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ASSISTED REPRODUCTION TECHNOLOGIES

Does reducing gamete co-incubation time improve clinical outcomes: a retrospective study

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Abstract
Purpose The objective of this retrospective study was to determine whether patients undergoing in vitro fertilization (IVF) benefit from reducing the gamete co-incubation time.
Methods Patients (n=570) were enrolled, including 281 patients in the reduced incubation time group (2-h incubation) and 289 patients in the standard IVF group (18-h incubation).
Results The observed outcomes, including the clinical pregnancy rate (CPR), implantation rate (IR), live birth rate (LBR), and miscarriage rate (MR), were similar between the two groups. When the data were divided into two subgroups based on the maternal age (≤30 and >30 years), the rates of top-quality embryos (30.83 vs. 25.89 %; p=0.028), CPR (66.67 vs. 42.11 %; p=0.013), and IR (41.90 vs. 31.25 %, p=0.019) of the 2-h incubation group were significantly higher in the younger subgroup. However, for older patients, only a lower MR (7.59 vs. 20.83 %; p=0.019) was achieved. Reducing the time of incubation still improved the CPR (OR=1.993, 95 % CI 1.141–3.480) and MR (OR=3.173, 95 % CI 1.013–9.936) in the younger and older subgroups, respectively, after it was adjusted for potential confounders.
Conclusions Reducing incubation time improves the clinical results of IVF, although the LBR is not statistically different between the 2- and 18-h incubation time groups. And the specific clinical outcomes of reducing incubation time varied between the >30-year-old and the ≤30-year-old.

Keywords Fertility · Gamete co-incubation · Live birth rate · Miscarriage rate

Capsule Reducing the incubation time favors the clinical results of IVF, although the manifestations vary with the different age groups.

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Introduction

During standard in vitro fertilization (IVF) in humans, oocytes are usually exposed to sperm for 16–18 h. Moreover, the number of sperm in proximity to oocytes during IVF is higher than during in vivo fertilization. Prolonged co-incubation of gametes and a high concentration of sperm may produce high levels of reactive oxygen species (ROS), which may decrease membrane fluidity by peroxidating polyunsaturated fatty acids [1], induce zona hardening, and affect embryo quality [2, 3].

There are several studies that show an enhanced clinical pregnancy rate (CPR) and implantation rate (IR) after reducing the co-incubation time of gametes (brief IVF) [2, 4–6]. However, only a few of them report the clinical results, including live birth rate (LBR) [7] and miscarriage rate (MR) [4]. Moreover, Barraud-Lange and co-workers [8] indicate that the fertility rate has been decreased in brief IVF compared with standard IVF; however, in their study, no specific clinical results, such as CPR, IR, and LBR, have been noted. Lundqvist and co-workers [7] showed that no beneficial effects were obtained from brief IVF, although the LBR was higher after reducing co-incubation versus standard IVF. The probable reason for this is because their study enrolled a smaller cohort of patients (87 patients in total), which was too small to show a difference in the two groups. Two meta-analyses recently showed that reducing the incubation time improved the CPR and IR; however, it was unknown whether or not reducing the incubation time improved the LBR and MR. [9, 10].

In this study, we compared the clinical outcomes in gametes with standard and brief IVF retrospectively. The diploid fertilization rate (two pronuclei, 2PN), polyspermy rate (>2PN), CPR, IR, LBR, and MR were analyzed in the two groups.

Materials and methods

Patients

This study was approved by the ethics committee of the authors' hospital (2011ECRM NO.4), and informed consent was obtained from all participating couples. Patients undergoing an IVF protocol were enrolled in the study between 1 October and 31 December 2011. The patients were excluded if one or more of the following criteria were present: (1) chromosome abnormality, (2) uterine abnormality, and (3) endometrial thickness <8 mm on the day of human chorionic gonadotropin (hCG) administration.

In addition, embryos are transferred routinely on day 3 in our clinic. Blastocyst transfer usually is used for patients with previously unsuccessful IVF-embryo transfer cycles because of repeated implantation failure. Thus, those patients who underwent blastocyst transfer were excluded to avoid possible bias.

Ovarian stimulation and oocyte handling

All patients undergoing the gonadotropin-releasing hormone agonist (GnRHa) long protocol were processed for pituitary downregulation during the luteal peak period with 0.93–1.25 mg of triptorelin (diphereline, 3.75 mg; Beaufour Ipsen, Paris, France) or a short-acting GnRHa (decapeptyl, 0.1 mg; Ferring Pharmaceuticals, Saint-Prex, Switzerland). A basic evaluation was conducted comprising an ultrasound examination and blood testing for hormone levels after 14 days. If downregulation standards were met, recombination FSH (Gonal-F; Merck-Serono, Rome, Italy) and/or human menopausal gonadotropin (Lizhu, Zhuhai, China) injections were administered. The starting doses (150–300 IU/L per day) were selected according to age, basal FSH level, and the antral follicle count. Ultrasonography and blood sex hormone testing were used to monitor the development of follicles, and 10,000 U of hCG (hCG; Lizhu) was injected intramuscularly for final oocyte maturation when at least one follicle reached 18 mm or two follicles reached 17 mm according to ultrasonography. Oocyte retrieval was carried out 36–38 h after hCG administration by transvaginal ultrasound-guided puncture of follicles.

Oocytes were collected in Gmops (10136; Vitrolife Sweden AB, Göteberg, Sweden), washed twice in equilibrated IVF medium (10086; Vitrolife Sweden AB), and then placed in equilibrated IVF medium in central well dishes (353075; BD Falcon Labware; BD Biosciences, San Jose, CA, USA) as described previously [7]. All of the oocytes in each group were incubated together in 1 mL of equilibrated IVF medium overlaid with paraffin oil (10029; Vitrolife Sweden AB) in a central well dish.

Sperm preparation and insemination procedures

Semen samples were collected by masturbation after 3 days of sexual abstinence. Samples were assessed for sperm concentration, motility, and vitality before and after selection according to the 2010 World Health Organization guidelines. After liquefaction, sperms were selected by gradient centrifugation (90 and 45 %, SpermGrad; 10099; Vitrolife-Sweden AB) and centrifuged at 500×*g* for 15 min. The sperm pellet was collected and washed in SpermRinse (10101; Vitrolife Sweden AB) at 300×*g* for 5 min and then IVF medium at 300×*g* for 5 min. The final sperm pellet was resuspended in 1 mL of IVF medium for insemination.

Before co-incubation of the gametes, the rate of the progressive sperm cells was evaluated and the oocytes were inseminated with 1.0×10^5 progressive sperm cells per milliliter 3–5 h after oocyte retrieval. The oocytes in the standard incubation time group were co-incubated with sperm in 1 mL of IVF medium for 16–18 h. In the reduced incubation time group, oocytes were removed and washed gently in 1 mL of fresh IVF medium and then placed into another central well

dish filled with 1 mL of IVF medium for 2 h. Eighteen hours later, all oocytes in both groups were denuded and assessed for fertilization. The zygotes then were cultured individually in 20 μL of G1 medium (10128; Vitrolife Sweden AB) and covered with paraffin oil. Over the next 2 days, the development and morphology of all embryos were evaluated.

Embryo score and embryo transfer

Embryos were scored according to the following criteria as described previously [11]: grade I, embryos have uniform blastomeres with no obvious fragmentation; grade II, blastomeres are of slightly uneven size or the cytoplasmic mass contains <10 % fragmentation; grade III, blastomeres have a cytoplasmic mass that contains 10–50 % fragmentation; and grade IV, blastomeres are of significantly uneven size and have >50 % cytoplasmic fragmentation. The number of blastomeres per embryo was also recorded. Embryos of grades I and II, with four cells on day 2 and eight cells on day 3, were defined as "top-quality embryos." Two or three embryos with the best morphology were transferred using a soft cook transfer catheter on day 3 after oocyte retrieval.

A clinical pregnancy was defined as an intrauterine gestational sac with a heart beat at 3 weeks after a positive hCG test. For the determination of CPR and LBR, the denominator was the number of patients who had received embryo transfer. For the determination of MR, the denominator was the number of clinically pregnant patients. The implantation rate was calculated as the ratio of gestational sacs with a heart beat observed by transvaginal ultrasonography in relation to the total number of embryos transferred.

Statistical analyses

The data were analyzed using SPSS statistical software (version 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). Continuous variables were presented as the mean±SD, and differences in the means between groups were determined using a two-sample t test. For categorical variables, a Pearson χ^2 test was used to analyze the differences between groups. Fisher's exact probability test was used in cases where the expected frequency was less than five. Logistic regression analyses were used to evaluate possible associations of clinical outcomes with co-incubation period and other confounding factors. Statistical significance was set at a $p<0.05$.

Results

Five hundred and seventy patients were enrolled in this study. The mean age of the patients was 32.04±4.34 years (range 21–44 years). There were 281 patients in the reduced incubation time group and 289 patients in the standard incubation time group. Demographic data are given in Table 1; all statistical categories were similar in the two groups. Although the top-quality embryo rate, CPR, IR, LBR, and MR were

Table 1 Demographic characteristics of patient (n=570)

	2 h (n=281)	18 h (n=289)	p
Maternal age	32.28±4.33	31.80±4.35	0.114
Duration of stimulation (days)	10.43±2.25	10.16±2.15	0.151
Total dose gonadotropin per cycle	2039.01±703.16	1991.44±968.72	0.503
Baseline FSH	8.20±3.35	8.11±3.34	0.751
Baseline LH	4.68±3.02	4.89±3.51	0.427
Endometrial thickness on hCG day (mm)	11.52±2.47	11.78±2.77	0.236
Duration of infertility (years)	5.27±3.58	4.88±3.24	0.172
BMI	21.14±2.52	21.02±2.81	0.585
Mean no. of oocytes	10.55±5.42	10.29±5.61	0.583
Embryo transfer number	2.25±0.55	2.18±0.56	0.157
Rate of GnRHa long protocol	90.75 %	87.20 %	0.177
Rate of recurrent abortions	1.78	1.73	0.964
Etiology			
Tubal factor (%)	140 (49.82)	153 (52.94)	0.456
Endometriosis (%)	10 (3.56)	5 (1.73)	0.173
Male factor (%)	16 (5.67)	17 (5.88)	0.923
Unexplained (%)[a]	4 (1.42)	2 (0.69)	–
Mixed factor (%)	109 (38.79)	107 (37.02)	0.664
Ovulation dysfunction (%)	2 (0.71)	5 (1.73)	0.451

[a] There is no statistical analysis because the statistics of the two groups are less than 5

Table 2 Outcomes of reducing co-incubation time on the clinical results

	2 h (n=281)	18 h (n=289)	p
2PN (%)	69.94	71.74	0.159
>2PN (%)	11.56	11.85	0.764
Rate of top-quality embryos (%)	26.13	24.38	0.211
Top-quality embryos transferred per ET cycles	0.32±0.62	0.35±0.67	0.590
CPR (%)	52.31	45.33	0.189
IR (%)	28.48	26.62	0.460
LBR (%)	40.93	34.26	0.100
MR (%)	11.56	15.27	0.401

ET embryonic transfer

improved in the reduced incubation time group, they were not significantly different between the two groups (Table 2).

We first divided the patients according to the age of the female into two subgroups: ≥35 and <35 years old. The results showed that the CPR was significantly higher after 2-h incubation in the <35-year subgroup (60.62 vs. 50.23 %, p=0.036) and that the MR was lower in the ≥35-year subgroup; however, the difference was not significant compared with the 18-h incubation group (10.00 vs. 29.17 %, p=0.072) (Supplementary Table S1).

Following the method of Menezo and Barak [12], patients were further categorized into three subgroups as follows: <30, 30–35, and >35 years. To determine the effect of reducing incubation time on the clinical results of different age subgroups, other subgroups (ages 30–38 and 38–44 years) also were analyzed. The results showed that when patients were aged <30 years, the CPR and IR were significantly higher in the 2-h incubation group, the same as observed in the ≤30-year subgroup. The rates of 2PN, >2PN, and top-quality embryo and CPR, IR, and MR were not significantly different in the three subgroups (>35, 30–38, and 38–44 years) (Supplementary Table S2).

The patients were then divided into two groups with the cut-off at age 30 years (Table 3). The top-quality embryo rate, CPR, and IR in the 2-h incubation time group were significantly higher than the rates in the 18-h incubation time group when the patients were ≤30 years of age (30.83 vs. 25.89 %, p=0.028; 66.67 vs. 42.11 %, p=0.013; and 41.90 vs. 31.25 %, p=0.019, respectively). In contrast, when the patients were >30 years of age, the CPR and IR were similar in the two groups (p>0.05); however, the MR was improved significantly (7.59 vs. 20.83 %, p=0.019). The 2PN rate, >2PN rate, and LBR did not differ significantly in the two subgroups, although the LBR was higher in both the study groups.

A logistic analysis was performed to adjust the risks for clinical outcomes after 18- and 2-h incubation in different maternal age subgroups. The results showed that reducing the time of incubation still significantly improved the CPR (OR=1.993, 95 % CI 1.141–3.480) and MR (OR=3.173, 95 % CI 1.013–9.936) in the younger and older subgroups, respectively, after adjusting for confounders (Table 4).

Discussion

During the period of co-incubation some metabolites, such as ROS [1], E_2, and P_4 [6], are typically produced by sperm and

Table 3 Outcomes of reducing co-incubation time on the clinical results in the different subgroups (n=570)

	Female >30 (n=350)			Female ≤30 (n=220)		
	2 h (n=179)	18 h (n=171)	p	2 h (n=102)	18 h (n=118)	p
2PN (%)	70.97	72.54	0.348	68.28	70.82	0.202
>2PN (%)	11.19	12.13	0.431	12.15	11.54	0.659
Rate of top-quality embryos (%)	23.31	23.08	0.890	30.83	25.89	0.028
CPR (%)	44.13	42.11	0.903	66.67	42.11	0.013
IR (%)	21.80	23.79	0.50	41.90	31.25	0.019
LBR (%)	36.31	30.41	0.288	50.00	39.83	0.130
MR (%)	7.59	20.83	0.019	16.18	8.47	0.192

Table 4 Adjusted risks for clinical outcomes after conventional and brief IVF in different maternal age subgroups

	≤30 years		>30 years	
	p	OR (95 % CI)[a]	*p*	OR (95 % CI)[a]
Clinical pregnancy	*0.015*	*1.993 (1.141–3.480)*	0.905	0.973 (0.617–1.533)
Live birth	0.123	1.538 (0.890–2.659)	0.350	1.254 (0.780–2.018)
Miscarriage	0.226	0.484 (0.150–1.565)	0.047	3.173 (1.013–9.936)

Italicized values indicate the main findings

[a] Adjusted for maternal age, IVF cycles of treatment, BMI, infertility duration, bFSH, BMI, amount of Gn, and endometrial thickness on hCG day, number of oocytes retrieved, number of embryos transferred

cumulus cells. These factors, which are increased in a conventional IVF procedure because of the length of incubation, may affect the vitality, even the epigenetics of the embryos [13, 14].

Our study suggested that reducing the co-incubation time improved IVF outcomes in different ways. For patients ≤30 years of age, CPR and IR were improved significantly in the reduced incubation time group. This result is in agreement with the observations of others [4, 6]; however, for patients >30 years of age, only the MR was reduced significantly by reducing incubation time. Our study also showed that the fertility rate was similar in the two groups, which was in agreement with previous studies using sibling oocytes [7, 15].

There are many factors that affect the outcome of IVF; the age of the female is considered to be one of the most important [16]. The impact of age is particularly evident in the increased percentage of fragmentation in embryos from older patients during preimplantation stages [16]. Thus, we further analyzed the data based on the age of the female.

Thirty-five years is usually considered the cut-off age in clinical pregnancy, but the sample size in the ≥35-year subgroup (n=164) may be too small to show a statistical difference in the two groups. As a result, 30 years of age has been used as a cut-off in some studies [12, 16]. In addition, we found that the MR was significantly reduced and that the CPR and IR were improved significantly in the >30- and ≤30-year subgroups, respectively (Table 3). This indicated that reducing the incubation time improved the clinical results in different ways.

Thouas and co-workers [17] report that aged oocytes were more sensitive to mitochondrial damage and more susceptible to ROS during the insemination process. According to these findings, oocytes from older women would benefit from reducing incubation time. Therefore, we assumed that an improvement would be achieved by reducing the gamete co-incubation time in the older subgroup of patients. It is noteworthy that our study showed that reducing the incubation time improved the MR but not the rates of high-quality embryos, pregnancy, and implantation for patients >30 years of age, implying a "delayed effect."

Why did a reduction in the incubation time not result in an improvement in the older subgroup at an earlier stage? Some studies have indicated that chromosomal abnormalities occur in 60–70 % of embryos resulting from IVF in older women [18, 19]. In addition, Friedman and co-workers [20] report that a hypoxic environment was associated with poor oocyte quality and elevations in vascular endothelial growth factor, which was increased in follicular fluid with advancing age [21]. Moreover, a number of studies revealed changes in DNA methylation with the aging process [22–24]. The highest amount of 5-methylcytosine was observed in embryos and then decreased gradually [25]. These studies indicate that the vitality of oocytes decreases with the genetic and epigenetic changes. Thus, we speculated that these oocyte factors in older women may compromise the results of reducing the incubation time.

The age of 35 years is usually considered the shift point in fertility in clinical pregnancies. It may be more reasonable for us to stratify by age 35 years. However, there were only 164 patients in the ≥35-year subgroup, which was not enough to show a significant difference in the 2- and 18-h incubation groups, which may be the main limitation of this study.

In conclusion, our study suggests that reducing the incubation time favors the clinical results of IVF, although the LBR is not different significantly between the 2- and 18-h incubation time groups. Moreover, the specific outcomes varied with the different age-groups. Additional studies, especially multicenter, randomized, controlled clinical studies, are needed to confirm the results. Moreover, current data have not established a causal relationship between altered clinical results and metabolic or epigenetic changes. Thus, the effect of reducing co-incubation time on embryonic metabolism and epigenetics should also be further studied.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval This study was approved by the ethics committee of the authors' hospital (2011ECRM NO.4), and informed consent was obtained from all participating couples.

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