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### COMMENTARY


# Are major defects in children conceived *in vitro* due to innate problems in patients or to induced genetic damage?

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**Abstract** Birth anomalies recently detected in epidemiological studies indicate greater risks following assisted human reproduction than with natural conception. Some of these conclusions and assumptions are questioned in this paper, and the effects of specific causative factors unique to some infertile couples are analysed. Other recent studies have identified imprinting defects as causes of birth disorders following IVF or intracytoplasmic sperm injection. While few in numbers, they apparently involve unusual factors in conception such as aberrant responses among preimplantation embryos to culture medium or serum. Various genetic and developmental factors in infertile couples influencing the origin of such birth outcomes are assessed, and the significance of imprinting and its embryological roles are discussed. 

**KEYWORDS:** assisted human conception, birth anomalies, ICSI, imprinting syndromes, IVF

### Introduction

Recognized since its initial years, the safety of children born after assisted reproduction technologies (ART) is of paramount importance. ART techniques rapidly became standardized worldwide, their greatest variations including the use of different culture media and their protein supplements such as human and fetal calf serum and serum albumin. Early follow-up studies on IVF children showed how frequencies of birth anomalies resembled those arising with natural conception (Steptoe *et al.*, 1986). More detailed analyses confirmed these findings, reinforcing the concept of the preimplantation period as teratologically 'safe'. The onset of ICSI (Palermo *et al.*, 1992) introduced another variable, its safety often criticized because the fertilizing spermatozoon neither binds to oolemma nor activates the oocyte. Calcium spiking and sperm decondensation are also compromised, and the persisting acrosomal vesicle partially constricts the early paternal pronucleus (reviewed by Ludwig *et al.*, 2001). Nevertheless, despite these apparent risk factors, birth anomalies were only slightly higher after intracytoplasmic sperm injection (ICSI) in follow-

up studies (Ludwig and Katalinic, 2002). Recent epidemiological studies on ART outcomes now question earlier findings, by reporting two-fold higher risks of infant malformations and the occurrence of syndromes related to errors in imprinting after assisted human conception. Are such increased risks genuine, due to ART or to endogenous causes in infertile couples, and can they be prevented?

### Recent epidemiological analyses

#### Clinical data

Two analyses published simultaneously indicated higher risks to ART children conceived by IVF or ICSI. One assessed major birth defects in 4000 1-year-old Australian children conceived naturally, 837 by IVF and 301 after ICSI (Hansen *et al.*, 2002). Odds ratios after adjusting for maternal age, parity, sex of children and correlation between siblings were 2.0 for ICSI (95% confidence interval 1.5–3.2) and 2.0 (1.5–2.9) for IVF. Causes included more children with multiple major defects, including musculoskeletal,

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cardiovascular, urogenital and chromosomal anomalies. The investigators accepting certain weaknesses in their study, such as differing diagnostic vigilance of ART children and those conceived naturally. They rebutted charges failing to adjust for specific causes of infertility, misclassification of major birth defects and sibling correlations.

The second analysis (Schieve *et al.*, 2002) compared low (<2500 g) and very low (<1500 g) singleton birth weights in 42,463 ART infants with >3,300,000 in the general US population. Singleton ART births at 37 weeks of gestation or later had a 2.6 times greater risk (2.4–2.7) of low birth weights. Curiously, twins displayed similar risks with both forms of conception (1.0–1.1). ART mothers delivered 0.6% of all children in the population, but 3.5 and 4.3% of those with low and very low birthweights respectively. ART was suggested as being a major factor in increases in birth defects and for many infants born with low and very low birthweights, additional to those due to more multiple gestations. These investigators also rebutted charges including their overlooking multiple male and female factors and different ratios of monozygotic:dizygotic ART twins.

Slightly lower risks were identified in two Swedish analyses reporting a relative risk (RR) of ~1.4, and both ascribing increased ART risk to due to multiple births and other associated risk factors (Ericson *et al.*, 2002; Strömberg *et al.*, 2002). Likewise, a prospective analysis comparing ICSI children with those conceived naturally reported an RR of 1.25 (1.10–1.46) (Ludwig and Katalinic, 2002). It involved very large cohorts of children and controls, with standardized examination procedures for both cohorts. Pregnancy data were collected once from control cohorts, versus up to five times from ICSI cohorts, biasing the statistics in favour of natural conception. Also, ICSI data were collected 38 days post-partum versus 7 days in controls, again favouring controls, since some defects become apparent some time after birth. Despite these statistics, an increased risk of major malformations could not be excluded definitely, since background factors or increased risks due to ICSI were not definitively measured. Recent comparisons between IVF and ICSI children revealed no differences in malformation rates (Hansen *et al.*, 2002; Bonduelle *et al.*, 2002). This implies that should ART involve extra birth risks, they do not depend on the technique used to achieve fertilization.

### Reservations over epidemiological risks reported for ART

Doubts additional to those already discussed arise about aspects of the study presented by Schieve *et al.* (2002). ART twins had equal risks to controls, which questions their findings on singletons since twins face much higher risks than those conceived naturally.

These include some twin survival in pregnancies originating as triplet or higher grade multiples; others are conceived in mothers secreting vast amounts of ovarian hormones. ART would hence be expected to result in more anomalies, especially among multiple births, yet they did not happen. Moreover, other findings in this study indicated ART was safe. These data arose when Schieve *et al.* (2002) identified no higher risks with ART embryos conceived *in vitro* and then transferred to surrogate mothers. ART infants now had an RR for low birth weight of 1.2 (0.6–1.8). This strongly suggests higher risks were not due to ART, although the sample of 16 observed surrogacy cases among a total of 180 surrogate mothers is too few for certainty. Even so, this analysis implies that low birth weights originate via oviductal or uterine conditions rather than by ART *per se*. The normal gestational environment offered by multiparous surrogate mothers is seemingly not matched in some IVF mothers.

Can such risk factors unique to infertile patients be identified in oviduct or uterus? Higher risks of pre-eclampsia, placental abruption and premature delivery characterize women with idiopathic infertility who conceived naturally or using ART (Pandian *et al.*, 2001). Conclusions were unchanged after adjusting for age, parity or fertility treatment, the perinatal outcome being similar in the two groups. Ludwig and Diedrich (2002) interpret such reports as evidence of infertility-related risks especially in uterine environments contributing to higher prematurity independent of ART, proposing such factors in some patients compromise infant safety rather than ART itself. Genital infections, more frequent in anamnesis of infertile as compared with fertile patients, might lead to endometrial micro-scars to impair implantation, placentation and increase risks of pre-eclampsia. The incidence of neuroblastoma in neonates is raised by prenatal infections and use of infertility hormones, indicating a causative combination of innate and external factors (e.g. Michalek *et al.*, 1996). This New York study on 183 cases in children aged 1–14 years revealed elevated odds ratios for several factors, including vaginal infections during pregnancy (2.2, 1.2–4.0), medical treatments for this condition (2.4, 1.2–4.9) and using sex hormones during pregnancy (3.0, 1.3–6.9) (Michalek *et al.*, 1996). Males seemed to be more affected. Analyses for any hormone use showed only those used in relation to infertility were significant (10.4, 1.2–89.9). Vitamins protected against this situation. The investigators were confident of an association between prenatal hormone exposure and risk of neuroblastoma. Despite these data and reports of other cases, risks of cancer in ART children seem to be no greater than after natural conception (Bruinsma *et al.*, 2000), perhaps due to the rarity of such conditions or a failure to detect them.

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The use of IVF or ICSI might also be a factor leading to other highly unusual occurrences in the female genital tract, which harm offspring of assisted conception. Depriving the uterus of exposure to partner-specific spermatozoa could raise risks of pre-eclampsia, compromising uterine immunological adaptation and pregnancy (Wang *et al.*, 2002). This proposal seems unlikely, since the use of testicular sperm extraction or ejaculated spermatozoa leads to similar degrees of risk (Ludwig and Katalinic, 2003). Moreover, the routine use of spermatozoa in IVF, without ICSI, is also associated with higher risks of pre-eclampsia and other complications (Ludwig, 2002). Thus, even in IVF, without ICSI (and of course testicular spermatozoa), the risk is higher which supports data of Ludwig and Katalinic (2003) and not the suggestions of Wang *et al.* (2002).

Finally, various reports have implied rates of spontaneous abortion are higher following ART as compared with natural conception. Answering this question really demands a prospective controlled study in which pregnancies are also followed up after natural conception from about 14 days post-conception. To the best of our knowledge, these data are not yet available. A retrospective study did assess those patients who conceived either spontaneously before the onset of infertility treatment with others who conceived after ART (Pezeshki *et al.*, 2000). Spontaneous abortion rates in the treated groups was 22.4% as compared with 26.2% in the non-treated patients. It is essential to bear in mind that this non-treated group consisted of patients originally defined as infertile. Nevertheless, it clearly shows that infertility treatment *per se* does not increase the risk of spontaneous abortions, and the group of infertile couples might have had an increased endogenous risk *per se*.

## Epidemiological risks of imprinting, imprinting syndromes and rare disease after ART

### Recent studies on imprinting defects in children

Serious recent doubts about children's safety after ART concern risks of imprinting defects. Well-characterized imprinting diseases in humans include Angelman and Beckwith–Wiedeman syndromes. Originally, single cases after assisted conception were identified by Sutcliffe *et al.* (1995) and by Olivennes *et al.* (2001). Two children with Angelman syndrome were then detected after assisted conception using ICSI (Cox *et al.*, 2002). Both inherited phenotypes characteristic of this syndrome, with a strong unmethylated band and a weak methylated band in chromosome 15. Both

fathers had severe oligozoospermia, due in one of them to hypogonadotrophic hypogonadism. Cox *et al.* (2002) stressed this number of cases in ART children over-represented this syndrome as compared with those conceived naturally. They surmised that ICSI may have disrupted trans-acting factors or other systems essential for imprinting, and proposed introducing a prenatal and preimplantation methylation test for all imprinted loci in mother and embryos. This full form of testing may not yet be feasible. The two most important loci could be considered if necessary, or prenatal tests on parents may be done for loci sensitive to perturbation such as 15q11–13 and 11pp15.5 and *LIT1* in particular.

A third Angelman case was reported by Ørstavik *et al.* (2003) in a 3-year-old girl conceived after a third attempt at ICSI. An initial ICSI attempt had resulted in spontaneous abortion, and the second in a healthy daughter. The girl suffered from an abnormal electroencephalogram, lack of language development, and mental retardation. Her *SNPRN* locus lacked a methylated maternal band, indicating an imprinting defect. Sperm numbers in the father were normal, indicating the abnormal numbers of spermatozoa in a case reported by Cox *et al.* (2002) did not relate to the syndrome. However, the case described by Ørstavik *et al.* (2003) may have been spontaneous, unlike the earlier cases. A history of spontaneous abortions in a grandmother implied the defect originated as a maternal mutation and not through ART. The implications of these cases for assisted conception nevertheless remain considerable (Edwards, 2002)

Two new groups of investigators then drew essentially the same doubts about ART safety when their studies revealed a five- to six-fold greater risk for Beckwith–Wiedemann syndrome after ART as compared with the general population. This disorder arises through mutations or epimutations affecting imprinted genes in a chromosome cluster at 11p15.5. Clinical features include macroglossia, pre- and/or postnatal overgrowth, defects of the anterior body wall and predisposition to embryonal cancer. Most cases are sporadic, with 20% of them inheriting uniparental disomy for a variable region of chromosome 11, including the cluster. This 11p15.5 region may be susceptible to several modifications of its methylated regions, which then influence the paternal expression of *IGF2* and *KCNQ10T* or the maternally expressed genes *H19* (involving risks of cancer) and *CDKN1C* (Maher *et al.*, 2003). The region *LIT1* within the *KvLQT1* maternal gene is also susceptible to aberrant hypo- or hypermethylation, to result in the silencing of *p57KIP2*, a cyclin-dependent kinase inhibitor associated with overgrowth and birth defects (DeBaun *et al.*, 2003). Different epigenetic alterations occur within these gene loci, including several perhaps arising through defects in

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the imprinting control centres BWSIC1 and BWSIC2. Curiously, the Beckwith–Wiedemann syndrome is also associated with monozyotic twinning in discordant female twin pairs, indicating it acts on the inner cell mass or earlier (Weksberg *et al.*, 2002). An imprinting defect at *KCNQ1OT1*, a locus especially vulnerable to loss of imprinting, was identified in each affected twin but not in their twin siblings.

For their study, Maher *et al.* (2003) studied medical records on the birth of six cases of Beckwith–Wiedemann syndrome from records of the Section of Medical Genetics, Birmingham University, UK. Using comparative birth data, they calculated an expected risk of 1.73 (1.5–8.8) in IVF children, which differed significantly from normal conception. One case arose among discordant identical twins, indicating the possible gene region involved. DeBaun *et al.* (2003) analysed data collected at the Genetic Epidemiology Branch of the National Cancer Institute, USA, and the University School of Medicine, Washington University, USA. Diagnoses involved close checks on several parameters in the children using data abstracted from records of patients treated with ICSI or IVF. Potential causes were listed as use of donor gametes, origin of spermatozoa (i.e. testicular, epididymal or ejaculated), and use of AID/AIH. Seven affected children were identified, all sporadic, without evidence of any family history. The data represented a six-fold increase in this condition above natural conception. Clinical features in these ART children resembled those arising after natural conception, five displaying abnormal imprinting of *LIT1* and one abnormal imprinting of *H19*. The investigators expect more cases as databases begin to include details of imprinting syndromes.

The involvement of maternal genes in many ART children with Beckwith–Wiedemann syndrome or Angelman syndrome implies the spermatozoon is not implicated, and disturbances arise in oocytes or embryos. This interpretation should be treated cautiously since the fertilizing spermatozoon is active genetically soon after its entry into the oocyte (Edwards, 2001). Possible confounding factors included a higher tendency for ART parents to hospitalize their affected children as compared with those in the general population. The cases of Beckwith–Wiedemann syndrome extend risks of imprinting to include both ICSI and IVF.

Caution is clearly needed in assessing imprinting risks. All cases of these syndromes and have arisen through aberrant hypomethylation of maternal alleles, arising through blocks to methylation or a demethylation of maternal imprints. Cases of Prader–Willi syndrome, due to hypermethylation of the paternal allele, have not been reported even though this system and maternal imprints arising during oocyte activation could be damaged by ICSI. Beckwith–

Wiedemann syndrome is associated with identical twinning, itself a risk of anomalies or death to developing fetuses that will not be recognized as originally being twins if one dies *in utero*.

The slight increase in imprinting disorders after ICSI may have been underestimated or overlooked, its full detection requiring larger analyses. Inevitably, many cases of anomalous imprinting after ART may also be lost as early abortions. So far unmeasured, such events could arise after ART or natural conception since many embryos die at these stage with both forms of conception. Imprinting mutations leading to Angelman syndrome have a frequency of 5–8% in patients (incidence 1/20,000), compared with 1/200,000–1/400,000 in the general population. In most reported cases, damage arises when the imprinting centre on maternal 15q11–13 is deleted (seen in maternal relatives or as maternal gonadal mosaicism in familial cases), which prevents resetting the imprint post-fertilization. Cases of Angelman syndrome arising after ICSI also display inappropriate hypomethylation but the pre-determining imprinting centre is not deleted. One case was mosaic, ca. 10% of maternal alleles being methylated, suggesting a somatic error in early embryos. Such cases are very novel and rare. Final maternal imprints at these sites were not gametic and arose during oocyte maturation or fertilization, perhaps due to ICSI or in-vitro culture. If this is the case, using immature male gametes with ICSI may not be a likely cause.

### Nature and origin of imprinting and other factors affecting preimplantation embryos *in vitro*

Most suspicion points to in-vitro culture as the primary cause of imprinting defects. Also, unsuspected over many years, the late pronuclear stage in mice is more sensitive than other early cleavage stages to teratogenic changes, even if effects are mild (Rutledge *et al.*, 1992). ICSI may induce more damage than IVF since Schröder *et al.* (2001) showed cleavages occurred more rapidly than after IVF, but retardation was greater in later in-vitro stages as fewer ICSI embryos became blastocysts. Responsibility was ascribed to a mixture of induced in-vitro damage and endogenous effects in male patients. Potential in-vitro causes included varying efficiency among embryologists, lack of oolemmal stimulation, embryonic mosaicism due to retention of acrosomal vesicles in ooplasm, fragmented DNA arising in infertile patients or during ICSI, and chromosomal imbalance in some sperm samples.

Concern about risks of imprinting errors arising in early embryos *in vitro* first arose when pronuclei were exchanged between mouse eggs, and resulting embryos displayed repressed transcription, methylated liver genes encoding urinary proteins

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and fetal growth retardation. Other genes could be methylated or demethylated *in vitro*, inducing epigenetic disorders in mice (Reik *et al.*, 1993) and in lambs and calves (Mayne and McEvoy, 1993). Mouse embryos cultured in M16 medium plus calf serum suffered high embryonic losses during cleavage and compromised growth to day 14 (Khosla *et al.*, 2001). The presence of serum in culture medium was considered the primary cause. Responsibility lay in a reduced expression of imprinted genes *H19* and *IGF2* genes, correlated with hypermethylation of a control gene upstream of *H19*, and the non-imprinted growth factor receptor-binding protein Grb7. Conversely, maternally expressed growth-suppressor Grb10 displayed heightened expression.

Experience in animals may help to avert clinical risks. Large offspring syndrome in cattle and sheep is ascribed to the addition of serum to culture medium. It hypomethylates the element controlling *Igf2r* imprinting in preimplantation embryos (reviewed by Hoshi, 2003). Avoiding serum increases yields of good quality bovine embryos and lowers risks of large calf syndrome and calf mortality. The nature of embryonic damage due to this cause includes large numbers of cytoplasmic lipid droplets and immature mitochondria, presumably lowering oxygen consumption. Highly viable embryos can be identified through scanning electrochemical microscopy to measure oxygen consumption quantitatively and non-invasively (Hoshi, 2003), an approach possibly of value in human assisted reproduction technology.

Imprinting risks also arise during later gestational stages (Mayer *et al.*, 2000). Examples include *mest* (*MEST* in humans), expressed paternally in mouse decidua and placenta, and human placenta. Spatial disturbances between maternal cell types at fetomaternal interfaces, and fetal death, arose when normally imprinted allelic differences were suppressed by the inheritance of two paternal copies of chromosome 12 (Georgiades *et al.*, 2001). Paternally expressed *Igf2* in mammalian placentae, along with other imprinted genes, regulates placental supply and fetal nutrition. Deleting a small *Igf2* segment regulating its specific expression in labyrinthine trophoblast reduced placental and then fetal growth, although simultaneous increases in placental permeability temporarily restored normal fetal supplies (Constancia *et al.*, 2002). This study was carried out in mice and is not relevant to humans, where *IGF2R* is not imprinted. These examples nevertheless indicate that imprinting disorders leading to problems in placentation could arise in the fetus rather than the mother.

### Are infertile patients more prone to imprinting disorders?

Are certain genes susceptible to imprinting modifications during preimplantation growth, and why

do epigenetic errors affect certain couples? Such queries demand an understanding the fundamental nature of imprinting and epigenesis. More than 50 years ago, Waddington (1942, 1953) and Waddington and Robertson (1966) showed how well established developmental systems could override the normal genetic control of individual phenotypes to result in new highly stable and persistent forms of expression ('canalization') persisting over many generations. Their overriding control could be broken by extreme shocks to eggs or embryos, which exposed 'hidden' genes. For example, exposing *Drosophila* to shocks such as prolonged elevated temperatures revealed previously unexpressed genes and characteristics, sometimes in unrelated organs, which were then transmitted through numerous generations. Phenocopies are another form of anomaly, distinct from imprinting and induced by environmental effects. They also involve the imposition of totally different phenotypes and disorders resembling existing mutations, but in contrast to imprints they are not heritable. So far, they have not been reported in clinical situations.

Some years after Waddington's original work, certain DNA gene sequences were found to be methylated, and their actions in tissues were enhanced or repressed. Major aspects of development were regulated in this manner (Holliday 1987, 1996). Terms such as 'epigenetics' and 'imprinting' were used in relation to methylation, and canalization was gradually clarified. By now, numerous studies have clarified relationships between systemic gene imprinting in relation to methylation or demethylation of specific genes, and how particular aspects of embryogenesis are regulated. Imprints arise in various forms, some related to the sex of the gamete, others imposed in oocytes and early embryos, and some arising during differentiation at particular developmental stages. Interference with such imprints can distort major growth patterns and lead to birth defects. Exposing animal and human embryos to media containing serum could have impaired some imprints just as they were being formed in oocytes and embryos.

Very little is known regarding the effects of environmental factors on methylation patterns and hence on imprinting defects. Imprints may be induced or disturbed by histone modifications, RNA interference, cellular errors, chromatin remodelling and gene mutations as with the *trithorax* gene in *Drosophila* (Sollars *et al.*, 2003). Each of them could impair imprinting after ART. Chromatin remodelling and histone modifications could lead to wide genomic defects, including imprinting disorders. Translocations, inversions, deletions, mono- and disomy arising in infertile patients could influence overall chromatin modelling far beyond their local actions. 'Silent' genes could be reactivated as recently illustrated

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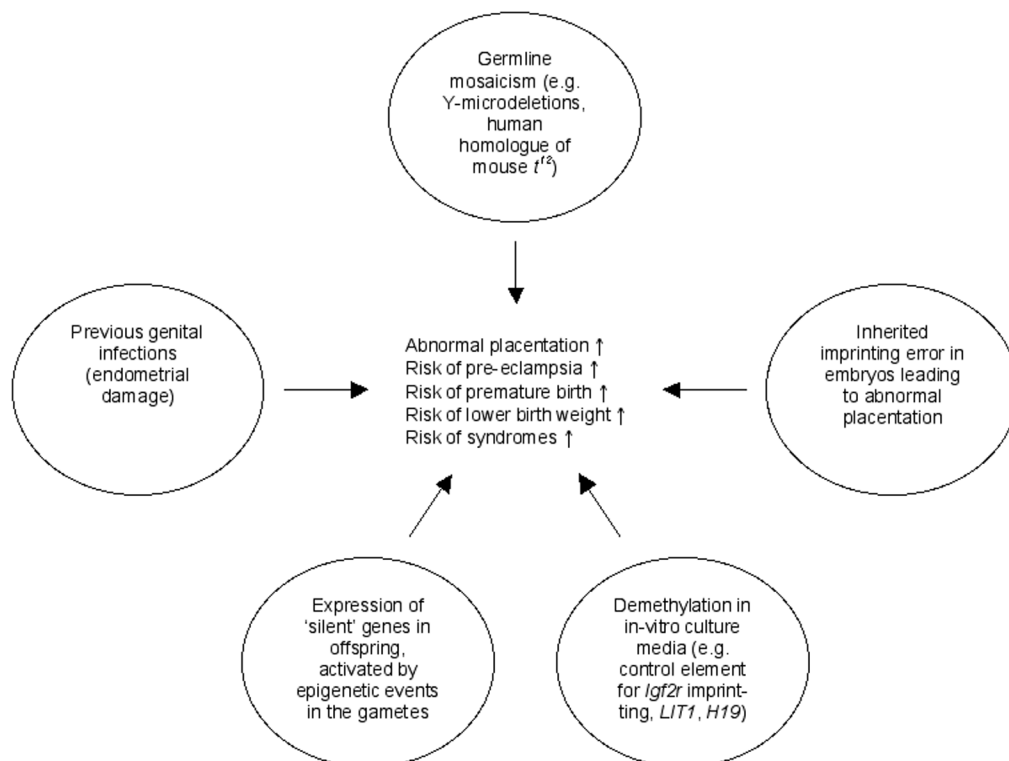


Fig. 1. Possible influence on the course and outcome of pregnancies after assisted reproduction technology (ART). The diagram shows how many factors may not be related to ART techniques, although such an effect cannot be excluded.

when various forms of chromatin imbalance led to abnormal expression of the gene *Krüppel* (*Kv*) in eyes of *Drosophila* offspring (Sollars *et al.*, 2003). The functions of heat shock protein 90 (Hsp90) in development were also compromised by epigenetic errors. The modified trait now became heritable, persisting even after the normal restoration of Hsp90 function.

### Other genetic and developmental risks influencing epidemiological analyses

Innate genetic changes leading to pleiotropic, genetic or epigenetic events in embryos of infertile patients could be mistakenly ascribed to ART. Changes in chromosome patterns, mutations, environmental agents or modified histones might invoke effects on embryonic or placental development or imprinting by modifying the nature of chromatin fibres, especially in heterochromatic regions (Horn and Peterson, 2002). Such effects could occur in infertile men with Y deletions (Siffroi *et al.*, 2000). Men carrying AZF microdeletions often present with structural chromosomal rearrangements including 45,X/46,XY mosaicism, isodicentric Y chromosomes and, if they are oligozoospermic, many spermatozoa devoid of complete Y chromosomes. Rare patients with deleted Y-chromosomes through natural transmission inherit ambiguous genitalia, chromosomal instability and

45,X/46XY mosaicism (Papadimas *et al.*, 2001). Sperm counts vary in response to microdeletions in various regions on the long arm of the Y chromosome. Part of this variation arises through the effects of different Y chromosome classes in the general population (McElreavy and Quintana-Murci, 2003). Diets, pollutants, and pesticides, and antagonists of sex steroids among other factors have long been suspected as causing hypospadias, disordered spermatogenesis, varied sperm counts and testicular cancer, again due to different interactions among various Y chromosome classes (Skakkebaek, 2002).

Incorrect use of tissue samples for diagnosis can mislead epidemiological interpretations. When testing for chromosomal and gene anomalies, parental genotypes are usually assessed using blood haemopoietic cells, which are mesodermal. In contrast, tests for the origin of inherited defects in children should be assessed in germline or gametes, where specific mutants may uniquely arise and differ from those in mesodermal derivatives. Examples abound. It emerged when Y chromosomal mosaicism in a child born of a normal father was found to originate through deletions in his germ-cell lineages when an early fetus (Kent-First *et al.*, 1996). Genetic anomalies in germline also stem from chromosome anomalies arising in embryos of patients lacking such defects, and as immature centrioles in many spermatozoa lead to high rates of mosaicism in embryos (Silber *et al.*, 2003). Frequent deletions, translocations and other

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chromosomal anomalies in infertile couples could arise independently in germline in either sex, consequential on external factors or specific mutations (Edwards and Bishop, 1997).

Gene mutations in germline might also lead to similar errors obscuring the origin of birth defects. Heterozygous familial germline mutations in the genes *MLH1* and *MLH2*, the latter leading to leukaemia and multiple café-au-lait spots, were associated with early-onset brain tumours. Children died at 15 months and 4 years respectively from T mediastinal lymphoma and temporal glioblastoma, yet parents had neither family history nor signs of disease. A case involving the heterozygous deletion of *MSH2* exons 1–6, corresponding to the control amplicon, originated in the father and transmitted to offspring (Bougeard *et al.*, 2003). Aberrant methylation in a p16 promoter Leiden mutation in a 54-year-old man who developed several tumours was absent from blood cells but present in tumours (der-Stock RS *et al.*, 2003). This mutation is known to occur in germline in Dutch people. Gene defects arising post-fertilization, after conception *in vitro* or *in vivo*, could explain discordancy in twins arising naturally or after ART (Edwards and Beard, 1998). An example involving imprinting defects leading to Beckwith–Wiedeman syndrome in discordant twins is given below.

Defects at implantation leading to embryonic disorders might also complicate and mislead epidemiological analyses. Retinoblastoma (*Rb*) is an example, a recent complication in ART statistics (Moll *et al.*, 2003). It was identified in five ART children, none with a family history. Calculations utilizing 1-year age-specific mortality rates in Holland, and assuming between ~1 and 1.5% of Dutch births arose through IVF, the defect had 4.9–7.2 times greater frequency with ART than with natural conception. All affected children were successfully treated. Some sporadic cases of retinoblastoma may arise through localized hypermethylation, as with other sporadic cancers. Mouse data then questioned such absolute reliance on epidemiological conclusions, since secondary causative effects arose during pregnancy (Wu *et al.*, 2003). In *Rb*<sup>-/-</sup> mice, an overgrowth of trophoblast in the early placenta led to anomalous development of extra-embryonic trophoblast within the labyrinthine layer of the placenta, occlusion of placental vessels, placental insufficiency and various developmental defects in fetuses (Wu *et al.*, 2003). The main cause of many fetal anomalies and lethality was thus an abnormal placental morphology, not the mutant gene. This mouse model may also explain small for gestational age infants and higher rates of prematurity in singletons born to previously infertile couples. Human information may be gained by addressing this issue in term placentae after ART.

Selective fertilization is a potential cause of unbalanced gene segregation, as in mice carrying the gene *t*<sup>12</sup>. *Tt*<sup>12</sup> heterozygotes transmit *t*<sup>12</sup> to offspring in far higher numbers than *T*. Similar systems in humans could distort IVF data via sperm selection *in vitro*. Segregation ratios might be modified in cases of severe oligozoospermia in humans if spermatozoa or earlier forms of germ cells carrying a particular allele are more or less resistant than those carrying the other allele. Using spermatozoa from severely oligozoospermic men carrying fresh deletions, translocations and other karyological modifications could have wide consequences for ART. Chromatid exchanges and some forms of heteroploidy (Wilton *et al.*, 2001; Munné, 2002) could compromise maternal inheritance in gametes and fertilized eggs, predicted by Reik and Dean (2002). Higher frequencies of monozygotic twinning in patients with Beckwith–Wiedeman syndrome is another indication of such pleiotropic effects affecting inner cell mass or earlier embryonic stages.

Clearly, considerable care is needed when apportioning embryonic damage to various causes. Shortcomings in methods in many published surveys include lack of proper controls, confounding variables, more vigorous surveying for anomalies after ART than after natural conception, inclusion or exclusion of minor abnormalities, and overlooking anomalies in terminated pregnancies (Simpson and Liebars, 1996). These authors insist on prospective surveillance and data collection from the first diagnosis of pregnancy, thorough pregnancy surveillance and exclusion of teratogenic influences.

## Summary

The epidemiological identification of higher frequencies of abnormal births after ART as compared with natural conception could be partly explained by factors independent of assisted conception. Preliminary data from surrogate mothers indicates relative risks after ART or natural conception are similar, indicating the uterus is a cause of increased anomalies. Unique factors in some infertile patients, e.g. higher risks of pre-eclampsia, infections and placental insufficiency could enhance anomalies among offspring by a mistaken attribution to ART in epidemiological analyses. High steroid levels and other hormones in ART cycles may impose specific risks absent under natural circumstances, and placental function and gene expression have to be related to its fetal and maternal components. Imprinting defects, serious for individual families, currently seem too rare to explain higher risks of birth anomalies with ART, although many more may be identified with stricter protocols in follow-up studies. Exposing early developmental

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stages to culture media and sera seems to be a major cause of some imprinting defects during ART, and is known to invoke immediate stress responses and metabolic modifications in embryos. A detail awaiting solution is why imprinting syndromes were not detected in original follow-up studies after ART, and whether numerous but so far unidentified imprinted loci in the genome may be affected *in vitro*.

Risk factors for embryos and fetuses may be heightened by the parental inheritance of chromosomal and other disorders, and such disorders and gene mutations may arise in germline rather than be due to ART. Unusual environmental effects may influence gene expression during preimplantation development, a sensitive phase involving chromatin remodelling, disordered chromosomal structures, and the onset of large-scale embryonic transcription. Placental insufficiency due to anomalous trophoblast development may invoke embryonic disorders secondary to the inheritance of defective genes. Simple therapies for some developmental irregularities may already be available. Imprinting errors in bovine embryos invoke the formation of immature mitochondria and poor oxygen consumption, and these may be avoided by omitting serum in medium and applying new forms of microscopy to assess their oxygen uptake in embryos. Risks of neuroblastoma in children may be modified by the use of vitamins.

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