Human Reproduction vol.15 no.2 pp.402–409, 2000

Comparison of in-vitro development of embryos originating from either conventional in-vitro fertilization or intracytoplasmic sperm injection

John C.M.Dumoulin[1,3], Edith Coonen[1], Marijke Bras[1], Lucie C.P.van Wissen[1], Rosie Ignoul-Vanvuchelen[1], J.Marij Bergers-Jansen[1], Josien G.Derhaag[1], Joep P.M.Geraedts[2] and Johannes L.H.Evers[1]

[1]Department of Obstetrics and Gynaecology and [2]Department of Molecular Cell Biology and Genetics, Academic Hospital, University of Maastricht, Maastricht, The Netherlands

[3]To whom correspondence should be addressed at: IVF-Laboratory, Department of Obstetrics and Gynaecology, Academic Hospital Maastricht, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands

In this retrospective study on 1628 consecutive cycles performed during a period of 4 years, development *in vitro* is compared of embryos obtained after either conventional in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). At 39–42 h after insemination or injection, embryos obtained after ICSI were significantly ($P < 0.01$) further developed (mean cell number 3.48 ± 0.03) as compared with those obtained after IVF (3.22 ± 0.03), whereas after 63–66 h of in-vitro development this difference was no longer present (mean cell number 6.11 ± 0.15 versus 6.09 ± 0.13 respectively). Culture of surplus embryos obtained after IVF resulted in a significantly higher ($P < 0.001$) mean incidence of blastocyst formation per cycle as compared with the ICSI group (31.8 ± 1.9 versus 23.0 ± 1.4 respectively). Blastocysts from both groups consisted of comparable numbers of cells. Blastocyst formation was also significantly higher when embryos were cultured in groups (31.2 ± 1.8) compared to single culture (23.1 ± 1.5; $P < 0.01$), in human tubal fluid (HTF) medium (29.2 ± 1.7) compared with IVF-50^{TM} medium (24.2 ± 1.6; $P < 0.01$), and when they were cultured under 5% O_2 (30.3 ± 1.5) compared with 20% O_2 (21.7 ± 1.7; $P < 0.01$). In all culture conditions used, the mean incidence of blastocyst formation per cycle showed comparable differences in favour of the IVF group as compared with the ICSI group.

Key words: human blastocysts/ICSI/preimplantation development

Introduction

Since 1992, when the first four pregnancies were reported (Palermo *et al.*, 1992), intracytoplasmic sperm injection (ICSI) has rapidly become the assisted reproduction procedure of choice for treatment of severe male-factor infertility (Van Steirteghem *et al.*, 1993, 1998). In a recent review, the ESHRE Task Force on ICSI (Tarlatzis and Bili, 1998), reported that in 1995 a total of 23 932 ICSI cycles were performed worldwide by 101 centres. The success rate obtained by ICSI is high, illustrated by a viable pregnancy rate of 21% per cycle reported in the above study (Tarlatzis and Bili, 1998). The procedure seems also to be safe, as the incidence of major and minor congenital malformations in the group of children born after ICSI is similar to those reported for in-vitro fertilization (IVF), which is in the range observed in the general population (Bonduelle *et al*., 1996; Tarlatzis and Bili, 1998). The incidence of de-novo chromosomal aberrations in children born after ICSI is slightly higher than expected in the general population, but this fact is probably linked directly to the characteristics of the infertile men treated rather than to the ICSI procedure itself (Bonduelle *et al*., 1996).

In spite of these reassuring results, concern has been raised about the potential dangers of the ICSI procedure. Theoretically, both the injection technique itself as well as the possible injection of abnormal spermatozoa can affect oocyte and subsequent embryo quality. During injection, it cannot be avoided that a small quantity of medium, which contains potentially harmful components, is injected into the oocyte. Also, physical damage to cytoplasmic structures can possibly be inflicted by a traumatic injection.

In view of these possible risks, it may be postulated that zygotes originating from ICSI have a higher incidence of sublethal cell damage and subsequent impaired embryonic development as compared to zygotes originating from conventional IVF. In this study, we present a retrospective analysis of the development *in vitro* of embryos derived from conventional IVF in comparison with ICSI. Developmental stages and morphological qualities of surplus embryos at the second and third day after insemination or injection, and embryonic development to the blastocyst stage were assessed.

Materials and methods

Patients

During a period of 4 years, 1628 consecutive IVF and ICSI treatment cycles performed in a total of 920 patients were included in this study. ICSI treatment was performed in cases of male infertility (478 patients), and in cases of failed fertilization in previous IVF cycles (33 patients). Male infertility was defined as a progressively motile sperm concentration of $<3\times10^6$/ml in combination with <5% morphologically normal spermatozoa, evaluated using strict criteria (Enginsu *et al.*, 1992). Males presenting on several occasions with severe oligozoospermia ($<5\times10^6$/ml total sperm concentration) had cell karyotypes performed, and if an abnormal karyotype was found, were excluded from treatment. Only ejaculated spermatozoa were used for ICSI. A conventional in-vitro insemination procedure was

used in a total of 409 patients (tubal factor infertility in 46%, unexplained infertility in 42%, mild male infertility in 3%, and other causes of infertility in 8% of the cases respectively).

The stimulation protocol used has been described previously (Land *et al*., 1996). In summary, the gonadotrophin-releasing hormone (GnRH) agonist nafarelin (Synarel; Searle BV, Maarssen, The Netherlands) was used in combination with human menopausal gonadotrophin (HMG, Pergonal; Serono, Amsterdam, The Netherlands; or Humegon; Organon, Oss, The Netherlands) to stimulate multiple follicular development. Follicle growth was monitored by ultrasound and 5000 IU of human chorionic gonadotrophin (HCG, Pregnyl; Organon) was given as soon as the dominant follicle was judged to be mature (>18 mm diameter), to induce final follicular and oocyte maturation. Ultrasound-guided oocyte retrieval was performed 34–36 h after HCG administration. Insemination or ICSI was performed ~5 h after oocyte retrieval.

IVF procedure

Oocytes were inseminated with a calculated volume of a swim-up sperm suspension which resulted in a concentration of ~50 000/ml progressively moving spermatozoa as described (Enginsu *et al*., 1992). After incubation for 18–20 h, corona cells that remained attached to the oocyte were removed using drawn Pasteur pipettes. Oocytes were checked for the presence of pronuclei, washed once, and transferred to fresh medium.

ICSI procedure

For the ICSI procedure, a previously published protocol was followed (Van Steirteghem *et al*., 1993). Briefly, after oocyte retrieval, cells of the cumulus and corona were removed by incubation for 30–60 s in HEPES-buffered human tubal fluid (HTF) medium (Quinn *et al*., 1985) supplemented with 9% (v/v) of a pasteurized human plasma protein solution (PPS) obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands) and with 80 IU hyaluronidase/ml (type VIII; Sigma Chemical Co., St Louis, MO, USA, cat. no. H-3757), followed by aspiration of the oocytes in and out of hand-drawn glass pipettes. Oocytes were rinsed several times in culture medium and incubated until the injection procedure. Micro-injection pipettes used were purchased from a commercial supplier (Humagen; Gynotech, Malden, The Netherlands). ICSI was performed using hydraulic remote control joystick manipulators (Narishige; Paes Nederland BV, Zoeterwoude, The Netherlands) mounted on an Olympus IX-70 inverted microscope equipped with a Linkam heated stage (Paes Nederland BV). Just before the ICSI procedure, 1 µl of a seminal plasma-free sperm suspension was pipetted into a 5 µl droplet of a 10% polyvinylpyrrolidone (PVP) solution (MediCult; Innogenetics, Nijmegen, The Netherlands). Injection was performed in 15 µl droplets of HEPES-buffered HTF solution. All droplets were covered with embryo-tested mineral oil (Sigma, cat. no. M-8410). For ICSI, only oocytes at metaphase of the second meiotic division were used. Only motile spermatozoa were used for injection, and care was taken to select spermatozoa that were morphologically apparently normal, if available. They were immobilized by touching the tail with the micro-injection needle and injected head-first into the oocyte with the polar body at either 6 or 12 o'clock to avoid the passage of the pipette through the cytoplasmic region containing the meiotic spindle with the oocyte chromosomes. After the pipette was inserted into the oocyte, gentle suction was applied until the breakage of the oocyte membrane was observed. Care was taken to aspirate as little as possible cytoplasm into the injection pipette, and the spermatozoon was injected into the oocyte with the smallest volume of the PVP solution possible.

Culture procedures

Oocytes and embryos were cultured in 50 µl and 20 µl droplets respectively, under mineral oil. During the first half of the study period, embryos were cultured separately, in order to study development of each embryo individually in relation to features of the injection procedure. These results will be published separately. During the second half of the study period, embryos were cultured communally with a maximum of five embryos per drop.

During most of the present study period, two other studies concerning culture procedures were performed. In one study, oocytes and embryos were alternately allocated per set of two treatment cycles to culture either under ambient (≈20%) or reduced (5%) O_2 (Dumoulin *et al.*, 1999). After ending this study, 5% O_2 was used for all subsequent cycles. In the second study, oocytes and embryos from each consecutive treatment cycle were alternately allocated to the use of either of two culture media: a ready-to-use commercially available medium: IVF-50™ (Scandinavian IVF Science AB, Göteborg, Sweden) or 'in-house' prepared HTF medium (Quinn *et al.*, 1985), supplemented with 9% (v/v) PPS. During a short period in the beginning of the present study period, HTF medium was used for all cycles. By this allocation procedure, a random distribution of treatment cycles over the four different combinations of culture techniques was ensured and the studies were independent.

After incubation for 18–20 h, the oocytes were checked for the presence of pronuclei as proof of fertilization, washed once and, after transfer to fresh medium, cultured for another day. At the second and third day after oocyte recovery, developmental stages and morphological aspects of all embryos originating from normally fertilized oocytes were assessed under an inverted microscope with ×200 magnification according to published criteria (Bolton *et al*., 1989). For each embryo, an embryo score was calculated by multiplying the morphological grade (values of 1 for poorest and 4 for best grade) by the number of blastomeres (Steer *et al.*, 1992). For each treatment cycle, the score of all embryos was averaged to obtain a mean embryo score (MES). Embryo transfer was routinely performed on day 2 after ovum retrieval, or, in a minority of the cases, on day 3 for reasons of convenience. If available, two or three embryos, depending on the developmental stage and morphological appearance of the embryos, as well as on the age of the patient, were transferred. After transfer, any supernumerary embryos were cultured until the third day after ovum retrieval. Cryopreservation of supernumerary embryos was performed on the morning of the third day if one or more embryos had reached the 8-cell stage, and if they were of good morphological quality (grades 3 and 4, Bolton *et al*., 1989).

Culture of human surplus embryos

If cryopreservation was not deemed feasible, surplus embryos originating from normally fertilized oocytes were used in the present study, or in two other small studies running in our centre (Dreesen *et al*., 1998; Dumoulin, *et al*., 1998). These studies have been approved by the local Ethics Committee. To avoid selection bias, surplus embryos of all treatment cycles during prearranged periods were used in only one study. In the present study, surplus embryos were left in their original culture medium for another 2 or 3 days. Developmental stages were recorded on each day of in-vitro development. On the morning of day 5 after ovum retrieval, surplus embryos that cavitated to form blastocyst-like structures (defined as a rim of cells surrounding a large cavity of extracellular fluid accumulated within the embryo) were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described earlier (Coonen *et al*., 1994). The number of nuclei stained with DAPI was taken as the number of cells of the embryo. All other embryos, including those that had only just started to form a small blastocoelic cavity, were

Table I. Characteristics of the two study groups treated with IVF or ICSI

Treatment procedure	IVF	ICSI
Number of cycles	653	975
Number of patients	409	511
Age (years)[a]	33.3 ± 0.1	32.7 ± 0.1[b]
First attempts[c]	307 (47)	452 (46)
Duration of infertility (years)[a]	5.4 ± 0.1	5.6 ± 0.1
Type of infertility[d]		
Primary	253 (62)	388 (76)[e]
Secondary	156 (38)	123 (24)[e]
Gas phase		
5% O_2		
no. of cycles[c]	353 (54)	565 (58)
no. of cycles with at least one surplus embryo cultured[f]	148 (42)	262 (46)
20% O_2		
no. of cycles[c]	300 (46)	410 (42)
no. of cycles with at least one surplus embryo cultured[f]	126 (42)	167 (41)
Culture medium		
IVF-50™		
no. of cycles[c]	307 (47)	468 (48)
no. of cycles with at least one surplus embryo cultured[f]	137 (45)	203 (43)
HTF		
no. of cycles[c]	346 (53)	507 (52)
no. of cycles with at least one surplus embryo cultured[f]	138 (40)	221 (44)
Day of embryo transfer[g]		
Day 2	493 (82)	762 (82)
Day 3	108 (18)	167 (18)

HTF = human tubal fluid.
[a]Mean number of years ± SEM with regard to the female partner at the time of ovum retrieval.
[b,e]Significant difference when compared with the group treated with conventional IVF: [b]unpaired Student's *t*-test, $P < 0.01$; [e]χ^2-test, $P < 0.001$.
[c]Values in parentheses are percentages of total number of treatment cycles.
[d]Values in parentheses are percentages of total number of patients.
[f]Values in parentheses are percentages of total number of treatment cycles in the indicated culture condition.
[g]Values in parentheses are percentages of total number of embryo transfers.

cultured for another day and were subsequently fixed on day 6 when they had developed to the full blastocyst stage. Fixation of embryos was performed only when the patients had given written consent.

Analysis of results

Data were analysed by χ^2 test or unpaired Student's *t*-test where appropriate.

Results

Patients' characteristics and other factors are summarized in Table I. No significant differences were found between the two groups for most of the parameters studied, except for the mean age (patients treated with ICSI were slightly younger in our programme), and the type of infertility (significantly more patients in the ICSI group were treated for primary infertility). Male factor infertility is predominantly congenital, as opposed to female factor infertility, where the condition is partially acquired, hence giving rise to a higher proportion of secondary infertility cases in the IVF group. The distribution over the different culture conditions of treatment cycles and cycles in which surplus embryos were cultured was not significantly different.

As can be seen in Table II, the fertilization rate expressed as a percentage of the total number of oocytes collected per cycle was significantly lower in the ICSI group as compared with the IVF group. On the second day of development, embryos obtained after ICSI were significantly further developed, whereas on the third day this difference was no longer present. Treatment outcomes in terms of implantation and pregnancy rates (Table III) were significantly better in the ICSI group. The mean number of embryos transferred per cycle was slightly but significantly lower in the ICSI group. This can be explained by the fact that the choice between transferring two or three embryos in our transfer policy was mainly dictated by the age of the patient and the developmental stage of the embryos, resulting in more cases of only two embryos being transferred in the ICSI group.

In 274 of 653 IVF cycles (42%) and in 429 of 975 ICSI cycles (44%), at least one surplus embryo was cultured to day 5 or 6. In the other 925 cycles, either no fertilization took place, available embryos were all transferred, surplus embryos were cryopreserved, or they were used in another study. In Table IV, development of surplus embryos to the blastocyst stage is summarized. In contrast to the better results obtained in the ICSI group with respect to embryonic development on the second day, as well as implantation and pregnancy rates, embryonic development of surplus embryos *in vitro* to the blastocyst stage was significantly lower in the ICSI group as compared with the IVF group. In the ICSI group, slightly but significantly fewer surplus embryos were obtained per cycle (Table IV). This can be explained by the fact that the fertilization rate in ICSI cycles was significantly lower than

Table II. Fertilization and embryo development at day 2 and day 3 of in-vitro development in the IVF and ICSI groups

Treatment procedure	IVF	ICSI
Cycles (*n*)	653	975
No. of oocytes per cycle[a]	9.6 ± 0.2	9.6 ± 0.2
No. of oocytes injected[b,c]	–	8.3 ± 4.5
Fertilization rate per cycle[a,d]	0.64 ± 0.01	0.57 ± 0.01[e]
Fertilization rate per cycle per injected oocytes[b,f]	–	0.67 ± 0.26
Embryonic development at day 2[g]		
Mean cell number per embryo[a]	3.22 ± 0.03	3.48 ± 0.03[e]
Cleavage to ⩾4-cell stage[a,h]	42.0 ± 1.2	51.3 ± 1.4[e]
Mean morphological grade per embryo[a,i]	2.45 ± 0.02	2.46 ± 0.02
Morphologically normal embryos[a,j]	53.3 ± 1.3	53.7 ± 1.1
Mean embryo score per cycle (MES)[a]	7.91 ± 0.11	8.56 ± 0.12[e]
Embryonic development at day 3[k]		
Mean cell number per embryo[a]	6.11 ± 0.15	6.09 ± 0.13
Cleavage to ⩾6-cell stage[a,h]	43.3 ± 3.0	41.1 ± 2.4
Mean morphological grade per embryo[a,i]	2.52 ± 0.05	2.44 ± 0.05
Morphologically normal embryos[a,j]	55.0 ± 3.0	51.6 ± 2.5
Mean embryo score per cycle (MES)[a]	15.5 ± 0.6	14.8 ± 0.5

[a]Results are expressed as means ± SEM.
[b]Results are expressed as means ± SD.
[c]Only oocytes in metaphase II at the time of ICSI were injected.
[d]Expressed as percentage: number of fertilized oocytes/total number of oocytes per cycle.
[e]Significant difference when compared with the group treated with conventional IVF: unpaired Student's *t*-test ($P < 0.01$).
[f]Expressed as percentage: number of fertilized oocytes/total number of injected oocytes per ICSI cycle.
[g]Assessment of developmental stage and morphological aspect of all embryos at 39–42 h after insemination or injection.
[h]Percentage of embryos developed to the indicated cell stage or further per cycle.
[i]Values of 1 for poorest and 4 for best morphological grade.
[j]Percentage of embryos of good morphological quality (grades 3 and 4) per cycle.
[k]Assessment of developmental stage and morphological aspect of all embryos at 63–66 h after insemination or injection; only the 275 cycles in which the embryo transfer was performed on day 3 (i.e. all embryos were cultured until day 3) were taken into account.

Table III. Comparison of pregnancy and implantation rates in the IVF and ICSI groups

Treatment procedure	IVF	ICSI
Cycles (*n*)	653	975
Embryo transfers[a]	601 (92.0)	929 (95.3)[b]
Pregnancies (%)[a,c]	158 (24.2)	282 (28.9)[b]
Ongoing (⩾12 weeks) pregnancies (%)[a,d]	129 (19.8)	249 (25.5)[b]
Multiple pregnancies (%)[d,e]	38 (29.5)	80 (32.1)
Mean no. of embryos transferred per cycle (± SEM)	2.61 ± 0.03	2.49 ± 0.02[f]
Implantation rates in all cycles		
Total no. of transferred embryos	1571	2312
Implantation sites (%)[d,g]	207 (13.2)	381 (16.5)[b]
Viable fetuses (⩾12 weeks) (%)[d,g]	171 (10.9)	333 (14.4)[b]
Implantation rates in pregnancy cycles		
Total no. of transferred embryos	423	728
Implantation sites (%)[d,g]	207 (48.9)	381 (52.3)
Viable fetuses (⩾12 weeks) (%)[d,g]	171 (40.4)	333 (45.7)

[a]Values in parentheses are percentages of total number of treatment cycles.
[b,f]Significant difference when compared with the group treated with conventional IVF: [b]χ^2-test ($P < 0.05$), or [f]unpaired Student's *t*-test ($P < 0.01$).
[c]Positive urinary pregnancy test (sensitivity 50 IU/l HCG) at 16–18 days after ovum retrieval.
[d]As determined by ultrasound at 5 and ⩾12 weeks after ovum retrieval.
[e]Values in parentheses are percentages of total number of ongoing pregnancies.
[g]Values in parentheses are percentages of total number of embryos transferred.

in IVF cycles, resulting in a lower mean number of embryos obtained in ICSI cycles (Table II). It could be postulated that the observed differences in blastocyst formation would be influenced by this fact, as after transfer of two or three of the best quality embryos, the fewer supernumerary embryos available in the ICSI group could be expected to be of inferior morphological quality. However, as can be seen in Table IV, no differences in either cell stage or morphological quality

Table IV. Development to the blastocyst stage of surplus embryos in the IVF and ICSI groups

Treatment procedure	IVF	ICSI
Cycles with at least one surplus embryo cultured	274	429
Total number of surplus embryos cultured	1253	1662
No. of surplus embryos per cycle[a]	4.57 ± 0.20	3.88 ± 0.13[b]
Development of surplus embryos at day 3		
Mean cell number per embryo[a]	4.00 ± 0.12	3.91 ± 0.10
Mean morphological grade per embryo[a,c]	1.70 ± 0.04	1.65 ± 0.04
Development of surplus embryos at day 5 or 6		
Incidence of blastocyst formation per cycle[a]	31.8 ± 1.9	23.0 ± 1.4[d]

[a]Results are expressed as means ± SEM.
[b,d]Significant differences (unpaired Student's *t*-test) when compared with the group treated with conventional IVF: [b]$P < 0.01$, [d]$P < 0.001$.
[c]Values of 1 for poorest and 4 for best morphological grade.

Table V. Development to the blastocyst stage of surplus embryos in the IVF and ICSI groups: effect of the number of surplus embryos available for culture per cycle

Surplus embryos cultured per cycle (*n*)	IVF			ICSI		
	No. of cycles with surplus embryos	Mean no. of surplus embryos per cycle	Incidence of blastocyst formation per cycle	No. of cycles with surplus embryos	Mean no. of surplus embryos per cycle	Incidence of blastocyst formation per cycle
1–5	192	2.68 ± 0.09	27.6 ± 2.4	339	2.68 ± 0.07	21.9 ± 1.6[a]
6–10	60	7.43 ± 0.17	41.7 ± 3.5	77	7.53 ± 0.13	26.2 ± 2.7[b]
>10	22	13.32 ± 0.48	41.5 ± 5.9	13	12.75 ± 0.58	30.1 ± 5.9
Total	274	4.57 ± 0.20	31.8 ± 1.9	429	3.88 ± 0.13[c]	23.0 ± 1.4[b]

[a,b,c]Significant differences (unpaired Student's *t*-test) when compared with the group treated with conventional IVF: [a]$P < 0.05$, [b]$P < 0.001$, [c]$P < 0.01$.

were found between the surplus embryos of the IVF and ICSI groups. The mean number of cells per embryo and the quality of the surplus embryos was low when compared with the embryos of those cycles in which all embryos were cultured until the third day of development (Table II), which was only to be expected in view of the fact that the best embryos were transferred or cryopreserved. It must be mentioned that the group of surplus embryos consisted of all supernumerary embryos originating from normally fertilized oocytes which were not transferred or cryopreserved, including also zygotes that were fragmented, degenerated, or remained uncleaved. Furthermore, when we adjusted our data by grouping the cycles according to the number of surplus embryos that were cultured (Table V), it was seen that the difference in the mean number of surplus embryos cultured per cycle was mainly the result of the fact that more ICSI cycles resulted in only a few surplus embryos being cultured as compared to the IVF group [339 of 429 ICSI cycles (79%) versus 192 of 274 IVF cycles (70%)]. In all subgroups, the mean incidence of blastocyst formation per cycle turned out to be higher in the IVF groups as compared to the ICSI groups, although in the subgroups of >10 surplus embryos per cycle, this difference was not statistically significant, probably due to the relatively small group sizes.

Besides the origin of surplus embryos (IVF or ICSI), other culture conditions that were used during the present study period were shown to influence blastocyst formation (Table VI). Blastocyst formation was significantly higher when embryos were cultured in groups in comparison with single culture, in HTF medium as compared with IVF-50™ medium, and when they were cultured under 5% O_2 compared with 20% O_2.

Of the embryos that developed a blastocoele, 326 of a total of 407 (80%) embryos resulting from a conventional IVF treatment, and 303 of a total of 389 (78%) embryos resulting from an ICSI treatment were fixed (Table VII). Reasons for not fixing were either that no consent was obtained from the patients, or that embryos classified as early blastocysts on day 5 and subsequently cultured for another day were found to be completely degenerated on day 6. The mean number of cells per blastocyst was not significantly different between the groups.

Discussion

The overall results of our IVF and ICSI treatment programme are similar to those of large series previously published. After ICSI a fertilization rate of 64–65%, expressed in relation to the number of metaphase II oocytes injected, has been reported (Tarlatzis and Bili, 1998; Van Steirteghem *et al.*, 1998). Pregnancy rates per cycle were reported to be 28% (Tarlatzis and Bili, 1998) and 34% (Van Steirteghem *et al.*, 1998).

Compared with the IVF group, embryonic development in our study was more advanced in the ICSI group on the second

Table VI. Development to the blastocyst stage of surplus embryos in the in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) groups: effect of different culture methods as possible confounding factors

Culture method[a]	IVF		ICSI		Total	
	No. of cycles with surplus embryos	Incidence of blastocyst formation per cycle	No. of cycles with surplus embryos	Incidence of blastocyst formation per cycle	No. of cycles with surplus embryos	Incidence of blastocyst formation per cycle
Single or group culture						
Single	159	28.6 ± 2.4[b]	202	17.6 ± 1.7[c,d]	361	23.1 ± 1.5[c]
Group	115	37.4 ± 3.1	227	28.2 ± 2.1[e]	342	31.2 ± 1.8
Gas phase						
5% O_2	148	37.9 ± 2.7[c]	262	26.0 ± 1.8[b,e]	410	30.3 ± 1.5[c]
20% O_2	126	24.7 ± 2.7	167	19.4 ± 2.1	293	21.7 ± 1.7
Culture medium						
IVF-50™	136	30.2 ± 2.7	208	20.2 ± 1.9[b,d]	344	24.2 ± 1.6[c]
HTF	138	33.8 ± 2.8	221	26.4 ± 2.0	359	29.2 ± 1.7

[a]For an explanation of the different culture methods and the distribution of the treatment cycles over the culture methods, see Materials and methods.
[b,c]Within a column, significant differences (unpaired Student's *t*-test) between each pair of compared culture methods: [b]$P < 0.05$, [c]$P < 0.01$.
[d,e]Within a row: significant differences (unpaired Student's *t*-test) between the group treated with conventional IVF and the group treated with ICSI: [d]$P < 0.01$, [e]$P < 0.05$.

Table VII. Mean cell number of blastocysts in the IVF and ICSI groups

Treatment procedure	IVF	ICSI[a]
Fixation of blastocysts on day 5		
Blastocysts fixed	163	145
Fixation successful[b]	143 (88)	114 (79)
Blastocysts consisting of at least 25 cells[c]	107 (75)	85 (75)
Cells per confirmed blastocyst[d]	46.9 ± 1.6	43.1 ± 1.5
Fixation of blastocysts on day 6		
Blastocysts fixed	163	158
Fixation successful[b]	149 (91)	144 (91)
Blastocysts consisting of at least 25 cells[c]	111 (74)	105 (73)
Cells per confirmed blastocyst[d]	55.9. ± 2.2	53.5 ± 2.1

[a]No significant differences were noted between the two groups.
[b]All individual nuclei of the blastocysts could be counted; values in parentheses are percentages of total number of blastocysts fixed.
[c]Values in parentheses are percentages of successfully fixed blastocysts.
[d]Expressed as mean number ± SEM of cells per embryo, only blastocysts with a minimum of 25 cells were taken into account.

day of development, which can be explained by the findings of Nagy *et al.* that the pronuclear development and cleavage to the 2-cell stage take place several hours earlier after ICSI than after IVF (Nagy *et al*., 1998). One day later the embryos derived from ICSI seem to have lost their head start advantage (Table II), whereas their late preimplantation development was even impaired (Table IV), relative to the IVF group. These results confirm in a large series of consecutive treatment cycles the earlier reports on a lower rate of blastocyst formation of embryos derived from ICSI treatment as compared with IVF. Eighty-five blastocysts out of 173 supernumerary embryos obtained in 45 cycles after IVF (47%), and 31 blastocysts out of 120 supernumerary embryos obtained in 46 cycles after ICSI (27%) have been reported (Shoukir *et al*., 1998). Also several preliminary reports have been published showing lower blastulation rates of embryos obtained after ICSI (abstract by Peters and Catt, 1998; abstract by Moreno *et al*., 1998). Also in the cat, indications of impaired blastocyst development of embryos obtained after ICSI were found (Pope *et al.*, 1998).

As during the 4-year period described in this study we used several culture conditions that are known to affect human blastocyst formation, we carefully examined them for their impact on the comparison of development between IVF- and ICSI-derived embryos. It has been shown in several studies that preimplantation embryos benefit from grouped culture (Moessner and Dodson, 1995; Almagor *et al*., 1996). These findings were confirmed in our study, although our data must be viewed with caution as they were collected in two different study periods. As explained earlier, it was not our intention to study the effect of group culture on embryonic development. The embryos were cultured singly in the first half of the study period in order to examine development of each embryo individually in relation to features of the injection procedure. A second culture condition that affects embryonic development *in vitro* is the use of a low oxygen concentration, which has been shown to be beneficial for development to the blastocyst stage in the human (Dumoulin *et al*., 1999), and in various animal species, e.g. mouse, hamster, rabbit, rat, pig, sheep, cow, and goat (for references, see Dumoulin *et al*., 1999). The third culture condition known to affect development to the blastocyst stage is the culture medium used (Jones *et al*., 1998; Martin *et al*., 1998). In our study, significantly more embryos developed to the blastocyst stage when cultured in HTF medium as compared with IVF-50™ medium. It must be noted that both culture media used in the present study are probably not as suitable as the recently developed sequential media to support the development of viable blastocysts *in vitro* (Jones *et al.*, 1998). The use of these suboptimal media for blastocyst development, as well as the use of only minor quality surplus embryos (being all untransferred embryos that were considered unsuitable for cryopreservation because of their major fragmentation and/or low cell number at the third day of development) can explain the rather low number of cells per blastocyst in the present study (54 and 56 on day 6, Table VII). The mean cell number of human blastocysts on day 6 of development has been shown to be related to culture conditions used (e.g. co-culture and type of medium) and quality of embryos included

in the study, and consequently varies considerably between different reports and between different study groups (41–73, Hardy *et al*., 1995; 42 and 87, Vlad *et al*., 1996; 64, Evsikov and Verlinsky, 1998; 44–286, Fong and Bongso, 1998; 61 and 74, Devreker *et al*., 1999; 54, Jurisicova *et al.*, 1999). However, the purpose of our study was not to achieve high blastulation rates *per se*, but to study differences in blastulation rates between the IVF and ICSI groups. As our experimental design ensured an equal distribution of the treatment cycles over the different culture conditions, resulting also in a comparable random distribution of surplus embryos (Table I), the use of these different culture techniques does not influence the comparison of the blastocyst formation of surplus embryos resulting from IVF or ICSI. Furthermore, the mean incidence of blastocyst formation per cycle showed similar differences in favour of the IVF group as compared with the ICSI group in all culture conditions used.

The discrepancy between our findings in the ICSI group of on the one hand higher pregnancy and implantation rates, and on the other hand impaired embryonic development to the blastocyst stage *in vitro* seems hard to explain. A relationship has been suggested between poor sperm quality and embryo quality, i.e. poor embryo morphology on the second day of development (Parinaud *et al*., 1993), and impaired blastocyst formation (Janny and Ménézo, 1994). It cannot be ruled out, however, that the observed effects in our studies using a conventional IVF technique were caused by a relative delay in the fertilization process which does not apply to the situation in our ICSI group in which fertilization is forced by injection. However, as discussed recently (Edwards, 1999), the ICSI technique harbours several risks and potential dangers from different sources so that one might expect that embryonic development would be impaired. First of all, the use of spermatozoa from men with severe sperm defects must be taken into consideration. Several lines of evidence have indicated that semen from infertile men contains an increased frequency of spermatozoa with genetic defects, and it was recently shown (Twigg *et al*., 1998) that such abnormal spermatozoa are well capable of forming normal pronuclei after ICSI. Spermatozoa from infertile men having a normal somatic karyotype show a slightly but significantly increased frequency of chromosomal abnormalities (Moosani *et al*., 1995). A recent study (Pang *et al*., 1999) reported a total aneuploidy frequency in the range of 33–74% in the spermatozoa from nine randomly selected patients with oligoasthenoteratozoospermia undergoing ICSI, while the total aneuploidy in spermatozoa of controls ranged from 4.1 to 7.7%. In an earlier study (Lee *et al*., 1996) it was demonstrated that the incidence of numerical and structural chromosome abnormalities in spermatozoa with highly aberrant head morphologies was about four times higher than in those with morphologically normal heads. Although we took care to select apparently morphologically normal spermatozoa, it could not be avoided in cases of extreme oligoteratozoospermia that only morphologically abnormal spermatozoa were found and injected. Other studies found higher DNA damage in spermatozoa from infertile males. It has been demonstrated (Hughes *et al*., 1996) that, although the baseline level of DNA damage in normozoospermic fertile men was similar to that in asthenozoospermic infertile men, the latter group was more susceptible to damage. Also oxidative DNA damage (Kodama *et al*., 1997; Twigg *et al*., 1998) as well as sperm chromatin anomalies (Sakkas *et al*., 1996) were found to be increased in spermatozoa of infertile male patients. Furthermore, defects of the sperm centrosome may lead to abnormal or irregular cleavage of embryos (Sathananthan, 1998). Such defects were observed more often in asthenozoospermic sperm samples (Sathananthan, 1998).

Besides the fact that an abnormal spermatozoon may be used for ICSI, the intracytoplasmic injection technique itself might lead to fertilization abnormalities in the zygote. It has been demonstrated in the rhesus monkey that ICSI resulted in abnormal sperm decondensation (Hewitson *et al*., 1999). Furthermore, these workers demonstrated the variable position of the second meiotic spindle in relation to the first polar body, which could result in damage to the spindle during the ICSI procedure as the polar body is used for microinjection targeting (Hewitson *et al*., 1999).

Thus it can be postulated that the reasons for the impaired blastocyst development of embryos obtained after ICSI are that the spermatozoa used for ICSI are selected from sperm populations with relatively high incidences of fragmented DNA and chromosomal abnormalities, and/or that some oocytes are injured sublethally during the injection procedure. Both factors would result in a slightly higher incidence of embryos that would be abnormal from the start, resulting in the noted decreased incidence of blastocyst formation, but also in a higher chance of transferring a non-viable embryo into the uterus. The fact that the pregnancy and implantation rates in the ICSI group were nevertheless higher than in the IVF group can be explained by the possibly higher fertility potential of the female partners in the ICSI group as compared with those of the IVF group. Patients undergoing ICSI are selected on the basis of severe male infertility and it can be expected that most of the female partners are normally fertile. Indeed, it has been demonstrated (Le Lannou *et al*., 1995) that conception rates following donor insemination of women whose partners are azoospermic were significantly higher than those for women whose husbands had only moderate oligozoospermia, reflecting compromised fertility in the female partners of moderate oligozoospermic males. Furthermore, in cases of unexplained infertility, which make up 42% of our IVF group, subtle oocyte dysfunction (Hull *et al*., 1998) and defective uterine receptivity (Lessey *et al*., 1995) are demonstrated, leading to a reduction (Lessey *et al*., 1995) are demonstrated, leading to a reduction In conclusion, we demonstrate in this study that embryos obtained after ICSI have a decreased potential to develop into blastocysts. Uncompromised fertility in the partners of severely fertility impaired men, relative to the female partners of IVF couples, appears to make up for this developmental impairment of ICSI embryos.

Acknowledgements

The authors thank the following members of the IVF-team, Academic Hospital Maastricht, Maastricht, The Netherlands, for their clinical

and technical assistance: Gerard A.J. Dunselman, M.D.; Tanja Gijsen, M.D.; Germaine Kengen, R.N.; Renate Kuijper, R.N.; Jacques Maas, M.D.; Chantal J.J. Delnoy-Meijers, M.T.; Nienke Muntjewerff, M.D.; Jolande A. Land, M.D.; and Anja Winkens-Tersteeg, R.N.

References

Almagor, M., Bejar, C., Kafka, I. and Yaffe, H. (1996) Pregnancy rates after communal growth of preimplantation human embryos *in vitro*. *Fertil. Steril.*, **66**, 394–397.

Bolton, V.N., Hawes, S.M., Taylor, C.T. and Parsons, J.H. (1989) Development of spare human preimplantation embryos *in vitro*: an analysis of the correlations among gross morphology, cleavage rates, and development to the blastocyst. *J. In vitro Fert. Embryo Transf.*, **6**, 30–35.

Bonduelle, M., Wilikens, A., Buysse, A. *et al.* (1996) Prospective follow-up study of 877 children born after intracytoplasmic sperm injection (ICSI), with ejaculated epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. *Hum. Reprod.*, **11** (Suppl. 4), 131–155.

Coonen, E., Dumoulin, J.C.M., Ramaekers, F.C.S. *et al.* (1994) Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescence in-situ hybridization. *Hum. Reprod.*, **9**, 533–537.

Devreker, F., Van den Bergh, M., Biramane, J. *et al.* (1999) Effects of taurine on human embryo development *in vitro*. *Hum. Reprod.*, **14**, 2350–2356.

Dreesen, J.C.F.M., Bras, M., de Die-Smulders, C. *et al.* (1998) Preimplantation genetic diagnosis of spinal muscular atrophy. *Mol. Hum. Reprod.*, **4**, 881–885.

Dumoulin, J.C.M., Bras, M., Coonen, E. *et al.* (1998) Effect of Ca^{2+}/Mg^{2+}-free medium on the biopsy procedure for preimplantation genetic diagnosis and further development of human embryos. *Hum. Reprod.*, **13**, 2880–2883.

Dumoulin, J.C.M., Meijers, C.J.J., Bras, M. *et al.* (1999) Effect of oxygen concentration on human in-vitro fertilization and embryo culture. *Hum. Reprod.*, **14**, 465–469.

Edwards, R.G. (1999) Widening perspectives of intracytoplasmic sperm injection. *Nature Med.*, **5**, 377–378.

Enginsu, M.E., Pieters, M.H.E.C., Dumoulin, J.C.M. *et al.* (1992) Male factor as determinant of in-vitro fertilization outcome. *Hum. Reprod.*, **7**, 1136–1140.

Evsikov, S. and Verlinsky, Y. (1998) Mosaicism in the inner cell mass of human blastocysts. *Hum. Reprod.*, **13**, 3151–3155.

Fong, C-Y. and Bongso, A. (1998) Comparison of human blastulation rates and total cell number in sequential culture media with and without co-culture. *Hum. Reprod.*, **14**, 774–781.

Hardy, K., Robinson, F.M., Paraschos, T. *et al.* (1995) Normal development and metabolic activity of preimplantation embryos in vitro from patients with polycystic ovaries. *Hum. Reprod.*, **10**, 2125–2135.

Hewitson, L., Dominko, T., Takahashi, D. *et al.* (1999) Unique checkpoints during the first cell cycle of fertilization after intracytoplasmc sperm injection in rhesus monkeys. *Nature Med.*, **5**, 431–433.

Hughes, C.M., Lewis, S.E.E., McKelvey-Martin, V.J. and Thompson, W. (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol. Hum. Reprod.*, **2**, 613–619.

Hull, M.G.R., Williams, J.A.C., Ray, B. *et al.* (1998) The contribution of subtle oocyte or sperm dysfunction affecting fertilization in endometriosis-associated or unexplained infertility: a controlled comparison with tubal infertility and use of donor spermatozoa. *Hum. Reprod.*, **13**, 1825–1830.

Janny, L. and Ménézo, Y.J.R. (1994) Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. *Mol. Reprod. Dev.*, **38**, 36–42.

Jones, G.M., Trounson, A.O., Gardner, D.K. *et al.* (1998) Evolution of a culture protocol for successful blastocyst development and pregnancy. *Hum. Reprod.*, **13**, 169–177.

Jurisicova, A., Antenos, M., Kapasi, K. *et al.* (1999) Variability in the expression of trophectodermal markers β-human chorionic gonadotrophin, human leukocyte antigen-G and pregnancy specific β-1 glycoprotein by the human blastocyst. *Hum. Reprod.*, **14**, 1852–1858.

Kodama, H., Yamaguchi, R., Fukuda, J. *et al.* (1997) Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile patients. *Fertil. Steril.*, **68**, 519–524.

Land, J.A., Yarmolinskaya, M.I., Dumoulin, J.C.M. *et al.* (1996) High-dose human menopausal gonadotropin stimulation in poor responders does not improve in vitro fertilization outcome. *Fertil. Steril.*, **65**, 961–965.

Le Lannou, D., Gastard, E., Guivarch, A. *et al.* (1995) Strategies in frozen donor semen procreation. *Hum. Reprod.*, **10**, 1765–1774.

Lee, J.D., Kamiguchi, Y. and Yanagimachi, R. (1996) Analysis of chromosome constitution of human spermatozoa with normal and aberrant head morphologies after injection into mouse oocytes. *Hum. Reprod.*, **11**, 1942–1946.

Lessey, B.A., Castelbaum, A.J., Sawin, S.W. and Sun, J. (1995) Integrins as markers of uterine receptivity in women with primary unexplained infertility. *Fertil. Steril.*, **63**, 535–542.

Martin, K.L., Barlow, D.H. and Sargent, I.L. (1998) Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum. Reprod.*, **13**, 1645–1652.

Moessner, J. and Dodson, W.C. (1995). The quality of human embryo growth is improved when embryos are cultured in groups rather than separately. *Fertil. Steril.*, **64**, 1034–1035.

Moosani, N., Pattinson, H.A., Carter, M.D. *et al.* (1995) Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence *in situ* hybridization. *Fertil. Steril.*, **64**, 811–817.

Moreno, C., Mercader, A., Ruiz, A. *et al.* (1998) Preimplantation development outcome of embryos after in-vitro fertilization is superior to that after intracytoplasmic sperm injection. *Abstracts from the 14th Annual Meeting of the ESHRE*, Göteborg, Sweden. *Hum. Reprod.*, **13**, Abstract Book, **1**, 164.

Nagy, Z.P., Janssenswillen, C., Janssens, R. *et al.* (1998) Timing of oocyte activation, pronucleus formation and cleavage in humans after intracytoplasmic sperm injection (ICSI) with testicular spermatozoa and after ICSI or in-vitro fertilization on sibling oocytes with ejaculated spermatozoa. *Hum. Reprod.*, **13**, 1606–1612.

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.

Pang, M.G., Hoegerman, S.F., Cuticchia, A.J. *et al.* (1999) Detection of aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y by fluorescence in-situ hybridization in spermatozoa from nine patients with oligoasthenoteratozoospermia undergoing intracytoplasmic sperm injection. *Hum. Reprod.*, **14**, 1266–1273.

Parinaud, J., Mieusset, R., Vieitez, G. *et al.* (1993) Influence of sperm parameters on embryo quality. *Fertil. Steril.*, **60**, 888–892.

Peters, K. and Catt, J.W. (1998) Routine observation of developing human zygotes. *Abstracts from the 14th Annual Meeting of the ESHRE*, Göteborg, Sweden. *Hum. Reprod.*, **13**, Abstract Book, 1, 217.

Pope, C.E., Johnson, C.A., McRae, M.A. *et al.* (1998) Development of embryos produced by intracytoplasmic sperm injection of cat oocytes. *Anim. Reprod. Sci.*, **53**, 221–236.

Quinn, P., Kerin, J.F. and Warnes, G.M. (1985) Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil. Steril.*, **44**, 493–498.

Sakkas, D., Urner, F., Bianchi, P.G. *et al.* (1996) Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum. Reprod.*, **11**, 837–843.

Sathananthan, A.H. (1998) Paternal centrosomal dynamics in early human development and infertility. *J. Assist. Reprod. Genet.*, **15**, 129–139.

Shoukir, Y., Chardonnens, D., Campana, A. and Sakkas, D. (1998) Blastocyst development from supernumerary embryos after intracytoplasmic sperm injection: a paternal influence? *Hum. Reprod.*, **13**, 1632–1637.

Steer, C.V., Mills, C.L., Tan, S.L. *et al.* (1992) The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. *Hum. Reprod.*, **7**, 117–119.

Tarlatzis, B.C. and Bili, H. (1998) Survey on intracytoplasmic sperm injection: report from the ESHRE ICSI Task Force. *Hum. Reprod.*, **13** (Suppl. 1), 165–177.

Twigg, J.P., Irvine, D.S. and Aitken, R.J. (1998) Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum. Reprod.*, **13**, 1864–1871.

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

Van Steirteghem, A., Nagy, P., Joris, H. *et al.* (1998) Results of intracytoplasmic sperm injection with ejaculated, fresh and frozen-thawed epididymal and testicular spermatozoa. *Hum. Reprod.*, **13** (Suppl. 1), 134–142.

Vlad, M., Walker, D. and Kennedy, R.C. (1996) Nuclei number in human embryos co-cultured with human ampullary cells. *Hum. Reprod.*, **11**, 1678–1686.

Received on June 10, 1999; accepted on November 5, 1999