Factors affecting low birthweight after assisted reproduction technology: difference between transfer of fresh and cryopreserved embryos suggests an adverse effect of oocyte collection

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BACKGROUND: Data show that differences exist in the birthweight of singletons after frozen embryo transfer (FET) compared with fresh transfer or gamete intra-Fallopian transfer (GIFT). Factors associated with low birthweight (LBW) after assisted reproduction technology (ART) were studied. METHODS: Birthweight, distribution of birthweight, z-score, LBW (<2500 g), gestation and percentage preterm (<37 weeks) for singleton births >19 weeks gestation, conceived by ART or non-ART treatments (ovulation induction and artificial insemination) between 1978 and 2005 were analysed for one large Australian clinic. RESULTS: For first births, the mean birthweight was significantly (P<0.005) lower, and LBW and preterm birth more frequent for GIFT (mean 3133 g, SD 549, n = 109, LBW = 10.9% and preterm = 10.0%), IVF (3166, 676, 1615, 11.7, 12.5) and ICSI (3206, 697, 1472, 11.5, 11.9) than for FET (3352, 615, 2383, 6.5, 9.2) and non-ART conceptions (3341, 634, 940, 7.1, 8.6). Regression modelling showed ART treatment before 1993 and fresh embryo transfer were negatively related to birthweight after including other covariates: gestation, male sex, parity, birth defects, Caesarean section, perinatal death and socio-economic status. CONCLUSIONS: Birthweights were lower and LBW rates higher after GIFT or fresh embryo transfer than after FET. Results for FET were similar to those for non-ART conceptions. This suggests IVF and ICSI laboratory procedures affecting the embryos are not causal but other factors operating in the woman, perhaps associated with oocyte collection itself, which affect endometrial receptivity, implantation or early pregnancy, may be responsible for LBW with ART.

Keywords: assisted reproduction technology; low birthweight; oocyte collection

Introduction

There is general agreement from national databases and other studies that singleton infants born after IVF, ICSI and gamete intra-Fallopian transfer (GIFT), called collectively assisted reproduction technology (ART), are slightly lighter and more likely to be of low birthweight (LBW), defined as <2500 g. However, it is not clear whether this is caused by factors intrinsic to the ART procedures or related to characteristics of the ART-seeking population (Doyle et al., 1992; Wang et al., 1994, 2005; Petersen et al., 1995; Tallo et al., 1995; Tanbo et al., 1995; Dawood, 1996; Tanbo and Abyholm, 1996; Bernasko et al., 1997; Aytoz et al., 1998; Moise et al., 1998; Schieve et al., 2002, 2004a,b; Lambert, 2003; Ochsenkühn et al., 2003; Puttermann et al., 2003; Helmerhorst et al., 2004; Jackson et al., 2004; Bower and Hansen, 2005; Ludwig et al., 2006; Halliday, 2007). There are several meta analyses for this issue (Lambert, 2003; Helmerhorst et al., 2004; Jackson et al., 2004; Bower and Hansen, 2005). Some find birth morbidities from treatments of infertile couples not involving IVF or GIFT (hereafter called non-ART treatments) were not higher than with ART suggesting that the IVF laboratory procedures would not account for the increased LBW in ART singletons (Lambert, 2003). Subfertility itself is a risk factor for LBW as natural conception after a diagnosis of infertility or a prolonged time to pregnancy increase the rates of adverse birth outcomes, particularly preterm birth (Kramer, 1987; Ghazi et al., 1991; Ollivennes et al., 1993; Joffe and Li, 1994; Henriksen et al., 1997; McElrath and Wise, 1997;
Bergh et al., 1999; Draper et al., 1999; Pandian et al., 2001; Basso and Baird, 2003; Axmon and Hagmar, 2005). However, other studies of ART and non-ART conceptions in subfertile women suggest that there is an additional specific adverse effect of ART on gestation and birthweight (Doyle et al., 1992; Olivennes et al., 1993; Wang et al., 1994, 2002; Petersen et al., 1995; Tallo et al., 1995; Tanbo et al., 1995; Aytouz et al., 1998; Moise et al., 1998; Schieve et al., 2004a,b; De Geyter et al., 2006). Although some studies show significant differences in birthweight with different types of infertility, the findings are inconsistent (Doyle et al., 1992; Wang et al., 2005).

ART related factors reported in some studies to affect birthweight include: type of ovarian stimulation, type of ART, origin of sperm used to fertilize the oocytes, number of embryos formed, number of embryos transferred, type of luteal support, number of implantations, vanishing twins and year that the procedure was performed (Doyle et al., 1992; Tanbo et al., 1995; Tallo et al., 1995 #328; Wennerholm et al., 1997; Aytouz et al., 1998; Wennerholm et al., 2000; Wennerholm, 2000; Klemetti et al., 2002; Schieve et al., 2002 #960; Dean and Sullivan, 2003; Lambert, 2003; Jackson et al., 2004; Schieve et al., 2004a,b; Pinborg et al., 2005, 2007). Whether the embryos were transferred fresh or after cryopreservation (hereafter called frozen embryos or frozen embryo transfer—FET) has also been noted to affect birthweight. In Australia, all ART births of 20 or more weeks gestation are reported in the National Perinatal Statistics Unit (NPSU) database on Assisted Conception run by the Fertility Society of Australia and the Australian Institute of Health and Welfare (www.npsu.unsw.edu.au). In the NPSU data, it has been shown consistently from 1986 that singletons from FET procedures have higher mean birthweight and less LBW than singletons from fresh embryo transfers or GIFT. This was first noted in the 1991 report for treatments up to 1989. Wennerholm et al. noted that singleton infants born after FET had significantly higher median birthweight than did those from fresh embryo transfers (Wennerholm et al., 1997, 2000; Wennerholm, 2000). It appears to us that the important implications and significance of this difference in outcomes for transfer of fresh and frozen embryos have largely escaped notice.

The aim of this study was to investigate the reasons for LBW in ART conceptions by detailed analysis of the results of ART and other treatments for infertility in a single infertility service. Our hypothesis was that reduced birthweight after ART is determined by factors involved in the ART process.

Materials and Methods

Subjects and treatment procedures

The subjects were treated for infertility by single service (Melbourne IVF) operating with the same staff and procedures at two sites at the Royal Women’s Hospital (RWH) from 1978 and at Epworth Freemasons Hospital (EFH) from 1989, until 31 December 2005. Oocytes were collected by laparoscopy under general anaesthesia until transvaginal ultrasound guided oocyte collections under neurolept analgesia were begun in March 1986. GIFT was introduced in 1985 but its use became infrequent after 1989. GIFT was performed under general anaesthesia and involved laparoscopic placement of the oocytes and spermatozoa into the ampulla of a fallopian tube via the fimbrial end of the tube. ICSI was introduced in 1993. Treatment of male infertility was attempted with standard IVF before the introduction of ICSI. Stimulation regimens involved clomiphene, HMG and hCG between 1982 and 1993. The oral contraceptive pill was used increasingly after 1995 to regulate the starting time of gonadotrophin treatment. Long down-regulation with GnRH analogues was used after 2001. Recombinant FSH was used after 1994. GnRH antagonists were used in small numbers of patients after 2000. Culture media changed from Ham’s F10 to human tubal fluid in 1987, then to multistage G media in 2000. The protein supplement was maternal or donor blood serum until 1990, then human serum albumin until 2000 and highly purified albumin thereafter. Embryo cryopreservation started in 1985 with a variety of protocols but cryoprotection with propandiol and embryo freezing on Day 2 (or rarely 3 or 6) was used after July 1986. Generally, transfers were performed and embryos frozen on Day 2. However, Day 3 transfers were performed with ICSI embryos until 1997. The first births from FET were in 1987. Cryopreserved embryos were thawed and transferred on the same day as their age after ovulation in natural or artificial cycles until 1996. After September 1997, all Day 2 and 3 cryopreserved embryos were thawed the day before transfer and cultured overnight so they would be on average 10 h more advanced than embryos transferred fresh. No luteal phase support was used initially but after 1990 hCG or progesterone was usually given.

There has been a progressive rise in the number of oocyte collections and embryo transfers per year (1980: 207, 18; 1985: 611, 424; 1990: 1121, 1404; 1995: 2067, 3474; 2000: 2392, 4118; 2005: 3097, 5554). Generally lower numbers of embryos were transferred in each procedure than in other Australian clinics (average number of embryos per transfer—1980: 1.1, 1985: 2.7, 1990: 1.9, 1995: 1.8, 2000: 1.7, 2005: 1.5) and more embryos were frozen. About 47% of births were from FET. Multiple births were more frequent before 1990 (23%) than subsequently (16%), and there were 37 triplet and one quadruplet births. There have been few (9) selective reductions of multiple pregnancies. National health funding of ART started in 1989 and has been increased so that about half to two-thirds of the costs were covered. Patients without their own specialist gynaecologist were classified as semiprivate and this is an indicator of socioeconomic status.

In compliance with Victorian State regulations and the Fertility Society of Australia’s Reproductive Technology Accreditation Committee requirements for reporting to the NPSU, infertility clinics are required to keep comprehensive records of all procedures that result in birth. Patients sign consent forms acknowledging this.

Data management and strategy for statistical analysis

The database used dBASE originally in Clipper from 1979 and then FoxPro from 1999. It was regularly checked for inconsistent and incorrect entries and, for this study, crosschecked with entries in the Victorian Health Department’s Perinatal Data Collection Unit database which has records for births in the state dating from 1983. Data were extracted for 10,039 births of >19 weeks gestation that resulted from IVF or ICSI with fresh (4406) or frozen (3708) embryo transfers, GIFT (164) and non-ART treatments (1307). Births from donated oocytes or embryos (375), after preimplantation genetic diagnosis (57), oocyte freezing (1), mixed transfer of fresh and cryopreserved embryos (3) and from embryos imported from other clinics (18) were excluded from analysis. The non-ART births were from ovulation induction with gonadotrophins or clomiphene (194), artificial insemination with partner’s sperm (111) and donor insemination 1645

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(1002). Stillbirths, late terminations and neonatal deaths (within 28 days of birth) were included.

Five outcome measures were analysed: birthweight in grams, birthweight expressed as a z-score using LMS British growth reference data from 1990 which adjusts for gestation after 23 weeks and sex (Freeman et al., 1995), percentage LBW defined as a birthweight <2500g, gestation calculated from 14 days before the estimated day of ovulation in the implantation cycle to the date of birth and percentage preterm defined as births <37 weeks gestation. The method of Wilcox (2001) was used to compare the predominant and residual distributions of birthweight for first ART singletons from fresh transfers and FET (eb.niehs.nih.gov/bwt).

There were 8179 singleton births, 1609 of which were second or subsequent births for the same woman with either ART or non-ART treatment. The effect of fresh embryo transfer and FET on the differences in birthweight of second versus first singleton birth was examined for 1219 women. Most analyses were only conducted for the 6570 first singleton births resulting from ART or non-ART treatment. We were concerned that selecting only singleton ART births for study might lead to a bias towards LBW. Such a bias would arise if the capacity for normal birth outcomes were related to the ability to implant multiple embryos. This could affect the comparison of the ART singletons with those in the general population or where there is variation in multiple birth rate as seen between fresh and FETs. Therefore, we compared results for first and second ART singletons from women with more than one birth, one or more of which was multiple, with those who had repeated singleton births. Results for 1254 multiple pregnancies were analysed for comparison of combined birthweights of 429 sets of twins from FET and 825 sets from fresh embryo transfers. Regression analyses were performed on the 5497 first singleton births from transfer of fresh or frozen embryos after IVF or ICSI. There were missing data for indeterminate sex (3), gestation (23) and birthweight (27), mostly for births in the early 1980s, births overseas and for stillbirths or babies with severe abnormalities where weight was not recorded (6). Analyses were performed after coding the latter six with missing birthweights as LBW and preterm, and the others as not LBW or preterm.

Non-parametric tests were used for birthweight, z-score and gestation and chi-squared and McNemar tests for paired data for LBW and preterm births. Linear regression analysis was used for birthweight, z-score and gestation. Logarithmic transformation of birthweight and fractional polynomial square and cube of gestation were used to linearize the relationship with gestation and reduce heteroscedasticity. Logistic regression analysis was used for LBW and preterm birth. To reduce the risk of obtaining results significant by chance alone (type I error) from the multiple potentially explanatory and dependent factors available for analysis, the data set was randomly divided into two groups with only birthweight analysed initially on half of the data (Group A). All subsets regression analysis was used to assist in making an objective selection of factors for analysis. Graphs were plotted to check the linearity and nature of the relationships between the data and the fit of the models. For example, maternal age was not significant despite testing several different patterns, but there was an upward trend in birthweight with year of treatment that was best modelled by a dichotomous variable before 1993, hereafter referred to as an era effect. The regression models chosen were parsimonious, including only significant factors (P < 0.05). The significant factors identified in Group A were then tested in the other half of the data (Group B) and the whole data set for birthweight. These factors were then tested with all the data for z-score, gestation, percentage LBW and preterm birth. To study the possible effects of ovarian stimulation, oocyte numbers, fertilization rate and embryo numbers, births resulting from fresh embryo transfers were analysed separately. Similarly, the effect of overnight culture of embryos before transfer was tested for FET births.

Results

Singleton births from ART and non-ART treatments

The differences in the results for IVF, ICSI and GIFT procedures were not significant (Table I). However, outcomes from FET were significantly better than for fresh embryo transfers or GIFT, except for gestation. FET results were similar to those for the non-ART conceptions but gestation was significantly shorter and z-score higher.

Twins: differences for fresh and FETs

The difference in birthweights following fresh and FETs was also present in twins. The combined birthweight for twins from fresh embryo transfer was significantly lower than for those from FET [4737 g (SD 1272) n = 825 versus 4926 (1210) 429, P = 0.005] and percentage combined LBW (<5000 g) was higher (418/835, 50%, versus 184/431, 43%, P = 0.013). Gestation [35.7 weeks (SD 3.4) n = 829 versus 35.9 (3.2) 431] and percentage preterm (428/828, 52%, versus 206/431, 48%) were not significantly different.

Two singleton ART births in the same woman

The differences in outcomes of two successive singleton births resulting from the same (fresh/fresh or FET/FET) or different (fresh/FET or FET/fresh) transfers show that the adverse effect of fresh embryo transfer operated within woman. The parity effect on the second birth was clear in the results from the same type of embryo transfer for two pregnancies in 600 women: on average a significant 89 (SD 706)g heavier and 0.578 (2.8) weeks. The differences for 390 s births from FET after a first ART birth from a fresh embryo transfer were greater: 244 (697)g heavier and 0.298 (969) higher z-score, lower LBW (6.0% versus 8.8%) and preterm birth (7.8% versus 10.5%) with no significant difference in gestation −0.02 (2.8) weeks. The differences for 390 s births from FET after a first ART birth from a fresh embryo transfer were greater: 244 (697)g heavier and 0.578 (1.02) higher z-score, LBW (2.6% versus 9.5%) and preterm birth (7.7% versus 10.8%) with no significant difference in gestation −0.02 (2.8) weeks. There were only 158 patients with second singleton births from fresh procedures following a first birth from FET and the difference in birthweight 57 (677)g was not significant and difference in z-score 0.214 (951) was less.

Singleton ART births in women with more than one ART birth

In the 1404 patients with more than one birth from ART, 265 had at least one multiple birth. There was no evidence that women who had multiple births from ART were more likely to have higher birthweight babies. The average singleton birthweights (SD) for first and second births were 3269(688) and 3406(585) g, respectively, for those who only had singleton births and 3315(603) and 3360(779) for those who also had one or more multiple births. The corresponding z-scores were −0.057(0.987) and 0.332(0.957), and −0.055(0.885) and 0.462(1.015). Adjusting for fresh versus FET and sex of baby by regression analysis did not alter this conclusion.
Singleton first ART births

Of the 5497 singleton first ART births, 56% were from transfer of fresh embryos, 84% of women were primiparous, 11% were semiprivate, 14% were treated before 1993 and 51% of the babies were boys (Table II). Regression analysis of birthweights for the randomly selected half of the first singleton ART births (Group A) showed the expected effects of the covariates: gestation, sex, parity, birth defect, perinatal death and semi-private socio-economic status (Table III). Caesarean section was also significant in the final model, with a slight semi-private (2.7% lower), Caesarean section (2.0% higher), era (3.6% lower before 1993) and fresh embryo transfer (3.3% lower than FET). Gestation was particularly affected by fetal factors: birth defect (0.8 weeks shorter) and perinatal death (10 weeks shorter), but after allowing for these and parity and Caesarean section, fresh embryo transfer was associated with significantly shorter gestation (0.25 weeks).

In summary, after adjustment for significant covariates, singleton first babies born after fresh embryo transfer had a mean birthweight 3.3% lower [95% confidence interval (CI) 2.3–4.3%] and a mean z-score 0.233 SD lower (95% CI 0.181–0.285) than did those born after FET. Births from fresh embryo transfer were more likely to be LBW [odds ratio (OR) 1.9, 95% CI 1.6–2.4] than those from FET. Births from fresh embryo transfer had 1.7 (95% CI 0.9–2.6) days shorter gestation and were more likely to be preterm (OR 1.9, 95% CI 1.6–2.4) compared with those from FET.

Factors not affecting birthweight or gestation

These analyses were performed for birthweight for all singleton first ART births from fresh and FET in Group A, for all the data and separately for only fresh transfers and FET. Each factor was tested on its own and with the other significant covariates (Table III). They were also tested with the other covariates with the exclusion of birth defects and perinatal death, which could obscure relevant relationships with birthweight if caused by components of the ART procedures or patient factors.
The following factors did not significantly affect birthweight: diagnosis of infertility (male, ovulatory, tubal, endometriosis, idiopathic), maternal or paternal age and difference between the couple’s ages, site (RWH, EFH), previous numbers of ART treatments, oocyte collections or embryo transfers, average number of oocytes collected or embryos formed per oocyte collection, type of stimulation used for oocyte collection for the pregnancy (natural cycle or FSH stimulation) and components of stimulation (oral contraceptive regulation of cycles, GnRH agonists in long or short protocols, GnRH antagonists and FSH), laparoscopic or transvaginal oocyte collection, type of sperm used (fresh or frozen from partner’s semen, testis or epididymis, frozen donor sperm), IVF or ICSI, type of culture medium or protein supplement, fertilization rate, number of usable embryos (transferred and frozen), number of embryos transferred in the implantation cycle, age of development of embryo at transfer, morphological quality or number of cells in the embryos transferred, clinician performing the transfer, overnight culture before FET, natural cycle or artificial hormone simulated cycle for FET, luteal support (none, progesterone and hCG) or numbers of sacs and fetal hearts seen on ultrasound. Although not

### Table III. Factors affecting birthweight in single (unadjusted) and final multiple linear regression models (covariate adjusted) of birthweight (log\(y\)) for singleton first births after fresh or FETs from IVF or ICSI.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Group A (n = 2733)</th>
<th>Group B (n = 2729)</th>
<th>All (n = 5462)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
<td>Adjusted</td>
<td>Unadjusted</td>
</tr>
<tr>
<td></td>
<td>(r^2 = 0.80)</td>
<td>(r^2 = 0.76)</td>
<td>(r^2 = 0.78)</td>
</tr>
<tr>
<td>Constant</td>
<td>1.66(0.040)</td>
<td>&lt;0.001</td>
<td>1.63(0.039)</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>3.34(0.070)</td>
<td>3.18(0.084)</td>
<td>3.04(0.088)</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>0.016</td>
<td>&lt;0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Birth defect</td>
<td>-0.14(0.016)</td>
<td>&lt;0.001</td>
<td>-0.099(0.016)</td>
</tr>
<tr>
<td>Perinatal death</td>
<td>-0.62(0.015)</td>
<td>&lt;0.001</td>
<td>-0.41(0.021)</td>
</tr>
<tr>
<td>Caesarean section</td>
<td>0.018(0.0070)</td>
<td>0.017(0.0064)</td>
<td>0.017(0.0032)</td>
</tr>
<tr>
<td>Era &lt;1993</td>
<td>0.012</td>
<td>&lt;0.001</td>
<td>0.007</td>
</tr>
<tr>
<td>Fresh embryo transfer</td>
<td>-0.0340(0.0051)</td>
<td>&lt;0.001</td>
<td>-0.020(0.0047)</td>
</tr>
<tr>
<td></td>
<td>0.016</td>
<td>&lt;0.001</td>
<td>0.011</td>
</tr>
</tbody>
</table>

A random selection of approximately half the data (Group A) was analysed first. The same covariates were then tested in the other half (Group B) and in all the data (All). Constant/regression coefficient (SE) and \(P\)-value are shown. Coefficients for gestation squared and cubed.

### Table IV. Adjusted coefficients (SE) and \(P\)-values for analysis by linear regression for \(z\)-score and gestation, and logistic regression for LBW and preterm, for singleton first births after fresh or FETs from IVF or ICSI.

<table>
<thead>
<tr>
<th>Factor</th>
<th>(z)-score n = 5438</th>
<th>(r^2 = 0.051)</th>
<th>LBW n = 5497</th>
<th>(r^2 = 0.090)</th>
<th>Gestation (weeks) n = 5474</th>
<th>(r^2 = 0.23)</th>
<th>Preterm n = 5497</th>
<th>(r^2 = 0.067)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Constant)</td>
<td>0.047(0.25)</td>
<td>-3.17(0.11)</td>
<td>&lt;0.001</td>
<td>39.5(0.06)</td>
<td>&lt;0.001</td>
<td>2.75(0.092)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Birth defect</td>
<td>0.060</td>
<td>1.04(0.23)</td>
<td>&lt;0.001</td>
<td>0.80(0.20)</td>
<td>&lt;0.001</td>
<td>0.94(0.22)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Perinatal death</td>
<td>-0.61(0.13)</td>
<td>3.32(0.27)</td>
<td>&lt;0.001</td>
<td>-10.0(0.26)</td>
<td>&lt;0.001</td>
<td>3.22(0.27)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Semi-private</td>
<td>-0.20(0.42)</td>
<td>0.48(0.15)</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Parity (&gt;0)</td>
<td>-0.31(0.036)</td>
<td>-0.39(0.15)</td>
<td>&lt;0.001</td>
<td>0.01</td>
<td>&lt;0.001</td>
<td>0.08</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Caesarean section</td>
<td>0.19(0.027)</td>
<td>0.55(0.10)</td>
<td>&lt;0.001</td>
<td>-0.71(0.064)</td>
<td>&lt;0.001</td>
<td>0.62(0.091)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Era &lt;1993</td>
<td>-0.27(0.038)</td>
<td>0.53(0.13)</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>0.32(0.12)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Fresh embryo transfer</td>
<td>-0.23(0.026)</td>
<td>0.64(0.10)</td>
<td>&lt;0.001</td>
<td>-0.25(0.063)</td>
<td>&lt;0.001</td>
<td>0.31(0.093)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Sex of baby was not significant in any model.
significant in the birthweight model in Group A, some other factors were significant in the model for all the first ART singletons. These were: diagnosis of polycystic ovary syndrome with a significant positive effect \((P = 0.018)\), clomiphene treatment before embryo transfer with a negative effect \((P = 0.040)\) and number of fetal hearts was close to a significant negative effect \((P = 0.061)\). Data on height, weight and smoking were only available for a small proportion of the subjects: weight \((n = 357, P = 0.006)\) and height \((n = 161, P < 0.001)\) had positive effects on birthweight. Smoking \((n = 336)\) was not significant.

**Nature and magnitude of the effect of fresh embryo transfer**

The lower birthweights for singleton first ART births after fresh embryo transfers is contributed to both by prematurity (shown by the shorter gestation and higher preterm rate) and also by LBW for gestation (indicated in the regression model for birthweight incorporating gestation and the lower z-score). Both these contributions were also demonstrated in the analysis of the predominant and residual distributions of birthweight by the method of Wilcox. The predominant distribution had a mean of 3366 g and SD of 496 g for the fresh embryo transfers, significantly lower \((P < 0.001)\) than for FET (3477, SD 494 g). The residual distribution in the LBW tail for the fresh transfers \((7.5\%)\) was significantly higher \((P < 0.001)\) than for FET \((4.4\%)\). All methods produced a consistent difference in mean birthweight at term for fresh embryo transfers and FET: 111 g for the birthweight regression model, 116 g for z-score and 111 g for the Wilcox method.

**Discussion**

Birthweight and preterm birth are used as individual and community indicators of successful pregnancy because of their relationship with perinatal mortality and morbidity (Robinson, 1979; Goldstein, 1981). However, Kramer and Wilcox have identified a number of problems with the seeming simplicity of birthweight and assumptions about the mechanisms of its relationship with mortality and morbidity (Kramer, 1987; Wilcox, 2001). We have used all the common measures of birthweight and gestation to avoid the problems involved in using only percentage LBW. LBW and preterm birth have multiple known causes. Fetal factors, such as sex and birth defects, affect birthweight and it is proposed that the fetus determines the length of gestation, whereas the mother’s size determines the size of the baby at birth (Robinson, 1979; Snow, 1981; Kramer, 1987; Nathanielsz, 1995). Other factors are known to affect birthweight. Some act via the mother, such as the parity effect that increases average birthweight from the first to the second pregnancy, and the age effect where both younger and older mothers have lower birthweight babies (Goldstein, 1981). The mechanisms of these well-known effects of parity and age are uncertain. Many inter-related health, lifestyle and socio-economic factors are also important, such as low maternal weight and weight gain during pregnancy, infections, hypoxia, anaemia, smoking, alcohol, drugs, physical and social abuse, surgical abortions, poor obstetric history, short inter-pregnancy interval and first or second trimester bleeding (Goldstein, 1981; Kramer, 1987; Parker et al., 1994; Cogswell and Yip, 1995; Tanbo et al., 1995; Reubinoff et al., 1997; Thame et al., 1997; Ehrenberg et al., 2003; Lambert, 2003; Jackson et al., 2004; Magee et al., 2004; Schieve et al., 2004a,b). These factors are unlikely to contribute to the association of LBW with ART because women pregnant by ART are usually more careful about adverse lifestyles and are of higher socio-economic status than those conceiving naturally. However, highly pertinent are the observations that subfertility itself is a risk factor for LBW (Kramer, 1987; Ghazi et al., 1991; Olivennes et al., 1993; Joffe and Li, 1994; Henriksen et al., 1997; McElrath and Wise, 1997; Bergh et al., 1999; Draper et al., 1999; Pandian et al., 2001; Basso and Baird, 2003; Axmon and Hagmar, 2005).

The current study was undertaken to investigate the cause of the LBW associated with ART. The major finding is that mean birthweight, z-score and gestation were lower, and percentage LBW and preterm birth were higher for fresh embryo transfers from IVF or ICSI than for FET. Thus, as well as increased prematurity, there was also lower birthweight at each gestation for fresh embryo transfers. After adjusting for the known covariates of birthweight (gestation, sex, parity, socio-economic status, perinatal mortality, birth defects and Caesarean section) only two factors related to ART were significant: era and fresh embryo transfer. Procedures performed before 1993 had lower birthweights than those did after this time, and fresh embryo transfer resulted in lower birthweights than did FET. The poorer outcome of births from fresh embryo transfer compared with FET was also seen in the analysis of differences between first and second pregnancies in the same woman, indicating that it is not the result of inter-patient variability. In addition, the average birthweights of ART twins were also higher for FET.

The strengths of this study are the large study population and detailed information available on the treatments from the one infertility service, the comparison of non-ART and ART
conceptions in subfertile women and, more particularly, the high usage of FET and low multiple pregnancy rate compared with most reported studies. To avoid the problem of analysis of subsequent pregnancies in the same woman and the high within subject birthweight correlations, the regression analysis was only performed on singleton first ART births. There are a number of limitations. The quality of the data is variable. For example, there were inevitable time related changes over the 25 years of data collection, with differences in patient mix and the approach to diagnosis and procedures with the evolution of ART. This makes separation of time effects from changes in patient mix, methods of ovarian stimulation, anaesthesia and oocyte collection impossible, or at least difficult. The effects of maternal complications of pregnancy on birthweight were not analysed, and these could result in earlier delivery and reduced birthweight. There was little information on important covariates such as maternal weight and weight gain or smoking during pregnancy. However, these are unlikely to confound the effects era and fresh embryo transfer. We were concerned that selecting singletons might cause a bias towards LBW, and we therefore compared the results for women with more than one ART birth, one or more of which was multiple, with those who had repeated singleton births. We found there was no significant difference in mean birthweights or z-scores of first singletons for women with either multiple or singleton subsequent births, or for a subsequent singleton if the first was multiple or singleton. Thus, there was no evidence that women who had multiple births had heavier singletons. Although these results are reassuring, this possible source of bias needs checking in larger data sets with higher proportions of multiple births.

Using a strategy to reduce Type I error, many factors were not significant in the regression models. A higher frequency of LBW singletons in women >40 years was found in the Australian ART data from 1996 to 2000 (Wang et al., 2005). In our data, female age at transfer or when embryos were formed was not significant. The lack of an age effect may be due to few young women in the data, and also exclusion of many older women who used donor oocytes or embryos. Higher numbers of embryos transferred, fetal sacs or hearts have been inversely related to birthweight or directly related to LBW in studies by other groups (Pinborg et al., 2005, 2007; Wang et al., 2005). ‘Vanishing twin’ pregnancies that start multiple but reduce naturally or are reduced have been shown to contribute to LBW in ART singletons (Pinborg et al., 2005, 2007). In these data, number of fetal hearts was close to significant in the regression model. This lack of significance may be related to the relatively low proportion (3.7%) of pregnancies with more than one fetal heart ending in singleton births.

**Effect of era**

Most national ART databases indicate improvements in birth outcomes with time. For example, the NPSU data for singleton births, including stillbirths between 1979 and 1989 from fresh embryo transfers from IVF, had percentage LBW 15.5% and preterm birth 17.8%. In 1989, LBW declined to 13.4%; the rates varied between 11.0% and 14.7% between 1990 and 1993, and then between 8.2% and 10.1% between 1994 and 2002. There was also a fall in the rate of preterm births from 14–15% to ~11% over the same period (www.npsu.unsw.edu.au). Some of the more recent data have also been published (Wang et al., 2005). In the British Human Fertilisation and Embryology Authority (HFEA) data, which records singleton live births after 22 weeks gestation, the mean birthweights and percentage of preterm (23–36 week) births were: 3200 g, 11.8%, n = 2708 in 1994–1995, 3196 g, 11.2%, n = 4713 in 1998–1999 and 3283 g, 9.1%, n = 5948 in 2002–2003. Schieve et al. (2002) reported a decrease in the overall rate of LBW with ART in the USA from 1996 to 2000. In singletons born in Finland from two ART year groups, 1991–1993 and 1998–1999, the OR for LBW compared with those who had naturally conceived births decreased from 2.4 (95% CI 1.9–3.1) to 1.7 (95% CI 1.4–2.1) (Klemetti et al., 2002). There has been no similar change in birthweight in the general population. In the State of Victoria, the mean birthweight for the ~60 000 singleton live and stillbirths after 19 weeks each year from 1982 to 2003 has remained between 3385 and 3399 g and the percentage LBW between 4.5% and 5.1%.

Several explanations are possible for this temporal trend in results. It may be explained to some extent by a parity effect, with increasing proportions of patients having second and subsequent ART births. However, the temporal trend was seen in the current data when only first ART births were analysed. Curiously this trend stopped after 1993. Whether this is a peculiarity specific to this data set requires more study. The possibility that the era effect is due to changes in stimulation drugs was analysed but the result was inconclusive. For example, clomiphene was used commonly in early stimulation regimens but not recently. In some regression models, clomiphene had a just significant negative effect without changing the era effect. The era effect and the introduction of ICSI coincide but seem unrelated, as ICSI was not significant in any analysis. Schieve et al. (2004a,b) proposed that technological advances, such as ultrasound-guided embryo transfer, leading to more optimal embryo placement and subsequent implantation in more advantageous sites, may be responsible for improvements in fetal growth. However, ultrasound-guided embryo transfer was not adopted universally and not used by our group. Although it may be impossible to disentangle changes in patient mix, treatments and other factors as the cause of this improvement with time, it suggests adverse effects were more marked in the early days of ART and that more analysis of larger data sets would be worthwhile as this may indicate mechanisms that could be modified to improve birthweight outcomes in the future.

**Effect of fresh embryo transfer**

The most striking finding in this study is that births from fresh embryo transfers had a lower mean birthweight and z-score and were more likely to be LBW and preterm than those from FETs. The 111 g difference in birthweights with fresh ART procedures and FET is important because LBW babies are major users of neonatal intensive care. Although current management means that many now survive and lead normal lives, there remains considerable mortality and morbidity especially
with neurological deficits (Robinson, 1979). Although not statistically significant in these data, birth defects and perinatal death tended to be higher with fresh embryo transfers. A difference in birth defect rates between fresh embryo transfer and FET has been found in some, but not all, data sets (Wada et al., 1994; Wennerholm et al., 1998; Olson et al., 2005).

In the NPSU report for births from ART to 1989, it was noted briefly that there were lower frequencies of LBW (8.4%) and preterm births (13%) in singleton pregnancies from FET compared with those from transfer of fresh embryos (www.npsu.unsw.edu.au). Subsequently, the results up to 1991 were compared for fresh and FETs, respectively: LBW (14.7% and 8.4%) and preterm births (14.7% and 13%). In 1996–2000 data, LBW occurred in 10.8% of singleton births from fresh transfers and 7.2% of those from FETs, and preterm birth occurred in 13.1% and 11.4%, respectively (Wang et al., 2005). Others have reported higher birthweights for FET after both IVF and ICSI (Wennerholm et al., 1997, 2000; Kallen et al., 2005; De Geyter et al., 2006). For twins, a significantly higher frequency of LBW with fresh transfer (55.2%) than with FET (47.3%) has also been reported before (Wang et al., 2005).

The FET results were similar to those of patients who conceived naturally with ovulation induction or with insemination using partner or donor sperm. The slightly longer gestation for these non-ART pregnancies may be explained by the less precise timing of gestation from the time of ovulation or commencement of last menstrual period in the non-ART treatments than from the time of embryo transfer in the ART pregnancies. GIFT results were similar to those for fresh embryo transfers. Although the GIFT numbers are small in the current data set, the fact that the results of GIFT are not better than those of fresh IVF or ICSI embryo transfers is clear in the NPSU data (www.npsu.unsw.edu.au). For example, for 1221 singleton births including stillbirths between 1985 and 1989 from GIFT, the percentage LBW was 15.6% and preterm birth 14.7%. There appeared to be a drop in LBW to 11.1% and preterm birth to 11.5% in 1992–1993 for 1171 singletons. Between 1994 and 2000, the rates varied between 9.4% and 13.9% for LBW and 12.5% and 16.4% for preterm birth but the numbers were few because GIFT was used less often. The fact that a similar or higher frequency of LBW occurs with GIFT singletons indicates that the effect of fresh embryo transfer on birthweight is not caused by the fertilization procedures performed in the laboratory, as these do not occur with GIFT. It must presumably result from the characteristics common to GIFT and fresh embryo transfers: ovarian stimulation and oocyte collection.

Various explanations for the difference in birthweight outcomes for fresh and FETs have been offered. It is possible that early observers considered it merely a parity effect, assuming the births from FET were more likely to result from transfer of stored embryos after previous successful pregnancies from fresh embryo transfers. The current results indicate that this is not the case as the effect of parity from previous ART pregnancies was eliminated by only analysing first ART pregnancies. Moreover, analysis of the differences in birthweights between first and second ART singleton births indicated a specific adverse effect with fresh embryo transfers. Several authors have suggested the better outcomes with frozen embryos can be explained as a patient effect, where patients who produce more and higher quality embryos are less likely to have LBW babies (Schieve et al., 2004a,b; Kallen et al., 2005; Wang et al., 2005). However, we find no significant effect on birthweight of average oocyte or embryo number per oocyte collection or quality of embryos transferred. Also, a persisting patient effect is unlikely because we found the difference between outcomes with fresh embryo transfers and FETs was seen within patients who had two singleton ART pregnancies. Another source of difference is a possible bias caused by only studying singleton births in a situation where the rate of multiple birth varies. In these data, the multiple birth rates were GIFT 29%, fresh transfers 20% and FET 12%. As mentioned above, by analysing patients with more than one ART birth, we found no evidence for women who had multiple pregnancies also having better birth outcomes of their singletons. Because many of the patients treated are not completely infertile the effect of natural conception during an ART procedures should also be considered (Baker, 2006). Although it is likely that such natural pregnancies would be more frequent with FET than with fresh embryo transfers and GIFT, we believe this too rare to be the explanation for the difference in birth outcomes.

Mechanisms mainly affecting the embryos have also been suggested. FET may have a protective effect in that superior embryos may result from selection of better quality embryos for freezing or because of the better response to ovarian stimulation necessary to provide excess embryos for cryopreservation (Wang et al., 2005). Alternatively, the physical effect of the freezing and thawing process on embryos may filter ‘weak’ embryos and allow only superior embryos to survive, resulting in babies with better fetal growth and higher birthweights. An effect of the cryoprotectants used during embryo freezing affecting DNA methylation and protecting against adverse effects on imprinting has also been postulated (De Geyter et al., 2006). If there are embryonic mechanisms operating, these do not appear to involve cell number or morphological grade, as we did not find these factors affect birthweight significantly. The practice of culturing the frozen embryos overnight and transferring them on Day 2 of the luteal phase means that many will have undergone an additional cleavage and be more advanced in development than the fresh embryos transferred on Day 2. Whether this could affect birthweight in humans is uncertain but it is known to occur in laboratory animals (Snow, 1981). We cannot find a significant difference in birthweight outcomes for FET before and after introduction of this practice in the present data, and the consistency of the difference between fresh and FET results in other data collections makes it unlikely that this could provide the whole explanation.

We conclude that the results of this study indicate a real adverse effect of fresh embryo transfer. We suspect that the outcomes with frozen embryos is equivalent to that expected for women conceiving naturally with the same characteristics (age, parity and time trying for pregnancy) and it is being overlooked that the poor birthweight outcomes with ART are related only to fresh embryo transfers and GIFT. The results
confirm that LBW in singleton infants born after ART is not caused by the IVF and ICSI laboratory procedures because results with frozen embryos are not similarly affected. We hypothesize that the mechanism involves some aspect of treatment common to IVF, ICSI and GIFT and distinct from FET, which must operate via the mother and is associated with oocyte collection including: controlled ovarian stimulation, anaesthesia and needle aspiration of the ovarian follicles. These occur in fresh embryo transfer and GIFT but not FET cycles and could affect endometrial receptivity, implantation and early pregnancy. Because FSH ovarian stimulation versus natural cycle without FSH stimulation and numbers of oocytes collected had no significant effects in the regression models for fresh embryo transfers, we think the difference in hormone levels in fresh and FETs are less likely to be involved. We suspect anaesthesia and surgery for oocyte collection might affect embryo implantation and early pregnancy with later effects on birthweight. Illness, injury, surgery and anaesthesia during pregnancy have been reported to increase LBW (Mazze and Kallen, 1989; Slap and Schwartz, 1989). However, many other factors may also contribute such as overnight culture before FET. Thus, further studies in a larger data set are required. We are currently involved in a collaborative project with the other infertility clinics in Melbourne and the State Perinatal Data Collection Unit to do linkage studies of the ART singletons and control singleton pregnancies in subfertile couples who conceived without ART. Improved understanding of the cause of LBW after fresh embryo transfer could have profound implications, as simple modifications of oocyte collection or identification of patients likely to be affected may lead to improvement in the birth outcomes.

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