Short gamete co-incubation during in vitro fertilization decreases the fertilization rate and does not improve embryo quality: a prospective auto controlled study

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Abstract

Purpose Evaluate the effect of short gamete incubation on fertilization rate and embryo quality.

Methods A prospective study has been performed. Two thousand five hundred and forty seven sibling oocytes from 240 couples undergoing IVF attempts were allocated to a short (1 h) or a standard (18 h) insemination procedure. Diploid fertilization rate (two pronuclei, 2PN), polyspermy (>2PN) and embryo quality were compared.

Results The fertilization rate was statistically lower in the short insemination group compared to the standard insemination one (64.9% and 70.1%; $P=0.039$), with a similar polyspermy rate observed between the two groups. A slight, but non significant, increase was observed concerning good embryo quality rate in the short insemination group when compared to the standard insemination, both at day 2 (60.1 vs. 58.1%; $P=0.06$) and day 3 (53.2 vs. 48.5%; $P=0.22$).

Capsule This study highlights that a 1 h gamete exposure decreases the fertilization rate and does not improve embryo quality compared with a standard 18 h insemination procedure.

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Conclusion This new study highlights that a 1 h gamete exposure decreases the fertilization rate and does not improve embryo quality compared with a standard 18 h insemination procedure.

Keywords Fertilization rate · Embryo quality ·
Short gamete insemination · Human IVF

Introduction

In vitro fertilization (IVF) techniques are continuously progressing since the 1980s. A way to improve their results consists in the optimization of methodologies to achieve better embryo quality. The actual standard IVF protocol recommends an overnight gamete co-incubation. Fertilization is assessed by the observation of two pronuclei 18 h later. However, several studies of gamete kinetic interaction *in vivo* have shown that (1) gamete contact and fertilization occur within 20 min post coitus in mammals [1, 2] and (2) an average number of 15 spermatozoa are observed, *in vitro*, in the cumulus after 15 min of gamete contact. Furthermore, 80% of oocytes are fertilized after 1-h exposure to spermatozoa [3–5]. On the other hand, reactive oxygen species (ROS) released by the cellular metabolism have been implicated in (1) fatty acid peroxydation causing a decrease in membrane fluidity [6, 7], (2) DNA fragmentation [8] and (3) embryo developmental arrest [9]. It is therefore possible that unnecessary long inseminations expose the oocyte and the zygote to the detrimental effect of ROS released by the spermatozoa, in a concentration dependant manner [6]. Based on this hypothesis, some investigators tried to test the beneficial effect of a shortened gamete contact on embryo quality and pregnancy rate. Some of them have shown an enhanced embryo quality and

pregnancy rate after a brief gamete contact of 1 to 4 h [3, 10, 11], while others have described an equivalent embryo morphology or pregnancy rate compared to a 18 h incubation [12–16]. The fertilization rate in case of shortened gamete contact was usually unchanged except as reported by Lundqvist *et al.*, which has described a decreased fertilization rate in the short insemination group compared to the standard one [14]. These results, obtained with varying IVF protocols and study designs, are contradictory and suggest that the improvement of IVF outcome by a reduction of the oocytes's insemination duration is still an open debate.

The aim of this new study was to investigate, on a large series, whether a short time of gamete co-incubation would improve embryo quality, without any detrimental effect concerning the fertilization rate. We performed a prospective auto controlled study and compared on sibling oocytes the following endpoints: diploid fertilization rate (two pronuclei, 2PN), polyspermy rate ($>2PN$) and embryo quality, according to the insemination protocol. To eliminate any possible bias which could influence the IVF outcome, such as etiology and duration of the infertility, age of women or ovarian stimulation protocol, we allocated every other sibling oocyte of all patients to either short exposure or to conventional insemination protocol; so the patients were used as their own controls. As far as we know, this work proposes the largest series ever published on this field.

Materials and methods

Patients

Between June 2004 and November 2005, 240 couples referred in our Infertility clinic for standard IVF were involved in the prospective auto controlled study (Table 1). This study was approved by the local ethical desk. Furthermore, each included couple has signed an approval consent form before being enrolled in the study. Patients with less than four oocytes at the time of ovarian puncture were excluded since

this number is required to expect at least one embryo in each group. Patients with fertilization failure in both groups were excluded as well. The demographic characteristics of the patients are presented in the Table 1. Mild male factor infertility is defined when the sperm concentration ranges from $10.10^6/ml$ to $20.10^6/ml$ and/or when percentage of grade “a” motility ranges from 10% to 25% and/or when percentage of normal head form ranges from 15% to 30%, according to WHO criteria [17]. Each patient could contribute with more than one cycle.

Patient stimulation and oocyte recovery

Based on prior evaluation of the ovarian function, three protocols of controlled ovarian stimulation (COS) were used. (1) The long agonist protocol used a first step of pituitary desensitization by GnRH agonist (Decapeptyl®, Ipsen-Biotech, Paris, France) followed by COS. (2) The short protocol used a GnRH agonist administration started 2 days before COS. (3) The antagonist protocol including COS associated to a daily GnRH antagonist injection (Cétrotide®, Serono, France) when the leader follicle reached about 14 mm in diameter. In all cases, COS was performed with s.c. administration of recombinant human FSH (75 IU per ampoule; Puregon®, Organon, Saint Denis, France; Gonal-F®, Serono, Boulogne, France) starting with one ampoules per day and adapted from day 6 onwards on an individual basis according to the ovarian response as assessed by sequential transvaginal ultrasonography and serum estradiol measurements. Human chorionic gonadotrophin (hCG; 10,000 IU, Organon, France) was administered i.m. when a consistent rise in serum oestradiol concentration was associated with the presence of ≥ 4 follicles ≥ 16 mm in diameter. Transvaginal oocyte retrieval was scheduled 36 h after hCG administration under ultrasound guidance and usual IVF techniques were performed. The luteal support was initiated on day 1 after oocytes retrieval with 400 mg/day of progesterone. In the absence of ovarian hyperstimulation syndrome (OHSS) risk, 2,500 and 1,500 units of hCG were administered 1 and 3 to 6 days after oocytes retrieval, respectively.

Insemination and culture procedures

Semen samples were collected by masturbation after 3 days of sexual abstinence and sperm were selected on a two density (90 and 45%) Puresperm® gradient (Nidacon International, Gothenburg, Sweden) centrifugation. Sperm concentration, motility and vitality were assessed before and after selection, according to WHO guidelines (WHO 1999). Each oocyte was inseminated, 4 h after ovarian puncture, in a $20 \mu l$ equilibrated drop of culture medium (Ferticult®, Fertipro, Beernem, Belgium), overlaid

Table 1 Demographic characteristics of the patients

Patients (n)	240
Female age, mean $\pm$ SD (years)	34.0 $\pm$ 4.2
Infertility etiology, n (%)	
Tubal disease	140 (58.3)
Endometriosis	16 (6.7)
Dysovulation	5 (2.1)
Mild male factor	41 (17.1)
Idiopathic	38 (15.8)
Number of retrieved oocytes	2,547
Number of oocytes per patient, mean $\pm$ SD	9.5 $\pm$ 5.1
Metaphase II stage oocytes (%)	78.4

with paraffin oil (MediCult®, Lyon, France) in a 5% CO₂ atmosphere. For each patient, every other cumulus–oocyte complex was allocated either to a 1-h insemination group (Short Insemination group) or to an overnight standard procedure (Standard Insemination group), in the same Petri dish (Falcon®, Becton-Dickinson, Le Pont de Clay, France). When the number of oocytes in the cohort was an odd number, the last was systematically allocated to the standard reference insemination group. Oocytes were inseminated with 1 to 2 µl of sperm preparation, at a final concentration of 150,000 progressive motile sperm cells/ml, according to the French Guidelines (Arrêté du 12 Janvier, 1999). One hour after the insemination, cumulus from the short insemination group were gently washed in a 20 µl drop of clean culture medium by driving them in and out of a sterile cone using a 20 µl pipette (Pipetman®, Gilson) adjusted on 4 µl. Eighteen to 20 h after the oocyte insemination, fertilization was assessed by the presence of two pronuclei (2PN) and two polar bodies, defining the fertilization rate [(number of 2PN/number of metaphase II stage oocytes)×100]. Polyspermy was defined as more than two visible pronuclei. Embryos were cultured using commercially available media for two more days, with development monitored daily.

Embryo development

Embryo morphology was assessed every day of culture. Embryos were scored according to criteria previously described [18]. Briefly, embryos were classified as: grade A, no cellular fragmentation; grade B, <20% fragmentation; grade C, 20% to 50% fragmentation; and grade D, >50% fragmentation. The number of blastomeres per embryo was also recorded. Grade A and B embryos constituted the “good quality embryo”. For A, B and C embryo qualities, a cellular index was defined as the ratio of the total number of blastomeres over the total number of embryos obtained for each grade. The ideal index was four cells at day 2 and eight cells at day 3 of culture. Percentage of good quality embryos were compared among the cohorts when the patients had obtained at least one embryo in each group. Cellular indexes were compared for the patients who had good quality embryos in both groups.

Statistical analysis

Data are presented as mean±SD or counts and percentages unless otherwise stated. The difference in fertilization rate, polyspermy rate and percentage of good quality embryos between short and standard insemination times were analyzed using conditional logistic regression models with generalised estimating equations to account for both correlation between observations from the same patient

and the size of the cohort of embryos from each patient. Differences were considered as statistically significant when $P<0.05$. Analyses were performed using R 2.2.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria).

Results

In all, 2,547 inseminated oocytes from 266 IVF attempts were analyzed. One thousand two hundred and thirty two and 1,315 oocytes were inseminated with the short and the standard insemination protocol, respectively. According to the exclusion criteria of fertilization failure within the two groups, 104 and 130 oocytes were not analyzed in the short and the standard insemination group, respectively (Fig. 1). There was a similar percentage of metaphase II stage oocytes (78.7% vs. 78.1%) in the short and standard insemination groups, respectively. Sperm samples at the day of oocyte retrieval presented a WHO grade “a” motility (17) of $24.3\pm 10.8\%$ and $81.4\pm 11.1\%$ before and after selection, respectively. There was a statistically significant difference between the fertilization rates of short and standard insemination groups (64.9% vs. 70.1%; $P=0.039$; OR=1.39, 95%CI: 1.11–1.74), respectively (Table 2). As we have included male factor infertility, we tested for the possible influence of such an indication to explain the lower fertilization rate observed in the short insemination group. Analysing the IVF attempts with normal sperm, we observed a fertilization rate of 66.7% and 69.9% in the short and standard insemination groups respectively, which still showed a statistically significant decrease ($P=0.03$; OR=1.32; 95% CI: 1.03–1.70). Polyspermy rates were similar in short and standard insemination groups (3.6% vs. 3.7%, $P=0.94$, respectively) (Table 2). A trend for better embryo quality was observed for embryos resulting from the short insemination group compared to those from the standard insemination group (Day 2: 60.1% vs. 58.1%; $P=0.06$; Day 3: 53.2% vs. 48.5%; $P=0.22$) (Table 2), but the difference was not significant. The mean number of blastomeres per embryo at day 2 and day 3 of culture was also similar between short and standard insemination groups (Day 2: 3.2 ± 1.0 vs. 3.3 ± 1.1 ; $P=0.64$; Day 3: 5.8 ± 2.0 vs. 6.1 ± 1.6 ; $P=0.13$), respectively (Table 2).

Discussion

The aim of this study was to investigate whether a short gamete insemination time would improve embryo quality without any deleterious effect on the fertilization rate. We showed that the shortening of gamete co-incubation decreased the fertilization rate and did not improve embryo

Fig. 1 The consort flowchart of oocytes through the trial

quality. As we have included in our study mild male factor infertility, we tested for the possible influence of such altered sperms, linked to a risk of oocyte fertilization failure [19, 20], to explain the lower fertilization rate in the short insemination group. In this regard, the persistence of a significantly decreased fertilization rate in the attempts performed with normal sperm confirmed the specific deleterious effect of a shortened gamete exposure.

The lower fertilization rate observed in the short insemination group of our study agrees with the findings of Lundqvist *et al.* [14] (72.5% vs. 80.5%, $P=0.02$); although this work compared a 2-h, and not a 1-h, co-incubation to an overnight protocol. Most of the others studies reported a similar fertilization rate in the short insemination group and in the standard one. However, in these last studies, the fertilization rate was lower in the group of short insemination time compared to the classical

18 h insemination procedure (fertilization rates ranged from 56 to 79% vs 61 to 87%, respectively), but the difference was not statistically significant [10–13]. We hypothesized two possible explanations for such conflicting results. Firstly, we designed our study as an auto-controlled one when some studies performed a prospective trial among patients [11, 13]. In these cases, the two groups could not be as similar as those resulting from the use of sibling oocytes. Secondly, in our study, the statistical analysis was performed at the oocyte level using conditional logistic regression models with generalised estimating equations taking into account the size of each oocyte cohort to prevent small cohorts from having the same impact on the result than larger ones. Moreover, the study was designed to allow within-subject comparison, as each patient had oocytes allocated to both short and standard insemination. The conditional regression we used was thus well-suited for

Table 2 Oocyte maturity, fertilization, polyspermy and embryo quality according to gamete co-incubation protocol (short vs standard insemination)

	Short insemination	Standard insemination	<i>P</i>
Metaphase II stage oocytes, <i>n</i> (%)	888 (78.7) ^a	926 (78.1) ^a	0.82
Diploid fertilization rate (%)	576/888 (64.9) ^b	649/926 (70.1) ^b	0.039
Polyspermy rate (%)	32/888 (3.6) ^a	34/926 (3.7) ^a	0.94
Day 2			
Grade A+B embryos (%)	311/518 (60.1) ^c	347/597 (58.1) ^c	0.06
No of blastomeres per A+B embryos, mean ± SD	3.2±1.0 ^a	3.3±1.1 ^a	0.64
Day 3			
Grade A+B embryos (%)	201/378 (53.2) ^d	206/425 (48.5) ^d	0.22
No of blastomeres per A+B embryos, mean±SD	5.8±2.0 ^a	6.1±1.6 ^a	0.13

^a Paired Wilcoxon test^b Conditional logistic regression: odds-ratio: OR=1.39, 95%CI: 1.11–1.74^c Conditional logistic regression: OR=0.75, 95% CI: 0.56–1.01.^d Conditional logistic regression: OR=0.80, 95% CI: 0.57–1.14.

this subject-specific analysis [21]. Actually, should the analysis be performed patient per patient, using a paired *t*-test as in all previous studies, no significant difference would be detected between the fertilization rate of our two groups ($P=0.076$) as in previous studies [10–13]. Nevertheless, it should be noted that such an analysis using a paired *t*-test would suffer two biases. First, rates in our study were obviously not Gaussian variables. This could have been overcome by using nonparametric tests. Second, a 100% fertilization rate obtained with one embryo for one inseminated mature oocyte would have the same impact in the analysis that one obtained with five for five, which is obviously not reasonable. This could have been overcome by using an analysis of variance weighted by the numbers of inseminated oocytes; but this would have relied again on the assumption of Gaussian data.

Following a short gamete exposure, we have observed no statistically significant improvement of the embryo quality, regarding the morphological parameters. This result supports the data of previous studies [3–5, 12–15] but is called into question by others [10, 11, 22], which have shown an improvement of the morphological embryo quality and of the pregnancy and implantation rates. The difference methods used for gamete co-incubation could explain these conflicting results. Standard insemination procedures, described in the studies which reported an improvement of embryo quality associated with a short gamete co-incubation time, took place with a higher sperm concentration (0.5 to 5.0×10^6 motile spermatozoa/ml) than in our study (0.15×10^6 motile spermatozoa/ml), increasing the amount of dead sperm cells close to the zygote and consequently affecting embryo development. This is why shortening sperm insemination, in these conditions, could explain the improvement of the embryo development ability. In our study, the number of motile inseminated sperm was reduced to a minimum concentration to obtain a fertilization rate comparable to those of most French ART centers (FIVNAT 2002). Consequently, sperm metabolic waste products should be lower in the culture medium. Therefore, we conclude that a reduction of gamete contact period has no significant beneficial effect concerning the embryo quality when compared with our standard IVF conditions.

The best quality embryos were transferred regardless of their insemination group to provide the patient with the highest chance of pregnancy. Accordingly, this transfer guideline led to three groups of transfer: transfers performed with (1) embryos from short insemination procedure (group 1, $n=32$), (2) embryos from standard insemination procedure (group 2, $n=62$) or (3) embryos from both groups (group 3, $n=145$). We did not observe statistical differences between pregnancy and implantation rates regarding the group of transfer (data not shown). Unfortunately, the low number of homogeneous transfers in groups 1 and 2 meant

that the comparison of the results between these two groups led to no conclusive results.

In conclusion and to the best of our knowledge, this is the second work highlighting that the decrease of the insemination time significantly reduces the fertilization rate and is not linked to an improvement of embryo quality. In this regard, the short gamete co-incubation protocol is not of clinical relevance.

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