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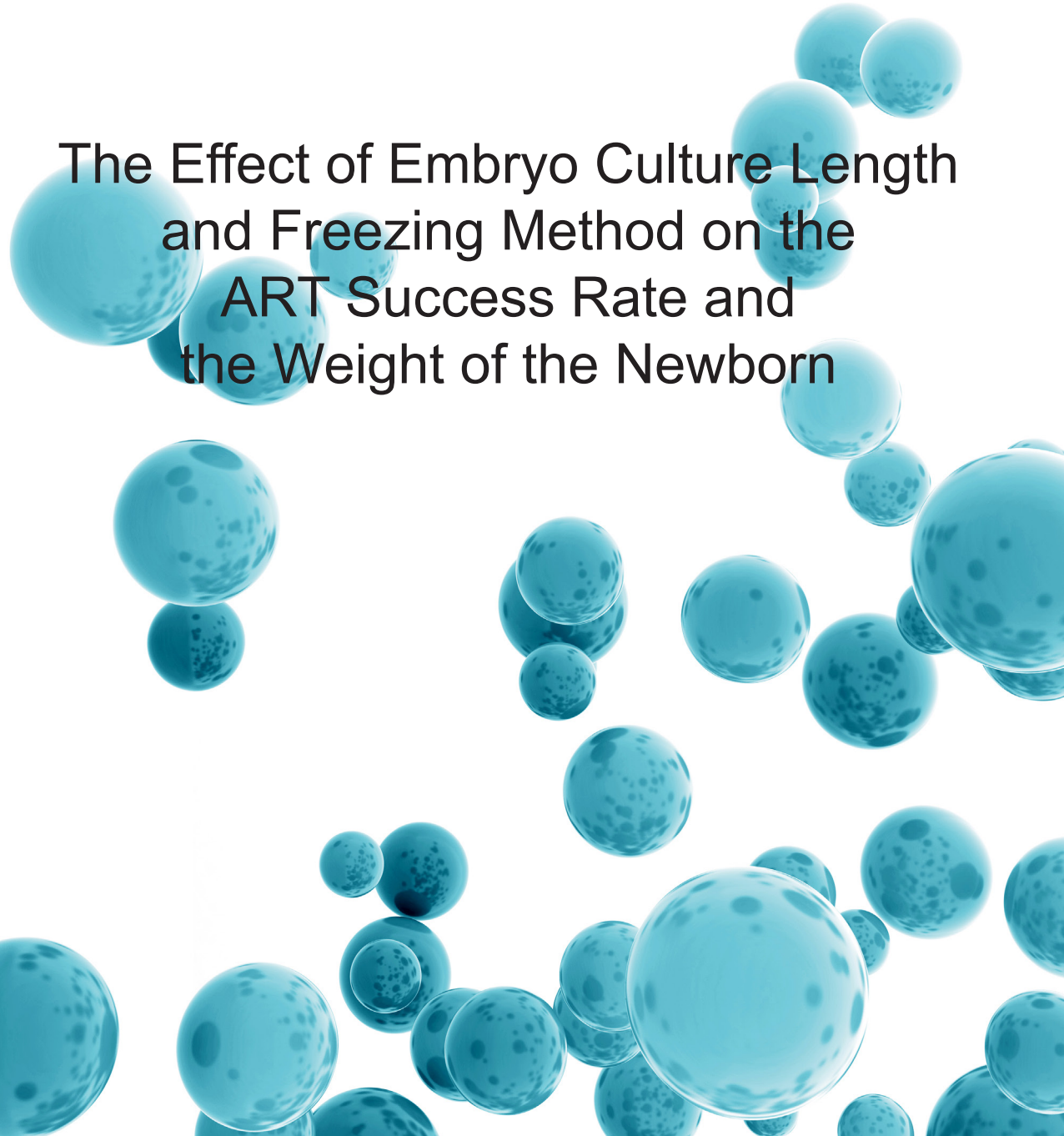

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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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ABSTRACT

The true indicator of assisted reproductive technology (ART) success is the cumulative live birth rate, reflecting the real chance of a couple to parent a child. The efficacy of IVF/ICSI treatments depends on an optimal ovarian stimulation, a high quality embryo culture and cryopreservation, and a skilled embryo transfer. Both the fresh embryo transfer and the subsequent frozen–thawed embryo transfers contribute to the result. The time period of early embryo development is characterized by intense epigenetic reprogramming (Clark, 2015). An embryo culture and cryopreservation during the early embryo development may predispose the embryo to epigenetic changes possibly affecting the phenotype.

The aim of the present study was to assess the effect of the embryo culture length and the type of cryopreservation (slow freezing/vitrification) on the clinical outcomes and on the weight of the newborn. The special interests were to evaluate the value of the extended culture of poor quality cleavage stage embryos and the use of Day 4 embryos.

The study design is a retrospective cohort study and the research material was collected from medical records over the period of 1.1.2008–31.12.2014. The IVF-laboratory work was produced by the Infertility clinic Ovumia during the study period. Additionally, in the study there are patients from the Infertility Clinic Fertinova (Tampere and Helsinki) and Infertility Clinic of Karolinska Hospital Huddinge, Stockholm, Sweden.

From 604 IVF/ICSI cycles 1879 poor quality cleavage stage embryos not eligible for transfer or cryopreservation were cultured until Day 5 or 6 (I). In 193 cycles (32%), at least one blastocyst stage embryo could be vitrified. The transfer of these blastocysts (n=134) has so far led to a delivery rate of 17.2%, increasing the cumulative delivery rate from 43% to 47%. Fifty-three repetitive IVF/ICSI cycles were estimated to be avoided (I).

The transfer of fresh (n=360) and vitrified-warmed (n=216) Day 4 embryo transfers compared to Day 3 (n=1371/635) and Day 5 (n=326/654) embryo transfers appeared to generate at least comparable clinical results. The embryos reaching the early blastocyst stage by Day 4 were associated with the highest live birth rate and the lowest miscarriage rate (47.4% and 6.7%), compared to the slower developing Day 4 embryos (19.4% and 15.4%) (IV).

To survey the effect of the IVF culture length on the birth weight, altogether 142 fresh and 135 frozen–thawed (FET) blastocyst transfers leading to a singleton live full-term

delivery were compared with Day 2–3 transfers matched with a newborn of same sex and gestational weeks. The weight of the babies born from fresh blastocysts was higher than the weight of the controls (the mean weight difference 111.8gr, p= 0.047), 4.9% of cases and 1.4% of controls being large for gestational age. When the gender of the baby was considered, the difference remained only in boys (weight difference between fresh blastocyst boys and cleavage stage boys 195.2g, p=0.023) but not in girls (weight difference 37.2g) (II). The freezing method did not seem to influence the birth weight. The post-thaw viability of the vitrified embryos was significantly higher than that of the slow frozen embryos (88.4% vs. 67.7% p<0.001). 7.4 vitrified embryos were needed to be thawed to produce one delivery while in the slow freezing group the number was 11.9 (III).

This study shows that the cumulative delivery rate can be increased, and repetitive IVF/ICSI treatments avoided by using the blastocysts developing from poor-quality cleavage stage embryos, which otherwise would have been discarded. The Day 4 embryo transfers produced good clinical outcomes and should be considered for wider utilization in IVF-clinics. However, a randomized controlled study with a larger study material is needed to verify the results. In some previous studies, the incidence of large for gestational age (LGA) babies has been increased after blastocyst stage transfers. The novel finding of the study II was the increased birth weight after blastocyst culture among boys but not among girls. Vitrification of cleavage stage embryos seems to be associated with a higher survival rate and a lower miscarriage rate compared to slow freezing.

TIIVISTELMÄ

Kumulatiivinen synnytysprosentti on lapsettomuushoitojen onnistumisen tärkein mittari. Se heijastaa pariskunnan todellista mahdollisuutta saada oma lapsi lapsettomuushoitojen tuloksena. IVF/ICSI-hoitojen tehokkuuteen vaikuttavat mm. munasarjojen optimaalinen stimulaatio, korkeatasoinen alkionviljelylaboratorio pakastusmenetelmineen sekä teknisesti korkeatasoinen ja oikein ajoitettu alkionsiirto. Kumulatiivisen raskausprosentin muodostavat tuoresiirron lisäksi pakastealkionsiirrot. Alkion viljely sekä pakastaminen ja sulattaminen varhaisen alkionkehityksen aikana altistavat alkion mahdollisesti epigeneettisille muutoksille, joilla voi olla vaikutusta jopa syntyvän lapsen fenotyyppiin.

Tutkimuksen tarkoituksena oli arvioida alkion viljelyn pituuden sekä käytetyn pakastusmenetelmän (hidas pakastus/vitrifikaatio) vaikutusta kliinisiin raskaustuloksiin, sekä vastasyntyneen painoon. Erityisenä mielenkiinnon kohteena oli tutkia huonolaatuisten jakaantumisvaiheen alkioiden jatkoviljelyn vaikutusta raskaustuloksiin, sekä neljän päivän ikäisten alkioiden käyttöä IVF-hoidoissa. Tutkimuksien potilasmateriaali on kerätty ajanjaksolta 1.1.2008–31.12.2014. Tampereen yliopistollisen sairaalan lapsettomuusklinikka osti tutkimusajanjaksolla IVF-laboratoriopalvelut Hedelmöitysklinikka Ovumialta. Lisäksi tutkimuksessa on mukana potilaita Tampereen ja Helsingin Fertinovasta sekä Karolinska sjukhuset Huddingen lapsettomuuspoliklinikalta. Kyseessä on retrospektiivinen kliininen tutkimus joka koostuu neljästä osatyöstä (I–IV).

604 IVF/ICSI syklistä valittiin jatkoviljelyyn huonolaatuiset jakaantumisvaiheen alkiot (n=1879), jotka eivät olleet siirtokelpoisia eivätkä pakastuskelpoisia tuoresiirtopäivänä. 193 syklistä 32%:ssa ainakin yksi alkio kehittyi hyvälaatuiseksi blastokystaksi ja pakastettiin. Näiden blastokystien siirroista 17.2% on tähän mennessä johtanut synnytykseen ja nämä ”ylimääräiset” synnytykset nostivat kumulatiivisen synnytysprosentin 43%:sta 47%:iin. Laskelmien mukaan 53 uutta IVF/ICSI sykliä pystyttiin välttämään käyttämällä näitä jatkoviljeltyjä alkioita, jotka muuten olisi tuhottu (I).

Tuoreiden (n=360) ja pakastettujen (n=216) päivän 4 alkioiden siirrot johtivat vähintään yhtä hyviin raskaustuloksiin verrattuna päivän 3 (n= 1371/635) ja päivän 5 (n=326/654) alkionsiirtoihin. Niiden alkioiden siirrot, jotka saavuttivat varhaisen blastokystavaiheen jo päivänä 4, johtivat korkeimpaan synnytysprosenttiin ja alhaisimpaan keskenmenoprosenttiin (47.4% ja 6.7%) (IV).

Tutkiaksemme alkionviljelyn pituuden vaikutusta lapsen syntymäpainoon, vertasimme 142:sta tuore- ja 135:sta pakasteblastokystasiirrosta syntyneiden täysiaikaisten yksöslasten painoa samalla raskausviikolla syntyneisiin, samaa sukupuolta oleviin, päivän 2-3 alkioista syntyneisiin lapsiin. Tuoreista blastokystistä syntyneet lapset olivat painavampia kuin jakaantumisvaiheen alkioista syntyneet (painoero 111.8g, p=0.047). Viiden ja kuuden päivän ikäisistä alkioista syntyneistä lapsista 4.9% kasvoi 95:n persentiilin käyrän yläpuolella kun taas vain 1,4% jakaantumisvaiheen alkioista syntyneistä lapsista kasvoi käyrän yläpuolella. Kun analyysissä huomioitiin lasten sukupuolet, jäi painoero merkittäväksi vain pojilla (painoero blastokystapoikien ja päivän 2-3 alkioista syntyneiden poikien välillä 195.2g, p=0.023, tyttöjen välillä 37.2g) (II). Pakastusmenetelmä ei näyttänyt merkittävästi vaikuttavan syntymäpainoon. Sulatuksen jälkeen vitrifioiduista alkioista oli käyttökelpoisia 88.4% ja hitaasti pakastetuista 67.7% (p<0.001). Yhden synnytyksen aikaansaamiseksi piti sulattaa 7.4 vitrifioitua alkiota, kun hitaasti pakastettuja piti sulattaa 11.9 (III).

Tutkimus osoittaa että kumulatiivinen synnytysprosentti nousee ja uusia hoitoja voidaan välttää siirtämällä jatkoviljeltyjä alun perin huonolaatuisia jakaantumisvaiheen alkioita, jotka muuten olisi tuhottu. Neljän päivän ikäisten alkioiden käyttö johtaa hyviin kliinisiin raskaustuloksiin ja niiden käyttöä tulisi harkita laajemminkin. Tarvitaan kuitenkin satunnaistettu, kontrolloitu tutkimus suuremmalla aineistolla tulosten varmistamiseksi. Aikaisemmissa tutkimuksissa on huomattu blastokystaviljelyn aiheuttavan suuren syntymäpainon lisääntymistä vastasyntyneillä. Tutkimuksessamme havaitsimme, että pitkä alkionviljely vaikuttaa johtavan suurempaan keskimääräiseen syntymäpainoon pojilla, mutta ei tytöillä. Jakaantumisvaiheen alkioiden pakastaminen vitrifioimalla näyttää johtavan korkeampaan sulatuksesta selvinneiden osuuteen kuin hitaalla pakastuksella. Vähemmän alkioita tarvitsee sulattaa yhtä synnytystä kohden vitrifikaatioryhmässä verrattuna hitaasti pakastettuihin.

LIST OF ORIGINAL COMMUNICATIONS

The present study is based on the following articles, which have been referred to in the text by their Roman numerals (I–IV)

I Kaartinen N, Das P, Kananen K, Huhtala H, Tinkanen H. Can repeated IVF-ICSI-cycles be avoided by using blastocysts developing from poor-quality cleavage stage embryos? Reprod Biomed Online 2015; 30(3):241-7.

II Kaartinen NM, Kananen KM, Rodriguez-Wallberg KA, Tomás CM, Huhtala HS, Tinkanen HI. Male gender explains increased birthweight in children born after transfer of blastocysts. Hum Reprod 2015; 30(10):2312-20.

III Kaartinen N, Kananen K, Huhtala H, Keränen S, Tinkanen H. Freezing method has no effect on birth weight of cleavage stage embryos. J Assist Reprod Genet 2016; 33(3): 393-9

IV Kaartinen N, Kananen K, Tomás C, Tinkanen H. The Day 4 embryos should not be underestimated in IVF. Submitted on 30th of June 2016.

ABBREVIATIONS

AFC	Antral follicular count
AMH	Antimullerian hormone
ART	Assisted reproductive technology
ARR	Adjusted relative risk
CPR	Clinical pregnancy rate
CSE	Cleavage stage embryos
DMR	Differentially methylated regions
FET	Frozen embryo transfer
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
ICM	Inner cell mass
IGF 2	Insulin like growth factor 2
LBR	Live birth rate
LBW	Low birth weight
LGA	Large for gestational age
LH	Luteinising hormone
M II	Metaphase II
MCR	Miscarriage rate
OPR	Ongoing pregnancy rate
PB	Polar body
PN	Pronucleus
RCT	Randomized controlled trial
SF	Slow frozen
SGA	Small for gestational age
SNRPN	Small nuclear ribonucleoprotein polypeptide N

CONTENTS

1 INTRODUCTION

The first IVF-child was born in 1978 (Steptoe & Edwards, 1978). The embryo resulted from the in vitro fertilization of a single oocyte, derived from a natural cycle. Since then, IVF technology has accomplished several improvements, such as controlled ovarian hyperstimulation, the development of sequential media, and embryo cryopreservation, to mention only a few. The IVF remains the most effective method for the treatment of infertility. Approximately 1,5 million assisted reproductive technology (ART) cycles were undertaken in 2006 and more than 5 million infants have already been born as a result of ART (Mansour et al., 2014).

IVF requires collaboration between several professionals, and the equipment is expensive. Additionally, the medications used in controlled ovarian hyperstimulation are moderately costly. The restricted amount of resources usually limit the amount of IVF cycles undertaken. In Finland, public health care usually provides three IVF/ICSI cycles per a couple, sometimes less if the first treatment attempts prove poor prognosis. Due to the increased risk of perinatal complications associated with multiple pregnancies, the Nordic countries especially have mainly adopted a single embryo transfer policy in IVF treatments. The change of pregnancy after a single embryo transfer is approximately 30%, and the cumulative pregnancy rate is dependent on the number of embryos cryopreserved after one IVF cycle, as well as the quality of the embryos. Implantation remains the stumbling block in IVF treatments. The high hormonal levels at the time of the fresh embryo transfer may hamper the implantation, which has raised the idea of freezing all embryos and transferring them during a later phase (Roque, 2015). Due to the limited availability of IVF cycles and due to the complications associated with ovarian hyperstimulation and oocyte retrieval, embryo culture and embryo cryopreservation should be refined to support the development of all viable embryos in the cohort. The embryo scoring is undertaken to select the good quality embryos for transfer or cryopreservation, while the global practice is usually to discard the poor quality embryos (Poulain et al., 2014).

In vivo the embryo arrives at the uterus 4–5 days after conception at the blastocyst stage. In ART, the embryos are usually transferred into the uterus on Day 2, Day 3 or Day 5. The embryo developmental status changes over the course of the culture, and embryo transfer can produce different pregnancy results at different stages. The blastocyst culture is

usually associated with a high implantation potential, explained by the natural selection of embryos with a superior developmental potential, after activation of the embryo´s genome in the age of three days (Glujovsky et al., 2012). The use of Day 4 embryos in clinical practice decreases the need to work on weekends. However, the results of Day 4 embryo transfers are rarely reported in the literature. The morphology of the cleavage stage embryo has been considered as the basis of the embryo quality assessment. Due to the supposed poor pregnancy potential and higher risk of abnormal karyotype, the poor quality embryos have often been discarded at cleavage stage.

The phenotype of a human is largely dependent on the genes inherited from the parents. The activity of the genes is controlled by the epigenetic marks, such as DNA methylation, histone modifications and micro RNA:s. The epigenetic marks are erased in the oogonia phase and rearranged during the period of early embryo development. This phase of epigenetic modifications is a delicate period in the embryo development, and one which may be easily disturbed by the unnatural environment associated with the in vitro culture. The birth weight of a newborn can be used as one measure to estimate whether ART has resulted in epigenetic changes affecting the phenotype. An increased risk for LGA has been observed among babies born from blastocyst stage embryo transfer compared to cleavage stage embryo transfer (Mäkinen et al., 2013). Also the embryo freezing has been found to increase the mean birth weight and the incidence of LGA among newborns (Wennerholm et al., 2013; Zhu et al., 2014).

One of the purposes of the present study was to examine the effect of the length of embryo culture on the clinical outcomes. The special interest was to evaluate the effect of the extended culture of poor quality embryos on the cumulative live birth rate, the most important endpoint of assisted reproduction. In our clinic the Day 4 embryo transfers have been used in addition to Day 2, Day 3 and Day 5 transfers to increase variety of tranfer days. The Day 4 embryo transfers have been an option when a longer embryo culture period has been considered appropppriate. We were interested to compare the clinical outcomes of Day 4 embryo transfers to Day 3 and Day 5 transfers. Furthermore, in addition to verifying the possible effect of the culture length and the freezing method on the birth weight of the newborn, we were interested in the possible effect of the gender on the phenomenon.

2 REVIEW OF THE LITERATURE

2.1 The embryo development

2.1.1 The oocyte

During fetal life, the oocyte, in the primordial follicle, enters meiosis and is thereafter arrested at the metabolically inactive diplotene stage for decades. It is unclear which factors promote its development to reach the growing phase, which ends at the early antral follicular stage. The diameter of the oocyte during the arrested meiotic phase is 120 μm. Meiosis is arrested until the ovulation. After the LH-surge the first polar body (PB) is extruded and the oocyte enters the metaphase II (MII). The cytoplasm undergoes many processes as well, such as the rearrangement of the tubules and microfilaments, to establish asymmetry for polar body extrusion. The reorganization of the mitochondrias and the endoplasmic reticulum are crucial for the chromosome segregation and prepare the oocyte for intracellular calcium oscillations, which in turn activate the oocyte. The co-operation between the cumulus cells and the oocyte is pivotal for the oocyte maturation and quality. (Coticchio et al., 2015.)

2.1.2 The Tsygote

The fertilization of an oocyte is confirmed approximately 17 (IVF) or 18 (ICSI) hours after insemination. In a normally fertilized embryo, two centrally positioned, juxtaposed pronuclei (PN) and two polar bodies can be detected. In some studies, a correlation between zygote morphology and embryo chromosomal integrity and implantation rate has been detected (Balaban et al., 2004; Gianaroli et al., 2007), whereas in others, the zygote quality doesn't predict the implantation rate (Brezinova et al., 2009). In a normally fertilized zygote, the longitudinal axis of pronuclei is parallel to the plane of polar bodies, and an abnormal position of the polar bodies in relation to the pronuclei can indicate aneuploidy of the embryo (Gianaroli et al., 2003). Pronuclear scoring by means of presence of cytoplasmic halo, and the size, number and pattern of distribution of nucleolar precursor bodies (NPB), has been related to further embryo development and embryo morphology on Day 3 and Day 5 (Scott, 2003). Berger et al. compared the pregnancy results of two groups of embryos, of which the other had been scored in the pronuclear phase, and the

other group was assessed on the transfer Day (Day 3). The pronuclear scoring did not, however, correlate with the clinical pregnancy rate while the Day 3 embryo grading served as an independent predictor of the pregnancy rate (Berger et al., 2014).

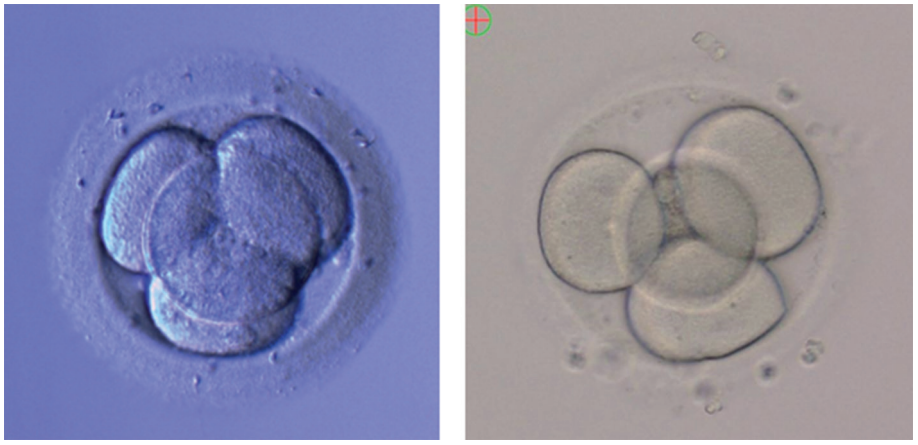
The timing of the first and second blastomere divisions has been proposed as the predictor of good embryo quality, though in some studies the late stage embryo development has been regarded as more efficient in predicting superior pregnancy results (Rehman et al., 2007). If the first cleavage of the embryo, i.e. the time of the first mitotic division occurs within 25–27 hours post insemination, a higher implantation rate can be expected compared to slowly cleaving embryos. Early cleaving embryo transfers result in higher overall pregnancy rates and develop into good quality embryos more often than late cleaving embryos (Lundin et al., 2001). Neuber et al. showed a positive relationship between PN symmetry at the time of the fertilization check and the early cleaving 2-cell embryo, and the development of good quality ≥ 4-cell and ≥ 7-cell embryos and blastocysts (Neuber et al., 2003).

2.1.3 The Cleavage stage embryo

2.1.3.1 Cleavage rate

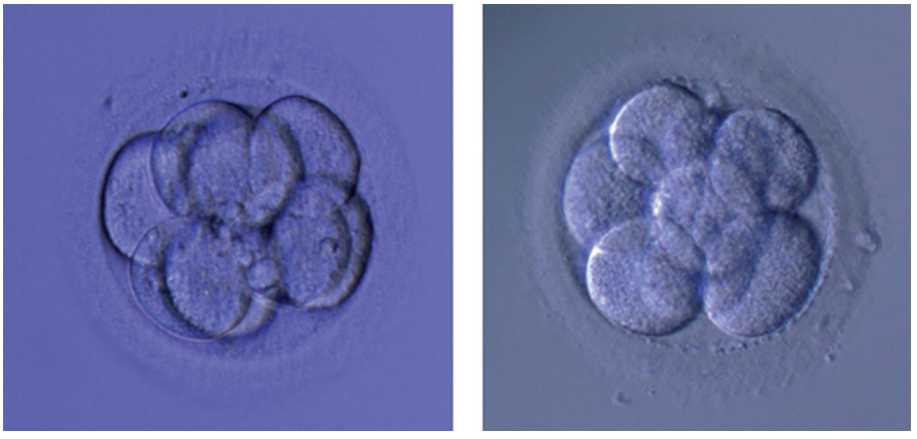
The cleavage stage begins at the 2 cell stage and ends at the compacted morula stage, composed of 8–16 cells. Cleavage stage embryo morphology has been studied widely and a large range of different parameters has been proposed for the prediction of the outcomes of the embryo transfers. The parameters which are most commonly involved in embryo evaluation and associated with good embryo quality and superior pregnancy rates, have been discovered to be the cleavage rate, blastomere symmetry, cytoplasmic appearance, the extent of fragmentation and the blastomere nuclear status (Meseguer et al., 2011; Sela et al., 2012; Stylianou et al., 2012). Abnormal cleavage rate, either too slow or too fast, has been associated with decreased developmental potential (Giorgetti et al., 1995). Other parameters such as the presence of morula on Day 3 has been proposed for embryo scoring (van Loendersloot et al., 2014). A large number of parameters has been suggested for cleavage stage embryo scoring, yet the only validated independently predictive variables are the number of cells, the extent of fragmentation, cell symmetry and nucleation (Lundin & Ahlström, 2015).

The embryo shape has been shown to have only minimal correlation with the implantation potential, and is not recommended for use as a variable on embryo scoring (Kamran et al., 2012). According to the Istanbul consensus workshop, the optimal cleavage rates of an embryo would be 2 cells on Day 1, 4 cells on Day 2 and 8 cells on Day 3 (Figures 1 and 2). A slow cleavage rate has traditionally been associated with an increased risk of aneuploidy, as has the increased blastomere number (Alikani et al., 2000; Magli et al., 2007). More than 9 blastomeres on Day 3 are associated with significantly increased aneuploidy (Kroener et al., 2015).



Figures: Fimlab.

Figure 1. Two top quality embryos; Day 2, 4 cells, score 3.5.



Figures: Fimlab.

Figure 2. Two top quality embryos; Day 3, 8 cells, score 3.5.

2.1.3.2 Embryo fragmentation

Fragmentation is a common phenomenon of in vitro conceived preimplantation embryos. The fragmentation of embryos has been suspected to be related to the progression of meiotic and mitotic cell cycles. Fragments are composed of small areas of cytoplasm surrounded by a cell membrane not including a nucleus. Extensive fragmentation is associated with poor embryo quality, and a negative correlation between the Day 3 embryo fragmentation and the rate of blastocyst development has been discovered. (Stone et al., 2005; Wu et al., 2011.) However, localized or small scattered fragments have no effect on the implantation potential of an embryo (Alikani et al., 1999). The spatial means of fragmentation are found to impact the embryo viability more than the occurrence of fragments per se, especially in the case of moderate fragmentation, and, moreover, small fragments may disappear during

the course of development (Van Blerkom et al., 2001). Embryos with heavy fragmentation are associated with an increased rate of chromosome abnormalities, mainly mosaicism (Plachot et al., 1987).

2.1.3.3 Embryo nucleation

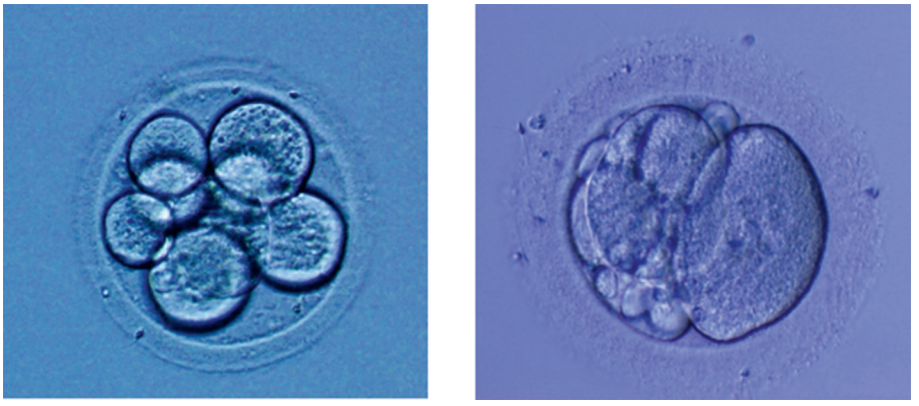
Multinucleation can be defined as the presence of more than one nucleus within at least one of the blastomeres of a two-cell embryo (Ergin et al., 2014). Multinucleation is associated with a higher rate of chromosomal abnormalities (Kligman et al., 1996). Embryos with a low multinucleation rate cleave normally more often, while an increased multinucleation rate is associated with a higher than optimal number of blastomeres. This effect has been detected both in Day 2 and Day 3 embryos. The factors predisposing to multinucleation include short stimulation, a high number of oocytes derived from thee oocyte pick-up, and high FSH doses. Multinucleation, which is associated with increased fragmentation and an abnormal cleaving rate, impairs the embryo implantation (Van Royen et al., 2003). In addition to multinucleation, other types of nuclear abnormalities, such as micronuclei and nucleoplasmic bridges, have been noted. An increased rate of mitotic chromosomal abnormalities is associated with anomalies in the structure of the nuclei. Nuclear abnormalities are more frequent at the cleavage stage (16%) embryos, compared with the blastocyst stage (5%) (Kort et al., 2016). The highest frequency of nuclear abnormalities has been discovered among the arrested cleavage stage embryos. DNA damage is more common in embryos with microscopic nuclear abnormalities, compared with normal nuclear morphology 87.1% vs 9.3% (Kort et al., 2016).

2.1.3.4 The embryo validation

A recent large prospective study was performed to find the most important embryo variables to predict the live birth rate after a single Day 2 embryo transfer. Typically, several morphologic criteria are used simultaneously in embryo scoring. Therefore, there was determination to create a method for embryo scoring, including various degrees and types of morphological deviation. In this study, five variables of embryo grading were tested univariately, but also in a multivariate model. The variables studied were the number of blastomeres, the proportion of mononucleated blastomeres, the degree of fragmentation, variation in the blastomere size, and the symmetry of the cleavage. In the study, the cleavage stage was detected to be the most powerful predictor of live birth, while the symmetry of the cleavage did not show any significant association with the live birth rate. The number of blastomeres, the nucleus score and the degree of fragmentation were independently significant. A ranking tree was presented to show the effects relating to the combinations

of values of the number of the blastomeres, blastomere size, nucleus score and the degree of fragmentation (Rhenman et al., 2015).

Compaction typically begins at the 8-cell stage, defined as an increased cell-to-cell adherence. Early compaction on Day 3 has been linked with embryonic quality, improving the implantation rates (Le Cruguel et al., 2013). An uneven blastomere cleavage has a negative effect on the pregnancy rates, probably due to the increased aneuploidy associated with these embryos (Hardarson et al., 2001). Other factors associated with the decreased cleavage stage embryo quality include cytoplasmic anomalies, abnormal spatial distribution of the cells and non-stage-specific uneven blastomere size (Figure 3) (Prados et al., 2012).



Figures: Fimlab.

Figure 3. On the left: Unequal size blastomeres, granularity; Day 3, score 2.0. On the right: a poor quality cleavage stage embryo; Day 2, 4cells, score 1.0.

2.1.4 The Morula stage

Embryo usually reaches the morula stage on the fourth day after insemination. The cleavage stage ends when the compaction starts. In an early compacted embryo the blastomeres form a clustered cell mass in which the cells have started to compact tightly. In a fully compacted morula, the cell boundaries are no longer visible, but the nuclei can be identified. At the late compact stage the cell boundaries become visible again and the cell number is markedly increased (Tao et al., 2002). The compaction precedes the development of a transporting epithelium, enabling the embryo to regulate its own homeostasis (Feil et al., 2008). An early compacted embryo is found to be associated with increased implantation potential (Figure 4) (Skiadas et al., 2006).

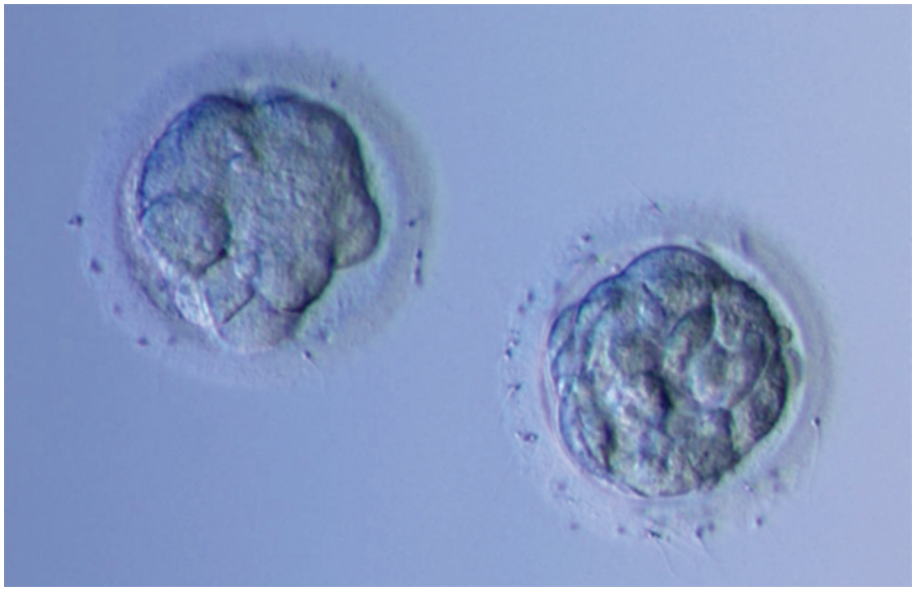


Figure: Fimlab.

Figure 4. Two embryos on Day 4; On the left: a compacted morula, score C90/75. On the right: an early blastocyst, score 1B.

2.1.5 The Blastocyst stage

On the Day 5 of embryo culture the embryo development can vary from morula and morula-blastula stage (differentiating the morula without a visible blastocoel) to a blastocyst of various phases. After compaction, spindle-shaped cells can be noticed at the edge of the embryo, implying to the onset of blastulation. Fluid starts to accumulate between the cells at the morula stage, usually between Days 4 and 5, and a cavity begins to appear forming the blastocoel. The number of the cells increases as the blastocoel expands, leading to the thinning of the zona pellucida (Hardarson et al., 2012). At this point, the number of cells can vary from 24 to 322 cells (Hardarson et al., 2003). The time up to the blastocyst expansion has been used to describe the developmental speed of an embryo, and a higher developmental speed has been associated with higher clinical and ongoing pregnancy rates (Kato et al., 2014).

Blastocysts have traditionally been classified according to Gardner and Schoolcraft (Gardner & Schoolcraft, 1999). Blastocysts are first given a numerical score from 1 to 6 on the basis of the extent of the blastocoel expansion and the hatching status. For full blastocysts (grades 3–6) the development of the inner cell mass (ICM) (the first letter) and the development of the trophectoderm (the second letter) are assessed further (Table 1). A significant correlation between the blastocyst score and the pregnancy outcome has been detected (Balaban et al., 2000; Goto et al., 2011). In a study by Honnma et al. (2012), the trophectoderm morphology was the only parameter statistically significantly associated

Table 1. The blastocyst classification according to Gardner and Schoolcraft (Gardner & Schoolcraft, 1999).

Blastocoel Expansion Number	The blastocoel size	The inner cell mass	The cells of the inner cell mass	The troph-ectoderm	The cells of the trophectoderm
1	Less than half of the volume of the embryo.	**A**	Tightly packed, many cells	**A**	Many cells forming a cohesive epitelium
2	Half of or larger than half of the volume of the embryo	**B**	Loosely grouped, several cells	**B**	Few cells forming a loose epithelium
3	Full blastocyst; Blastocoel fills the embryo	**C**	Very few cells	**C**	Very few large cells
4	An expanded blastocyst; The volume of the blastocyst greater than the volume of the early embryo				
5	A hatching blastocyst; trophectoderm partly herniating trough the zona pellucida				
6	A hatched blastocyst; Blastocyst has escaped from the zona pelucida.				

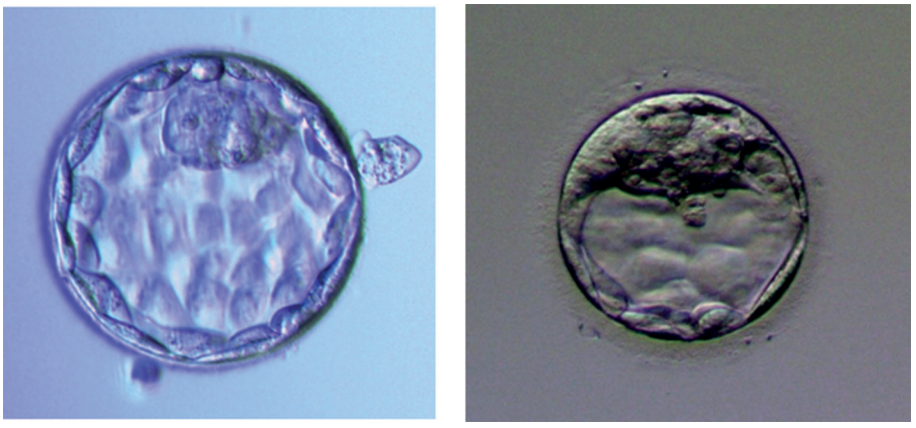
with ongoing pregnancy and miscarriage rates. ICM and the blastocyst expansion weren't related to these outcomes in the study (Honnma et al., 2012). This was assumed to indicate the importance of the successful implantation of the trophectoderm cells into the endometrium in order to form a well functioning placenta with a satisfying pregnancy outcome. In another study, the trophectoderm was the only independent predictor of live birth with statistical significance (Ahlström et al., 2011). Ebner et al. and Thompson et al. found the trophectoderm morphpology to present the highest predictive value of live birth (Ebner et al., 2016; Thompson et al., 2013), while Du et al. (2016) reported the highest predictive value of the live birth rate of blastocoele expansion and re-expansion, compared to trophectoderm or ICM grades (Du et al., 2016). Contradictory findings of the blastocyst development rate on pregnancy potential have been presented. El-Toukhy detected a comparable live birth rate after good quality frozen–thawed Day 5 and Day 6 blastocysts, while in Levens' study, superior pregnancy outcomes were observed after cryopreserved Day 5 blastocysts, compared to Day 6 blastocysts (El-Toukhy et al., 2011; Levens et al., 2008).

In a secondary analysis of a large prospective study all the three blastocyst morphology parameters were statistically significantly related to a positive pregnancy test, clinical pregnancy rate (CPR), ongoing pregnancy rate (OPR) and live birth rate (LBR). The inner cell mass was the only parameter associated significantly to the miscarriage rate. The early hatching status was the only parameter significantly associated with the live birth rate (Van den Abbeel et al., 2013).

For a long period of time, the transfer of Day 2 and Day 3 embryos was global practice in IVF. This was partially due to the lack of culture medias capable of supporting the blastocyst development in vitro. The essential problem with the blastocyst culture was not exactly the inability of embryos to reach the blastocyst stage, but rather the poor implantation potential of the embryos reaching Day 5. The development of sequential media enabled the culture of viable blastocysts, though the number of embryos reaching the blastocyst stage was not remarkably increased (Desai et al., 1997; Gardner & Lane, 1997). Nowadays, however, an effective blastocyst culture can be undertaken using a single medium from Day 0 up until the blastocyst stage (Costa-Borges et al., 2015).

Several advantages have been related to the blastocyst culture, of which the endometrial synchronization remains the most unequivocal. In vivo the oocyte is fertilized in the ampulla of the oviduct, and the generated embryo travels for 4–5 days before reaching the uterine cavity. Hence, the transfer of embryos on Day 2 or Day 3 can be perceived as unphysiologic, whereas the blastocyst arrives into the uterine cavity at the optimal moment. The implantation of a human embryo is a complex phenomena involving various hormone receptors, mainly the estrogen- and progesterone receptors (Vasquez & DeMayo, 2013).

Weather blastocyst culture and transfer improves the implantation, clinical pregnancy rate, delivery rate and cumulative pregnancy rate, still remains uncertain. A blastocyst transfer has been considered to best serve young, healthy patients of a good prognosis with a good ovarian capacity and an increased risk of multiple pregnancies. The natural selection of embryos, caused by the five days culture eliminates a proportion of the embryos. The remaining embryos have a higher change of a normal chromosome and thereby higher implantation potential (Harton et al., 2013). However, aneuploidy still exists in blastocyst stage embryos. A recent study observed that as many as 50% of blastocysts are affected by aneuploidy. In fact, it has been stated that the major selection of chromosome abnormalities would take place around the implantation time or shortly thereafter (Fragouli & Wells, 2011). A correlation between the blastocyst morphological score and euploidy rate has been detected. Blastocysts with the highest morphological scores are associated with a higher euploidy rate in comparison with embryos of lower quality (Figures 5, 6 and 7). The euploidy rates of excellent, good, average and poor blastocyst morphology groups were 56.4%, 39.1%, 42.8% and 25.5%, respectively in a study by Capalbo (Capalbo et al., 2014). No difference was observed in the aneuploidy rate of faster and slower growing embryos. In the implantation rates (around 50%), no significant difference was observed between poor and average quality euploid embryos and blastocysts with good and excellent quality.



Figures: Fimlab.

Figure 5. On the left: A top-quality blastocyst stage embryo; Day 5, score 5AA. On the right: A poor quality blastocyst stage embryo; Day 5, score 3BC.

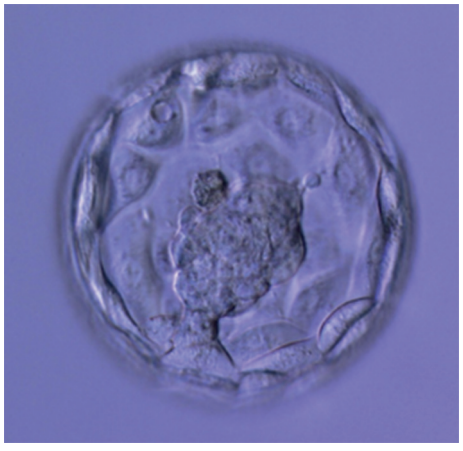


Figure: Fimlab.

Figure 6. A blastocyst stage embryo; Day 5, score 4AB.

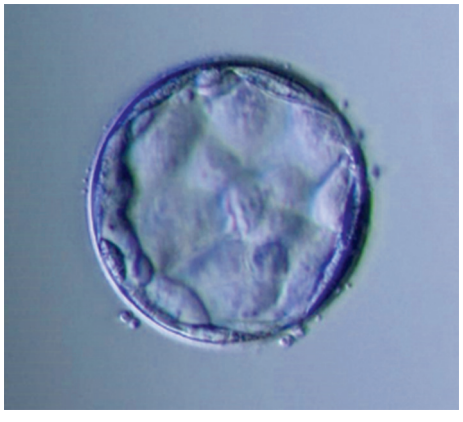


Figure: Fimlab.

Figure 7. A poor-quality blastocyst stage embryo; Day 5, score 4CC.

Embryo morphology is the most important method of embryo selection. The rationale behind embryo evaluation and scoring is to reduce the number of transfers a patient needs to go through, in order to minimize the financial burden and emotional stress. Embryo selection can shorten the time to pregnancy if the embryos with highest implantation potential are transferred first, while the cumulative pregnancy rate is unlikely to be increased by the embryo selection (Wong et al., 2014). Rijnders et al. observed a poor predictive value of Day 3 embryo morphology on the subsequent blastocyst stage morphology. Due to the high implantation potential of blastocysts Rijnders recommended embryo culturing to the blastocyst stage to successfully select the right embryos for transfer (Rijnders & Jansen, 1998).

Time-lapse monitoring has been introduced to evaluate the embryo morphology and developmental kinetics. Its role in ART and decisions concerning embryo selection still remains unsolved. According to the studies by Kirkegaard and Rienzi, morphokinetic characteristics are not useful in the selection of euploid blastocysts, and the timing of the full blastocyst formation does not predict live birth (Kirkegaard et al., 2016; Rienzi et al., 2015).

2.2 Embryo cryopreservation

2.2.1 General considerations

An ongoing pregnancy after the transfer of a frozen–thawed human embryo was first introduced on year 1983 by Trounson et al. (Trounson & Mohr, 1983). The pregnancy terminated on week 24 due to a premature rupture of the membranes with infection and premature delivery, resulting in loss of the infant. Embryo cryopreservation has since become a routine practice, entailing multiple advantages to IVF-treatments. The opportunity to use the whole embryo cohort, transferring one embryo at a time maximizes the change of pregnancy from a single oocyte pick-up procedure. Still the risk of multiple pregnancies with the associated complications increasing the hidden costs of IVF-treatments, remains low. Furthermore, the endometrium receptivity in a frozen embryo transfer cycle is suspected to be preferable compared to the fresh embryo transfer cycle possibly due to the more physiological hormone concentrations in the endometrium (Check et al., 1999; Shapiro et al., 2011). This phenomenon might be reflected by the lower incidence of premature births after frozen embryo transfers as well as the higher mean birth weight of newborns after frozen embryo transfer cycles (Wennerholm et al., 2013). Ovarian hyperstimulation syndrome can be almost completely prohibited by triggering the ovulation with GnRH agonist in GnRH antagonist cycles and freezing all the available embryos (Boothroyd et al., 2015). The 'freeze all' policy has been under debate recently but it can be supported by the elimination of the risk of OHSS. However, the effect of the 'freeze all' policy on the cumulative pregnancy rate needs to be thoroughly evaluated before implementing the

protocol into an every day practice. The embryos can be cryopreserved by two different methods: slow freezing and vitrifcation. The latter has several advantages compared to slow freezing, concerning the laboratory work.

2.2.2 The slow freezing

The slow freezing protocol was introduced by Whittingham on year 1972 (Whittingham et al., 1972). The protocol requires the dehydration of the embryo by exposure to both permeating (dimethyl sulphoxide, ethylene glycol, propanediol) and non-permeating cryoprotectants (sucrose), prior cooling. After the dehydration process the embryos are placed in plastic straws and the temperature is first slowly reduced to -30°C and then rapidly to -150°C before the embryos are preserved in liquid nitrogen (Edgar & Gook, 2012). The nonpermating cryoprotectants osmotically draw the water out of the cell, while the pearmeating cyoprotectants enter the cell and replace the intracellular water. Cryoprotectants are used to prohibit intracellular ice crystal formation. The destruction and impairment of function of cell organelles can be caused by the formation of ice crystals, which is the major adverse effect of embryo freezing (Mazur, 1984). The ice formation starts usually at -5 to -15°C when individual water molecules join to form an ice nuclei. The ice nuclei itself is too small to cause damage to the cell, even inside the cell. However, the water molecules start to bond with the ice nuclei to form large ice crystals causing intracellular damage by accumulation of salts, solutes and gases possessing toxic effects. The dehydration caused by the cryoprotectants necessitates the ice formation outside the cell but not inside it. The difference in the ice nucleation temperature between the cells and the surrounding solutions allows the extracellular solution to freeze before the intracellular environment (Shaw & Jones, 2003). A balance between the optimal dehydration, to prohibit ice crystal formation, and the optimal amount of cryoporotectants used is needed. Very high concentrations of cryoprotectants have a toxic effect on the embryo, though the low temperetures decrease the risk of toxic effects (Vajta & Kuwayama, 2006). This challenge is more demanding in the vitrification procedure, another cryopreservation method, established by Rall and Fahy year 1985, in which higher concetrantions of cryoprotectants are needed (Rall & Fahy, 1985).

2.2.3 The vitrification

Vitrification doesn't require expensive equipment, is not time-consuming and uses small amounts of liquid nitrogen (Loutradi et al., 2008). In vitrification the solidification of solutions occur by high cooling rate to low temperature. The embryo is solified into a glass-like state reducing the risk of formation of ice crystals (Palasz & Mapletoft, 1996). High concentrations of the same cryoprotectants as in the slow freezing procedure are

needed to achieve extreme dehydration and a viscous cytoplasm. Several cryoprotectants are used to avoid the toxic effect of high concentrations of a single cryoprotectant (Ali & Shelton, 1993). Optimal exposure time to cryoprotectants is also needed to avoid the toxic effects. The embryo is then exposed to liquid nitrogen either in an open or closed system to rapidly decrease the temperature to the storage temperature of liquid nitrogen -196 °C. The warming of the cryopreserved embryo includes the rehydration by means of gradually removing the permeating cryoprotectants.

Oocyte cryopreservation trough vitrification may be superior to slow freezing, and comparable IVF-results have been achieved in a selected population with cryopreserved and fresh oocytes (Paramanantham et al., 2015). Embryo cryopreservation can be undertaken at all stages of preimplantation, from Day 1 to Day 6. Some controversy still exists on the preferable cryopreservation method at different stages of development of a preimplantation embryo. At the cleavage stage however, the current literature refers to a higher cryosurvival after vitrification compared to slow freezing (Table 2). The size of the blastocysts, the presence of a blastocoel and the multicellular structure form a challenge for the blastocyst cryopreservation.

In terms of a post-thaw embryo survival the literature quite unanimously report higher survival rates after vitrification both at the cleavage stage (Table 2) and the blastocyst stage (Table 3) (Balaban et al., 2008; Chong et al., 2014; Fasano et al., 2014; Lin et al., 2010; Pouget et al., 2015; Rezazadeh Valojerdi et al., 2009; Van Landuyt et al., 2013; Zhu et al., 2015). However, some further controversy exists concerning the effect of cryopreservation method on the pregnancy results. Especially the delivery- and miscarriage rates are seldom reported in the studies. Cobo et al. (2012) reported comparable delivery rates after Day 2, Day 3, Day 5 and Day 6 vitrified-warmed embryo transfers (Cobo et al., 2012). Several factors affect the outcomes of frozen–thawed embryo transfer cycles including the female age, and the cause of infertility (Wang et al., 2001).

The transfer of fully intact embryos results in higher pregnancy rates compared to partially damaged embryos (Abbeel et al., 1997; Burns et al., 1999). A feasible mechanism behind the lower survival rate of a partly damaged embryo is the toxic effect of the destroyed blastomere on the sibling cells (Alikani et al., 1993). The removal of necrotic blastomeres has been shown to improve the outcome of the transfers of partially damaged frozen–thawed embryos (Rienzi et al., 2002).

Table 2. Studies addressing the cryopreservation of cleavage stage embryos by slow freezing and vitrification.

Author, year	Setting	Number of participants and the age of the embryos	Survival rate	Pregnancy results
Fasano et al., 2014b	RCT	645 IVF-cycles CSE	Vitr 89.4-87.6% vs SF 63.8% (p<0.001)	No significant difference in IR or LBR
Rezazadeh Valojerdi et al., 2009	Retrospective	Vitr 153 IVF/ICSI cycles SF 152 IVF/ ICSI cycles CSE	Vitr 96.9% vs SF 82.8% p<0.0001	CPR Vitr 40.5 vs SF 21.4 p<0.001
Chong et al., 2014	Retrospective	Modified one-step SF 231 cycles vs conventional SF 295 cycles CSE	Modified One-step SF 86.9% vs conventional SF 83.1 p=0.04	CPR /LBR one-step SF 29.5%/20.7% vsconventional SF 34.0% /21.8% p=0.31/0.86
Lin et al., 2010	Retrospective	Vitr 59 cycles vs SF 45 cycles CSE	Vitr 96.4% vs SF 65.9% p<0.001	CPR/OPR Vitr 35.6%/33.9% vs SF15.6%/13.3% p=0.04/0.03
Levron et al., 2014	Retrospective observational study	Vitr 327 cycles vs SF 212 cycles CSE	Vitr 81.6% vsSF 70.0% p<0.0001	CPR Vitr 20.0% vs SF 11.9% p=0.02
Zhu et al., 2015	Retrospective cohort study	Vitr 1455 cycles vs SF 3054 cycles Day 3 embryos	Vitr 97.4% vs SF 91.5%	PR/IR /LBR Vitr 58.8/34.6%/28.8% vs SF58.4%/38.8%/29.6% p>0.05/<0.001/>0.05
Balaban et al., 2008	RCT	Vitr 234 embryos vs SF 232 embryos Day 3 embryos	Vitr 94.8% vsSF 88.7% p<0.05	Progression to the blastocyst stage Vitr 60.3% vs SF 49.5% p=0.02
Van Landuyt et al., 2013	Retrospective	Vitr 1827 vs SF 7664 Day 3 embryos	Vitr 94.3%/77.5% vs SF 64.4%/39.2% p<0.001	IR/LBR Vitr 20.7%/13.0% vs SF 20.5%/16.1%
Debrock et al., 2015	RCT	Vitr 217 embryos vs SF 200 embryos Day 3 embryos	Vitr 84.3% vs SF 52.5% p<0.0001 Fully intact embryos Vitr 75.4% vs SF 28.6% p<0.0001	IR/LBRVitr 20.7%/16.1% vs SF 7.5%/5.0% p=0.0012/<0.0022
Wilding et al., 2010	Prospective randomized study	Vitr 320 embryos vs SF 382 embryos Day 3 embryos	Vitr 93.1% vs SF 87.0% p=0.89	CPR Vitr 35.3% vsSF 35.4% p=0.16

Vitr = vitrified embryos
SF= slow frozen embryos
CSE= Cleavage stage embryos
LBR= Live birth rate
IR= Implantation rate
RCT= Randomized, controlled trial

As the embryo cryopreservation has become an intrinsic part of the IVF, the optimal timing for the embryo cryopreservation has been debated. A higher live birth rate after the blastocyst stage compared to the pronuclear stage in spite of the higher cryosurvival rate among pronuclear stage embryos was observed in Keller's study using the slow freezing technique (Surrey et al., 2010). A higher survival rate and ongoing pregnancy rate was likewise discovered after blastocyst freezing, compared to Day 3 cleavage stage

embryo freezing (Anderson et al., 2004). However, a recent randomized controlled study comparing the effect on the outcomes of 'freeze all' embryos at the blastocyst stage and pronuclear stage detected no difference between the two different stages on the ongoing pregnancy rate or the cumulative ongoing pregnancy rate (Shapiro et al., 2015). In the study, the cryopreservation protocol changed from slow freezing to vitrification mid-study, making it impossible to evaluate the effect of the cryopreservation method on the results.

Table 3. Studies addressing the cryopreservation of blastocyst stage embryos by slow freezing and vitrification.

Author, year	Setting	Number of participants	Survival rate	Pregnancy results
Pouget et al., 2015	Retrospective	Vitr n=86 SF n=86	Vitr 97% vs SF 86% (p<0.0001)	IR/CPR Vitr 32%/43% vs SF 20%/28% p=0.02/0.04
Kuć et al., 2010	Retrospective	Vitr n=58 SF n=189		PR Vitr 54% vs SF 25.9%
Li et al., 2014	Population based cohort study	Vitr cycles n=20 887 vs SF n= 12 852		Vitr vs SF CPR ARR 1.47 DR ARR 1.41
Bernal et al., 2008	Prospective randomized study	Vitr n=64 SF n=51	Vitr 93% vs SF 76% p= <0.0001	CPR Vitr 65% vs SF 55% (NS)

Vitr = Vitrified embryos
SF = Slow frozen embryos
ARR = Adjusted relative risk
IR= Implantation rate
CPR= Clinical pregnancy rate

2.3 Factors affecting the IVF-results

2.3.1 The length of the embryo culture

The embryo transfer at the cleavage stage was the prevailing routine in IVF for a long period of time before the implementation of the blastocyst culture. In vivo the embryo enters the uterine cavity at the blastocyst stage and the embryo transfer at cleavage can therefore be considered as unphysiologic (Croxatto et al., 1978). In other mammalian species the transfer of a cleavage stage embryo to uterine cavity leads to significantly impaired pregnancy results compared to the blastocyst transfers (Bavister, 1995; Marston et al., 1977). A number of studies have found preferable pregnancy results after a blastocyst transfer in comparison with a cleavage stage embryo transfer (Karaki et al., 2002; Papanikolaou et al., 2006; 2005; Sotiroska et al., 2015; Balaban et al., 2004). In other studies, however, similar results between cleavage stage and blastocyst stage embryo transfers have been presented (Emiliani et al., 2003; Guerif et al., 2009; Han et al., 2012). In a Cochrane Database Systematic Review by Glujovsky et al. (2012), a higher live birth rate was observed after blastocyst

stage transfer compared to Day 2–3 transfers. The cumulative clinical pregnancy rate was nevertheless higher in the cleavage stage embryo group. (Glujovsky et al., 2012.) This can be at least partially explained by the loss of embryos during the blastocyst culture. The embryo genome activates approximately at the 8-cell stage, prior to compaction before which the embryo development is controlled by the genome of the oocyte (Braude et al., 1988). After the activation of the genome, the developmental potential of the embryo becomes tested and, as a consequence, a proportion of the chromosomally abnormal embryos discontinue the development. In the worst case this leads to a situation with no embryos for a transfer after a culture of five days. The rationale behind higher implantation rates after blastocyst transfer compared to cleavage stage reported in some studies, could be the natural selection of embryos. At the blastocyst stage, the possibility to transfer a chromosomally intact embryo may, in theory, be higher than at the cleavage stage because of the deletion of chromosomally aberrant embryos during the culture. In a study by Gardner et al. high pregnancy rates were reported after a double embryo transfer of top-scoring blastocysts (87%) with a twinning rate of 61%. When two low-scoring blastocysts were transferred, the pregnancy rate was 44% and twinning rate 29% (Gardner et al., 2000). Gardner concluded that sufficient pregnancy rates could be achieved by single embryo transfer of blastocyst, since the double embryo transfers were this exceptionally high.

The outcomes of IVF treatments are dependent on multiple factors, ranging from the etiology of the infertility to the controlled ovarian hyperstimulation, oocyte and embryo quality, to endometrial receptivity and various other factors. Two large studies by Roberts et al. investigated the factors affecting outcomes of IVF. The age of the woman has a detrimental effect on the success of IVF and was found to be the most important predictor of live birth. Age mostly affected the embryo, not the uterine receptivity. Blastocyst culture was associated with increased embryo viability, associated both with the natural selection of the embryos and uterine receptivity. Factors, such as the number of embryos, the number of attempts, previous history of pregnancy, duration of infertility, the culture length and the tubal cause of infertility proved to be significant prognostic factors for IVF results (Roberts et al., 2015; 2010). Additionally, adequate ovarian responsiveness and the number of oocytes retrieved have an effect on the IVF success (Pouly et al., 2012; Qublan et al., 2005). In contrast to maternal age, paternal age has no effect on the IVF outcome (Meijerink et al., 2016).

Embryo quality affects the success rate. One or more top quality embryos at the ransfer results in a significant increase in live birth rate (OR 3.41) (Veleva et al., 2013). Early pregnancy miscarriage increases the change of pregnancy in the subsequent cycle among patients with repeated IVF failures (Haas et al., 2012). Dhillon et al. studied the effect of ethnicity on the IVF outcome. According to the results, Black and South Asian women have a lower clinical pregnancy rate and live birth rate compared with white women. The explanation for this difference remained unclear and needs to be studied further (Dhillon et al., 2015).

2.3.2 Endometrium

The implantation of the conceptus occurs normally 8–10 days after ovulation (Wilcox et al., 1999). In fresh IVF cycles the endometrium is approximately 2–3 days advanced compared to natural cycles induced by the human chorionic gonadotropin (Papanikolaou et al., 2010). A premature expression of pinobodes and integrins has been detected in an advanced endometrium (Bourgain & Devroey, 2003). This shift in the implantation window may have a detrimental effect on the success of the implantation as well as on the development of the fetus. This altered window of implantation in the fresh embryo transfer cycles is probably due to the superovulation caused by the supraphysiologic concentrations of hormones caused by the controlled ovarian hyperstimulation of the exogenous FSH. No difference in the luteal phase endometrial histology and the serum progesterone concentration prior to oocyte retrieval was detected between the GnRH agonist and antagonist cycles (Saadat et al., 2004). A high progesterone level on the day after hCG administration is the main reason for the advanced endometrium (Liu et al., 2015). High estradiol concentrations during the ovarian hyperstimulation cause the progesterone levels to rise high before the oocyte retrieval, thus hampering the implantation. The chance of an ongoing pregnancy is significantly impaired when the endometrial advancement of the oocyte retrieval is 3 days compared to the expected chronological rate (Kolibianakis et al., 2002). This adverse effect can be overtaken by the 'freeze all' strategy. In a prospective randomized study by Shapiro et al. (2011), a significantly higher CPR was detected after a frozen–thawed blastocyst transfer compared to fresh transfers (84% vs 54.7%). This was suspected to be related to an impaired endometrial receptivity after ovarian stimulation in fresh cycles (Shapiro et al., 2011).

The histological changes caused by the superovulation on the endometrium has been found to affect the trophoblast differentiation and placental gene expression in mice (Mainigi et al., 2014). In a human study, endometrial biopsies were performed 6 days after the oocyte pick-up and the global miRNA and mRNA gene expressions were examined. Differences in the gene expression in 4 miRNA and 22 mRNA was found in respect to the progesterone level on the day of the hCG injection (Li et al., 2011). Increased risk for low birth weight associated to ART-conceived children and higher mean birth weight after frozen embryo transfers compared to fresh embryo transfers could originate from this altered endometrial status around the superovulation. In a recent study equivalent live birth rates were observed between fresh and frozen–thawed single embryo transfers. The newborns from the vitrified-thawed embryo transfers were 145g heavier than the ones from fresh embryo transfers (Roy et al., 2014).

Endometrium priming prior the frozen–thawed embryo transfer is usually undertaken by means of hormonal substitution using estrogen and natural progesterone, in the natural cycle with or without hCG or in a clomiphene/letrozol induced cycle. No significant difference in the results between the protocols in the frozen–thawed transfer cycles have been observed (Ghobara & Vandekerckhove, 2008). In a study by Tomás et al. a higher

number of positive pregnancy tests, yet a higher number of early pregnancy losses in hormonally substituted cycles, were observed as leading to similar delivery rates between the protocols (Tomás et al., 2012).

2.4 Miscarriage

Miscarriage is a common pregnancy complication, affecting as much as 31% of all pregnancies (Wilcox et al., 1988). The incidence of clinically recognized spontaneous abortion varies in studies approximately from 10 to 20%. In Wang's prospective study, the incidence of an early pregnancy loss was 24.6%, while the incidence of clinical spontaneous abortions was 7.9% (Wang et al., 2003). In a prospective observational study by Zinaman, the total pregnancy loss was 31% of the pregnancies detected. 41% of these were recognized only biochemically (Zinaman et al., 1996). Approximately 80% of all miscarriages occur within the first trimester (Wilcox et al., 1988). The most common cause of the miscarriage are the chromosomal abnormalities of the fetus, the trisomy being the most frequent form of aneuploidy. The frequency of euploid miscarriages is higher among younger women (less than 36 years of age) (Stephenson et al., 2002). Approximately 60% of miscarriages are associated with sporadic chromosomal abnormalities studied from the examined products of conception. The most common aneuploidies in miscarriages of the IVF pregnancies are trisomies in the chromosomes 15, 16, 18, 21 and 22 (Rodriguez-Purata et al., 2015).

Factors such as the age of the woman, antiphospolipid syndrome, uterine anomalies, trombophilias, hormonal or metabolic disorders, parental chromosomal abnormalities, such as translocations, infection e.g. endometritis, autoimmunity and lifestyle habits have been found behind recurrent early pregnancy losses (Practice Committee of the American Society for Reproductive Medicine, 2012).

A large population study including more than 52 000 pregnancies was undertaken to examine the effect of embryo freezing and blastocyst culture on the miscarriage rate of IVF pregnancies. The overall miscarriage rate in the study was 18.7%. Singleton pregnancies occuring after a fresh double embryo transfer had a 1.43 times higher risk to become terminated into a miscarriace, compared with singleton pregnancies after a fresh single embryo transfer. Fresh blastocyst transfers were associated with 8% less of a hazar of a miscarriage, when compared with cleavage stage embryo transfers, while thawed blastocysts were associated with a higher hazard of a miscarriage (14%). The authors suggested a practice of transferring fresh blastocysts and frozen–thawed cleavage-stage embryos in order to reduce the miscarriage rate after ART (Wang et al., 2011).

Frozen embryo transfers have been discovered to increase the miscarriage rate compared to fresh embryo transfers. Aflatoonian et al detected a higher risk of a miscarriage after a frozen embryo transfer (14.5%) compared to fresh embryo transfer (9%) (Aflatoonian et al., 2010). In Veleva's study the respective numbers were 23% and 13.8% (Veleva et al.,

2008). Nevertheless, contradictory results about the effect of embryo cryopreservaton on the miscarriage rate have been presented (Hipp et al., 2015).

A previous abortion has been studied to be a positive (Bates & Ginsburg, 2002; Haas et al., 2012) as well as a negative (Yang et al., 2015) predictor of succes in the subsequent IVF cycles. Rittenberg et al. studied the impact of BMI on the miscarriage rate after a single blastocyst transfer. Of the study population 27% suffered a miscarriage before 23 weeks of gestation. The increase of the BMI ≥25 on the OR of miscarriage before 23 weeks in fresh and frozen–thawed cycles were 2.7 and 6.8 respectively (Rittenberg et al., 2011). The risk of a miscarriage is likewise increased in underweight women (Veleva et al., 2008).

2.5 Factors affecting the weight of the newborn

2.5.1 The lifestyle factors

Birth weight is known to have a multifactorial etiology, the gestational age explaining approximately 80% of the total variation in birth weight (Amini et al., 1994). In a Swedish study by Clausson, genetic factors were found to explain 25–40% of the variations in birth weight (Clausson et al., 2000). A low maternal birth weight and a 'small for gestational age' status are associated to the low birth weight or SGA of the children (Shah et al., 2009). Maternal obesity and excess weight gain during the pregnancy of mothers with a normal pre-pregnancy weight are risk factors for a birth weight >4000gr and 'large for gestational age' (LGA) (Crane et al., 2009; Rode et al., 2007) while a low pre-pregnancy BMI predisposes newborns to a low birth weight (<2500g) (Han et al., 2011; Murakami et al., 2005; Viswanathan et al., 2008). Maternal smoking increases the risk of a low birth weight of newborns (Einarson & Riordan, 2009; Knopik, 2009). Gestational diabetes is known to increase intrauterine growth with the associated perinatal complications (Simmons, 2011; Yessoufou & Moutairou, 2011). Boys have a higher mean birth weight compared to girls and younger siblings weigh more at birth compared to the first born (Oken et al., 2003).

Several studies have shown that the singletons born after in vitro fertilization are lighter and more likely to be small for gestational age (SGA), compared to naturally conceived infants (Dawood, 1996; Doyle et al., 1992; Schieve et al., 2004; Tan et al., 1992). In a meta-analysis by Helmerhorst et al. the relative risk (RR) of low birth weight (<2500gr) among IVF-singletons was 1.70 and 1.40 for SGA compared with children born from natural pregnancies. The relative risk of a very low birth weight (<1500gr) was 3.00 (Helmerhorst et al., 2004). Subfertility per se is suspected to increase the risk of a low birth weight (Joffe & Li, 1994; McElrath & Wise, 1997).

2.5.2 The effect of cryopreservation on birth weight

The effect of embryo freezing on the health of the newborn has been studied, using the birth weight as one parameter. A higher birth weight has been detected among children born from both vitrified-warmed cleavage stage embryo transfers compared to fresh embryo transfers and from blastocyst transfers compared to cleavage stage embryo transfers (Kato et al., 2012; Liu et al., 2013; Shi et al., 2012), yet contradictory results have been published as well (Rama Raju et al., 2009). A large Japanese study of frozen–thawed blastocyst transfers found an increased risk for LGA in children born after frozen–thawed single blastocyst transfers (Ishihara et al., 2014). A Swedish registry study found an increased rate of LGA and preterm birth after cryopreserved/thawed transfers, while a Danish study observed a higher mean birth weight after cryopreservation (Pinborg et al., 2010; Sazonova et al., 2012).

2.5.3 The effect of the culture length on birth weight

In a blastocyst culture the embryos are exposed to an unphysiological environment for a longer period of time than when transferred at the cleavage stage. An increased rate of preterm deliveries and congenital malformations have been detected in children born from a blastocyst transfer compared to cleavage stage babies (Dar et al., 2013; Källén et al., 2010; Kalra et al., 2012; Maheshwari et al., 2013). In some studies such a difference was not detected (Fernando et al., 2012). Mäkinen et al. was the first to discover an increased rate of LGA in newborns originating from blastocyst transfers compared to cleavage stage transfers (Mäkinen et al., 2013). Not only was the number of LGA babies increased but the mean birth weight in general was higher after a blastocyst transfer, compared to a cleavage stage embryo transfer, as found in a study by Zhu (Zhu et al., 2014).

2.5.4 The effect of the culture media on the birth weight

In the literature, contradictory views have been presented on the question of whether the IVF culture media affect the birth weight of the newborn. Other researchers have found significant differences between the birth weight of children from embryos cultured in different culture media, while some have detected weight differences as early as on the second trimester, lasting until 2 years of age (Kleijkers et al., 2014; Nelissen et al., 2012b; 2013; Zhu et al., 2014). On the other hand, increasing evidence supports the perception of the culture media having no impact on the birth weight (Dumoulin et al., 2010; Lemmen et al., 2014; Lin et al., 2013; Yin et al., 2015). A recently published Cochrane review evaluated the safety and effectiveness of different culture medias used in IVF. Due to the

wide heterogeneity and poor quality among the studies there wasn't enough evidence to support or to discard the use of any of the media (Youssef et al., 2015).

2.6 Epigenetics

The genome undergoes several phases of epigenetic programming during the early embryonic development and gematogenesis. During the gametogenesis, a massive epigenetic reprogramming is undertaken, including the deletion of parental imprints, which are later replaced by new imprints during the spermatogenesis in male or oogenesis in female. The genome is demethylated at the fertilization followed by a wave of the novo methylation. The human genome is mainly regulated by the epigenetic modifications of which the DNA methylation at cytosine residues of CpG dinucleotides, in the gene promoting regions, is one of the most important.

Another epigenetic mechanism, genomic imprinting, is a means of epigenetic regulation by which certain genes are expressed monoallelically, depending on their parental origin. (Le Bouc et al., 2010.)

Epigenetic modifications, in general, affect the activity of the genome without interfering with the DNA sequence. Due to the simultaneous timing of the active epigenetic changes, controlled ovarian hyperstimulation, fertilization by IVF or ICSI, and the embryo culture and freezing are thought to have an impact on the epigenome and possibly later on the phenotype. The possible effect of the ART on the epigenome has been discussed widely in the literature. Superovulation, with the associated effects on the endometrium has been considered as a potential factor for epigenetic changes possibly affecting the conceptus or the placentation. Abnormal placentation is a possible explanation for the increased incidence of a low birth weight among IVF/ICSI-children.

More than 5 million children have been born as a result of assisted reproduction technology since the born of Louse Brown in 1978. The accumulated information on the health of these children has raised rare imprinting disorders, such as Beckwith-Wiedemann syndrome and Angelman, of which prevalence has been detected to be increased among children conceived trough IVF/ICSI (Amor & Halliday, 2008; Odom & Segars, 2010).

When combining the information of eight epidemiological studies, the relative risk of the birth of a child with Beckwith-Wiedemann syndrome following IVF/ICSI was 5.2. Angelman or Prader-Willi syndromes were not associated with IVF and ICSI treatments but rather with subfertility of the parents. Disentangling the causes of subfertility from the ART-procedures as the ultimate cause of the epigenetic changes presents a challenge. Weiderman et al. concluded that no proof of a causal relationship between imprinted diseases and IVF/ICSI treatments exists, even though imprinting disorders are more prevalent after IVF/ICSI. The infertility problems behind the imprinting disorders should be assessed (Vermeiden & Bernardus, 2013). Song et al. tried to solve this problem by comparing the placental DNA methylation levels at 37 CpG sites in 16 genes between

children that were conceived naturally, children conceived by ART with autologous oocytes, and children conceived through ART with donated oocytes from fertile donors. According to the results of the data analysis, the differences in the DNA methylation between naturally and in vitro conceived children are associated with the ART protocol, not the infertility (Song et al., 2015).

Nonetheless, in a recent meta-analysis, comprising 18 studies, an increased risk of imprinting disorders in children conceived trough ART was detected.

However, if there are changes in imprinting epigenetic status between children conceived spontaneously or as a result of ART, the effects are small in magnitude (Lazaraviciute et al., 2014). In samples taken from the cord blood evidence of DNA-methylation defects in children born after IVF/ICSI has been found in some (Zheng et al., 2011) but not in all studies (Melamed et al., 2015), in which the study populations tend to be small. On the other hand, the DNA methylation of SNRPN (small nuclear ribonucleoprotein polypeptide N), an imprinted gene in buccall cell samples taken from 20 children born after ICSI, was higher than in spontaneously conceived children (Whitelaw et al., 2014). Placental hypomethylation has been examined to occur after IVF/ICSI (Nelissen et al., 2013a). The manipulation of embryos and oocytes in the laboratory, such as exposing them to an unnatural milieu through cryopreservation, different culture lengths and culture media could, in theory, cause epigenetic alterations during the time period of intense epigenetic activity of early embryonic development. Genomic imprinting affects genes that are known to affect the placental and fetal growth.

A moderate increase in the risk (adjusted OR 1.24) of congenital anomalies in children born from a fresh or frozen embryo transfer, compared to the naturally conceived embryos, has been found. The effect of subfertility on the results was not assessed (Pelkonen et al., 2010).

One of the most important factors of fetal growth is the Insulin-like growth factor 2 (IGF2), also known as the somatomedin. The expression of the IGF2 gene is regulated by the epigenetic marks (methylation) of the IGF2/H19 differentially methylated regions (DMRs). The placental DNA methylation changes at the IGF2/H19 imprinted locus are related to birth weight and fetal development (St-Pierre et al., 2012). A systematic review, however found only a weak and inconsistent association between the epigenetic abnormalities of IGF-related genes and LBW or SGA (Toure et al., 2016).

3 AIMS OF THE STUDY

The poor quality cleavage stage embryos are often discarded due to the supposed poor implantation potential. The poor quality cleavage stage embryos can, however, develop to good quality blastocysts. The effect of the extended culture of poor quality Day 2–3 embryos on the cumulative live birth rate has not been previously studied.

The embryos are most often transferred on Day 2, Day 3, Day 5 or Day 6. The Day 4 embryo transfers are rarely reported in the literature.

Increased risk of congenital anomalies and low birth weight as well as preterm birth has been associated with ART. In some studies blastocyst culture has been associated with increased birth weight, while in other studies this association was not detected. The effect of the cryopreservation method on the birth weight has been studied with conflicting results.

The present study was undertaken to answer the following questions:

1. How often do the poor quality cleavage stage embryos reach the blastocyst stage, and what is the effect of the extended culture on the cumulative delivery rate? (Study I)
2. Are the Day 4 embryo transfers (both fresh and vitrified-warmed) associated with similar clinical outcomes compared to Day 3 and Day 5 embryo transfers? (Study IV)
3. Does the blastocyst culture affect the birth weight of the newborn and is the possible phenomenon associated with the sex of the newborn? (Study II)
4. How does the cryopreservation method of cleavage stage embryos affect the clinical outcomes and does it have an effect on the birth weight of the newborn? (Study III)

4 SUBJECTS AND METHODS

4.1 Patients

All the studies were conducted retrospectively using the patient records as a data source. All the IVF/ICSI treatments of studies I, III and IV were undertaken at the Infertility clinic of Tampere University Hospital (TAYS) in collaboration with Infertility Clinic Ovumia, which provided the laboratory services during the study period. Of the IVF/ICSI cycles undertaken at the Infertility clinic of Tampere Iniversity Hospital, the controlled ovarian hyperstimulation was commenced and monitored either at the Infertility Clinic of Tampere Universty Hospital, Vaasa Central Hospital, Etelä-Pohjanmaa Central Hospital, Satakunta Central Hospital or Kanta-Häme Central Hospital.

For study II the study material of fresh embryo transfers was collected from TAYS, Infertility Clinic of Karolinska University Hospital Huddinge and Infertility Clinic Fertinova (Tampere and Helsinki) (Table 4). The newborns from cleavage stage embryo transfers and blastocyst transfers were matched by the gender and the pregnancy week of the delivery. The distribution of the study material (II) is presented in Table 4.

A total of 135 frozen–thawed blastocyst babies, with 135 cleavage stage frozen–thawed controls, were included in the study, all from TAYS. The characteristics of the study groups are presented in the Tables 5 and 6.

Table 4. The distribution of cases and controls of Study II by the clinic of the infertility treatment

	Fresh D 2–3	Fresh D 5–6	FET D 2–3	FET D5–6
TAYS	75	75	135	135
Karolinska	53	53		
Fertinova	14	14		
All	142	142	135	135
Girls	75	75	67	67
Boys	67	67	68	68

Table 5. Characteristics of the study populations of the four studies as well as the main objectives and the main outcome measures.

Groups	Study I D2–3/D5–6	Study II D2–3/D5–6	Study III SF/Vitr	Study IV Day 3/4/5
Study design	Retrospective	Retrospective	Retrospective	Retrospective
Number of women	512	554	871	
Number of cycles	604	554	418/453	
Number of fresh embryo transfers	604	142/142	848	1371/369/326
Number of frozen-thawed embryo transfers	134/306	135/135	663/683	635/216/654
Mean age of women (years)	32.0 ± 4.1	32.6 ± 4.0/ 31.6 ± 4.0	31.4 ± 4.0/ 32.1 ± 4.2	32.2 ± 4.2/ 32.0 ± 4.1 / 31.6 ± 4.1
Main objectives	To examine the effect of extended culture of poor quality cleavage stage embryos on the cumulative pregnancy rate	To examine the effect of blastocyst culture on the weight of the newborns boys and girls separately	To study the effect of the cryopreservation method of cleavage stage embryos on the birth weight of the newborns.	To investigate the effect of embryo culture length on the clinical outcomes of fresh and vitrified-thawed embryos
Study period	1.8.2008-30.11.2011	1.1.2008-31.3.2014	1.4.2009-30.10.2013	1.1.2009-31.12.2014
Main outcome measures	The live birth rate of good quality fresh and FET and poor quality FET	The weight of the newborns from fresh and FET cleavage stage and blastocyst transfer cycles due the gender	The weight of the newborns from vitrified-warmed and frozen–thawed embryo transfer cycles as well as clinical outcomes	CPR, LBR, MCR of fresh and vitrified-warmed embryo transfers

SF = slow frozen embryos
Vitr = vitrified embryos
FET = Frozen–thawed embryo transfers
CPR = Clinical pregnancy rate
LBR = live birth rate
MCR = Miscarriage rate

Table 6. The proportion of the antagonist protocol used in the studies as well as the proportion of the main causes of the infertility.

	Study I	Study II D2–3/ D5–6	Study III SF/ Vitr	Study IV D3/4/5
Antagonist %	46.0	42.3 / 40.1	45.5 / 52.3	58.7 / 61.1 / 45.5
Main causes of infertility				
Male origin %	32.3	30.7 / 30.9	30.9 / 26.3	36.8
Unexplained %	30.0	33.6 / 27.3	29.2 /29.8	30.5
Endometriosis %	13.4	10.0 / 7.2	12.4 / 16.1	3.9
Anovulation %	10.9	9.3 / 12.9	12.9 / 11.0	3.4
Multiple reasons %	8.0	12.1 / 15.1	8.4 / 9.1	46.3

4.2 Methods

The study plan for all four studies were approved by the Institutional Review Board of Tampere University Hospital. The study material was collected from the medical records. Only cycles with autologous oocytes were included in the study.

4.2.1 The controlled ovarian hyperstimulation protocol

The ovarian stimulation was performed at each center accordingly to their routines. At all centers, the controlled ovarian stimulation was accomplished using either antagonist (Orgalutran® Organon, Ireland or Cetrotide® Merck Serono, Switzerland), long agonist (Synarela® Pfizer, Belgium), or short agonist protocols (Table 6). The study material has been collected from patient records and the controlled ovarian hyperstimulation has been described more accurately in the original communications (I–IV).

4.2.2 The culture conditions

All the embryos of studies I, III and IV were cultured in the laboratory of Ovumia, with which Tampere University Hospital collaborated during the study period. In study II, all the frozen–thawed embryos were cultured in the Ovumia laboratory whereas a part of the fresh embryos were cultured in the laboratories of Karolinska University Hospital Huddinge, Stockholm, Sweden and Infertility clinic Fertinova Tampere and Helsinki.

The culture media were supplied by Origio (Måløv, Denmark) or Vitrolife (Gothenburg, Sweden). Successful fertilization was confirmed 16–18 h after the insemination (hpi). The normally fertilized zygotes were cultured in sequential media, using ISM1™ (Origio) as the cleavage stage medium until Day 2 or 3, or G-1™ PLUS (Vitrolife) until Day 3. In case of the blastocyst culture, the cleaved embryos were moved from ISM1™ to blastocyst culture

in BlastAssist™ (Origio) on Day 2, or in ISM2™ (Origio) on Day 3, or from G-1™ PLUS to G-2™ (Vitrolife) on Day 3. The blastocyst culture was performed in a reduced oxygen atmosphere (6% CO2/6% O2/88% N2) until Day 5 or 6. The low oxygen culture was used from Day 2 or 3 onwards as the embryos were moved from the cleavage stage culture to the blastocyst culture. The low oxygen culture was not used for the entire length of the culture period owing to the limited bench top incubator capacity available.

4.2.3 The embryo evaluation at the cleavage stage

Embryo evaluation was carried out on Day 2 (44–46 hpi) and Day 3 (68–70 hpi) and a modified scoring system was applied (Fridström et al., 1999; Mohr et al., 1985). In the scoring system (Table 7) an embryo with the expected developmental stage (i.e. four cells on Day 2 or eight cells on Day 3) received a starting score of 3.5, whereas slowly or fast cleaving embryos received a starting score of 3.0. The starting score was then reduced in increments of 0.5 for every reducing factor observed: the presence of more than 10% fragmentation, an unequal size of blastomeres, a large perivitelline space or cytoplasm abnormalities. Drastic reducing factors subtracting 1.0 point were the presence of more than 25% of fragmentation or the presence of multinucleated blastomeres.

Table 7. The embryo evaluation schema for cleavage stage embryos. In study I the embryo was classified as poor quality with a score below 2.0 or a score of 2.0 with more than 25% fragmentation or presence of multinucleated blastomeres.

Starting score	
3.5	Embryo with expected developmental stage: 4 cells on Day 2 or 8 cells on Day 3
3.0	Slowly or fast cleaving embryo
Reduction parameters	
-0.5	Presence of more than 10% fragmentation Unequal size of blastomeres A large perivitelline space Cytoplasm abnormalities
-1.0	Presence of more than 25% of fragmentation Presence of multinucleated blastomeres

In study I one or two embryos with the highest score were transferred on Day 2 or 3 using ultrasound guidance and a Sydney® catheter (Cook). On the day of the fresh embryo transfer, the selected good-quality embryos with a minimum score of 2.0 and no drastic reducing factors were frozen, using an Embryo Freezing Pack (Origio) and the standard slow-freezing method. Poor quality embryos, with a score below 2.0, or a score of 2.0 with more than 25% fragmentation or a presence of multinucleated blastomeres, were cultured to the blastocyst stage (Figure 8).

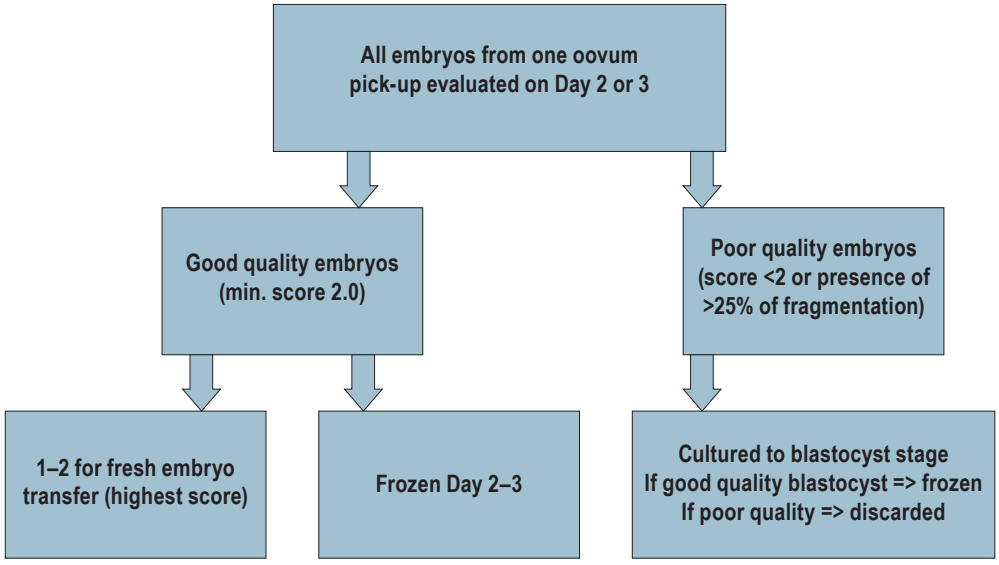


Figure 8. The flow-chart describing the fate of embryos in Study I.

The reassessment of the embryos, as defined by Gardner and Schoolcraft, (1999) was carried out on Day 5 or 6 (Gardner & Schoolcraft, 1999). An embryo was selected for cryopreservation by vitrification if it had reached at least a fully compacted morula stage by Day 5 or the true blastocyst stage (i.e. stage 3) by Day 6. At the blastocyst stages 2–6, the inner cell mass and trophectoderm class A or B had to be present.

4.2.4 The freezing and thawing protocols

In studies I, II and III, cleavage stage embryos (except the vitrified embryos in study III) were frozen using an Embryo Freezing Pack (Origio) and the standard slow-freezing method. The cooling rate was controlled by the Freeze Control™ (Cryologic Ltd., Mulgrave, Australia) liquid nitrogen freezing apparatus. Thawing was performed using the Embryo Thawing Pack. The cryoprotectants included propylene glycol and sucrose. The survived

embryos were cultured overnight in BlastAssist, and those that had cleaved were selected for embryo transfer.

The vitrification and warming of the vitrified Study III embryos, and Study II and IV embryos were performed according to the instructions of the manufacturer (VitriFreezeES™, VitriThawES™, Fertipro, Beernem, Belgium). In studies II, III and IV, the embryos that were vitrified, were vitrified using CBS™ High Security Vitrification straws (Cryo Bio Systems, Láigle, France), and a VitriFreeze ES™ vitrification kit (FertiPro, Beernem, Belgium). In vitrification, the main cryoprotectants were dimethyl sulfoxide and ethylene glycol.

The embryos that were vitrified or slow-frozen on Day 2, Day 3 or Day 4 as well as the compacted morulas and early blastocysts (studies I, II, III and IV) were warmed/thawed 1 day before the embryo transfer and the survived embryos (minimum of 50% of blastomeres alive) were cultured overnight in BlastAssist. The embryos that had cleaved were evaluated and selected for embryo transfer. Embryo survival was defined as cleavage during the overnight culture, except in study IV in which the survival was defined more accurately: The percentage of embryos surviving the warming, the percentage of intact embryos after the warming, and the proportion of embryos transferred/embryos warmed. The developmental stages 2–6, as defined by Gardner and Schoolcraft 1999, i.e. the true blastocysts, were thawed on the day of transfer, using the VitriThaw ES™ thawing kit.

4.2.5 Frozen–thawed embryo transfers

The frozen–thawed embryotransfers (FET) were performed in spontaneous or hormone-substituted cycles. In hormone-substituted cycles, estradiol hemihydrate as tablets (Zumenon® Abbot Biologicals BV, Neatherlands, 2 mg three times a day) or transdermal patches (Estradot® Novartis Pharma GmbH, Germany, 100–150 μg twice a week) were used from the beginning of the menstrual cycle, in addition to natural vaginal progesterone tablets (Lugesteron® Besins Healthcare Ltd, France, 200 mg x 3 a day), starting when the endometrial thickness measured 7–8 mm. The age of the embryo determined the day of embryo transfer. In substituted cycles the length of the progesterone use along with the estrogen was adjusted considering the age of the embryo. One or two embryos were transferred using ultrasound guidance.

In the present study, a clinical pregnancy was assessed with a transvaginal ultrasound 5 weeks after the embryo transfer and defined as the presence of at least one gestational sac on the ultrasound scan. A spontaneous abortion was considered to be a pregnancy loss until week 22 of gestation, after a previous confirmation of a clinical pregnancy. The clinical outcomes are defined in the study as follows: the clinical pregnancy rate as the proportion of clinical pregnancies of the embryo transfers; The miscarriage rate as the proportion of miscarriages of the clinical pregnancies and the live birth rate as the proportion of live births of the embryo transfers.

4.3 Statistical analyses

The statistical analyses are more accurately described in the original communications (I–IV). In the study I the results are detailed in terms of SD or percentage. When evaluating the factors affecting the success of an extended culture, a binary logistic regression was used.

For study II a pairwise t-test was used for a matched analysis between weights of cleavage stage embryo babies, and blastocyst stage embryo babies. Student's t-test was used for comparing the weights between the genders, and also between fresh and frozen–thawed embryo transfers.

In study III the difference between birth weight and the national gestational age and gender-adjusted reference weight is expressed as the standard deviation score (SD-score). The difference between mean weights of the babies from frozen–thawed and vitrified-warmed embryo transfers was considered clinically significant when exceeding 100gr. A t-test for independent samples was used when comparing the mean birth weights between the groups. A chi-square test was used to compare the delivery rates, clinical pregnancy rates and miscarriage rates between the groups.

In study IV the qhi-square test was used to compare the delivery rates, clinical pregnancy rates and miscarriage rates between the groups. The Kruskal-Wallis test was used for statistical comparison of the cryosurvival rates. All statistical analysis were performed using the IBM SPSS software (v19.0 Armonk, NY, USA).

5 RESULTS

5.1 The effect of the culture length on the clinical outcomes of the IVF/ICSI treatments (Studies I and IV)

In the Study I altogether 1879 surplus embryos were cultured further (48.2% of all the embryos). The percentage of the poor quality cleavage stage embryos, that reached the blastocyst stage and became chosen for vitrification due to the good quality, was 19.7%. These originally poor quality embryos, improving the quality during the culture, resulted in a clinical pregnancy rate of 24.6% and a delivery rate of 17.2%. The miscarriage rate was 27.3%. The 23 deliveries from the transfers of extendedly cultured embryos increased the cumulative delivery rate from 43% to 47% (Figure 9). With a delivery rate of 47%, 53 repeated IVF/ICSI cycles would be required to achieve the observed 23 ”extra” deliveries, leading to the conclusion that 53 repeated new IVF/ICSI cycles were avoided due to the extended culture of poor quality cleavage stage embryos (Figure 9).

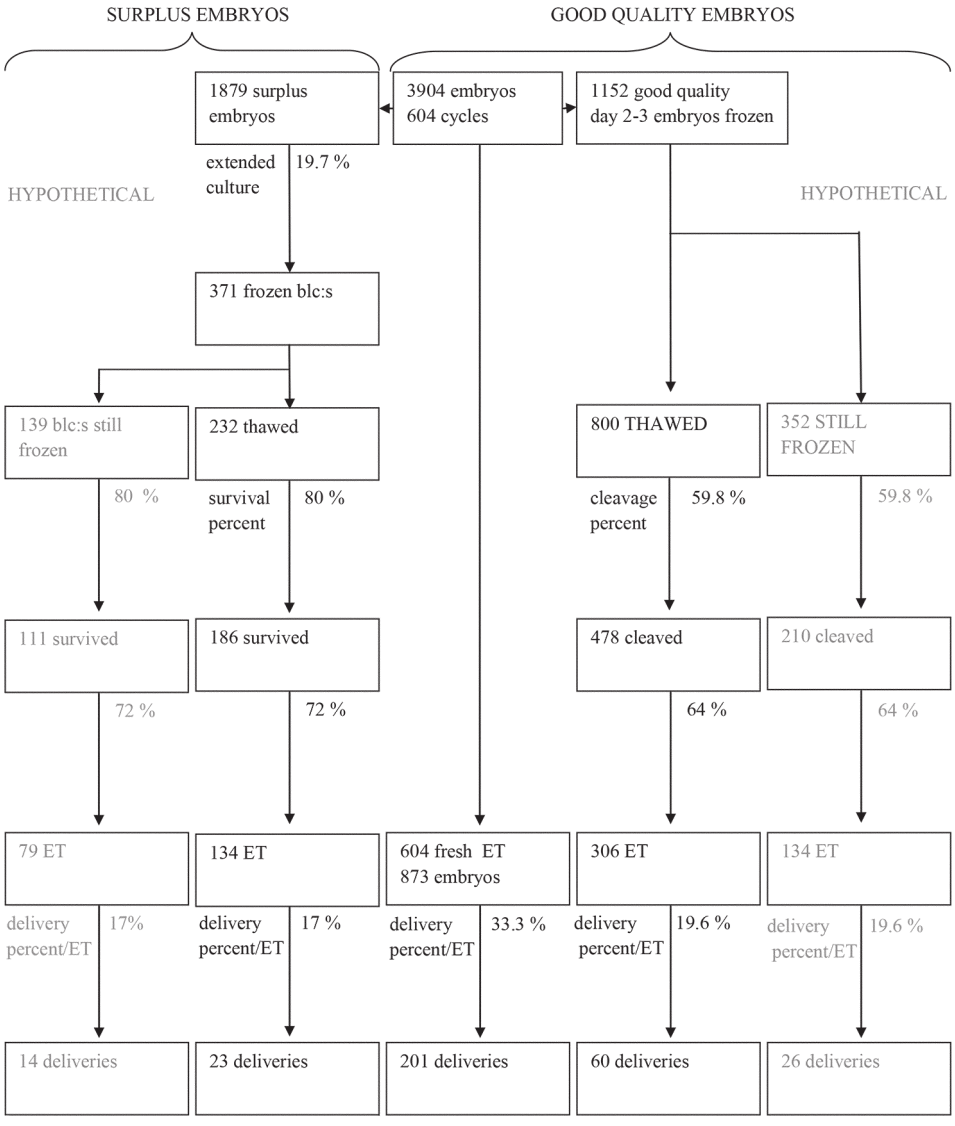


Figure 9. The flowchart depicting the fate of poor and good quality embryos as well as the hypothetical outcome of poor and good quality embryos remaining in storage. Hypothetical values are expressed as numbers followed with 95 % confidence intervals. ET = embryo transfer.

5.2 The Day 4 embryo transfers are associated with good pregnancy results (Study IV)

The fresh Day 4 embryo transfers resulted in higher live birth rates (31.4%) compared to fresh Day 3 (26.5%) or fresh Day 5 (24.5%) embryo transfers (p= 0.032 and 0.026 respectively) (Table 8). The clinical pregnancy rate was higher after fresh Day 4 embryo transfers compared to fresh Day 3 or fresh Day 5 embryo transfers without a statistical significance. In addition, the miscarriage rate was statistically significantly lower after the fresh Day 4 embryo transfers compared to fresh Day 5 embryo transfers.

The Day 4 vitrified-warmed embryo transfers resulted in a higher live birth rate and lower miscarriage rate compared with Day 3 and Day 5 vitrified-thawed embryo transfers. (Table 8).

Table 8. The clinical outcomes of fresh and FET Day 3, Day 4 and Day 5 transfers. The p-values for comparison with Day 4 results.

	Day 3 %	p-value for Day 3 vs Day 4	Day 4 %	Day 5 %	p-value for Day 5 vs Day 4
Fresh					
CPR	33.9	0.142	38.1	34.7	0.356
LBR	26.5	0.032	31.4	24.5	0.026
MCR	16.6	0.165	11.7	23.0	0.017
FET					
CPR	29.9	0.017	39.4	32.0	0.63
LBR	22.0	0.016	30.9	22.6	0.024
MCR	21.6	0.317	15.9	27.8	0.049

The fresh Day 4 embryos were devided in three groups according to the developmental stage; Group 1 (embryos at the early morula stage), Group 2 (partially or fully compacted embryos) and Group 3 (embryos at the early blastocyst stage). The live birth rate was lowest in Group 1 (19.4%) and highest in Group 3 (47.4%, p<0.001). The same phenomenon was seen in terms of the clinical pregnancy rate (25.2% vs 52.6%, p<0.001).

The rate of survival after warming was the highest in the Day 3 embryo group and the lowest in the Day 5 embryo group (96.9% vs 93.5%, p= 0.001). A similar effect was detected in the percentage of intact embryos after thawing (80.3% vs 72.7%, p= 0.001). The proportion of embryos transferred, of the total number of embryos thawed was the lowest in the Day 4 group (88.1%) and the highest in the Day 3 group (93.4%, p=0.003).

5.3 The effect of the culture length on the weight of the newborns (Study II)

The birth weight of the babies from fresh blastocyst transfers was higher than the birth weight of babies from fresh cleavage stage embryo transfers (weight difference 111.8g, p=0.047). However, when the study material was divided by the sex, this effect remained statistically and clinically significant in boys but not in girls (Table 9).

Table 9. The mean weights (g) of the newborns born after fresh and frozen–thawed Day 2–3 and Day 5–6 embryo transfers. P-values when comparing the weights of cleavage stage and blastocyst stage newborns

	Day 2–3 Fresh	Days 5–6 Fresh	Weight difference	p-value	Day 2–3 FET	Day 5–6 FET	Weight difference	p-value
Total	3418.8	3530.6	-111.8	0.047	3650.9	3647.5	3.4	0.95
Boys	3447.1	3642.3	-195.2	0.023	3657.3	3767.9	-110.6	0.159
Girls	3393.5	3430.8	37.2	0.610	3644.4	3525.2	119.2	0.108

In the frozen–thawed cycles, no significant differences were detected between cleavage stage and blastocyst stage newborns' weights when boys and girls were analyzed in one group. The mean birth weight of the newborns from frozen–thawed blastocysts was similar to the mean weight of newborns from frozen–thawed cleavage stage embryo transfers (weight difference 3.4 gr, p=0.95) (Table 9). However, when the sex-spesific weight differences were considered, a clear difference between the effect of blastocyst culture on the birth weight was found. The heaviest babies were FET boys from blastocyst transfers. The FET girls from blastocyst transfers were 242.7g lighter than the boys (p= 0.002) (Table 10). In the cleavage stage only 12.9g weight difference between FET boys and girls was detected (Table 11).

When the weights were compared with the gender- and pregnancy week -adjusted reference weights, the fresh cleavage stage boys were 176.6g lighter and girls 101.3gr lighter than the reference weight (Table 10). The fresh blastocyst boys were 18.6g heavier but the fresh blastocyst girls were 64.1g lighter than the gender and pregnancy week adjusted reference weight. The FET blastocyst boys were 103.8g heavier, whereas the FET blastocyst girls 24.6g lighter than the gender- and pregnancy week adjusted reference weight.

Table 10. The mean deviation (g) from the sex- and gestational age adjusted population-based reference weight (by Sankilampi et al. 2013)

	Day 2–3 Fresh	Day 5–6 Fresh	Weight difference	p-value	Day 2–3 FET	Day 5–6 FET	Weight difference	p-value
Boys	-176.6	+18.6	195.2	0.015	-6.8	+103.8	-110.6	0.125
Girls	-101.3	-64.1	37.3	0.596	+94.9	-24.6	119.2	0.066

Table 11. The differences in the mean birth weights between boys and girls in fresh and frozen–thawed Day 2–3 and Day 5–6 cycles.

	Total mean weight	Boys mean weight	Girls mean weight	Weight difference between boys and girls	p-value
Fresh Day 2–3	3418.8	3447.1	3393.5	53.6	0.478
FET Day 2–3	3650.9	3657.3	3644.4	12.9	0.863
Fresh Day 5–6	3530.6	3642.3	3430.8	211.5	0.011
FET Day 5–6	3647.5	3767.9	3525.2	242.7	0.002

5.4 The effect of embryo cryopreservation method on the weight of the newborn (Study III)

In study III, the mean weights of the babies born after Day 2 and 3 fresh, frozen–thawed and vitrified-warmed embryo transfers were 3454.1g, 3670.3g and 3588.7g, respectively. The mean SD-scores were -0.6, -0.22 and -0.27, respectively. The differences between the mean weights (81.6g), or between the mean SD-scores were not statistically different (p=0.263 and p=0.893 respectively). The babies born after cryopreserved embryo transfers were 173.1g heavier than the ones from fresh embryo transfers (p=0.003). The percentages of SGA and LGA did not differ significantly between the two cryopreservation methods.

5.5 The effect of embryo cryopreservation method on the pregnancy rates (Study III)

The viability-rate of Day 2–3 vitrified-warmed embryos at 88.4% and 67.7% respectively (p<0.001), was significantly higher, compared to frozen–thawed embryos. By viability we refer to the rate of embryos surviving thawing/warming (≥50%) and resuming cleavage in an overnight culture.

The live birth rates were similar between the two cryopreservation methods. However, among single embryo transfers, the miscarriage rate was higher in the slow freezing group (29.0%) compared to vitrification group (15.7%, p=0.021). When surveying the single and double embryo transfers together, a statistically significant difference between miscarriage rates was further detected (27.8 vs 18.0, p=0.028). In order to produce one delivery, the number of embryos needed to thaw/warm were 11.9 vs 7.4 in the slow-freezing and vitrification groups, respectively.

Table 12. The clinical outcomes of slow-frozen and vitrified-warmed embryo transfers.

	Slow freezing		Vitrification		
	n	%	n	%	p-value
Embryo transfers	663		683		
Mean number of embryos transferred	1.46		1.29		
CPR	194	29.3	183	26.8	0.314
MCR	54	27.8	33	18.0	0.028
LBR	134	20.2	142	20.8	0.792

CPR = Clinical pregnancy rate
MCR = Miscarriage rate
LBR = Live birth rate

6 DISCUSSION

6.1 The effect of the embryo culture length on the clinical outcomes of the IVF/ICSI treatments (Studies I and IV)

6.1.1 The extended culture of poor quality cleavage stage embryos

The actual indicator of the IVF success is the cumulative live birth rate compromising the results of the fresh embryo transfer as well as the subsequent frozen embryo transfers. This parameter reflects the real change of a couple to have a baby as a result of an IVF cycle (Maheshwari et al., 2015). The cumulative live birth rate for the first IVF/ICSI cycle in women younger than 40 years, using their own oocytes was 32.3% in a recent large British study. Six repetitive IVF-cycles reached a cumulative prognosis-adjusted live-birth rate of 68.4% (A. D. A. C. Smith et al., 2015). McLernon et al. detected, in a large population-based observational cohort study, a cumulative delivery rate of 42.3% after the third complete IVF cycle, over the time period of 1999–2007. He estimated that, assuming all patients would have continued the IVF/ICSI treatments the cumulative live birth rate would have reached 82.4% after eight complete cycles (McLernon et al., 2016). According to these studies, the cumulative live birth rate seems to increase further after the three standard cycles usually provided for a couple in the public health care in Finland. The Finnish national data does not report cumulative pregnancy rates. The cumulative live birth rate per one cycle increases along with the number of oocytes retrieved (Drakopoulos et al., 2016), implying a need for effective stimulation with a high number of oocytes and several IVF/ICSI cycles to maximize the chances of delivering a baby. However, the resources limit the number of IVF/ICSI cycles available. The restricted amount of IVF/ICSI cycles demands the most effective possible use of the embryos resulting from a single ART-cycle. The balance of cryopreserving all the viable embryos from a cohort, while eradicating the ones with no pregnancy potential is a matter of high quality embryo scoring, requiring high sensitivity and accuracy. This challenge can be at least partially overcome by the extended culture of the poor quality cleavage stage embryos. Balaban et al. reported of a higher implantation rate of poor quality cleavage stage embryos cultured to blastocyst stage before a transfer, compared to a cleavage stage transfer, even though there were fewer embryos available for transfer at the blastocyst stage (Balaban et al., 2001). This refers to selection of viable embryos by the extended culture.

In our study (I) 19.7% of the poor quality cleavage stage embryos reached the blastocyst stage. This number is significantly smaller number than in a previous study, in which 66% of cleavage stage embryos with normal chromosomy reached the blastocyst stage. The poor morphology detected at the embryo scoring in cleavage stage, in our study, is probably explained by the increased prevalence of chromosomal aberrations resulting in the termination of the development. The blastocyst culture, however, does not prohibit the development of all chromosomally abnormal embryos to the blastocyst stage (Sandalinas et al., 2001). In a cohort of blastocysts of women aged mean 39 years, the aneuploidy rate at the blastocyst stage was 58%. When divided by age in two categories, the proportion of aneuploidy was 48% in the younger population, while it was 65% in the older one (Fragouli et al., 2013). In the same study the proportion of aneuploidic oocytes was 78% while at the blastocyst stage the rate was 58% referring to the natural selection of embryos during the course of the culture. The genomic instability observed at the cleavage stage, presenting as high rate of chromosome breakage and mitotic chromosome malsegregation decreases, when reaching the blastocyst stage. This phenomenon has been suspected to result from the activation of cycle-regulatory mechanisms maintaining the accurate chromosome segregation and other aspects of genomic integrity (Fragouli et al., 2013).

The development of good quality blastocysts from poor quality cleavage stage embryos might reflect the capacity of an embryo to out-compete aneuploid cells by euploid cells in case of the mosaicism. This phenomenon has been demonstrated by Greco et al., who transferred a mosaic blastocyst resulting in a healthy euploid newborn (Greco et al., 2015).

6.1.2 The Day 3, Day 4 and Day 5 embryo culture

In some previous studies the prolonged culture until the blastocyst stage before a transfer has been associated with improved pregnancy results compared to a transfer at the cleavage stage (Elgindy et al., 2011; Glujovsky et al., 2012). In some studies, however, no significant differences were observed neither after fresh embryo transfers (Aziminekoo et al., 2015; Karacan et al., 2014) nor after vitrified-warmed embryo transfers (Cobo et al., 2012). These latter results are in line with our study (IV), in which the live birth rates between Day 3 and Day 5 tranfers were similar both after fresh (26.5% vs 24.5%) and vitrified-warmed (22.0% vs 22.6%) transfers. The mean number of embryos transferred was slightly higher in the Day 3 transfer group compared to Day 5 transfers. The live birth rate was significantly higher after fresh and vitrified-warmed Day 4 transfers compared to Day 3 and Day 5 transfers. The Day 4 embryos had the lowest rate of embryos transferred/embryos warmed. The embryos that had reached the early blastocyst stage on Day 4 had the highest live birth rate of all groups (47.4%). The overnight culture after the warming of the Day 4 embryos seemed to have selected the viable embryos effectively.

The superior outcomes of the Day 4 embryo transfers, compared to Day 3 transfers, could result from better embryo-endometrial synchrony, better embryo selection due to

the longer culture period and, possibly, a higher rate of euploid embryos in the cohort of early blastocysts on Day 4.

The reason behind the higher live birth rates compared to Day 5 embryos is unknown. Perhaps the structure of Day 4 embryo was more tolerant towards manipulation, in comparison with blastocysts. The results encourage to increase the transfers of Day 4 embryos.

6.2 The effect of the culture length and cryopreservation on the weight of the newborn

6.2.1 Fresh embryo transfers

In our study, the percentage of LGA babies after a blastocyst culture was 4.9%, while the rate was 1.4% for the cleavage stage transfer babies (II). In the Finnish study by Mäkinen et al. (2013) the proportion of LGA babies after blastocyst transfers was 18.8%, 11.5% after Day 3 transfers and 9.4% after Day 2 transfers (Mäkinen et al., 2013). This difference was statistically significant. They did not however, detect a difference between mean birth weight between groups. In our study the mean birth weight of babies from blastocyst transfers was significantly higher than the mean birth weight of babies from cleavage stage embryo transfers. This phenomenon has also been detected by Zhu et al. (Zhu et al., 2014). However, when our study material was divided by sex, the significant weight difference was only detected among boys (195.2g), but not girls (37.2g). The increased length of the embryo culture seems to increase the weight difference between girls and boys after fresh embryo transfers. The boys born from blastocyst transfers were 230g heavier than girls born from blastocyst transfers. After cleavage stage transfers the boys weighed only 53.6g more than girls. In general, the male fetuses gain weight 0,5g per day faster than the females (Nahum et al., 1995). Different factors such as gestational age, pre-eclampsia, diabetes mellitus type 1 and parity do have an impact on the birth weight of the newborn, but for one sex more than the other (Lehre et al., 2013). In cattle and sheep, large offspring syndrome has been detected to be associated, for example, with an in vitro embryo culture, maternal exposure to excessively high urea diets and an asynchronous embryo transfer into an advanced uterine environment (Young et al., 1998). It has also ben shown in animal studies that embryos of different sexes may respond differently to various environmental conditions (Bermejo-Alvarez et al., 2008). In bovines, suboptimal culture conditions have been detected to induce more deviations in gene expression in male than female blastocysts, implying a different response of embryos of different sexes to epigenetic alterations (Heras et al., 2016). The different effect of a blastocyst culture on a girl and a boy newborn mean birth weight detected in our study may result from different epigenetic changes. The other

effects associated with a blastocyst culture such as the increased risk of a preterm birth and congenital anomalies may also be due to the epigenetic alterations (Dar et al., 2014).

In our study the effect of the culture medium on the birth weight was not considered in the analysis. This is partially due to the retrospective study method. However, it remains unclear if the culture media has any effect on the birth weight. In a meta-analysis from 2015 there were 11 studies addressing the question whether the culture media has an influence on the birth weight. Among the 11 studies, 5 supported the theory of culture media affecting the birth weight while the rest of the studies didn't detect an impact (Zanstra et al., 2015).

6.2.2 Frozen–thawed embryo transfers

Frozen embryo transfers are associated with a higher mean birth weight compared to fresh embryo transfers (Pelkonen et al., 2010; Pinborg et al., 2010). Additionally, frozen embryo transfers result in a decreased rate of preterm birth, a decreased rate of low birth weight (<2500g) and decreased rate of SGA compared to fresh embryo transfers, whereas LGA is increased after frozen embryo transfer (Ishihara et al., 2014). In our study (II) embryo cryopreservation at the cleavage stage significantly increased the mean birth weight of both girls and boys. FET blastocyst girls weighed slightly less than the FET cleavage stage girls. On the contrary FET blc boys were heavier than the cleavage stage FET boys. In several studies, IVF-children have been associated with an increased rate of perinatal mortality, preterm delivery, a low birth weight, a very low birth weight, and SGA (Jackson et al., 2004; McDonald et al., 2009; Tomic & Tomic, 2011). The opposite effect of embryo cryopreservation on the IVF outcomes has raised a question whether the fresh embryo transfers should be totally abandoned and the freeze-all policy be implemented into routine practice, as nowadays the pregnancy success rates resemble those from fresh embryo transfers.

The long term consequences of the low or high birth weight is not fully known, yet adverse outcomes has been associated with both extremities of birth weight. A low birth weight has been observed to increase the risk of cardiovascular disease later in life (Bayman et al., 2014; C. J. Smith et al., 2015). On the other hand, an increased risk of childhood obesity and mental health disturbances has been associated with a LGA-birth weight (Lahti et al., 2015; Nicklas & Barbour, 2015). The increased birth weight of babies born from frozen–thawed cycles might be explained by the better growth potential associated with the capacity to survive the freezing thawing process. The preferable endometrial synchrony probably at least partially explains the increased birth weight as well as the lower incidence of preterm deliveries, associated with frozen–thawed embryo transfers.

Epigenetic changes might be one explanation for the changed mean birth weight, affecting either the fresh or frozen–thawed embryo transfers more. Potentially all aspects of the in vitro fertilization can cause epigenetic changes affecting the phenotype of the newborn. The period of preimplantation embryo development is crucial for the epigenetic

reprogramming. Predisposing the oocytes and the embryo to an unnatural milieu during this delicate period may cause changes in the epigenome with a possible impact on the phenotype and the baby's future health.

The effect of cryopreservation method on the birth weight was studied in the study III. The embryos were all at the cleavage stage to avoid the effect of the blastocyst culture on the birth weight. There was a weight difference of 81.6g for the benefit of slow freezing, but the difference wasn't statistcally significant. The babies born from fresh embryo transfers were lighter than those from the frozen embryo transfers. The rate of a low birth weight was significantly higher in the vitrification group compared, to the slow freezing group (4.3% vs 0%). Our results contradict those of Liu, who detected a higher birth weight after vitrification compared to slow freezing at the cleavage stage (Liu et al., 2013). The high concentrations of cryoprotactants used in the vitrification technique have been suspected to predispose the embryos to epigenetic disturbances. The subject warrants studies with a larger study population controlling the confounding factors.

6.3 The effect of embryo cryopreservation on the survival rate and clinical outcomes of the different stages of embryo development (I, III, IV)

6.3.1 Embryo survival

The embryos that were cryopreserved by vitrification had a higher survival rate of warming, 88.4%, compared the ones cryopreserved by slow freezing, 67.7% at the cleavage stage (III). The term survival here refers to survival after thawing and resuming cleavage after an overnight culture. A higher survival rate after vitrification has been detected in several previous studies, both in retrospective and prospective randomized trials (Balaban et al., 2008; Debrock et al., 2015; Fasano et al., 2014; Levron et al., 2014; Lin et al., 2010; Rezazadeh Valojerdi et al., 2009; Van Landuyt et al., 2013). The quality of pre-freeze embryos affects the survival rate, and different clinics have different ratings for embryos qualified for freezing. Therefore the comparison between survival rates at different clinics may not be reliable. The lower survival rate after slow freezing is probably associated with the lower cryoprotectant concentrations causing formation of ice-crystals and leading to cell death and deteorioration of the function of the cell organelles. Not only survival, but also the recovery of the normal physiologic function of the cells is crucial for the successful implantation of the embryo. An increased miscarriage rate after slow freezing compared to the vitrification of cleavage stage embryos in our study (III), might refer to disturbed cell function and subsequent lower quality implantation of the embryo. The instant survival from thawing was not included in the data collection in the study III. In the sudy IV, however, dealing only with embryos cryopreserved by vitrification, the instant survival of warming of the Day 3 embryos was remarkably high, 96.9%. The

instant survival from warming as well as the rate of transferred embryos/thawed embryos were higher than that of Day 4 or Day 5 embryos. The differences seem clinically small but reach statistical difference. Contradicting our results, the higher survival rates after blastocyst cryopreservation, compared to cleavage stage embryo cryopreservation has been detected by Anderson et al. and Moragianni et al. (Anderson et al., 2004; Moragianni et al., 2010). In both studies slow freezing was the method of cryopreservation. Our survival rate of blastocysts after vitrification and warming are similar to the survival rates reported by Mukaida et al. (93.5% vs 93.8% respectively) (Mukaida & Oka, 2012). Both in our laboratory as well as in Mukaida's, the blastocoel of the true blastocyst is collapsed prior to vitrification to prohibit the formation of ice crystals.

Of the blastocysts originating from poor quality embryos (I) only 80.2% survived thawing and resumed cleavage after an overnight culture. This is lower rate than the rate of all vitrified blastocysts (89.6%) reported in study IV. This may reflect the lower viability of the blastocysts originating from poor quality cleavage stage embryos. Survival rates for early morula, compacted morulas and late morula, of 90.2%, 87.8% and 92.2%, respectively, have been reported (Tao et al., 2004). In our study only the survival rate of all Day 4 embryos was analyzed (94.3%), which was slightly higher than the previously reported. The percentage of intact embryos after warming in our study (80.1%) was similar to the rate of intact embryos after warming the compacted morulae in Tao's study (80.9%), in spite of the cryopreservation having been undertaken by slow freezing in their study and by vitrification in ours (Tao et al., 2004).

6.3.2 The pregnancy rates

The superior live birth rates after fresh and vitrified-warmed Day 4 embryos compared to Day 3 and Day 5, is a relatively new finding (IV). In a study by Tao et al. (2002) a higher pregnancy rate was detected after Day 4 good quality embryo transfers, compared to Day 3 embryo transfers, despite the lower number of embryos transferred. The quality of the morula was mainly dependent of the rate of compaction. In a prospective randomized study a trend toward a higher clinical and ongoing pregnancy rate after Day 4 compared to Day 3 embryo transfers was detected (Pantos et al., 2008). In our study (IV) the compacted morulae produced a higher live birth rate in comparison to the ones still in the non-compaction stage. However, the highest live birth rate among the fresh Day 4 embryo transfers was achieved by the early blastocysts (47.4%). In Kang's study the live birth rate after an elective single morula transfer (Day 4) was 39.2% and 44.7% after an elective single blastocyst transfer (Day 5) (p=0.36) (Kang et al., 2012). In a study by Lee et al. the clinical pregnancy rates after Day 4 and Day 5 transfers didn't differ significantly either (40.7% vs 44.6%, p=NS) (Lee et al., 2013). The reason for the higher live birth rate after Day 4 embryo transfer compared to Day 5 embryo transfer, detected in our study is unknown. The live birth rate of fresh Day 4 early blastocysts was particularly high (47.4%).

This fast development of an embryo to the early blastocyst stage on Day 4 might suggest a low proportion of aneuploidity in the cohort, further evidence resulting from the lowest miscarriage rate in the same group (6.7%). The high success rates of these early blastocysts partially explain the results of the fresh Day 4 embryo transfers.

The highest miscarriage rate after a blastocyst transfer of both fresh and frozen compared to Day 3 and Day 4 is a surprising finding in our study (IV). Due to the natural selection taking place during the five days culture the risk of a miscarriage could be supposed to be lower after a blastocyst transfer than after cleavage stage transfer. On the other hand, a recent data refers to main selection taking place around the time of implantation or soon thereafter (Fragouli & Wells, 2011) In the cochrane meta-analysis comparing the cleavage stage and blastocyst stage embryo transfers, no difference was observed in miscarriage rate (Glujovsky et al., 2012). In a study by Emiliani et al. the miscarriage rate was 15.4% after blastocyst transfer and 8.5% after Day 2 embryo transfer but the difference was not statistically significant (Emiliani et al., 2003). In our study (I) the miscarriage rate after a transfer of blastocysts, originating from poor quality cleavage stage embryos (27.3%) was higher than the miscarriage rate from fresh cleavage stage embryos (15.8%) but not from frozen-thawed cleavage stage embryos (30.0%).

6.4 The strengths and shortcomings of the study

The shortcoming of all the studies is the retrospective setting. In study II many factors affecting the birth weight such as BMI of the mother and the length of the gestation, the presence of gestational diabetes, as well as the sex of the newborn were controlled, whereas the smoking status of the mother wasn't included in the analysis. In study III the BMI of the mother or the smoking status was not included in the study material collection, which is a clear shortage when comparing the birth weights. The strength of the study was that the clinical outcomes were reported as live birth rates and miscarriage rates instead of implantation rates as these outcomes are more informative, and live birth rate represents the main goal of the IVF-treatments.

7 CONCLUSIONS

The main findings of the present study were:

Approximately 20% of poor quality cleavage stage embryos develop to good quality blastocysts eligible for cryopreservation. By transferring these blastocysts, repetitive IVF/ICSI cycles with the associated complications and economical burden can be avoided. (I)

The fresh embryos transferred on Day 4 and the vitrified embryos warmed on Day 4 and cultured overnight before transfer reach at least comparable live birth rates compared to Day 3 and Day 5 embryos. The use of Day 4 embryos can be increased. (IV)

The study verifies the finding of extended culture increasing the mean birth weight of the newborn. The novel finding is that the blastocyst culture significantly increases the birth weight of only the boys but not the girls. (II)

The method of the cryopreservation doesn't seem to significantly affect the birth weight, albeit a tendency for a slightly lower mean birth weight after vitrification compared to slow freezing was detected in our study. With a higher survival rate and a lower miscarriage rate, vitrification seems to be superior in cleavage stage embryo cryopreservation. (III)

8 FUTURE PERSPECTIVES

The effect of a Day 4 transfer on the clinical outcomes, such as the live birth rate and miscarriage, should be studied in a prospective randomized trial in which the patients be allocated according to the cause of the infertility and the age of the patients. The developmental status of the Day 4 embryos should be included in the study.

The weight difference in girls and boys, born after blastocyst transfer, could be due to epigenetic factors. This subject should be examined further by analyzing the methylation status of genes associated with fetal growth, such as IGF2 from both newborn girls and boys, born from the cleavage stage and blastocyst stage transfers.

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ARTICLE

Can repeated IVF–ICSI-cycles be avoided by using blastocysts developing from poor-quality cleavage stage embryos?

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Abstract In many clinics, good-quality embryos are selected for embryo transfer and cryopreservation at the cleavage stage, and poor-quality embryos are discarded. The aim of this retrospective study was to examine how many repeated IVF cycles could be avoided by culturing the cleavage stage poor-quality embryos to blastocyst stage and transferring them after vitrification and warming (604 IVF and intracytoplasmic sperm injection [IVF–ICSI] cycles were included). Poor-quality cleavage stage embryos not eligible for transfer or cryopreservation were cultured until day 5 or 6, and those developing to the blastocyst stage were vitrified. The rate of vitrified blastocysts and clinical pregnancy and delivery rate of the warmed blastocysts was evaluated. The effect of the extended culture on the cumulative delivery rate, and the number of avoided new treatment cycles was calculated. The surplus blastocysts resulted in clinical pregnancy, spontaneous abortion and delivery rates of 24.6%, 27.3% and 17.2% respectively. The use of surplus blastocysts raised cumulative delivery rate from 43% to 47% and 53 repeated new cycles were avoided. This study shows that the cumulative delivery rate can be increased, and repeated IVF–ICSI treatments avoided by using blastocysts developing from poor-quality cleavage stage embryos, which otherwise would have been discarded.

KEYWORDS: blastocyst, cleavage stage, cryopreservation, cumulative delivery rate, embryo quality, poor quality

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Introduction

The most effective treatment for infertility is IVF, with cumulative delivery rates of 47–51% per cycle (De Neubourg et al., 2010; Stewart et al., 2011). The process of an IVF cycle is psychologically stressful, and ovarian stimulation and oocyte retrieval are associated with risks of ovarian hyperstimulation syndrome, infection and haemorrhage (Dicker et al., 1993). The costs of IVF are high, limiting the number of affordable cycles for a couple.

Embryo morphology correlates with embryo quality, and is one of the most important factors determining the survival and implantation potential of fresh and frozen-thawed embryos (Lee et al., 2012; Lundin et al., 2001; Scott et al., 2000; Solé et al., 2011). In addition to choosing the highest quality embryos for transfer, the number of embryos available for freezing and subsequent transfers determines the cycle's probability of pregnancy. The good-quality embryos are transferred in the fresh and frozen-thawed embryo transfer cycles, but the practice of using the poor-quality cleavage stage embryos varies. At some clinics, poor-quality cleavage stage embryos are routinely cultured to blastocyst stage and then transferred, whereas, at others, they are discarded or used in research programmes (Poulain et al., 2014; Wang et al., 2012).

The practice of discarding poor-quality cleavage stage embryos is based on their supposed low implantation potential. A blockage of cleavage often associated with poor-quality embryos may indicate developmental disturbances owing to chromosomal abnormalities (Gardner et al., 1998). The static microscopic observation of embryos, however, is insufficient for an accurate assessment of an embryo's developmental potential, as has been shown with time-lapse imaging (Meseguer et al., 2012). Because of embryo plasticity, the proportion of chromosomally abnormal cells varies during the culture, and the corrupted cells can be eliminated resulting in a good-quality blastocyst developing from a poor-quality cleavage stage embryo (Fragouli et al., 2013). On average, one-half of the cleavage stage embryos reach the blastocyst stage and can be frozen and transferred (Jones et al., 1998).

At our clinic, the extended culture of poor-quality cleavage stage embryos was introduced in 2008 to support the major practice of embryo transfer and cryopreservation of good-quality cleavage stage embryos. At the same time, blastocyst culture and transfer was introduced as a treatment for a selected group of patients.

In the present study, the number of poor-quality cleavage stage embryos reaching the blastocyst stage and which qualified for cryopreservation was investigated, in addition to how they survived thawing and warming and resulted in a live birth. The main aim was to calculate the effect of the blastocyst transfers on the cumulative pregnancy rate and, finally, to estimate how many repeated IVF and intracytoplasmic sperm injection (ICSI) treatments could be avoided.

Materials and methods

This retrospective study was approved by the Ethics Committee of Tampere University Hospital in November 2011 and carried out on data collected between August 2008 and November 2011, at the infertility clinic of Tampere University Hospital. All the couples included in the study had low-quality surplus embryos on the day of fresh embryo transfer (day 2 or 3), in addition to the good-quality embryos that were transferred or frozen. Extended culture of these poor-quality embryos was carried out in 604 cycles for 512 couples, out of which 51.5% were ICSI and 48.5% IVF treatments. The mean age of patients was 32.0 ± 4.1 years. The cause of infertility in these cycles was male origin (32.3%), unexplained (30.0%), endometriosis (13.4%), anovulation (10.9%), multiple reasons (8.0%) and tubal origin (5.3%). The mean duration of infertility at the time of the first visit to the infertility clinic was 3.52 (range 0.1–15) years. The stimulation protocol was long agonist, antagonist and short agonist in 52%, 46% and 2% of cycles, respectively.

Ovarian stimulation protocol

Ovarian stimulation was carried out either by a long agonist protocol, antagonist protocol or short agonist protocol. In the long agonist protocol, the nafarelin (Synarela® Pfizer, Belgium) was started on the cycle day 21 to supress the ovaries. About 2 weeks after starting nafarelin, the ovarian suppression was confirmed by transvaginal ultrasound scanning. The recombinant FSH (Gonal F® Merck Serono, Italy or Puregon® Organon, Ireland) was started at 125–350 IU per day based on the antral follicle count, woman's age and previous ovarian response. Ovarian response was monitored once or twice during stimulation. In the antagonist protocol, the recombinant FSH was started on the cycle day 2. Ganirelix (Orgalutran® Organon, Ireland) or setrorelix (Cetrotide® Merck Serono, Swizerland) was combined with recombinant FSH around cycle day 7. In the short agonist protocol, nafarelin was started on cycle day 1 and recombinant FSH on cycle day 2. The human chorionic gonadotrophin (HCG) was administered when dominant follicles measured over 18 mm in diameter in the long agonist cycle and over 17 mm in the antagonist cycle. Oocyte retrieval was carried out 36 h after the HCG injection.

Culture conditions

The culture media was supplied by Origio (MålØv, Denmark) or Vitrolife (Gothenburg, Sweden).

Successful fertilization was confirmed 16–18 h after the insemination. The normally fertilized zygotes were cultured in sequential media using ISM1™ (Origio) as the cleavage stage medium until day 2 or 3 or G-1™ PLUS (Vitrolife) until day 3. The cleaved embryos were moved from ISM1™ to blastocyst culture in BlastAssist ™ (Origio) on day 2 in 437 cycles, in ISM2™ (Origio) on day 3 in 142 cycles or from G-1™ PLUS to G-2™ (Vitrolife) on day 3 in 25 cycles. The blastocyst culture was carried out in a reduced oxygen atmosphere (6% CO_2/6% O_2/88% N_2) until day 5 or 6. The low oxygen culture was used from day 2 or 3 onwards as the embryos were moved from cleavage stage culture to blastocyst culture. The low oxygen culture was not used for the entire culture period owing to limited bench top incubator capacity available.

Embryo evaluation

Embryo evaluation was carried out on day 2 (44–46 hpi) and day 3 (68–70 hpi). A modified scoring system was applied (Fridström et al., 1999; Mohr et al., 1985). In brief, an embryo with the expected developmental stage (i.e. four cells on day 2 or eight cells on day 3) received a starting score of 3.5, whereas slowly or fast cleaving embryos received a starting score of 3.0. The starting score was then reduced in increments of 0.5 for every reducing factor observed: the presence of more than 10% fragmentation, an unequal size of blastomeres, a large perivitelline space or cytoplasmic abnormalities. Drastic reducing factors subtracting 1.0 point were the presence of more than 25% fragmentation or the presence of multinucleated blastomeres.

From all 604 cycles of the study group, one or two embryos with the highest score were transferred on day 2 or 3 with ultrasound guidance using a Sydney® catheter (Cook). On the day of a fresh embryo transfer, the selected good-quality embryos with a minimum score of 2.0 and with no drastic reducing factors were frozen using an Embryo Freezing Pack (Origio) and the standard slow-freezing method. Poor-quality embryos with a score below 2.0, or a score of 2.0 with more than 25% fragmentation or a presence of multinucleated blastomeres were cultured to the blastocyst stage.

The reassessment of the embryos was carried out on day 5 and 6 according to Gardner and Schoolcraft (1999). An embryo was selected for cryopreservation by vitrification if it had reached at least a fully compacted morula stage by day 5 or the true blastocyst stage (i.e. stage 3) by day 6 (Gardner and Schoolcraft, 1999). At the blastocyst stages 2–6, the inner cell mass and trofectoderm class A or B had to be present. The vitrification was carried out using CBS™ High Security Vitrification straws (Cryo Bio Systems, Paris, France) and a VitriFreeze ES™ vitrification kit (FertiPro, Beernem, Belgium).

Cryopreserved embryo transfers

The cryopreserved embryo transfers were carried out in hormone-substituted cycles using oestradiol hemihydrate as tablets (2 mg three times a day, Zumenon®; Abbot Biologicals BV, Netherlands) or transdermal patches (100–150 μg twice a week Estradot® Novartis Pharma GmbH, Germany) from the beginning of the menstrual cycle, in addition to natural vaginal progesterone tablets (200 mg × 3 a day Lugesteron® Besins Healthcare Ltd, France), starting when the endometrial thickness measured 7–8 mm. One or two embryos were transferred using ultrasound guidance. Clinical pregnancy was assessed with a transvaginal ultrasound 5 weeks after the embryo transfer and defined as the presence of at least one gestational sac on the ultrasound scanning. A spontaneous abortion was considered to be a pregnancy loss until week 22 of gestation after previous confirmation of a clinical pregnancy.

The cleavage stage embryos were thawed 1 day before the day of transfer (Embryo Thawing Pack, Origio), cultured overnight BlastAssist ™ (Origio) and evaluated for cleavage on the morning of the embryo transfer. The compacted morulae and early blastocysts were warmed one day before the transfer, and the true blastocysts were warmed on the day of the transfer (VitriThawES, FertiPro). The embryos with a minimum of 50% viability were transferred.

Table 1 Availability of good-quality and poor-quality embryos in 604 cycles.

	Total number	*Mean*	*%*
Good-quality fresh day 2/3 embryo transfers	604	–	–
Good-quality fresh embryos transferred day 2/3	873	1.45	–
Good-quality embryos cryopreserved day 2/3	1152	3.84	–
Cycles with good-quality embryos cryopreserved on day 2/3	299	–	49.5
Cycles with poor quality embryos for extended culture	604	–	100
Poor-quality embryos available for extended culture on day 2/3	1879	3.11	–
Number of poor-quality embryos reaching blastocyst stage and eligible for vitrification	371	1.63	19.7
Number of cycles with poor-quality embryos Reaching blastocyst stage and eligible for vitrification	193	–	32.0

Data analysis

A statistical analysis was carried out using the IBM SPSS software (v19.0 Armonk, NY, USA). The results are detailed in terms of SD or percentage. When evaluating factors affecting the success of extended culture, a binary logistic regression was used. The results are shown as odds ratios with 95% confidence interval.

Results

The extended culture of poor-quality cleavage stage embryos

A total of 1879 originally poor-quality embryos were cultured until day 5 or 6, and 371 (19.7%) were selected for vitrification at the blastocyst stage (Table 1). In 411 (68.0%) cycles, no embryos reached the blastocyst stage or the blastocysts were not qualified for vitrification, whereas in 193 cycles (32.0%), at least one blastocyst stage embryo could be vitrified. By March 2013, 232 (62.5%) of the blastocysts had been warmed. A total of 186 embryos (80.2%) survived warming and 134 embryo transfers were made, with an average of 1.39 embryos per embryo transfer. These transfers resulted in 33 clinical pregnancies (24.6% per embryo transfer), 23 deliveries (17.2% per embryo transfer) and 25 newborns, with a delivery rate of 9.9% per warmed blastocyst (Table 2). In the data analysis, no factors in the background or in the IVF–ICSI treatment related to the success of the extended culture of the surplus embryos, the survival after warming or cryopreserved pregnancy rates.

Table 2 Pregnancy outcome data for the poor-quality cleavage stage embryos cultured t blastocyst stage and qualified eligible for cryopreservation.

	Surplus blastocysts (vitrified day 5/6)	
	n	%
Total embryo number	371	–
Warmed	232	–
Survived	186	80.2
Embryo transfers	134	–
Clinical pregnancy rate	33	24.6
Delivery rate	23	17.2
Spontaneous abortion rate	9	27.3
Tubal pregnancy	1	3.0
Embryos transferred per embryo transfer (mean)	1.39	–

Table 3 Pregnancy outcome data for the good-quality fresh and frozen day 2/3 embryo transfers.

	Fresh day 2/3		*Frozen day 2/3*	
	n	%	n	%
Total embryo number	873	–	1152	–
Thawed	–	–	800	–
Cleavage in culture after thawing	–	–	478	59.8
Embryo transfers	604	–	306	–
Clinical pregnancy rate	240	39.7	90	29.4
Delivery rate	201	33.3	60	19.6
Spontaneous abortion rate	38	15.8	27	30.0
Tubal pregnancy	1	0.4	3	3.3
Embryos transferred per embryo transfer (mean)	1.45	–	1.56	–

Good-quality cleavage stage embryos

A fresh embryo transfer on day 2 or 3 in the 604 cycles resulted in 240 pregnancies (39.7%) and 201 deliveries (33.3%/cycle). In 49.5% of cycles ($n = 299$), 1152 good-quality embryos were frozen on the fresh embryo transfer day (day 2 or 3) (Table 3). By March 2013, 800 of these cleavage-stage frozen embryos had been thawed, 59.8% of them cleaved in an overnight culture, and 306 transfers have been carried out. The clinical pregnancy rate of these good-quality cleavage stage frozen–thawed embryos was 29.4% per embryo transfer and the delivery rate was 19.6% (60 deliveries).

Cumulative delivery rate

The observed number of deliveries after the transfer of fresh and frozen–thawed day 2 and 3 embryos was 201 and 60, respectively. This corresponds to a cumulative delivery rate of 43% for the 604 treatments included in the study. The blastocysts originating from poor-quality cleavage-stage embryos resulted in 23 additional deliveries, increasing the cumulative delivery rate from 43% to 47%. With this delivery rate, 53 repeated IVF–ICSI cycles would be required to achieve the observed 23 deliveries, which resulted from the surplus day 2 or 3 cultured embryos.

Discussion

The clinically most important finding of the present study was the number of repeated new IVF–ICSI cycles that could be avoided with the extended culture of poor-quality cleavage stage embryos. During the 3-year study period, this practice resulted in 23 additional deliveries, leading to an increase of cumulative delivery rate from 43% to 47% and estimated 53 repetitive IVF–ICSI cycles were avoided.

Recently Poulain et al. (2014) reported on extended culture of poor-quality cleavage stage embryos with a smaller number of cultured embryos. Their results are comparable with this study in percentage of blastocysts surviving thawing–warming (85% versus 80%), clinical pregnancy rate (30.3% versus 24.6%), delivery rate per embryo transfer (15.2% vs 17.2%) and the delivery rate per thawed–warmed blastocyst (12.5% versus 9.9%) (Poulain et al., 2014). The spontaneous abortion rate was nevertheless higher in Poulain's study (50% versus 27.3%). The results of the study by Shaw-Jackson et al. (2013) are also in line with the present study; 73% of the blastocysts originating from poor-quality cleavage stage embryos survived warming and the live birth rate per warmed blastocyst was 15.9%. They vitrified fewer blastocysts than in the current study (16% versus 19.7%), which may reflect the selection of better quality blastocysts, affecting pregnancy rates. In the study by Ren et al. (2012), stricter selection criteria were used, and only 6.6% of the cultured surplus embryos were vitrified, which is less than in the present study (19.7%). The birth of a healthy child can be considered as the main goal in IVF treatment and, therefore, the delivery rate rather than implantation rate reflects the true value of the practice of using poor-quality embryos. All these publications show the value of extended culture of surplus embryos in the light of increased number of cryopreserved embryos, but they have not paid attention to the number of avoided repeated new IVF cycles and the economical aspect, which we consider as the most important implication of this practice.

The results of the present investigation are in line with the findings of an earlier study, in which 47% of good-quality embryos reached the blastocyst stage by day 5 compared with 21% of poor-quality embryos (Rijnders and Jansen, 1998). Higher clinical pregnancy rates than in this study have been reported earlier, especially for good-quality blastocysts, but in these reports the numbers of transferred embryos per cycle have been high (up to four blastocysts), resulting in several multiple pregnancies (68.9–90.9%) (Balaban et al., 2000; Glujovsky et al., 2012). A recent study (Oron et al., 2014) found similar results to this study regarding the delivery rates after single fresh good-quality embryo transfers (32.3% versus 33.3%). The poor-quality embryo transfers also resulted in similar delivery rates (15.5% versus 17.2%). They transferred both cleavage stage and blastocyst stage embryos, however, and carried out only fresh embryo transfers. In their study, only single embryo transfers were carried out, whereas in the present study not all the transfers were single, the mean number of embryos transferred being 1.39. The effect of

different culture media used in blastocyst culture has been investigated, and differences in the blastocyst development rates has been detected with no unequivocal results (Van Langendonckt et al., 2001; Wirleitner et al., 2010).

Extended culture and cryopreservation can be seen as means of selecting good embryos. The survival after thawing and warming is better in blastocysts than in cleavage stage embryos, and in some studies cryopreserved blastocysts have yielded better pregnancy rates than cleavage stage cryopreserved embryos (Anderson et al., 2004; Moragianni et al., 2010). In the present study, the survival rate of vitrified and warmed blastocysts was 80.2%, which is a little lower than in Moragianni et al.' study (2010) (90.1%). Many embryos, however, did survive vitrification and warming, indicating good development potential despite their origin.

In the present study, the overnight cleavage rate of good-quality cleavage stage embryos seems to be low. It was, however, calculated from the total number of thawed embryos, including those not surviving and those not cleaving. By culturing overnight, we confirmed the continued capacity to cleave and avoided transferring embryos without developmental potential.

The IVF treatment is expensive and requires collaboration of several professionals and high-quality laboratory facilities. The expenses of an IVF cycle consist of direct and indirect costs, and it has been calculated that, in the private sector, costs of IVF treatment can amount to 10% of annual household expenditure in Europe (Collins, 2002). In publicly financed clinics, the number of IVF–ICSI cycles is usually limited (e.g. usually to three in Finland). Maximizing the number of embryos available for transfer is therefore justifiable and discarding poor-quality embryos at cleavage stage may increase the risk of permanent childlessness of some couples. In Scandinavia, the cost of a standard IVF-cycle was €4275 in 2004 (Chambers et al., 2009). With this average cost, it could be calculated that by avoiding 53 repeated IVF–ICSI cycles, at least €226,575 was saved. The expenses caused by the extended culture of poor-quality embryos need to be deducted from this sum.

In addition to economical aspects, there are also other considerable factors supporting the use of surplus embryos; psychological stress, for instance has been shown to be high in women participating in IVF treatment (Salvatore et al., 2001).

One noteworthy advantage of avoiding new IVF–ICSI cycle is avoiding the risks associated with the treatment, such as severe forms of ovarian hyperstimulation syndrome, intraperitoneal haemorrhage, pelvic organ injury and postoperative pelvic infection not underrating the pain in oocyte retrieval, which is considered severe in 3% of patients (Bennett et al., 1993; Brinsden et al., 1995; Delvigne and Rozenberg, 2002; Ludwig et al., 2006).

In spite of the clear advantages associated with extended culture of poor-quality cleavage stage embryos, some controversies have been attached to poor-quality embryo culture and transfer. Compared with good-quality cleavage-stage embryos, poor-quality embryos are more often related to chromosomal abnormalities, resulting in lower implantation and pregnancy rates (Márquez et al., 2000; Ziebe et al., 1997). Blastocyst morphology has been shown to correlate with the euploidy rate. In a study by Capalbo et al. (2014), the euploidy rate was 56.4% if the blastocyst morphology was good. Only 6.8% had complex aneuploidity if the blastocyst morphology was excellent. In the poor morphology group, the euploidy rate was only 25.5% and the percentage of complex aneuploidity was 27.5%. Hardarson et al. (2003) found significantly more chromosomal abnormalities in blastocysts derived from poor-quality embryos compared with those derived from good-quality embryos when seven chromosomes were tested. In this study, only 42% of the surplus blastocysts were chromosomally normal. The morphology of the surplus blastocysts was also inferior to the control group and the improvement in morphology during the extended culture of the poor-quality embryo group did not predict a chromosomally normal blastocyst. Hardarson et al. (2003) did not recommend transferring originally poor-quality surplus embryos, regardless of the quality of the blastocysts they would turn out to become. The investigated embryos were not transferred and hence no pregnancy results were reported. In the present study, only good-quality blastocysts were cryopreserved.

On the other hand, the blastocyst culture can be perceived as a means of embryo selection. Mosaicism and aneuploidies are common in cleavage stage embryos, but this does not necessarily define their ultimate development, which is dependent on the degree of the mosaicism and the involved chromosome. The euploid cells proliferate faster than aneuploidic cells, and mosaicism can later be limited to a confined area that does not decrease the possibility of implantation and the birth of a healthy child (Taylor et al., 2014). In the study by Fragouli et al. (2013), the highest rate of complex aneuploidity was detected in the cleavage stage embryos, whereas in the blastocyst stage, the aneuploidity rate was lower (Fragouli et al., 2013). This can be explained by the loss of affected embryos or loss of the abnormal cells during the extended culture (Ebner et al., 2003). This view is supported by Balaban et al. (2001) who found that blastocysts from poor-quality cleavage stage embryos had a higher implantation rate than similar quality cleavage stage embryos transferred at cleavage stage.

The spontaneous abortion rate among assisted reproduction pregnancies in our study (27.3%) as well as Poulain's study (50%) was higher than that reported in earlier studies (13.6–22.4%), in which the quality of the embryos transferred was not reported (Sunkara et al., 2014; Tomás et al., 2012). As chromosomal abnormalities are the main reason for early spontaneous abortions, the most likely reason for the elevated spontaneous abortion rate in our study material could be the higher incidence of chromosomal abnormalities in the blastocyst population derived from the poor-quality cleavage stage embryos reflecting the natural selection process of embryos in the early pregnancy (Hassold et al., 1980). In the present study, all the children born from the poor-quality cleavage stage embryos cultured to blastocyst stage were healthy and no chromosomal abnormalities or malformations were reported, except for one child with a bilateral cleft lip. Therefore we can consider this practice safe, as concluded by Shaw-Jackson et al. (2013).

Chromosomal screening has been shown to result in higher delivery rates after blastocyst transfers compared with morphology based embryo selection (Scott et al., 2013), and is a powerful tool for embryo selection. This kind of protocol, however, cannot be applied to the most patients owing to economical aspects, missing resources and prevailing legislation. Therefore, extended culture serves as a practical means of embryo selection.

In the present study, 19.7% of the cultured surplus embryos qualified for vitrification as blastocysts. If the rest of the embryos survive warming with a similar survival rate (80%) as those already warmed, we can calculate that 15.8% of the cultured poor-quality embryos can develop to blastocysts suitable for transfer after warming. The surplus embryos in the current study have so far resulted in 23 deliveries and 25 newborns, and it could be calculated that 53 repeated IVF–ICSI treatments have been avoided. The restricted availability of IVF, the high costs of the treatments and the risks associated with the procedure support the maximal use of available embryos. In terms of increased cumulative delivery rate and healthy newborns, we conclude that the blastocyst culture of surplus embryos qualified as inferior for freezing at an early developmental stage is a valid practice.

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ORIGINAL ARTICLE *Infertility*

Male gender explains increased birthweight in children born after transfer of blastocysts

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STUDY QUESTION: Does extended embryo culture have a different effect on the birthweight of girls and boys?

SUMMARY ANSWER: The mean birthweight of boys born after fresh and frozen–thawed blastocyst transfer was increased compared with those born after cleavage stage embryo transfer. This effect was not detected among girls.

WHAT IS KNOWN ALREADY: Previous studies indicate that newborns from frozen–thawed cleavage stage embryos may present with a higher weight than newborns from fresh embryo transfers. With regard to fresh embryos, newborns after a blastocyst transfer have been reported as having higher birthweights than newborns from cleavage stage embryos.

STUDY DESIGN, SIZE, DURATION: Retrospective multicentre case–control cohort study. All IVF/ICSI treatments were performed in the time-period from January 2008 to March 2014.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Birthweight of singletons born at full-term (≥37 weeks), after fresh or frozen blastocyst embryo transfers ($n = 277$), were compared with weights of children born after fresh or frozen cleavage stage embryo transfers (Day 2–3) ($n = 277$). The cases and controls were matched by delivery week, and by gender. Data of IVF/ICSI treatments, and the treatments' outcomes were collected and analysed.

MAIN RESULTS AND THE ROLE OF CHANCE: The birthweight after a fresh blastocyst transfer was significantly higher (mean 3530.6 g) than that after a transfer of cleavage stage embryos (mean 3418.8 g; weight difference 111.8 g, $P = 0.047$). The weights of newborns after frozen–thawed blastocyst transfers (mean 3647.5 g) and the frozen–thawed cleavage stage embryo transfers (mean 3650.9 g), were similar (weight difference 3.4 g, $P = 0.95$). The boys born after transfer of frozen–thawed blastocysts had a significantly higher birthweight (mean 3767.9 g) than girls (3525.2 g; weight difference 242.7 g, $P = 0.002$), whereas the difference of birthweights between genders was only 13.5 g in cleavage stage ($P = 0.863$). The same effect was seen after fresh blastocyst transfers (weight difference 211.5 g, $P = 0.011$), but not after fresh Day 2–3 embryo transfers (weight difference 53.6 g, $P = 0.478$).

LIMITATIONS, REASONS FOR CAUTION: The study material was large enough to detect differences between birthweights as a whole, but a larger study group would confirm these new findings. To avoid selection bias, the next possible control candidate, fulfilling the selection criteria, was included for matching cases and controls. We have matched the cases and controls by gender and gestational week at birth, with an aim to reduce their impact as confounding factors.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings of a similar weight at birth of newborns after frozen–thawed blastocysts and frozen–thawed cleavage stage embryos, when matching for age and duration of pregnancy, are novel. The gender of the newborn has an impact on the birthweight, and the extended embryo culture increases the weight difference between the genders, which is a new finding as well.

STUDY FUNDING/COMPETING INTERESTS: The study was funded by the Fertility Society of Finland.

Key words: blastocyst culture / IVF/ICSI / infertility / birthweight / preimplantational period

Introduction

Fertility treatments, using *in vitro* assisted reproductive techniques (ART), have resulted in over 4 million babies born worldwide (Biggers, 2012). Continuous and rigorous quality control of all procedures involved in ART is needed, as well as the investigation of possible associated health issues to these treatments. An excess of congenital abnormalities has been observed among ART children, in comparison to spontaneously conceived pregnancies (Qin *et al.*, 2015). Furthermore, IVF/ICSI treatments are related to an increased risk of adverse perinatal outcomes, such as very low birthweight, low birthweight and small for gestational age (SGA) newborns (Helmerhorst *et al.*, 2004). It is not clear whether these are associated with the ART's procedures, or to patient-related factors.

The birthweight is related to the children's future health. For example, children large for gestational age (LGA) have a risk of increasing accumulation of fat in early childhood, and LGA is associated with higher risk of severe obesity (Hediger *et al.*, 1999; Cnattingius *et al.*, 2012). In addition, an increased risk of autism and depression later in life has been reported to be associated with LGA (Ornoy, 2011; Colman *et al.*, 2012; Moore *et al.*, 2012). Both SGA and LGA children have been shown to have an increased risk of adverse cardio-metabolic profile during childhood and adolescence, referring to an increased risk of cardiovascular disease later in life (Chiavaroli *et al.*, 2014).

Some studies have investigated the effect of the length of embryo culture on birthweight. In Mäkinen's study, the extended culture of embryos was associated with increased incidence of LGA children (Mäkinen *et al.*, 2013). Zhu *et al.* discovered a higher birthweight and more LGA children in singletons from blastocyst transfer, compared with Day 3 embryo transfer (Zhu *et al.*, 2014). Another study by DeVos could not detect a difference in the mean birthweight between Day 3 and Day 5 embryo transfers (De Vos *et al.*, 2014). A possible explanation for the increased birthweight after blastocyst transfer could be linked to the epigenetic reprogramming of the embryo genome in the extended culture. The culture environment during blastocyst culture can never completely match the endogenous milieu, and therefore the embryo is predisposed to stress for a longer period of time than during the conventional culture of two or three days. Epigenetic reprogramming influences the expression of genes, and establishes the epigenetic profile of the offspring during the preimplantation period. DNA methylation, the most accurately studied epigenetic mechanism, either promotes or suppresses gene expression, depending on the operating region on the genome. The *in vitro* culture of an embryo may increase the risk of methylation reprogramming defects leading to abnormal somatic development (Laprise, 2009). About 50% of the variation in birthweight can be explained by genetic factors (Lunde *et al.*, 2007), but epigenetic mechanisms have also been discovered to have an impact on the weight of a newborn (St-Pierre *et al.*, 2012; Engel *et al.*, 2014).

The effect of culture length on birthweight has been studied with conflicting results, but the impact of gender on birthweight has not been considered in the previous studies. We conducted a retrospective cohort study to survey the effect of the IVF culture length on birthweight, both in fresh and frozen–thawed transfers. The secondary aim was to investigate whether gender had an impact on the birthweight of newborns.

Materials and Methods

This is a retrospective multicentre case–control cohort study, undertaken in Tampere University Hospital, Finland; Reproductive Medicine, Karolinska University Hospital, Stockholm, Sweden; and infertility clinic Fertinova, Finland.

The ethical approval

The study plan was approved by the Ethics Committee of Tampere University Hospital, Finland, and the Regional Ethics Committee in Stockholm, Sweden.

Power calculation

The power calculation was based on a difference between experimental and control means of 200 g with SD of 400. The number of subjects needed for the experimental group and control group is 64 + 64 to reject the null hypothesis stating that the population means of the experimental and control groups are equal with probability (power) 0.8. The type I error probability associated with this test of this null hypothesis is 0.05.

Patients

We analysed all the Day 5–6 fresh and frozen–thawed embryo transfers leading to a singleton live full-term delivery ≥37 weeks, in the time-period from January 2008 to March 2014. As a control we used Day 2–3 fresh and frozen–thawed transfers from the same centre and time-period, also leading to a live full-term singleton delivery with a newborn of same sex and with birth at the same full gestational week than the case.

Of the 142 fresh blastocyst cases and Day 2–3 controls 75 were collected from Tampere University Hospital, 53 from Karolinska University Hospital, and 14 from Fertinova clinic, Finland. The frozen–thawed blastocyst cases and Day 2–3 controls ($n = 135$) were collected from Tampere University Hospital. There were 67 boys and 75 girls in the fresh blastocyst group ($n = 142$) and 68 boys and 67 girls in the frozen–thawed blastocyst group. Information about the infertility treatments, and patient characteristics, were obtained from the medical records. Day 4 embryo transfers were not included, and only cycles with autologous oocytes were included.

The blastocyst transfers and controls were adjusted by gender and gestational age in terms of full gestational weeks. The study group consisted of 277 Day 5–6 transfers and 277 Day 2–3 controls all leading to a singleton full-term delivery. Of these, 142 were fresh blastocyst transfers with 142 fresh Day 2–3 controls and 135 frozen blastocysts with 135 frozen Day 2–3 controls. In addition to comparing the weights of the babies born after blastocyst replacement with babies born after cleavage stage replacement with adjusted gender and gestational age, we compared the weights to the population-based references for birthweight by Sankilampi *et al.* (2013). SGA (small for gestational age) and LGA (large for gestational age) were defined as −2 SD or +2 SD above the sex, and gestational age-specific reference mean. The primary outcome was the difference in weight at birth, between children born from fresh and frozen–thawed cleavage stage (Day 2–3) and blastocyst stage (Day 5–6) embryos. Secondary outcomes were the differences in the deviation from the gender and gestational week adjusted reference weights between groups.

The controlled ovarian hyperstimulation protocol

The treatments were performed at each centre accordingly to their routines. In brief, the controlled ovarian stimulation was accomplished using either antagonist (Orgalutran® Organon, Ireland or Cetrotide® Merck Serono, Switzerland), long agonist (Synarela® Pfizer, Belgium), or short agonist protocols. Recombinant FSH (Gonal F® Merck Serono, Italy or Puregon®

Organon, Ireland) was used to induce follicular growth, and the hCG was administered when the leading follicle reached 18 mm in the agonist protocol, and 17 mm in the antagonist protocol. Thirty-six hours after the hCG injection, the oocytes were collected, using transvaginal ultrasound-guided puncture. The luteal support consisted of vaginally administered progesterone, and the embryo replacement was performed under ultrasound monitoring.

Culture conditions

The culture media were supplied by Origio (Måløv, Denmark) or Vitrolife (Gothenburg, Sweden). Successful fertilization was confirmed 16–18 h after the insemination (hpi). The normally fertilized zygotes were cultured in sequential media, using ISM1™ (Origio) as the cleavage stage medium until Day 2 or 3, or G-1™ PLUS (Vitrolife) until Day 3. The cleaved embryos were moved from ISM1™ to blastocyst culture in BlastAssist™ (Origio) on Day 2, or in ISM2™ (Origio) on Day 3, or from G-1™ PLUS to G-2™ (Vitrolife) on Day 3. The blastocyst culture was performed in a reduced oxygen atmosphere (6% CO_2/6% O_2/88% N_2) until Day 5 or 6.

The freezing and thawing protocols

Cleavage stage embryos were frozen using an Embryo Freezing Pack (Origio) and the standard slow-freezing method. Thawing was performed using the Embryo Thawing Pack. The survived embryos were cultured overnight in BlastAssist, and those that had cleaved were selected for embryo transfer. Blastocyst stage embryos were vitrified using CBS™ High Security Vitrification straws (Cryo Bio Systems, Paris, France), and a VitriFreeze ES™ vitrification kit (FertiPro, Beernem, Belgium). They were thawed the day before transfer, and cultured overnight in Blast Assist (compacted morulae or early blastocysts), or on the day of the transfer (developmental stages 2–6 according to Gardner and Schoolcraft, 1999), using the VitriThaw ES™ thawing kit.

Frozen–thawed embryo transfers

The frozen–thawed embryo transfers (FET) were performed in spontaneous or hormone-substituted cycles. In hormone-substituted cycles, estradiol hemihydrate as tablets (Zumenon® Abbot Biologicals BV, Neatherlands, 2 mg three times a day) or transdermal patches (Estradot® Novartis Pharma GmbH, Germany, 100–150 μg twice a week) from the beginning of the menstrual cycle were used, in addition to natural vaginal progesterone tablets (Lugesteron® Besins Healthcare Ltd, France, 200 mg × 3 a day), starting when the endometrial thickness measured 7–8 mm. One or two embryos were transferred using ultrasound guidance. The clinical pregnancy was assessed with a transvaginal ultrasound 5 weeks after the embryo transfer.

Statistical analyses

A pairwise *t*-test was used for a matched analysis between weights of cleavage stage embryo babies, and blastocyst stage embryo babies. Student's *t*-test was used for comparing weight between genders, and also between fresh and frozen–thawed embryo transfers. One-way ANOVA was used to test the effect of stimulation, IVF/ICSI, gestational diabetes or parity on the results and the Spearman's correlation was calculated to find any correlation between mothers age and weight of the newborns. A statistical analysis was performed using the IBM SPSS software (v19.0 Armonk, NY, USA).

Results

We compared the weights of the newborns between the study groups, and population-based references for birthweight at the corresponding week (Sankilampi *et al.*, 2013).

The maternal characteristics

Clinical characteristics of the patients are summarized in Table I. There were no patient differences between the blastocyst and cleavage stage embryo groups, in terms of maternal age, BMI, length, height and parity. In all groups the most common causes of infertility were male and unexplained infertility. The distribution of IVF/ICSI cycles was between 40 and 50% in all groups. Long agonist was the most frequently used stimulation protocol in all groups, while short agonist was used only in five cycles in total. Only full-term deliveries were included. More than half of the deliveries occurred in the full pregnancy weeks 39 and 40, both in fresh and frozen embryo transfer pregnancies. The sex ratio of the newborns was quite similar both in fresh and frozen blastocyst transfer pregnancies (percentage of boys 47.2 and 50.4%, respectively). In the FET cycles, the hormonal preparation of endometrium predominated in both the cleavage stage and blastocyst stage groups (81.1 and 81.8%, respectively).

Day 2–3 and Day 5–6 transfers

The weight of the babies born from fresh blastocysts was higher, in comparison to the fresh cleavage stage embryo infants (weight difference 111.8 g, $P = 0.047$). When the gender of the baby was considered, the difference remained in boys (weight difference 195.2 g, $P = 0.023$), but not in girls (weight difference 37.2 g, $P = 0.610$).

No weight difference was detected between newborns from frozen–thawed Day 2–3 and frozen–thawed Day 5–6 transfers as a group (weight difference 3.4 g, $P = 0.95$). When the genders were considered separately, the blastocyst boys were heavier than the Day 2–3 boys (weight difference 110.6 g) whereas the blastocyst girls were lighter than the Day 2–3 girls (weight difference 119.2 g). These differences did not reach statistical significance.

When fresh and frozen–thawed embryos were assessed together the blastocyst boys were significantly heavier than Day 2–3 boys (weight difference 152.6 g, $P = 0.009$). In the girls group almost no difference existed (weight difference 36.6 g, $P = 0.49$) (Fig. 1). This effect was also observed when the weights were compared with the gender and pregnancy week adjusted population-based reference weights (Fig. 2). The boys from fresh Day 2–3 embryos weighed 176.6 g less than the sex- and pregnancy week adjusted reference weight, but the boys from fresh blastocysts were slightly heavier than the reference weight (difference in the deviation from the reference weight 195.2 g, $P = 0.015$). Both the cleavage stage embryo and blastocyst girls were lighter than the reference weight (Table II).

4.9% of the fresh blastocyst newborns were large for gestational age (LGA), defined as >2 SD more than reference mean weight, compared with 1.4% of the cleavage stage embryos

Fresh and frozen–thawed embryo transfers

Day 2–3 frozen–thawed babies were heavier than babies from fresh Day 2–3 embryo transfers (weight difference 232.1 g, weight difference $P < 0.001$) (Table III). This difference was statistically significant both in girls (weight difference 250.9 g, $P = 0.001$), and boys (weight difference 210.2 g, $P = 0.006$). Also Day 5–6 frozen–thawed boys were heavier than boys born from fresh Day 5–6 embryos, but the difference was not statistically significant.

Table I Characteristics of the mothers and the newborns.

	Fresh				FET			
	Day 2–3 $n = 142$ n (SD)	%	Day 5–6 $n = 142$ n (SD)	%	Day 2–3 $n = 135$ n (SD)	%	Day 5–6 $n = 135$ n (SD)	%
Age of the mother (years)	32.6 (4.0)		31.6 (4.0)		31.6 (4.0)		31.1 (4.7)	
Height of the mother (cm)	167 (6.7)		167 (5.5)		167.2 (5.9)		167.2 (6.5)	
BMI (kg/m^2)	24.6 (4.5)		24.4 (3.9)		25.1 (4.4)		23.7 (4.1)	
Parity								
Primipara	98	69.0	90	63.4	70	51.9	83	62.4
Previous miscarriage/eu	19	13.4	16	11.3	23	17.0	15	11.3
Multipara	25	17.6	36	25.4	42	31.1	35	26.3
Stimulation								
Antagonist	60	42.3	57	40.1	62	46.3	64	48.1
Long agonist	82	57.7	85	59.9	69	51.5	67	50.4
Short agonist	0		0		3	2.2	2	1.5
Main cause of the infertility								
Anovulation	13	9.3	18	12.9	14	10.4	16	12.0
Endometriosis	14	10.0	10	7.2	27	20.1	17	12.8
Tubal origin	6	4.3	9	6.5	18	13.4	11	8.3
Male origin	43	30.7	43	30.9	37	27.6	37	27.6
Unexplained	47	33.6	38	27.3	31	23.1	39	29.3
Multiple reasons	17	12.1	21	15.1	7	5.2	13	9.8
Week of the delivery								
37	12	8.5			10	7.4		
38	30	21.1			23	17.0		
39	42	29.6			29	21.5		
40	43	30.3			39	28.9		
41	12	8.5			29	21.5		
42	3	2.1			5	3.7		
Gestational diabetes								
Yes, dietary treatment	6	4.2	2	1.4	6	4.5	12	9.0
Yes, medication	1	0.7	0	0.0	5	3.8	3	2.3
No	87	63.1	116	81.7	56	42.1	52	39.1
No information available	48	33.8	24	16.9	66	49.6	66	49.6
IVF	76	53.5	65	45.8	63	46.7	66	48.9
ICSI	66	46.5	77	54.2	72	53.3	69	51.1
Sex of the newborn								
Boy	67		47.2		68		50.4	
Girl	75		52.8		67		49.6	
LGA	2	1.4	7	4.9	4	3.0	4	3.0
SGA	3	2.1	2	1.4	1	0.7	3	2.2
Normal weight	137	96.5	133	93.7	130	96.3	128	94.8
FET cycle								
Hormonal					99	81.1	108	81.8
Spontaneous					23	18.9	24	18.2
d2	111	78.2			22	16.3		
d3	31	21.8			113	83.7		
d5			142	100			106	78.5
d6			0				29	21.5

SGA (small for gestational age) and LGA (large for gestational age) defined as −2 SD or +2 SD above the sex, and gestational age-specific reference mean.
FET, frozen–thawed embryo transfer.

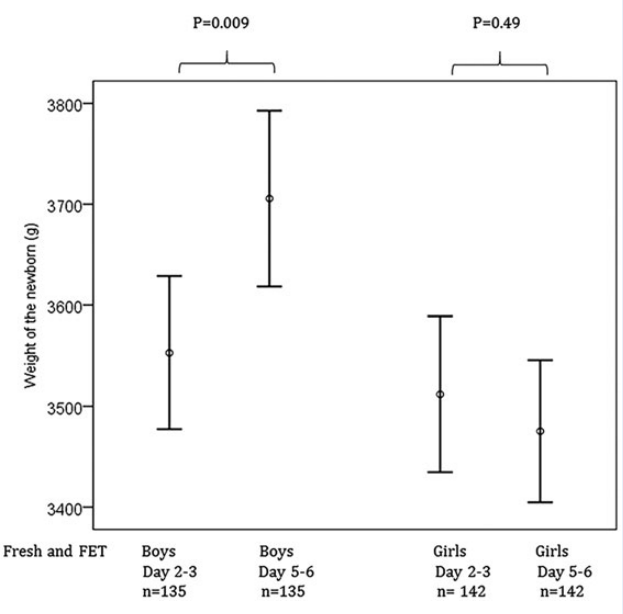

Figure 1 Weights (g) of Day 2–3 and Day 5–6 newborns by gender. FET and Fresh combined. Mean and 95% CI are presented.

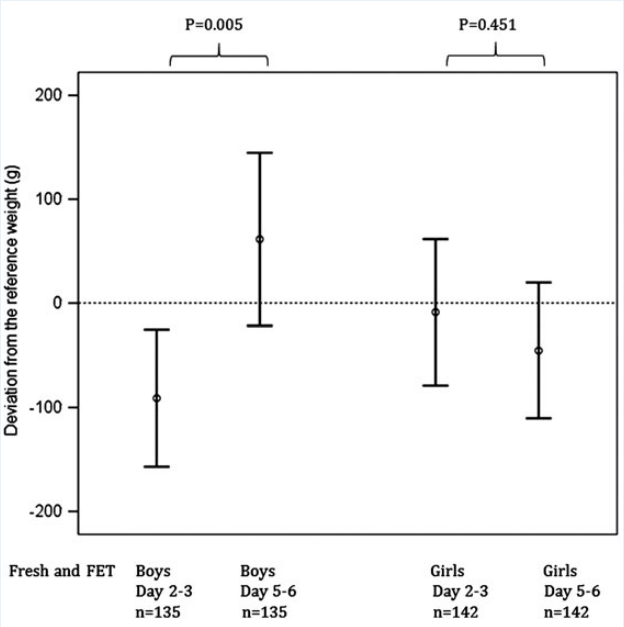

Figure 2 The mean deviation (g) of the weights of the newborns from the sex- and gestational age-adjusted reference weights (Sankilampi *et al.*, 2013). FET and Fresh combined. Mean and 95% CI.

Boys and girls

Boys presented with higher weights at birth than girls in all the groups investigated. The boys born after blastocyst (both fresh and frozen–thawed included) replacement were 230 g heavier than girls born after blastocyst replacement ($P < 0.001$) (Table IV). The weight difference between girls and boys was even more prominent in the newborns born after frozen–thawed blastocyst replacement (mean difference of 242.7 g, $P = 0.002$) (Fig. 3). The weight difference between girls and boys in cleavage stage (both fresh and frozen–thawed included) was markedly less prominent, 41.1 g ($P = 0.453$).

No associations were observed between weight differences of the newborns and the mothers' age, parity, gestational diabetes, stimulation protocol or performance of IVF or ICSI.

Discussion

The most important finding of our study was the increasing weight difference between the genders emerging after extended embryo culture. In fresh embryo group the blastocyst boys were significantly heavier than Day 2–3 boys, whereas in the girls no significant weight difference existed. In the frozen–thawed embryo group, the blastocyst boys tended to be heavier but the blastocyst girls lighter than the newborns from Day 2–3 frozen–thawed embryos. The boys born after blastocyst replacement were significantly heavier than girls born after blastocyst replacement, but also heavier than Day 2–3 boys. Our study showed that there was no difference between the birthweights of babies born from frozen–thawed cleavage stage embryos or frozen–thawed blastocysts. The results of our study confirm the earlier findings stating, that newborns from fresh blastocyst transfers are heavier than ones born from fresh cleavage stage embryos (Pelkonen *et al.*, 2010; Pinborg *et al.*, 2010; Mäkinen *et al.*, 2013; Zhu *et al.*, 2014). Similarly, the earlier finding of heavier babies born from frozen–thawed cleavage stage embryos, compared with babies born from fresh cleavage stage embryos was confirmed in the study at hand. In our study, the boys from frozen blastocyst transfers were 242.7 g heavier than girls from frozen blastocyst transfers while the boys from fresh blastocyst transfers were 211.5 g heavier than girls from fresh blastocyst transfers. In cleavage stage, the difference between boys and girls was less evident. Boys are heavier than girls in spontaneous pregnancies, and the same difference is seen in IVF fresh embryo transfer pregnancies (Cogswell and Yip, 1995; O'Neill *et al.*, 2014). Factors affecting the birthweight, such as gestational age, pre-eclampsia, maternal diabetes and parity, have been discovered to influence genders unequally, affecting one sex more than the other (Lehre *et al.*, 2013). Our results could indicate that the longer culture affects boys more than girls.

We took into the analysis many factors affecting the birthweight, the most important being the gestational week at birth and the gender of the newborn, to which the controls were matched. Only singleton full-term pregnancies from own gametes were included, and also other possible factors such as the mother's age, parity, BMI, the treatment method (IVF/ICSI), aetiology of the infertility, ovarian stimulation protocol, and the diabetic tendency during the pregnancy, were considered in the data analysis.

The pre-pregnancy BMI affects the newborns weight, so that one unit increase in the mother's BMI increases the baby's weight by 20.3 g (Fleten *et al.*, 2010; Yu *et al.*, 2013). Weight gain during pregnancy, maternal age, height and gestational duration are related to increased birthweight, whereas primiparity, smoking and the female gender are associated with a decrease in birthweight (Pavić *et al.*, 2011; Terada *et al.*, 2013; Suzuki *et al.*, 2014). Compared with spontaneously conceived newborns, children born from assisted reproductive technology are smaller at birth, partly due to increased rate of multiple pregnancies,

Table II The mean deviation (g) from the sex- and gestational age-adjusted population-based reference weight by Sankilampi *et al.* (2013).

	Fresh				FET			
	Day 2–3	Day 5–6	Difference (g)	*P*-value	Day 2–3	Day 5–6	Difference (g)	*P*-value
All	−136.9	−25.1	11.8	0.034	+43.7	+40.2	3.4	0.944
Boys	−176.6	+18.6	−195.2	0.015	−6.8	+103.8	−110.6	0.125
Girls	−101.3	−64.1	37.2	0.596	+94.9	−24.6	119.2	0.066

Table III The mean weights (g) (SD) of fresh and frozen Day 2–3 and Day 5–6 transfer newborns.

	Day 2–3				Day 5–6			
	Fresh mean (g) (SD)	FET mean (g) (SD)	Difference	*P*-value	Fresh mean (g) (SD)	FET mean (g) (SD)	Difference	*P*-value
Total	3418.8 (448)	3650.9 (433)	232.1	<0.001	3530.6 (496)	3647.5 (462)	116.9	0.04
Boys	3447.1 (446)	3657.3 (420)	210.2	0.006	3642.3 (531)	3767.9 (448)	125.6	0.155
Girls	3393.5 (450)	3644.4 (449)	250.9	0.001	3430.8 (441)	3525.2 (403)	94.4	0.187

and the trend to deliver at earlier weeks, partly being related to the procedure of assisted reproduction or the subfertility itself (Schieve *et al.*, 2002; Jackson *et al.*, 2004; Allen *et al.*, 2008). Furthermore, singleton pregnancies after assisted reproduction are related to higher rates of complications, such as prematurity and malformations, in comparison with naturally conceived babies. In O'Neill's study, IVF reduced birthweight by 81 g in comparison with newborns from spontaneous pregnancies (O'Neill *et al.*, 2014), but also no difference in birthweight has been reported (Romundstad *et al.*, 2008). In our study material, Day 2–3 fresh embryo newborns weighed 136.9 g less than the reference children at birth, whereas the weight of newborns from blastocysts deviated only 25.1 g from the reference weight.

In several studies the newborns from frozen–thawed embryo transfers have been found to be heavier than babies from fresh embryo transfers, considering both the cleavage stage and blastocyst embryos (Pelkonen *et al.*, 2010; Wikland *et al.*, 2010; Wennerholm *et al.*, 2013). In our study, the babies born from frozen–thawed embryos were significantly heavier, than babies from fresh embryos, both in cleavage stage and blastocyst stage. Many causes have been suggested for the higher weight in newborns from frozen embryos, such as the hormonal excess in fresh embryo transfers, compared with the more natural milieu in the frozen embryo transfer cycles. It could be speculated that the freezing-thawing process itself can influence the embryo in the early preimplantation period, predisposing it for epigenetic reprogramming and changing the embryo's metabolism. The freezing-thawing process eliminates embryos with lower adaptation capacity, which can theoretically select embryos with better growth potential (Wennerholm *et al.*, 2013). This theory is supported by the finding, that less preterm births and low birthweights were found among children born after frozen embryo transfers, compared with those born after fresh embryo transfers (Wennerholm *et al.*, 2009). Vitrification may also have an impact on the newborns' weights. In a recent study, Day 3 embryo vitrification resulted in increased mean birthweight, compared with slow freezing, but no difference was found in the mean weight of babies born from fresh and slow frozen–thawed Day 3 embryos (Liu *et al.*, 2013). In another study, the mean weight of the vitrified-thawed Day 3 embryo babies was higher than fresh Day 3 embryo babies (Shi *et al.*, 2012). The slow freezing method has been used with cleavage stage embryos, but the vitrification, used with blastocysts, is increasing regardless of the age of the embryos. The impact of the embryo freezing method on the newborns' weight is to be verified in the future. The effect of the embryo culture media on the birthweight, has been investigated with conflicting results (Dumoulin *et al.*, 2010; Nelissen *et al.*, 2012; Carrasco *et al.*, 2013).

In a study comparing the effect of the embryo culture length on the newborns' weight, using the growth charts of the general population as reference, no differences in the mean weights of babies was found, but the percentage of LGA infants in the blastocyst children was high, 18.8% (Mäkinen *et al.*, 2013). In the study by Ishihara, the percentage of LGA children was 12.4 in newborns from fresh, and 18.2 from frozen–thawed blastocysts (Ishihara *et al.*, 2014). Also in the study by Zhu, a higher proportion of LGA children was born from blastocysts, compared with cleavage stage embryos, and the absolute birthweight of babies born from fresh blastocysts was significantly higher (Zhu *et al.*, 2014). Contradictory results has also been reported; Fernando did not observe differences in proportions of LGA or SGA newborns in a study of 4200 women, comparing the outcomes of blastocyst and cleavage stage embryo transfer pregnancies (Fernando *et al.*,

Table IV Differences between the mean weights (g) of girls and boys by culture length and embryo transfer at fresh or frozen–thawed stage.

	All	Boys	Girls	Weight difference (g) between boys and girls	*P*-value
Day 2–3					
Fresh	3418.8	3447.1	3393.5	153.6	0.478
FET	3650.9	3657.3	3644.4	13.5	0.863
Day 5–6					
Fresh	3530.6	3642.3	3430.8	211.5	0.011
FET	3647.5	3767.9	3525.2	242.7	0.002
Fresh and FET					
Day 2–3		3553.0	3511.9	41.1	0.453
Day 5–6		3705.6	3475.3	230.3	<0.001

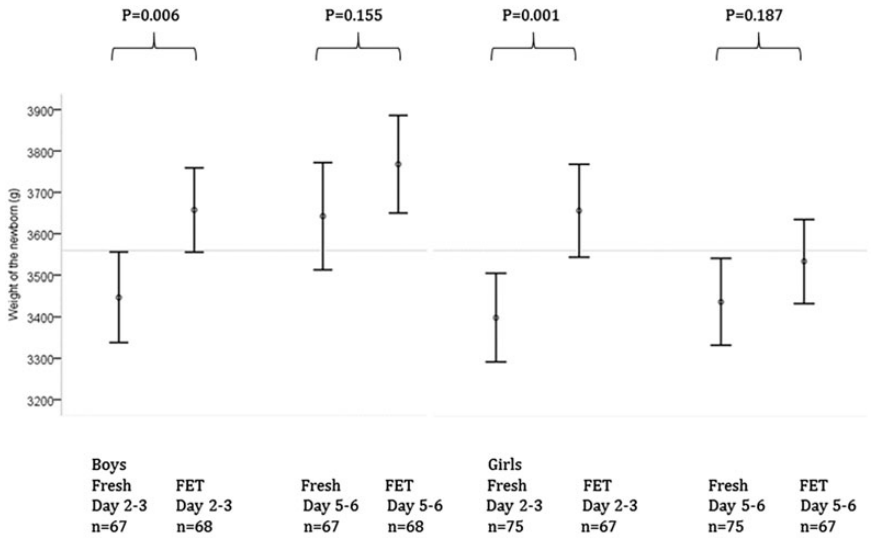

Figure 3 Plots of weight (g) at birth (mean with 95% CI) of boys and girls born after replacement of fresh or frozen–thawed (FET) embryos either Day 2–3 or Day 5–6.

2012). In our study, only 4.9% of the fresh blastocyst transfer newborns were LGA children, probably because the used LGA definition was more strict (mean + 2 SD) than in Mäkinen's study. We could assume that blastocyst babies are heavier than cleavage stage embryo babies throughout, since those few LGA babies cannot explain the weight differences between the groups.

The strengths of our study include gender and gestational age adjusted cleavage stage embryo controls, and the fact that all the children were born full-term, referring to somewhat uncomplicated pregnancies. Our study was limited by the retrospective design, which prevented collecting a complete data of factors affecting the birthweight, such as smoking or presence of hypertension or pre-eclampsia, and the data regarding gestational diabetes remained unclear in almost 50% of cases.

Our study verifies the earlier observations about weight difference between babies born from fresh blastocyst stage and cleavage stage embryos, and also between babies born from frozen–thawed cleavage stage compared with fresh cleavage stage. New finding was the increase of the gender difference in the birthweight favouring boys in blastocyst children. It is not known, what consequences the increased birthweight carries, considering the future health of the child, and this question remains a subject for follow-up studies. Further studies are also needed to reveal the different mechanisms of the culture milieu, which may affect embryo development. The reason for the weight differences among newborns remains unclear, but it could be caused by extended exposure and handling of the embryo during the sensitive preimplantation period. These preliminary results need to be confirmed in prospective studies with a larger study material.

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Authors' roles

N.M.K., H.I.T., K.M.K., K.A.R.-W., C.M.T. and H.SA.H. participated with substantial contributions to conception and design, collecting the data, analysis and interpretation of the data, writing the article, or revising the draft critically and the final approval of the version.

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Conflict of interest

None declared.

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ASSISTED REPRODUCTION TECHNOLOGIES

The freezing method of cleavage stage embryos has no impact on the weight of the newborns

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Abstract
Purpose The aim of this study was to study the effect of the embryo freezing method on the birth weight of newborns from frozen embryo transfer (FET) cycles, and the pregnancy results of cleavage stage embryos cryopreserved by slow freezing or vitrification.
Methods This is a retrospective cohort study undertaken in a University Hospital IVF unit using concurrently both the slow-freezing and the vitrification techniques. All frozen-thawed and vitrified-warmed day 2 and day 3 embryo transfers during the time period from 1 April 2009 to 31 November 2013 were included in the study.
Results There was no statistically significant weight difference between newborns from vitrified or slow-frozen embryos (3588 vs 3670 g). A higher post-thaw viability rate was achieved after cryopreservation by the vitrification technique compared to the slow-freezing protocol (83.4 vs 61.4 %). The miscarriage rate was lower in the vitrification group (15.7 vs 29.0 %). The live birth rates were similar (19.5 vs 19.1 %) in the slow-freezing and vitrification groups, respectively. Among vitrified embryos, 7.4 embryos needed to be thawed to produce one delivery; in the slow-freezing group, that number was 11.9.
Conclusions The freezing method has no impact on the weight of the newborn.

With lower post-thaw survival rates and higher miscarriage rates, the slow-freezing cryopreservation protocol is inferior to the vitrification technique.

Keywords Cryopreservation · Vitrification · Slow freezing · Frozen embryo transfer

Capsule The freezing method has no impact on the weight of the newborn. With lower post-thaw survival rates and higher miscarriage rates, the slow-freezing cryopreservation protocol is inferior to the vitrification technique.

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Introduction

Since the early days of the in vitro fertilization technology, a remarkable progress has been accomplished in the process of embryo culture while the implantation has remained the critical point limiting the pregnancy rate. Embryo freezing has revolutionized the entire process by enabling the use of the whole embryo cohort obtained from a single oocyte pick-up. The opportunity to transfer a single embryo at a time has diminished the risk of multiple pregnancies resulting in a better obstetrical outcome and increased cumulative delivery rate from the maximal utilization of embryos. The possibility to freeze all of the embryos in case of threatening ovarian hyperstimulation has reduced the complication rate associated with ovarian stimulation. The potential adverse effect of the superovulation on the endometrium receptivity may be avoided by cryopreserving all of the embryos and replacing them later in a more physiological milieu [1, 2].

Two cryopreservation techniques are mainly used in the IVF laboratories. In the slow-freezing protocol the temperature is decreased step by step first slowly to −30 °C and then rapidly to −150 °C, whereas in vitrification, the temperature is

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reduced to −196 °C by means of immediate exposure to liquid nitrogen [3]. The former technique requires costly equipment, while the latter one engages more labor force. The disadvantage of the slow freezing is the formation of ice crystals predisposing the embryo to cell damage during thawing. Cryoprotectants are used to cause expulsion of intracellular water, thus decreasing the ice crystal formation. In vitrification, higher concentrations of cryoprotectants are used for a short period of time before rapid cooling compared to slow freezing. The formation of ice crystals is inhibited as the solutes turn into a glass-like structure, i.e., the solutes vitrify [4].

Numerous studies have detected higher post-thaw survival rates in cleavage stage embryos cryopreserved by vitrification compared to embryos cryopreserved by slow freezing [5–12].

The efficiency of these two freezing methods has been studied in blastocyst stage embryos showing higher implantation and clinical pregnancy rates by vitrification compared to slow freezing (32 vs 20 % and 43 vs 28 %, respectively) [13]. A large population-based cohort study detected a significantly higher clinical pregnancy rate (adjusted relative risk ARR 1.47) and delivery rate (ARR 1.41) in benefit of vitrification [14]. No difference in the implantation rates of day 3 embryos was detected between the slow-freezing and vitrification techniques in two randomized, controlled studies (35.3 vs 35.4 % [15] and 21.4 vs 15.8 % [9]). A retrospective study found not only a significantly higher live birth rate after vitrification (35.3 %) compared to slow freezing (25.8 %) but also a higher miscarriage rate higher in the vitrification group (16.5 vs 7.7 %) [16]. In the only controlled, randomized study comparing the two cryopreservation methods at cleavage stage by Debrock et al. [17], a significant difference in live birth rate per embryo thawed/warmed for the benefit of vitrification was observed (16.1 vs 5.0 %). The use of vitrification at cleavage stage has raised interest in the possible epigenetic effects of the freezing method, the weight of the newborn as one expression.

The occurrence of increased birth weight after frozen-thawed embryo transfer compared to fresh embryo transfers has been observed in several studies [18–20]. However, the outcome of studies comparing the effect of the freezing method on birth weight is conflicting. Wang et al. [16] reported no statistically significant differences in birth weights after fresh, slowly frozen-thawed, or vitrified-warmed embryo transfers. Contradicting these results, Liu et al. [21] reported an increased birth weight after vitrified-warmed embryo transfers compared to slowly frozen thawed embryos, whereas Shi et al. [22] suggested a higher birth weight after vitrified-warmed day 3 embryos compared to fresh day 3 embryo.

Several IVF laboratories apply both cryopreservation techniques. The possible effect of the cryopreservation techniques on the birth weights could indicate epigenomic changes in the embryo, with further implications. We conducted a retrospective study to investigate the effect of the freezing method on the birth weight. The possible effect the cryopreservation technique used had on the miscarriage rate and delivery rates was also studied.

Materials and methods

The study plan was approved by the Institutional Review Board of Tampere University Hospital. We analyzed all the frozen-thawed day 2–day 4 transfers from the time period from 1 April 2009 to 31 November 2013. The study material was collected from medical records. Only cycles with autologous oocytes were included in the study. The study included vitrified embryos and embryos cryopreserved by slow freezing. The difference between mean weights of the babies from frozen-thawed and vitrified-warmed embryo transfers was considered clinically significant when exceeding 100 g. The difference between the birth weight and the national gestational age- and gender-adjusted reference weight is expressed as the standard deviation score (SD-score). The SD-score is calculated using the formula $Z=(x-\mu):\delta$, where x is the birth weight of a newborn, μ the mean weight of babies born at the same gestational age and gender, and δ is the standard deviation in the reference group. Only weights of newborns from single pregnancies were included.

The controlled ovarian hyperstimulation protocol

The controlled ovarian hyperstimulation was accomplished using either antagonist, long agonist, or short agonist protocol. A recombinant FSH was used to induce follicular growth, and the hCG was administered when the leading follicle reached 18 mm in the agonist protocol and 17 mm in the antagonist protocol. Thirty-six hours after hCG injection, the oocytes were collected using a transvaginal ultrasound-guided puncture. The luteal support consisted of vaginally administered progesterone and the embryo transfer was made under ultrasound monitoring.

Culture conditions

The culture media was supplied by Origio (MålØv, Denmark) or Vitrolife (Gothenburg, Sweden). Successful fertilization was confirmed 16 to 18 h after the insemination (hpi). The normally fertilized zygotes were cultured in sequential media using ISM1™ (Origio) until day 2 and BlastAssist ™ (Origio) from day 2 to 3, or in G-1™ PLUS (Vitrolife) until day 3.

Embryo evaluation and selection

Cleavage stage embryos were evaluated on the day of the fresh embryo transfer on day 2 or 3 of the culture. The evaluation was performed as previously reported [23]. One or two of the best quality embryos were selected for fresh embryo

transfer and the remaining top or good quality embryos were selected for cryopreservation by either slow freezing or vitrification.

The cryopreservation and thawing protocols

The slow freezing was performed according to the instructions of the manufacturer using the Embryo Freezing Pack (Origio) and the standard slow-freezing method. The cooling rate was controlled by the Freeze Control (Cryologic Ltd., Mulgrave, Australia) liquid nitrogen freezing apparatus. Thawing was performed using the Embryo Thawing Pack (Origio). The cryoprotectants included propylene glycol and sucrose. Vitrification and warming were performed according to the instructions of the manufacturer (VitriFreezeES™, VitriThawES™, FertiPro, Beernem, Belgium). High security vitrification straws (Cryo BioSystems, L'aigle, France) were used as a vitrification device. In vitrification, the main cryoprotectants were dimethyl sulfoxide and ethylene glycol.

The embryos were thawed 1 day before the embryo transfer and the survived embryos (minimum of 50 % of blastomeres alive) were cultured overnight in BlastAssist. The embryos that had cleaved were evaluated and selected for embryo transfer. Embryo survival was defined as cleavage during an overnight culture.

The frozen-thawed embryo transfers

The frozen-thawed embryo transfers (FET) were performed in spontaneous or hormone-substituted cycles. In hormone-substituted cycles estradiol hemihydrate as tablets (2 mg three times a day) or transdermal patches (100–150 μg twice a week) from the beginning of the menstrual cycle was used, in addition to natural vaginal progesterone tablets (200 mg × 3 a day), starting when the endometrial thickness measured 7–8 mm. One or two embryos were transferred using ultrasound guidance.

A clinical pregnancy was assessed with a transvaginal ultrasound 5 weeks after the embryo transfer and defined as the presence of at least one gestational sac on the ultrasound scan. A spontaneous abortion was considered to be a pregnancy loss until week 22 of gestation after a previous confirmation of a clinical pregnancy.

Statistical analyses

The distribution of the birth weight of the babies was normal in all groups. A *t* test for independent samples was used when comparing the mean birth weights between the groups. When comparing the differences in median birth weight standard deviations calculated from the gender- and gestational age-adjusted population-based reference weight, a nonparametric test (Mann-Whitney) was used because of the nongaussian distribution. A chi-square test was used to compare the delivery rates, clinical pregnancy rates, and miscarriage rates between the groups. All statistical analyses were performed using the IBM SPSS software (v19.0 Armonk, NY, USA).

Results

The study consisted of 871 IVF/ICSI cycles with 848 fresh embryo transfers resulting in 160 live births (18.9 %). There were 1346 days 2–3 frozen-thawed embryo transfers in the study, of which 663 were cryopreserved by slow freezing and 683 were vitrified. The frozen-thawed embryo transfers resulted altogether to 276 live births. The stimulation protocols, IVF/ICSI distribution, and the cause of infertility were comparable between the groups as well as the ages of the mothers (31.4 vs 32.1 years) (Table 1). The results of three patients were lost to follow up.

The mean weight of the babies born after fresh, slowly frozen, and vitrified embryo transfers were 3454.1, 3670.3, and 3588.7 g, respectively. Only babies from singleton deliveries were included in the analysis. We analyzed the weights

Table 1 The characteristics of the cycles of the two cryopreservation methods

	Slow freezing		Vitrification		*p* value
	n	%	*n*	%	
Number of cycles	418		453		
Age (years) mean (SD)	31.4 (4.0)		32.1 (4.2)		0.012
Stimulation protocol					0.032
Antagonist	190	45.5	237	52.3	
Long agonist	223	53.3	205	45.3	
Short agonist	5	1.2	11	2.4	
Insemination					0.961
IVF	206	49.3	228	50.3	
ICSI	211	50.5	224	49.4	
Shared	1	0.2	1	0.2	
Infertility					0.104
Primary	253	60.5	261	57.6	
Secondary	165	39.5	185	40.8	
Unknown			7		
Cause of infertility					0.094
Tubal	24	5.7	23	5.1	
Male factor	129	30.9	119	26.3	
Anovulation	54	12.9	50	11.0	
Unexplained	122	29.2	135	29.8	
Multiple reasons	35	8.4	41	9.1	
Endometriosis	52	12.4	73	16.1	
Poor ovarian reserve	2	0.5	12	2.6	

of the newborns using standard deviations from the national gestational age- and gender-adjusted population-based reference weight. The SD-scores in fresh, slow-freezing, and vitrification groups were −0.6, −0.22, and −0.27, respectively (Table 2). Considering the two freezing methods, these SD-scores did not differ statistically ($p = 0.893$). The difference between the mean weights of the babies born after slow-frozen and vitrified embryo transfers was 81.6 g, which is not statistically significant ($p = 0.263$ with 95 % CI −61.5–224.7). The babies born after fresh embryo transfers were lighter than the babies born after frozen embryos transfers (3454.1 vs 3627.2 g, weight difference 173 g, $p = 0.003$). The length of the gestation was similar in both FET groups, the mean delivery week being 39.8 in slow-freezing and 39.5 in vitrification group ($p = 0.979$).

The percentages for SGA (small for gestational age) and LGA (large for gestational age) were 1.6 vs 3.6 % and 2.4 vs 5.0 % for slow freezing and vitrification, respectively.

The ratio of boys was 54.4 vs 50.7 %, respectively.

The slow-freezing group consisted of 359 (54.2 %) single and 304 (45.8 %) double embryo transfers, and the vitrification group consisted of 487 (70.9 %) and 196 (29.1 %) single and double embryo transfers, respectively. The viability rate of the embryos was 67.7 % in the slow-freezing group and 88.4 % in the vitrification group ($p < 0.001$ with 95 % CI of 0.175–0.236).

The pregnancy results of the frozen embryo transfers are listed in Table 3. There was no statistical difference in the clinical pregnancy or live birth rates between the freezing methods. However, in pregnancies resulting from single embryo transfers, the miscarriage rate was significantly higher in the slow-frozen transfers compared to the vitrified embryo transfers (29 vs 15.7 %, $p = 0.021$).

To produce one delivery, 7.4 vitrified embryos were needed to be thawed. In slow-freezing group, the corresponding number was 11.9.

Discussion

In this study, no statistically significant weight difference was observed in the newborns from slow-frozen or vitrified embryo transfers. Even though the freezing method did not have an influence on live birth rates, vitrification was shown to be more effective freezing method for cleavage stage embryos. The survival rate of thawed vitrified embryos was significantly higher and fewer frozen embryos were needed to produce a delivery.

In the present study, the clinical pregnancy and delivery rates were similar for both freezing methods. The miscarriage rate (the proportion of miscarriages in all clinical pregnancies) in the slow-freezing group was higher (29 vs 15.7 %), especially in the case of single embryo transfers. The miscarriage rate were higher than presented in previous studies in the case of day 3 FET cycles by Liu et al. (12.6 vs 12.1 %) or Rama Raju et al. (7.71 vs 9.24 %) [21, 24], but it did not differ from the results of a study on the miscarriage rate after fresh embryo transfers (17.4–22.2 %) in PCO and tubal infertility [25]. The most common reason for early pregnancy miscarriages is the chromosomal abnormalities irrespective the mode of fertilization (spontaneous or ART) [26–28]. Slow freezing and thawing has been detected to induce numerical chromosomal changes in human embryos, which could be explained by increased ice crystal formation [28]. In frozen-thawed blastocyst transfers, no difference has been detected in the miscarriage rates between the two cryopreservation methods [14]. In a study by Li et al. [29], the embryo DNA integrity index was higher in vitrified than in slow-frozen human blastocysts which could explain the lower miscarriage rate in vitrified blastocyst pregnancies compared to slow freezing.

The weight difference between newborns from fresh and frozen embryo transfers does not seem to be related to maternal factors, as the increased risk of LGA and macrosomia in singletons after FET has been detected in a sibling cohort as

Table 2 Neonatal characteristics of the live born singletons from frozen embryo transfer cycles

	Slow freezing *n* (%)	Vitrification *n* (%)	*p* value
Boys	68 (54.4)	71 (50.7)	
Gestational age at birth (weeks)	39.8	39.5	0.979
Preterm births (<37 weeks)	6 (4.8)	12 (8.6)	0.328
Very preterm births (<32 weeks)	1 (0.8)	1 (0.7)	1.000
Birth weight (g)	3670.3	3588.7	0.263
Low birth weight (<2500 g)	0	6 (4.3)	0.032
Low birth weight in term births (≥37 weeks)	2 (1.6)	3 (2.1)	1.000
Very low birth weight (<1500 g)	0	1 (0.7)	1.000
High birth weight (>4500 g)	5 (4.0)	10 (7.1)	0.3
Small for gestational age (<−2SD)	2 (1.6)	5 (3.6)	0.452
Large for gestational age (>+2SD)	3 (2.4)	7 (5.0)	0.342
Z score (mean)	−0.22	−0.27	0.893

Table 3 The pregnancy results after the transfer of slow-frozen/thawed and vitrified/warmed embryos, both single and double embryo transfers

	Slow freezing		Vitrification		*p* value
	n	%	*n*	%	
Single embryo transfers	359		487		
Clinical pregnancy	100	27.9	115	23.6	0.175
Miscarriage	29	29.0	18	15.7	0.021
Extrauterine pregnancy	0		3	2.6	
Induced abortion	0		1		
Still birth	1		0		
Live birth	70	19.5	93	19.1	0.87
Singletons	70		92		
Gemini	0		1		
Double embryo transfers	304		196		
Clinical pregnancy	94	30.9	68	34.7	0.381
Miscarriage	25	26.6	15	22.1	0.582
Extrauterine pregnancy	3	3.2	3	4.4	0.696
Induced abortion	1		1		
Still birth	1		0		
Live birth	64	21.1	49	25.0	0.325
Singletons	55		47		
Gemini	9		2		

well [30]. In a study by Wikland et al., no difference in birth weight was observed after the transfer of vitrified blastocysts or slow-frozen early cleavage stage embryos [31]. The blastocyst culture itself has been found to have an impact on the birth weight of newborns, complicating the comparison of two cryopreservation techniques using embryos from different stages [32, 33]. In Liu's study, the median birth weight of babies born from a vitrified cleavage stage embryo transfer was higher by a weight difference of 103 g, than the birth weight after the transfer of slow-frozen cleavage stage embryos. In their study, no weight difference existed between babies from fresh or slow-frozen cleavage stage embryos. This contradicts the study by Pelkonen et al. in which 134-g higher birth weight was detected among slow-frozen FET singletons compared to fresh singletons [19]. In Liu's study, the birth weights of the babies were obtained through patient questionnaires which may cause some uncertainty in the results. In our study, the precise length of the pregnancy of all of the newborns was known, and the weights were compared to the gender- and gestational age-adjusted population-based reference values, increasing the reliability of the results. The mean birth weight of the newborns was approximately 80 g lower in the vitrification group compared to slow-freezing group ($p = 0.263$). We considered the weight difference more than 100 g clinically significant. The same tendency was seen in the study of Wang et al. of day 3 FET pregnancies; the 140-g difference in birth weights for the benefit of slow freezing compared to vitrification was not statistically significant ($p = 0.307$) [16]. The lack of significant difference in the weights of babies born after these two different cryopreservation techniques could indicate a somewhat similar effect on the epigenetic processes or similar embryo selection caused by the cryopreservation. On the other hand, it could also refer to minimal epigenetic effect of either one of the techniques.

The embryo survival has usually been defined as survival of at least 50 % of the pre-freeze blastomeres. A survival as high as 95 % has been associated with the cryopreservation of cleavage stage embryos by the vitrification technique and a higher survival rate after the vitrification of human cleavage stage embryos compared to the slow freezing has been detected in many studies [5, 6, 10, 11, 21]. This was also observed in our study (61.4 vs 83.4 %). Our viability rate refers to the percentage of embryos surviving the thawing and resuming cleavage after an overnight culture. Embryos with damaged cells after thawing have been shown to have a lower overnight developmental potential compared to intact cells both after slow freezing and vitrification [11]. Certainly, the decreased survival associated with slow freezing is caused by increased ice formation during freezing and thawing physically damaging cell membranes. Conversely, cryoprotectants and osmotic stress can have potential negative impact on the bioenergetic function of mitochondria [34].

It has been supposed that better endometrial receptivity could be one reason for the increased birth weight after FET compared to fresh embryo transfers. Another explanation could be the stress caused by the embryo freezing-thawing resulting in a selection of embryos with better growth potential [20]. Epigenetic mechanisms have also been proposed to explain the weight difference. The freezing and thawing during the delicate period of early embryo development, i.e., the activation of embryonic genome, may cause epigenetic alterations affecting the phenotype at birth.

The strength of the present study is the accurate birth weights compared to the population-based reference weight expressed as the SD-score from the reference weight taking into account the gestational age and the sex of the newborn. A shortcoming in this study was the retrospective design of the study. Furthermore, the maternal BMI and smoking were not included in the data collection. These factors are known to have impact on the birth weight. Especially, the smoking habits of the patients are poorly registered in the patent records.

In conclusion, no significant birth weight difference exists between the two cryopreservation techniques, although there seems to be a tendency toward a slightly lower mean birth weight after vitrification. Undoubtedly, the benefits of vitrification technology for preserving cellular integrity outweigh the trend toward a slightly lower live birth weight. Vitrification is a more efficient procedure, yielding higher rates of cell survival and viability which translates into greater

embryo utilization per IVF cycle and fewer pregnancy losses upon transfer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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