Original Article

Two-six Hours is the Optimal Timing of Intracytoplasmic Sperm Injection After Oocyte Pickup: Single-centre 10 Years Cohort Study

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Abstract

Background: Optimal incubation period for oocyte competence remains contentious despite intracytoplasmic sperm injection(ICSI) being in practice for 34 years. Dilemma exists as the current literature favors both early and delayed denudation with equivocal results. With ever-rising demand for the procedure this conundrum continues to plague the clinical outcomes. **Aims:** This study attempts to provide a consensus regarding optimal time duration required for incubating the oocytes after oocyte pickup(OPU) and time to perform ICSI. **Settings and Design:** A retrospective study in a tertiary centre. **Materials and Methods:** A retrospective 10-year cohort study including 726 ICSI cycles was conducted in a single tertiary care infertility centre. All cycles comprised at least one metaphase-II oocyte injected with one good quality sperm followed by fresh embryo transfer. The cohort was broadly divided into two groups: (a) Group 1: OPU-ICSI <4 hours(n=466) and (b) Group 2: OPU-ICSI>4 hours(n=260). **Statistical Analysis Used:** The fertilization(FR) and clinical pregnancy rates(CPR) were compared using the Pearson Chi-square test. The OPU-ICSI interval were subdivided into one-hourly intervals and CPR was compared after adjustment for multiple comparisons by holm method. **Results:** The FR and CPR were similar between Group 1 and Group 2(p>0.05). Comparing CPR for each one-hourly OPU-ICSI interval revealed no significant clinical difference (p>0.05) amongst one another, however, the CPR was maximum for 2-3 hours as the OPU-ICSI interval. **Conclusion:** With no significant clinical difference amongst various temporal groups, this study advocates and reinstates 2-6 hours as the optimal timing for ICSI after the OPU. This will also translate into better time management for both embryologists and clinicians and help them prioritise the laboratory workflow.

Keywords: *Intracytoplasmic sperm injection, oocyte pickup, optimal timing*

Introduction

Since the introduction of intracytoplasmic sperm injection (ICSI), it has revolutionised the process of assisted reproductive technology (ART). ICSI involves the injection of a single sperm into the cytoplasm of oocytes, thus bypassing the zona pellucida and normal fertilisation mechanisms.[1] Before performing ICSI, the oocyte needs to be in optimal condition. The oocyte development and maturation consist of a complex mechanism, involving molecular, structural and biochemical modifications, which are still not completely deciphered. This oocyte maturation results in metaphase II (MII) oocyte with one polar body extruded in the perivitelline space.[2] This maturation is mainly nuclear as evident by the extruded polar body. However, cytoplasmic maturation involving the accumulation of mRNA, proteins, substrates and nutrients is equally required to achieve oocyte developmental competence, which is difficult to assess.[3]

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Table 1: Baseline characteristics of the patients between Group 1 (oocyte pickup-intracytoplasmic sperm injection <4 h) versus Group 2 (oocyte pickup-intracytoplasmic sperm injection >4 h)

Baseline characteristics	OPU-ICSI time <4 h (Group 1) (*n*=466)	OPU-ICSI time >4 h (Group 2) (*n*=260)	*P*
Age (years), mean±SD	31.71±3.78	32.25±3.88	0.069
BMI (kg/m^2), mean±SD	25.10±4.0	25.52±4.02	0.177
Starting dose of FSH (IU) (mean)	290.80±64.38	284.02±71.79	0.192
Starting dose of HMG (IU), median (IQR)	75 (0.00-75.00)	75 (0.00-75.00)	0.053
Average number of oocytes retrieved, median (IQR)	8 (5-11)	8.5 (6-12)	0.177
Number of MII oocytes, mean±SD	4.09±2.66	4.40±3.92	0.201

OPU-ICSI: Oocyte pickup-intracytoplasmic sperm injection, SD: Standard deviation, BMI: Body mass index, IQR: Interquartile range, MII: Metaphase II, FSH:Follicle stimulating hormone, HMG: Human menopausal gonadotropins

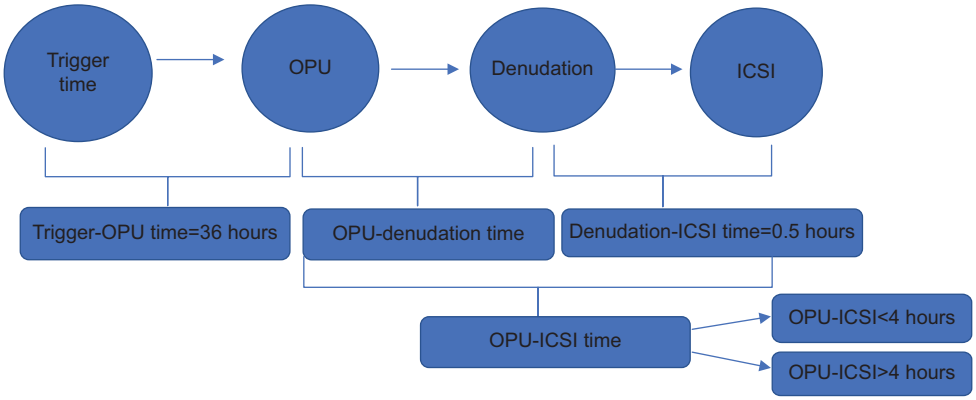

Figure 1: Cycles with this information were included in the study. OPU = Oocyte pickup, ICSI = Intracytoplasmic sperm injection

The incubation period required for this oocyte competence/maturation is based on original evidence by Trounson *et al*., who suggested delayed insemination after 5–6 ½ h of incubation to increase the fertilisation rate (FR).[4] Although the optimal window of 2–6 h has been shown to improve FR and pregnancy rates (PRs), there has been conflicting evidence regarding the timing of ICSI.[5-7] Further Patrat *et al*. narrowed denudation timings to be at least 2 h and up to 3 h after oocyte retrieval and ICSI to be completed as soon as denudation is completed for improved FR and implantation rate.[8] Subsequently, Pujol *et al*. showed that on average, each 1 h increase in the oocyte pickup (OPU)-ICSI time reduced the likelihood of biochemical pregnancy by 7.3% and of clinical pregnancy by 7.7% after the fresh ET.[9] On the contrary, Carvalho *et al*. demonstrated prolonged oocyte culture before denudation (>4 h) is associated with improved PR, live birth (LB) and cumulative LB.[10] A recent randomised controlled trial by Smith *et al*. demonstrated no difference in FR and blastulation rate (BR) between two arms of OPU-ICSI timings (2.5 h vs. 5 h) thus suggesting oocytes have a physiological tolerance for fertilisation during 2–6 h window.[11]

Although the literature is immense, still there is a lack of consensus regarding optimal timings about how long to incubate the oocytes after retrieval and when to perform ICSI. Furthermore, no such previous studies have been performed in ethnically and racially unique South-Asian population.

Materials and Methods

This was a retrospective single-centre cohort study including 726 ICSI cycles with at least one MII oocyte injected with either freshly ejaculated or surgically retrieved sperm followed by fresh embryo transfer, performed in an ART facility of a tertiary care hospital between January 2011 and October 2020. Those with the following parameters were included in the study: (a) Female age <40 years with male factor infertility (azoospermia or oligoasthenozoospermia [OATS]) (b) previous history of fertilisation failure with conventional *in vitro* fertilisation (IVF). Those females with polycystic ovarian syndrome, Grade III or IV endometriosis and poor ovarian responders according to Bologna criteria[12] were excluded from the study. Ethics committee approval was obtained for waiver of consent in view of the retrospective nature of the study (IECPG-782/30.11.2022). No formal power calculation was performed. All subjects have been treated according to the principles of Helsinki Declaration (2013).

GnRH agonist or GnRH antagonist protocol was used, depending on the patient's baseline characteristics. In GnRH agonist protocol, gonadotrophin stimulation was started after ensuring downregulation. Standard formulations of either recombinant follicle-stimulating hormone (FSH) (Injection Gonal-F, Merck Serono Specialties Pvt. Ltd., India) or highly purified human menopausal gonadotrophin (DIVA HMG, Bharat Serum, India) were used for stimulation with initial dosing ranging from 150 to 450 IU per day. Gonadotrophin dose was determined as per the individual physician's discretion based on ovarian reserve, patient age and body mass index (BMI). They were injected with 0.5–1 mg leuprolide acetate (Injection Lupride, Bayer Zydus Pharma Ltd., Mumbai) beginning from day 21 of menstruation for the next 14 days. Downregulation of gonadotrophin was ensured by evaluating biochemical markers (LH <2 IU/ml, E2 <30 pg/ml) as well as Transvaginal sonogram (TVS) assessment of endometrial thickness and ovarian status (endometrial thickness <4 mm, no ovarian cyst >2 cm). After the downregulation, the leuprolide dose was reduced to 0.1–0.5 mg/day, and patients were started on rFSH (Injection Gonal-F, Merck Serono Specialties Pvt. Ltd., India). The starting dose was between 150 IU/day and 300 IU/day depending on the patient's baseline characteristics.

In the antagonist group, patients were evaluated for the presence of an ovarian cyst which was done on the 1st day of the menstrual cycle and was started on injection Gonal F (150 IU to 300 IU) from the 2nd day. GnRH antagonist cetrorelix acetate (Injection Cetrotide, Merck Serono Specialties Pvt. Ltd., India) 250 ug was added on the 6th day of the menstrual cycle (fixed-dose regimen) or when the follicular size was 14 mm (flexible protocol). Follicular and estradiol monitoring was done in both groups using transvaginal ultrasound (TVS), and the dose of gonadotrophin was adjusted accordingly. If none of the follicles were more than 10 mm after 10 days of gonadotrophin stimulation, the cycle was terminated. Ovulation was triggered when the leading follicle reached 18 mm along with at least two follicles >16 mm, using 250 µg of recombinant human chorionic gonadotrophin (hCG) (Injection Ovitrelle, Merck Serono, UK) or dual trigger (injection leuprolide 1 mg and injection ovitrelle 250 µg). Serum E2, P4 and endometrial thickness were measured on the day of the trigger.

Only those cycles where the oocyte retrieval was performed at 36 h following the administration of hCG with the timing of OPU and ICSI mentioned in the embryology register were included in the study [Figure 1]. Freshly ejaculated sperm was processed using the double density gradient method followed by swim up in fertilisation media for 45 min at 37°. Surgically retrieved sperm was washed once with normal sperm washing media (Vitrolife) followed by swim up in fertilisation media (G-IVF™ Vitrolife). ICSI dish was prepared and incubated at 37° 1 h before the ICSI. The retrieved oocytes were incubated in G-IVF™ media (Vitrolife) for variable time intervals, as there was no consensus regarding the optimal duration between OPU and ICSI. There was minimal interval of 2 h which could be extended to 6 h depending on the embryologist and their laboratory workload on the day of OPU. This was followed by denudation with Hydase enzyme (Vitrolife) before half an hour of performing ICSI. Before performing ICSI, needle alignment was done (R I manipulator/Olympus inverted microscope). Single oocytes were injected with viable selected single sperm. All the injected oocytes were washed thrice in G-MOPS™ media (Vitrolife) and incubated in G-1™ PLUS Media (Vitrolife) for culture. Fertilisation was checked at 16–18 h of incubation. Oocytes with single or without pro-nuclei were separated and discarded. Embryo transfer of one or two embryos was done under ultrasound guidance between days 2 and 5 depending on the number of good-quality embryos. All patients were given luteal phase support in form of micronised progesterone vaginal pessary 300 mg twice a day with intramuscular progesterone 100 mg/day. Pregnancy was assessed on the 16th day of embryo transfer by serum beta HCG assay and this was confirmed after another 2 weeks by the presence of the gestational sac on TVS. Biochemical pregnancies were not included in our analysis. Clinical pregnancy was defined by a viable intrauterine gestation at 6 weeks scan. Multiple gestational sacs were counted as one clinical pregnancy. The clinical PR (CPR) was calculated as the number of clinical pregnancies per embryo transferred.[13]

Statistical analysis

The data were compiled on an Excel spreadsheet and analysed using RStudio software. The cohort was divided into two groups: (a) Group 1: OPU-ICSI <4 h and (b) Group 2: OPU-ICSI >4 h. Each group was further divided into hourly subgroups for subanalysis. Group 1 was divided into intervals such as 1–2 h, 2–3 h and 3–4 h. Similarly, Group 2 was divided into intervals such as 4–5 h and 5–6 h. Variables amongst the group were compared using the Pearson Chi-square test and a $P < 0.05$ was considered statistically significant. The CPR in the subgroups was compared after adjustment for multiple comparisons by the Holm method.

Results

A total of 726 ICSI cycles were included in the study. Due to heterogeneity in the time duration between OPU and ICSI, the cycles were divided into two broad groups: OPU-ICSI <4 h (Group 1) and OPU-ICSI >4 h (Group 2).

The baseline characteristics such as age, BMI, starting dose of FSH/HMG, average number of oocytes retrieved and number of MII oocytes were similar between the two groups ($P > 0.05$) [Table 1].

On comparison of clinical outcomes such as FR and CPR, there was no significant difference between the two groups ($P > 0.05$) [Table 2].

Both the groups (Group 1 and Group 2) were further categorised into 1–2, 2–3, 3–4, 4–5 and 5–6 h, and the CPR was calculated for each group as depicted in Table 3.

Both the groups were further subanalysed, and the CPR was compared between 1 and 2 h versus 2–3 h versus 3–4 h of OPU-ICSI interval in Group 1 (OPU-ICSI time <4 h). Although 2–3 h seems to be the ideal OPU-ICSI interval with the maximum CPR, it was not statistically significant from the subgroup intervals. Similarly, the CPR was compared between 4 and 5 h versus 5–6 h of OPU-ICSI interval in Group 2 (OPU-ICSI time >4 h). There was no significant difference in CPR on subanalysis in Group 2 also.

Table 2: Comparison of clinical outcome between the Group 1 (oocyte pickup-intracytoplasmic sperm injection <4 h) versus Group 2 (oocyte pickup-intracytoplasmic sperm injection >4 h)

	OPU-ICSI time <4 h (Group 1) (*n*=466), *n* (%)	OPU-ICSI time >4 h (Group 2) (*n*=260), *n* (%)	*P*
Fertilisation rate	80.3	79.9	0.73
Cleavage rate	91.3	93	0.08
CPR	108 (23.2)	60 (23.1)	1.00

OPU-ICSI: Oocyte pickup-intracytoplasmic sperm injection, CPR: Clinical pregnancy rate

Table 3: Clinical pregnancy rate for each one hourly oocyte pickup-intracytoplasmic sperm injection interval

One hourly OPU-ICSI interval (h)	Number of pregnant women	Total number of women in each OPU-ICSI interval	CPR (%)*
1-2	0	8	0
2-3	24	72	33.33
3-4	60	306	19.6
4-5	56	242	23.14
5-6	28	90	31.11

*P>0.05. OPU-ICSI: Oocyte pickup-intracytoplasmic sperm injection interval, CPR: Clinical pregnancy rate

Discussion

This study provides great insights into the highly debated topic of optimal timing for performing ICSI after the OPU. This is the first study to compare only the OPU-ICSI timings between the two groups after matching parameters such as the interval between trigger and OPU (36 h) and interval between denudation and ICSI (0.5 h), thus avoiding confounding effects of these intervals.

Although recommended indications of ICSI include male factor infertility, pre-implantation genetic testing, previous fertilisation failure and ICSI use have increased from 15.4% (4197/27,191) to 66.9% (42,321/63,250) between 1996 and 2012, for non-male factor infertility.[14] However, recent data refute the use of ICSI for normozoospermic males, as the PRs lowered by approximately 30% as compared to conventional IVF.[15] Furthermore, the CDC states that ICSI is associated with an increased risk for chromosomal abnormalities, autism, intellectual disabilities and birth defects compared with conventional IVF.[16] Therefore, ICSI for all is not advisable and should be done only in cases of male factor infertility (OATS or Obstructive azoospermia) or previous fertilisation failure. In our study also, ICSI was performed for the recommended indications only.

Before performing denudation and ICSI, there is a need to pre-incubate the oocytes to bring synchronicity between nuclear and cytoplasmic maturation. Nuclear maturation involves recovery from the first meiosis, germinal vesicle breakdown and the first polar body formation. Whereas during cytoplasmic maturation, the oocytes get prepared for fertilisation and subsequent embryonic development thus priming to provide enough energy, enzymes and protein synthesis reserve to meet the needs of new functional protein synthesis during embryonic development.[3] However, due to the complexity of the process, there is no clear standard for defining and detecting cytoplasmic maturation. If the cytoplasm of oocytes is not mature during ICSI, this may directly affect fertilisation and embryo development. It may also impair the supply of material to the embryo, resulting in early embryo death and pregnancy failure.[17]

But how long to pre-incubate the oocytes in IVF laboratory is the next question. There is mixed evidence regarding the OPU-denudation (OPU-DN) time. Many studies compared different time intervals between OPU-DN and concluded that oocyte maturity remarkably improved with the increase in OPU-DN time.[18-20] Furthermore, Hassan observed that oocyte maturation was remarkably lower with immediate denudation than with 4-h incubation with intact cumulus (80.5%

vs. 91.9%).[18] Whereas other studies have compared various OPU-DN intervals: 1–2 versus 5–6,[21] 1–3 versus 3–5 versus 5–7 versus 7–9 versus 9–11,[22] 0 versus 2,[23] 0 versus 0–2 versus 2–5,[24] <2 versus > 2[25] and no difference in oocyte maturation rate/FR or PR was seen. However, considering the differences in methodologies used, there may have been certain confounding factors, one of which was the HCG-OPU time. There was heterogeneity in the time of HCG trigger varying between 34 and 36 h, thus affecting the *in vivo* maturation time of oocytes and thus affecting the clinical outcome. Therefore, in our study, we included only those with HCG-OPU time at approximately 36 h to remove any confounding effect. Furthermore, our denudation to ICSI time was constant (0.5 h); therefore, the OPU-DN time in Groups 1 and 2 was comparable. The fertilisation and CPR are comparable in the two groups. Even on further subgroup – analysis of Group 1 (OPU-ICSI time <4 h) and comparing 1 versus 2 versus 3 versus <4 h, no difference in CPR was observed. Similarly in Group 2 (OPU-ICSI time >4 h), on comparing 4 versus 5 versus 6 h, no difference in CPR was observed. Although the CPR was maximum for 2–3 h as the OPU-ICSI interval, it was not clinically significant.

Since the consensus on pre-incubation interval is lacking, the next question that arises is whether to incubate with or without denudation. Trounson *et al*. in their pioneer study demonstrated a marked increase in the proportion of oocytes that fertilised and developed into normal embryos and maximum FR was seen if denudation and ICSI were done after 5–5 1/2 h in culture.[4] The prolonged incubation of cumulus–corona–oocyte complexes is necessary for cytoplasmic maturity, thus leading to a higher activation rate on microinjection.[26,27]

On the contrary, the animal model study demonstrated accelerated oocyte ageing on incubation with cumulus cells due to the release of ageing promoting factor[28] or soluble Fas ligand.[29] But any effect of cumulus cells on oocytes has been disregarded by Van de Velde *et al*. suggesting that the surrounding cells are not necessary for survival, fertilisation, and cleavage after ICSI.[21] Similarly, prolonging oocyte culture before denudation did not influence ICSI outcome since it does not compensate for insufficient post-priming exposure to the follicular environment.[30] However, a recent single-centre retrospective cohort analysis of 1378 ICSI cycles concluded that extended culture before denudation, especially >4 h, results in improved CPR, LB rate and cumulative LB rate.[10] However, this result is invalidated by a randomised controlled trial comparing OPU-denudation interval between 2.5 h versus 5 h post-pickup and observed no difference in FR and BR between the two arms.[11] However, this lacks clinical relevance since the CPR and LBR were not compared.[31] Whereas in our study, both fertilisation rate and CPR were similar between the two compared groups.

Although this study is limited by retrospective nature and LB rate is not assessed and compared, this study provides great insights into the flexible timings for ICSI after the OPU.

Conclusion

With no significant clinical difference amongst various temporal groups, this study advocates and reinstates 2–6 h as the optimal timing for ICSI after the OPU. This will also translate into better time management for embryologists as well as clinicians and help them prioritise the laboratory workflow.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Data availability statement

Data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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