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Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study

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BACKGROUND: Evaluation of relationships between assisted reproduction technologies (ART), fertility problems and disorders caused by disturbed genetic imprinting such as Angelman syndrome (AS) and Beckwith–Wiedemann syndrome (BWS). METHODS: A nation-wide questionnaire survey was performed regarding ART in families with a child with AS, BWS or Prader–Willi syndrome (PWS) including questions on fertility. Molecular data on the genetic disorder in affected children were gathered. RESULTS: Of the 220 affected children in this study, 14 (6.4%) were born following any form of ART compared with 83 818 (2.1%) in the Dutch population. Of AS, PWS or BWS children 15 (6.8%) were born after a fertility problem (Time To Pregnancy >12 months, no forms of ART) compared to 141,340 (3.5%) in the Dutch population. Maternal age in the individual syndromes was higher than in the Dutch population. Families with affected children were three times more likely to experience fertility problems than the general population. All three syndromes were also individually associated with increased fertility problems in the families. CONCULSIONS: After correction for the increased fertility problems of the parents, there is no increased incidence of ART related birth of AS, PWS or BWS children. ART does not seem to have a direct effect on the increase of imprinted diseases.

Keywords: genetic imprinting; imprinting disorders; artificial reproduction techniques; infertility; IVF

Introduction

Recently, a series of reports have raised concern about a possible relation between assisted reproduction technologies (ART) and genomic imprinting disorders (Chang *et al*., 2005; Cox *et al*., 2002; Gosden *et al*., 2003; Horsthemke and Ludwig, 2005; Ludwig *et al*., 2005; Maher *et al*., 2003; Maher, 2005; Sutcliffe *et al*., 2006). For years it has been known that IVF and embryo culture can affect the methylation status of some genes in mice, cattle and sheep, and it was postulated to happen in humans as well (Ceelen and Vermeiden, 2001; Horsthemke and Ludwig *et al*., 2005). This raised the issue of a possible increased risk in ART for conceiving a child with disorders that can be caused by a disturbed imprinting, such as Beckwith–Wiedemann syndrome (BWS) and Angelman syndrome (AS) (Maher, 2005). We studied the incidence of children with these imprinting disorders and also of children with Prader–Willi syndrome (PWS), following ART in the Netherlands, and the association with impaired fertility.

Materials and Methods

All families of children with BWS, AS and PWS known to the Dutch support groups born at or between 1 January 1983 and 31 December 2003 were informed of the study through the support groups and asked to participate. The year 1983 was chosen as this was the first year in which IVF children were born in the Netherlands. The families received a questionnaire regarding parental age, fertility (including impaired fertility related birth of siblings), pregnancy, birth and aetiology of the syndrome (Appendix 1). In order to confirm the aetiology, permission was asked to retrieve this information from the Dutch Diagnostic Molecular Genetic Laboratories.

Results were compared with all children born in the Netherlands in the same period. The total number of children born in the Netherlands in that period was 4 038 279 (Centraal Bureau voor de Statistiek; www.cbs.nl). In a large regional Dutch study Snick *et al*. (1997) showed that 9.9% of all couples had fertility problems and that 56.6% of these couples conceived at least one child, so 5.6% of all children were born after a fertility problem. From the same study, it is estimated that at least 5.9% of the families with children had at

least one child that was born after a fertility problem (Table 1, second row, last column). A German study provided support for these figures (Gnoth *et al*., 2003). It showed that 90% of all couples conceived within 12 months of unprotected intercourse, 5% became pregnant after >12 months of unprotected intercourse, whereas 5% of couples remained childless. Thus, in this study 5.3% (5/95*100) of all children were born after a fertility problem (Gnoth *et al*., 2003). To prevent underestimation of the percentage of children born after a fertility problem we used the higher figure (5.6%, Table 1, last figure).

We defined ART as either IVF, ICSI, the use of fertility drugs to induce ovarian stimulation and ovulation, intrauterine insemination (IUI) or donor insemination. 'Fertility problems' were defined as the inability to conceive within 12 months of unprotected intercourse (Time To Pregnancy longer than 12 months (TTP > 12 months) and/or if ART were used.

'Fertility problems of any kind' was defined as TTP > 12 months and/or the use of ART in conceiving the child with the imprinting disorder and/or fertility problems in conceiving a sibling (again defined as TTP > 12 months and/or ART in conceiving). It was assumed that there were 'no fertility problems' if all children in a family were conceived spontaneously within 12 months of unprotected intercourse and no ART was performed.

The number of Dutch children born following IVF/ICSI in 1983–2003 has been estimated to be 37 081 [0.92% of all children (Kremer *et al*., 2002; De Boer *et al*., 2004)], whereas 17 037 children (0.39%) were born after the use of ovulation inducing drugs (Steures *et al*., 2004), and 29 700 children (0.74%) were born after IUI or donor insemination (Janssens *et al*., 2005; Steures *et al*., 2006). In total, 83 818 children (2.1%) were conceived through ART. As 5.6% of children were born after a fertility problem and ART is rarely used in the absence of fertility problems, 3.5% (5.6 − 2.1%) of children must have been born after fertility problems but without using a form of ART.

For statistical analysis, STATA® (StataCorp LP) was used. As most of the variables were binary or categorical, we tested for significance with Pearson's Chi-squared test or Fisher's exact test if the numbers were small. Logistic regression was used in multivariate analyses. Analyses were carried out both on the individual syndromes and the total group (any of the three syndromes).

The Medical Ethical Committee of the Academic Medical Centre Amsterdam approved the study.

Results

The numbers of patients with AS, PWS and BWS known to the support groups were 135, 227 and 138, respectively. The response rate for children born in the study period 1983–2003 was 72.6% ($n = 98$; AS), 78.0% ($n = 177$; PWS) and 54.3% ($n = 75$; BWS). Of these 63 (AS), 86 (PWS) and 71 (BWS) were eligible for the study (born in the Netherlands in the period 1983–2003), in total, 220 affected children in 220 families. The combined incidence of AS, PWS and BWS for the Dutch population is 1:5769, indicating that of the 4 038 279 children born in the study period, 700 will have had one of the disorders. Therefore, it is estimated that 31.4% of the total group of AS, PWS and BWS patients participated in the present study.

Fourty Five mothers reported their own day of birth in the AS group, 54 in the PWS group and 46 in the BWS group. The maternal age of the study group was found to be significantly higher compared with the Dutch population, both for the total study group (30.82 versus 29.68 years, $P = 0.00$) and for the individual syndromes (AS, 30.64 years, $P = 0.03$;

Table 1: Results of questionnaire study regarding fertility and use of artificial reproduction techniques (ART) of all patients known to the Dutch support groups with AS, PWS and BWS born between 1 January 1983 and 31 December 2003, compared with data from the general Dutch population

		AS ($n = 63$)	PWS ($n = 86$)	BWS ($n = 71$)	Total ($n = 220$)	Dutch population 1983–2003 ($n = 4\,038\,279$)
1	Maternal age* (years) (SD, years)	30.64, 4.22, $P = 0.03$ ($n = 45$)	31.17, 5.19, $P = 0.01$ ($n = 54$)	30.59, 3.76, $P = 0.03$ ($n = 46$)	30.82, 4.45, $P = 0.00$ ($n = 145$)	29.68, 4.67
2	Families with fertility problems of any kind	12, 19.0%, RR = 3.4, $P = 0.00$	12, 14.0%, RR = 2.5, $P = 0.00$	15, 11.1%, RR = 2.0, $P = 0.00$	39, 17.7%, RR = 3.0, $P = 0.00$	5.9%
3	Affected children born after IVF/ICSI	0 (0%), RR = 0, $P = 0.44$	2 (2.3%), RR = 2.5, $P = 0.17$	4 (5.6%), RR = 6.1, $P = 0.00$	6 (2.7%), RR = 3.0, $P = 0.00$	37 081 (0.92%)
4	Affected children born after IUI/donor insemination	1 (1.6%), RR = 2.2, $P = 0.43$	1 (1.2%), RR = 1.6, $P = 0.64$	1 (1.4%), RR = 1.9, $P = 0.51$	3 (1.4%), RR = 1.9, $P = 0.28$	29 700 (0.74%)
5	Affected children born after ovulation induction by drugs	3 (4.8%), RR = 12.3, $P = 0.00$	1 (1.2%), RR = 3.0, $P = 0.25$	1 (1.4%), RR = 3.6, $P = 0.17$	5 (2.3%), RR = 5.8, $P = 0.00$	17 037 (0.39%)
6 (sum of 3,4 and 5)	Affected children born after any form of ART	4, 6.3%, RR = 3.0, $P = 0.02$	4, 4.7%, RR = 2.2, $P = 0.10$	6, 8.5%, RR = 4.0, $P = 0.00$	14, 6.4%, RR = 3.0, $P = 0.00$	83 818 (2.1%)
7	Affected children born after TTP > 12 months, without use of ART	4, 6.3%, RR = 1.8, $P = 0.22$	5, 5.8%, RR = 1.7, $P = 0.24$	6, 8.5%, RR = 2.4, $P = 0.02$	15, 6.8%, RR = 1.9, $P = 0.00$	3.5%
8 (sum of rows 6 and 7)	Affected children born after any fertility problem (TTP > 12 months + ART)	8 (12.6%), RR = 2.3, $P = 0.04$	9 (10.5%), RR = 1.9, $P = 0.24$	12 (16.9%), RR = 3.0, $P = 0.00$	29 (13.2%), RR = 2.5, $P = 0.00$	5.6%

*Number between parentheses: mothers who reported their day of birth.

PWS 31.17 years, $P = 0.01$; BWS 30.59, $P = 0.03$, Dutch population 29.68).

Of the 220 families, 39 families had fertility problems of any kind (17.7%). This was significantly higher compared with the Dutch population, (17.7% versus 5.9%, relative risk (RR) = 3, $P = 0.00$). Also each syndrome group had significantly more fertility problems of any kind than the Dutch population (AS 19%, RR = 3.4, $P = 0.00$; PWS 14.0%, RR 2.5, $P = 0.00$; BWS 11.1, RR = 2.0, $P = 0.00$, Dutch Population 5.9% see Table 1).

The percentage of children born after ART was increased compared with the Dutch population, both in the individual syndromes (AS 6.3% RR = 3.0, $P = 0.02$; PWS 4.7% RR = 2.2, $P = 0.10$; BWS 8.5%, RR = 4.0, $P = 0.00$) and in the total study group (6.4%, $P = 0.00$; RR = 3.0).

The results were also analysed for the individual syndromes and various modalities of ART. There were two significant associations: three AS children were born after ovulation induction by drugs (4.8% versus 0.39% Dutch population, RR = 12.3, $P = 0.00$) and four BWS children were born after IVF (5.6% versus 0.92% Dutch population, RR = 6.1, $P = 0.00$).

In Table 2, we depicted the number births and the number of affected children and their siblings born after a TTP > 12 months and born after any form of ART, and we calculated the ratios between them. We compared these numbers with the percentages of births and children born in the Dutch Population after TTP > 12 months and born after ART. We used percentages of the Dutch population because the percentage of children born after TTP > 12 months is based on the observations of Snick *et al*. (1997), and it is presumed that this percentages will be valid for the whole Dutch population. In the Dutch population, the ratio between children born after ART and TTP > 12 months was 0.59 and the ratio between the number of births after ART and TTP > 12 months was 0.52. It appeared that these ratios of the affected children and their siblings (between 1.38 and 0.93) were not significantly different from each other but significantly different from the ratios of the Dutch Population (1.09 versus 0.59, $P \subseteq 0.05$ (children) and 0.96 versus 0.52; $P \subseteq 0.05$ (birth)).

The results of the DNA analysis of 14 of the affected children which were available are depicted in Table 3.

Discussion

There are several suggestions in the literature of a causal relationship between ART and the increased incidence of AS and BWS after ART (Gosden *et al*., 2003; Maher, 2005). We report here a three-times increase of the incidence of imprinted diseases after ART (RR = 3.0, $P < 0.00$), confirming the increased incidence reported in literature. However, 17.7% of families with AS, BWS or PWS children have at least one child born after a fertility problem (siblings and/or affected children). This percentage is 5.9% in the general population (RR = 3.0, $P < 0.00$). So, the relative risks are the same: after correction for impaired fertility there is no increased incidence of AS, PWS and BWS after ART. We conclude that the increased incidences of imprinted diseases after ART can fully be explained by the increased fertility problems of the parents.

Studying siblings of affected children can confirm or reject this conclusion. If the birth of normal siblings is associated with fertility problems to the same extent as that of the Dutch population, and if only the birth of the affected children is associated with increased ART, it would be an argument in favour of a causal relationship between ART and imprinted diseases. However, if the birth of siblings is associated with fertility problems to the same extent as in the affected children, it points to fertility problems as the cause of the increased incidence of imprinted diseases after ART. Table 2 shows that normal siblings are conceived after fertility problems to the same extend as in affected children and both differ significantly from the Dutch population.

The maternal age of the affected group is increased by 1.14 years ($P < 0.00$). Whether this is a primary factor, is secondary to the decreased fertility or has a multi-factorial cause remains uncertain at present.

The increased risk for AS, PWS and BWS in couples with fertility problems has been suggested before (Buckett and Tan, 2005). The incidence of fertility problems was 20% in the AS study (Ludwig *et al*., 2005), very similar to the percentage in the present AS group (19%). The authors of the AS study suggested reporting bias as an explanation for the frequent infertility. Although a biased parental recollection of fertility in pregnancies of a child with an imprinting disorder cannot be excluded, we think this less likely to be the explanation: if recollection were

Table 2: Comparison of the fertility problems related number of children and number of births in families with affected children with that of the Dutch Population

	Affected children and siblings					Dutch population	
	Affected children (=birth)	Siblings (children)	Siblings (births)	Affected children + Siblings (children)	Affected children + Siblings (births)	Children	Births
TTP > 12 months	15	8	8	23	23	1 41 340 (3.5%)	1 41 340 (3.5%)
ART	14	11	8	25	22	83 818 (2.1%)	68 651 (1.7%)
Ratio	0.93	1.38	1.00	1.09[a]	0.96[b]	0.59[a]	0.52[a]

[a] $P \leq 0.05$ versus Dutch (children).
[b] $P \leq 0.05$ versus Dutch (births).

Table 3: Aetiology and type of ART[a] for individual patients with AS, PWS and BWS born after ART in the present study

Condition	Maternal age (years)	Time to pregnancy (months)	ART	Aetiology	Fertility problem
AS 1	32	72	HSO	Confirmed[b]	Ovulation
AS 2	36	156	AI	Del	Ovulation
AS 3	30	36	HSO	Del	Ovulation
AS 4	32	12	HSO	Unknown[c]	Ovulation
PWS 1	37	60	ICSI	Confirmed[b]	Paternal subfertility
PWS 2	Unknown	96	IVF	Del	Paternal subfertility
PWS 3	36	Unknown	AI	Del	Lesbian couple
PWS 4	28	7	HSO	Del	Ovulation
BWS 1	32	54	IVF	Hypomethylation LIT1	No cause detected
BWS 2	31	6	IVF	Hypomethylation LIT1	Paternal subfertility
BWS 3	41	60	IVF	Hypomethylaton LIT1	No cause detected
BWS 4	28	30	IVF	Hypomethylation LIT1	Endometriosis
BWS 5	31	18	HSO	Hypomethylation LIT1	POF[a]
BWS 6	37	Unknown	AI	Hypomethylation LIT1	Unknown

[a]HSO = hormonal stimulation of ovulation, AI = artificial insemination, POF = premature ovarian failure.
[b]Confirmed with molecular analysis, but not further specified (microdeletion/uniparental disomy/methylation defect).
[c]Clinical diagnosis, molecular analysis is pending.

to be biased, one would have expected that for pregnancies of healthy siblings. This was not biased, whereas this recollection was similar.

Up to now no significant association between PWS and ART has been reported (Sutcliffe *et al*., 2006). If the association between PWS and ART in the present study is observed separately no significant association is present (Table 1, row 8, RR = 1.9; $P = 0.25$), but if siblings are included a significant association between fertility problems and PWS does exist ($P = 0.00$).

We have presumed that the group that participated in the study is representative for the whole group of parents with affected children. Cases were recruited from parent support groups, and one must question what attracts parents to become member of such groups. However, it does not seem likely, that parents with infertility problems are more susceptible to join support groups.

The association between impaired fertility and disorders that can be caused by a disturbed imprinting such as AS, PWS and BWS remains difficult to explain. The number of patients in this study is too small to allow firm conclusions as to whether the association can be explained by a disturbed imprinting or whether other etiologies of the three entities (microdeletion/duplication; uniparental disomy; mutations in UBE3A) are more frequent than occurring in the three entities in general. This should be taken into account in explaining the data as well. It has been suggested that infertility in itself is also the risk factor explaining the increased rate of children with congenital abnormalities born after ART (Buckett and Tan 2005; Ludwig *et al*., 2005). Both oocyte development (Schultz, 2005) and spermatogenesis (Maclean and Wilkinson, 2005) are extremely complex processes that involve epigenetic influences including imprinting.

We suggest that only a large, carefully designed international study that will include all couples both with and without fertility problems and that also will include all disorders that can be explained by disturbed imprinting could definitively settle the cause of the relationship of these disorders with impaired fertility and ART.

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Appendix 1

Questionnaire

Data on your child		
1.	Name:	
2.	Birth date:	
3.	Sex:	
4.	Birth weight:	
5.	Pregnancy term (weeks):	
6.	Diagnosis of your child:	Angelman syndrome/Prader–Willi syndrome/ Beckwith–Wiedemann syndrome
7.	This diagnosis was made by:	Name medical doctor: Department and Hospital:
8.	This diagnosis was confirmed by:	DNA/chromosomal tests/not confirmed
9.	What is found at this investigation?	Imprinting/deletion/UPD/different/unknown
Data on your pregnancy		
10.	Did you ever have a miscarriage?	Yes/no If so, how many times?
11.	Have you ever had a period of unwillingly not getting pregnant/problems with getting pregnant?	Yes/no
12.	If so, how long was this periods?	…. (months)
13.	How many months did it take to become pregnant of your child with a syndrome?	… (months)
14.	If there was a problem, has there been an investigation of this problem?	a. Yes/no b. And if so, what was the result of this investigation?
15.	Were there any treatment or medications used to become pregnant of your child with a syndrome?	Nothing/IVF/ICSI/IUI/stimulation of ovulation/ other medication:
16.	Was there any treatment and medications used in your other pregnancies?	Nothing/IVF/ICSI/IUI/stimulation of ovulation/ other medication:
17.	If you had an investigation, treatment or use of medication in becoming pregnant, by which medical doctor was this performed, when and in which hospital?	
18.	Birth date of mother:	

(This questionnaire was sent with a letter of informed consent and information about the study. Also an 'agreement-form' requested confirmation of agreement to participate in the study and to allow confirmation of the data by collecting test results and data from the medical doctors, laboratories and hospitals concerned.)