Human Reproduction vol.13 no.8 pp.2223–2226, 1998

Influence of oocyte preincubation time on fertilization after intracytoplasmic sperm injection

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During the intracytoplasmic sperm injection (ICSI) procedure, the collected oocytes are incubated until just before ICSI. The ideal preincubation time of oocytes was investigated in 544 treatment cycles. Oocyte retrieval was carried out 35 h after human chorionic gonadotrophin administration. Oocytes were cultured for between 1 and 11 h before ICSI. Embryo transfer was performed 48 h after oocyte collection. The survival, fertilization and cleavage rates of injected oocytes indicated no statistically significant differences between oocytes preincubated for different lengths of time. The proportion of good-quality embryos (grades 1 and 2) was lower at 9–11 h of preincubation time than for all the other preincubation times ($P < 0.001$). No statistically significant differences were detected in the pregnancy rate between each group (mean: 15.9%), although the pregnancy rate at 9–11 h of preincubation time appeared to be low (7.7%). These results suggest that the oocyte retained sufficient potential for fertilization between 1 and 9 h after oocyte collection in ICSI. For the researchers who practise more complex ICSI procedures than IVF, it would be convenient to be able to perform ICSI at any time between 1 and 9 h after oocyte collection.

Key words: delayed insemination/ICSI/incubation/oocyte

Introduction

Intracytoplasmic sperm injection (ICSI) is a common method of micro-insemination (Van Steirteghem *et al*., 1996). In this procedure, the collected oocytes are incubated until just before microinjection of a spermatozoon. In most hospitals, this incubation period (preincubation time) is from 3 to 7 h (Van Steirteghem *et al*., 1993, 1996; Payne *et al*., 1994; Sherins *et al*., 1995; Tucker *et al*., 1995) and the reasons behind this preincubation time are not clear. The preincubation time is considered to be the cause of delayed insemination (Trounson *et al*., 1982) by in-vitro fertilization (IVF) in the ICSI procedure. During delayed insemination, it has been reported that the maturity of collected oocytes is increased and that the fertilization rate is improved in the IVF procedure. In normal fertilization, a mature plasma membrane of the oocyte is necessary to achieve sperm–egg fusion. After sperm–egg fusion, the Ca^{2+} influx pathway is activated in the cytoplasm (Miyazaki *et al*., 1986). In immature oocytes, there may be some problems with the Ca^{2+} influx pathway in the IVF procedure. Since the sperm–egg fusion is bypassed during fertilization by ICSI, these problems are resolved. The relationship between the preincubation time and the fertilization after ICSI is not clear. Therefore the influence of oocyte preincubation time on the results of ICSI was examined.

Materials and methods

In 242 infertile couples with severe oligozoospermia or failed IVF, ICSI was performed between June 1994 and April 1996 in the hospital of Fukushima Medical College. Only those cases in which motile spermatozoa could be collected were used in this investigation. The total number of treatment cycles was 544.

Oocyte collection

Ovulation was induced using a combination of gonadotrophin-releasing hormone (GnRH) analogue (buserelin acetate, Suprecur; Hoechst Japan Co., Tokyo, Japan), follicle stimulating hormone (FSH, Fertinorm P; Serono Japan Co., Tokyo, Japan), human menopausal gonadotrophin (HMG, Pergonal; Teikokuzoki Co., Tokyo, Japan), human chorionic gonadotrophin (HCG; Mochida Pharmaceutical Co., Tokyo, Japan). The patients were administered with buserelin acetate after day 21 in the previous luteal phase. They were injected daily with 300 IU FSH on days 3 and 4 of the treated cycle and with 150 IU FSH on days 5 and 6 and with 150 IU HMG from day 7 until maturation of the follicles. When two leading follicles reached a mean diameter of 18 mm, 5000–10 000 IU HCG was administered. Oocyte retrieval was carried out under transvaginal ultrasound 35 h later.

Handling of oocyte

The oocytes were cultured in human tubal fluid (HTF; Irvine Scientific, CA, USA) for 1–11 h. The cumulus was removed from the oocytes just before ICSI by brief treatment with 0.025% hyaluronidase (H3757, type 8, 330 unit/mg; Sigma, St Louis, MO, USA) in HEPES-buffered HTF (mHTF; Irvine Scientific) with a pipette. The maturity of oocytes (metaphase II or not) were assessed under the inverted microscope with Nomarsky modulation. All of the metaphase II oocytes were used for ICSI. We did not remove cumulus until the start of ICSI in order to promote oocyte maturation.

Preparation of spermatozoa

Semen samples were collected by masturbation and were liquefied at room temperature. Motile spermatozoa were isolated by the swim-up method after washing by centrifugation at 350 *g* for 5 min. If motile spermatozoa could not be collected by the swim-up method, sperm suspensions were prepared by simple sperm wash procedure in mHTF by centrifugation at 350 *g* for 10 min.

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Table I. Summary of 544 intracytoplasmic sperm injection treatment cycles

Total patients	242
No. of treatment cycles	544
Mean maternal age (years)	34.1
Total no. of oocytes recovered	3297
Total no. of metaphase II oocytes (% of recovered) (all injected)	2864 (88.6)
No. surviving oocytes (% of injected)	2434 (85.0)
No. fertilized oocytes (% of injected)	1809 (63.2)
No. cleaved oocytes (% of injected)	1495 (52.2)
Total no. of embryo transfers (% per retrieval)	515 (94.7)
No. of pregnancies (% per embryo transfer)	82 (15.9)

Method of ICSI

ICSI was performed essentially as described by Perreault *et al*. (1982) and Yanagida *et al*. (1992). The outer diameter of the injection needle was 5–6 μm and the inner diameter of the holding pipette was 15 μm. A micromanipulator (Model MO-102, Narishige, Tokyo, Japan) and an inverted microscope (Olympus IMT2, Tokyo, Japan) with Nomarsky modulation or Hoffman modulation were used for ICSI. The motile spermatozoon was immobilized just before ICSI. Immobilization was achieved by repeatedly drawing a spermatozoon in and out of an injection needle in HTF medium containing 10% polyvinylpyrrolidone (PVP-360, Sigma Co., Tokyo, Japan). The immobilized spermatozoon was drawn tail-first into the injection needle, and the needle was transferred to a drop for ICSI. An oocyte was held against the holding pipette so that the polar body was in the 6 or 12 o'clock position, then the oocyte was punctured by the needle and a small amount of cytoplasm was sucked into the needle to confirm rupture of the membrane. The spermatozoon was expelled into the oocyte, and the needle was withdrawn from the oocyte. The oocytes after ICSI were cultured for 18–24 h in HTF containing 10% cord serum. After confirmation of fertilization, normal fertilized oocytes were placed into HTF media supplemented with 15% cord serum. Embryos were cultured until embryo transfer, and cleaved embryos were transferred to the uterus 48 h after oocyte retrieval. The number of transferred embryos was three or fewer.

The oocytes collected from the patients in 544 treatment cycles were divided retrospectively into five groups according to differences in preincubation time as follows: group A: 1–3 h; group B: 3–5 h; group C: 5–7 h; group D: 7–9 h; and group G: 9–11 h. The fertilization rate, cleavage rate, quality of embryo and pregnancy rate of each group were analysed. Embryo quality was classified as grade 1 to grade 5 according to Veeck's (1991) classification. Grade 1 was the embryo of highest quality, and grade 5 indicated severe fragmentation that comprised >75% of oocyte.

Statistical significance was assessed using the χ^2-test and one-way analysis of variance (ANOVA). At $P < 0.05$, the difference was considered statistically significant.

Results

The results of ICSI from 544 treatment cycles are shown in Table I. A total of 3297 oocytes were obtained from 544 cycles, and 2864 oocytes (86.9%) were metaphase II oocytes just before ICSI. All of the metaphase II oocytes were injected with a spermatozoon; of these, 430 were damaged by the injection procedure and the overall survival rate was 85.0%. The overall fertilization rate of the injected oocytes was 63.2% and the overall cleavage rate of the injected oocytes was 52.2%. The cleaved oocytes did not include the oocytes with severe fragmentation. These results were the same as the data of Van Steirteghem *et al*. (1996).

Table II shows the profile of treatment cycles according to the preincubation time. Patients undergoing oocyte retrieval ranged from 21 to 46 years in age (34.0 ± 4.3, average ± SD). For maternal age, there were no significant differences between each preincubation time. The causes of infertility in patients undergoing ICSI were homogeneously distributed among groups. From this ICSI procedure, no statistical difference was obtained with regard to number of retrieved oocytes, proportion of metaphase II oocytes, survival rate, fertilization rate and cleavage rate among groups (Table III).

The fertilized oocytes that were observed at the embryo transfer were at the stages of pronuclei (PN), 2- or 4-cell or fragmentation. The percentages of these stages of oocytes in each group are shown in Figure 1. The percentage of the 4-cell stages in group A was 73.1% and this value decreased according to the preincubation time. Statistically significant differences of the 4-cell stage were detected between group A and other groups. The percentage of the 2-cell stage oocytes in group A was 14.8%. This value was increased according to preincubation time. Statistically significant differences of the 2-cell stage were detected between group A and other groups ($P < 0.001$). In percentages of PN stage and fragmentation, there were no significant differences between groups. The quality of embryos during embryo transfer is shown in Figure 2. The proportion of grade 1 embryos in group E was low (9.4%), and this was significantly different from group D ($P < 0.05$). Furthermore, the proportion of good-quality embryos (grade 1 + grade 2) in group E was significantly lower than in the

Table II. Patient age and causes of infertility according to the different preincubation periods

				Indication for ICSI	
Group	Preincubation period (h)[a]	No. of treatment cycles	Age[b] (years)	Male factor (%)[c]	Failure of fertilization (%)[d]
Group A	1–3	75	34.4 ± 4.7	85.3	14.7
Group B	3–5	167	34.5 ± 4.3	83.2	16.8
Group C	5–7	141	34.0 ± 4.2	88.7	11.3
Group D	7–9	107	33.2 ± 3.7	85.0	15.0
Group E	9–11	54	33.7 ± 4.2	87.5	12.5

[a]Preincubation period means the interval from oocyte retrieval to intracytoplasmic sperm injection (ICSI), e.g. '1–3' means '1 < preincubation period ≤ 3'.
[b]Values are mean ± SD. No significant difference between any groups (analysis of variance).
[c,d] No significant difference between each group (χ^2-test).

Table III. Metaphase II oocytes, oocyte survival, fertilization and cleavage of oocytes in intracytoplasmic sperm injection procedures according to preincubation time[a]

Group	*n*	No.of retrieved oocytes per cycle	Metaphase II oocytes[b] (%)	Surviving oocytes (%)	Fertilized oocytes[c] (%)	Cleaved oocytes[d] (%)
Group A	75	5.9 ± 3.7	85.4 ± 20.0	82.6 ± 27.5	58.7 ± 34.3	53.1 ± 34.8
Group B	167	6.1 ± 3.8	86.7 ± 18.0	85.9 ± 20.8	66.8 ± 29.2	59.7 ± 29.5
Group C	141	5.8 ± 3.7	88.9 ± 17.5	86.1 ± 18.7	65.3 ± 27.9	56.1 ± 30.8
Group D	107	6.3 ± 3.5	87.5 ± 16.8	83.4 ± 22.7	61.1 ± 28.9	51.1 ± 29.2
Group E	54	6.1 ± 4.1	90.0 ± 12.9	89.1 ± 17.3	67.3 ± 28.2	56.1 ± 31.4

Values are mean ± SD. For the survival rate, fertilization rate and cleavage rate, there were no significant differences between any groups (analysis of variance).
[a]See Table II for details of preincubation periods.
[b]All of the metaphase II oocytes were injected.
[c,d]Percentages of injected oocytes.

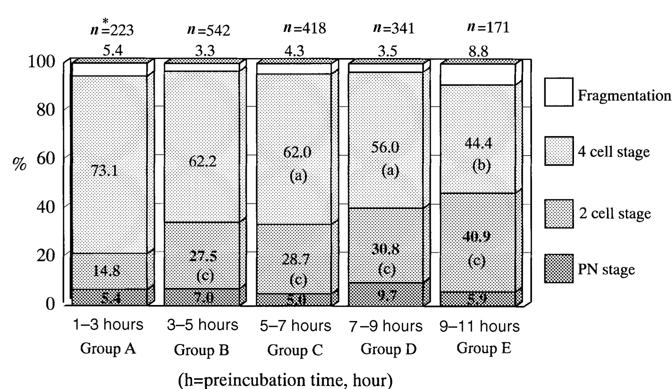

Figure 1. Status of embryos at the embryo transfer according to the different preincubation times. Transfer was performed 48 h after oocyte retrieval. Group A was the longest (for 45–47 h) and group E was the shortest (for 37–39 h). **N* is the number of oocytes examined (*N* = fertilized oocytes – cryopreserved oocytes). [a]$P < 0.01$, [b]$P < 0.001$, [c]$P < 0.001$.

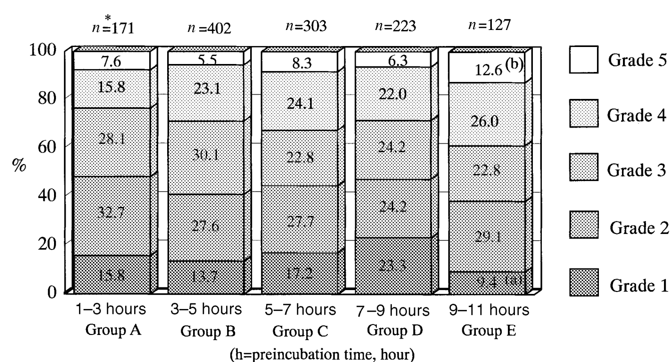

Figure 2. Quality of embryos during transfer classified according to Veeck (1991). **N* is the number of oocytes examined out of the fertilized oocytes. [a]$P < 0.05$, compared with group D (χ^2-test). [b]Percentage of grade 1 + grade 2 embryos in group E was significantly different from other groups ($P < 0.001$, χ^2-test).

other groups ($P < 0.001$). The proportion of grade 5 embryos in group E was significantly higher than in the other groups ($P < 0.001$).

Table IV shows the rates of the embryo transfer and pregnancy. The number of embryo transfers carried out as a percentage of the number of treatment cycles was 82.7% in group A, the lowest of all groups statistically. The corresponding percentage in the other groups ranged from 95.2 to 98.1%. The mean number of oocytes transferred per cycle was 2.2–2.4 and no statistically significant differences were detected between the groups. For pregnancy rate, no statistically significant differences were detected between any groups, but the pregnancy rates appeared lower for preincubation times of >9 h (7.7%).

Discussion

In standard IVF–embryo transfer, delayed insemination is currently practised in most IVF centres. In delayed insemination, insemination is performed several hours after collection of the oocytes. Trounson *et al.* (1982) introduced this method and reported that culture for 5–6.5 h following collection of the oocytes was beneficial for the completion of oocyte maturation and for increasing fertilization and pregnancy rates. The maturity of the oocytes is generally evaluated according to the appearance of cumulus–corona complexes. When the collected oocytes were immature, it was reported in some studies that the fertilization was improved by preincubation of oocytes. Marrs *et al.* (1984) reported that preincubation intervals of 8–24 h, depending upon the estimated maturity of the oocyte, increased fertilization rate (50% versus 71%, $P < 0.05$). Veeck *et al.* (1982) examined the relationship between various periods of preincubation and the fertilization in the procedure of IVF–embryo transfer, and reported that the proportion of fertilization and development to embryos maximized after 5–5.5 h in culture (89% versus 0–0.5 h, 26%; 4–4.5 h, 50%; 6–6.5 h, 69%). As oocytes are collected before ovulation in the procedure of IVF, some immaturity is thought to exist in collected oocytes. The nature of this immaturity is as yet unclear.

The cumulus–corona complex was removed just before ICSI in this study. The purpose of this procedure was to promote oocyte maturity in the cumulus–corona–oocyte complex. As for the ratio of the oocytes that were at metaphase II after preincubation (for 1–11 h), there were no significant differences in any groups. Culture for >15 h was generally necessary for an oocyte of metaphase I to mature (Veeck, 1991). For this reason, it was thought that a ratio of metaphase II oocytes did not increase during preincubation time for 11 h. For the results

Table IV. Results of embryo transfer according to preincubation periods[a]

Group	No. of treatment cycles	No. of embryo transfers[b] (%)	No. of embryos transferred per embryo transfer[c]	No. of pregnancies[d] (%)
Group A	75	62 (82.7)	2.4 ± 0.9	11 (17.7)
Group B	167	159 (95.2)**	2.3 ± 0.8	25 (15.7)
Group C	141	137 (97.2)*	2.3 ± 0.9	26 (19.0)
Group D	107	105 (98.1)*	2.2 ± 0.9	16 (15.2)
Group E	54	52 (96.3)***	2.3 ± 0.9	4 (7.7)

[a]See Table II for details of preincubation periods.
[b]Significant difference compared with group A: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (χ^2-test).
[c]Values are mean ± SD. There were no significant differences between any groups (analysis of variance).
[d]Values in parentheses are percentages of number of embryo transfers. No significant differences between any groups (χ^2-test).

of survival rate, it was thought that there was no change in the function of restoration at the cell membrane after being punctured by a needle.

In the protocol of this study, embryo transfer was performed 48 h after oocyte retrieval. Accordingly, as for incubation time of oocytes after ICSI, group A was the longest (for 45–47 h), and group E was the shortest (for 37–39 h). This information influences the distribution of the fertilized oocyte stages at the time of embryo transfer. In group A the incubation time was long, there were few 2-cell stage oocytes, and many 4-cell stage oocytes. As for the quality of embryos, the profile of good-quality embryos (grade 1 + grade 2) in group E was low and indicated significant differences between other groups ($P < 0.001$), although there were no significant differences in fertilization rate and cleavage rate. The reason for the low profile of good-quality embryos was thought to be the fact that the long culture influenced the quality of the embryos. An accumulation of oxidative damage in a cultured oocyte with preincubation time may have an influence on embryo development. Oxidative stress damages the mitochondrial DNA and induces the decrease in intracellular ATP and glutathione/glutathione disulphide ratio. This may cause the disorder of cytoskeletal fibres, fertilization and embryo development (Tarin, 1996). Also Igarashi *et al.* (1997) reported that aged mouse oocytes (20 h after HCG administration) have a dysfunction of the Ca^{2+} pump of the endoplasmic reticulum that induces disordered calcium oscillations after fertilization. These abnormal mechanisms during fertilization are likely to influence the embryo quality.

These results suggest that the oocyte maintains sufficient fertilization ability between 1 and 9 h after oocyte collection in ICSI procedure. There is no change in the quality of oocytes in preincubation times under 9 h. Finally, pregnancy rate is maintained at a good level within 9 h of preincubation time. If ICSI is applied within 9 h of oocyte collection, it is confirmed that it is possible to obtain good embryos. For researchers who practise more complex ICSI procedures than IVF, it is convenient to be able to perform ICSI at any time between 1 and 9 h after oocyte collection.

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Received on October 7, 1997; accepted on April 14, 1998