Effects of Reducing Insemination Time in Human In Vitro Fertilization and Embryo Development by Using Sibling Oocytes[1]

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Purpose: *Recent studies showed a beneficial effect of reducing the time of sperm–oocyte interaction on fertilization, division, and implantation rates of the oocytes obtained from randomized patients. In the present study, the effects of reduced insemination time on fertilization and embryo development were evaluated by using sibling oocytes from the same patient.*

Methods: *A total of 464 oocytes from 36 patients was randomly allocated to be inseminated for either 1 hr (reduced) or 18 hr (regular).*

Results: *Fertilization rates were not significantly different between reduced (135/229; 59%) and regular (150/235; 64%) groups. Cleavage rates and embryo quality were similar in both groups. A total of 135 embryos (73 from the reduced and 62 from the regular group) was transferred to 36 patients. Thirty-four embryos implanted in 18 patients (25.2% implantation and 50.0% pregnancy rates).*

Conclusions: *Fertilization, cleavage, and embryo development from 1-hr insemination is comparable, not superior, to those from an 18-hr insemination time, which is commonly used in in vitro fertilization programs. These data suggest that reduced insemination time can be used during in vitro fertilization to avoid unnecessarily longer exposure to spermatozoa.*

KEY WORDS: embryo; fertilization; human; in vitro fertilization; reduced insemination.

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INTRODUCTION

Successful fertilization of a human oocyte requires only a single spermatozoon to penetrate through the cumulus cells, zona pellucida, and oolemma. No more than several hundred spermatozoa can be found at the site of natural fertilization in the Fallopian tubes, although millions are deposited into the vagina (1). Currently, oocytes are exposed to an excessive number of spermatozoa during in vitro fertilization (IVF) for an extended period.

It has been reported that cumulus cells sequester spermatozoa before spermatozoa penetrate through the zona pellucida in mice (2). Furthermore, Gianaroli *et al.* (3) have reported that approximately 15 spermatozoa were observed inside the cumulus mass after 15 min and the number did not change significantly 4 hr after insemination. The fertilizing spermatozoon was found inside the zona pellucida after 4 hr of insemination (3). All these findings have implied that long-term exposure of oocytes to sperm may not be necessary or may even be harmful. Human spermatozoa are known to produce reactive oxygen species (4). The generation of reactive oxygen species not only reduces the sperm fertilizing potential (5) but also may affect the subsequent embryo quality and development (6).

Recently, a reduced insemination time was used in human IVF to avoid the aforementioned concerns (3,7,8). Results from these studies were not conclusive. Although fertilization rates were not significantly different between long-time and short-time exposure (3,7), short-time exposure resulted in a significantly better fertilization rate (8). The significance was even more prominent in male-factor patients (8). The first paper by Gianaroli *et al.* (3) caused a discussion in the Letters to Editor section in *Human Reproduction* about the significance of the fertilization rate (9,10).

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In three reports published so far, about the effect of reduced insemination time on fertilization rate, oocytes from randomized patients were used (3,7,8).

In this study, the effects of a reduced insemination time (1 hr) vs. a longer insemination time (18 hr) on fertilization and embryo development were evaluated by using randomized sibling oocytes from the same patients.

MATERIALS AND METHODS

Patients. All patients with six or more oocytes retrieved on the day of oocyte recovery during in vitro fertilization treatment were included in the sibling oocyte randomization study. Patients with no fertilization in both group were excluded from the study. All data were recorded prospectively in a computer database (Microsoft Access). The diagnosis of infertility is distributed as following: 24 had tubal disease; 6 had unexplained infertility; and 3 had male factor, 1 with endometriosis, 1 with polycystic ovary, and 1 with cervical factor infertility.

Patient Stimulation. Ovarian stimulation was performed by gonadotropin-releasing hormone (GnRH) agonist, long protocol. Patients were given 3.75 mg of Lupron Depot (Abbott Laboratories, Chicago, IL) on day 3 of the cycle. On day 3 of the following cycle, human menopausal gonadotropin (hMG) (Pergonal; Serono Laboratories) injections were started, along with Suprefact nasal spray (400 μg; Hoechst UK Limited, Middlesex, UK) until the day of human chorionic gonadotropin (hCG) administration. Ten thousand IU of hCG was given intramuscularly when the leading follicle size reached 18 mm and estradiol levels were appropriate.

Oocyte Pickup. Oocyte pickup was performed with transvaginal ultrasound guidance using an aspiration needle (Svemed) under routine intravenous sedation (5 mg midazolam and 100 mg fentanyl) 36 hr after hCG injection. The follicular aspirate was poured into 60-mm dishes (No. 3004 Falcon dish) and cumulus–oocyte complexes were transferred into another dish with modified HTF–HEPES medium (Irvine, Santa Ana, CA) supplemented with 10% synthetic serum substitute (SSS) (Irvine). Each complex was evaluated for maturity based on cumulus–corona cell morphology. Two to four cumulus–oocyte complexes were transferred into 100 μl of HTF (Irvine) supplemented with 10% SSS (referred as culture medium) under mineral oil. They were incubated in 5% CO_2 in air with saturated humidity until the time of insemination.

In Vitro Fertilization. All the semen samples were allowed to liquefy for 15–30 min. A sperm count and motility analysis were performed, and depending on the result, the motile sperm fraction was enriched by using 2–4 discontinuous Percoll tubes (95 and 47.5%; Sigma, St. Louis, MO). One-half to two milliliters of raw semen was layered over the Percoll and preparations were centrifuged at 300*g* for 20 min. At the end of centrifugation, the base of 95% from each tube was collected into 5 ml of culture medium and centrifuged for another 10 min at 300*g*. Each drop, containing three or four oocytes, was inseminated with $0.7–3 \times 10^6$ motile spermatozoa/ml as a final concentration in the drop. Half of the oocytes from each patient were randomly removed from insemination drops after 1 hr and washed four times in culture medium before they were transferred into 100 μl of equilibrated culture medium under oil. The other half of the oocytes were kept in the insemination drops for 18 hr. Both groups of oocytes were decoronated and checked for the presence of two pronuclei and two polar bodies to confirm fertilization. All other outcomes (i.e., no fertilization, one pronucleus, polyspermia, degeneration) were also recorded.

Embryo Development. The number of blastomeres, degree of fragmentation, and evenness of blastomere size for each embryo were recorded after 42 and 66 hr of insemination. Embryos were graded as good, fair, and poor. Good embryos included embryos with even-sized blastomeres with no obvious fragmentation or even-sized blastomeres with less than 10% fragmentation or uneven-sized blastomeres with no obvious or less than 10% fragmentation. Fair embryos had 10 to 30% fragmentation and poor embryos were heavily fragmented (greater than 30%). The best quality of embryos regardless of group was transferred to the patient 72 hr after insemination. Patients were supplemented with progesterone (100 mg/daily intramuscularly; Steris Laboratories Inc., Phoenix, AZ). Positive pregnancy was confirmed first with a Tandom Icon urine hCG test (Hybritech, San Diego, CA) and serum β-hCG levels at 13 days and then with ultrasound 5 weeks after embryo transfer, respectively.

Statistical Analysis. Comparisons between groups were done using the Fisher exact test or χ^2 analysis. A P value of less than 0.05 was considered significant.

RESULTS

The mean age of the patients in the study was 32.1 ± 4.9 years, with 9.6 ± 4.9 years of infertility. The

estradiol level on the day of hCG injection was 10,587 ± 6012 p*M*. A total of 464 oocytes from 36 patients was randomly allocated to be inseminated for either 1 hr (reduced) or 18 hr (regular). Fertilization rates were not significantly different between the reduced (135/229; 59%) and the regular (150/235; 64%) insemination groups (Table I). Similar cleavage rates were obtained in both groups [reduced, 128/135 (95%); regular, 135/150 (90%)]. The number of embryos with more than three cells was comparable between the reduced (64/128; 50%) and the regular (55/135; 41%) insemination group on day 2 after insemination (Table I). The rates of good embryos were 39.8 and 43.7% in reduced and regular insemination groups, respectively, on day 3 after insemination. Also, the number of embryos with more than six cells was similar between the reduced (51/128; 40%) and the regular (50/135; 37%) insemination groups on day 3 after insemination (Table I). None of the differences were significant between the two groups. A total of 135 embryos (73 from reduced and 62 from regular group) was transferred to 36 patients (Table I). Thirty-four embryos implanted in 18 patients (25.2% implantation and 50.0% pregnancy rates; Table II).

DISCUSSION

The results of this study demonstrate that overnight incubation of oocytes with a supraphysiological number of spermatozoa, as is widely practiced in in vitro fertilization laboratories, is not necessary. Reduction of insemination time can yield similar fertilization rates. Gianaroli *et al.* (8) reported even better fertilization rates with the shorter insemination time. However, Waldernstrom *et al.* (7) and Gianaroli *et al.* (3) have reported that reduced insemination times resulted in similar fertilization rates compared with longer insemination times. All these studies were designed to use oocytes from randomized patients. The present study was conducted by using sibling oocytes from the same patient to test the effects of reduced insemination time in a better controlled study. The results were in agreement with the results of Waldernstrom *et al.* (7) and Gianaroli *et al.* (3), showing that fertilization rates were similar. Fertilization is the result of a series of processes that should take place in sequence. From insemination to the formation of two pronuclei several steps have to be completed. First, the fertilizing spermatozoon has to penetrate into cumulus cells and bind to the zona pellucida. Then it has to pass through the zona pellucida into the perivitelline space to fuse the oolemma (for review, see Ref. 11). Once a spermatozoon goes through the zona pellucida, no more spermatozoa are required for the completion of fertilization. Gianarolli *et al.* (3) have shown that sperm entry into cumulus cells reaches saturation after 15 min of insemination and the number of spermatozoa trapped into the cumulus mass did not change during 4 hr of culture. Moreover, the fertilizing spermatozoa can be seen at the subcortical area of oocytes 4 hr following insemination (3). It is well-known that spermatozoa as well as white blood cells originating from the ejaculate are capable of producing reactive oxygen species (4,5,12). Moreover, it has been reported that the production of reactive oxygen species not only impaired sperm function but also reduced fertilization rates in in vitro fertilization cycles (5). It has been also shown that the generation of reactive oxygen species could be damaging to embryos (6). In this study, semen was processed by discontinuous Percoll gradient, and only the base of the 95% fraction was collected to be used for insemination. This fraction is an enriched motile spermatozoon fraction, although the presence of white

Table I. Fertilization and Early Embryo Development from Sibling Oocytes that Were Inseminated for Either 1 or 18 hr[a]

	Insemination duration	
	18 hr	1 hr
Number of		
Oocytes	235	229
Fertilized	150 (63.8)	135 (58.9)
Divided	135 (90)	128 (94.8)
Embryos ≥ 4-cell[b]	55 (41.4)	64 (50)
Embryos ≥ 7-cell[c]	50 (37.0)	51 (39.8)
Good embryos[c]	59 (43.7)	51 (39.8)
Transferred embryos	62	73

[a] Numbers in parentheses are percentages. None of the values are significantly different from each other.
[b] Forty-two hours after insemination.
[c] Sixty-six hours after insemination.

Table II. Pregnancy and Implantation Rates After the Mixed Transfer of Embryos Generated from 1 or 18 hr of Insemination

No. cycles with transfer	36
No. embryos transferred	135
No. gestational sacs	34 (25.2)[a]
Pregnancies	
Biochemical	1 (2.7)
Clinical	18 (50.0)
Aborted	3 (15.8)
Delivered	15 (78.9)
No. multiple gestation	10 (52.6)

[a] Numbers in parentheses are percentages.

blood cells has been reported after Percoll preparation (4). Nevertheless, it is not evident from the present study that long-term incubation with sperm results in reduced fertilization. However, the results of this study as well as others (3,7,8) confirmed that the overnight incubation of sperm and oocytes is not required or beneficial.

Cleavage rates, the number of blastomeres in each embryo, and the embryo quality were similar in the reduced and regular groups. Results about cleavage rate and the embryo quality are in agreement with earlier reports (3,8). In the present study, the number of embryos with four or more cells or seven or more cells was not significantly different on day 2 and day 3 after insemination between the two groups, respectively. However, it has been reported previously that a short-time insemination group had more fast-cleaving embryos compared to regular insemination (3,8). Nonetheless, if this is true, one might expect that embryos generated by intracytoplasmic sperm injection should be superior to in vitro fertilization embryos. This issue is controversial in the literature. Hall *et al.* (13) reported no difference in embryo quality between sibling embryos obtained from either intracytoplasmic sperm injection or in vitro fertilization with a high insemination concentration. On the other hand, Oehninger *et al.* (14) have reported that intracytoplasmic sperm injection produced a significantly higher number of morphologically superior embryos compared with a similar group of patients who had in vitro fertilization. Yang *et al.* (15) have also reported improved quality of embryos generated from intracytoplasmic sperm injection compared with sibling embryos obtained from in vitro fertilization. The differences may result from either experimental design or patient population among the studies.

The results of this study as well as others (3,7,8) show that the shorter exposure time to sperm is enough to achieve a fertilization rate comparable to that with the current long-term exposure time. There is no need to expose oocytes for an unnecessarily longer time to spermatozoa. The current in vitro culture conditions for human embryos are neither ideal nor optimal. The presence of an extraphysiological number of spermatozoa for an extended period may further increase embryonic stress in culture.

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