Different Timings of Early Cumulus Cells Removal Have Different Multiple Pronuclei Rates in Human in Vitro Fertilization

Zhiren Liu
the First Affiliated Hospital of Fujian Medical University
Qicai Liu
the First Affiliated Hospital of Fujian Medical University
Mingting Jiang
Institute for Translational Medicine, Fujian Medical University
Xingting Chen
the First Affiliated Hospital of Fujian Medical University
Chen Lin
the First Affiliated Hospital of Fujian Medical University
Yujia Guo (guoyujiaszzx@163.com)
Center for Reproductive Medicine, the First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian, People's Republic of China.

Research

Keywords: timing, cumulus cells removal, multiple pronuclei, fertilization, oocyte quality

Posted Date: January 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-136801/v1

Abstract

Background: Cumulus cells removal 4 h post-insemination has a significantly higher multiple pronuclei (MPN) rate than cumulus cells removal 20 h post-insemination. And, cumulus cells removal 6 h post-insemination has a significantly lower MPN rate than cumulus cells removal 20 h post-insemination. However, it remains unclear whether the different timings of early cumulus cells removal, such as the timings of 4, 5 and 6 h post-insemination, have significantly different MPN rates.

Methods: This was a retrospective study. The included cycles were early cumulus cells removal cycles (n=752) at our center from January 2015 to August 2020. The included cycles were divided into two groups according to whether MPN exist (MPN=0% and MPN>0%). The patient and cycle stimulation characteristics of the two groups were compared. Binary logistic regression was performed to investigate the correlation between the timing of early cumulus cells removal and MPN. The cohort study was also performed to compare the patient characteristics, cycle stimulation characteristics, fertilization outcomes, and cultivation outcomes.

Results: In the population of our study, the timing of early cumulus cells removal had a significant effect on the MPN. The cumulus cells removal ≤4 h post-insemination group had a high MPN rate, and the 5.5<time≤6 h group had a high fertilization failure rate. However, 2PN rate was not significantly different among the different timings of early cumulus cells removal. In addition, the ≤4 h post-insemination group had a high grade 1–2 embryo rate at day 3.

Conclusion(s): Even if all the timings of cumulus cells removal are early, the different timings of early cumulus cells removal still have a significant effect on the MPN.

Background

Short co-incubation of gametes combined with early rescue intracytoplasmic sperm injection (ICSI) has been shown to be useful in the treatment of total fertilization failure (TFF) and near-TFF(1–3). The incidence of TFF is 1–10% in conventional in vitro fertilization (IVF) cycles(4–6). If we want to perform the early rescue ICSI for these TFF cycles, early cumulus cells removal is needed. In the early rescue strategy, cumulus cells are usually removed at an early stage to observe the polar bodies. If there are two polar bodies in the perivitelline space, the oocytes are considered to be fertilized. If most or all oocytes don't extrude the second polar body, the early rescue ICSI is performed in time.

Compared with late rescue ICSI (rescue ICSI is performed on day 1 after ovum pick-up), the early rescue ICSI has better fertilization, implantation and pregnancy rates(1, 7, 8). It can be explained by two causes. First, the early rescue has younger oocytes than late rescue. These younger oocytes have better developmental potential and fertilization ability than 1-day-old ones. Second, short co-incubation of gametes can reduce the detrimental effects of sperm metabolic waste products, such as free oxygen radicals(9, 10).

Therefore, the early rescue ICSI is helpful for the TFF and near-TFF cycles. However, the TFF and near-TFF cycles are a minority. And most of conventional IVF cycles can get a normal fertilization rate and do not need the early rescue ICSI. Thus, we have to consider the effect of early cumulus cells removal on these normal fertilization cycles. It has been reported that cumulus cells removal 4 h post-insemination group has a significantly higher multiple pronuclei (MPN) rate than cumulus cells removal 20 h post-insemination group(11). However, another study showed that cumulus cells removal 6 h post-insemination group has a significantly lower MPN rate than cumulus cells removal 20 h post-insemination group(1). Remarkably, when the MPN rates of early cumulus cells removal (4 h and 6 h) were compared with that of late cumulus cells removal (20 h), the results of these two studies were different. Why? Although both 4 h and 6 h belong to the early cumulus cells removal, they still had a significant difference in MPN rate? Or the MPN rate was only affected by other factors, such as age, duration of ovarian stimulation or quality of oocytes? All of these are still unknown. Therefore, the unanswered question is that whether the timing of early cumulus cells removal has an influence on the MPN.

In our study, after adjustment for the other patient and cycle stimulation variables, the correlation between the timing of early cumulus cells removal and the MPN was determined. It would help us to optimize the early cumulus cells removal strategy and to know the formation causes of the MPN in this strategy.

Materials And Methods

Study design and patient selection criteria

A retrospective study was performed. The included cycles in this study were recruited from the Center for Reproductive Medicine at our hospital from January 2015 to August 2020. The inclusion criteria of cycle were set as follows: 1) It was an early cumulus cells removal cycle. 2) It was a first conventional IVF treatment in our center. 3) According to World Health Organization guidelines (WHO, 1999), before preparation, the rate of normal morphology sperm ≥ 15%. And after preparation, progressive sperm concentration could reach 300,000/ml when inseminating. The exclusion criteria of cycle: 1) Cycles didn't have any metaphase II (MII) oocytes. 2) It was TFF Cycles. 3) Cycles involving frozen-thawed gamete. Gamete donation cycles were also excluded, because all of these cycles were the frozen-thawed gamete cycles in our center. The freezing of gamete may have an effect on the fertilization. Preimplantation genetic testing (PGT) were not performed in our center. In addition, about the cycles which were performed rescue ICSI due to low fertilization rate, only the outcomes of the conventional IVF before rescue ICSI were counted in our study, the outcomes of the rescue ICSI were not counted.

Stimulation, sperm preparation and insemination

Ovarian stimulation protocol included GnRH-agonist prolonged protocol, GnRH-agonist long protocol, GnRH-antagonist protocol, mini-stimulation protocol, luteal phase ovarian stimulation protocol(12), and natural cycle. Human chorionic gonadotropin was administered when at least one follicle diameter reached 17 mm. Approximately 36 hours later, cumulus-oocyte complexes were retrieved using transvaginal ultrasound–guided aspiration.

Sperm preparation was divided into two steps, gradients centrifugation and swim-up. Frist, the semen sample was centrifuged with a discontinuous SpermGrad gradient (90% and 45%, Vitrolife, Sweden). After centrifugation, the sperm pellet was washed once in IVF medium (Vitrolife, Sweden). And then, the sperm pellet was transferred into the bottom of a new centrifuge tube which had 0.5 ml of IVF medium. This new centrifuge tube was kept in an incubator at 6% CO_2, 37 ° C for 1 hour. Second, after incubating for 1 hour, the upper layer of medium was collected from the centrifuge tube. Concentration of sperm in the collected medium was counted by Makler Counting Chamber (Sefi Medical Instruments, Israel). Cumulus-oocyte complexes were inseminated in 0.5 ml of IVF medium at 38 ~ 40 h post-HCG. The insemination concentration was 300,000 progressive sperms per ml.

Cumulus cells removal, fertilization check and embryo evaluation at day 3

After short co-incubation, cumulus cells were mechanically removed by a standardized pipette with an inner diameter of 145 um (EZ-tip and EZ-Grip, RI, Britain). Cumulus-oocyte complexes were gently aspirated and blew out repeatedly by EZ-Grip until most of the cumulus cells were removed. The decision of the timing of early cumulus cells removal was ruleless, the difference of the timings was usually caused by workloads and the number of embryologists on duty. Fertilization was assessed at 16–18 h post-insemination. The number of pronuclei was recorded. ≥3PN was defined as multiple pronuclei (MPN). On day 3 (67–69 h) post-fertilization, the embryos were scored as previously described by the Istanbul consensus workshop(13). And the grade 1–2 embryo at day 3 was defined as well as the Istanbul consensus workshop too(13).

The definition of each rate and primary outcome

The MPN rate was defined as the number of MPN zygotes divided by number of MII oocytes. As well as 2PN rate, 1PN rate, 0PN with cleavage rate, and 0PN without cleavage rate. On day 3, grade 1–2 embryo rate was defined as the number of grade 1–2 embryos divided by number of cleaved embryos(14). Blastocyst formation was defined as the presence of a formed blastocoel at day 5/6. Blastocyst formation rate was defined as the number of blastocysts divided by number of the embryos which were cultured to form blastocysts.

There were 752 MPN rates (752 cycles) in our study. The mean and median of these rates was 8.71% and 0.00%, respectively. In order to analyse by binary logistic regression, the MPN rates were divided into two groups according to the median. Therefore, the primary outcome was defined as the presence of MPN in a cycle (MPN = 0% or MPN > 0% in a cycle).

Statistical analyses

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA, version 19.0). Cycles were divided into two groups according to whether MPN exist (MPN = 0% and MPN > 0%). The patient and cycle stimulation characteristics of the two groups are compared in Table I. Student's t-test and Mann–Whitney test were used for the data with homogeneous variance and heterogeneous variance, respectively. The chi-square test was used for categorical variables. Binary logistic regression was performed to control for those factors with greater clinical importance and p values less than 0.1 in Table I. The odds ratio (OR) and 95% confidence interval (CI) was also calculated. Cycles were also categorized into six groups according to the timings of early cumulus cells removal. The patient and cycle stimulation characteristics of these six groups were compared by One-way ANOVA, Kruskal-Wallis test and Pearson X^2 test. The outcomes of fertilization and cultivation of these six groups were compared by Kruskal-Wallis test.

Results

During the study period, a total of 815 early cumulus cells removal cycles were performed in our center. According to the exclusion criteria, 3 of these cycles were excluded due to they didn't have any eligible MII oocytes, and 60 of these cycles were excluded due to TFF. Therefore, 752 cycles were analyzed in our study. In these included cycles, the mean of time-intervals between the cumulus cells removal and insemination was 4.92 ± 0.77 h, and the shortest and longest intervals were 1.75 and 10.80 h, respectively. At 16–18 h post-insemination, one or more zygotes with MPN could be observed in 369 of these included cycles, and the remaining 383 cycles didn't have any zygotes with MPN.

The patient and cycle stimulation characteristics with respect to whether MPN exist are summarized in Table I. For MPN = 0%, significantly higher mean values were found for female age, male age and gonadotrophins per oocyte, and significantly lower mean values was found for duration of ovarian stimulation and number of oocytes retrieval. Ovarian stimulation protocol was significantly different between the two groups. There were no significant differences in female infertility type, female infertility duration, female BMI, male infertility type, male infertility duration, male BMI, MII rate, or timing of early cumulus cells removal between the MPN = 0% group and MPN > 0% group (Table I).

The cycles were also divided into six groups according to the timing of cumulus cells removal, and the patient and cycle stimulation characteristics are summarized in Table II. There were significant differences in female infertility type, male infertility type, and ovarian stimulation protocol among the six timing groups. The other characteristics were not significantly different among the six timing groups.

Binary logistic regression was performed using to control for those factors with greater clinical importance and p values less than 0.1 in univariate analysis (Table I). According to clinical experience, the fertilization of immature oocytes was easier to be abnormal. Therefore, the MII rate was included in the logistic regression although its p value is larger than 0.1 in Table I. The GnRH-agonist prolonged protocol and 5.5 < time ≤ 6 categories were used as references. This analysis revealed that the timing of early cumulus cells removal had a significant effect on the MPN. After adjustment for the other variables, all the adjusted ORs for MPN of time ≤ 4, 4 < time ≤ 4.5, 4.5 < time ≤ 5 and 5 < time ≤ 5.5 categories were larger than 1, and these categories were significantly different from the referenced category. The largest adjusted OR was 4.568 (1.855–11.245) in the time ≤ 4 category. Although the adjusted OR of time > 6 was larger than 1 too, its p value was larger than 0.05. The other variables, such as gonadotrophins per oocyte, number of oocytes retrieval and MII rate, also had significant effects on the MPN. But for female age, male age, duration of ovarian stimulation, or ovarian stimulation protocol, the effect was not significant (Table III).

The cycles were divided into six groups according to the timing of cumulus cells removal, and the outcomes of fertilization and cultivation are summarized in Table IV. 1 of these cycles was excluded due to all zygotes could not cleave when we analysed the grade 1–2 embryo rate at day 3. 23 of these cycles were excluded due to they didn't have any embryos for blastocyst culture when we analysed the blastocyst formation rate. There were significant differences in MPN rate, 0PN without cleavage rate, and grade 1–2 embryo rate at day 3 among the six timing groups. The time ≤ 4 group had the highest MPN rate and the lowest 0PN without cleavage rate among these six groups, and the 5.5 < time ≤ 6 group was just the opposite. In addition, the time ≤ 4 group had the highest grade 1–2 embryo rate among the six groups. There were no significant differences in 2PN rate, 1PN rate, 0PN with cleavage rate, or blastocyst formation rate among the six groups.

Figure 1 show the rate of MPN > 0% cycle for each timing of early cumulus cells removal category.

Discussion

Early rescue ICSI is effective for TFF and near-TFF. It can provide more transplantable embryos and get a normal neonatal outcome for the TFF cycles(2, 3, 6, 15, 16). However, the early rescue ICSI must be combined with early cumulus cells removal. And previous studies only compared the MPN rate and other outcomes of the early cumulus cells removal (4–6 h post-insemination) with those of the late cumulus cells removal (18–20 h post-insemination)(1, 11, 15). They did not compare the MPN rate among the different timings of the early cumulus cells removal, such as the timings of 4, 5 and 6 h post-insemination. Therefore, when all the removal timings belong to the early cumulus cells removal, it is still unknown whether the early removal timing has a significant effect on the MPN. Another study showed that the different timings of early rescue ICSI (< 6 h, 6–8 h, and > 8 h post-insemination) have significant effects on the outcomes of rescue ICSI (3). Although the different timings of early rescue ICSI usually be combined with the different timings of early cumulus cells removal, the previous study still did not analyzed whether the timing of early cumulus cells removal have a significant effect on the MPN. Therefore, our study tried to answer this question.

In our study, after adjustment for the other variables, we found that the timing of early cumulus cells removal was associated with the MPN. And time ≤ 4 group had the highest rate of the cycles with MPN > 0%, 5.5 < time ≤ 6 group had the lowest rate of the cycles with MPN > 0% (Fig. 1). In cohort study, MPN rate also had a significant difference among the six timing groups, the highest and lowest mean of MPN rate were also the time ≤ 4 group and 5.5 < time ≤ 6 group, respectively (Table IV). These results were consist with the two previous studies (1, 11) and could explain the conflicting results of the two studies. Even if both the two studies were early cumulus cells removal, the timing of early cumulus cells removal still had a significant effect on MPN. In the Hong paper(1), the authors had a speculation about that why the studies of Zhang Wei and Sun Ying-pu (cumulus cells were removed at 2–4 h of insemination) had a higher MPN rate than their study (cumulus cells were removed at 6 h of insemination). They speculated that it is due to the different timings of early cumulus cells removal. And our results confirmed the speculation.

Interestingly, in our study, the 2PN rate was not significantly different among the different timings of early cumulus cells removal. And previous studies(1, 11) showed that the 2PN rate was also not significantly different between the early and late cumulus cells removal groups. It seems that the timing of cumulus cells removal does not have a significant effect on the 2PN. In addition, compared with the two previous studies, our study had a further analysis on the 0PN with cleavage rate and 0PN without cleavage rate, and found that 0PN without cleavage rate had a significant difference among the six timing groups. The 0PN without cleavage oocytes are usually the fertilization failure oocytes.

According to the result of 2PN rate, we speculated that the oocytes which can be normally fertilized did not affected by the timing of cumulus cells removal. The affected oocytes were the poor quality ones. These poor quality oocytes manifested MPN when the cumulus cells were removed at time ≤ 4 h post-insemination and manifested fertilization failure when the cumulus cells were removed at 5.5 < time ≤ 6 h post-insemination.

In the Kotil study(17), the authors evaluated fine structural morphology and cytoskeletal features of 3PN oocytes after ICSI(17). They found that the 3PN oocytes have the features of poor oocyte quality, such as lipofuscin granules, lack of cytoplasmic halo, multilamellar body formation, diminished cytoskeletal fine filaments, disrupted γ-tubulin accumulation and degenerated mitochondria(17–19). These results confirmed our speculation that most of the MPN oocytes in the time ≤ 4 group are the poor quality oocytes. And only the poor quality oocytes are easy to be affected by the early cumulus cells removal.

There are three major reasons for the formation of MPN. 1) Polyspermy. 2) Failure of the second polar body (PBII) extrusion. 3) Abnormal pronucleus formation(17, 20). In the Hong study(1), the authors speculated that the high MPN rate is due to the damage on oocytes, when the cumulus cells are completely removed at 2 ~ 4 h post-insemination. They speculated that the early cumulus cells removal maybe damages the zona penucida, and makes the zona penucida lost the ability to prevent polyspermy. However, in our study, the oocytes were washed and transferred into a new medium without sperm immediately after early cumulus cells removal. Therefore, even though the zona penucida was damaged, no a new sperm could enter into the oocytes. Thus, the main cause of the highest MPN rate in time ≤ 4 groups is not the polyspermy.

In the Coticchio study(21), the authors showed that the timing of PBII extrusion is 3.3 ± 1.1 h post-insemination. In the Kotil study(17), their results indicated that MPN oocytes are the poor quality oocytes with diminished cytoskeletal fine filaments and disrupted γ-tubulin accumulation. According to these studies, we speculated that one main cause of the highest MPN rate in time ≤ 4 group is the failure of PBII extrusion. Because the timing of cumulus cells removal was exactly the timing of PBII extrusion in time ≤ 4 groups, and the mechanical operation of cumulus cells removal would damage the cytoskeleton organization of the poor quality oocytes. These would result in the failure of PBII extrusion. Note that the damage generally only happened to the poor quality oocytes, it was the cause that why the time ≤ 4 group had a normal 2PN rate.

In addition, the damage of the cytoskeleton organization also affects the pronucleus formation. And assembly error of pronucleus can result in MPN too(20). Therefore, abnormal pronucleus formation is another main cause of the highest MPN rate in the time ≤ 4 group. The Mutia study showed that 33.3% of 3PN embryos had no chromosomal abnormalities(22). The formation cause of these 3PN embryos may be the abnormal pronucleus formation.

Coticchio et al(21) also showed that the female and male PN appearance are 6.2 ± 1.4 and 6.3 ± 1.4 h post-insemination, respectively. Therefore, in 5.5 < time ≤ 6 group, the mechanical operation of cumulus cells removal would interfere the pronucleus formation. It might be the reason why this group had the highest 0PN without cleavage rate. As well as the time ≤ 4 group, most of the affected oocytes were the poor quality oocytes, because this gourp also had a normal 2PN rate.

In binary logistic regression, after adjustment for the other variables, number of oocytes retrieval, gonadotrophins per oocyte, and MII rate also had significant effects on the MPN. Our results showed that the more oocytes are retrieved in a cycle, the more chance of MPN > 0% in the cycle. It is due to the number of poor quality oocytes often increases with the number of retrievable oocytes. And the poor quality oocytes are easy to be fertilization failure or fertilization abnormality. The previous research had a similar result(23), it showed that the fertilization rate decreases with the number of oocytes retrieval(23). Although our study showed that the MPN > 0% group had a higher MII rate than the MPN = 0% group, it was unclear whether the MII oocytes had a mature cytoplasmic in the MPN > 0% group. Only when the cytoplasmic maturation is concomitant with nuclear maturation can the oocyte achieve its full fertilization and developmental potential(24, 25). And the cytoplasmic maturation is regulated by microenvironmental factors, such as gonadotrophin and growth factors, in the follicles(26). However, in our study, gonadotrophin dose per oocyte was lower in the MPN > 0% group. The lower gonadotrophin dose per oocyte might not be enough to support the cytoplasmic maturation of all oocytes. Therefore, some of these MII oocytes did not complete the cytoplasmic maturation and were easy to be MPN in the MPN > 0% groups. In a word, these results showed that if there are too many retrievable oocytes in a cycle, the gonadotrophin dose per oocyte will be low. Even though the MII rate is high in the cycle, some of these MII oocytes do not complete the cytoplasmic maturation and are poor quality. These oocytes can be fertilized, but they often are MPN. These results confirmed our speculation that the affected oocytes by the timing of cumulus cells removal are the poor quality oocytes. In addition, a high dose of gonadotrophins can affect the oocyte quality too(27). However, there was no significant difference in the total dose of gonadotrophins between MPN = 0% and MPN > 0% group (33.25 ± 13.41 vs 33.53 ± 10.74, ampoule, Mann-Whitney Test, $P = 0.713$).

The highest grade 1–2 embryo rate at day 3 in time ≤ 4 group was due to the shortest co-incubation of gametes. A high concentration of sperm and the corresponding metabolic products have a negative effect on embryo quality(28, 29). Our result was in consistent with the previous studies(28, 29).

There were some important limitations in this study due to it was a retrospective study. First, some confounding factors might be missed due to the data could not be collected. Second, we decided the removal timing according to workloads and the number of embryologists on duty. And the cycles could not be randomly assigned to the six removal timing groups. It might lead to a population selection bias. Therefore, a randomized controlled trial should be done to confirm our results. Third, we speculated that one main cause of the highest MPN rate in time ≤ 4 group is the failure of PBII extrusion. However, we could not collect the data of PBII extrusion to verify the speculation. It was due to the data were not recorded in the clinical work. In addition, some good quality embryos were selected to transfer or freeze at day 3 in some cycles, but in the other cycles, all embryos were cultured to blastocyst stage. Therefore, the result of blastocyst formation rate also had a bias. It needed to be confirmed in a targeted research.

Conclusion

In the population of our study, the timing of early cumulus cells removal had a significant effect on the MPN. The cumulus cells removal ≤ 4 h post-insemination group has a high MPN rate, and the 5.5 < time ≤ 6 h group has a high fertilization failure rate. However, 2PN rate is not significantly different among the different timings of early cumulus cells removal. In addition, the ≤ 4 h post-insemination group has a high grade 1–2 embryo rate at day 3. These indicate that selecting an earlier timing to removal the cumulus cells in the early rescue ICSI strategy may have a better outcome.

Abbreviations

MPN
multiple pronuclei; ICSI:intracytoplasmic sperm injection; IVF:in vitro fertilization; TFF:total fertilization failure; MII:metaphase II; PGT:preimplantation genetic testing; GnRH:gonadotropin-releasing hormone; 3PN:three pronuclei; 2PN:two pronuclei; 1PN:one pronucleus; 0PN:zero pronucleus; HCG:human chorionic gonadotropin; PBII:second polar body.

Declarations

Ethical approval

Before beginning the study, approval was obtained from the Reproductive Medicine Ethics Committee of the First Affiliated Hospital of Fujian Medical University and signed and informed consent was obtained from the patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

No

Authors' contributions

All of the authors (Zhiren Liu, Qicai Liu, Mingting Jiang, Xingting Chen, Chen Lin and Yujia Guo) made substantial contributions to the conception of the study and to the design or acquisition of the data, as well as to the analysis and interpretation of the data. They drafted the article or revised it critically for important intellectual content. All of the authors gave final approval of the version to be published.

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Tables

Table I Patient and cycle stimulation characteristics of the MPN=0% and MPN>0% groups

Characteristics	MPN=0% n=383, 50.93%	MPN>0% n=369, 49.07%	*P*-value
Female age	32.57±5.25	31.37±4.87	*P*=0.001[a]
Male age	34.42±6.05	33.49±5.88	*p*=0.032 [a]
Female infertility type, n (%)			
primary infertility	243 (63.45)	243 (65.85)	
secondary infertility	140 (36.55)	126 (34.15)	*p*=0.490 [c]
Female infertility duration (years)	4.14±2.83	3.84±2.53	*p*=0.332 [b]
Female BMI	22.01±3.34	21.82±3.23	*P*=0.429 [b]
Male infertility type, n (%)			
primary infertility	270 (70.50)	276 (74.80)	
secondary infertility	113 (29.50)	93 (25.20)	*p*=0.186 [c]
Male infertility duration (years)	3.98±2.82	3.89±2.39	*p*=0.468 [b]
Male BMI	23.85±3.15	24.26±3.92	*P*=0.200 [b]
Duration of ovarian stimulation (days)	10.44±2.58	10.99±2.12	*P*=0.002 [b]
Gonadotrophins per oocyte (ampoule [d])	6.30±8.53	3.45±4.60	*P*<0.001 [b]
Number of oocytes retrieval	10.82±7.78	16.05±8.99	*P*<0.001 [b]
MII rate (%)	87.40±16.23	89.96±12.50	*P*=0.282 [b]
Ovarian stimulation protocol			
GnRH-agonist prolonged protocol	87 (22.72)	99 (26.83)	
GnRH-agonist long protocol	101 (26.37)	149 (40.38)	
GnRH-antagonist protocol	11 (2.87)	11 (2.98)	
Mini-stimulation protocol	96 (25.07)	66 (17.89)	
Luteal phase ovarian stimulation protocol	84 (21.93)	43 (11.65)	
Natural cycle	4 (1.04)	1 (0.27)	*P*<0.001 [c]
Timing of early cumulus cells removal (h) [e]			
time≤4	20 (5.22)	28 (7.59)	
4<time≤4.5	90 (23.50)	85 (23.04)	
4.5<time≤5	140 (36.55)	135 (36.59)	
5<time≤5.5	72 (18.80)	87 (23.58)	
5.5<time≤6	34 (8.88)	17 (4.61)	
time>6	27 (7.05)	17 (4.61)	*P*=0.058 [c]

[a] Two-sample t-test. Values are mean+SD.

[b] Two-sample Mann–Whitney test. Values are are mean+SD.

[c] Pearson X^2 test. Values are number (percentage).

[d] 75 IU per ampoule.

[e] The timing is defined as the time-interval between cumulus cells removal and insemination.

MPN: multiple pronuclei.

Table II Patient and cycle stimulation characteristics of the different cumulus cells removing timing groups

	patient groups (according to cumulus cells removing timing [e])						
	time≤4 n=48, 6.38%	4<time≤4.5 n=175, 23.27%	4.5<time≤5 n=275, 36.57%	5<time≤5.5 n=159, 21.14%	5.5<time≤6 n=51, 6.78%	time>6 n=44, 5.85%	*P*-value
Female age	32.04±5.20	31.86±5.06	32.26±5.27	32.01±4.67	31.43±5.55	31.18±5.09	*P*=0.762 [a]
Male age	33.35±5.17	33.58±6.14	34.55±5.98	33.73±5.79	33.73±6.53	33.66±6.21	*P*=0.515 [a]
Female infertility type, n (%)							
primary infertility	35 (72.92)	122 (69.71)	159 (57.82)	103 (64.78)	33 (64.71)	34 (77.27)	
secondary infertility	13 (27.08)	53 (30.29)	116 (42.18)	56 (35.22)	18 (35.29)	10 (22.73)	*P*=0.034 [c]
Female infertility duration (years)	4.58±3.22	4.28±2.74	3.68±2.46	4.12±2.68	3.54±2.62	4.22±3.13	*P*=0.092 [b]
Female BMI	21.17±2.90	21.92±2.97	22.18±3.49	21.50±3.14	22.05±3.58	22.41±3.55	*P*=0.136 [b]
Male infertility type, n (%)							
primary infertility	42 (87.50)	134 (76.57)	187 (68.00)	116 (72.96)	32 (62.75)	35 (79.55)	
secondary infertility	6 (12.50)	41 (23.43)	88 (32.00)	43 (27.04)	19 (37.25)	9 (20.45)	*P*=0.021 [c]
Male infertility duration (years)	4.42±3.20	4.09±2.64	3.68±2.46	4.11±2.45	3.43±2.26	4.37±3.43	*P*=0.134 [b]
Male BMI	24.80±5.53	24.00±3.80	23.96±3.24	23.99±2.97	23.98±3.62	24.32±3.64	*P*=0.990 [b]
Duration of ovarian stimulation (days)	10.67±2.04	10.77±2.53	10.58±2.31	10.92±2.32	10.78±2.62	10.50±2.57	*P*=0.852 [b]
Gonadotrophins per oocyte (ampoule [d])	5.36±7.05	6.22±9.10	4.95±7.58	3.87±3.72	4.07±5.45	3.55±2.97	*P*=0.380 [b]
Number of oocytes retrieval	12.38±9.00	12.65±8.37	13.24±8.98	13.89±8.34	14.41±8.81	15.30±10.37	*P*=0.397 [b]
MII rate (%)	84.51±14.20	88.68±15.30	89.45±14.14	89.69±13.08	87.87±17.27	85.32±15.82	*P*=0.057 [b]
Ovarian stimulation protocol							
GnRH-agonist prolonged protocol	14 (29.17)	43 (24.57)	64 (23.27)	39 (24.53)	11 (21.57)	15 (34.09)	
GnRH-agonist long protocol	22 (45.83)	60 (34.29)	93 (33.82)	49 (30.82)	14 (27.45)	12 (27.27)	
GnRH-antagonist	1 (2.08)	0 (0.00)	8 (2.91)	10 (6.29)	3 (5.88)	0 (0.00)	

protocol							
Mini-stimulation protocol	1 (2.08)	30 (17.14)	73 (26.55)	38 (23.90)	15 (29.41)	5 (11.36)	
Luteal phase ovarian stimulation protocol	10 (20.83)	41 (23.43)	34 (12.36)	23 (14.47)	7 (13.73)	12 (27.27)	
Natural cycle	0 (0.00)	1 (0.57)	3 (1.09)	0 (0.00)	1 (1.96)	0 (0.00)	*P*=0.001 [c]

[a] One-way ANOVA. Values are mean+SD.

[b] Kruskal-Wallis Test. Values are mean+SD.

[c] Pearson X^2 test. Values are number (percentage).

[d] 75 IU per ampoule.

[e] The timing is defined as the time-interval between cumulus cells removal and insemination.

Table III Multivariable analysis of variables for multiple pronuclei

predictors	OR(95% CI)	*P*-value
Female age	0.987 (0.934-1.043)	0.646 [a]
Male age	1.027 (0.982-1.074)	0.245 [a]
Duration of ovarian stimulation (days)	1.055 (0.972-1.146)	0.203 [a]
Gonadotrophins per oocyte (ampoule [c])	0.961 (0.926-0.997)	0.035 [a]
Number of oocytes retrieval	1.074 (1.044-1.103)	<0.001 [a]
MII rate (%)	6.758 (2.136-21.382)	0.001 [a]
Ovarian stimulation protocol		
GnRH-agonist prolonged protocol	1	
GnRH-agonist long protocol	1.486 (0.985-2.244)	0.059 [b]
GnRH-antagonist protocol	0.995 (0.377-2.629)	0.992 [b]
Mini-stimulation protocol	1.611 (0.929-2.796)	0.090 [b]
Luteal phase ovarian stimulation protocol	1.111 (0.635-1.945)	0.712 [b]
Natural cycle	1.006 (0.087-11.584)	0.996 [b]
Timing of early cumulus cells removal (h) [d]		
5.5<time≤6	1	
time≤4	4.568 (1.855-11.245)	0.001 [b]
4<time≤4.5	2.551 (1.257-5.176)	0.009 [b]
4.5<time≤5	2.270 (1.156-4.456)	0.017 [b]
5<time≤5.5	2.740 (1.351-5.554)	0.005 [b]
time>6	1.367 (0.551-3.392)	0.500 [b]

[a] *P*-value of each variable's overall effects after adjusting for the other variables.

[b] *P*-value between each variable's subgroups and reference group.

[c] 75 IU per ampoule.

[d] The timing is defined as the time-interval between cumulus cells removal and insemination.

TableIV Fertilization and cultivation outcome characteristics of the different cumulus cells removing timing groups

	patient groups (according to cumulus cells removing timing)						
	time≤4 n=48, 6.38%	4<time≤4.5 n=175, 23.27%	4.5<time≤5 n=275, 36.57%	5<time≤5.5 n=159, 21.14%	5.5<time≤6 n=51, 6.78%	time>6 n=44, 5.85%	*P*-value
Timing of early cumulus cells removal (h) [b]	3.71±0.39	4.31±0.14	4.83±0.15	5.28±0.16	5.79±0.16	6.96±0.95	*P*<0.001 [a]
MPN rate (%)	9.45±10.30	8.99±15.12	9.22±16.84	9.45±12.71	4.94±8.42	5.33±10.00	*P*=0.038 [a]
2PN rate (%)	71.82±20.71	69.24±23.89	66.62±24.63	66.05±23.18	60.39±25.95	63.67±28.21	*P*=0.158 [a]
1PN rate (%)	6.71±14.18	4.58±8.81	3.90±6.91	3.72±7.43	4.01±6.38	3.85±9.35	*P*=0.808 [a]
0PN with cleavage rate (%)	0.62±3.00	1.16±6.63	0.87±3.52	1.32±4.70	0.55±2.03	0.22±1.00	*P*=0.491 [a]
0PN without cleavage rate (%)	11.39±15.17	16.04±19.50	19.40±21.54	19.47±23.27	30.10±26.84	26.93±27.88	*P*=0.001 [a]
Grade 1–2 embryo rate at day 3 (%)	60.99±35.66	45.06±30.49	46.53±31.10	42.83±30.07	46.72±30.94	41.21±30.02	*P*=0.032 [a]
Blastocyst formation rate (%)	71.89±67.30	57.16±48.50	64.74±54.29	72.53±56.65	61.83±45.73	69.31±75.69	*P*=0.180 [a]

[a] Kruskal-Wallis Test. Values are mean+SD.

[b] The timing is defined as the time-interval between cumulus cells removal and insemination.

Figures

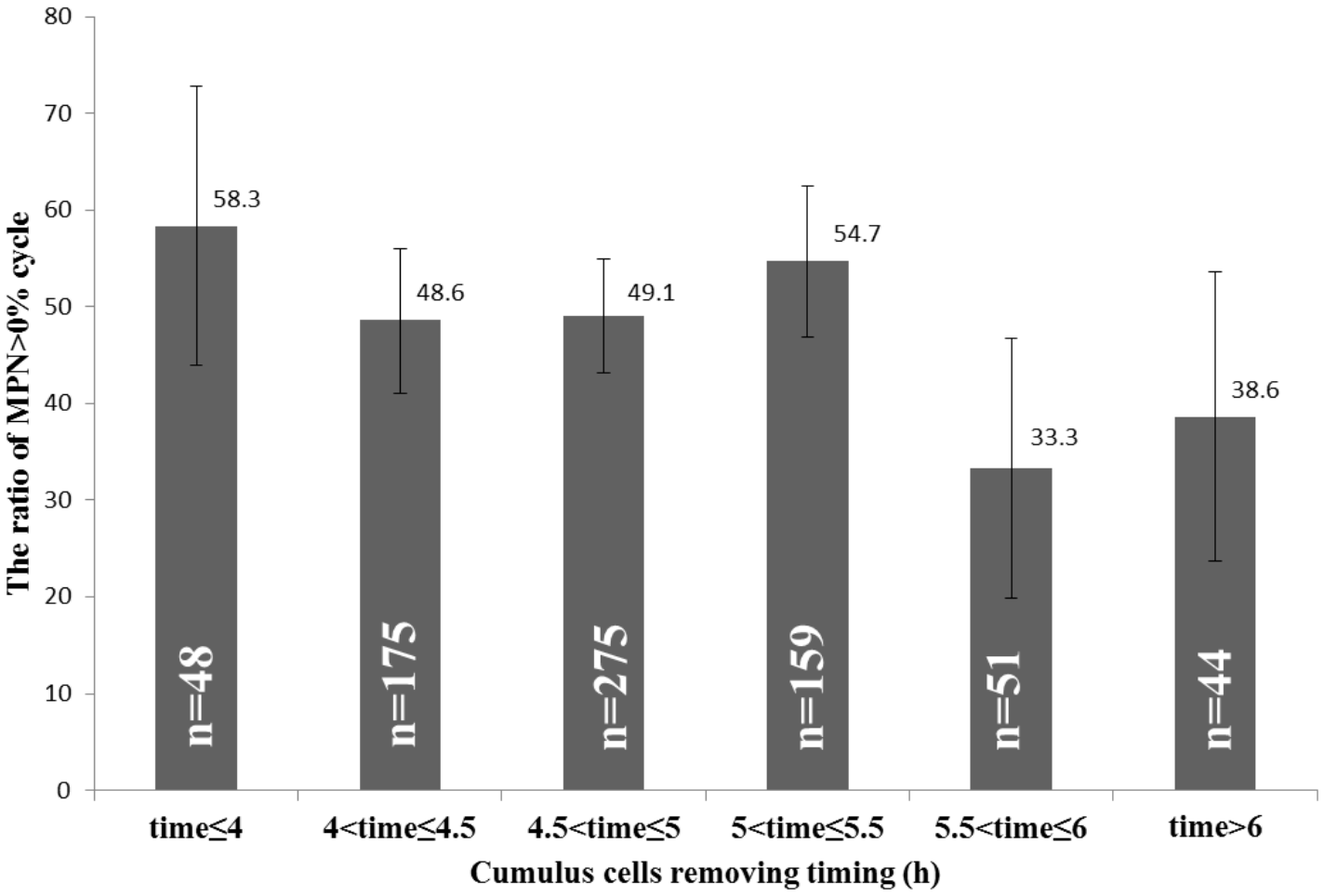

Figure 1

The rate of MPN>0% cycle for each timing of early cumulus cells removal category. Each bar represents a 95% CI. MPN: multiple pronuclei.