Human Reproduction, Vol. 14, (Suppl. 1), pp. 243–264, 1999

Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children

Maryse Bonduelle[1], Michel Camus[2], Anick De Vos[2], Catherine Staessen[2], Herman Tournaye[2], Elvire Van Assche[1], Greta Verheyen[2], Paul Devroey[2], Inge Liebaers[1] and André Van Steirteghem[2,3]

[1]Centre for Medical Genetics and [2]Centre for Reproductive Medicine, University Hospital and Medical School, Dutch-speaking Brussels Free University (Vrije Universiteit Brussel), Belgium

[3]To whom correspondence should be addressed at: Centre for Reproductive Medicine, Academisch Ziekenhuis VUB, Laarbeeklaan 101, B-1090 Brussels, Belgium

Intracytoplasmic sperm injection (ICSI) with ejaculated, epididymal or testicular spermatozoa was first successful in 1992 and has since become the widely accepted treatment for couples with severe male-factor infertility. The outcome of several thousands of ICSI cycles in terms of fertilization, embryo cleavage and implantation is similar to that for conventional in-vitro fertilization in couples with tubal or idiopathic infertility. To evaluate the important issue of safety of the new technique of ICSI, a prospective follow-up study of 1987 children born after ICSI was carried out. The aim was to compile data on karyotypes, congenital malformations, growth parameters and developmental milestones. Parents' agreement to genetic counselling was obtained as well as prenatal diagnosis, followed by a physical examination of the children at 2 months, 1 year and 2 years. Between April 1991 and August 1997, 1699, 91 and 118 children were born after ICSI with ejaculated, epididymal and testicular spermatozoa respectively; 79 children were born from cryopreserved ICSI embryos. In all, 1082 karyotypes were determined by prenatal diagnosis, 18 of which were abnormal and *de novo* (1.66%) (nine each of autosomal and sex chromosomal aberrations), and 10 karyotypes (0.92%) were inherited structural aberrations. Of these, nine (eight balanced structural aberrations and one unbalanced trisomy 21) were transmitted from the father. Ten pregnancies were terminated after prenatal karyotyping or DNA testing. Forty-six major malformations (2.3%) were observed at birth. Seven malformations, observed by prenatal ultrasound, were terminated. Twenty-one (1.1 %) stillbirths, including four with major malformations, occurred later than 20 weeks of pregnancy. Mean gestational age at birth was 38.7 weeks for singletons, 36.0 weeks for twins and 32.0 weeks for triplets. No specifically higher incidence of malformations was found in any given subgroup.

Key words: congenital malformations/fetal karyotype/genetic counselling/ICSI/ male infertility

Introduction

Shortly after its introduction, it became clear that in-vitro fertilization (IVF) had limitations in the alleviation of long-standing infertility. Couples were not accepted for IVF if the semen parameters were too impaired and a substantial number of IVF cycles carried out for male infertility did not result in embryo transfer. In 1992, our group reported the first pregnancies and births to result from replacement of embryos generated by intracytoplasmic sperm injection (ICSI), which involved injection of a single spermatozoon through the zona pellucida directly into the oocyte (Palermo *et al.*, 1992). The experience with ICSI has been that the fertilization rate is considerably better than after other assisted-fertilization procedures and that more embryos with implantation potential have been produced. Since July 1992, ICSI has been the only procedure used in our Centre when some form of assisted fertilization is necessary (Van Steirteghem *et al.*, 1993a,b,c).

When ICSI was introduced, there was major concern about its safety. ICSI is indeed a more invasive procedure than routine IVF, since one spermatozoon is injected through the oocyte membrane and since fertilization can be obtained from spermatozoa which could never have been used previously in fertility treatment. Even more questions were raised and concern was again expressed when ICSI with non-ejaculated spermatozoa, either epididymal or testicular, was introduced. Emphasis was put on the fact that the risk of chromosomal aberration might be even higher in men with non-obstructive azoospermia (Van Assche *et al.*, 1996). On the other hand, it was suspected that imprinting might be less complete at the time of fertilization if testicular spermatozoa were used (Tesarik and Mendoza, 1996). If this were so, it would be unlikely to impair fertilization and early development, but anomalies might become manifest at birth or only later in life.

The safety of this novel procedure of assisted fertilization had therefore to be assessed carefully (Van Steirteghem *et al.*, 1993a,b). In previous publications, we (and other groups) failed to find any increased risk of major congenital malformations as compared with the general population (Bonduelle *et al.*, 1994, 1995, 1996a,b; Palermo *et al.*, 1996; Wennerholm *et al.*, 1996). The results of the ICSI procedure were also evaluated by looking at the first 130 children born after ICSI and comparing this group of children to a control group of 130 matched children born after IVF pregnancies in the same period of time and after the same ovarian stimulation and in-vitro culture conditions (Bonduelle *et al.*, 1995). We compared the data on karyotypes, congenital malformations, growth parameters and developmental milestones in the two groups of children and could find no statistically significant differences. We therefore concluded

from a limited number of children that when ICSI was carried out and compared with standard IVF procedure, no additional risk was observed.

In the present review, we report our experience of 7 years of ICSI practice in terms of indications, origin of spermatozoa used, oocyte damage and pronuclear status after ICSI, in-vitro embryo development of normally fertilized oocytes and the outcome of embryo transfers. We further report on the follow-up of a large cohort of 1987 children born after ICSI on the basis of data from genetic counselling, prenatal diagnosis, neonatal data, congenital malformations, growth parameters and developmental milestones.

Seven years of ICSI practice (1991–1997)

In the course of 7 years of ICSI practice (between January 1991 and December 1997), 7374 ICSI cycles involving 74 520 metaphase II oocytes were carried out and evaluated in our centre.

ICSI indications

Most couples with severe male-factor infertility can now be treated by ICSI, which requires only one spermatozoon with a functional genome and centrosome for the fertilization of each oocyte. ICSI can also be used with spermatozoa from the epididymis or testis when there is an obstruction in the excretory ducts. If a patient is azoospermic because of reduced germ cell production, ICSI can be used if enough spermatozoa can be retrieved in testicular tissue samples.

ICSI can be used with ejaculated spermatozoa in cases of oligo-, terato- or asthenozoospermia, in the presence of high titres of antisperm antibodies, in cases of repeated fertilization failure after conventional IVF, in cancer patients in remission using spermatozoa which were frozen prior to chemotherapy, and in ejaculatory disorders.

ICSI can be used with epididymal spermatozoa in cases of congenital bilateral absence of vas deferens (CBAVD), Young's syndrome (Le Lannou *et al.*, 1995), failed vaso-epididymo- or vasovasostomy, bilateral herniovaphy or obstruction of both ejaculatory ducts.

ICSI can be used with testicular spermatozoa in all indications for epididymal spermatozoa, in the presence of extensive scar tissue preventing aspiration of spermatozoa from the epididymis, in azoospermia caused by testicular failure and in necrozoospermia.

Artificial insemination with donor spermatozoa (AID) is currently used only when ICSI with spermatozoa from the ejaculate, the epididymis or the testis cannot be used or has failed and for couples who prefer AID to other procedures because of financial, psychological, ethical or genetic considerations or who consider the ICSI procedure too sophisticated.

ICSI results

A total of 7610 ICSI cycles was scheduled in couples with long-standing infertility. The ICSI procedure could not be carried out in 236 cycles (3.1%) because there were no cumulus–oocyte complexes (COC) or metaphase II oocytes (106 cycles), or because no spermatozoa were available for the microinjection procedure (130 cycles). The latter condition occurred especially in patients with non-obstructive azoospermia who were scheduled for ICSI with testicular spermatozoa.

The ICSI procedure was performed with spermatozoa from the ejaculate in 6397 cycles (86.8%), with freshly collected epididymal spermatozoa in 161 cycles (2.2%), with frozen–thawed epididymal spermatozoa in 130 cycles (1.8%) and with testicular spermatozoa in 686 cycles (9.3%).

After ovarian stimulation using gonadotrophin releasing hormone agonist (GnRHa)–human menopausal gonadotrophin (HMG)–human chorionic gonadotrophin (HCG) in most cycles, a total of 92 838 COC were retrieved in 7610 cycles, i.e. a mean of 12.2 COC per cycle. Cumulus and corona cells were removed by means of a combination of enzymatic and mechanical procedures. Microscopic observations revealed that 95.0% of the COC contained an oocyte with an intact zona pellucida, 81.5% contained metaphase II oocytes that had extruded the first polar body, 9.8% contained germinal-vesicle stage oocytes and 3.7% contained metaphase II oocytes that had undergone breakdown of the germinal vesicle but had not yet extruded the first polar body (metaphase I oocytes). The distribution of oocytes with different nuclear maturity was quite variable between patients. ICSI was carried out only on metaphase II oocytes.

Assessment of freshly ejaculated spermatozoa used in 6180 cycles revealed that all three semen parameters (sperm concentration, motility and morphology) were abnormal in 44.0% of the cycles, two semen values were abnormal in 30.4% of the cycles, one semen value was abnormal in 18.4% of the cycles and all three semen values were normal in 7.2% of the cycles. Most of the couples with normal semen values had previously undergone conventional IVF treatments without success.

In total, ICSI was performed in 7374 cycles on 74 520 metaphase II oocytes (a mean of 10.1 metaphase II oocytes per cycle). Sixteen to 18 h after ICSI, oocytes were inspected for integrity and fertilization. The number of intact oocytes was 67 708 (90.9% of the oocytes that had received injections), i.e. an injection damage rate of 9.1%. The mean number of successfully injected oocytes was 9.2 per treatment. Oocytes were considered to be normally fertilized when two individual or fragmented polar bodies were present together with two clearly visible pronuclei containing nucleoli. Normally fertilized oocytes resulted from 72.2% of the successfully injected oocytes, from 65.6% of the injected metaphase II oocytes and from 52.6% of the retrieved COC. Abnormal fertilization occurred as one-pronuclear (1PN) oocytes in 2.8% (2090) of the injected metaphase II oocytes, and 3.7% (2741) of the injected metaphase II oocytes revealed three

Table I. Sperm origin, oocyte damage and pronuclear status and embryo development after intracytoplasmic sperm injection (ICSI)

	Ejaculated semen	Epididymal		Testicular
		Fresh	Frozen–thawed	
No. of cycles	6397	161	130	686
No. of oocytes undergoing ICSI	63 645	1877	1374	7624
Inact oocytes (%)	9.8	92.3	90.8	
Injected oocytes (%) with				
one pronucleus	2.6	4.0	3.2	4.2
two pronuclei	67.0[a]	59.8[b]	57.3[b]	56.6[b]
three or more pronuclei	3.7	4.9	4.8	3.3
No. of two-pronuclear oocytes	42 648	1122	787	4315
Excellent embryos (%)	7.0	7.9	3.9	5.9
Good-quality embryos (%)	58.3[a]	49.4[b]	45.2[b]	49.7[b]
Fair-quality embryos (%)	15.8	14.6	18.7	19.3
Transferred or frozen embryos (%)	65.7	64.8	59.6	62.7

[a,b]Values with different superscripts within rows were significantly different ($P < 0.001$; χ^2 contingency table).

pronuclei. If such abnormally fertilized 1PN or 3PN oocytes cleaved further, they were not transferred.

Oocyte damage, pronuclear status and further in-vitro embryo development after ICSI for all four types of spermatozoa used (ejaculated, fresh and frozen–thawed epididymal, and testicular) were analysed (Table I). The percentage of oocytes that remained intact after ICSI varied between 89.8 and 92.3% and was similar for the four types of spermatozoa. The percentages of oocytes that had fertilized normally (2PN) varied between 56.6 and 67.0%. The normal fertilization rate for ICSI was higher when ejaculated spermatozoa were used than when other types of spermatozoa were used ($P < 0.001$ by χ^2 contingency table). The percentages of oocytes with one pronucleus after ICSI varied between 2.6 and 4.2%. The percentages of oocytes with three pronuclei after ICSI varied between 3.3 and 4.9%. Abnormal fertilization occurred to a similar extent in the four different sperm groups. The exceptional circumstances in which no injected oocytes had fertilized normally were associated with the availability of only very few metaphase II oocytes for ICSI, the availability of only totally immotile spermatozoa for the injection, with gross abnormalities in the oocytes, with round-headed spermatozoa being injected or with all oocytes being damaged in the injection procedure. In this last case, most of the patients achieved fertilization in subsequent cycles (Liu *et al.*, 1995a; Vandervorst *et al.*, 1997).

Embryo cleavage was evaluated after a further 24 h of in-vitro culture. The cleaving embryos were categorized according to equality of size of the blastomeres and proportion of anucleate fragments as excellent type A embryos (no anucleate fragments), good quality, type B embryos (between 1 and 20% of the volume filled with anucleate fragments) and fair quality, type C embryos (between 21 and 50% of the volume filled with anucleate fragments). Cleaved embryos with less than one-half of their volume filled with anucleate fragments were eligible

Table II. Sperm origin and outcome of embryo transfers after intracytoplasmic sperm injection in the period 1991–1997

	Ejaculated semen	Epididymal		Testis
		Fresh	Frozen–thawed	
No. of cycles	6397	161	130	686
No. of transfers	5949	149	112	624
Transfer rate (%)	93.0	92.5	86.2	91.0
No. of embryos transferred				
Mean ± SD	3.0 ± 0.9	2.7 ± 1.3	2.8 ± 1.1	2.9 ± 1.3
Range	1–8	1–11	1–8	1–12
No. of pregnancies	2128	64	35	201
No. of transfers with known HCG outcome	5888	149	108	613
Pregnancy rate per transfer[a] (%)	36.1	43.0	32.4	32.8
one embryo	13.9	20.0	21.4	11.3
two embryos	24.7	33.3	17.6	27.3
two embryos (elective)	42.6	31.8	33.3	31.1
three embryos	38.0	43.2	31.0	32.9
three embryos (elective)	45.4	59.4	45.5	46.5
more than three embryos	34.6	44.0	41.2	38.4
Pregnancy rate per cycle[a] (%)	33.6	39.8	27.8	29.8
Implantation rate[b] (%)	16.5	17.4	16.1	11.5

[a]With known serum HCG outcome; [b]no. of fetal sacs as percentage of no. of embryos replaced.

for transfer. Supernumerary embryos with <20% anucleate fragments were cryopreserved on day 2 or 3 after oocyte retrieval. The total numbers of embryos of transfer quality, i.e. those with <50% anucleate fragments, was 39 135: 80.0% of the 2PN oocytes, 57.8% of the successfully injected (intact) oocytes, 52.5% of the injected metaphase II oocytes and 42.2% of the retrieved COC. The percentages of 2PN oocytes developing into excellent, good quality and fair quality embryos according to the different types of spermatozoa used for ICSI are summarized in Table I. A statistically significant higher percentage of good quality embryos was obtained in the group using ejaculated spermatozoa ($P < 0.001$ by χ^2 contingency table). The percentages of embryos actually transferred or frozen as supernumerary embryos was similar for the four types of spermatozoa and varied between 59.6 and 65.7% of the 2PN oocytes.

Replacement of at least one embryo was possible in 6834 of the 7374 ICSI treatment cycles (92.7%). This may be considered a high transfer rate, since it includes couples with previous fertilization failure in conventional IVF, ejaculated spermatozoa too poor to be included in IVF or men with obstructive or non-obstructive azoospermia. As indicated in Table II, the transfer rate was similar across the four sperm groups used for ICSI, varying from 86.2 to 93.0% of the 6834 embryo replacement cycles; 6758 transfers were with known serum HCG outcome. For the other 76 transfers (61, four and 11 respectively in the ejaculated, frozen–thawed epididymal, and testicular sperm groups) the subsequent serum HCG was unknown. The overall pregnancy rates per transfer with known serum HCG outcome and the pregnancy rates per number of embryos transferred were

similar for the four types of spermatozoa. Especially high pregnancy rates have been observed when elective transfer of two or three embryos was performed (Staessen *et al.*, 1993, 1995). The pregnancy rate per cycle with known serum HCG outcome varied from 27.8 to 39.8%.

Follow-up of 1987 ICSI children

From April 1991 onwards, 2375 pregnancies, leading to the births of 1987 children before September 1997, were studied. The follow-up of this cohort of children was carried out by the Centre for Medical Genetics in collaboration with the Centre for Reproductive Medicine.

Prospective follow-up study design

Before starting ICSI, couples were asked to agree to the follow-up conditions of our study. These conditions included genetic counselling and agreement to prenatal karyotype analysis as well as participation in a prospective clinical follow-up study of the children. This included completing a standardized questionnaire (Wisanto *et al.*, 1995), returning it to the research nurse, and, where possible, visiting the Centre for Medical Genetics with the child after birth.

All couples referred for assisted fertilization were evaluated for possible genetic problems, either before starting in cases of maternal age >35 years, positive family history or a chromosomal aberration carried by a parent, or at 6–8 weeks of pregnancy. A history, including a pedigree, was obtained in order to identify genetic risks or possible causes of congenital malformations. This history included details of medication, alcohol abuse and environmental or occupational risk factors and socio-economic status. A karyotype was routinely performed for the couple. In view of possible risk factors due to the new techniques of assisted fertilization and taking into account the previous results of prenatal diagnosis obtained in our ICSI patients, couples during the first year of our programme were counselled to have a prenatal test (Bonduelle *et al.*, 1996a,b). Eventually it was possible to inform patients more precisely about the different types of risk factor and they were then left free to opt for a prenatal test procedure or not. Pros and cons of the different types of prenatal diagnosis were discussed in detail at ~6–8 weeks of gestation; amniocentesis was suggested for singleton pregnancies, while chorionic villous sampling was proposed for multiple pregnancies. Chromosome preparations were obtained from cultured amniocytes according to a modified technique (Verma *et al.*, 1989). Chromosome preparations from non-cultured and cultured chorionic villous cells were obtained by means of the techniques described by Gibas *et al.* (1987) and Yu *et al.* (1986) respectively. If indicated, prenatal tests or preimplantation diagnosis for other genetic diseases were planned.

The follow-up study of the expected child was further explained: it was to

consist of a visit to the geneticist–paediatrician at 2 and 12 months of age, and then once a year.

For all pregnancies, written data concerning pregnancy outcome with regard to the babies were obtained from the gynaecologists in charge. Perinatal data, including gestational age, mode of delivery, birthweight, Apgar scores, presence or absence of malformations and neonatal problems were recorded. If any problem was mentioned, detailed information was also requested from the paediatrician in charge. For babies born in our university hospital, a detailed physical examination was done at birth, looking for major and minor malformations and including evaluation of neurological and psychomotor development.

For babies born elsewhere, written reports were obtained from gynaecologists as well as from paediatricians, while a detailed morphological examination by a geneticist–paediatrician from our centre was carried out at 2 months whenever possible. Additional investigations were carried out if the anamnestic data or the physical examination suggested them.

At follow-up examination at 12 months and 2 years, the physical, neurological and psychomotor examinations were repeated by the same team of geneticist–pediatricians. At ~2 years or more, a Bayley test was performed in order to quantify the psychomotor evolution of the children (Van der Meulen and Smrovsky, 1983). Further psychomotor evaluation and social functioning will be evaluated at the age of 4–6 years. If parents did not come spontaneously to the follow-up consultations, they were reminded by phone to make an appointment.

A widely accepted definition of major malformations was used, i.e. malformations that generally cause functional impairment or require surgical correction. The remaining malformations were considered minor. A minor malformation was distinguished from normal variation by the fact that it occurs in $\leq 4\%$ of the infants of the same ethnic group. Malformations or anomalies were considered synonymous with structural abnormality (Smith, 1975; Holmes, 1976).

Children born

In the group of 1987 children studied, 1072 were singletons, 816 were from twin pregnancies and 99 were from triplet pregnancies. Of the 1987 children, 1699 were born after a cycle using fresh embryos obtained by ICSI with ejaculated spermatozoa; 91 after ICSI using epididymal spermatozoa obtained after microsurgical epididymal sperm aspiration (including 58 with fresh spermatozoa and 33 with frozen spermatozoa), 118 after ICSI using testicular spermatozoa and 79 after replacement of cryopreserved embryos obtained after ICSI with ejaculated spermatozoa (Table III). Of the 1987 children born, 1966 were livebirths and 21 were stillbirths (defined as fetal death ≥ 20 weeks gestation or ≥ 400 g). The stillbirth rate was 1.06%, varying from 0.83% in the singletons to 1.34% in the twins. For 1951 of the 1987 children (98.2%) we had complete information at birth: for 36 children, data remained incomplete, even after several attempts to obtain the missing information (Table III).

Overall, the mean maternal age as regards the children born was 32.4 years

Table III. Number of children born after replacement of intracytoplasmic sperm injection (ICSI) embryos and follow-up rate of the cohort of 1987 children born

	Ejaculated Spermatozoa	Epididymal spermatozoa		Testicular spermatozoa	Cryopreserved embryos[a]	Total
		Fresh	Frozen			
Singleton	893	31	51	65	63	1072
Twin	734	18	10	38	16	816
Triplet	72	9	3	15	–	99
Total	1699	58	33	118	79	1987
Children born	1699	91		118	79	1987
liveborn	1680	90		118	78	1966
stillbirth	19	1		–	1	21
Complete information at birth	1674/1699 (98.52)	87/91 (95.60)		117/118 (99.15)	77/79 (97.46)	1951/1987 (98.18)
Follow-up at 2 months	1432/1680 (85.23)	64/90 (71.11)		90/118 (76.27)	66/78 (84.61)	1652/1966 (84.02)
Follow-up at 1 year						868/1409 (61.60)

Values in parentheses are percentages.
[a]Transfer of frozen–thawed supernumerary embryos obtained after ICSI with ejaculated spermatozoa.

(range 19.9–45.1) 32.6 years for the singleton pregnancies, 31.9 years for multiple pregnancies. The overall mean paternal age was 35.0 years (range 25.5–64.7).

We obtained data from the physical examination at birth for all the children. We compiled this information from the medical records as well as from careful questioning of the parents during follow-up consultations. For the children living further away, or where the parents were no longer willing to come to the clinic, detailed histories (except for one major malformation where we were given only the name of the malformation) were obtained from the paediatrician if any problem was mentioned in response to the questionnaire. During the follow-up at 2 months, 1652 of the 1966 liveborn children (84.02%) were examined by one of the geneticists; 868 of the 1409 children (61.60%) who reached 1 year have so far been examined a second time at 1 year (Table III).

Genetic counselling

At the genetic counselling session, we saw 1298 of the 1519 couples (85%) and concluded that there was an increased genetic risk (Table IV) for 557 children. This increased risk was due to maternal age, 404; paternal age, nine; chromosomal aberrations, 27; monogenic disease, 79; multifactorial disease, 32 and consanguinity, seven. We found 20/415 (4.8%) abnormal karyotypes in the tested men and 7/480 (1.5%) in the tested women. Within the monogenic diseases, cystic fibrosis (CF)-related problems were encountered in 60 couples, seven of whom were carriers of CF detected on routine CF screening offered for ICSI couples. Most of the 18 other monogenic diseases were found in couples who came to the Centre with a request for preimplantation diagnosis for the disease (Table IV).

Table IV. Genetic and environmental problems encountered in the genetic counselling sessions early in pregnancy for 1304 couples

	Ejaculated spermatozoa	Epididymal spermatozoa	Testicular spermatozoa	Cryopreserved embryos	Total
No. of couples seen at the genetic counselling session					
No. of couples	1284	69	89	77	1519
Not seen	180	10	25	6	221
Seen (%)	1104 (86)	59 (86)	64 (72)	71 (92)	1298 (85)
Parental age at birth					
Maternal age ⩾35 years	347	14	27	16	404
Paternal age ⩾50 years	7	–	2	–	9
Total	354	14	29	16	413
Karyotype anomalies in parents					
Normal 46,XX	411	9	22	31	473
Abnormal (%)	7 (1.07)	–	–	–	7 (1.5)
Total	418	9	22	31	480
Normal 46,XY	347	8	17	23	395
Abnormal (%)	18 (4.9)	–	1	1	20 (4.8)
Total	365	8	18	24	415
Monogenic disease					
Cystic fibrosis (CF)-related					
CBAVD	1	24	11	1	37
CF patients (+ CBAVD)	–	2	2	–	4
CF 1/4 risk (+ CBAVD)	–	1	1	–	2
CAVD	1	8	1	–	10
Epididymal agenesis	–	–	1	–	1
CF carrier (– CBAVD)	2 males 5 females	–	–	–	2 males 5 females
Total	9	35	16	1	61
Other					
Adult polycystic kidney disease	3	–	–	–	–
Fragile X premutation	3	–	–	–	–
Haemophilia A	3	–	–	–	–
Duchenne's muscular dystrophy	2	–	–	–	–
Huntington disease	2	–	–	–	–
X-linked ichthyosis	1	–	–	–	–
Myotonic dystrophy	1 + 1	–	–	–	–
X-linked retinitis pigmentosa	1	–	–	–	–
X-linked mental retardation	1	–	–	–	–
Total	18	–	–	–	18
Multifactorial disease					
Cleft lip and palate	–	–	–	–	–
RR >1%	3	–	–	–	3
RR <1%	–	–	–	1	1
Neural tube defect	–	–	–	–	–
RR >1%	4	–	–	–	4
RR <1%	6	–	–	1	7
Epilepsia	5	–	–	1	6

Table IV. *Cont.*

	Ejaculated spermatozoa	Epididymal spermatozoa	Testicular spermatozoa	Cryopreserved embryos	Total
Diabetes type I	2	–	1	–	3
MODY diabetes	1	–	–	–	1
Bechterew's reflex	3	–	–	1	4
Manic depression[a]	–	–	–	–	2
Schizophrenia[a]	1	–	–	–	1
Total	27	–	1	4	32
Level of consanguinity					
Third	2	–	–	1	3
Fourth	1	–	–	–	1
Fifth	3	–	–	–	3
Total	6	–	–	1	7

[a]Before ICSI was used, the referring psychiatrist agreed to the infertility treatment.
RR = relative risk; CBAVD = congenital bilateral absence of the vas deferens; CAVD = congenital absence of the vas deferens; MODY = maturity onset diabetes of the young.

Prenatal diagnosis

Abnormal fetal karyotypes were found in 28 cases out of 1082 tested fetuses: 690 amniocenteses (15 of which were abnormal), 392 chorionic villous sampling (CVS) biopsies (13 of which were abnormal) and seven cord blood punctures which were control samples of previous amniopunctures and were normal (Table V).

Overall, the mean maternal age of the mothers undergoing a prenatal test procedure was 33.3 years (range 21.4–45.1); the mean maternal age was 33.5 years for mothers undergoing amniocentesis (SD 4.06) and 32.9 years for mothers undergoing CVS (SD 4.08). In these 1082 tests we observed 18 (1.66%) de-novo chromosomal aberrations: nine of these (0.83%) were sex-chromosomal aberrations and another nine (0.83%) were autosomal (trisomies and structural aberrations) (Table VI).

Table VII gives more details of the type of abnormal results, maternal age and the outcome of the pregnancies after an abnormal result. In all, nine pregnancy interruptions were carried out after an abnormal karyotype result (five trisomies and four sex-chromosomal aberrations) and one after a DNA diagnosis for fragile X syndrome, where the fetus was affected. For one mother, a preimplantation diagnosis with amplification of the non-deleted region of the healthy allele was performed as she was a carrier of Duchenne's muscular dystrophy and had chosen to undergo this treatment rather than a prenatal diagnosis for ethical reasons (Liu *et al.*, 1995b). For one other mother with a one-in-four risk of cystic fibrosis, a preimplantation diagnosis for cystic fibrosis was performed (Liu *et al.*, 1994). In a study group of 460 consecutive ICSI singleton pregnancies with amniocentesis and 360 consecutive ICSI singleton pregnancies without amniocentesis, there was no statistically significant difference in outcome measured in terms of prematurity, low birthweight, very low birthweight or loss

Table V. Prenatal diagnosis: normal and abnormal results after chorionic villous sampling (CVS), amniocentesis and cord blood puncture

	Normal	Abnormal	Failure	Total
Amniocentesis	675	15	(2)[a]	690
CVS	379	13	–	392
Cord blood puncture	(7)[b]	–	–	(7)[b]
Total	1054	28	(2)[a]	1082

[a]In two amniotic fluid punctures no result was obtained; they were therefore not added to the total number of results.
[b]Cord blood puncture was done in all cases as a control of amnio puncture and results were normal in all cases. For this reason they were not added to the total number of tests.

Table VI. Prenatal diagnosis in 1082 children: abnormal results

Type of abnormal results	No.	Percentage	95% CI
De-novo	18	1,66	1.0–2.7
Autosomal	9	0.83	0.3–1.6
Structural	4	0.36	
Trisomies	5	0.46	
Sex chromosomal	9	0.83	0.3–1.6
Inherited	10	0.92	0.4–1.7
Balanced	9	0.83	
Non-balanced	1	0.09	

CI = confidence interval.

of pregnancy. The same findings were observed in 109 consecutive ICSI twin pregnancies with CVS and 174 ICSI twin pregnancies without CVS (Aytoz *et al.*, 1998).

The values in Table VI show a slight increase in sex-chromosomal aberrations (0.83%), which is statistically significant since the 95% confidence limits of this percentage (0.3–1.6%) do not contain the percentage of aberrations (0.19–0.23%) described in the literature in a neonatal population (Nielsen and Wohlert, 1991; Jacobs *et al.*, 1992). The increase in autosomal aberrations is due partly to the increase in trisomies, linked with higher maternal ages. On the other hand, there is also an increase in structural de-novo aberrations (0.36% compared with 0.07% in the literature), which is also significantly higher. The number of inherited aberrations, one of which was unbalanced, is of course higher than in the general population but was predictable for the individual couples, in all but one of whom the father was a carrier of the structural anomaly.

It is interesting to observe that all de-novo sex-chromosomal aberrations were found in cases using spermatozoa from men with extreme oligoasthenoteratozoospermia (concentration $0.1–4.6\times10^6$ spermatozoa/ml; normal morphology 0–40%; a progressive motility 0–18%) (Devroey, 1998). So far, however, there is no known statistical correlation between standard semen parameters (concentration, motility, normal morphology) and de-novo chromosomal aberrations.

Table VII. Prenatal diagnosis: type of abnormal results from 1082 tests

	Maternal age (years)	Outcome
De novo (n = 18)		
Sex chromosomes (n = 9)		
45,X	37	CVS → interruption
46,XX/47,XXX	44	CVS → intrauterine death (>40 weeks)
47,XXX	37	AC → born
47,XXX	32	CVS (twin) → born
47,XXY	32	CVS (twin) → 2 affected[a] → interrupted
47,XXY	28	CVS (twin) → born
47,XXY	28	AC → interrupted
47,XXY	26	AC → interrupted
47,XYY	25	AC → born
Autosomal trisomies (n = 5)		
47,XY+21	41	CVS (twin) → selective reduction
47,XY+21	41	CVS → interrupted
trisomy 21	37	AC → outcome unknown
47,XY+21	32	CVS (twin) → 2 affected[a] → interrupted
47,XY (+ 18)	33	AC → intra uterine death 37 weeks
Structural anomalies (n = 4)		
46,XY,t(4;5)	30	CVS (twin) → born 28 weeks
46,XX,t(2;5)	30	AC → born
46,XX,t(2;13)	36	AC → born
46,XX,inv(1qh)	39	AC → born
Inherited structural aberrations (n = 10)		
Balanced (n = 9)		
46,XY,inv(1) (p22p23.1)		AC/pat. origin
46,XY,inv(5) (p13q13)		AC/pat. origin
46,XX,t(14;15)		AC/pat. origin
46,XX,t(13;14)		CVS/mat. origin
46,XX,+invdup(15p)		CVS/pat. origin
46,XX,+invdup(15p)		CVS/pat. origin
45,XY,t(13;14)		CVS/pat. origin
45,XX,t(14;15)		AC/pat. origin
45,XY,t(13q;14q)		AC/pat. origin
Unbalanced (n = 1)		
46,XY,t(14;21)+21		CVS → pat.inherited → interrupted

[a]Same pregnancy with two affected fetuses.
CVS = chorionic villous sampling; AC = amniotic fluid puncture; mat. = maternal; pat. = paternal.

Neonatal data

Neonatal measurements for 1966 liveborn children of 20 or >20 weeks of gestation are listed in Table VIII. For the total group, mean birthweight was 2818 g, mean length was 47.9 cm and mean head circumference was 33.5 cm.

Prematurity (birth before or at 37 weeks of pregnancy) was observed in 11.8% of the singletons, 59% of the twin children and 96% of the triplet children. Birthweight <2500 g was observed for 8.2% of the singletons, 51.6% of the twin children and 84.8% of the triplet children. Very low birthweight (<1500 g)

Table VIII. Neonatal measurements in 1966 children born after the replacement of embryos using intracytoplasmic sperm injection

	Overall		
	Weight (g)	Length (cm)	Head circumference (cm)
Singleton	3220.1 ± 583.6 (610–4970)	49.6 ± 3.1 (31–59)	34.3 ± 1.8 (21.5–43)
Twin	2421.8 ± 518.1 (520–4080)	46.2 ± 3.1 (30–54)	32.6 ± 2.1 (22–44)
Triplet	1724.1 ± 565.7 (610–3100)	41.4 ± 4.4 (31–49.5)	29.6 ± 3.0 (22–34)
Total	2818.5 ± 720.1 (520–4970)	47.9 ± 3.8 (30–59)	33.5 ± 2.3 (21.5–44)

Values are mean ± SD (range).

was observed for 1.8% of the singletons, 5.0% of the twin children and 36% of the triplet children.

Sex ratio of male/female was 0.98 in the total group and 0.98, 1.39, 0.73 and 1.0 in the different subgroups of ICSI children, born after ICSI with ejaculated, epididymal or testicular spermatozoa or after cryopreservation respectively.

Major malformations

Major malformations were found in seven terminations and in four intrauterine deaths in a total of 21 stillbirths after 20 weeks. No other malformations were detected prenatally, apart from one twin child with a holoprosencephaly detected at the age of 15 weeks of pregnancy, where the multiplicity and the risk involved in a selective abortion led to the option of continuing the pregnancy. This child died at birth.

Major malformations were found in 22/1063 (2.1%) singleton children, 22/805 (2.7%) twin children and 2/98 (2.0%) triplet children. This was 46/1966 or 2.3% of all babies born alive. The major malformations were distributed among different organ systems: limb malformations 28.2%, nervous system 17.4%, cleft palate and lip 10.9%, cardiac system 10.9%, internal urogenital malformations 10.9%, musculo-skeletal system 10.9%, external urogenital malformations 8.6% and other less frequently occurring malformations. If we define the malformation rate as (affected livebirths + affected fetal deaths + induced abortions for malformations) divided by (livebirths + stillbirths) the values were: (46+4+7)/(1966+21) = 2.9% (Lechat and Dolk, 1993).

During the follow-up consultations at 2 months and 1 year, 10 more major malformations were detected. This gave a total malformation rate after 1 year of 67/1987 or 3.4%, taking into account that not all the children have reached 1 year at the time of writing.

Further medical and developmental outcome of ICSI children

Two recent *Lancet* publications report on the further development of ICSI children. A study in Australia (Bowen *et al.*, 1998) compared the medical and developmental outcome at 1 year of 89 children conceived by ICSI with 84 children conceived by conventional IVF, and with 80 children conceived naturally. Developmental assessment was done with Bayley Scales for Infant Development. There was no significant difference in the incidence of major congenital malformations or major health problems in the first year of life. However, the Bayley-derived Mental Development Index (MDI) was significantly lower for ICSI children (especially boys) than for IVF or naturally conceived children. More children conceived by ICSI experienced mildly or significantly delayed development. In the same *Lancet* issue, our own group reported that at 2 years of age the mental development as tested by the mental scale of 201 ICSI and 131 routine IVF children was similar to that for the general population (Van der Meulen and Smrovskyk 1983; Bonduelle *et al.*, 1998). We concluded that there is no indication, so far, that ICSI children have a slower mental development than the general population. Both articles as well as the commentary in the *Lancet* suggested that further case–control studies taking into account parental background and other confounding variables are needed before conclusions can be drawn (Te Velde *et al.*, 1998).

Discussion

Although all pregnancy outcomes were recorded, the data are not analysed in the present review. In a previous article (Wisanto *et al.*, 1995), the incidence of pregnancy loss, i.e. subclinical pregnancies, clinical abortions and ectopic pregnancies, was reported as 21.9% in the group with ejaculated spermatozoa, 37.8% in the group with epididymal spermatozoa, 33.3% in the group with testicular spermatozoa and 61.4% in the pregnancies from frozen and thawed embryos; perinatal mortality was 1.71%. The stillbirth rate of 1.06% observed is not higher than the number reported in the literature for IVF pregnancies (Rizk *et al.*, 1991).

From the beginning of our ICSI treatments, nearly all patients have been seen at the Centre for Medical Genetics either before starting treatment or at 6–8 weeks of pregnancy. Since increasing numbers of our patients come from abroad, they tend to leave the country early and not to attend the genetic counselling session. Even so, we have seen 86% of the couples and have estimated an increased risk (of 1/4 to 1/2) for 78 children due to monogenic disorders. As mentioned earlier, in this group of the monogenic disorders a number of couples were included in our ICSI patients because they requested a preimplantation genetic diagnosis which was performed in combination with ICSI (in order to reduce the risk of contamination). For 27 children, there was an increased risk due to the karyotype anomalies in their parents, most often the fathers with either

sex-chromosomal aberrations or structural anomalies (4.8%). This percentage is much higher than the expected figure of 0.5% in the general population (Jacobs *et al.*, 1992) and is associated with the severe male-factor infertility often present in the patient population presenting for ICSI. The different possibilities for the offspring were explained to all the parents carrying a structural aberration, in terms of the specific chromosomal aberration and the sex of the parent: a normal karyotype is of course possible, but so is a higher miscarriage rate and perhaps a lower implantation rate, both leading to a lower success rate for the ICSI procedure, a risk of chromosomally unbalanced offspring which can be detected by a prenatal diagnosis, and a risk of transmitting exactly the same structural aberration as that present in the parent. As well as being told about the risk of transmitting the same chromosomal abnormality to the offspring, leading to greater genetic risks for the latter, parents were also informed about the possible higher risk of infertility, mainly for their male children. When indicated, a preimplantation diagnosis for sex chromosomal aneuploidy was discussed or a specific diagnosis for translocation carriers was evaluated and further discussed if technically feasible.

We think it necessary to continue to perform parental karyotypes, since for the couples with a structural aberration the reduction of success rate of the ICSI treatment procedure should be explained, as well as the strict indications for a prenatal test and the risks for the offspring. It would be of help for future counselling if chromosome analysis of germ cells was possible on a routine basis, since so far some studies have described a higher percentage of gonosomal aneuploidy present in males with severe oligoasthenoteratozoospermia, and this could perhaps lead to a more clearly differentiated way of counselling for a prenatal diagnosis (Bernardini *et al.*, 1997).

For the first 2 years, 85% of the counselled pregnant patients participated in the prenatal diagnosis programme. At that time we were still in the experimental stage of our ICSI programme and no data on prenatal karyotypes in ICSI were available besides our own. Patients had to agree to a prenatal diagnostic procedure as an entry criterion for the treatment. Now, however, we discuss our actual risk figures and can offer a free choice for testing. Under these conditions only 54.5% of the couples accept either CVS or amniocentesis. Patients also take into consideration that the increased risk of, mainly, sex-chromosomal aberrations is more acceptable, since children with sex-chromosomal aneuploidies usually have a normal physical appearance and are likely to have an IQ within the normal range of the population, since mental retardation, defined as IQ of <70, is not typically associated with sex chromosome aneuploidy. There is, however, a moderate risk of developmental problems in the areas of speech, motor skills and learning abilities. Infertility is often present (Linden *et al.*, 1997).

It is interesting to note that the mean age of our ICSI patient population is 32.4 years and that 49% of the patients under 35 years of age accept prenatal diagnosis, while 66% of those of 35 years or older are prepared to do so. It is clear that the risk perception of the prenatal procedure increases as the chances

of getting pregnant (again) decrease or that the risk of a problem child becomes more acceptable if the parents think that this is their last chance of having a child.

More singletons than twins were tested, since parents of a multiple pregnancy were afraid of the test procedure as we counselled them to have a CVS rather than amniocentesis and attributed during the counselling a higher risk (of 1%) of miscarriage to the latter. As a recent study by our group has demonstrated that there is no difference in outcome either with or without a prenatal test procedure, we shall also discuss these data with our patients in the future (Aytoz *et al.*, 1998).

Abnormal fetal karyotypes were found in 28 cases out of 1082 tested fetuses, 18 of which (1.66%) were de-novo chromosomal aberrations The mean maternal age of the mothers at conception was 32.5 years, which does not explain the higher rate of chromosomal aberrations found. For a mean maternal age of 32 years we would expect a value of ~0.3% chromosomal aberrations at the time of prenatal diagnosis (Ferguson-Smith, 1983) rather than 1.66%, including the 0.83% of sex-chromosomal aberrations. The incidence of these sex-chromosomal aberrations at the time of prenatal diagnosis is comparable with the incidence at birth (since these aberrations are not critical to survival) (Nielsen and Wohlert, 1991; Jacobs *et al.*, 1992). The value of 0.83% of sex- chromosomal aberrations can thus be compared with the total new-born population and is ~4-fold higher than the values of 0.19% (Jacobs *et al.*, 1992), 0.2% (Hook and Hamerton, 1977) and 0.23% (Nielsen and Wohlert, 1991) found in an unselected newborn population. The diffences between these values are also statistically significant since the mean incidence is outside the 95% confidence interval.

The hypothesis for the higher incidence of chromosomal aberrations, i.e. that spermatozoa from men with a fertility problem contain a higher frequency of chromosomal abnormalities, is now increasingly well supported (Hoegerman *et al.*, 1995; Pang *et al.*, 1995; Bernardini *et al.*, 1997). Even if data from the literature are somewhat contradictory, since our own observations are going in the same direction, we conclude that the higher frequency of chromosomal aberrations in spermatozoa from men with oligoasthenoteratozoospermia (OAT) is a risk factor in ICSI treatment and is in itself the origin of the higher percentage of chromosomal aberrations observed. This might also be the explanation for the observation of a significantly higher number of structural aberrations. On the other hand, there is experimental evidence that epigenetic development mechanisms are altered in this means of conception (Tesarik, 1995; Tesarik and Souza, 1995), leading to a higher degree of occurrence of aneuploidy because of errors of mitosis during the early cleavage divisions. This mechanism could lead to a higher number of post-meiotic errors, such as in the one case of mosaicism 46,XX/47,XXX from an older woman (Table VII). We did not observe an abnormally high incidence of such post-meiotic errors, but we think it is interesting to follow this in data compiled from all laboratories, since inter-laboratory differences might play a role in the occurrence of such problems.

Ten out of a total of 1082 results (0.92%) were familial structural aberrations. These were certainly not induced by the microinjection technique, since they

were all detected in the infertile males (or partners) before the treatment. Statistically, familial structural aberrations can lead to normal karyotypes, to exactly the same structural aberration as in the parent, or to a percentage from 0 to 50% of unbalanced karyotypes. In this group of parents carrying a structural aberration, however, only one unbalanced fetus was found, a child with a trisomy 21 due to a paternal translocation 14;21.

Neonatal data indicate that prematurity, low birthweight and very low birthweight are due mainly to multiple pregnancies. For singletons, the rates of low birthweight (8.2%) and very low birthweight (1.8%) are comparable to or lower than the percentages described in the IVF population in the literature (Rizk *et al.*, 1991; Bachelot *et al.*, 1995; Lancaster *et al.*, 1995) but are still higher than in cases of natural conception.

The value of 2.3% major malformation rate is similar to that found in most of the general population national registries and assisted reproduction surveys [Andrews *et al.*, 1986; Saunders and Lancaster, 1987; Beral and Doyle, 1990; Bachelot *et al.*, 1995; Lancaster *et al.*, 1995; Society for Assisted Reproductive Technology (SART) and American Fertility Society, 1995]. We have here taken the livebirth malformation rate as this is most frequently used, rather than a more precise calculation of the ratio, taking fetal deaths and interruptions of affected fetuses into account, which is used in only a very few malformation surveys. Anomalies are most often nationally registered at birth or during the first week of life, whereas in this study the follow-up is carried through to 2 years and the higher value at the age of 1 year (3.8%) should be compared with like data. Belgium is one of the countries where registration for Eurocat is carried out. In the Province of Antwerp, registered major anomalies up to the age of 1 year were 2.28% from 1989 to 1996. This is lower than the value we found, but risk figures in the national statistics will probably also be somewhat lower than in reality, as it is unlikely that malformations generally are looked for as carefully as in the current survey.

Assisted reproduction surveys have their limitations too: data were obtained through standard data collection forms, most often filled in at birth. The children born after assisted procreation were not examined in a systematic manner and no follow-up was provided to detect congenital malformations or developmental problems, which become manifest only later. There is no system in place to check the reported results and the missing data. This explains why we expect to find malformation rates to be lower in the surveys reported after IVF than in this detailed prospective follow-up study of children after ICSI.

A few smaller studies have been done to compare outcomes of IVF to natural conception (Morin *et al.*, 1989): 83 IVF children and 93 matched controls were studied and a systematic examination for 130 major and minor malformations showed no difference between the IVF and the control group. In a recent retrospective study in the USA by (Schattman *et al.*, 1995), 3.6% (11 out of 303 children) had major anomalies after routine IVF within the first year of life, attested by questionnaires (with a 68% response). These rates were considered comparable to those observed in the New York population (New York State

Department of Health, 1990). Even though data from only a small number of good studies on malformation rates after assisted reproduction treatment are available, it is generally accepted that there are no more malformations than in the general population.

A recent article (Kurinczuk *et al.*, 1997) provided a less reassuring interpretation of our data. Using the classification scheme from the Western Australia birth defects registry the writers noted that many (mostly cardiac) major defects in the Belgian series had been incorrectly classified as minor. In the commentary to this article we replied that most of the minor heart defects were found by routine heart ultrasonography, and that the disproportionate numbers of cardiac malformations were due to overreporting (as minor malformations), rather than to the ICSI technique itself (Bonduelle *et al.*, 1997; Sutcliffe *et al.*, 1998).

The increase in de-novo chromosomal aberrations and the higher frequency of transmitted chromosomal aberrations are probably linked directly to the characteristics of the infertile men treated rather than to the ICSI procedure itself. Compared to most registers of children born after assisted reproduction and to registers of malformations in the general population, the malformation rate of 2.3% is within the expected range. These observations should be completed by others and by collaborative efforts. In the meantime and before any treatment is started, patients should be informed of the available data: the risk of transmitting chromosomal aberrations, the risk of de-novo, mainly sex chromosomal, aberrations and the risk of transmitting fertility problems to the offspring. Patients should also be reassured that there seems to be no higher incidence of congenital malformations in children born after ICSI.

Low percentages of major malformations were observed in all the different subgroups: 3.3% (3/90) in cases of ICSI with epididymal spermatozoa, 1.6% (2/118) in the testicular spermatozoa group and 1.26% (1/78) in the children born after replacement of frozen–thawed supernumerary ICSI embryos. As the totals in the subgroups are still low, it is too early to reach conclusions on any differences in offspring due to the origins of the spermatozoa or to additional techniques but there seems at present to be no particular reason for concern.

The developmental and genetic disorders in spermatogenesis were recently reviewed, including the ethical dilemmas (Diemer and Desjardins, 1999).

Acknowledgements

We are indebted to many colleagues: the clinical, scientific, nursing and technical staff of the Centre for Medical Genetics and the Centre for Reproductive Medicine, Hubert Joris, for his efforts in computing these data, M.-Paule Derde for statistical calculations and Frank Winter for reviewing the manuscript. Research grants from the Belgian Fund for Medical Research and an unconditional educational grant from Organon International are kindly acknowledged.

References

Andrews, M.C., Muasher, S.J., Levy, D.L. *et al.* (1986) An analysis of the obstetric outcome of 125 consecutive pregnancies conceived in-vitro and resulting in 100 deliveries. *Am. J. Obstet. Gynecol.*, **154**, 848–854.

Aytoz, A., De Catte, L., Bonduelle, M. *et al.* (1998) Obstetrical outcome after prenatal diagnosis in intracytoplasmic sperm injection pregnancies. *Hum. Reprod.*, **13**, 2958–2961.

Bachelot, A., Thepot, F., Deffontaines, D. *et al.* (1995) Bilan FIVNAT 1994. *Contracept. Fertil. Sex.*, **23**, 7–8, 490–493.

Beral, V. and Doyle, P. (1990) Report of the MRC Working Party on Children Conceived by In-vitro Fertilization. Births in Great Britain resulting from assisted conception, 1978–87. *Br. Med. J.*, **300**, 1229 – 1233.

Bernardini, L., Martini, E., Geraedts, J. *et al.* (1997) Comparison of gonosomal aneuploidy in spermatozoa of normal fertile men and those with severe male factor detected by in-situ hybridisation. *Mol. Hum. Reprod.*, **3**, 431–438.

Bonduelle, M., Desmyttere, S., Buysse, A. *et al.* (1994) Prospective follow-up study of 55 children born after subzonal insemination and intracytoplasmic sperm injection. *Hum. Reprod.*, **9**, 1765–1769.

Bonduelle, M., Legein, J., Derde, M.P. *et al.* (1995) Comparative follow-up study of 130 children born after ICSI and 130 children born after IVF. *Hum. Reprod.*, **10**, 3327–3331.

Bonduelle, M., Legein, J., Buysse, A. *et al.* (1996a) Prospective follow-up study of 423 children born after intracytoplasmic sperm injection. *Hum. Reprod.*, **11**, 1558–1564.

Bonduelle, M., Willikens, J., Buysse, A. *et al.* (1996b) Prospective study of 877 children born after intracytoplasmic sperm injection, with ejaculated epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. *Hum. Reprod.*, **11** (Suppl. 4), 131–159.

Bonduelle, M., Devroey, P., Liebaers, I. and Van Steirteghem, A. (1997) Commentary: Major defects are overestimated. *Br. Med. J.*, **7118**, 1265–1266.

Bonduelle, M., Joris, H., Hofmans, K. *et al.* (1998) Mental development of 201 ICSI children at 2 years of age. *Lancet*, **351**, 1553.

Bowen, J.R., Gibson, F.L, Leslie, G.I. *et al.* (1998) Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. *Lancet*, **351**, 1529–1534.

Devroey, P. (1998) Clinical application of new micromanipulative technologies to treat the male. *Hum. Reprod.*, **13** (Suppl. 3), 112–122.

Diemer, T. and Desjardins, C. (1999) Developmental and genetic disorders in spermatogenesis. *Hum. Reprod. Update*, **5**, 120–140.

Ferguson-Smith, M. (1983) Prenatal chromosomal analysis and its impact on the birth incidence of chromosomal disorders. *Br. Med. Bull.*, **3**, 355–364.

Gibas, L., Gruyic, S., Barr, M. and Jackson, L. (1987) A simple technique for obtaining high quality chromosome preparations from chorionic villus samples using Fdu synchronization. *Prenat. Diagn.*, **7**, 323–327.

Hoegerman, S., Pang, M.-G. and Kearns, W. (1995) Sex chromosome abnormalities after intracytoplasmic sperm injection Letter to the editor. *Lancet*, **346**, 1095.

Holmes, L.B. (1976) Congenital malformations. *N. Engl. J. Med.*, **295**, 204–207.

Hook, E. and Hamerton, J. (eds) (1977) *Population Cytogenetics: Studies in Humans*. Academic Press, New York, pp. 63–79.

Jacobs, P., Browne, C. Gregson, N. *et al.* (1992) Estimates of the frequency of chromosome abnormalities detectable in unselected newborns using moderate levels of banding. *J. Med. Genet.*, **29**, 103–106.

Kurinczuk, J. and Bower, C. (1997) Birth defects conceived by intracytoplasmic injection: an alternative interpretation. *Br. Med. J.*, **7118**, 1260–1264.

Lancaster, P., Shafir, E. and Huang, J. (1995) *Assisted Conception Australia and New Zealand 1992 and 1993*. AIHW National Perinatal Statistics Unit, Sydney, pp. 1–71.

Lechat, M.F., Dolk, H. (1993) Registries of congenital anomalies: Eurocat. *Envirn-Health-Persect.*, **101** (Suppl. 2), 153–157.

Le Lannou, D., Jézéquel, P., Blayau, M. *et al.* (1995) Obstructive azoospermia with agenesis of the vas deferens or with bronchiectasia (Young's syndrome): a genetic approach. *Hum. Reprod.*, **10**, 338–341.

Linden, M.G., Bender, B.G. and Robinson, A. (1997) Intrauterine diagnosis of sex chromosome aneuploidy. *Obstet. Gynecol.*, **87**, 468–475.

Liu, J., Lissens, W., Silber, S. *et al.* (1994) Birth after preimplantation diagnosis of the cystic fibrosis ÆF508 mutation by the polymerase chain reaction in human embryos resulting from intracytoplasmic sperm injection with epididymal sperm. *J. Am. Med. Assoc.*, **272**, 1858–1860.

Liu, J., Nagy, Z., Joris, H. *et al.* (1995a) Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. *Hum. Reprod.*, **10**, 2630–2636.

Liu, J., Lissens, W., Van Broekhoven, C. *et al.* (1995b) Normal pregnancy after preimplantation DNA diagnosis of a dystrophin gene deletion. *Prenat. Diagn.*, **15**, 351–358.

Morin, N.C., Wirth, F.H., Johnson, D.H. *et al.* (1989) Congenital malformations and psychosocial development in children conceived by in-vitro fertilization. *J. Pediatr.*, **115**, 222–227.

National Perinatal Statistics Unit and The Fertility Society of Australia (1992) *IVF and GIFT pregnancies, Australia and New Zealand, 1990.* National Perinatal Statistics Unit, Sydney.

New York State Department of Health (1990) *Congenital Malformations Registry Annual Report. Statistical Summary of Children Born in 1986 and Diagnosed Through 1988.*

Nielsen, J. and Wohlert, M. (1991) Chromosome abnormalities found among 34 910 newborn children: results from a 13-year study in Arhus, Denmark. *Hum. Genet.*, **87**, 81–83.

Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340**, 17–18.

Palermo, G., Colombero, L., Schattman, G. *et al.* (1996) Evolution of pregnancies and initial follow-up of newborns delivered after intracytoplasmic sperm injection. *J. Am. Med. Assoc.*, **276**, 1893–1897.

Pang, M., Zackowski, J., Hoegerman, S.F. *et al.* (1995) Detection by fluorescence *in situ* hybridisation of chromosome 7, 11, 12, 18, X and Y sperm abnormalities in of an in-vitro fertilization program. *J. Assist. Reprod. Genet.*, **12**, OC 105, 1995.

Rizk, B., Doyle, P., Tan, S.L. *et al.* (1991) Perinatal outcome and congenital malformations in in-vitro fertilization babies from the Bourn–Hallam group. *Hum. Reprod.*, **6**, 1259–1264.

Saunders, D.M. and Lancaster, P. (1987) Congenital malformations after in-vitro fertilization. *Am. J. Perinat.*, **6**, 252–255.

Schattman, G., Rosenwaks, Z., Berkely, A. *et al.* (1995) Congenital malformations in children born utilizing the assisted reproductive technologies. *Serono International Symposium on Genetics of Gametes and Embryos*, June 2–5, New York, Serono Publications, Rome, p. 36.

Smith, D.W. (1975) Classification, nomenclature, and naming of morphologic defects. *J. Pediatr.*, **87**, 162–164.

Society for Assisted Reproductive Technology (SART) and The American Fertility Society (1995) Assisted reproductive technology in the United States and Canada: results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. *Fertil. Steril.*, **64**, 13–21.

Staessen, C., Janssenswillen, C., Van den Abbeel, E. *et al.* (1993) Avoidance of triplet pregnancies by elective transfer of two good quality embryos. *Hum. Reprod.*, **8**, 1650–1653.

Staessen, C., Nagy, Z.P., Liu, J. *et al.* (1995) One year's experience with elective transfer of two good quality embryos in the human in-vitro fertilization and intracytoplasmic sperm injection programmes. *Hum. Reprod.*, **10**, 3305–3312.

Sutcliffe, A.G., Taylor, B., Grudzinskas, G. *et al.* (1998) Children conceived by intracytoplasmic sperm injection. *Lancet*, **352**, 578–579.

te Velde, E.R., van Baar, A.L. and van Kooij, R.J. (1998) Concerns about assisted reproduction. Commentary. *Lancet*, **351**, 1524–1525.

Tesarik, J. (1995) Sex chromosome abnormalities after intracytoplasmic sperm injection. Letter to the editor. *Lancet*, **346**, 1095.

Tesarik, P. and Sousa, M. (1995) Key elements of a highly efficient intracytoplasmatic sperm injection technique: Ca^{2+} fluxes and oocyte cytoplasmic dyslocation. *Fertil. Steril.*, **64**, 770–776.

Tesarik, J. and Mendoza, C. (1996) Genomic imprinting abnormalities: a new potential risk of assisted reproduction. *Mol. Hum. Reprod.*, **2**, 295–298.

Van Assche, E., Bonduelle, M., Tournaye, H. *et al.* (1996) Cytogenetics of infertile men. *Hum. Reprod.*, **11** (Suppl. 4), 1–26.

Van der Meulen, B.F. and Smrovsky, M. (1983) *BOS 2-30 Bayley ontwikkelingsschalen.* Swets and Zietlinger.

Van Steirteghem, A.C., Liu, J., Joris, H. *et al.* (1993a) Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum. Reprod.*, **8**, 1055–1060.

Van Steirteghem, A.C., Nagy, Z., Joris, H. *et al.* (1993b) High fertilization and implantation rates after intra-cytoplasmic sperm injection. *Hum. Reprod.*, **8**, 1061–1066.

Van Steirteghem, A., Nagy, Z., Liu, J. *et al.* (1993c) Intracytoplasmic sperm injection. *Assist. Reprod. Rev.*, **3**, 160–163.

Vandervorst, M., Tournaye, H., Camus, M. *et al.* (1997) Patients with absolutely immotile spermatozoa and intracytoplasmic sperm injection. *Hum. Reprod.*, **12**, 2429–2433.

Verma, R. and Babu, A. (eds) (1989) *Human Chromosomes; Manual of Basic Techniques.* Pergamon Press, New York, pp. 13–15.

Wennerholm, U.B., Bergh, C., Hamberger, L. *et al.* (1996) Obstetric and perinatal outcome of pregnancies following intracytoplasmic sperm injection. *Hum. Reprod.*, **11**, 1113–1119.

Wisanto, A., Magnus, M., Bonduelle, M. *et al.* (1995) Obstetric outcome of 424 pregnancies after intracytoplasmic sperm injection (ICSI). *Hum. Reprod.*, **10**, 2713–2718.

Yu, M., Yu, C., Yu, C. *et al.* (1986) Improved methods of direct and cultured chromosome preparations from chorionic villous samples. *Am. J. Hum. Genet.*, **38**, 576–581.