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A review of ten years experience of ICSI

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This review summarizes the introduction of ICSI in the early 1990s as an assisted fertilization procedure in couples with severe male factor infertility, who could not be helped by conventional IVF. As for current practice, the indications for ICSI using fresh or frozen–thawed ejaculated, epididymal or testicular sperm are reviewed as well as some reports on the use of ICSI in non-male infertility. The main steps in an ICSI cycle are well standardized by now; it is rare that ICSI cannot be carried out and the results in terms of fertilization, embryo transfer and clinical pregnancy rate have been consistent for many years, indicating that a substantial number of couples can now have their own genetic child instead of having to use artificial insemination with donor sperm. This review also emphasizes the importance of assessing the risk of ICSI for the children: there is a slight increase in *de novo* chromosomal abnormalities, the major congenital malformation rate is similar for IVF and ICSI (between 3 and 4%), and at ~2 years of age the developmental outcome as assessed by the Bayley scale is similar for IVF and ICSI. Recent publications mention that a few children are affected by diseases caused by imprinting disorders. Future studies are needed to assess the association between assisted reproductive technologies and imprinting disorders. ICSI is frequently used in couples undergoing preimplantation genetic diagnosis. PGD *stricto sensu* as well as PGD for aneuploidy screening and for Klinefelter patients are reviewed using the ESHRE PGD Consortium data.

Key words: ICSI/IVF/male factor infertility/preimplantation genetic diagnosis/risk assessment

Early considerations and developments preceding ICSI

The aim of this review is to describe the current practice and assessment of risks related to ICSI as well as other applications. This review is partly the result of discussions held at the Serono Symposia International Conference on ICSI, held at the Vrije Universiteit Brussel (VUB) April 12–14, 2002, 10 years after the first birth. The conference was supported by grants from Serono Symposia and the Fund for Scientific Research Flanders (FWO – Vlaanderen). Participants are listed in the Appendix.

Since the birth of Louise Brown in July 1978, IVF has proven to be an efficient treatment to alleviate female factor infertility, especially tubal infertility (Edwards *et al*., 1980). In subsequent years IVF was also successfully applied in couples with unexplained infertility, with male infertility as well as endometriosis (Mahadevan *et al*., 1983). Semen characteristics from the male partner have been scored according to World Health Organization (1999) criteria.

It became apparent for many groups including our own that the results of conventional IVF were much less efficient when the semen characteristics of the male partner were well below the reference values concerning concentration, morphology and motility. The percentage of oocytes which fertilized normally were significantly lower, resulting in the formation of many fewer embryos; this meant that embryos were not available for transfer in a substantial number of cycles (Tournaye *et al*., 1992).

A number of couples with <500 000 progressive motile sperm available for insemination could not be included in an IVF programme. This also includes patients with azoospermia. Therefore at the end of the 1980s several procedures of assisted fertilization were developed and applied in couples where conventional IVF could not be used. In a first technique—partial zona dissection, PZD—a small opening was made in the zona pellucida allowing the sperm direct access to the oolemma. Overall PZD results were generally inconsistent and rather disappointing. The next technique to be introduced was subzonal insemination (SUZI). A few motile sperm were microinjected through the perivitelline space. After SUZI, ~20% of the microinjected fertilized oocytes became normally fertilized (Ng *et al*., 1991; Fishel *et al*., 1993). The overall experience with PZD and SUZI was that the percentage normal fertilization was too low. As a consequence, only one or two embryos became available for transfer in approximately two-thirds of the patients. Percentages in pregnancies and deliveries were too low to consider PZD and SUZI for routine clinical application. In July 1992, our group published the first pregnancies and deliveries after replacement of embryos generated after ICSI, a novel assisted fertilization procedure (Palermo *et al*., 1992). In ICSI, a single spermatozoon

is microinjected into the oocyte after passage through the zona pellucida and the membrane of the oocyte (oolemma). Prior to this first application in the human, live young were born after ICSI in rabbits and cattle. Two other IVF groups, one in the USA and one in Singapore, had applied ICSI on 134 oocytes. Four two-pronuclear (2PN) oocytes were replaced in two patients and 11 cleaving embryos in seven patients. However, no pregnancy was established (Lanzendorf *et al*., 1988). The initial observations with ICSI demonstrated that fertilization was significantly better after ICSI than SUZI and more embryos suitable for embryo replacement were obtained (Van Steirteghem *et al*., 1993a).

Since the second half of 1992, only ICSI has been applied in our centre when assisted fertilization was indicated (Van Steirteghem *et al*., 1993b).

Current practice of ICSI

Indications and procedure

Most couples with severe male factor infertility can be treated with ICSI. In order to generate normally fertilized oocytes after ICSI a spermatozoon containing a functional genome and centriole is required. ICSI can also be applied with sperm from the epididymis and testis in case of obstruction of the seminal excretory ducts. ICSI can be applied in the case of azoospermia caused by impaired spermatogenesis if sufficient sperm can be retrieved from testicular tissue (Craft *et al*., 1993; Schoysman *et al*., 1993; Devroey *et al*., 1994, 1995, 1996; Tournaye *et al*., 1994).

ICSI using ejaculated sperm can be applied in the presence of oligoasthenoteratozoospermia, in cases of repeated fertilization failure after conventional IVF, in the presence of a high concentration of antisperm antibodies, in cancer patients in remission where sperm were cryopreserved prior to chemo- and radiotherapy, in patients with spinal cord injury, in patients with ejaculatory disturbances, in patients with retrograde ejaculation and in patients where semen was banked prior to vasectomy (Lahteen Maki *et al*., 1995; Nagy *et al*., 1995; Chung *et al*., 1998; Benadiva *et al*., 1999; Nikolettos *et al*., 1999; Schatte *et al*., 2000). When preimplantation genetic diagnosis (PGD) is applied for monogenetic diseases and PCR is used, ICSI is also indicated (Liebaers *et al*., 1998).

ICSI can be applied with sperm from the epididymis in cases of obstructive azoospermia: congenital bilateral absence of the vas deferens (CBAVD), Young syndrome, failed vaso-epididymostomy, failed vasovasostomy, and a bilateral (iatrogenic) inguinal obstruction of both ejaculatory ducts (Tournaye *et al*., 1994).

ICSI can also be applied with testicular sperm in all cases where epididymal sperm can be used, in the presence of excessive scar tissue preventing retrieval of sperm from the epididymis, in cases of testicular failure due to maturation arrest, partial germ cell aplasia or tubular sclerosis as well as in the exceptional case of necrozoospermia (Silber *et al*., 1995, 1996; Tournaye *et al*., 1996).

Epididymal sperm can be obtained by means of microsurgical epididymal sperm aspiration (MESA). In MESA, thousands of sperm are retrieved in patients with obstructive azoospermia. Excess sperm can be cryopreserved for eventual later use (Devroey *et al*., 1995a). Epididymal sperm can also be obtained by percutaneous sperm aspiration (PESA) (Tsirigotis *et al*., 1996). In the case of epididymal fibrosis, testicular sperm can be isolated from shredded pieces of testicular tissue obtained by open biopsy (TESE: testicular sperm extraction) (Devroey *et al*., 1994). In patients with obstructive azoospermia and normal spermatogenesis, a testicular biopsy can also be obtained by percutaneous aspiration of sperm by means of fine needle aspiration (FNA) (Bourne *et al*., 1995; Tournaye *et al*., 1998).

In approximately half of patients with non-obstructive azoospermia, sperm can also be found; it may take the embryologist and medical technologist hours of careful searching in order to find vital sperm in several testicular biopsies (Devroey *et al*., 1995b; Verheyen *et al*., 1995, 1997; Crabbé *et al*., 1997; Schlegel *et al*., 1997, 1999; Tournaye *et al*., 1997; Amer *et al*., 1999, 2002).

In all cases where fresh sperm can be used, it is also possible to use frozen–thawed sperm: ejaculated, epididymal and testicular. This may especially avoid repeated surgery, which is not without potential risks (Devroey *et al*., 1995a; Tournaye *et al*., 1999; Chan and Schlegel, 2000; Gil-Salom *et al*., 2000; Habermann *et al*., 2000; Kupker *et al*., 2000).

Before the first clinical application the ICSI procedure was evaluated and approved by the Ethics Committee of the Medical Campus of the Dutch-speaking Brussels Free University. Before starting treatment the couples were informed about the novel aspects of the treatment, the available data on ICSI treatment and the so far unknown possible later risks. Patients were asked to have prenatal diagnosis and to participate in a prospective follow-up study of the children born (Bonduelle *et al*., 1994).

Reviewing several thousands of ICSI procedures, ICSI could not be carried out in 1–3% of oocyte retrievals because of absence of mature oocytes and/or sperm; the latter was especially the case in couples with non-obstructive azoospermia (Liu *et al*., 1995; Vandervorst *et al*., 1997). In the authors' experience ~85% of ICSI cycles were carried out with fresh and frozen–thawed ejaculated sperm and in 15% with fresh and frozen–thawed epididymal testicular sperm (Bonduelle *et al*., 1999).

Ovarian stimulation and oocyte retrieval were similar as for conventional IVF. In the majority of cases the combination of GnRH agonist/antagonist and urinary or recombinant gonadotrophins was used. Ovulation induction was done by urinary hCG.

Approximately 12 cumulus–oocyte complexes (COC) were retrieved. Cumulus oophorus and corona radiata cells were removed by mechanical and enzymatic procedures. Microscopic evaluation revealed on average that 95% of COC contained oocytes with an intact zona pellucida; 81.5% of COC had metaphase II oocytes with one polar body, 9.8% of the COC contained germinal vesicle stage oocytes and 3.7% metaphase I oocytes. ICSI was only carried out on metaphase II oocytes (Bonduelle *et al*., 1999).

For the ICSI procedure, the oocyte is immobilized using a holding pipette; an injection pipette with an internal diameter of 6 μm is used to aspirate a single spermatozoon. These micropipettes are commercially available and can also be made in the laboratory. Before aspiration the sperm is immobilized in polyvinylpyrrolidone. A morphologically normal sperm is aspirated into the injection needle, tail first. Immobilization of the sperm can also be achieved by crushing the tail with the injection pipette. The injection pipette is passed through the zona pellucida and the membrane of the oocyte into the cytoplasm in a position sufficiently distant from the first polar body.

Table I. IVF and ICSI results in Europe in 1998[a]

	IVF	ICSI
Cycles started	51 471	34 576
Aspirations	46 474	33 133
Aspirations/cycle started (%)	90.3	95.8
Embryo transfers	40 980	30 460
Embryos transferred/aspiration (%)	88.2	91.9
Pregnancies	11 384	8419
Pregnancies/embryos transferred (%)	27.8	27.6
Deliveries	8950	6510
Deliveries/cycle started (%)	17.4	18.8

[a]For those 12 countries for which sufficient data were available: Czech Republic, Denmark, Finland, Greece, Hungary, Italy, Norway, Portugal, Russia, Sweden, Switzerland and the UK (ESHRE EIM consortium). Values calculated from Nygren and Nyboe Andersen (2001).

After ICSI in ejaculated sperm, more than two-thirds of the injected oocytes became normally fertilized. The fertilization rate with surgically retrieved sperm in non-obstructive azoospermia was less than with ejaculated sperm but still >50% (Joris *et al*., 1998; Van Steirteghem *et al*., 1998).

Further development of the normally fertilized oocytes after ICSI was evaluated in a similar fashion as for IVF. More than 80% of normally fertilized oocytes developed further to embryos of sufficient morphological quality to be replaced. For all types of sperm the percentage of embryos replaced or frozen was between 60 and 65% of the normally fertilized oocytes. In the case of non-obstructive azoospermia, normal fertilization is compromised (Vernaeve *et al*., 2003). Also in non-obstructive azoospermia, testicular tissue can be damaged after repeated surgery (Schlegel and Su, 1997).

Absence of fertilization occurred after ICSI when only a few oocytes were available, only totally immotile sperm were present, all sperm had no acrosome, all oocytes had abnormal morphology and were damaged by the injection itself. Fertilization did mostly occur in a subsequent cycle (Liu *et al*., 1995; Nagy *et al*., 1995; Staessen *et al*., 1995; Vandervorst *et al*., 1997).

According to the published reports from IVF/ICSI registries, in a substantial proportion of assisted reproductive technology cycles, i.e. 40% in Europe, ICSI was applied as the procedure of fertilization. It became apparent that worldwide a number of groups have abandoned conventional IVF and use ICSI as a standard procedure even when sperm parameters are normal (Bhattacharya *et al*., 2001; Oehninger, 2001; Nygren and Anderson, 2002). As indicated in Table I, extracted from the European registry on IVF and ICSI, there are more oocyte retrievals per started cycle in the ICSI group, more embryo transfers per oocyte retrieval, a similar number of pregnancies per embryo transfer, and more deliveries per started cycle. The fact that most patients reach oocyte retrieval in the ICSI group may reflect the unimpaired fertility of the female partner (Table I).

There are fewer unexpected fertilization failures in the ICSI group, but if embryos are obtained, ICSI embryos generated a similar percentage of pregnancies as IVF embryos did (Staessen *et al*., 1999).

A meta-analysis of sibling oocytes studied in patients with moderate oligoasthenoteratozoospermia (OAT) revealed that the odds of fertilization after ICSI are 3.9-fold greater than IVF. The number needed to treat (NNT) in order to prevent one complete fertilization failure after IVF could be three, indicating that three ICSI procedures would have to be performed instead of conventional IVF in couples with moderate OAT, to prevent one complete fertilization failure (Tournaye *et al*., 2000).

A large randomized controlled trial (RCT) from the UK of 435 treatment cycles in 415 couples with non-male factor subfertility (IVF = 224; ICSI = 211) showed that the implantation rate was higher in the IVF group than in the ICSI group [95/318 (30%) versus 72/325 (22%); relative risk (RR) 1.35 (95% confidence interval 1.04–7.6)]. The pregnancy rate per cycle started was also higher after IVF [72 (33%) versus 53 (26%); RR 1.17 (0.97–1.35)]. They concluded that ICSI offers no advantage over IVF in terms of clinical outcome in cases of non-male factor subfertility. They support the current practice that ICSI should be reserved only for severe male factor problems (Bhattacharya *et al*., 2001; Van Steirteghem and Collins, 2003).

It has been suggested that ICSI should be the treatment of choice in all assisted reproduction cycles. If this would be introduced without further studies, such policy could have a serious impact on laboratory time, on medical resources, and above all perhaps on overall safety because of bypassing the natural selection mechanisms of the gametes and because of the invasiveness of the technique itself (Oehninger, 2001).

Medical risks

In cases of male infertility due to severe OAT or non-obstructive azoospermia, a peripheral karyotype and a search for AZF deletion on Yq11 should be performed. In men with CBAVD, mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene should be looked for (Silber *et al*., 1998; Tuerlings *et al*., 1998; Van Landuyt *et al*., 2000).

It is well known that constitutional chromosomal aberrations increase as sperm counts decrease. It is also clear that the incidence of numerical sex chromosome aberrations such as 47,XXY and 47,XYY are elevated in males with azoospermia. Apparently structural chromosomal aberrations of autosomes such as Robertsonian and reciprocal translocations are not infrequent in oligozoospermic males (Van Assche *et al*., 1996; Yoshida *et al*., 1997).

In some males with non-obstructive azoospermia the histology reveals a cessation of spermatogenesis, resulting in the failure of germ cell development to progress to the formation of 'testicular' sperm. Attempts to mature germ cells in culture have been extremely disappointing and the inability to define conditions to allow *in vitro* completion of meiosis and spermatogenesis remains a substantial block to expand an understanding of spermatogenesis. There is considerable scope to improve the scientific approach to the issue of using immature germ cells for ICSI. Moreover, there is an increasing number of mutations being recognized as a cause of disordered spermatogenesis, i.e. Y-chromosome deletions. Male children conceived after ICSI will carry the same Y-chromosome deletion as their father (Kent-First *et al*., 1996; Silber and Repping, 2002). Until a better definition of the cause of disordered spermatogenesis is obtained, there will also be a substantial risk of transmitting genetic defects that may underlie the pathology (Katz *et al*., 2002). Sperm from men with non-obstructive azoospermia do have a higher incidence of chromosomal aneuploidy, of which sex chromosome aneuploidy is the most

likely (Mateizel *et al*., 2002; Palermo, 2002). Non-human primate models are of paramount importance to a greater understanding of the gametes' interaction (Hewitson and Schatten, 2002).

The major risk after ICSI is the occurrence of multiple pregnancies, since 50% of all assisted reproductive technology children are not from singleton pregnancies. Elective single embryo transfer associated with a good cryopreservation programme could avoid this risk (Gerris and Van Royen, 2000; Tiitinen *et al*., 2001).

Prenatal data

Prenatal diagnosis was performed between 1991 and 2000 in 47% of 2622 consecutive established ICSI pregnancies; prenatal diagnosis was done in 49% of singleton pregnancies and 43.5% of multiple pregnancies (Bonduelle *et al*., 2002a). The main reason for having a prenatal diagnosis was the possible higher risk related to ICSI; for 37% (n = 588) there was also a maternal age-related risk (⩾35 years of age). In all, 1586 fetuses obtained after fresh embryo transfer were tested by chorionic villus sampling (CVS) (n = 698) or by amniocentesis (n = 888). Abnormal fetal karyotypes were found in 47 samples (2.96%); 25 anomalies (1.58%) were *de novo*. There were 10 sex chromosomal anomalies and 15 autosomal anomalies, either numerical (n = 8) or structural (n = 7), and 22 inherited abnormalities (1.39%) (21 balanced and 1 unbalanced). In 17/22 cases the chromosomal structural defect was inherited from the father. A significantly higher percentage of 2.1% *de novo* prenatal chromosomal anomalies was observed for sperm concentrations of $<20\times10^6$ sperm cells per ml, as compared to 0.24% if sperm concentration was $\geqslant20\times10^6$ sperm cells per ml (Fisher's exact test P = 0.006). For lower cut-off values of sperm concentration ($<1\times10^6$, $<5\times10^6$, $<10\times10^6$, $<15\times10^6$), no statistical difference in frequency of chromosomal anomalies was observed. Statistical difference was also observed for motility criteria, but not for morphology. One chromosomal anomaly was found prenatally after use of epididymal or testicular sperm in a total of 94 samples (Aytoz *et al*., 1998; Bonduelle *et al*., 2002a).

Postnatal data

In the two cohorts of 2889 ICSI and 2995 IVF pregnancies, similar rates of multiple pregnancies were observed. ICSI and IVF maternal characteristics were comparable for medication taken during pregnancy, pregnancy duration and maternal educational level, whereas maternal age was higher in ICSI and a higher percentage of first pregnancies and first children born was observed in the ICSI mothers (Bonduelle *et al*., 2002b). Birthweight, number of neonatal complications, low birthweight, stillbirth rate and perinatal death rate were compared between the ICSI and the IVF groups and were similar for ICSI and IVF. Prematurity was slightly higher in the ICSI children (31.8%) than in the IVF children (29.3%) (P = 0.046). Very low birthweight was higher in the IVF pregnancies (5.6%) compared to ICSI pregnancies (4.4%) (P = 0.031). Major malformations (defined as those causing functional impairment or requiring surgical correction), were observed at birth in 3.4% of the ICSI live-born children and in 3.8% of the IVF children (P = 0.538, not significant). Malformation rate in ICSI was not related to sperm origin or sperm quality. The number of stillbirths (born ⩾20 weeks of pregnancy) was 1.69% in the ICSI group and 1.31% in the IVF group (P = 0.241, not significant). Total malformation rate taking into account major malformations in stillborns, in terminations and in live-borns was 4.2% in ICSI and 4.6% in IVF (P = 0.482, not significant).

Couples should be informed about the risks of an abnormal prenatal result, about the risk linked to a prenatal procedure as well as about the relatively benign character of some chromosomal anomalies such as the *de novo* structural anomalies or sex chromosomal anomalies in order to be able to make a choice for prenatal testing or not. Couples should be reassured that neonatal outcome of ICSI is comparable with regular IVF and that there is no increased risk for major malformation in ICSI children (Bonduelle *et al*., 2002b).

Developmental outcome

Since the introduction of ICSI in 1991, few studies have addressed developmental outcome. By performing a standard developmental test on children born in Belgium after ICSI as compared with children born after IVF, at the age of 2 years no difference in psychomotor development of ICSI children has been observed (Bonduelle *et al*., 2003). In a prospective study, the medical and developmental outcome of 439 children born after ICSI (378 singletons, 61 twins) were compared with those of 207 children born after IVF (138 singletons, 69 twins), at the age of 24–28 months. These children were part of a cohort of children followed since birth. Of children reaching the age of 24–28 months between May 1995 and March 2002, 44.3% (2375/5356) were examined by a paediatrician who was unaware of the type of treatment used for each couple. Of all the children born, 12.2% (439/3618) in the ICSI group and 11.9% (207/1738) in the IVF group underwent a formal developmental assessment using the Bayley Scale of Infant Development (mental scale) by a paediatrician blinded to the type of treatment. There was no significant difference in maternal educational level, maternal age, gestational age, parity, birthweight, neonatal complication rate or malformation rate at 2 years between ICSI and IVF singletons, or between ICSI and IVF twins. No significant difference was observed in the developmental outcome using the Bayley Scale at the age of 24–28 months between ICSI children or IVF children. A multivariate regression analysis for the singleton children indicated that parity, sex (boys had lower scores than girls) and age had a significant influence on the test result, but that the fertility procedure (ICSI versus IVF) did not influence the test result. ICSI children from fathers with low sperm concentration, low sperm motility or poor morphology had a similar developmental outcome to that of children from fathers with normal sperm parameters. There were no significant differences between the initial cohort and the group lost to follow-up, nor between the psychologically tested and the non-tested group for a number of variables such as maternal educational level, birthweight in singletons and neonatal malformation rate. Although only some of the cohort of ICSI children were evaluated, a representative sample of both ICSI an IVF children was compared (Bonduelle *et al*., 2003).

Specific issues in ICSI

Imprinting

It has been shown in animal studies that the ICSI process is vulnerable to external factors. Particularly the use of spermatids

Table II. The preimplantation genetic diagnosis procedure at Vrije Universiteit Brussel

Centre for Reproductive Medicine	Centre for Medical Genetics
IVF–ICSI	Diagnosis
	Accurate single-cell Δ
	Counselling:
	Geneticists
	Nurse counsellor
Embryo biopsy	Genetic diagnosis
	FISH
	PCR
Embryo transfer of unaffected embryo(s)	

instead of mature sperm or the use of testicular sperm has been considered as a higher risk for imprinting disorders (Simerly *et al*., 1995); but also the use of ICSI or IVF itself could increase the risk for imprinting disorders considering that the mammalian embryo, cultured *in vitro*, is susceptible to changes in imprinting control (Young *et al*., 2001). Two children with Angelman syndrome (a neurogenetic disorder) due to a sporadic imprinting defect have been reported so far after use of the ICSI procedure (Cox *et al*., 2002). The lack of an imprinting centre mutation and the detection of a mosaic methylation pattern in one of the two patients make an inherited defect unlikely and point to a postzygotic epigenetic defect (De Rycke *et al*., 2002). The authors speculated that ICSI disrupts the production or function of transacting factors necessary for imprinting of the maternal chromosome 15. On the other hand, Manning *et al*. (2000) did not find an abnormal methylation pattern on chromosome 15 in 92 cases of ICSI children, but such a small sample size does not exclude a small but increased risk for imprinting disorders. In a small case control series of assisted reproductive technology cryopreserved offspring, a single case of Beckwith–Wiedemann syndrome (a human overgrowth syndrome) (BWS) was reported among 91 cases (Sutcliffe *et al*., 1995). One case of BWS was reported in a study of Olivennes *et al*. (2001) on assisted reproductive technology children, where no further genetic origin (*de novo* or inherited) could be determined. A recent article of DeBaun *et al*. (2003) reports evidence that assisted reproductive technology is associated with BWS, observing a higher frequency of history of assisted reproductive technology in a BWS registry than in the general population. In all, seven children with BWS were found to be born after assisted reproductive technology, of whom four were born after ICSI with ejaculated sperm and one after ICSI with testicular sperm. Overestimation of the number of cases related to case identification series has been taken into account. The prevalence of the BWS is considered to be 1/15 000. Molecular studies indicated that five of the six children studied had specific epigenetic alterations associated with BWS (5/6 had imprinting mutations of the LIT1 and/or H19 genes, two distinct methylation alterations specific for BWS). Based on these findings and the observation that Angelman syndrome occurred after ICSI, there is sufficient evidence that some aspects of the ICSI procedure could increase the frequency of epigenetic anomalies leading to congenital malformation syndromes. As all imprinting disorders are rare disorders (BWS affects ~1/15 000 newborns, Angelman affects ~1/30 000, Prader–Willi affects 1/15 000) a large sample size is needed to detect minor increases. Given the known association between BWS, cancer and an increased frequency of H19 methylation abnormalities, we can postulate that assisted reproductive technology could be associated with embryonal cancers of childhood where methylation abnormalities have been implicated. Since the incidence of cancer in BWS in children aged <4 years is 0.02 per 110 patient-years (DeBaun *et al*., 1998), one would expect to observe these problems from the moment the patient groups are sufficiently large and when follow-up is also beyond the neonatal period (~1/1000 at the age of 5 years). So far ~50 imprinted genes have been identified, many have not been cloned or still have unknown function. In our study of 2840 live-born ICSI children, no diseases relating to imprinting disorders have been observed, except for one BWS with omphalocoele in the ICSI group and one case of omphalocoele in the IVF group, where the diagnosis of BWS is not proven; no further cases were observed in our study on psychomotor development of 2 year old ICSI children (n = 439) (Bonduelle *et al*., 2002b, 2003). However, for some imprinting disorders such as Angelman syndrome the diagnosis may not yet have been made before the age of 2 months or even before 2 years. One more case of BWS in ICSI children was encountered at 9 months of age (with a small umbilical hernia and macroglossia) in our further data collection after closure of the study on 2840 live-born ICSI children. In another follow-up study of 300 ICSI children compared to spontaneously conceived children at the age of 5 years, performed by the Study Group of Brussels, Gothenburg and New York (2002), no imprinting disorders have been reported.

From all these data, we can conclude that there is evidence for an increased risk of imprinting disorders in ICSI children and that childhood cancers could be associated, but only a few observations in the literature provide data on possible frequencies of these events. Only a systematic survey aimed at those syndromes/diseases which have a known, defined phenotype linked to imprinted genes may clarify whether epigenetic anomalies play a role in ICSI (and IVF) more often than in the general population. In both reports on the Angelman syndrome and on the BWS, it is the maternal allele that is affected (unmethylated). This makes it unlikely that the problem is related to sperm differentiation and more likely that ICSI or some other aspects of the assisted reproductive technology used disturbs methylation in the maternal genome or early embryo. Further biological studies are required to understand the pathogenesis of these events and to find out whether precautions can be taken to prevent their occurrence.

Preimplantation genetic diagnosis

PGD procedure

The PGD procedure normally consists of the steps shown in Table II. Potential parents referred for PGD are first seen at a Centre for Medical Genetics. PGD can be offered for carriers of specific genetic defects, sex-linked disorders or chromosomal rearrangements. The genetic diagnosis must be confirmed and the possibility of carrying out a reliable diagnosis on a single cell must be available. If, after careful genetic counselling on the advantages and disadvantages of PGD versus prenatal diagnosis, the couple opt for PGD, the couple is referred to the Fertility Clinic for further evaluation. This evaluation consists of the reproductive history of the couple; a gynaecological examination of the wife; blood tests of the female partner including at least oestradiol, LH and FSH.

Sometimes screening for infectious diseases completes this evaluation. In some clinics, karyotyping of both partners is standard even where fluorescence *in situ* hybridization (FISH) is not used for the PGD. For the male partner at least a sperm analysis is necessary. More tests are performed where indicated. Finally, in many centres a specific informed consent is signed. In almost all centres belonging to the ESHRE PGD Consortium, embryo biopsy for PGD takes place on the third day of early embryonic development (ESHRE Preimplantation Genetic Diagnosis Consortium, 1999). To obtain the embryos at this early developmental stage, IVF with or without ICSI is necessary. ICSI is preferred over conventional IVF in monogenic disorders requiring PCR-based DNA diagnosis to prevent contamination from sperm still attached to the zona pellucida. After hormonal stimulation, on average ~10 oocytes are obtained, of which six or seven become fertilized after the assisted conception procedure. The sperm is produced by masturbation immediately preceding IVF or ICSI. On the third day post insemination, one or two blastomeres are biopsied from embryos, which have reached about the 8-cell stage. Either the cells are spread onto a slide and analysed by FISH or the DNA is extracted and amplified using PCR methodology. Although the latter is technically more difficult, the detection method at the single cell level is principally that applied to other tissues for prenatal diagnosis, although working with blastomeres has specific difficulties including lysis, multinucleation and allele drop-out, where one of the two alleles under study is preferentially amplified. Other diagnostic problems can result from embryonic mosaicism. DNA contamination from sperm cells attached to the zona pellucida after IVF can be overcome by ICSI. Some centres consider it advisable to diagnose two cells. Only those embryos with a concordant result for both cells would be considered for transfer to the uterus or for cryopreservation for transfer at a later date. Generally no more than two unaffected embryos are transferred to the uterus, thus reducing the risks of prematurity with triplets, and limiting the inevitable risk of misdiagnosis. Serum hCG is determined at day 12. If a pregnancy is established, an ultrasound examination at 7 weeks is performed to confirm the presence of a fetal heart beat. Couples are advised strongly to undergo prenatal diagnosis by CVS or amniocentesis because of the novelty of the PGD procedure and the risk of misdiagnosis (Geraedts *et al*., 2001).

From the most recent referral data collected by the ESHRE PGD Consortium (2002) it is clear that the majority of patients has had one or more pregnancies already although the vast majority has no healthy children. About a quarter of the couples has one or more affected children while even a larger percentage of the couples have experienced spontaneous abortions or terminations of pregnancy after prenatal diagnosis.

Genetic risk and objection to termination of pregnancy still is the most important reason for PGD. About a quarter of the couples need artificial reproductive treatment and want to combine this with PGD.

Other reasons for PGD were recurrent abortion and objection to presymptomatic testing in case of Huntington's disease. Two couples wanted an HLA-identical donor for a child suffering from Fanconi anaemia.

The genuine medical indications can be divided into two broad groups: chromosomal and monogenic. Mitochondrial disease is the indication in <0.5%.

The referrals for chromosomal disorders can be divided into two groups as well: structural and numerical abnormalities. Reciprocal translocation remains the most important abnormality among the first group. All these rearrangements are private, i.e. they have a sporadic nature, except the reciprocal translocation (11;22) which is known to be recurrent. There are four times as many reciprocal than Robertsonian translocations. Inversions and deletions are reasons for referral in <1% of the cases each. Aneuploidy screening is done for different reasons: maternal age, repeated IVF failure (i.e. implantation failure), recurrent spontaneous abortion or combinations of these.

The relative proportions of the monogenic disorders have changed slightly in comparison to previous years. There is a relative increase of dominant disorders and a decrease of referrals for X-linked disease. The top three in each disease group remains constant over the years: cystic fibrosis, thalassaemia and spinal muscular atrophy as autosomal recessive diseases; myotonic dystrophy, Huntington's disease and Charcot–Marie–Tooth disease as autosomal dominant; and Duchenne's muscular dystrophy, Fragile-X syndrome and haemophilia as X-linked disorders.

The centres' decisions show, if the unknowns are not taken into account, that >80% of the cases were technically possible, that ~90% of the patients can be accepted for IVF or ICSI and that only a few cases are rejected on ethical grounds. The two most important reasons for not accepting women for IVF were high FSH and increased maternal age. In one centre, women with myotonic dystrophy were not accepted for IVF because of the risks involved in the infertility treatment.

In this Consortium report, only 13 referrals were not accepted on ethical grounds. The reasons were the cases looking for an HLA-identical donor mentioned above, low risk of affected progeny, AZFc deletion and achondroplasia. In one case a single woman was not accepted. Finally the request of an Islamic couple, of which the husband did not want daughters, was dishonoured.

Examination of the cumulative data shows that a total of 1197 cycles went on beyond oocyte retrieval, with a clinical pregnancy rate of 17% per oocyte retrieval and 22% per embryo transfer procedure. The biopsy was successful in 97% of cases and the diagnosis obtained in 86% of successfully biopsied blastomeres.

For the Robertsonian translocations, a total of 51 cycles reached the oocyte retrieval stage, and the average female age was 35 years. A high number of patients were also infertile. ICSI was undertaken in most cases. All embryo biopsies were performed at the cleavage stage, using blastomere aspiration. From 768 oocytes, 414 fertilized (54%), 314 were suitable for biopsy and 312 were successfully biopsied (100%). The diagnosis was successful in 252 cases (81% of embryos successfully biopsied) and 85 embryos were diagnosed as transferable (11% of oocytes collected). Thirty-eight embryo transfer procedures were conducted (74% of oocyte retrievals), and 11 clinical pregnancies resulted (22% of oocyte retrievals and 29% of embryo transfers).

For the reciprocal translocations, a total of 96 cycles reached the oocyte retrieval stage, and the average female age was 34 years. In this case only 25 patients were infertile, which was much lower than in the case of Robertsonian translocations. All embryo biopsies were performed at the cleavage stage, using mostly blastomere aspiration. The cumulative data show that 368 cycles have now been performed, with 689 embryos being suitable for transfer (13% of the oocytes collected), 290 embryo transfer

procedures and 62 clinical pregnancies (17% per oocyte retrieval and 21% per embryo transfer).

Preimplantation genetic diagnosis by aneuploidy screening (PGD-AS)

Two new techniques have been introduced into the assisted reproductive technology laboratory: (i) the use of sequential media makes possible the growth of human blastocysts, and (ii) using PGD for aneuploidy screening (PGD-AS) it has become possible to analyse the ploidy status of several chromosomes of *in vitro*-generated embryos before transfer. It is well known that aneuploidy increases with maternal age and is correlated with reduced implantation and a higher rate of abortion. Several assisted reproductive technology centres have already introduced PGD-AS with the aim of increasing success rates, although no controlled randomized prospective studies have been reported (Munné *et al*., 1999).

Preimplantation screening for chromosome aneuploidy is carried out at many centres throughout the world in order to enhance IVF success. It is the most common reason for embryo diagnosis (Verlinsky *et al*., 2001). There is disagreement on whether to distinguish PGD for inherited genetic diseases and PGD for the detection of sporadic chromosomal abnormality, to enhance assisted reproductive technology success. This is the reason why the latter procedure has been designated as PGD-AS by the ESHRE Consortium on PGD or as PGS (preimplantation genetic screening) by the Human Fertilisation and Embryology Authority, while it has been included in the definition of PGD in the USA (American Society for Reproductive Medicine, 2001). Candidate couples for PGD-AS are infertile and undergoing IVF/ICSI to overcome their infertility—the success of which is heavily influenced by maternal age (>36 years) and the previous reproductive history of the couple (e.g. repeated IVF failure) (Templeton, 2000). Older patients at risk for Down's syndrome or other age-related chromosomal abnormalities might opt for PGD instead of prenatal diagnosis and possible abortion.

As far as the final assessment of PGD-AS is concerned, abundant doubt still remains. Although several indications have been put forward, there is a lack of scientific evidence proven by randomized controlled trials. Besides the indication of maternal age, others have been proposed such as in the cases of recurrent miscarriage, bad implanters and non-obstructive azoospermia. For none of the above-mentioned indications has any randomized controlled trial been published so far. One can speculate that if more than two embryos are available for transfer, PGD-AS will be able to increase the pregnancy rate and avoid the occurrence of high rank multiple pregnancies (Gianaroli *et al*., 2001; Rubio *et al*., 2003; Silber *et al*., 2003).

PGD for Klinefelter patients

Even azoospermic patients with focal spermatogenesis in the testis may benefit from the ICSI technique in order to father a child. As the use of ICSI has become more widespread, centres have already started with infertility treatment of Klinefelter patients. At the time of writing, the births of 26 healthy children have been reported, while in one case the conception of a 47,XXY fetus has been reported. In view of the possible risk of an increased number of gonosomes in the sperm of Klinefelter patients, a safer approach offering these couples ICSI in combination with PGD has been taken by a limited number of centres, resulting in the birth of three healthy children (Staessen *et al*., 2003).

Conclusion

ICSI is the method of choice in cases of oligoasthenoteratozoospermia, obstructive and non-obstructive azoospermia. In the case of OAT, there is a need for performing a parental karyotype. In the case of obstructive azoospermia due to bilateral absence of the vas deferens, genetic screening for cystic fibrosis is indicated. ICSI should not be performed in the presence of normal sperm parameters.

There is a significant increase in *de novo* sex and autosomal chromosome aberrations after ICSI.

The major malformation rate of children born after ICSI and IVF is similar.

There is evidence for an increased risk of imprinting disorders in ICSI children.

To avoid contamination in case of preimplantation genetic diagnosis, ICSI is indicated.

Further clinical research is needed to evaluate the correct indication for PGD-AS in advanced female age, in recurrent abortion, if testicular sperm of patients with non-obstructive azoospermia is used and in patients with repeated implantation failure.

Further research is needed on the follow-up of the medical and psychological development and fertility potential of children born after ICSI.

Since ICSI has been introduced in 1992, the need for donor sperm has been drastically reduced. More than 95% of males can father their own genetic child.

In order to judge the congenital malformation rate after IVF/ICSI, follow-up studies are needed in infertile couples obtaining spontaneous pregnancies, pregnancies after reduction of ovulation and after mild ovarian stimulation.

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Appendix

Serono Symposia International Conference on ICSI, held at the Vrije Universiteit Brussel (VUB), April 12–14, 2002

Participants: Patricia Baetens (Belgium), Maryse Bonduelle (Belgium), Michel Camus (Belgium), Jacques Cohen (USA), John Collins (Canada), David M.de Kretser (Australia), Martine De Rycke (Belgium), Paul Devroey (Belgium), Klaus Diedrich (Germany), José Egozcue (Spain), Yvon Englert (Belgium), José A.Enriquez (Spain), Johannes L.H.Evers (The Netherlands), Bart C.J.M.Fauser (The Netherlands), Joep Geraedts (The Netherlands), Lars Hamberger (Sweden), Pierre Jouannet (France), Ingeborg Liebaers (Belgium), Willy Lissens (Belgium), David Page (USA), Gianpiero Palermo (USA), Guido Pennings (Belgium), Budhan Pukazhenti (USA), Raphael Ron-El (Israel), Gerald Schatten (USA), Rik Schots (Belgium), Paul Schotsman (Belgium), Sherman Silber (USA), Johan Smitz (Belgium), Basil C.Tarlatzis (Greece), Herman Tournaye (Belgium), André Van Steirteghem (Belgium), Hilde Van de Velde (Belgium) and Greta Verheyen (Belgium).