The Effects of Brief Gamete Co-incubation in Human In Vitro Fertilization

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Purpose: To investigate whether very short exposure of mature oocytes to sperm in vitro may affect the fertilization rates, embryo cleavage rates, and embryo quality between sibling oocytes in the same patient.

Methods: Sibling oocytes of the same patient from 23 oocyte collection cycles were randomly allocated to the study group, with a 1-hr or 3-hr sperm–oocyte incubation, or the control group with the standard overnight gamete co-incubation. The fertilization rates, cleavage rates, and subsequent embryo quality were evaluated.

Results: Our results showed no statistically significant differences in fertilization rates, embryo cleavage rates, and quality of the embryos between the study group and the control group.

Conclusions: Since the present study showed that long exposure of the oocyte to sperm has no advantage over short exposure, we prefer shortening the oocyte–sperm incubation period for reducing the negative effect induced by nonphysiologically high concentrations of spermatozoa.

KEY WORDS: Embryo quality; gamete co-incubation; IVF.

INTRODUCTION

The overnight sperm–oocyte co-incubation method is used as a standard in vitro fertilization protocol. However, the possible detrimental effects of the relatively high concentration of spermatozoa over the long incu-

bation time used in this procedure have drawn a lot of attention recently (1–3). The reactive oxygen species present in high concentrations of spermatozoa after a prolonged culture period is harmful to oocytes and zygotes. Free oxygen radicals may peroxidate polyunsaturated fatty acids in cell membranes, causing a decrease in the flexibility and fluidity of the membrane (4, 5). Actually, it has been noted previously that the arrest of development of mouse embryos might be due to cell membrane malfunction following an increase in the concentration of these oxidative species. In fact, the addition of antioxidants to the culture media promotes embryo development (6).

Based on this assumption, investigators tried to shorten the gamete coculture period to see whether this could result in a better in vitro fertilization (IVF) outcome (7–10). In those studies, patients were randomly allocated into the control (conventional) and study (shortened co-incubation period) groups. Similar or better fertilization rates, cleavage rates, and increased implantation rates were obtained in the study group.

In order to provide a better control group to determine whether brief co-incubation of gametes might affect the fertilization, cleavage rates, and embryo quality, we allocated sibling oocytes of the same patient to either the short-exposure (1 hr or 3 hr co-incubation of sperm and eggs) or the conventional overnight co-incubation groups, instead of using oocytes from different patients as described in previous reports (7–10).

MATERIALS AND METHODS

Patient Selection and Characteristics

Following patient consent, 23 subfertile couples without male infertility factors were selected for this

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study. The sperm count and motility were classified based on the criteria specified by the World Health Organization (WHO) (11), while the sperm morphology was evaluated according to the strict criteria provided by Kruger *et al.* (12). The patients were randomly allocated to two groups. In group 1, with 9 couples, approximately one third of each patient's oocytes were exposed to spermatozoa for only 1 hr, while the remaining oocytes were exposed to spermatozoa for 16 hr as in the standard procedure. In group 2, with 14 couples, a 3 hr and an overnight sperm-oocyte co-incubation were compared within each couple.

Ovarian Stimulation and Oocyte Retrieval

Induction of multiple follicular growth was obtained according to protocols described previously (13). Briefly, 15–20 days after pituitary desensitization with gonadotropin-releasing hormone analogue (GnRHa) (Leuprolide acetate; TAP Pharmaceuticals Inc., USA), follicle-stimulating hormone (FSH) (Metrodin; Serono, Switzerland), and human menopausal gonadotropin (hMG) (Humegon, N.V. Organo, Holland) were administered in a dosage adjusted to the individual's response. The estradiol assessment and transvaginal ultrasound examinations were processed to monitor follicular growth. Thirty-six hours after human chorionic gonadotropin (hCG) injection, oocytes were aspirated under the guidance of transvaginal ultrasound. Oocyte maturity was assessed according to the morphological appearance of the cumulus–corona complex (14). Oocytes were then washed and placed in 50 μ l drops of prebalanced IVF medium (Medi-Cult, Denmark), and overlaid with washed and preequilibrated light mineral oil (Sigma, USA) in a 5% CO₂, 5% O₂, and 90% N₂ humidified gas atmosphere at 37°C.

Insemination Procedures

All semen samples were prepared by the conventional swim-up procedure (15), approximately 90–120 min before oocyte insemination. At 5–7 hr after oocyte retrieval, each matured oocyte was inseminated with approximately 50,000–100,000 motile spermatozoa. One or three hours after insemination, approximately one third of the matured oocytes were randomly selected from group 1 or group 2, respectively. Each oocyte was gently washed through two organ dishes containing 1.5 ml of equilibrated medium, and then transferred to the corresponding microdroplet of equilibrated fresh medium. The remaining two thirds of the

sibling oocytes were exposed to spermatozoa for 16 hr with the standard procedure as the control.

Fertilization and Embryo Evaluation

Evaluation for the presence of pronuclei was performed 16–18 hr after insemination, at which time all oocytes from both groups were transferred to equilibrated fresh IVF medium (Medi-Cult, Denmark). On the day of embryo transfer, i.e., 40–42 hr after insemination, morphological evaluation of the developing embryos was assessed blindly under an inverted microscope (Model-135M, Zeiss, Germany). The embryos were scored according to the following criteria. Grade I (score 4), was given to the best-quality embryos containing even-sized, symmetrical blastomeres with no obvious fragments. Grade II (score 3) was given if the embryos had < 10% fragmentation. Grade III (score 2) was given if they had uneven size blastomeres. Grade IV (score 1) was given when 10–50% of their cytoplasm was fragmented, grade V (score 1) was given if they showed > 50% cytoplasmic fragmentation (16).

A score was then calculated for each embryo by multiplying the morphological value described above by the number of blastomeres observed to be present in each embryo. Thus, for example, a four-cell embryo with < 10% fragmentation would have an embryo score of $4 \times 3 = 12$. The average embryo score for all embryos in the study group was calculated and compared with the control group from the same patient.

Statistical Analysis

The differences in fertilization rates, embryo cleavage rates, and percentage of good-quality (grades I and II) embryos between matched groups were analyzed by χ^2 analysis 2×2 contingency tables with Yate's correction. The average embryo scores were compared in matched groups using the Wilcoxon matched-pairs signed-rank test.

RESULTS

The fertilization rates of the oocytes in the various treatment groups are shown in Tables I and II. When comparing the 1-hr insemination group with the overnight insemination control group, we found that the fertilization rates in the 1-hr group (79%) were lower than the control group (87%), but this did not achieve statistical significance. In group II, the 3-hr sperm–

Table I. The Fertilization Rates of Oocytes Co-incubated with Spermatozoa for 1 Hr and Overnight

	1-hr gamete co-incubation	16- to 18-hr gamete co-incubation
Number of patients (8)		
Total oocytes inseminated	34	83
Normal fertilization (2PN ^a)	27	72
Abnormal fertilization (3PN)	3	7
Normal fertilization rate (%)	79 ^b	87 ^b

^a PN, pronuclei.^b $P = 0.47$ (χ^2 analysis with Yate's correction).**Table II.** The Fertilization Rates of Oocytes Co-incubated with Spermatozoa for 3 Hr and Overnight

	3-hr gamete co-incubation	16- to 18-hr gamete co-incubation
Number of patients (14)		
Total oocytes inseminated	78	133
Normal fertilization (2PN)	66	112
Abnormal fertilization (3PN)	5	2
Normal fertilization rate (%)	85 ^a	84 ^a

^a $P = 0.38$ (χ^2 analysis with Yate's correction).

oocyte co-incubation group had a fertilization rate of 84.6%, which was very similar to the overnight exposure control group (84.2%).

In one case of polycystic ovarian syndrome (PCOS), 12 eggs were divided into two groups: 5 in the 1-hr co-incubation group and the others in the overnight exposure control group. While all seven oocytes in the control group were fertilized successfully, none of the oocytes in the 1-hr group were fertilized (Table III).

In Tables IV and V, we demonstrated that the embryo cleavage rates were very similar under all treatment conditions. Both the percentage of good-quality embryos and the embryo morphology index were higher in the 1-hr co-incubation group compared to the control group, but these improvements did not reach statistical significance by χ^2 and two-tailed Wil-

Table III. The Fertilization Rates of Oocytes from a PCOS Patient after 1 Hr and Overnight Sperm-Oocyte Co-incubation

	1-hr gamete co-incubation	16- to 18-hr gamete co-incubation
Total oocytes inseminated	5	7
Normal fertilization (2PN)	0	7
Normal fertilization rate (%)	0	100

Table IV. The Cleavage Rates and Embryo Qualities Following 1-Hr and Overnight Gamete Co-incubation

	1-hr gamete co-incubation	16- to 18-hr gamete co-incubation
Embryo cleavage rate (%)	22/24 (92) ^a	63/65 (96) ^a
Good quality embryos (grade I & II) (%)	18/22 (82) ^b	44/63 (70) ^b
Average embryo morphology index	11.54 ^c	9.64 ^c

^a $P = 0.62$ (χ^2 analysis with Yate's correction).^b $P = 0.42$ (χ^2 analysis with Yate's correction).^c $P = 0.12$ by two-tailed Wilcoxon matched-pairs signed-rank test.

coxon matched-pairs signed-rank test (Table IV). The embryo quality of the 3-hr sperm-oocyte co-incubation group was similar to the control group, since both the morphology index and percentage of good quality embryos were similar between these two subgroups (Table V).

DISCUSSION

The standard 16-hr incubation period of oocytes with spermatozoa was originally established for practical reasons. This generally corresponds to the time for observation of pronuclei. However, some studies suggest that a long oocyte exposure to spermatozoa may be harmful (1–5). The primary goal of this study therefore was to see whether brief exposure of human oocytes to spermatozoa in vitro results in equivalent fertilization rates and possibly better quality embryos compared with overnight co-incubation of gametes from the same couple.

Our results showed no statistically significant difference in the fertilization rates between the shortened sperm-oocyte exposure group (both 1-hr and 3-hr subgroups) and the control groups. These results con-

Table V. The Cleavage Rates and Embryo Qualities Following 3-Hr and Overnight Gamete Co-incubation

	3-hr gamete coincubation	16- to 18-hr gamete co-incubation
Embryo cleavage rate (%)	58/61 (95) ^a	104/110 (94.5) ^a
Good-quality embryos (grade I & II)(%)	43/58 (74.1) ^b	76/104 (73) ^b
Average embryo morphology index	9.89 ^c	11.2 ^c

^a $P = 0.86$ (χ^2 analysis with Yate's correction).^b $P = 0.97$ (χ^2 analysis with Yate's correction).^c $P = 0.75$ by two-tailed Wilcoxon matched-pairs signed-rank test.

firmed previous observations (7,8,15), which suggested that the interaction between sperm and oocyte–cumulus complex took place within 1 hr. In fact, an in vitro study using fluorescent-labeled human spermatozoa to inseminate normal oocyte–cumulus complexes confirmed this hypothesis (7). The authors demonstrated that spermatozoa enter the cumulus complex within 15 min, traverse the cumulus layer within 3 hr, and first appear in the oocyte cortex at 4 hr postinsemination.

However, care should be taken in oocytes from PCOS patients, since we demonstrated failure of fertilization in oocytes from one patient in the 1-hr exposure subgroup despite a 100% fertilization rate in the standard overnight co-incubation group. It was claimed that oocyte maturation could be disturbed in PCOS patients (16). Therefore, the timing for fertilization might be delayed leading to fertilization failure in the 1-hr gamete co-incubation group.

The results reported herein also demonstrated equivalent embryo cleavage rates and embryo quality between the control and shortened sperm–oocyte co-incubation group. Although the morphological index of the 1-hr sperm–oocyte exposure group was numerically greater than that of the control group, the difference was not statistically significant. Most previous studies also demonstrated an equivalent or better embryo quality in the shortened sperm–oocyte co-incubation group, in regard to the morphological evaluation (7,8,10,15). Only some of the differences achieved statistical significance (8,15). In our study, the shortened sperm–oocyte interaction period did not give a dramatic improvement in embryo quality, possibly for the following reasons. First, since previous studies have shown that the level of reactive oxygen species formation was inversely correlated to semen parameter (17–19), the normal semen samples (according to the criteria specified by WHO (11) used in this study may have produced less, and thus provided a less significant improvement in the shortened sperm–oocyte interaction groups. In fact, Gianaroli *et al.* (8) demonstrated that long exposures of oocytes to male factor spermatozoa were more detrimental than long exposures to normal sperm. Second, the oxygen tension in our incubator was reduced to 5% in order to minimize the oxidative stress. Therefore, the detrimental effect caused by long-term sperm–oocyte interaction could be partially overcome in our system.

Following embryo morphology evaluation, we chose the best embryos for transfer from both groups in the same patient. Therefore, we could not compare the pregnancy outcome between them. However, the

overall pregnancy rate in this study was 20%. According to previous studies in which patients were randomly allocated to the control (conventional) and study (shortened co-incubation period) groups, increased implantation rates were obtained in the study group. This improvement most likely resulted from minimizing the oxidative stress caused by long-term exposure to a high concentration of sperm (7,8). It was also claimed that high concentrations of spermatozoa may induce a zona hardening effect through the enzymes within the semen samples (such as hyaluronidase and acrosine, substances suspected to enhance zona hardening) and through the release of zona-hardening factors from oocytes upon activation in vitro by sperm (9,10). Embryos with a hardened zona pellucida have reduced hatching rates and impaired implantation rates. Therefore, short-time gamete co-incubation may also have a favorable effect on implantation by preventing zona hardening.

Using sibling oocytes from the same patient, the present study confirmed that a shortened gamete co-incubation period of 1 or 3 hr gave equivalent fertilization, cleavage rates, and embryo qualities compared to standard overnight sperm–oocyte exposure. Our observation along with the results of Gianaroli *et al.* (7,8), which demonstrated an improved implantation rate in the shortened gamete exposure group, recommend that shortening the oocyte–sperm incubation period may reduce the negative effects induced by nonphysiologically high concentrations of spermatozoa, including free radicals and zona hardening effects. However, special care should be taken for oocytes from PCOS patients because a 1-hr sperm–oocyte co-incubation may not be enough to accomplish a normal fertilization reaction.

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REFERENCES

1. Aitken JR, Clarkson JS: Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1987;81:459–469
2. Dumoulin JCM, Bras M, Land JA, Pieters MH, Engelsu ME,

- Gerardts JP, Evers JL: Effect of the number of inseminated spermatozoa on subsequent human and mouse embryonic development in vitro. *Hum Reprod* 1992;7:1010–1013
3. Parinaud J, Labal B, Mieusset R, Richoille G, Vieitez G: Influence of sperm parameters on embryo quality. *Fertil Steril* 1993;60:888–892
  4. Aitken JR: A free radical theory of male infertility. *Reprod Fertil Dev* 1994;6:19–24
  5. Aitken JR: Pathophysiology of human spermatozoa. *Curr Opin Obstet Gynecol* 1994;6:128–135
  6. Nasr-Esfahani M, Johnson MH, Aitken JR: The effect of iron chelators on the in vitro block to development of the mouse pre-implantation embryo: BAT6 a new medium for improved culture of mouse embryos in vitro. *Hum Reprod* 1990;5:997–1003.
  7. Gianaroli L, Magli MC, Ferraretti AP, Fiorentino A, Tosti E, Panzella S, Dale B: Reducing the time of sperm-oocyte interaction in human in vitro fertilization improves the implantation rate. *Hum Reprod* 1996;11:166–171
  8. Gianaroli L, Fiorentino A, Magli MC, Ferraretti AP, Montanaro N: Prolonged sperm-oocyte exposure and high sperm concentration affect human embryo viability and pregnancy rate. *Hum Reprod* 1996;11:2507–2511
  9. Waldenstrom U, Hamberger L, Nilsson L, Ryding M: Short-time sperm egg exposure to prevent zona hardening. *J Assist Reprod Genet* 1993;10:OC–125
  10. Koifman M, Dirnfeld M, Goldman S, Gonen Y, Calderon I, Abramovici H: Very short exposure of oocytes to normal sperm in vitro improves implantation rates: a prospective randomized study. *J Assist Reprod Genet* 1995;12:OC–216 (Suppl)
  11. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction. Cambridge, Cambridge University Press, 1992
  12. Kruger TF, Menkveld R, Stander FSH, Lombard CJ, Van der Merwe JP, van Zyl JA, Smith K: Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril* 1986;46:1118–1123
  13. Lin TK, Lee RKK, Su JT, Liu WY, Lin MH, Hwu YM: A successful pregnancy with in vitro fertilization and embryo transfer in an infertile woman with Kartagener's syndrome: A case report. *J Assist Reprod Genet* 1998;15:625–627
  14. Hill GA, Freeman M, Bastias MC, Rogers BJ, Herbert CM III, Osteen KG, Wentz AC: The influence of oocyte maturity and embryo quality on pregnancy rate in a program for in vitro fertilization–embryo transfer. *Fertil Steril* 1989;52:801–806
  15. Quinn P, Lydic ML, Ho M, Bastuba M, Hendee F, Brody SA: Confirmation of the beneficial effects of brief coincubation of gametes in human in vitro fertilization. *Fertil Steril* 1998;69:399–402
  16. Ludwig M, Finas DF, Al-Hasani S, Diedrich K, Ortmann O: Oocyte quality and treatment outcome in intracytoplasmic sperm injection cycles of polycystic ovarian syndrome patients. *Hum Reprod* 1999;14:345–358
  17. Aitken RJ, Irvine PS, Wu FC: Prospective analysis of sperm–oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol* 1991;164:542–551
  18. Iwasak A, Gagnon C: Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992;57:409–416
  19. Mazzilli F, Rossi Y, Marchesini M, Ronconi C, Dondero F: Superoxide anion in human semen related to seminal parameters and clinical aspects. *Fertil Steril* 1994;62:862–868