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Neonatal outcome of 937 children born after transfer of cryopreserved embryos obtained by ICSI and IVF and comparison with outcome data of fresh ICSI and IVF cycles

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BACKGROUND: To evaluate the safety of cryopreservation in combination with IVF and ICSI, prenatal diagnosis and neonatal outcome were investigated in children conceived from frozen-thawed ICSI embryos (cryo ICSI) and frozen-thawed IVF embryos (cryo IVF). Data were also compared with earlier published results from fresh ICSI and IVF embryos. METHODS: Questionnaire data and results of physical examination at 2 months of 547 cryo ICSI children and 390 cryo IVF children were compared, and these were also compared with those of infants born after transfer of fresh embryos. RESULTS: Birth characteristics were comparable for cryo ICSI and cryo IVF infants. Cryo singletons showed a trend towards higher mean birthweight compared with fresh singletons, in ICSI and IVF, reaching significance when all cryo (ICSI plus IVF) singletons were considered. Low birthweight rate according to multiplicity was comparable between the fresh and the cryo groups, in ICSI and IVF. Non-statistically significantly increased rates of *de novo* chromosomal anomalies (3.2%) were found in cryo ICSI fetuses/children compared with the fresh ICSI group (1.7%) (OR 1.96; 95% CI 0.92–4.14). Major malformations were more frequently observed in cryo ICSI live borns (6.4%) than in cryo IVF live borns (3.1%) (OR 2.15; 95% CI 1.10–4.20) and fresh ICSI live borns (3.4%) (OR 1.96; 95% CI 1.31–2.91). CONCLUSIONS: In cryo ICSI compared with cryo IVF, prenatal and neonatal outcome results were comparable, except for a higher major malformation rate in the cryo ICSI group. In the total cryo group compared with the total fresh group, we found a higher mean birthweight in singletons and a higher major malformation rate in live borns.

Keywords: cryopreservation; ICSI; IVF; outcome; children

Introduction

The first human pregnancy following the transfer of a cryopreserved embryo was described in 1983 by Trounson *et al*. and the first live birth was reported in 1984 (Zeilmaker *et al*., 1984).

Embryo cryopreservation followed by thawing and transfer into the uterus offers several advantages in assisted reproductive technology (ART) programmes. It can provide an increased cumulative pregnancy rate while decreasing the risk of multiple gestations and the risk of ovarian hyperstimulation syndrome (Trounson, 1986; Bergh *et al*., 1995). Since multiple gestation, with its inherent risk for adverse outcome, remains a major concern in ART practice, the single-embryo transfer (SET) strategy has become increasingly accepted (Gerris *et al*., 1999, 2003; Vilska *et al*., 1999; Tiitinen *et al*., 2001; Ombelet *et al*., 2005; Van Landuyt *et al*., 2006). One consequence of SET is an increased availability of supranumerary embryos for freezing (De Neubourg *et al*., 2002), resulting in more children born after cryopreservation and a decrease of multiple pregnancies (Gerris *et al*., 2003). Cryopreservation will also increase the chance of pregnancy in a natural cycle without additional ovarian stimulation and oocyte retrieval.

The pregnancy rate after transfer of frozen-thawed embryos depends on the freezing programme used, the stage of the embyro at freezing, the quality of the frozen embryo and the survival rate after thawing (Lasalle *et al*., 1985; Hartshorne *et al*., 1990) as well as the number of frozen-thawed embryos transferred. Pregnancy and birth rates per transfer of one or more frozen-thawed embryos have been reported as 25–30% and 15–20%, respectively (Cohen *et al*., 1988; Sathanandan *et al*., 1992, Van der Elst *et al*., 1997).

Although cryopreservation and thawing involve important cellular changes, there were initially no reports of adverse outcome in mammalian embryos (Dulioust *et al*., 1995; Whittingham *et al*., 1997). However, a detailed study of adult mice born after cryopreservation as embryos found morphological and behavioural disorders (Dulioust *et al*., 1995) and since only some of these findings were in adult animals only, the authors concluded that the effects of embryo freezing and thawing may be delayed.

Although cryopreservation of embryos is part of most IVF programmes, only limited studies on perinatal outcome of children born after replacement of cryopreserved embryos are available today (Frydman *et al*., 1989; Wada *et al*., 1994; Heijnsbroek *et al*., 1995; Sutcliffe *et al*., 1995a,b; Olivennes *et al*., 1996; Bonduelle *et al*., 1998; Wennerholm *et al*., 1997, 1998, 2000). Moreover, the number of children studied is small and no distinction is made between ICSI and conventional IVF. Aytoz *et al*. (1999), when describing the initial pregnancies of the cohorts studied here, were the first to compare the obstetric outcome of cryo ICSI and cryo IVF related to fresh ICSI and fresh IVF, respectively. No pathological features in terms of prematurity, low birthweight or intrauterine death were found in the frozen group compared with the fresh group. Reassuring data in terms of gestational age, birthweight and perinatal mortality were also published by Wada *et al*. (1994) comparing birth characteristics in a series of children conceived from cryopreserved IVF embryos and children born after fresh IVF. Wennerholm *et al*. (1997) described the obstetric and neonatal outcome of 270 children conceived from cryopreserved IVF embryos in comparison with children born after IVF with fresh embryos as well as in comparison with children born after spontaneous conception: gestational age at delivery, birthweight, the incidence of malformations and the perinatal mortality of children born after cryopreservation were comparable with the two control groups both for singletons and for twins. Major malformations rates in children conceived from cryopreserved embryos are reported to be similar to those in children after spontaneous conception and to those in children born after transfer of fresh IVF embryos (Sutcliffe *et al*., 1995b; Wennerholm *et al*., 1997, 1998; Bergh *et al*., 1999; Westergaard *et al*., 1999).

The aim of this report was to evaluate the safety for offspring born from frozen-thawed embryos after ICSI or IVF compared with children born from fresh embryos from the same techniques.

Prenatal findings and neonatal findings of 937 children born after transfer of frozen-thawed ICSI (1993–2006) and IVF (1986–2006) embryos will be compared with neonatal data of 2889 infants born after fresh ICSI (1991–1999) and of 2995 infants born after fresh IVF (1983–1999) (Bonduelle *et al*., 2002a,b).

Materials and Methods

Ovarian stimulation, oocyte retrieval

Female patients underwent ovarian stimulation using urinary or recombinant FSH in combination with GnRH antagonists, agonists or clomiphene citrate. Oocyte retrieval was carried out 36 h after hCG injection (Smitz *et al*., 1988a,b; Camus *et al*., 1989; Kolibianakis *et al*., 2004). IVF treatment was predominantly applied for patients with tubal or idiopathic infertility indications, whereas ICSI was predominantly performed for male factor indications.

Embryos for transfer and for freezing

Fertilization was checked 16–19 h post-microinjection or post-insemination. Embryos were evaluated daily until transfer. From 1984 up to 1999, oocytes and embryos were cultured in home-made media formulations from the day of pick-up till Day 3 of embryo culture as described by Staessen *et al*. (1990) and from 1999 onwards in sequential culture media preparations from the day of pick-up till Days 5–6 of embryo culture as described by Van Landuyt *et al*. (2005). Intrauterine embryo replacement was carried out mainly on Day 1, 2, 3 or 5 of the oocyte collection cycle (OCC). Up to three (occasionally more than three) of the best-quality available embryos were transferred during the OCC. Supernumerary embryos were frozen on Day 1, 2, 3, 5 or 6 of the OCC, according to morphological criteria previously described in detail (Van den Abbeel *et al*., 1988, 1997, 2005; Van der Elst *et al*., 1995).

The freezing procedure depended on the day of freezing in the OCC and on the developmental stage of the embryos: Day 1 embryos were frozen with a slow controlled-rate freezing procedure with 1,2-propanediol as the cryoprotectant or with an ultra-rapid freezing procedure as described in detail by Van den Abbeel *et al*. (1988, 1997); Day 2/3 embryos were frozen with a slow controlled-rate freezing procedure with either 1,2-propanediol or dimethylsulphoxide as the cryoprotectant as described in detail by Van den Abbeel *et al*. (1988, 2000) and Van der Elst *et al*. (1995); Day 5/6 embryos were frozen with a slow controlled-rate freezing procedure with glycerol as the cryoprotectant as described by Van den Abbeel *et al*. (2005).

Evaluation of thawing and transfer of thawed embryos

Day 1 embryos were scored immediately after thawing and dilution of the cryoprotectant, and again after a 24 h post-thaw *in vitro* culture. Day 2/3 embryos were scored immediately after thawing, and again after either a 4 h (up to 1996) or a 24 h (from 1996 onwards) post-thaw *in vitro* culture (Van der Elst *et al*., 1997. Day 5/6 embryos were scored immediately after thawing, and occasionally again after a 24 h post-thaw *in vitro* culture. Surviving embryos were transferred according to methods previously described by Van den Abbeel *et al*. (1988, 1997, 2000, 2005) and Van der Elst *et al*. (1995, 1997).

Frozen embryos were mainly transferred during the course of a natural cycle. Other transfer cycles involved the use of clomiphene citrate or human menopausal gonadotrophins in association with hCG.

Clinical follow-up study

Inclusion criteria

Irrespective of the cryoprocedure used, all pregnancies after transfer of frozen-thawed embryos obtained by conventional IVF or ICSI were consecutively included. Pregnancies obtained after mixed ICSI–IVF procedures were not taken into account in this study. Cycles with preimplantation genetic diagnosis were excluded. Children born after ICSI were conceived using ejaculated, epididymal or testicular spermatozoa (fresh or frozen).

Study design

The study set-up is similar to earlier follow-up studies and has been extensively described in previous publications (Bonduelle *et al*., 2002a,b). Briefly, data on pregnancies, deliveries and neonatal histories were retrieved from written information obtained from the gynaecologists, paediatricians and parents. At the child's age of ~2

months, the information given by the gynaecologists/paediatrician was double-checked with the parents and completed with additional information on illness, hospital admissions, surgery and medication intake, either at the consultation in the genetic department or over the phone by the research nurse. At this time, when possible, a detailed physical examination of the babies with attention to minor and major malformations was performed. If no prenatal test had been carried out, some children had a routine karyotype at that moment with their parents' consent.

Statistical analysis

Categorical data are presented as number of children and percentages for each group of interest. Comparisons of percentages among groups are presented as odds ratios (ORs) with corresponding 95% confidence intervals (95% CIs) for each comparison made (Altman, 1991). In doing so, we provide information to the reader on magnitude and precision of the difference between groups for each comparison, as well as the role that chance may play in the observed study results (Altman, 1991). Continuous data on birthweight, gestational age and maternal age are presented as mean and standard deviation (SD). Statistical analysis of continuous data is performed by two-way analysis of variance (with cryo ICSI/cryo IVF, singletons/multiples and their interaction), using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Definitions

Biochemical pregnancy: significant increase in hCG levels (>10 IU/ml) between Day 10 and Day 20 after LH surge (Camus *et al*., 1989). Ongoing pregnancy: pregnancy with fetal heartbeat. Spontaneous abortion: loss of a fetus with a gestational age <20 weeks. Gestational age: calculated from the day of oocyte aspiration which was defined as Day 14 of the cycle. Stillbirth: intrauterine or intrapartum death of a child born with a gestational age ≥ 20 weeks (active terminations after 20th week of gestation are not included). Early pregnancy termination: termination before 20th week of gestation. Late pregnancy termination: termination after 20th week, 20 weeks included. Preterm birth: delivery before 37 completed weeks of gestation. Low birthweight: <2500 g at birth. Very low birthweight: <1500 g at birth. Early neonatal death: death of a child before Day 7. Late neonatal death: death of a child from Day 7 until Day 28 inclusive. Infant death: death of a child after Day 28. Total infant death: number of early and late neonatal and infant deaths. Total perinatal death: number of stillborns and early neonatal deaths. Total malformation rate: (affected live births + affected stillborns + induced abortions for malformations) divided by (live births + stillbirths) (Eurocat, 1993; Lechat and Dolk, 1993).

Identical guidelines and classifications in the two study populations were used as extensively described in previous publications (Bonduelle *et al*., 2002a,b). A widely accepted definition of major malformations was used, i.e. malformations that generally cause functional impairment or require surgical correction. The remaining malformations were considered minor according to an extensive checklist for minor congenital anomalies on the basis of a textbook by Aase (1990).

Results

Evolution of pregnancies and number of children

In cryo

Data on early pregnancy loss in terms of biochemical pregnancy, spontaneous abortion, ectopic pregnancy, terminations and pregnancies lost to follow-up are detailed in Table I. Reasons for early and late terminations are described in detail in Appendix 1. In cryo ICSI, 470 ongoing pregnancies led to the birth of 556 children (547 live born and 9 stillborn), of which 384 (70.2%) were singletons and 163 (29.8%) were multiples (Appendix 2). In cryo IVF, 342 ongoing pregnancies led to the birth of 397 children (390 live born and 7 stillborn), of which 284 (72.8%) were singletons and 106 (27.2%) were multiples.

Biochemical pregnancy rates and spontaneous abortion rates were comparable between cryo ICSI and cryo IVF (Table I). No higher frequency of ectopic pregnancy was documented in cryo ICSI in comparison with cryo IVF. Comparable percentages of pregnancies led to the birth of a child in the cryo ICSI pregnancies compared with the cryo IVF pregnancies. Stillbirth rate and multiple birth rate were comparable between cryo ICSI and cryo IVF [OR 0.92 (0.34–2.48) and OR 1.14 (0.85–1.52), respectively] (Appendix 2).

In cryo versus fresh

Statistically significantly higher rates of biochemical pregnancies were observed in the cryo ICSI and cryo IVF group compared with the fresh ICSI and fresh IVF cohort, respectively (Table I). No higher incidence of spontaneous abortion or ectopic pregnancy was found in the frozen groups compared with the fresh groups, both for ICSI and for IVF. Significantly lower percentages of pregnancies led to the birth of a child in the cryo ICSI (59.2%) and cryo IVF group (61.2%)

Table I. Early pregnancy loss in cryo and fresh pregnancies.

	Cryo ICSI $N = 793$ (%)	Fresh ICSI $N = 3073$ (%)	OR (95% CI) Cryo ICSI versus Fresh ICSI	Cryo IVF $N = 558$ (%)	Fresh IVF $N = 3329$ (%)	OR (95% CI) cryo IVF versus fresh IVF	OR (95% CI) cryo ICSI versus cryo IVF
Biochemical pregnancy	119 (15.0)	251 (8.2)	1.99 (1.57–2.51)	98 (17.5)	394 (11.8)	1.59 (1.25–2.02)	0.83 (0.62–1.11)
Spontaneous abortion	129 (16.2)	428 (13.9)	1.20 (0.97–1.49)	79 (14.1)	439 (13.2)	1.09 (0.84–1.41)	1.18 (0.87–1.60)
Ectopic pregnancy	17 (2.1)	49 (1.6)	1.35 (0.78–2.36)	17 (3.0)	74 (2.2)	1.39 (0.81–2.36)	0.70 (0.35–1.38)
Lost to follow-up	48 (6.1)	73 (2.4)	2.65 (1.82–3.85)	18 (3.2)	86 (2.6)	1.26 (0.75–2.11)	1.94 (1.12–3.36)
Terminated pregnancy	10 (1.2)	18 (0.6)	2.17 (0.99–4.72)	4 (0.7)	22 (0.7)	1.09 (0.37–3.16)	1.77 (0.55–5.67)
Ongoing pregnancy	470 (59.2)	2254 (73.3)	0.53 (0.45–0.62)	342 (61.2)	2314 (69.5)	0.70 (0.58–0.84)	0.92 (0.74–1.15)

compared with the fresh ICSI (73.3%) and fresh IVF group (69.5%), respectively. A statistically significantly higher proportion of singletons were born in cryo ICSI (70.2%) and cryo IVF (72.8%) than in fresh ICSI (52.8%) and fresh IVF (52.7%) [ICSI: OR 2.11 (1.73–2.57); IVF: OR 2.41 (1.91–3.05)]. Comparable numbers of stillbirths were found in the frozen groups compared with the fresh groups, both for ICSI and for IVF (Appendix 2).

Maternal characteristics

In cryo

Maternal educational level, classified to a five-level status was comparable between cryo ICSI and cryo IVF (Wilcoxon two-sample test: $P = 0.340$) (data not shown). More mothers (64.0%) of cryo ICSI children were nulliparous compared with mothers (58.4%) of cryo IVF children, but this difference was not statistically significant. Mean maternal age and mean pregnancy duration were comparable between cryo ICSI and cryo IVF mothers (Appendix 3). Mean maternal age was also comparable in multiple versus singleton pregnancies in both groups (data not shown). However, pregnancy duration differed significantly between singleton and multiple pregnancies both in cryo ICSI and in cryo IVF (data not shown). Mode of delivery differed significantly between cryo ICSI and cryo IVF ($P = 0.028$). Of the cryo ICSI, children 41.3% were delivered by Caesarean section compared with 33.3% of the cryo IVF children.

In cryo versus fresh

Mothers of newborns conceived by fresh ICSI and fresh IVF embryos were significantly older than mothers of newborns conceived by frozen ICSI and frozen IVF embryos, respectively (Appendix 3). Pregnancy duration, however, did not differ between the fresh and frozen groups, both for ICSI and for IVF.

Prenatal diagnosis

In cryo

Prenatal diagnosis was performed in 173 out of the 480 (36.0%) ongoing cryo ICSI pregnancies and in 64 out of the 346 (18.5%) ongoing cryo IVF pregnancies.

The median maternal age of the mothers who underwent prenatal testing in the cryo ICSI group was 31.8 years (4.36) and was 34.7 years (3.45) in the cryo IVF group ($P < 0.001$).

For 274 fetuses tested prenatally, a karyotype was obtained in 200 cryo ICSI fetuses and in 72 cryo IVF fetuses. No results were obtained in one cryo ICSI fetus and in one cryo IVF fetus, but the two pregnancies led to two live borns without clinical abnormality at birth. Of the tested fetuses in the cryo ICSI pregnancies, 147 fetuses were singletons and 53 were multiples. Of the tested fetuses in the cryo IVF pregnancies, 56 were singletons and 16 were multiples. Abnormal fetal karyotypes (Table II) were found in eight cases in 200 prenatally tested cryo ICSI fetuses (4%); seven (3.5%) anomalies were *de novo* and one (0.5%) was an inherited anomaly from the mother. All eight of the pregnancies were terminated. It is worth mentioning that one trisomy 21 and one Klinefelter syndrome issued of the same twin pregnancy. Abnormal fetal karyotypes were found in 2 cases in 72 prenatally tested cryo IVF fetuses (2.8%); 1 was a *de novo* numerical anomaly which was terminated and one was a structural defect inherited from the mother, which led to the birth of a live born child without clinical abnormality at birth.

Table II. Chromosome abnormalities pre- and post-natally in cryo ICSI versus cryo IVF and fresh ICSI.

	ICSI			IVF	
	Cryo ICSI *N* (%)	Fresh ICSI *N* (%)	OR (95% CI) cryo ICSI versus fresh ICSI	Cryo IVF N (%)	OR (95% CI) cryo ICSI versus cryo IVF
Prenatal	***N* = 200**	***N* = 1586**		***N* = 72**	
De novo	*7 (3.5)*	*25 (1.5)*	*2.27 (0.97–5.31)*	*1 (1.4)*	*2.51 (0.30–20.71)*
Sex		10			
Klinefelter	3				
Autosomal numerical		8			
Trisomy 18				1	
Trisomy 21	3				
Autosomal structural	1	7			
Inherited	*1 (0.5)*	*22 (1.4)*	*0.36 (0.05–2.67)*	*1 (1.4)*	*0.35 (0.02–5.62)*
Prenatal total	8 (4)	47 (2.9)	1.36 (0.64-2.93)	2 (2.8)	1.42 (0.29-6.84)
Post-natal	***N* = 81**	***N* = 338**		***N* = 40**	
De novo	*2*	*7*	*1.20 (0.24–5.87)*		
Sex		2			
Autosomal numerical		3			
Trisomy 21	1				
Autosomal structural	1	2			
Inherited	*1*	*6*			
Post-natal total	3 (3.7)	13 (3.8)	0.96 (0.27-3.46)	0	3.60 (0.18-71.21)
Overall (prenatal and post-natal) anomalies in tested group	***N* = 281**	***N* = 1924**		***N* = 112**	
De novo	*9 (3.2)*	*32 (1.7)*	*1.96 (0.92–4.14)*	*1 (0.9)*	*3.68 (0.46–29.33)*
Total	11 (3.9)	60 (3.1)	1.27 (0.66–2.44)	2 (1.8)	2.24 (0.49–10.28)

There were more cryo ICSI fetuses with chromosomal anomalies than cryo IVF fetuses, but this difference was not statistically significant (OR 1.42; 95% CI 0.29–6.84). Likewise, there were more cryo ICSI fetuses with *de novo* aberrations than cryo IVF fetuses, but again this difference was not statistically significant (OR 2.51; 95% CI 0.3–20.71). For 71/201 (35.3%) of the mothers of tested cryo ICSI fetuses, there was a maternal age-related risk (maternal age ≥35 years) compared with 46/73 (63%) for mothers from tested cryo IVF fetuses. There were seven mothers of cryo ICSI fetuses with an abnormal result who were younger than 35 years. All mothers of cryo IVF fetuses with an abnormal result were older than 35 years. For 1 of the 71 cryo ICSI fetuses tested for a maternal age risk, the result was a *de novo* anomaly (trisomy 21). Likewise, for one of the cryo IVF fetuses tested for maternal age risk, the result was a *de novo* anomaly (trisomy 18). For 5/109 (4.6%) of the fetuses of mothers aged <35 years, who were tested only because of the ICSI procedure, the result was a *de novo* anomaly.

Abnormal karyotypes were also found in 3 out of 81 cryo ICSI children (3.7%) karyotyped at birth (and not tested prenatally) (Table II). One inherited (from the father) balanced karyotype and two *de novo* anomalies were found. No abnormal karyotypes were found in the 40 post-natally karyotyped (and not tested prenatally) cryo IVF children.

Overall, chromosomal anomalies (pre- and post-natal) in the tested fetuses/children were more frequent in cryo ICSI (3.9%) compared with cryo IVF (1.8%), but this difference was not statistically significant (OR 2.24; 95% CI 0.49–10.28).

In cryo versus fresh

More *de novo* karyotype anomalies were found prenatally in the cryo ICSI group compared with the fresh ICSI group, but this difference was not statistically significant (OR 2.27; 95% CI 0.97–5.31) (Table II). Comparable rates of abnormal karyotypes were found in cryo ICSI and fresh ICSI post-natally.

Overall, pre- and post-natally, there was a trend to more karyotype anomalies in cryo ICSI compared with fresh ICSI (OR 1.27; 95% CI 0.66–2.44). This non-statistically significant increase was also found in cryo ICSI versus fresh ICSI concerning *de novo* anomalies (OR 1.96; 95% CI 0.92–4.14).

Neonatal data

In cryo

For 572 cryo ICSI children (99.3%) and 399 cryo IVF children (99.2%), we collected complete information at birth.

Neonatal measurements for cryo ICSI and cryo IVF live born children are listed in Table III. Birthweight of all children in the cryo ICSI and cryo IVF group was not found to be statistically different. A significant difference between the birthweight of singletons and multiples was observed, but this difference was comparable in cryo ICSI versus cryo IVF children.

Low birthweight, very low birthweight and prematurity were present in comparable rates in cryo ICSI and cryo IVF (Table IV).

Admission to a neonatal care unit did not occur more often in cryo ICSI neonates than in cryo IVF children (Table IV). Multiples were more often admitted to neonatal care units than singletons, both in cryo ICSI and in cryo IVF newborns. Total infant death rate was 0.1% in cryo ICSI and 0.5% in cryo IVF; this difference was not statistically significant ($P = 0.313$).Total perinatal death rate was 2.3% in cryo ICSI and 3% in cryo IVF which was not statistically significantly different ($P = 0.539$) (data not shown).

In cryo versus fresh

Neonatal measurements for cryo ICSI, cryo IVF, fresh ICSI and fresh IVF live born children are listed in Table III. Mean weight, length and head circumference at birth of all children in the frozen groups were higher than in the fresh groups, both for ICSI and for IVF ($P < 0.001$). Differences in birthweight between cryo singletons and fresh singletons, both in ICSI and in IVF, were borderline significant ($P = 0.059$ and

Table III. Birth characteristics of children born after transfer of cryopreserved and fresh embryos.

	ICSI					IVF				
	Cryo ICSI		Fresh ICSI		*P*-value	Cryo IVF		Fresh IVF		*P*-value
	N	Mean (SD)	*N*	Mean (SD)		*N*	Mean (SD)	*N*	Mean (SD)	
Mean birthweight										
Total group	539	3067.9[a] (666.6)	2799	2806.7 (719.1)	<0.001	384	3023.8[a] (689.1)	2920	2765.3 (725.0)	<0.001
Singletons	381	3301.2[b] (571.9)	1476	3224.3 (581.9)	0.059	281	3269.7[b] (535.2)	1523	3176.4 (582.6)	0.046
Twins	155	2505.3[b] (533.2)	1211	2394.5 (522.0)	0.013	98	2365.6[b] (623.7)	1251	2382.5 (560.9)	0.776
Mean birth length										
Total group	507	48.9[c] (3.1)	2452	47.9 (3.7)	<0.001	365	48.7[c] (3.5)	2697	47.7 (3.7)	<0.001
Mean head-circumference										
Total group	440	34.1 (1.8)	2164	33.4 (2.3)	<0.001	306	33.9 (2.2)	2336	33.4 (2.3)	<0.001

[a]ANOVA No difference in birthweight for cryo ICSI and cryo IVF children ($P = 0.329$).
[b]ANOVA Difference in birthweight between singletons and multiples ($P < 0.001$).
[b]ANOVA The pattern of difference in birthweight between singletons and multiples is the same in cryo ICSI and cryo IVF ($P = 0.176$).
[c]ANOVA No difference in birth length for cryo ICSI and cryo IVF ($P = 0.132$).

Table IV. Low birthweight (<2500 g), very low birthweight (<1500 g), prematurity (<37 weeks) and admission to neonatal care units (NCU) in liveborns after transfer of cryopreserved and fresh embryos.

	ICSI					IVF					
	Cryo ICSI *N*	%[a]	Fresh ICSI *N*	%	OR (95% CI) cryo ICSI versus fresh ICSI	Cryo IVF *N*	%	Fresh IVF *N*	%	OR (95% CI) cryo IVF versus fresh IVF	OR (95% CI) cryo ICSI versus cryo IVF
Low birthweight	96	17.8	760	27.1	0.58 (0.46–0.74)	73	19.0	784	26.8	0.64 (0.49–0.84)	0.92 (0.66–1.29)
Singletons	24	6.2	106	7.2	0.87 (0.55–1.37)	20	7.1	121	7.9	0.64 (0.54–1.45)	0.88 (0.48–1.62)
Twins	70	45.1	593	48.1	0.86 (0.61–1.20)	49	50.0	568	45.1	1.21 (0.80–1.81)	0.82 (0.50–1.37)
Very low birthweight	13	2.4	125	4.4	0.53 (0.30–0.94)	17	4.4	167	5.6	0.76 (0.46–1.27)	0.54 (0.26–1.11)
Singletons	6	1.6	22	1.5	1.06 (0.43–2.63)	2	0.7	28	1.8	0.39 (0.09–1.61)	2.23 (0.45–11.14)
Twins	7	4.5	64	5.2	0.85 (0.38–1.88)	15	15.3	96	7.6	2.18 (1.21–3.92)	0.26 (0.10–0.67)
Prematurity	139	25.4	902	31.8	0.73 (0.59–0.90)	102	26.1	867	29.3	0.86 (0.67–1.08)	0.96 (0.72–1.29)
Singletons	44	11.4	126	8.4	1.41 (0.98–2.03)	34	11.9	140	9.0	1.38 (0.92–2.05)	0.95 (0.59–1.53)
Twins	92	57.5	669	54.6	1.13 (0.81–1.58)	62	62.0	600	47.6	1.77 (1.17–2.69)	0.83 (0.50–1.38)
Admission NCU	171	31.3	1149	40.5	0.67 (0.55–0.81)	140	35.9	1288	43.6	0.73 (0.58–0.90)	0.81 (0.62–1.07)
Singletons	77	20.1	261	17.5	1.18 (0.89–1.57)	66	23.2	289	18.7	1.31 (0.97–1.78)	0.83 (0.57–1.20)
Twins	91	56.9	782	63.4	0.78 (0.56–1.10)	69	69.0	853	67.7	1.11 (0.71–1.74)	0.60 (0.35–1.03)

[a] % = % children in each group indicated in Table III.

$P = 0.046$ respectively), but as a total group ICSI plus IVF cryo versus ICSI plus IVF fresh, the differences were highly significantly different ($P < 0.001$). Cryo ICSI twins, but not cryo IVF twins, had a significantly higher mean birthweight compared with their fresh peers (ICSI: $P = 0.013$; IVF: $P = 0.776$).

Overall rates of low birthweight (<2500 g) were statistically significantly higher in the fresh groups compared with the frozen groups, both for ICSI and for IVF, reflecting the higher proportion of multiple births in the fresh group (Table IV). In singletons and in twins, comparable rates of low birthweight were found in the frozen groups versus the fresh groups, both for ICSI and for IVF.

Very low birthweight (<1500 g) was more often the case in the fresh ICSI neonates compared with cryo ICSI neonates, but not in fresh IVF compared with cryo IVF. Twins in the cryo IVF group showed a significantly higher risk for very low birthweight compared with their peers born after transfer of fresh embryos.

Prematurity was more frequently observed in the total fresh ICSI group compared with the total cryo ICSI group reflecting the different proportions of twins in both groups. A similar but non-significant trend to more prematurely born infants in the total fresh IVF group compared with the total cryo IVF group was found. In singletons, comparable prematurity rates were found between fresh and cryo children, both for ICSI and for IVF. Significantly more cryo IVF twins were prematurely born compared with fresh IVF twins.

Admission to a neonatal care unit did occur more often in fresh ICSI and fresh IVF neonates compared with cryo ICSI and cryo IVF, respectively. Stratification according to birth status (singletons versus twins) showed that this is likely to be simply a reflection of a higher proportion of twins in the fresh population.

Total perinatal death rate was comparable in the frozen groups and the fresh groups. Likewise, total infant death rate was comparable between the frozen and the fresh group (data not shown).

Malformations

In cryo

Major malformations in early and late terminated cryo fetuses are listed in Appendix 1.

The major malformation rate in live births is 6.4% in cryo ICSI children, which is statistically significantly higher than 3.1% in cryo IVF children (OR 2.15; 95% CI 1.10–4.20). For details on major malformations in cryo live borns, see Appendix 4. A higher malformation rate in live born multiples compared with live born singletons was observed in cryo IVF but not in cryo ICSI (Table V).

The total malformation rate taking into account major malformations in stillborns, in terminations and in live borns was 8.4% in cryo ICSI and 4.1% in cryo IVF, which was a statistically significant difference (OR 2.15; 95% CI 1.20–3.85). Adjusted analysis for variables such as maternal age, term and birthweight did not change the result [adjusted OR 2.48; 95% CI (1.25–4.90)].

All major malformations were classified as malformations of different organ systems, and frequencies were similar between cryo ICSI and cryo IVF for most organ systems (data not shown). Apparent, but non-significant, higher percentages of cardiac (1.8% versus 0.3%) and genital (1.6% versus 0.5%) problems were observed in the cryo ICSI group versus the cryo IVF group (OR 7.25; 95% CI 0.92–56.82 for cardiac problems and OR 3.5; 95% CI 0.70–15.10 for genital problems).

Minor malformations were observed in 154/547 (28.2%) live born cryo ICSI children and in 111/390 (28.5%) live born cryo IVF children (OR 0.99; 95% CI 0.74–1.31), which was comparable.

Table V. Major malformations in liveborns after transfer of cryopreserved and fresh embryos.

	ICSI			IVF			
	Cryo ICSI *N* (%)	Fresh ICSI *N* (%)	OR (95% CI) cryo ICSI versus fresh ICSI	Cryo IVF *N* (%)	Fresh IVF *N* (%)	OR (95% CI) cryo IVF versus fresh IVF	OR (95% CI) cryo ICSI versus cryo IVF
Liveborn group							
Total	35 (6.4)	96 (3.4)	1.96 (1.31–2.91)	12 (3.1)	112 (3.8)	0.81 (0.44–1.48)	2.15 (1.10–4.20)
Singleton	24 (6.3)[a]	46 (3.1)	2.11 (1.27–3.50)	6 (2.1)[b]	49 (3.1)	0.67 (0.28–1.56)	3.09 (1.25–7.66)
Multiples	11 (6.7)[a]	50 (3.6)	1.87 (0.95–3.67)	6 (5.7)[b]	63 (4.5)	1.27 (0.54–3.01)	1.21 (0.43–3.37)
All groups*							
Total	46 (8.4)	122 (4.2)	2.05 (1.44–2.91)	16 (4.1)	135 (4.6)	0.86 (0.51–1.46)	2.15 (1.20–3.85)

*All groups include liveborns, stillborns and terminations.
Comparing major malformations in multiples versus singletons in cryo ICSI [OR 1.09 (0.47–2.37)][a] and in cryo IVF [OR 2.78 (0.88–8.22)][b].

In cryo versus fresh

The incidence of 6.4% of major malformations in the cryo ICSI live born group is significantly higher than 3.4% in the fresh ICSI live born group (OR 1.96; 95% CI 1.31–2.91) but not more major malformations were found in the cryo IVF group compared with the fresh IVF group (Table V). The major malformation rate in the total live born cryo group (ICSI plus IVF) (5.0%) was significantly higher than in the total live born fresh group (ICSI plus IVF) (3.6%) (OR 1.42; 95% CI 1.03–1.96). A significantly higher rate of major malformations was found in cryo ICSI singletons compared with fresh ICSI singletons (OR 2.11; 1.27–3.50). The increased rate of major malformations in cryo ICSI twins versus fresh ICSI twins did not reach statistical significance (OR 1.87; 95% CI 0.95–3.67).

The total major malformation rate was significantly higher in cryo ICSI (8.4%) than in fresh ICSI (4.2%) (OR 2.05; 95% CI 1.44–2.91) but not in cryo IVF compared with fresh IVF (OR 0.86; 95% CI 0.51–1.46).

Medical history of cryo infants

Up to the age of 2 months, significantly more cryo ICSI children (129/530; 24.3%) had to deal with an illness compared with cryo IVF children (66/387; 17.1%) (OR 1.57; 95% CI 1.12–2.18) (data not shown in the table). The major cause of illness in both groups was an infectious disease. Other causes were non-infectious illnesses, e.g. eczema, obstipation, oesophagitis and gastro-oesophageal reflux. Not more cryo ICSI children (64/234; 27.4%) had to take medication (mostly antibiotics, aerosol, mucolytica and laxativa) compared with cryo IVF children (24/96; 25.0%) (OR 1.13; 95% CI 0.66–1.95). Although more cryo ICSI children (27/264; 10.2%) were at least once admitted to the hospital compared with cryo IVF children (8/112; 7.1%), this difference was not significant. In both groups, most admissions were because of an infectious condition. Surgery was performed more often in the cryo ICSI [pyloric stenosis (2), inguinal hernia (2), nasolacrimal duct stenosis (1), naevus excision (1), excision of a xanthogranuloma (1), congenital glaucoma (1)] compared with the cryo IVF group [inguinal hernia (2)], although this difference was not statistically significant (OR 2.74; 95% CI 0.58–12.96), reflecting the small numbers involved in this comparison.

Follow-up of cryo children at 2 months

For the cryo ICSI children, the follow-up rate at 2 months was 84.6% (463/547) of the total number of live born children. Of these, 76.6% were examined by a geneticist, for 5.6% written information was obtained from the paediatrician/general practitioner and for 17.8% written information was given by the parents. For the cryo IVF children, the follow-up rate at 2 months was 88.9% (347/390) of the total number of live born children. Of these, 90.5% were examined by a geneticist, for 3.6% written information was obtained from the paediatrician/general practitioner and for 5.9% written was given by the parents.

Not previously mentioned minor anomalies were found in 18 cryo ICSI children and 12 cryo IVF children.

Discussion

This study was set up to follow-up the total cohort of children conceived from cryopreserved embryos since the introduction of this technique in our centre. We focused on the outcome of children born after transfer of cryopreserved and thawed embryos obtained through the ICSI technique. Outcome measures were primarily the neonatal outcome of children born after the use of cryopreserved ICSI embryos in comparison with the outcome after the use of cryopreserved IVF embryos. Secondly, we evaluated the possible influence of the cryopreservation technique by comparing our results in children born after cryopreservation with previously published data from our centre concerning neonatal outcome of 2889 ICSI and 2995 IVF children born after transfer of fresh embryos (Bonduelle *et al*., 2002a) and karyotype anomalies (Bonduelle *et al*., 2002b). In this report, only data concerning children's health and malformations up to 6 months were analysed, although more parameters concerning pregnancy and concerning the children were recorded. Other variables such as pregnancy complications and details on the transfer of frozen-thawed embryos are not reported here.

Comparison of study populations

The cryo ICSI and cryo IVF group were a homogeneous population in which a number of variables such as maternal educational level, maternal age and parity were comparable. In both groups, similar rates of multiple births were observed, so that we could evaluate different parameters in the whole cohort as well as in the singletons or multiple cohorts.

Pregnancy outcome in terms of risk of pregnancy loss (biochemical, ectopic and spontaneous abortion) was similar in cryo ICSI compared with cryo IVF. Comparable rates of early fetal abortions for malformations or karyotype anomalies in the total cryo ICSI group and the total cryo IVF group point out that there is no bias in the cryo ICSI pregnancies towards the fresh ICSI pregnancies due to elimination in early pregnancy of an amount of anomalies possibly as a result of the ICSI procedure. Since the follow-up rates in the pregnancy cohorts and for the neonatal data in the cryo groups are high, a bias because of missing data is unlikely. Even if all those lost to follow-up were cryo ICSI pregnancies leading to early pregnancy loss or to a live born with a major malformation, this would only strengthen the outcomes reported in this paper.

Although the cryo IVF cohort (1986–2006) is born partly prior to the cryo ICSI cohort (1993–2006) and this difference in treatment period is impossible to evaluate, the fresh ICSI and IVF cycles, that are used for comparison (Bonduelle *et al*., 2002a) show a great overlap in time (ICSI 1991–1999; IVF 1983–1999).

Pregnancy outcome

Biochemical pregnancy rates were comparable between cryo ICSI and cryo IVF but significantly higher than in fresh ICSI and fresh IVF, confirming the findings of Aytoz *et al*. (1999) on the first part of the studied cohorts.

Spontaneous abortion rates in our studied frozen groups were found to be similar and were also comparable with spontaneous abortion rates in the fresh groups. Delivery rates were comparable in the cryo ICSI and the cryo IVF group, but were both significantly lower than in the fresh ICSI and fresh IVF group, again confirming the results published by Aytoz *et al*. (1999). A lower delivery rate in the frozen population is a reflection of a significant higher percentage of biochemical pregnancies and a trend to a higher spontaneous abortion rate in the frozen groups compared with the fresh groups. A less favourable pregnancy outcome after cryopreservation might reflect a less favourable selection of embryos for freezing when compared with fresh embryos or a negative impact from the freezing procedure itself. A higher risk of pregnancy loss after transfer of cryopreserved ICSI embryos compared with cryopreserved IVF embryos is still controversial in the literature (Van Steirteghem *et al*., 1994; Kowalik *et al*., 1998; Aytoz *et al*., 1999) as is the difference in abortion rate between fresh and frozen embryos (Palermo *et al*., 1996; Wisanto *et al*., 1996; Wennerholm *et al*., 2000).

Prenatal diagnosis

Comparing cryo ICSI and cryo IVF, a non-statistically significant increase was found in the chromosomal anomaly rate (*de novo*/inherited) prenatally as well as post-natally. Overall (pre- and post-natal), cryo ICSI fetuses/infants were slightly more likely to have a karyotype anomaly than were fresh ICSI fetuses/infants. Considering pre-and post-natal samples, cryo ICSI fetuses/infants were almost twice as likely to have a *de novo* karyotype anomaly compared with the fresh ICSI group. The incidence of *de novo* chromosomal anomalies in cryo ICSI fetuses/children was also higher than in the general population (Jacobs *et al*., 1992).

Neonatal data

Neonatal measurements (birthweight, length and head circumference) were comparable between cryo ICSI and cryo IVF children for singletons as well as for multiples.

Comparison of these data with values of birth characteristics of all children born after fresh cycles shows that these measurements were all significantly higher in the cryo ICSI as well as in the cryo IVF group, probably due to the higher rate of twins in the fresh groups. In accordance with results from FIVNAT (1996) and Wennerholm *et al*. (2000) concerning mean birthweight in singletons, our results show that the mean birthweight of singletons in the cryo IVF and cryo ICSI group is higher compared with the reference fresh cohorts. This difference was significant for IVF singletons and reached borderline significance for the ICSI singleton offsprings. In addition, comparing the total group of cryo singletons (ICSI plus IVF) with the total group of fresh singletons (ICSI plus IVF), newborns born after transfer of cryopreserved embryos had a higher mean birthweight. A trend towards a higher mean birthweight in singletons and twins born after cryopreservation and IVF has previously been described (Wennerholm *et al*., 1997). Speculation of an optimized environment for the early developing embryo in the cryo pregnancies can be made since most of the frozen-thawed embryos are replaced in natural or minimally stimulated cycles. The striking favourable outcome is nevertheless in contrast to the high risk of early pregnancy loss after transfer of frozen-thawed embryos.

The mean gestational age according to multiplicity was not different when comparing the cryo ICSI with the cryo IVF group and was similar to the corresponding values for babies resulting from transfer of fresh embryos after ICSI and IVF. The same findings on gestational age were reported previously (Wada *et al*., 1994; Wennerholm *et al*., 1997; 2000; Aytoz *et al*., 1999).

A trend to lower preterm birth rates in the cryo group compared with the fresh group in singletons (Wennerholm *et al*., 2000) and in twins (Wada *et al*., 1994; Wennerholm *et al*., 2000) could not be found in our study.

The frequency for low birthweight was similar in the total cryo ICSI group compared with the total cryo IVF group, but was significantly lower than in the total fresh groups, respectively, probably due the different twinning rates in the cryo and fresh populations. Low birthweight rate according to multiplicity was however comparable between cryo and fresh, both in ICSI and in IVF, which is in line with literature data from Wennerholm *et al*. (1997, 2000).

Major malformations

A 2-fold increase of major malformations was found in cryo ICSI children, compared with cryo IVF children and compared with fresh ICSI children. Moreover, a significantly higher major malformation rate was found in the total cryo group (ICSI plus IVF) compared with the total fresh group (ICSI plus IVF). Literature data suggest that malformation rates after cryopreservation seems to be comparable with those of fresh ICSI and fresh IVF (Wennerholm *et al*., 1997, 1998; Aytoz *et al*., 1999; Westergaard *et al*., 1999) and vary between 1% (Wada *et al*., 1994) and 4.7% (Sutcliffe *et al*., 1995; Aytoz *et al*., 1999;

Wennerholm *et al*., 1997, 2000; Bergh *et al*., 1999; Westergaard *et al*., 1999). However, the number of infants born after cryopreservation is rather small in the previous studies, ranging from 105 to 270 and data on the combination of cryopreservation and ICSI are scarce (Aytoz *et al*., 1999; Wennerholm *et al*., 2000). Most studies report on cryopreservation and IVF without distinction between ICSI and conventional IVF. Factors that might influence the malformation rate, but beyond the scope of this study, are different cryopreservation protocols, difference in freezing day (Day 2, 3, 5 or 6) and difference in number and quality of frozen-thawed embryos transferred. Since these factors change over time, as well as across fertility centres, it will be challenging to tease out their effects on infant health outcome.

In our study, the cryo IVF cohort is born partly prior to the cryo ICSI which could induce a bias. The impact of advanced ovarian stimulation and freezing-thawing protocols cannot be ignored. From 1986 to 1991, IVF treatment was the sole ART procedure applied, predominantly for patients with tubal or idiopathic infertility indications, whereas ICSI was introduced in 1991 and mainly performed for male factor infertility and gradually for non-male factor infertility. Taking the above weaknesses into account, we still consider our cohorts provide a good opportunity to study additional risk factors for the offspring from cryopreserved embryos since all patients were recruited from the same centre and definitions and study protocol were equally applied in all four studied cohorts.

Conclusion

This study of the total cohort of children conceived from cryopreserved embryos in our centre showed that the neonatal outcome after ICSI is similar to that of IVF, except for a higher malformation rate in cryo ICSI. Once the first trimester is passed with its inherent risks of pregnancy loss, cryopreservation does not adversely affect neonatal outcome in terms of mean birthweight, risk of (very) low birthweight and risk of prematurity in singletons compared with singleton offspring from fresh embryos. Cryo IVF twins, but not cryo ICSI twins, are more at risk for very low birthweight and prematurity. Higher rates of *de novo* chromosomal anomalies were found in cryo ICSI fetuses/children compared that in the fresh ICSI group, although this difference did not reach statistical significance.

We observed more major malformations in cryo ICSI children in comparison with cryo IVF children and in comparison with the reference cohort using fresh embryos. This new finding of increased frequency of birth defects after the combination of cryopreservation and ICSI warrants further attention and understanding. Long-term follow-up studies are needed to ascertain that there are no late consequences for the children conceived from cryopreserved and thawed embryos.

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References

Aase JM. *Diagnostic Dysmorphology*. New York and London: Textbook Plenum Medical Book Company, 1999.

Altman DG. *Practical Statistics for Medical Research*. London, UK: Chapman and Hall, 1991.

Aytoz A, Van den Abbeel E, Bonduelle M, Camus M, Joris H, Van Steirteghem A, Devroey P. Obstetric outcome of pregnancies after the transfer of cryopreserved and fresh embryos obtained by conventional in-vitro fertilization and intracytoplasmic sperm injection. *Hum Reprod* 1999;**14**:2619–2624.

Bergh C, Werner C, Nilsson L, Hamberger L. Cumulative birth rates following cryopreservation of all embryos in stimulated in vitro fertilization (IVF) cycles. *J Assist Reprod Genet* 1995;**12**:191–194.

Bergh T, Ericson A, Hillensjo T, Nygren KG, Wennerholm UB. Deliveries and children born after in-vitro fertilisation in Sweden 1982–1995: a retrospective cohort study. *Lancet* 1999;**354**:1579–1585.

Bonduelle M, Wilikens A, Buysse A, Van Assche E, Devroey P, Van Steirteghem A, Liebaers I. A follow-up study of children born after intracytoplasmic sperm injection (ICSI) with epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. *Hum Reprod* 1998;**13**(Suppl 1):196–207.

Bonduelle M, Liebaers I, Deketelaere V, Derde MP, Camus M, Devroey P, Van Steirteghem A. Neonatal data on a cohort of 2889 infants born after ICSI (1991–1999) and of 2995 infants born after IVF (1983–1999). *Hum Reprod* 2002a;**17**:671–694.

Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A, Liebaers I. Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. *Hum Reprod* 2002b;**17**:2600–2614.

Camus M, Van den Abbeel E, Van Waesberghe L, Wisanto A, Devroey P, Van Steirteghem A. Human embryo viability after freezing with dimethylsulfoxide as a cryoprotectant. *Fertil Steril* 1989;**51**:460–465.

Cohen J, De Vane GW, Elsner CW, Kort HI, Massey JB, Norbury SE. Cryopreserved zygotes and embryos and endocrinologic factors in the replacement cycle. *Fertil Steril* 1988;**50**:61–67.

De Neubourg DD, Mangelschots K, Van Royen E, Vercruyssen M, Ryckaert G, Valkenburg M, Barudy-Vasquez J, Gerris J. Impact of patients' choice for single embryo transfer of a top quality embryo versus double transfer in the first IVF/ICSI cycle. *Hum Reprod* 2002;**17**:2621–2625.

Dulioust E, Toyama K, Busnel MC, Moutier R, Carlier M, Marchaland C, Ducot B, Roubertoux P, Auroux M. Long-term effects of embryo freezing in mice. *Proc Natl Acad Sci USA* 1995;**92**:589–593.

Eurocat (European Registration of Congenital Anomalies). Report 5, Surveillance of Congenital Anomalies 1980–1990. Edited by a Eurocat Working Group, 1999.

FIVNAT. Bilan des transferts d'embryons congelés de 1987 à 1994. *Contracept Fertil Sexual* 1996;**24**:700–705.

Frydman R, Forman R, Belaisch-Allart J, Hazout A, Fernandez H, Testart J. An obstetric analysis of fifty consecutive pregnancies after transfer of cryopreserved human embryos. *Am J Obstet Gynecol* 1989;**160**:209–213.

Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerssche M, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. *Hum Reprod* 1999;**14**:2581–2587.

Gerris J, De Neubourg D, De Sutter P. Cryopreservation as a tool to reduce multiple birth. *Reprod Biomed Online* 2003;**7**:286–294.

Hartshorne GM, Wick K, Elder K, Dyson H. Effect of cell number at freezing upon survival and viability of cleaving embryos generated from stimulated IVF cycles. *Hum Reprod* 1990;**5**:857–861.

Heijnsbroek I, Helmerhorst FM, van den Berg-Helder AF, van der Zwan KJ, Naaktgeboren N, Keirse MJ. Follow-up of 30 pregnancies after embryo cryopreservation. *Eur J Obstet Gynecol Reprod Biol* 1995;**59**:201–204.

Jacobs P, Browne C, Gregson N, Joyce C, White H. Estimates of the frequency of chromosome abnormalities detectable in unselected newborns using moderate levels of banding. *J Med Genet* 1992;**29**:103–106.

Kolibianakis E, Zikopoulos K, Verpoest W, Camus M, Joris H, Van Steirteghem AC, Devroey P. Should we advise patients undergoing IVF to start a cycle leading to a day 3 or a day 5 transfer. *Hum Reprod* 2004;**19**:2550–2554.

Kowalik A, Palermo G, Barmat L, Veeck L, Rimarachin J, Rosenwaks Z. Comparison of clinical outcome after cryopreservation of embryos obtained from intracytoplasmic sperm injection and in vitro fertilization. *Hum Reprod* 1998;**13**:2848–2851.

Lasalle B, Testart J, Renard JP. Human embryo features that influence the success of preservation with the use of 1,2 propanediol. *Fertil Steril* 1985;**44**:645–651.

Lechat MF, Dolk H. Registries of congenital anomalies: Eurocat (19993). *Envir Health Perspect* 1993;**101**(Suppl 2):153–157.

Olivennes F, Schneider Z, Remy V, Blanchet V, Kerbrat V, Fanchin R, Hazout A, Glissant M, Fernandez H, Dehan M *et al.* Perinatal outcome and follow-up of 82 children aged 1–9 years old conceived from cryopreserved embryos. *Hum Reprod* 1996;**11**:1565–1568.

Ombelet W, De Sutter P, Van der Elst J, Martens G. Multiple gestation and infertility treatment: registration, reflection and reaction—the Belgian project. *Hum Reprod Update* 2005;**11**:3–4.

Palermo G, Colombero L, Schattman G, Davis OK, Rosenwaks Z. Evolution of pregnancies and initial follow-up of newborns delivered after intracytoplasmic sperm injection. *J Am Med Assoc* 1996;**276**:1893–1897.

Sathanandan M, Mcnamee MC, Wick K, Mathews CD. Clinical aspects of human cryopreservation. In: Brinsden PR, Rainsbury PA (eds). *A Textbook of In Vitro Fertilization and Assisted Reproduction.* Carnforth, UK: Parthenon, 1992,259–263.

Smitz J, Devroey P, Camus M, Khan I, Staessen C, Van Waesberghe L, Wisanto A, Van Steirteghem A. Addition of Buserelin to human menopausal gonadotrophins in patients with failed stimulations for IVF or GIFT. *Hum Reprod* 1988a;**3**(Suppl 2):35–38.

Smitz J, Devroey P, Camus M, Deschacht J, Khan I, Staessen C, Van Waesberghe L, Wisanto A, Van Steirteghem A. The luteal phase and early pregnancy after combined GnRH-agonist/HMG treatment for superovulation in IVF or GIFT. *Hum Reprod* 1988b;**3**:585–590.

Staessen C, Van den Abbeel E, Carlé M, Khan I, Devroey P, Van Steirteghem A. Comparison between human serum and Albuminar-20 (TM) supplement for in-vitro fertilization. *Hum Reprod* 1990;**5**:336–341.

Sutcliffe AG, D'Souza SW, Cadman J, Richards B, McKinlay IA, Lieberman B. Outcome in children from cryopreserved embryos. *Arch Dis Child* 1995a;**72**:290–293.

Sutcliffe AG, D'Souza SW, Cadman J, Richards B, McKinlay IA, Lieberman B. Minor congenital anomalies, major congenital malformations and development in children conceived from cryopreserved embryos. *Hum Reprod* 1995b;**10**:3332–3337.

Tiitinen A, Halttunen M, Harkki P. Elective single embryo transfer: the value of cryopreservation. *Hum Reprod* 2001;**16**:1140–1144.

Trounson A. Preservation of human eggs and embryos. *Fertil Steril* 1986;**46**:1–12.

Van den Abbeel E, Van der Elst J, Van Waesberghe L, Camus M, Devroey P, Khan I, Smitz J, Staessen C, Wisanto A, Van Steirteghem A. Hyperstimulation: the need for cryopreservation of embryos. *Hum Reprod* 1988;**3**(Suppl 2):53–57.

Van den Abbeel E, Camus M, Van Waesberghe L, Devroey P, Van Steirteghem AC. Viability of partially damaged human embryos after cryopreservation. *Hum Reprod* 1997;**12**:2006–2010.

Van den Abbeel E, Camus M, Joris H, Van Steirteghem A. Embryo freezing after intracytoplasmic sperm injection. *Mol Cell Endocrinol* 2000;**169**:49–54.

Van den Abbeel E, Camus M, Verheyen G, Van Waesberghe L, Devroey P, Van Steirteghem A. Slow controlled-rate freezing of sequentially cultured human blastocysts: an evaluation of two freezing strategies. *Hum Reprod* 2005;**20**:2939–2945.

Van der Elst J, Camus M, Van den Abbeel E, Maes R, Devroey P, Van Steirteghem AC. Prospective randomised study on the cryopreservation of human embryos with dimethylsulfoxide or 1,2 propanediol protocols. *Fertil Steril* 1995;**63**:92–100.

Van der Elst J, Van den Abbeel E, Vitrier S, Camus M, Devroey P, Van Steirteghem A. Selective transfer of cryopreserved human embryos with further cleavage after thawing increases delivery and implantation rates. *Hum Reprod* 1997;**12**:1513–1521.

Van Landuyt L, De Vos A, Joris H, Verheyen G, Devroey P, Van Steirteghem AC. Blastocyst formation in IVF versus ICSI cycles: influence of the fertilisation procedure. *Fertil Steril* 2005;**83**:1397–1403.

Van Landuyt L, Verheyen G, Tournaye H, Camus M, Devroey P, Van Steirteghem A. New Belgian embryo transfer policy leads to sharp decrease in multiple pregnancy rate. *Reprod Biomed Online* 2006;**13**:765–771.

Van Steirteghem A, Van der Elst J, Van den Abbeel E, Joris H, Camus M, Devroey P. Cryopreservation of supernummerary multicellular human embryos obtained after intracytoplasmic sperm injection. *Fertil Steril* 1994;**62**:775–780.

Vilska S, Tiitinen A, Hyden-Granskog C, Hovatta O. Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. *Hum Reprod* 1999;**14**:2392–2395.

Wada I, Macnamee MC, Wick K, Bradfield JM, Brinsden PR. Birth characteristics and perinatal outcome of babies conceived from cryopreserved embryos. *Hum Reprod* 1994;**9**:543–546.

Wennerholm UB. Cryopreservation of embryos and oocytes: obstetric outcome and health in children. *Hum Reprod* 2000;**15**(Suppl 5):18–25.

Wennerholm UB, Hamberger L, Nilsson L, Wennergren M, Wikland M, Bergh C. Obstetric and perinatal outcome of children conceived from cryopreserved embryos. *Hum Reprod* 1997;**12**:1819–1825.

Wennerholm UB, Albertsson-Wikland K, Bergh C, Hamberger L, Niklasson A, Nilsson L, Thiringer K, Wennergren M, Wikland M, Borres MP. Postnatal growth and health in children born after cryopreservation as embryos. *Lancet* 1998;**351**:1085–1090.

Westergaard H, Johansen A, Erb K, Andersen AN. Danish National In-Vitro Fertilization Registry 1994 and 1995: a controlled study of births, malformations and cytogenetic findings. *Hum Reprod* 1999;**14**:1896–1902.

Whittingham DG, Lyon MF, Glenister PH. Long term storage of mouse embryos at −196°C: the effect of background radiation. *Genet Res* 1977;**29**:171–181.

Wisanto A, Bonduelle M, Camus M, Tournaye H, Magnus M, Liebaers I, Van Steirteghem A, Devroey P. Obstetric outcome of 904 pregnancies after intracytoplasmic sperm injection. *Hum Reprod* 1996;**11**(Suppl 4):121–130.

Zeilmaker GH, Alberda AT, Van Gent I, Rijkmans CM, Drogendijk AC. Two pregnancies following transfer of intact frozen thawed embryos. *Fertil Steril* 1984;**42**:293–296.

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Appendix 1

Reasons for early and late terminations in cryo fetuses.

	Cryo ICSI $N^a = 576$		Cryo IVF $N = 402$	
	Early	Late	Early	Late
Major malformation	*8*	*3*	*2*	*2*
Cardiac	1			
Hypoplastic left heart		1		
Aplasia vena cava superior			1	
Unspecified				1
Chromosomal				
Trisomy 21	3			
Trisomy 18			1	
Inherited autosomal structural	1			
De novo autosomal structural		1		
Fragile X mutation		1		
Klinefelter	3			
Musculoskeletal				
Limb-bodywall defect	1			
Other malformation				
Severe intrauterine growth retardation and a single umbilical artery				1
Others	*8*	*1*	*1*	*0*
Maternal indication	3	1	0	0
Multiple gestation	5	0	1	0
Total terminations	*16*	*4*	*3*	*2*

[a]N = (liveborns + stillborns + terminations).

Appendix 2

Number (%) of livebirths and stillbirths in the cryo and fresh groups.

	ICSI					IVF					
	Cryo ICSI		Fresh ICSI		OR (95% CI) Cryo ICSI versus fresh ICSI	Cryo IVF		Fresh IVF		OR (95% CI) Cryo IVF versus fresh IVF	OR (95% CI) Cryo ICSI versus cryo IVF
	Livebirth	Stillbirth	Livebirth	Stillbirth		Livebirth	Stillbirth	Livebirth	Stillbirth		
Singletons	**384 (70.2)**	**2**	**1499 (52.8)**	**18**	2.11 (1.73-2.57)	**284 (72.8)**	**5**	**1556 (52.7)**	**6**	2.41 (1.91–3.05)	0.88 (0.66–1.17)
Multiples	**163 (29.8)**	**7**	**1341 (47.2)**	**31**	0.48 (0.39–0.58)	**106 (27.2)**	**2**	**1399**	**(47.3) 34**	0.42 (0.33–0.53)	1.14 (0.85–1.52)
Twins	160 (29.3)	1	1228 (43.2)			100 (25.7)	2	1250 (42.3)			
Triplets	3 (0.5)	6	113 (4.0)			6 (1.5)	0	145 (4.9)			
Quadruplets	0	0	0			0	0	4			
Total	**547**	**9 (1.6)**	**2840**	**49(1.7)**	0.96 (0.47–1.95)	**390**	**7(1.8)**	**2955**	40(1.3)	1.33 (0.59–2.98)	0.92 (0.34–2.48)

Appendix 3

Maternal characteristics in cryo and fresh.

	ICSI			IVF		
	Cryo ICSI *N* of observ = 480 (SD)	Fresh ICSI *N* of observ = 2134 (SD)	*P*-value	Cryo IVF *N* of observ = 341 (SD)	Fresh IVF *N* of observ = 2196 (SD)	*P*-value
Mean maternal age in years	31.2 (4.6)[a]	32.7 (4.3)	<0.001	31.1(4.2)[a]	32.2 (4.1)	<0.001
Mean pregnancy duration in weeks	37.7 (3.9)[b]	37.9 (2.6)	NS	37.7 (3.6)[b]	37.9 (2.8)	NS

Comparing cryo ICSI versus cryo IVF.
[a]$P = 0.575$; [b]$P = 0.321$.

Appendix 4

Major malformations in cryo liveborns.

Major malformation	Cryo ICSI ($N = 547$), no of cases	Cryo IVF ($N = 390$), no of cases
Cardiac	10	1
Foramen ovale*	3	1
Pulmonalis stenosis	2	
Coarctatio aortae	1	
Ductus arteriosus	1	
Tricuspidalis insufficiency	1	
Complex cardiopathy	1	
ASD	1	
Chromosomal	1	
Trisomy 21	1	
Cleft lip/palate	3	
Of which Pierre-Robin syndrome	1	
Ear, eye	1	1
Congenital glaucoma	1	
Oculo-cutaneous albinism		1
Gastrointestinal	5	2
Hirschprung disease	1	
Pyloric stenosis	3	1
Biliary tract atresy	1	
Duodenal atresy		1
Genital	9	2
Perineal hypospadias	1	
Penile hypospadias	1	
Hypospadias not specified or no follow-up	2	
Cryptorchidism	5	2
Musculoskeletal	3	2
Syndactyly	1	
Hernia inguinalis	1	1
Craniostenosis	1	
Polydactyly pre-axial		1
Nervous	2	2
Leucomalacy*	2	
Porencephaly		1
Ondine's curse		1
Urinary	2	
Hydronephrosis	2	
Skin		2
Ichtyosis		1
Large naevus		1
Total major malformations	35	12

*1 child had two major malformations.