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POLISH GYNECOLOGY
GINEKOLOGIA POLSKA
ORGAN POLSKIEGO TOWARZYSTWA GINEKOLOGICZNEGO
THE OFFICIAL JOURNAL OF THE POLISH GYNECOLOGICAL SOCIETY

ISSN: 0017-0011

e-ISSN: 2543-6767

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DOI: 10.5603/GP.a2021.0078

Article type: Research paper

Submitted: 2020-11-26

Accepted: 2021-03-11

Published online: 2021-05-14

Articles in "Ginekologia Polska" are listed in PubMed.

ORIGINAL PAPER / OBSTETRICS

The influence of preincubation time of prepared sperm before IVF on fertilization, embryo developmental competence and the reproductive outcomes

Short title: The influence of preincubation time on embryo development in assisted reproduction

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ABSTRACT

Objectives: It has been provided that if incubation time of prepared sperm can affect sperm motility and DNA fragment, but little is known about the influence of sperm preincubation time (SI) on the sperm's fertilizing ability, subsequent embryonic development and pregnancy outcomes in in vitro fertilization (IVF). The aim of this

study was to explore the association of SI with fertilization rate, embryo development and clinical outcomes in IVF, further, to find an optimal preincubation time for prepared sperm before insemination in IVF.

Material and methods: This retrospective cohort study included a total of 1453 infertile couples undergoing IVF in our center performed from January 2016 to January 2019. Sperm were preincubated at 37 ℃ 6% CO_2 for different times before insemination. Preincubation time associated with fertilization rate (FR), 2PN rate, D3 good quality embryo rate, fresh embryo implantation rate (IR), blastocyst formation rate, cumulative pregnancy rate (CPR), cumulative ongoing pregnancy rate (COPR), cumulative live birth rate (CLBR), newborn health and gender ratio were analyzed by chi-square analysis.

Results: FR and 2PN rate of SI more than four hours SI groups (> 4 h SI group) decreased significantly compared with other SI groups ($p < 0.01$). There were no significant differences of the D3 high quality embryo rate among five SI groups. The blastocyst formation rate of > 4 h SI group was significantly lower than that of 2–3 h SI group (45.5% vs 56.1%, $p < 0.05$); and 1–2 h SI group also had significant difference with 2–3 h and 3–4 h SI group (48.9% vs 56.1% and 54.6%, $p < 0.05$). There were a significant decrease of fresh IR and CPR in ≤1 h SI group compared with 1–2 h SI group (19.6% vs. 38.0%, $p < 0.05$; 62.7% vs 73.7%, $p < 0.05$); ≤ 1 h SI group also have the lowest CLBR (45.6%), it had statistic differences with 1–2 SI group and 3–4 SI group (45.6% vs 63.2%, $p < 0.01$; 45.6% vs 61.2%, $p < 0.05$).

Conclusions: The sperm preincubated time at 37℃6% CO_2 before insemination could influence sperm fertilizing ability, blastocyst formation, embryo implantation and CLBR in IVF cycles. The best time for prepared sperm preincubation at 37℃is one to four hours before insemination in IVF.

Key words: sperm; preincubation time; IVF; fertilization rate; embryo development; cumulative live birth

INTRODUCTION

Immediately following ejaculation, human sperm lack the ability to fertilize. To achieve fertilization-competence, sperm must undergo several metabolic and structural changes, collectively known as capacitation [1]. During capacitation, modification of membrane characteristics, enzyme activity, and motility property of spermatozoa render these cells responsive to stimuli that induce the acrosome reaction prior to fertilization [2]. Sperm capacitation is a temperature dependent phenomenon since variations in the incubation temperature cause alterations in key events associated with these processes. At room temperature, sperm cells are in a 'quiescent' state (non-capacitated, with low percentages of spontaneous AR), but when exposure to 37°C, capacitation-related events are activated [3].

Sperm quality is an important predictor of fertility and successful IVF outcomes. To date many studies have been conducted to evaluate the relationship between sperm parameters and sperm incubation time at 37°C. Several studies [4–6] have shown that there is a significant decrease in sperm motility when processed sperm are incubated > four hours before use in IVF. In other studies, a preincubation time of > 6 hours can have deleterious effects on sperm function whereby oxidative stress and reactive oxygen species production increases over a 24-hour period, promoting DNA fragmentation [7]. Further, two groups showed that sperm DNA fragmentation significantly increased, either after a two hour incubation period [8] or after a four-hour incubation period [9]. In other studies, when human sperm were incubated under capacitating conditions up to 24 h, a significant increase in acrosome loss over time was observed [10]. Further, in related experiments, after sperm were preincubated for three hours, it was observed that the percentage of acrosome reactions significantly increased but the spermatozoa ATP concentration did not change between one and three hours

[11].

In routine IVF, processed sperm are incubated at 37℃ incubator to promote capacitation. Currently, different laboratories select a range of incubation times from as short as under one hour to as long as five hours. To date, there have been no systemic studies to correlate the optimal time for sperm incubation and IVF outcomes. To address this issue, we retrospectively analyzed the relationship between sperm incubation time at 37℃ with fertilization, embryonic development and cumulative clinical outcomes in a large cohort of patients.

MATERIAL AND METHODS

IVF patients

This study enrolled 1453 women who had completed 1622 IVF cycles performed from January 2016 and January 2019 at the Department of Reproductive Medicine in Calmette Hospital, Kunming, China. We included hyperstimulation cycles in which the male partner had normal sperm parameters or only mild male factor. Couples were excluded from the study if they had complete fertilization failure, or their fertilization rate was less than 30%. IVF cycles were divided into five test groups according to the prepared sperm incubation time before insemination, namely, ≤ 1h, 1–2h, 2–3h, 3–4h and > 4h.

Ovarian stimulation and laboratory procedures

Women were treated with 1.5 mg of Diphereline (triptorelin acetate for injection; IPSEN PHARMA BIOTECH, France) on day 21 of their previous menstrual cycle. After the serum oestradiol concentration had decreased to < 50 pg/mL, 75–225 IU of recombinant human follitropin (GONAL-f: Merck Serono SA Aubonne Branch, Switzerland) was administered. Oocyte maturation was induced by injection of 5000–10,000 IU of chorionic gonadotrophin (Livzon Pharm, China) when more than three follicles of ≥ 18mm diameter had developed in both ovaries. Cumulus oocyte complexes (COCs) were then retrieved 36h post trigger by ultrasound-guided

transvaginal follicular aspiration. COCs were finally collected in buffered medium (G-MOPS® PLUS, Vitrolife, Göteborg) containing Human Serum Albumin (HSA) and then incubated at 37°C in 6% CO_2 and 95% relative humidity (RH) in culture medium containing HSA (IVF®, Vitrolife Sweden AB, Goteborg, Sweden).

On the day of oocyte pick up (OPU), sperm samples were collected from male partners by masturbation following two to seven days of sexual abstinence. Samples were analyzed for sperm count, motility and morphology according to the 2010 parameters of the World Health Organization [12]. After sperm liquefication sperm was purified through a density gradient comprising two 1 mL layers of 40% and 48% Sperm Isolate (Isolate, Ovrine). After deposition of 1–1.5 mL liquefied sperm onto the 40% layer, and centrifugation at 300xg for 10 minutes, the pellet was collected and washed with 2 mL of IVF Plus Medium at 200 g for two minutes. The final pellet was then resuspended in IVF Plus Medium (0.3–1.0 mL) at a final concentration of 3–5 $\times$ 10^6 sperm/mL. The whole process was performed at room temperature. Processed sperm was then preincubated at 36°C in 6% CO_2 and 95% relative humidity (RH) for either $\leq$ 1h, 1–2h, 2–3h, 3–4h or > 4h.

Approximately 10,000 motile sperm were added into 50 μL culture medium drops (IVF®,Vitrolife, Göteborg) four hours after COCs collection (each drop contained one or two COCs). Cumulus cells were subsequently removed at 16–19h post-insemination and the zygotes were washed and transferred to independent drops of 25 μL of culture medium (G1®, Vitrolife, Göteborg) covered with mineral oil (OVOIL®, Vitrolife, Göteborg) and then incubated at 37°C in 6% CO_2 and 95% RH. Fertilization was assessed by the presence of pronuclei (PN) and extrusion of two polar bodies.

Day three (D3) embryos were classified according to the Istanbul consensus Workshop on Embryo Assessment [13]. Grade 1 was defined as good quality embryos with fragmentation < 10%, 7–9 blastomeres and no multinucleation; grade 2 as fair quality embryos with 10–25% fragmentation, more than six blastomeres and no evidence of multinucleation and grade 3 as poor-quality embryos with > 25% fragmentation, less than five cells (or abnormal cell size) and evidence of multinucleation. On D3, two of the best quality embryos were transferred into the uterus

or frozen, and the rest were cultured to D5 or D6. Blastocysts reaching the expansion stage, with inner cell mass and trophectoderm layer Gardner scores of A or B [14], were frozen. On D3 or D5 1–2 optimal embryos were selected for transplantation and following transfer, women were given corpus luteum support.

Statistical analysis

Data is presented as the mean ± standard deviation (SD) for all the continuous variables whereas categorical variables were presented in percentage form. The difference of continuous variables between the study groups was evaluated by ANOVA and percentage data was evaluated by Chi-square test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS 16.0 (New York, USA) statistical software.

Normal fertilization (2PN) rate was calculated as number of 2PN zygotes/number of MII oocytes. The fertilization rate was calculated as number of multi-PN, 2PN zygotes and 1PN zygotes/number of MII oocytes. Blastocyst formation was defined as development past the early blastocyst sage. Clinical pregnancy was defined as the presence of one or more gestational sacs visualized on ultrasound two weeks after embryo transfer. CPR was based on a clinical pregnancy following the use of all fresh and frozen embryos derived from a single ovarian stimulation cycle (clinical pregnancy after embryo transfer performed between January 2016 to June 2020). The implantation rate was calculated as number of gestational sacs/number of embryos transferred; cumulative ongoing pregnancy was defined as cumulative pregnancy with a detectable heart rate after 12 weeks of gestation, CLBR was defined as the first live birth following the use of all fresh and frozen embryos derived from a single ovarian stimulation cycle (live birth after embryo transfer performed between January 2016 to June 2019).

RESULTS

Baseline characteristics of the patients and sperm incubation groups

Patients (n = 1453) were divided into five SI groups, namely ≤ 1 h, 1–2 h, 2–3 h,

3–4 h or > 4 h. The baseline characteristics and sperm parameters for each SI group are summarized in Table 1. For the female, age, BMI, dose and days of gonadotrophin stimulation and days of gonadotrophin showed no statistical difference between each SI group. For the male, age and sperm volume, concentration and morphology also showed no statistical differences between the SI groups. However, the mean number of MII oocytes and D3 good quality embryos per cycle were significantly fewer in the ≤ 1h and > 4 h SI groups.

Fertilization rate and embryo development in the SI groups

There was a significant correlation between the SI time for fertilization and embryo development outcomes (Tab. 2). The fertilization rate for ≤ 1 h and > 4 h SI groups was significantly lower ($p < 0.05$) than other SI groups. The 2PN rate of > 4 h SI group was also significantly lower than other SI groups ($p < 0.01$). The blastocyst rate of > 4 h SI group was significantly lower than 2–3 h SI group (45.5% vs 56.1%, $p < 0.05$); and 1–2 h SI group also had significant difference with 2–3 h and 3–4 h SI group (48.9% vs 56.1% and 54.6%, $p < 0.05$). However, there were no difference in the percentage of D3 good quality embryos amongst the SI groups ($p > 0.05$).

The influence of sperm incubation time to the IVF treatment and birth outcomes is shown in Table 3.

Embryos from the ≤ 1h SI group had a lowest implantation rate from fresh embryo transfer cycles (19.6%), and there was a significant difference compared with 1– 2 SI group ($p < 0.001$). Among 1946 ovarian stimulation cycles resulting in embryo transfers (fresh or/and frozen embryo transfer), 998 had one ET, 332 had 2 ETs, 75 has three ETs and 14 had more than three ETs. There were no differences in the mean embryo transfer cycles, ratio of single and two embryos transfer cycles and ratio of D3 cleavage embryo and D5 blastocyst embryo transfers among SI groups; however, the ≤ 1h SI group had the lowest cumulative clinical outcomes. The CPR and COPR were not significantly different among the five SI groups. However, ≤ 1h SI group also have the lowest cumulative live birth rate (CLBR) (44.0%), it had statistic differences with 1–2 SI group and 3–4 SI group (45.6% vs 63.2%, $p < 0.01$; 45.6% vs 61.2%, $p < 0.05$). There were

no significant differences for newborn health and gender ratio outcomes between the five SI groups.

DISCUSSION

In this retrospective study, we investigated the relationship between sperm incubation time and IVF outcomes. Our results showed that different preincubation time at 37°C 6% CO_2 for processed sperm before insemination can affect the fertilization rate, the 2PN rate, the blastocyst formation rate and embryo implantation rate in IVF. In addition, there were some differences in cumulative clinical outcomes such as cumulative pregnancy, cumulative ongoing pregnancy and cumulative live birth. For achieving improved IVF outcomes, our findings suggest that the optimal preincubation sperm time is one to four hours before insemination.

We separated the IVF cycles for five groups as ≤ 1 h, 1–2 h, 2–3 h, 3–4 h and > 4 h SI groups, and analyzed the effect of different incubation time on IVF fertilization rate. The results showed a significant decrease in fertilization rate and 2PN rate with > 4h SI time ($p < 0.01$), these results could be explained by two conclusions proved before, the sperm motility and viability decline rapidly when spermatozoa were incubated at 37℃ for more than four hours [4]. The fertilization rate will decrease with the sperm loss of motion [15]; and high sperm DNA fragmentation has a significant negative association with fertilization rate [16], more than four hour incubation of sperm will increase the DNA fragmentation [8, 9, 17].

The results also showed a significant decrease in fertilization rate with ≤ 1 h SI time ($p < 0.05$). this is a phenomenon we have not noticed before. However, try to look on the time course of sperm capacitation and AR, we could find the seasonality of this result. Prior to interaction with the oocyte, spermatozoa must undergo capacitation, then become able to undergo the acrosome reaction and to develop hyperactivation, these two capacitation-associated events will allow sperm to pass through the cumulus oophorous that surround the egg, to bind to and penetrate the zona pellucida, and finally to fuse with the egg plasma membrane. A series events happened during sperm

capacitation, many key events are time-dependent, for example, the superoxide production of human spermatozoa started immediately at the beginning of incubation under capacitating, reached a plateau 15–25min later, and was sustained for > 4 h [18]. Early events during capacitation are the production of ROS and activation of the PKA pathway, the PKA activity is maximal at 30 min of capacitation in human sperm [19–21]. Sperm hyperactivation peaked after one to three hours of incubation [18] and the phosphorylation significantly increased 1h after the beginning of the incubation, which play a role in the control of sperm motility [22]. There is also an increase in the SH content of Triton X-100 detergent-soluble proteins, which is time-dependent occurring during the first 30–60 min of capacitation [23, 24]. Therefore, it is possible that the sperm preincubation time less than one hour would be insufficient for capacitation.

We also analyzed the effect of sperm SI time on D3 embryo quality and blastocyst formation. > 4h SI time has effect on blastocyst formation rate, compared with 2–3 h SI group, the difference was significantly ($p < 0.05$), whereas there was no such effect on D3 embryo quality. Whether embryo quality in IVF cycles negatively correlated with sperm DNA fragmentation, however there is a controversy, Xue et al. [25], did not find DNA fragmentation affecting the effectiveness of IVF, however, several other studies confirmed sperm DNA fragmentation does have negative effect [16, 26–28]. If sperm incubation time truly associated with the increasing of DNA fragmentation, our results will support the conclusion of sperm DNA fragmentation have negative effect to embryo development and blastocyst formation in IVF.

In addition, we evaluated the effect of sperm on IVF outcome with different preincubation time. The results showed that shorter than one hour SI significantly effect IR in fresh embryo transfer cycles after IVF, the CPR and CLBR of ≤ 1 h SI group were also at the lowest level, there are no relative studies on this, one reason could be the quantity of good embryos in this SI group was significantly fewer, but it is far from adequate, we speculate that when sperms are preincubated for less than one hour, most of the optimized sperms not be stimulated enough, the competitive mode is not fully opened, some sub-optimal sperms have the opportunity to penetrate into the oocyte. These sub-optimal sperms decrease the embryos implantation ability, then decrease the

CPR and CLBR, it's just a speculation, to confirm this speculation, research on changes of sperm incubation time should be further shorten to less than one hour.

There were no differences in the effect of > 4h SI time on IR, CPR, COPR and CLBR, however, these parameters were lower among five SI groups. While the association between sperm DNA fragmentation and alterations in pregnancy or live birth rates in IVF also have contrary, a negative correlation between the intensification of DNA fragmentation and the achievement of pregnancy and live birth in IVF has been reported [16, 26–28], whereas other author reported there was no influence [29]. For the data we calculated were CPR and CLBR, and the outcomes they evaluated were pregnancy rate and live birth rate, there was an inconsistency in evaluation method. We cannot support the views of either side. However, there were a lot of early pregnancy loss in > 4h SI group (CORP minus CPR), this was inconsistent with previous research which demonstrated there was no increased risk of early miscarriage after IVF in high sperm DNA fragment groups [29].

CONCLUSIONS

In conclusion, the findings of this study demonstrate that sperm preincubation time has effect on the fertilization, embryo quality and clinical outcome after IVF. One to four hours preincubation time for prepared normozoospermic at 37°C 6% CO_2 prior to be used should be considered as a necessary procedure in IVF. In order to keep this preincubation time, we suggest that normozoospermic samples collection could be taken three to five hours before insemination, we'd better to guide couple males collect semen post oocyte pick up, after COCs checking and insemination plan making. Further research is required into the mechanisms responsible for effecting of short sperm incubation time on IVF.

Ethics approval and consent to participate

Not applicable.

Consent of publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. For more information, please contact Dr. Liu at liusai1316@163.com.

Conflict of interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Sai Liu and Yuanqing Yao designed this paper, Sai Liu, Guoxuan Wu, Yanyan Zhao, yongqing Lv and Nannan Dang collected data and performed data analysis, Sai Liu wrote this manuscript, Li Wang and Yao Yuanqing revised this manuscript, and and all authors read this paper.

Acknowledgements

Not applicable.

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Table 1. Baseline characteristics of the patients in the SI groups

	≤ 1h (n = 95)	1-2h (n = 523)	2-3h (n = 725)	3-4h (n = 226)	> 4h (n = 53)	P value
Female age (years)	34.9 ± 5.3	33.8 ± 5.2	33.9 ± 5.4	33.7 ± 5.4	34.6 ± 4.9	0.319
BMI (kg/m^2)	21.0 ± 3.1	22.1 ± 3.9	22.2 ± 3.1	23.5 ± 18.4	21.6 ± 2.9	0.253
Male age (years)	37.3 ± 5.4	36.2 ± 5.9	35.9 ± 6.2	35.5 ± 6.6	35.7 ± 5.2	0.231
Sperm Volume (mL)	2.7 ± 1.2	2.8 ± 1.4	2.7 ± 1.8	3.0 ± 4.4	2.8 ± 1.2	0.836
Sperm Concentration (10^6/mL)	63.4 ± 27.9	70.7 ± 31.8	73.3 ± 33.6	72.7 ± 34.6	68.6 ± 17.6	0.114
Sperm Morphology abnormal (%)	93.5 ± 2.5	93.1 ± 2.9	93.3 ± 2.8	93.4 ± 3.0	94.4 ± 1.9	0.269
WHO sperm a (%)	39.1 ± 9.9	40.0 ± 10.6	40.9 ± 10.0	40.6 ± 10.8	40.1 ± 7.3	0.085
Total dose of gonadotrophins (IU/mL)	2859.5 ± 1156.3	2587.8 ± 1065.1	2604.2 ± 1079.4	2580.2 ± 1202.6	2733.8 ± 1549.3	0.313
Days of gonadotropin stimulation	10.9 ± 1.7	11.1 ± 1.8	11.0 ± 1.9	10.9 ± 1.9	10.6 ± 2.5	0.801
Mean No. of MII oocytes	8.1 ± 5.8[a]	10.7 ± 6.3[b]	10.4 ± 6.5[b]	10.7 ± 6.6[b]	7.3 ± 4.1[a]	0.001
Mean No. of D3 good embryo	2.6[ac]	3.3[b]	3.2[ab]	3.4[b]	2.3[ac]	0.035

Data are presented as mean ± SD

Table 2. Fertilization rate and embryo development in the SI groups

	≤ 1 h	**1–2 h**	**2–3 h**	**3–4 h**	**> 4 h**	**total**
FR	90.6% (693/765)[a]	92.6% (5182/5594)[b]	91.9% (6956/7567)[ba]	92.3% (2231/2418)[ba]	85.5% (389/455)[c]	92.0% (15451/16799)
2PN rate	82.9% (634/765)[a]	82.3% (4603/5594)[a]	82.5% (6239/7567)[a]	82.8% (2001/2418)[a]	75.4% (343/455)[b]	82.3% (13820/16799)
D3 good embryo rate	40.5% (250/617)[a]	39.5% (1768/4477)[a]	38.4% (2341/6095)[a]	40.0% (776/1940)[a]	36.4% (122/335)[a]	39.0% (5257/13464)
Blastocyst formation rate	55.2% (116/210)[a]	48.9% (796/1629)[ab]	56.1% (1245/2220)[ac]	54.6% (341/625)[acd]	45.5% (45/99)[abd]	53.2% (2543/4783)

FR: a–b — P<0.05, a–c — p < 0.01, b–c — p < 0.01; 2PN rate: a–b — p < 0.01; blastocyst formation rate: b–c — p < 0.05, c–d — p < 0.05, b–d — p < 0.05

Table 3. The influence of sperm incubation time to the IVF treatment and birth outcomes

	≤ 1 h	**1–2 h**	**2–3 h**	**3–4 h**	**> 4 h**	**Total**
IR	19.6% (11/56)[a]	38.0% (89/234)[b]	35.0% (131/374)[ab]	34.2% (39/114)[ab]	33.3% (5/15)[ab]	34.7% (275/793)
Fresh ET /Frozen ET	25/68[a]	123/509[a]	195/700[a]	60/212[a]	8/45[a]	411/1535
D3ET/D5ET	78/15[a]	509/123[a]	722/173[a]	234/38[a]	43/10[a]	1586/360
Mean cycles of ET	1.24[a]	1.38[a]	1.39[a]	1.35[a]	1.29[a]	1.37
Single embryo/ two embryo ET	16/77[a]	99/533[a]	151/744[a]	34/238[a]	6/47[a]	307/1639
CPR	62.7% (47/75)[a]	73.7% (337/457)[b]	67.7% (435/643)[a]	72.1% (145/201)[ab]	68.3% (28/41)[ab]	70.0% (992/1417)
COPR	53.3% (40/75)[a]	65.0% (297/457)[a]	59.4% (382/643)[a]	63.2% (127/201)[a]	51.2% (21/41)[a]	61.2% (867/1417)
CLBR	45.6% (31/68)[a]	63.2% (265/419)[b]	56.7% (337//594)[ac]	61.2% (112/183)[bcd]	52.8% (19/36)[abcd]	52.8% (764/1300)
sex ratio (male: female)	0.76 (16:21)	1.06 (169：159)	0.92 (201：218)	1 (76:76)	1.46 (16:11)	0.99 (478:485)

Fetus malformation rate	0%（0/37）	0.3%（1/328）	0.95%（4/421）	0.7%（1/152）	0%（0/27）	0.6%（6/965）

IR: a–b — $p < 0.01$; CPR: a–b — $p < 0.05$; COPR: a–b — $p < 0.05$; CLBR: a–b — $p < 0.01$, b–c — $p < 0.05$, a–d — $p < 0.05$, c–d — $p > 0.05$