Study of DNA-methylation patterns at chromosome 15q11-q13 in children born after ICSI reveals no imprinting defects

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The introduction of intracytoplasmic sperm injection (ICSI) has raised concern about safety in terms of a possible increase in the incidence of major congenital malformations, chromosomal aberrations or developmental problems. The possible influence of genetic imprinting on an ICSI procedure has not yet been investigated. We therefore studied the DNA-methylation status at a defined region in chromosome 15q11-q13 in 92 children born after an ICSI procedure. Imprinting defects in this region are associated with neurogenetic disorders, e.g. Angelman syndrome (AS) and Prader–Willi syndrome (PWS). Blood samples were taken directly after birth and stored at –80°C. Genomic DNA purification was performed from 3–7 ml EDTA–blood. Sodium bisulphite treatment was carried out in order to distinguish methylated from unmethylated DNA by transferring the unmethylated nucleic acid cytosine into uracil and leaving the methylated cytosine unchanged. Subsequently, a methylation-specific polymerase chain reaction (M-PCR) was performed. In all 92 children (83 from ICSI with ejaculated spermatozoa and nine from ICSI with non-ejaculated spermatozoa), a regular DNA-methylation pattern was found in the PWS/AS region. In none of the children were clinical symptoms of PWS or AS present. In conclusion, the results of this study do not indicate a higher risk of DNA-methylation defects in children born after ICSI.

Key words: children imprinting/DNA-methylation/ICSI/male infertility

Introduction
Intracytoplasmic sperm injection (ICSI) enables formerly infertile couples to have their own genetic child and has in many ways revolutionized the understanding of fertilization procedures. ICSI has also raised concern about its safety in terms of a possible increase in the incidence of major congenital malformations, chromosomal aberrations or developmental problems. Two groups of genetic risks can be distinguished: (i) genetic risks as a result of the possible genetic causes of infertility present in the (infertile) population: the genetic disorders causing infertility would be transmitted to the offspring and thus would be found in increased numbers in such offspring as compared with a normal fertile population; and (ii) genetic risks as a result of ICSI itself: the procedure might cause damage to the germ cells, interrupt or interfere with the natural maturation process of the sperm cells and possibly lead to genetic defects.

Most extensive studies conducted with regard to the possible genetic risks are quite reassuring. However, data from a large follow-up series reveal a slight but statistically relevant increase in sex-chromosomal and de-novo structural chromosome abnormalities (Bonduelle et al., 1996, 1998). A possible influence of imprinting on an ICSI procedure has already been mentioned (Tesark et al., 1998), but has not yet been investigated in a specific follow-up study. Imprinting is a mechanism of gene regulation by which only one of the parental copies of a gene is expressed (Hall, 1990; Barlow, 1995; Latham et al., 1995; Bartolomei and Tilghman, 1997; Constancia et al., 1998). Although the imprinting mechanism is largely unknown, methylation at the CpG dinucleotide is involved (Glenn et al., 1996). The germline plays a key role in the imprinting process. Imprints inherited from the previous generation have to be erased and the imprints have to be re-established according to the sex of the germ line. The exact timing of this process is elusive. In the female, methylation occurs after oocyte growth (Chaillet et al., 1991, 1995; Howlett and Reik, 1991; Ueda et al., 1992; Brandeis et al., 1994). Less is known about the male germ line. However, methylation of imprinted genes is assumed to occur between the spermatogonial and the spermatocyte stages (Bestor, 1998).

The major aim of the present study is the investigation of genetic risks of the ICSI procedure associated with DNA methylation defects. We analysed the methylation status in an imprinted region at chromosome position 15q11-q13 in children born after ICSI, as this region is known to be involved in imprinting disorders causing two distinct neurobehavioural syndromes: Prader–Willi syndrome (PWS) with a deficient or absent paternal copy and the Angelman syndrome (AS) with a deficient maternal copy at the imprinted region (Clayton-Smith and Pembrey, 1992; Wagstaff et al., 1992; Reis et al., 1994; Buiting et al., 1995; Ledbetter and Ballabio, 1995; Saitoh et al., 1997).
Materials and methods

Blood samples from 92 children born after an ICSI procedure were taken directly after birth and stored at −80°C. Genomic DNA purification was performed from 3–7 ml EDTA-blood with the QIAmp DNA Blood Maxi Kit (Qiagen, Leusden, The Netherlands).

Sodium bisulphite treatment

Before methylation-specific polymerase chain reaction (PCR), a sodium bisulphite treatment was performed, converting unmethylated cytosine to uracil and leaving methylated cytosine unchanged. In a multistep procedure, the DNA samples (2.5 μg of DNA) were first diluted in 10 mmol/l Tris pH 7.5/0.1 mmol/l EDTA in a total volume of 50 μl. Denaturation was then performed by the addition of 5.5 μl of 3 mol/l NaOH and incubation at 37°C for 15 min and 95°C for 3 min. DNA samples were put on ice. Then 500 μl of a sodium bisulphite solution (667 μmol/l sodium bisulphite and 400 μmol/l hydroquinone, 10 ml of 2.8 mol/l sodium bisulphite and 400 μl of 10 mol/l NaOH) were added to each denatured DNA sample, reactions were overlayed with 50 μl of Biowax (Biozym, Landgraaf, The Netherlands) and incubated for 16 h at 55°C in the dark. DNA purification was carried out using the Wizard DNA Clean-Up System (Promega, Leiden, The Netherlands). Elution was done with 50 μl of 10 mmol/l Tris pH 7.5/0.1 mmol/l EDTA at 80°C and the purified DNA was desulphonated by the addition of 5.5 μl of 3 mol/l NaOH and 15 min incubation at 37°C. Ammonium acetate (55 μl) 6 mol/l pH 7 and 220 μl of pure ethanol were added to each sample for precipitation. The washing was performed by the addition of 500 μl of 70% ethanol. After centrifugation and drying, the DNA pellet was resuspended in 25 μl of 10 mmol/l Tris pH 7.5/0.1 mmol/l EDTA.

Methylation-specific PCR (M-PCR)

PCR can take advantage of this sodium bisulphite treatment to distinguish methylated from unmethylated DNA (Kubota et al., 1997; Zeschnigk et al., 1997). The primer sequences used were as follows (Sutcliffe et al., 1994).

Maternal primer pair (primers 1 and 2 in Figure 1):
SNRPN-Mfor: 5'-TAAATAAGTACGTTTGCGCGGTC-3' ('-Mfor' standing for maternal forward)
SNRPN-Mrev: 5'-AACCCTACCCGCTCCATCGCG-3' ('-Mrev' standing for maternal reverse)

Paternal primer pair (primers 3 and 4 in Figure 1):
SNRPN-Pfor: 5'-GTATGGTTTGGTTGTATGTATTAGGT-3' ('-Pfor' standing for paternal forward)
SNRPN-Prev: 5'-ACATCAAAACATCTCCAACAACCA-3' ('-Prev' standing for paternal reverse)

These four primers are localized in exon 1 and the 5′ flanking region of the small nuclear ribonucleoprotein-associated polypeptide N (SNRPN) gene in the PWS/AS region (Zeschnigk et al., 1997). The genomic DNA represented in Figure 1 corresponds with the sequence before modification by sodium bisulphite treatment. In normal individuals, the maternal and paternal copies of the region differ in methylation of the cytosines (Cs) in the CpG dinucleotides (underlined in Figure 1): in the maternal copy Cs in the CpG dinucleotides are methylated and will remain unchanged upon bisulphite treatment, while in the paternal copy no methylation is observed and the Cs will be modified into uracil. Likewise, no methylation of Cs outside CpG dinucleotides is observed and these Cs will also be changed into uracil upon bisulphite treatment.

Each sample was analysed in two independent M-PCR reactions. PCR reactions were carried out in a 30 μl volume containing 3 μl of PCR Buffer II (Perkin Elmer), 2 mmol/l MgCl2, 2 mmol/l of each dNTP, 1 μmol/l SNRPN-Mfor and 1 μmol/l SNRPN-Mrev in the PCR reaction amplifying the maternal imprint specifically or 1 μmol/l 1 SNRPN-Pfor and 1 μmol/l SNRPN-Prev in the paternal PCR. 0.6 IU of AmpliTaq Gold (Perkin Elmer) and 2 μl of bisulphite-modified DNA. The polymerase was activated at 95°C for 10 min. DNA was amplified in 35 cycles at 94°C, 62°C and 72°C for 30 s each, followed by a final extension at 72°C for 10 min. PCR products were separated on a agarose gel, stained with ethidium bromide and visualized under UV illumination. PCR reactions without DNA or with genomic DNA from an AS patient, a PWS patient and a normal control person were also carried out at the same time. In normal individuals, amplification results in a 174 bp PCR product from the methylated maternal SNRPN allele and a 100 bp product from the unmethylated paternal allele.

Results

Clinical data

Blood samples were collected from 92 children born after an ICSI procedure. The ICSI was performed with ejaculated spermatozoa in the majority of the children (n = 83, 90.2%), with fresh testicular spermatozoa after testicular sperm extraction (TESE) in six children (6.5%) and with epididymal spermatozoa after microsurgical epididymal sperm aspiration (MESA) in three children (3.3%).

The 92 ICSI children included in the present study were born to 72 couples. Of the 92 babies, 40 were delivered from 20 twin pregnancies. The remaining 52 children were born from singleton pregnancies.

The ICSI procedure was performed because of male factor infertility in all couples. The origin of this infertility was idiopathic oligoasthenoteratozoospermia (OAT) in 61 out of 72 couples. In another three couples, obstructive azoospermia was the reason for the ICSI and non-obstructive azoospermia in another six couples. Donor spermatozoa were used in two of them. In both these cases, the father appeared to have a 47,XXY-karyotype and no spermatozoa were found in several testicular biopsies.

All 92 children participated in a clinical follow-up. None of the children (aged between 5 months and 4 years in the follow-up) had clinical symptoms of PWS, e.g. feeding problems and hypotonia in newborns, later obesity, mental retardation, short stature, craniofacial dysmorphism and hypothalamism. Symptoms indicating AS (microcephaly, severe mental retardation, jerky movements, absence of speech and paroxysms of laughter) were also absent in all children.

DNA-methylation status in the children born after ICSI

The M-PCR on genomic DNA from normal individuals showed both a 174 bp product from the maternal chromosome and a 100 bp product from the unmethylated paternal chromosome. In a PWS patient (diagnosis based on characteristic symptoms and former PCR diagnosis), the 100 bp product from the paternal chromosome was missing and in an AS patient the 174 bp product from the maternal chromosome was absent. In all 92 children both the product from the methylated maternal chromosome and that from the unmethylated paternal chromosome have been detected. A representative example of the
DNA-methylation patterns in children born after ICSI

Figure 1. Sequence of part of the SNRPN gene amplified by methylation-specific polymerase chain reaction (M-PCR). The positions of the primers are indicated by arrows on the coding strand (5'-3'). All cytosines (Cs) in CpG dinucleotides (underlined) are methylated in the maternal copy and unmethylated in the paternal copy. All unmethylated Cs, including all Cs outside of CpG dinucleotides, will be modified into uracil by bisulphite treatment. The two primer pairs, SNRPN-Mfor/SNRPN-Mrev (arrows 1 and 2 respectively) and SNRPN-Pfor/SNRPN-Prev (arrows 3 and 4), are designed to fit with the sequence of the maternal and paternal copy respectively, after bisulphite treatment. M-PCR will result in the amplification of a 174 bp maternal-specific fragment and a 100 bp paternal-specific fragment.

M-PCR analysis in children born after ICSI and controls is shown in Figure 2.

Discussion

Imprinting in the germline

The germline plays a key role in the imprinting process since it is at this stage that the existing imprints inherited from the previous generation are erased and the new imprints are established according to the sex of the germline. Little is known about the exact timing of this resetting. Global demethylation and methylation events occur in germ cells (Razin and Shemer, 1995; Huntriss et al., 1998). This also includes imprinted genes (Brandeis et al., 1994). The timing of methylation establishment in imprinted genes is clearly defined for the female but not for the male germline. In the female, oocytes are not methylated until after birth, when methylation occurs after oocyte growth (Chaillet et al., 1991; Howlett and Reik, 1991; Ueda et al., 1992; Brandeis et al., 1994). In the male, the embryonic primordial germ cells migrate into the undifferentiated gonad (Loveland and Schlatt, 1997), then differentiate into prospermatogonia and reside in a quiescent state inside the testicular seminiferous tubules. Gonadotrophic stimulation at the onset of the puberty induces spermatogenesis (the meiotic divisions giving rise to the spermatozoa) followed by spermiogenesis (the differentiation of the sperm cell, from haploid round spermatid to flagellated spermatozoa). Methylation of imprinted genes is supposed to occur during spermatogenesis between the spermatogonial and the spermatocyte stages (Bestor, 1998).

Figure 2. Representative example of methylation-specific PCR (M-PCR) analysis in children born after ICSI and in controls. In the upper part of the gel maternal-specific M-PCR products (174 bp) are shown, while the paternal-specific products (100 bp) are shown in the lower part of the gel. Excess primers are also visible (lower bands). Lane 1 = normal control. Lane 2 = patient with Prader–Willi syndrome; the paternal-specific 100 bp fragment is absent. Lane 3 = Angelman syndrome patient; the maternal-specific 174 bp fragment is absent. Lanes 4–12 = results of M-PCR analysis of nine unrelated children born after ICSI; in all children both maternal- and paternal-specific bands are present, as in the normal control sample (lane 1).
**Imprinting and ICSI**

Although the methylation of the imprinted genes is assumed to take place at an earlier developmental stage (spermatogenesis) than the ICSI procedure (spermioogenesis), the germ cells remain vulnerable to dysregulated de-novo methylation in the latter period (Bestor and Tycko, 1996). Especially when testicular (immature) sperm cells are injected, the ICSI might interfere with the process of imprinting or with the imprint switch and, in a worst-case scenario, provoke failures at this level.

Although the follow-up data of the ICSI children investigated in the present study do not report AS or PWS, a silent transmission to further generations cannot be completely excluded. New failures might occur in the non-expressed region and so not give rise to any immediate symptoms. If transmitted to the next generation, the subsequent offspring might have a different sex and therefore the deficient gene would be expressed. However, there is no reason to believe that only these kind of silent imprinting switch failures would occur while immediately detectable failures, as searched for in this study, would not occur.

**No risk at all?**

In the present study, the M-PCR of all 92 children born after an ICSI procedure revealed a methylation status in the PWS/AS region in chromosome 15q11-q13 identical to the methylation pattern in the normal controls. Signs of PWS or AS, e.g. absence of the 100 bp PCR product (PWS) from the paternal chromosome or 174 bp PCR product (AS) from the maternal chromosome, have not been found in any of the children. Therefore, the results of the present study do not indicate a higher risk of methylation defects, at least in the PWS/AS region, in the DNA of children born after an ICSI procedure. The number of investigated babies (n = 92) seems to be small when compared to the fact that in a normal population only 1 in 15 000 newborns is affected. Nevertheless, one might expect to find abnormalities in methylation patterns in at least some of these 92 children, if they did have an increased risk of DNA-methylation defects. The group of children born from ICSI with non-ejaculated spermatozoa (TESE, MESA) is of special interest, as this procedure implies fertilization with possibly immature spermatozoa. Especially in these cases, one might be concerned about whether the imprinting process is still vulnerable to dysregulation or even not yet perfectly completed at this stage of maturation. However, the small number of children from this group (n = 9) also had a regular DNA-methylation pattern in the PWS/AS region. It is also of interest to mention here that the M-PCR will not detect patients with a mosaic pattern of normal and methylation-defective cells. In these patients, paternal and maternal bands produced by the normal cells would mask the absence of unmethylated or methylated copies, respectively, in the methylation-defective cells.

In conclusion, the results of this study do not indicate a higher risk of DNA-methylation defects at the PWS/AS region in children born after an ICSI procedure. However, more data are needed, especially from children born after ICSI with non-ejaculated spermatozoa.

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