J Assist Reprod Genet (2011) 28:1205–1211
DOI 10.1007/s10815-011-9630-9

ASSISTED REPRODUCTION TECHNOLOGIES

Effects of cumulus cells removal after 6 h co-incubation of gametes on the outcomes of human IVF

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Received: 11 April 2011 / Accepted: 24 August 2011 / Published online: 6 September 2011

Abstract
Purpose To investigate the effects of cumulus cells removal after 6 h co-incubation of gametes on the fertilization, polyspermy, multinucleation and clinical pregnancy rates in human IVF.
Methods A total of 1,200 IVF-ET cycles undergoing 6 h co-incubation of gametes in 2009 were included in this study. Inclusion criteria were: female age <38 years, first IVF treatment, with bi-ovary and normal ovarian response, e.g., 4~20 oocytes could be obtained. A 6 h period of co-incubation was applied in all IVF cycles. According to the history of infertility, cumulus cells were mechanically removed either 6 h post-insemination or 20 h post-insemination. For couples with primary infertility, or unexplained infertility, or mild oligospermia or asthenospermia, the cumulus cells were removed at 6 h of insemination for the polar body observation (6 h group, n=565). Of these, 80 cycles received early rescue ICSI due to fertilization failure or low fertilization rate at 6 h of insemination. For couples with secondary infertility and normal semen analysis, the cumulus cells were removed at 20 h of insemination as routine (20 h group, n=635). Of these, three cycles received late rescue ICSI due to fertilization failure at 20 h of insemination. Normal fertilization, polyspermy (≥3PN), multinucleation and clinical pregnancy rates were compared between the two groups (rescue ICSI cycles were not included in the comparison in both groups).
Results Significant difference (P<0.05) was observed between the groups regarding polyspermy rates (7.48% in 6 h group and 9.22% in 20 h group). No difference was observed between the groups regarding normal fertilization rates (2PN rate) (64.89% in 6 h group and 65.74% in 20 h group). No difference was observed between the groups regarding multinucleation and clinical pregnancy rates (11.01% and 65.15% in 6 h group, 10.75% and 66.93% in 20 h group, respectively). The clinical pregnancy rate was 51.43% in cycles receiving early rescue ICSI, while no clinical pregnancy was obtained in cycles receiving late rescue ICSI.
Conclusion The present results indicate that cumulus cells removal at 6 h of insemination is a relatively safe operation, which yielded comparable normal fertilization rate, multinucleation and clinical pregnancy rates compared with 20 h group. This protocol may be beneficial for early obsevation of fertilization failure and make early rescue ICSI possible.

Keywords In-vitro fertilization · Short co-incubation · Cumulus cells removal · Polyspermy · Clinical pregnancy · Rescue ICSI

Introduction

In traditional IVF, oocytes are exposed to excessive numbers of sperm for 16~20 h. Studies have shown possible detrimental effects on sperm-oocyte fusion and subsequent embryo development due to potential damage from sperm metabolic waste products such as free oxygen radicals [1]. Free oxygen radicals may peroxidate polyunsaturated fatty acids in cell membranes, causing a decrease

Capsule Removal of cumulus 6 hours after the initiation of IVF is practical, safe, and offers advantages when early rescue ICSI is indicated.

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in the flexibility and fluidity of the membrane [1, 2]. It seems logical that the harmful effects of these metabolites increase with time. Short co-incubation of gametes reduces the time of oocyte exposure to sperm from 16 to 20 h to 1~6 h, even to 30 s [3]. This may have favourable effects on pregnancy rates by reducing potentially damaging products produced by sperm [4–6].

The fertilization rate of traditional IVF is about 60%~70% [7]. However, there are cases of lower fertilization or even total fertilization failure due to various factors, such as defects in the sperm and/or oocytes. Attempts to rescue unfertilized oocytes by ICSI on day 1 after overnight co-incubation generally lead to poor results [8]. Better fertilization, implantation and pregnancy rates are obtained in early rescue ICSI compared with late rescue ICSI [9]. Short co-incubation of gametes provides the possibility of early rescue ICSI.

A 6 h period of co-incubation has been used in all IVF cycles in our center since 2008. For couples with primary infertility, or unexplained infertility, or mild oligospermia or asthenospermia, cumulus cells are removed at 6 h of insemination for the polar body observation. Oocytes absent of the second polar body are subjected to rescue ICSI. For couples with secondary infertility and with normal semen analysis, cumulus cells are removed at 20 h of insemination as routine. With the combination of short co-incubation and early rescue ICSI, the ICSI rate dropped from 27.79% in 2007 to 19.97% in 2009.

But the effects of early cumulus cells removal on the outcomes of human IVF remained unclear. Limited studies demonstrated the effects of cumulus cells removal on the outcomes of IVF and results remained controversy [10, 11]. The aim of this study was to investigate the effects of cumulus cells removal on fertilization, polyspermy, multinucleation and clinical pregnancy rates afte 6 h short co-incubation of gametes in human IVF.

Materials and methods

Patient selection and characteristics

A total of 1,200 cycles who received IVF-ET treatment in Chong Qing Reproductive and Genetics Institute between January and December in 2009 were included in the study. The inclusion criteria were as following: female age <38 years, first IVF treatment, with bi-ovary and normal ovarian response, e.g., 4~20 oocytes were obtained.

A 6 h period of co-incubation was applied in all IVF cycles. According to the history of infertility and semen analysis, cumulus cells were mechanically removed either 6 h post-insemination (6 h group, n=565) or 20 h post-insemination as traditional IVF (20 h group, n=635).

Indications for 6 h group were as following: primary infertility; unexplained infertility; mild oligospermia or asthenospermia, e.g. sperm density was $10\sim19\times10^6$/ml, and/or progressive sperm was 20%~50%. Of which, 80 cycles received early rescue ICSI after 6 h co-incubation due to fertilization failure or low fertilization rate.

Indications for 20 h group were as following: secondary infertility and with normal semen analysis. Of which, 3 cycles received late rescue ICSI after 20 h co-incubation due to total fertilization failure.

The patients gave their informed consent to receive rescue ICSI.

Protocols of ovarian stimulation and oocyte retrieval

Pituitary down-regulation and controlled ovarian stimulation was achieved according to our routine [12]. Briefly, after down-regulation with a gonadotrophin-releasing hormone (GnRH) agonist (Triptorelin Acetate, Ipsen Pharma, France), the ovaries were stimulated with recombinant FSH (rFSH) (Puregon; Organon, The Netherlands or Gonal-F, Merck Serono, Switzerland). Human chorionic gonadotropin (HCG) (Ovidrel, Merck Serono, Italy) was administered when at least three follicles measured > 18 mm. Transvaginal oocyte retrieval was performed 36 h after HCG injection. Cumulus-enclosed oocytes were collected in 2.5 ml IVF medium (G-IVF, Vitrolife Sweden AB, Sweden) and incubated at 5%O_2, 6%CO_2, 37°C incubators for insemination.

Sperm preparation and insemination

Semen samples were collected in sterile containers by masturbation. After liquefaction, semen samples were assessed for sperm density, motility and morphology. Sperm density $\geq20\times10^6$/ml, progressive sperm ≥50%, normal morphology ≥15% were regarded as normal according to World Health Organization guidelines (WHO, 1999). Sperm preparation was carried out by gradients centrifugation. Finally, sperm pellet was suspended in 0.5 ml of culture medium and incubated at 5%O_2, 6%CO_2, 37°C inbubators for insemination.

Each cumulus-oocyte complex was inseminated in a 50 ul equilibrated IVF microdroplet at 38~40 h post-HCG, using 10,000~15,000 motile sperm/microdroplet.

Cumulus cells removal

In 6 h group, cumulus cells were mechanically removed after 6 h of co-incubation. The method of cumulus cells removal was as following: Pipette Pasteurs were pulled to capillary pipette with the diameters 120~150 um, slightly larger than oocyte. Oocytes were aspirated and blew out

repeatedly until most of the cumulus cells were removed. The operation should be gentle to prevent the damage on zona pellucidas and oocytes. Fertilization was determined when two polar bodies were present in a zygote after cumulus cells removal. Zygotes with two polar bodies were transferred to another fresh microdroplet without sperm and cultured overnight. Total fertilization failure was determined when all of the oocytes did not present the second polar body. Low fertilization rate was determined when fertilization rate was lower than 30%. In cycles with lower fertilization rate or total fertilization failure, oocytes absent of the second polar body were subjected to rescue ICSI at 6 h of insemination.

In 20 h group, after 6 h of co-incubation, cumulus-oocyte complexes were transferred from the insemination medium to another fresh microdroplet without sperm and cultured overnight. Cumulus cells were removed at 20 h of insemination for fertilization assessment as routine.

All the procedures, such as insemination, cumulus cells removal, were performed by the same person.

Fertilization assess and embryo transfer

For practical reasons, fertilization assessment was performed at 20 h of insemination (day 1). All zygotes in two groups were transferred to fresh cleavage medium (G-1, Vitrolife Sweden AB, Sweden). Normal fertilization was determined when two pronuclei were present. Polyspermy was determined when ≥3 pronuclei were present. Embryo assessment was according to the criterion of Dale [13]. Embryos with more than 7 cells on day 3, fairly equal size blastomeres, few or no cytoplasmic fragments were defined as good quality embryos (grade 1). According to Dale criteria, embryos with grade 1~3 were transferrable embryos. Embryos with one or more multinucleated blastomeres either on day 2 or on day 3 or on both days were defined as multinucleated embryos. 1~3 trasferrable embryos were selected for transfer on day 3 under ultrasound guidance. Multinucleated embryos were transferred when there was no other choice. The surplus transferrable embryos were cryopreserved for further transfer.

Patients received luteal support (combination of estrogen and progesterone) starting on the oocytes retrieval day and continuing until the blood HCG test day (14 days after embryo transfer). A clinical pregnancy was established when gestational sac was detected by transvaginal ultrasound 28 days after embryo transfer.

Statistical analysis

Comparative data of the patients were expressed as mean +/− Sd. The quantity data was performed by *t*-test. Fertilization, polyspermy, multinucleation and pregnancy rates between 6 h group and 20 h group were compared using Chi square test. A P value<0.05 was defined as being statistically significant.

Results

Comparative data of the patients and cycle stimulation characteristics were shown in Table 1.

A total of 5,295 and 6,797 oocytes were retrieved in 6 h group and 20 h group respectively (rescue ICSI cycles were not included in two groups). Regarding the normal fertilization rate (2PN rate), a similar result was obtained (64.89% in 6 h group and 65.74% in 20 h group, P>0.05, Power=0.1577). The overall polyspermy rate was 8.46% (1023/12092) in two groups. The incidence of polyspermy was lower in 6 h group than in 20 h group (7.48% and 9.22%, respectively, P<0.05, Power=0.9245). The overall multinucleation rate was 10.87% (842/7749) in two groups. A similar multinucleation rate was observed in 6 h group and 20 h group (11.01% and 10.75%, respectively, P>0.05, Power=0.0515). The overall clinical pregnancy rate was 66.16% (739/1117) in two groups. A similar pregnancy rate was obtained in 6 h group and 20 h group (65.15% and 66.93%, respectively, P>0.05, Power=0.0810) (Table 2).

There were 3%~5% IVF cycles which received early rescue ICSI due to total fertilization failure or low fertilization rate in our center in recent 3 year. This rate was 5.07% in 2009. In 6 h group, 80 cycles received early rescue ICSI at 6 h of insemination due to total fertilization failure or low fertilization rate. A 3.82% (21/550) of polyspermy rate was obtained. Embryo transfer was cancelled in ten cycles because of abnormal fertilization (n=3), unfertilization (n=1) or embryo cryopreservation (n=6). This procedure resulted in 51.43% (36/70) of clinical pregnancy rate. In 20 h group, three cycles received late rescue ICSI on day 1 due to total fertilization failure. Embryo transfer was cancelled in one cycles due to abnormal fertilization. No clinical pregnancy was obtained in late rescue ICSI cycles (Table 3).

Discussion

In conventional human IVF, 16~20 h incubation of oocytes and sperm was originally established for practical reasons. This generally corresponded to the time for pronuclei observation. However, some studies suggested that an extensive time of oocyte exposure to sperm affected embryo viability and pregnancy rate [4, 5]. Compared with conventional IVF, shorter exposure of oocytes to sperm had favourable effects on embryo quality and pregnancy rate

Table 1 Comparative data of the patients and cycle stimulation characteristics*

	6 h group	20 h group	*P*-value
Patients	485	632	
Age (years, ±SD)	30.23±3.8	31.17±3.48	<0.05
Primary infertility	352	0	–
Secondary infertility	133	632	<0.05
Main cause of infertility			
Tubal disease	322	575	<0.05
Endometriosis	43	42	NS
Unexplained	25	3	<0.05
Anovulation	31	6	<0.05
Male factor	7	0	–
Complex	57	6	<0.05
Mean duration of infertility (±SD)	6.02±3.46	5.27±3.56	<0.05
No.of oocytes retrieved (±SD)	10.91±4.58	10.75±4.40	NS
No.of MII oocytes (±SD)	9.17±4.18	9.17±4.10	NS
Sperm concentration (10^6 /ml,±SD)	81.11±42.98	85.85±43.04	NS
Progressive sperm (%,±SD)	47.52±8.26	49.83±7.25	<0.05
Mean No. of embryo transferred (±SD)	2.08±0.39	2.12±0.44	NS

*Rescue ICSI cycles were not included in both groups

due to reduce the negative effects producing by high concentration of sperm and the corresponding metabolic products [5, 6]. Short co-incubation of gametes has been performed routinely in our center since 2008. About 90% of fertilized oocytes released the second polar body at 6 h of insemination and 80% presented two pronuclei at 8 h of insemination [14–17]. We therefore chose a 6 h period of co-incubation.

For couples with primary infertility, unexplained infertility or mild oligospermia or asthenospermia, cumulus cells were removed at 6 h of insemination for the purpose of polar bodies observation. Fertilization was determined when two polar bodies were present in a zygote. For cycles with low fertilization rate (<30%) or total fertilization failure, early rescue ICSI was performed at this time. But the effects of cumulus cells removal on the outcomes of IVF still kept controversy [10, 11]. This study analyzed the effects of early cumulus cells removal on the fertilization, polyspermy, multinucleation and clinical pregnancy rates.

The effect of early cumulus cells removal on the fertilization rate

During routine short co-incubation (1~6 h co-incubation), oocytes were withdrawn from insemination medium to fresh medium, without cumulus cells removal. This generally resulted in similar fertilization rates compared with conventional IVF (16~20 h co-incubation) [5, 6]. During this process, early cumulus cells removal contributed to fertilization observation and made the early rescue ICSI possible. But little attention has been paid to the effects of early cumulus cells removal on the fertilization rate.

Compared with conventional IVF, lower normal fertilization rates were observed by Lundqvist [10], Zhang Wei and Sun Yingpu (unpublished results). In these studies, part

Table 2 Details of treatment outcome in two groups*

	6 h group	20 h group	*P*-value	Power
Total No. of oocytes retrieved	5,295	6,797		
Normal fertilization rate	64.89% (3436/5295)	65.75% (4469/6797)	NS	0.16
Polyspermy rate	7.48% (396/5295)	9.22% (627/6797)	<0.05	0.92
Multinucleation rate	11.01% (370/3360)	10.75% (472/4389)	NS	0.05
Clinical pregnancy rate	65.15% (316/485)	66.93% (423/632)	NS	0.08

*Rescue ICSI cycles were not included in both groups

Table 3 Comparison between early rescue ICSI cycles and late rescue ICSI cycles

	Early rescue ICSI	Late rescue ICSI	P-value	Power
Patients	80	3	–	
Female age	31.31±4.17	35.33±3.21	NS	0.56
No.of oocytes retrieved	9.66±4.93	9.33±5.19	NS	0.03
No.of MII oocytes	7.88±4.51	6.67±6.35	NS	0.05
Sperm concentration 10^6 /ml	59.54±23.52	55±36.35	NS	0.04
Progressive sperm (%)	42.01±9.52	39.67±6.66	NS	0.08
IVF fertilization rate (%)	8.12	0	–	
ICSI fertilization rate (%)	76.5	87.67	NS	0.05
Mean No. of embryo transferred	2.09±0.62	1.5±0.71	NS	0.29
No.of ET cycles (n)	70	2	–	
Cycles of cancel ET (n)	10	1	–	
Cycle cancel reason				
Abnormal fertilization	3	1	–	
Unfertilized	1	0	–	
Froze due to uterine cavity fluid	6	0	–	
Clinical pregnancy rate (%)	51.43% (36/70)	0	–	

or all of the cumulus cells were removed after 2~4 h co-incubation. Whereas, a comparable normal fertilization was observed in 6 h group compared with 20 h group in our study. This discrepancy indicated that the time of cumulus cells removal might affect the normal fertilization rate.

Cumulus cells played an important role in modulating fertilization in human [18]: (1) cumulus cells caused mechanical entrapment of sperm and guided them towards the oocyte; (2) cumulus cells facilitated sperm capacitation, acrosome reaction and penetration into the oocyte; (3) cumulus cells prevented changes in the oocyte which were unfavourable for normal fertilization, for example, premature cortical exocytosis, resulting in zona hardening and subsequent decreased fertilization rate. Tao Tao et al. [11] reported that partial removal of cumulus cells immediately after oocyte retrieval decreased the fertilization rate, indicating the importance of sperm in modulating fertilization. Sperm entered cumulus cells within 15 min of in vitro insemination. Although oocytes were exposed to large quantity of sperm, only 10–20 sperm entered cumulus cells and further sperm did not enter later. Passage through cumulus cells in human oocytes was slow and appeared to require at least 2 h [19]. The sperm penetration in cumulus cells was gotten rid of when cumulus cells were removed at 2~4 h of insemination. This blocked the interaction of gametes, resulting in lower fetilization rates. This indicates that cumulus cells removal at 2~4 h of insemination exerts a negative effect on normal fertilization rate. A 6 h period of co-incubation may be enough for normal fertilization. Cumulus cells removal at this time does not influence normal fertilization rate.

The effect of early cumulus cells removal on the polyspermy rate

The polyspermy rate in conventional IVF was about 2%~9% [20]. A similar polyspermy rate was observed in routine short co-incubation when cumulus cells were left [6, 19]. But the effects of early cumulus cells removal on polyspermy rate remained conflict.

A lower polyspermy rate was obtained by Lundqvist [10]. In his study, the digested cumulus cells were removed but those cumulus cells attached to the corona radiata were left after 2 h co-incubation. In contrast, higher polyspermy rates were observed by Zhang Wei and Sun Ying-pu (17.86% and 11.6%, respectively, unpublished results), in which cumulus cells were completely removed after 2~4 h co-incubation. This discrepancy indicated that partial or complete removal of cumulus cells brought different polyspermy rates. We speculate that the higher polyspermy rates from Zhang Wei and Sun Ying-pu are attributed to the damage on oocytes: (1) zona penucida is an important barrier to prevent polyspermy. Fracture of zona penucida caused by the early removal of cumuls cells may result in polyspermy. This is an equivalent mechanism with partial zona dissection, which is known for causing an elevated polyspermy rate [21]; (2) the damage on oocyte organelles, such as spindle microtubule, may be another reason. The spindle microtubule fails to bind to the chromosome centromeres, resulting in detachment of the chromosomes from the metaphase plate. Each of these sets of detached chromosomes may be independently form pronuclei [22]. It seems logical that complete removal of cumulus cells produces severer damage on oocytes than partial removal, resulting in the higher polyspermy rates.

The polyspermy rates (17.86% and 11.6%) from Zhang Wei and Sun Ying-pu were significantly higher than conventional IVF. In their studies, the cumulus cells were removed at 2~4 h of insemination. The polyspermy rates (7.48% in 6 h group and 9.22% in 20 h group) in our study were comparable with conventional IVF. One possible explanation for this discrepancy may be the different time of cumulus cells removal: (1) It is known that cumulus cells are more difficult to remove at 2~4 h of insemination than at 6 h of insemination. A severer damage on oocytes may be produced, resulting in a higher polyspermy rate at 2~4 h of insemination than at 6 h of insemination; (2) A lower polyspermy rate was obtained in 6 h group than in 20 h group in our study. The maturity of the oocyte may account for this difference. There is the possibility that some oocytes that are in germinal vesicle or metaphase I stage when insemination, which become mature during incubation. Part of mature oocytes become post-mature during incubation. Immature or post-mature oocytes fail to block polyspermy due to the dysfunction of cortical granules [23]. The sperm attach to the cumulus cells continue to fertilize those oocytes, resulting in the increased polyspermy in 20 h group.

In addition, most couples in 6 h group were primary infertility. It needs further study to determine whether the causes of infertility are in correlation with the lower polyspermy rate. It is well known that the female age is not in correlation with the polyspermy rate [24]. So we do not think the lower polyspermy rate in 6 h group derived from the different female age between the two groups.

The effect of early cumulus cells removal on the multinucleation and pregnancy rate in short co-incubation of gametes

Multinucleation is a frequently observed phenomenon in IVF, which may be positively correlated with factors such as shorter stimulation, higher number of oocytes, higher FSH dosage [25]. Our study showed that a similar multinucleation rate between 6 h group and 20 h group, indicating that cumulus cells removal at 6 h of insemination did not increase the risk of multinucleation. It is well known that multinucleation was one of the factors affecting the pregnancy rate due to the reduced development potential. Embryos with multinucleated blastomeres are not suitable for transfer and should be excluded unless no other embryos are available.

Our study showed a similar pregnancy rate between 6 h group and 20 h group, indicating that cumulus cells removal at 6 h of insemination did not affect the clinical pregnancy rate.

The comparison of early rescue ICSI and late rescue ICSI

Fertilization failure occurred in 10%~25% of IVF cycles [26]. Attempts to rescue unfertilized oocytes by ICSI on day 1 after overnight co-incubation generally led to poor results [8]. Christopher Chen [9] showed rescue ICSI at 6 h of insemination gave better fertilization, pregnancy and implantation rates than rescue ICSI at 22 h of insemination. There were 3%~5% cycles which received rescue ICSI due to fertilization failure or low fertilization rate in our center. Of which, most were early rescue ICSI at 6 h of insemination. Early rescue ICSI generally resulted in 51% of clinical pregnancy rate. No clinical pregnancy was obtained in late rescue ICSI cycles in our study. This may be correlated with aged oocytes.

Furthermore, early rescue ICSI generally led to 3.82% of polyspermy rate in our study, indicating that early rescue ICSI at 6 h of insemination did not increase the risk of polyspermy. Some studies showed that oocytes had been observed to be fertilized 2~4 h after exposure to sperm, and the second polar body was released in ~90% of fertilized oocytes by 6 h [14–16]. These may be account for the low fertilization rate in our study. Nagy et al. [27] reported that polar body observation could provide an accurate tool to determine oocyte fertilization. In their study, oocytes that were identified with one polar body at 6 h of insemination but were not received rescue ICSI (50 oocytes) did not showed any signs of fertilization. But it should be noted that the judgement of two polar bodies is critical and difficult. This should be performed by experienced embryologists.

Conclusion

Cumulus cells removal at 6 h of insemination is a relatively safe operation, which yields comparable normal fertilization rate, similar multinucleation and clinical pregnancy rates compared with 20 h group. This protocol may be beneficial for early obsevation of fertilization failure and make early rescue ICSI possible.

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