.2–4.2)	6 (2.4, 1.1–5.2)
Urogenital, *n* (‰, 95%CI)	6 (1.5, 0.7–3.2)	8 (3.2, 1.6–6.2)
Musculoskeletal, *n* (‰, 95%CI)	13 (3.2, 1.9–5.4)	5 (2.0, 0.8–4.6)
Nervous, *n* (‰, 95%CI)	3 (0.7, 0.2–2.2)	1 (0.4, 0.1–2.2)
Respiratory, *n* (‰, 95%CI)	1 (0.2, 0.0–1.4)	0 (0.0, 0.0–1.5)
Other congenital abnormality, *n* (‰, 95%CI)	11 (2.7, 1.5–4.8)	4 (1.6, 0.6–4.1)
Unknown malformation, *n* (‰, 95%CI)	5 (1.2, 0.5–2.9)	5 (2.0, 0.8–4.6)
Minor birth defects, *n* (‰, 95%CI)	15 (3.7, 2.2–6.0)	8 (3.2, 1.6–6.2)
Major birth defects, *n* (‰, 95%CI)	85 (20.8, 16.8–25.6)	43 (17, 12.6–22.8)
Birth defects total, *n* (‰, 95%CI)	100 (24.4, 20.0–30.0)	51 (20.2, 15.0–26.0)

After decades of research, convincing evidence suggests that assisted reproduction techniques are associated with approximately a 30–40% increase in the rate of birth defects when compared to naturally conceived children [25]. Reasons behind this phenomenon are unclear but it is hypothesized that patient characteristics related to infertility rather than specific ART techniques might be mainly responsible for the higher malformation rate [26]. Birth defect rates in ART children vary considerably (1.09–6.2%) between different studies due to methodological limitations [27]. The overall malformation rate in our study was in a similar slightly lower range (2.3%). This could be at least partly related to the fact that our study included only singletons and thereby excluded any increase in birth defects related to multiple pregnancies [28]. Furthermore, to date there are very limited data on ART malformations from East Asia, with only two published Chinese studies reporting even lower (1.09–1.23%) congenital malformation rates [29,30]. Although it has been suspected that ART is associated with a higher prevalence of imprinting diseases [31] the single case of Beckwith–Wiedemann syndrome observed in our series (in the vitrification group) was still in the expected prevalence range (1/13,700 live births) for this disorder.

Our centre's approach to IVF treatment is unique in several aspects. In addition to an exclusive policy based on minimal ovarian stimulation and mandatory single-embryo transfer, blastocyst culture and elective vitrification are also used to increase success rates. A large population-based Australian study has already demonstrated that in the setting of SET blastocyst culture optimizes the chance of live birth [32]. Moreover recently available evidence suggests that embryonic implantation potential might be considerably improved with the systematic use of cryopreservation and subsequent transfer in FET cycles, which is very much in line with our own experience [33–36]. In this regard vitrification has a crucial role because it yields consistently high survival rates and reduces the risk of cycle cancellation [13].

The limitation of our study is that neonatal outcome data were obtained from parent questionnaires without direct access to the newborns' medical records. Although it is unlikely that this would have altered the basic neonatal outcome data such as birth-weight or gestational age it has previously been shown that birth defect rates might be underestimated if evaluated by questionnaires [37]. As a consequence it might be that if birth defects could have been ascertained more precisely the association between vitrification and congenital malformations could become significant (currently the lower confidence interval of the adjusted OR is slightly below 1.0). Furthermore, in our study some data on maternal characteristics were unavailable (e.g. infertility type and duration, smoking

Table 4
Breakdown of birth defects in 6623 singletons.

Malformation type	*n* (‰)
Chromosomal anomalies, *n* (‰, 95%CI)	14 (2.1, 1.3–3.5)
Down syndrome 11, Edwards–Patau syndrome 2, unbalanced translocation 1	
Congenital heart disease, *n* (‰, 95%CI)	45 (6.8, 5.1–9.2)
Double-outlet right ventricle 2, VSD/ASD 30, Fallot tetralogy 1, congenital pulmonary valve stenosis 2, congenital mitral insufficiency 1, PDA 5, pulmonary artery stenosis 2, total anomalous pulmonary venous connection 2	
Digestive system, *n* (‰, 95%CI)	15 (2.3, 1.4–3.7)
Cleft lip/palate 5, oesophagus atresia 4, imperforate rectum 2, [a]tongue tie 2, Hirschprung's disease 1, bile duct malformation 1	
Eye, ear, face, neck, *n* (‰, 95%CI)	15 (2.3, 1.4–3.7)
Accessory auricle 8, ear fistula 3, ear pinna aplasia 2, [a]congenital ptosis 1, dacryocystocele 1	
Urogenital, *n* (‰, 95%CI)	14 (2.1, 1.3–3.5)
[a]Cryptorchism 5, hydronephrosis 3, [a]hypospadias 2, kidney cyst 1, external female genital malformation 1, ovariocele 1, congenital chordee 1	
Musculo-skeletal, *n* (‰, 95%CI)	18 (2.7, 1.7–4.3)
Polydactily 5, syndactyly 3, torticollis 2, hypodactyly 1, congenital hip dislocation 2, pes valgus/varus 2, arthogryposis multiplex congenita 1, [a]pectus excavatum 1, diaphragmatic hernia 1	
Nervous, *n* (‰, 95%CI)	4 (0.6, 0.2–1.6)
Myelomeningocele 2, hydrocephaly 1, holoprosencephaly 1	
Respiratory, *n* (‰, 95%CI)	1 (0.1, 0.0–0.9)
Congenital laryngomalacia 1	
Other congenital malformation, *n* (‰, 95%CI)	15 (2.3, 1.4–3.7)
[a]Congenital naevus 12, congenital alopecia 1, congenital phlebectasia 1, Beckwith–Wiedemann syndrome 1	
Unknown malformation, *n* (‰, 95%CI)	10 (1.5, 0.8–2.8)
Minor birth defects total, *n* (‰, 95%CI)	23 (3.5, 2.3–5.2)
Major birth defects total, *n* (‰, 95%CI)	128 (19.3, 16.3–22.9)
Birth defects total, *n* (‰, 95%CI)	151 (22.8, 19.5–26.7)

[a] Minor birth defects according to Aaese [17].

and socioeconomic status), hence statistical adjustment for these potential confounders was not possible. In relation to maternal smoking, in our IVF programme we have observed only a negligible proportion of smoking women which might, at least partly, explain the low overall incidence of SGA infants (<10%) in both the fresh and vitrified embryo transfer groups. The strength of our dataset lies in its large size and that it represents the outcome of only singletons born after single-embryo transfer, therefore excluding the confounding effect of any multiple pregnancies (including vanishing twins).

In conclusion our large retrospective study found that singletons born after embryo vitrification had a higher birth weight and no increase in adverse perinatal outcomes or birth defects when compared to fresh embryo transfer cycles. Long-term follow-up studies are still needed to evaluate the health of children born after vitrified embryo transfer.

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