Comparison between day-2 embryos obtained either from ICSI or resulting from short insemination IVF: influence of maternal age*

Yves Ménezo¹,³ and Yona Barak²

¹Laboratoire Marcel Merieux, 1 Rue Laborde, 69500 BRON, France and ²In Vitro Fertilization Unit, Herzliya Medical Center, 7 Ramot-Yam Street, Herzliya-on-Sea 46851, Israel
³To whom correspondence should be addressed: Laboratoire Marcel Merieux, 1 Rue Laborde, 69500 Bron France, France.
E-mail: yves.menezo@insa-lyon.fr

Short incubation time prevents deleterious effects of cumulus cell degeneration and excess spermatozoa in IVF embryos. We performed a short incubation (3 h) protocol in 328 IVF cycles, in order to compare the developmental potential of regular IVF embryos with those originating from 316 cycles entered our intracytoplasmic sperm injection (ICSI) programme over the same period. Embryo transfers were performed in all patients on day 2. The mean number of embryos transferred was 1.92 for the ICSI group and 1.73 for the IVF group (P < 0.007). This was related only to the wishes of patients. However, the policy of the centre is to transfer a low number of embryos in young patients in order to avoid multiple pregnancies. All spare embryos were permitted to grow to the blastocyst stage for freezing. Shortening incubation time did not decrease fertilization rates. In our overall population, no difference was observed in the implantation rates per embryo for IVF (19%) or for ICSI (20%). An age-related decrease in embryo production was observed for both groups of patients (P < 0.01 for ICSI and P < 0.001 for IVF). The age-related decrease in embryo implantation was only significant for the IVF group (P < 0.03 for patients <30 and >35 years of age). A significant overall decrease in blastocyst formation was observed for spare embryos after ICSI versus IVF (34.2 versus 43.8%; P < 0.05). The significance of this observation is discussed.

Key words: embryo implantation/ICSI/IVF/short insemination time

Introduction

The paternal influence in embryogenesis has gained substantial attention since the appearance of techniques such as intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992). Treatment of men with poor quality spermatozoa by using ICSI has improved our knowledge of sperm biology and several points of negative impact, both genetic and epigenetic, have been identified (Ménézo and Dale, 1994; Ménézo and Janny, 1997). A recent study (Shoukir et al., 1998) noted that ICSI embryos have a lower developmental potential as measured by blastocyst formation. Jones et al. (1998) also confirmed the negative impact of male factors on blastocyst formation. Gardner et al. (1998) were the only ones who could not detect this observation. One study (Gardner et al., 1998), however, did not identify a negative effect of ICSI on blastocyst formation.

In order to understand properly the effect of the ICSI technique on the developmental potential of ICSI compared with IVF embryos, we performed the short insemination time protocol (Gianaroli et al., 1996; Quinn et al., 1998) in regular IVF. In fact, in terms of culture conditions, the short insemination protocol is similar to that of ICSI; the oocytes are rapidly denuded, as after 3 h most of the cumulus cells are detached and removed by rinsing. They are no longer submitted to the possible deleterious effects of degenerating cumulus cells, or spermatozoa. We believe, therefore, that a comparison such as this will lead to a better understanding of the paternal effect on the developmental potential of the embryo. In addition, in order to discriminate a possible maternal effect, the patients were ranked by partner ages: <30, 30–35 and >35 years, as the age of 30 was shown as a 'shifting point' in fertility (Piette et al., 1990; Janny and Ménézo, 1996).

Materials and methods

The present study, undertaken from April 1998 to the end of December 1998, was performed on patients who entered the IVF programme at the Institut Rhonalpin, Laboratoire Marcel Merieux in Bron (France). A total of 644 cycles was assessed, of which 316 were ICSI. In 328 IVF cycles a short insemination procedure took place during conventional IVF. IVF was performed on the first attempt when >1.5×10⁶ spermatozoa with at least 50% motility could be observed in the specimen. ICSI was performed: (i) when the concentration of motile spermatozoa was <1.5×10⁶ in the total ejaculate; (ii) when the percentage of abnormal forms was over 90% according to the World Health Organization criteria (WHO, 1992); (iii) after one previous fertilization failure with no spermatozoa attached to the zona pellucida in one IVF cycle, in which at least three metaphase II (MII) oocytes had been retrieved.

This proportion of ICSI/IVF corresponds to our regular activity. The patient population in the ICSI group for ages <30, 30–35 and >35 years comprised 94, 140 and 82 cycles respectively totalling 316, and in the IVF group there were 61, 143, and 124 cycles respectively, totalling 328 in all.

The stimulation protocol was identical to that already described (Janny and Ménézo, 1994). Briefly, it included the use of gonadotrophin-releasing hormone analogues (GnRHa) in semi-long and/or short protocols, followed by stimulation with urinary follicle stimulating hormone (uFSH), or recombinant FSH (rFSH). Ovulation was trig-
gered between day 11 and day 13 of stimulation, by 9000 IU of human chorionic gonadotrophin (HCG). The oestradiol concentration at the time of ovulation was ~150 pg/ml/follicle.

**IVF procedure and culture media**

The follicle-rinsing medium was prepared in the laboratory, i.e. Earle's medium (Sigma, St Quentin Fallavier, France) supplemented with 0.4% of human serum albumin (LFB, 78000, Les ULIS, France) and 80 mg/l of gentamycin. The same medium was used for washing the spermatozoa which were processed by a two-layer pure-sperm gradient (IVF Science, Gothenburg, Sweden) for ICSI and regular IVF.

Insemination of oocytes took place in 0.5 ml Universal-IVF (U-IVF, Medicult, Copenhagen, Denmark), in a 4-well dish (Nalge Nunc, Roskilde, Denmark). Three hours later, inseminated oocytes were placed in a fresh droplet of U-IVF medium. At this point, the spermatozoa and >90% of the cumulus were naturally removed. Only a few corona cells still surrounded the oocytes. Eighteen hours later, the oocytes were examined for the presence of pronuclei. As the oocytes were usually devoid of the majority of cumulus cells, a slight motion in a pipette totally removed the remaining corona cells around the zygotes. Observation of the fertilized oocytes was therefore easy and fast.

Zygotes were then transferred into fresh droplets of Universal IVF medium (Medi-Cult). Embryo transfer took place 44–48 h post-insemination. Supernumerary embryos were grown in a sequence of media similar to those previously described (Chouteau et al., 1998). Embryos that reached the blastocyst stage were frozen according to a protocol previously described (Ménéo et al., 1996; Ménéo and Veiga, 1997). All procedures were performed in droplets under oil (light oil; BDH, Poole, Dorset, UK), in a 5% CO2 atmosphere in air.

**ICSI**

Within 1 h after ovm retrieval, oocytes were denuded in a solution of 80 IU hyaluronidase/ml BM1 medium (Ellios Biomega, Paris, France). Sperm microinjection was performed in droplets under oil of BM1 medium containing 10% PVP, and the injected oocytes were then cultured in U-IVF (Medicult), for the subsequent 48 h. Fertilization was checked 20–22 h later in the same droplets. The remaining supernumerary embryos were cultured for freezing at the blastocyst stage. As mentioned above, all the procedures were performed under oil, in a 5% CO2 atmosphere in air.

**Embryo transfer**

All embryo transfers were performed in BM1 medium. Oocyte collection, maturation, fertilization, pregnancy rates per transfer and the implantation rates per embryo were recorded.

**Statistical analysis**

As it was not possible to determine precisely the number of MII oocytes in IVF, where they were cumulus-enclosed, in contrast to ICSI where MII oocytes had had cumulus cells removed, all calculations were made considering the number of collected cumulus–oocyte complexes (COC).

Statistical analysis was carried out using SPSS software. General descriptive statistics were performed for the entire population and Student’s *t*-test was used to compare the two treatments.

Quantitative data are presented as mean ± SD. Overall comparisons of means between the various groups were processed by analysis of variance (ANOVA), and two-by-two comparison by *t*-test was used to determine which groups were significantly different.

For qualitative data, the overall comparisons of the distribution between the groups were processed by *χ²* analysis. When a difference was found, a two-by-two comparison by *χ²* was used to determine which groups were significantly different.

**Results**

The entire population analysis was performed using ANOVA. Pairwise comparisons using the *t*-test were made in cases of significant *F*. The *t*-test that compared the whole population showed a higher maternal age for the IVF group.
33.93 ± 4.69 versus 32.43 ± 4.45 years for IVF and ICSI groups respectively ($P < 0.0001$).

The mean ages for the three IVF versus ICSI groups were as follows: (i) <30 years, 27.35 ± 1.86 versus 27.69 ± 1.53; (ii) 30–35 years, 32.45 ± 2.78 versus 32.17 ± 1.59; (iii) >35 years, 38.24 ± 3.87 versus 39.03 ± 3.31.

As shown in Figure 1, in the ICSI-treated group, the number of COC significantly decreased in the oldest group (>35 years), in comparison with the other two age groups ($P < 0.0005$). In the IVF group the decrease was significant between all three age groups ($P < 0.0001$). In both ICSI and IVF groups, an age-related decrease was also found in the number of embryos obtained. A higher embryo formation, per collected COC, was observed for IVF ($P < 0.0005$; Figure 2). The mean number of embryos obtained was higher for the IVF group ($P < 0.0001$; Table I).

The mean number of embryos transferred was lower in the IVF group (1.7 versus 1.9; $P < 0.0007$), but the pregnancy rate per transfer and the implantation rates per embryos were similar (Table I). No difference was noticed in the mean number of embryos transferred, by age group.

Differences were found between pregnancy rates (Figure 3) in the young group (42.6%), the 30–35 year group (31.2%) and the group of >35 years (24.8%; $\chi^2 = 12.95; P < 0.001$), in the entire population. Differences were also found in implantation rates between the youngest group and that of the >35 group (25 versus 16% respectively; $P < 0.02$; Figure 4). No significant differences were found in pregnancy or implantation rates when the ICSI and IVF groups were compared (Figures 3 and 4).

No differences in implantation rate were noticed between the various age groups within the ICSI population (Figure 4). However, a higher implantation rate was observed for the youngest group (<30 years) compared to the oldest group (>35 years) in the IVF patients ($P < 0.03$).

Regression and discriminate analyses gave no indication or model to predict success. Although it was not statistically significant, we observed an age-related increase in polyploidy for IVF embryos, that reached 10.7% for patients >35 years of age.

A significant decrease in blastocyst formation was observed.
in the supernumerary ICSI versus IVF embryos [130/380 (34.2%) and 543/1241 (43.8%) respectively; P < 0.05].

Discussion
The short insemination method did not decrease fertilization and cleavage rates. In 1997, during a corresponding period of time, when oocytes of 538 cycles were incubated with spermatozoa overnight as a routine procedure for insemination, our overall cleavage rate was 61%. In our current study, the cleavage rate was 57% in the 328 cycles, which were treated by the short insemination protocol. This confirms the results from previous short co-incubation time studies of oocytes and spermatozoa for fertilization (Gianaroli et al., 1996; Quinn et al., 1998). In general, for IVF embryos the polyploidy observed by us, of up to 10.7% for patients >35 years of age, is rather higher than that currently described in the literature (8–10%) in this age range (Ho et al., 1994). SpERMatozoa remained in contact with the oocytes for a shorter period, and most cumulus cells were removed while rinsing the oocytes 3 h post-insemination. Thus, the zygotes are ‘cleaner’ and a simple motion with the denuding pipette retrieves the few remaining corona cells. This smooth removal may also have a positive impact on embryo quality.

The age-related decline in ovarian production, determined by the number of COC collected, has been previously observed (Piette et al., 1990; Janny and Ménézo, 1996; Figure 1). A higher implantation rate was observed as an outcome of IVF with short co-incubation time, when compared with the results of routine IVF (e.g. overnight incubation with spermatozoa) in the previous year during the same period of time (19% versus 13.5%) in our Unit, (Dossier FIVNAT 1999).

The possibility that reduced zona hardening may facilitate hatching and implantation has previously been mentioned (Waldenstrom et al., 1993). It is possible that the early removal of excess spermatozoa may avoid exposure of the embryos to a high density of free radicals generated by supernumerary spermatozoa and degeneration of cumulus cells. Reactive oxygen species induce zona hardening and tanning. Apart from this aspect, they also have a deleterious effect on DNA. With regard to these biochemical aspects, the short insemination time is the only protocol which makes it possible to compare ICSI and IVF embryos. Our data show no difference in pregnancy outcome between IVF and ICSI embryos in the overall population.

The implantation rates per embryo of ~19% were similar for ICSI and IVF embryos in our overall population. At first sight, this similarity is surprising: epigenetic-related problems, such as centrosome function or dysfunction, (Sathananthan et al., 1996; Van Blerkom, 1996) are expected to interfere with embryo quality. As mentioned earlier (Ménézo and Janny, 1997), ICSI may rescue embryos in some patients by avoiding delays in cell cycles through a direct sperm deposition in the oocyte. The spare time helps to save maternal messenger ribonucleic acid (mRNA), which may partially overcome any epigenetic defects. This agrees with previous observations (Oehninger et al., 1996) that ICSI embryos, in cases of severe teratozoospermia, have higher morphological scores and higher developmental potential than their corresponding IVF embryos. Moreover, as fast-cleaving embryos lead to the best pregnancy rates after transfer on day 2 or 3, as observed (Shoukir et al., 1997), this aspect was confirmed.

Our lower embryo cleavage rate was observed for ICSI in this study, which is either related to technical problems, or to these epigenetic or genetic problems. It has also been observed (Asch et al., 1995) that a considerable proportion of the so-called unfertilized eggs are fertilized, but are unable to perform even the first division. In ICSI, the quality of the injected spermatozoon is randomly chosen; selection is performed on gross morphological aspect and motility (when possible). These sperm characteristics probably have nothing to do with the real potential of the spermatozoon, in terms of affecting the developing embryo. It is likely, therefore, that only some of the injected spermatozoa have a good developmental potential (Hewitson et al., 1997). Moreover, Lundin et al. (1998) claimed that ICSI can increase genetic risk (Lundin et al., 1998). Although ICSI is performed to increase the chance of fertilization, even with good spermatozoa, in most cases the real genetic capacity of the spermatozoon is unknown. It is admitted (Lundin et al., 1998) that, when possible, it is better to perform regular IVF in order to try and avoid genetic risk, as again nothing is known about the injected spermatozoon, suggesting that a study such as the current one to compare ICSI and IVF would be useful. Obviously it would be necessary, ideally, to compare ICSI and IVF in the case of good spermatozoa. This would facilitate determination of the contribution related to the extra manipulation of the embryos due to the injection only, in cases of ICSI, and the putative damage to the spindle, that follow (Hewitson et al., 1997). However, this kind of investigation is ethically questionable.

Thus, we assume that, in comparison to IVF, fewer embryos may reach the blastocyst stage in ICSI. In addition, when embryo transfers are performed on day 2, as in the study presented here, a partial selection of embryos based on cleavage speed and morphological appearance also occurs on that day. Therefore, even fewer ICSI embryos will be able to reach the blastocyst stage when compared to regular IVF.

Our data on spare embryos to some extent agree with these predictions. This simply indicates that not all the epigenetic and genetic problems mentioned earlier, and those related to poor sperm quality, can be overcome. The rescue is only partial for the overall ICSI embryo population. This is also in agreement with the observations of Shoukir et al. (1998) concerning decreased blastocyst formation for ICSI embryos, and it confirms the paternal effect on early embryogenesis (Janny and Ménézo, 1994; Jones et al., 1998). Moreover, the apparently (but not significantly) lower implantation rate per embryo observed for ICSI versus regular IVF in younger patients (Figure 4) (aged <30 years) may also be linked to a paternal effect in these couples, where the maternal effect is minimal.

According to the above data, we confirm that obtaining early-stage embryos does not necessarily lead to a positive end-point, and furthermore, ICSI cannot universally cure major sperm defects.
A study of blastocyst formation is currently taking place for ICSI and IVF patients who enter our blastocyst transfer programme. This will facilitate ultimate determination of whether blastocyst formation is impaired in ICSI patients, and whether blastocysts obtained for IVF and ICSI have the same developmental (implantation) potential.

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References


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