Review Article

Time line in IVF laboratory

Ethiraj Balaji Prasath

Thomson Fertility Centre, Singapore

Abstract The time line in IVF is critical, as the procedures are associated with the time of the ovulation trigger for follicular maturation, followed by sequential procedures in relation to it. Starting from oocyte recovery scheduled 36 hours post-trigger, the laboratory procedures are followed up sequentially. Oocyte cumulus complex (OCC) may be incubated until insemination or ICSI until 40 hours post-trigger. Conventional insemination may be done either short (4–6 hours) or long insemination (overnight). However, short insemination is associated with an increase in the 3-PN rate. OCC for ICSI may be denuded at least after 38 hours post-trigger, and ICSI may be performed within 2 to 4 hours post-denuding. ICSI done within 2 hours post-denuding appears to be yielding better-quality embryos. Time-lapse studies have indicated that it is possible to obtain higher clinical and live birth rates with embryos reaching the blastocyst stage faster than other embryos. Embryo transfers done with Day 3 embryos result in a higher pregnancy rate than Day 2 while blastocyst transfers perform better than day 3 transfers. Mobilization of patients after ET may not affect the outcome.

Keywords: Bedrest, denuding, embryo transfer, ICSI, insemination, IVF

Address for correspondence: Dr Ethiraj Balaji Prasath, Thomson Fertility Centre, #19-01, Paragon, 290 Orchard Road, Singapore 238859, Singapore.
E-mail: balaivf@thomsonmedical.com
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INTRODUCTION

IVF, or assisted reproduction, involves hormonal stimulation of the ovaries of the female partner to produce supernumerary follicles and thereby oocytes. These follicles mature with the help of Human Chorionic Gonadotropin (HCG) injection, leading to oocyte collection or retrieval (OPU) at 36 hours (h) after trigger. The following time line in IVF starts once the trigger is administered. The laboratory procedures of incubating oocytes, denuding them, inseminating or injecting sperm into them, fertilization, and cleavage checking need to be performed at fixed times in relation to the time of trigger. Although there is a time line to be followed in IVF, questions such as how strictly it has to be followed or whether there is any relaxation in such a time line are addressed in this paper.

Timing OPU

OPU is scheduled for 35 to 36 hours post-trigger. Performing OPU earlier than these timings may end up in retrieving more immature follicles. A recent retrospective study of 796 cycles with 4930 oocytes exhibited a significantly higher percentage of mature oocytes (79.3% vs. 77.0%; $P < 0.03$) in OPUs done after 34.5 hours post-HCG than OPUs done before ≥34.5 hours post-HCG.[1] The same authors also showed increased maturation at the longer intervals OPU performed much later than 36 hours post-trigger may result in ovulation of follicles, and hence a lesser

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number of follicles are retrieved than expected, based on follicular tracking scans. The follicles ovulate around 40 hours post-trigger, and hence an OPU done around 36 hours post-trigger may yield most of the follicles present. Our centre always schedules OPU at 36 hours post-trigger, except for patients with one follicle or with natural cycle IVF. As Empty Follicle syndrome is out of the scope of this article, it is not discussed here. The trigger is administered in the late evening or night so as to schedule OPU in the morning. HCG injected at other times of the day may cause OPU to be performed at an inconvenient time for all personnel involved. OPU has to be scheduled around 36 hours post-trigger to avoid spontaneous ovulation, which may occur between 38 and 41 hours post-trigger, and also to obtain more mature oocytes.[2] Hence, laboratory staff should be aware of the time of trigger and relate their procedures to it rather than the time of OPU done.

Duration of incubation of OCC before insemination or denudation or ICSI

Oocytes may be identified as mature with the presence of the first polar body but this indicates nuclear maturity only. Cytoplasmic maturity may take place sometime after nuclear maturity. It is widely accepted that cytoplasmic maturity is accomplished around 40 hours post-trigger. Now comes another question on the length of incubating OCC before insemination, denuding, or ICSI. Cumulus cells protect oocytes from ROS by secreting antioxidants, which in turn delay the aging of oocytes. The early removal of cumulus cells and incubation of oocytes for a longer duration may affect the quality of oocytes. A larger study done by Vandenberghe *et al.*[3] with 8811 cycles from 5651 patients involving 74,365 injected oocytes also showed a higher percentage of maturation when ICSI was done at 41 hours post-HCG compared to 38 hours post-HCG. Fertilization rate was also shown to increase when the time of incubation of oocytes between OPU and ICSI was around 4-5 hours, when OPUs are done at 36 hours post-HCG.[4]

Time line for processing and post-processing incubation of sperm

Obtaining sperm sample and processing it need a time line to be followed after OPU. The motility of sperm drops significantly in an ejaculated sample at 60 minutes and head defects increase 30 minutes after the sample is produced and kept at room temperature.[5] A significant drop was observed in rapid progression and viability after 2 hours. This study clearly demonstrates that analysis and processing of an ejaculated semen sample must start at 30 minutes after the sample is produced. A sample may be kept for 20 to 25 minutes for liquefaction.

The next question is how long to keep the sample after processing before using it for insemination or ICSI. Nabi *et al.*[6] have established that DNA fragmentation increases significantly in the processed sample after 2 hours and increases further after 3 hours when the sample is incubated at 37 °C temperature. Progressive motility and thereby total motile sperm count drop significantly ($P = 0$) after 2 hours of incubation after processing for intrauterine insemination.[7] The same can be applied for IVF or ICSI. However, survival of processed sperm has been shown to be significantly better at room temperature (23 °C) than at 35 °C irrespective of whether semen sample was processed by swim up or gradient ($P < 0.01$ and $P < 0.001$, respectively).[8] As most of the IVF laboratories keep sample at 37 °C, it is advisable to use the processed sperm within 2 hours after preparing. Therefore, time of obtaining sperm sample and processing it should be decided based on when insemination or ICSI has to be done, which is related to the time of the trigger injection. The lab may attempt to try keeping samples at room temperature to minimize drop in motility, survival, and DNA fragmentation.

Time line of co-incubation of OCC and sperm

The conventional insemination has been practiced as long insemination (OCC and sperm co-incubated overnight) or short insemination (OCC and sperm co-incubated for 4–6 hours). Denudation of oocytes is done either the next morning or after 4 to 6 hours according to the length of co-incubation between the OCC and sperm. A significant increase of the 3 PN rate ($P < 0.041$) has been observed in short insemination after denuding oocytes at 4 hours of co-incubation than at 20 hours (long insemination), and no difference was observed in rates of 2 PN, 1 PN, top-quality embryos, positive HCG, clinical pregnancy, and implantation.[9] A similar observation of high polyspermy and no difference in normal fertilization rate was confirmed in a meta-analysis.[10] This meta-analysis contrastingly exhibited an increase in good quality embryos, and clinical and on-going pregnancy rates with short insemination. It is surprising to observe a high level polyspermy in short insemination. Possibly there is damage to zona structural integrity caused by denuding, which is difficult due to tight bonding of cumulus cells with zona at 4 hours of co-incubation period compared to more than 6 hours, particularly when second polar body formation is delayed.[9]

Time line for denuding and ICSI

As previously stated, removing cumulus cells from oocytes may increase oocyte susceptibility to ROS and may accelerate oocyte ageing in culture. A minimal incubation of oocyte cumulus complexes for more than 2 hours between OPU and ICSI yielded a higher fertilization rate and a higher proportion of good-quality embryos without significant differences in cleavage, implantation, and pregnancy rates.[11] A significant increase in maturation (76.4% vs. 83.2%; $P < 0.001$) and fertilization (69.2% vs. 79.3%; $P < 0.001$) rates was observed when denuding, followed by ICSI, done at or more than 41 hours after ovulation trigger than <36 hour post-HCG.[3] However, no difference was found in the usable embryo rate, whether on Day 3 or on Day 5. Also, a statistically insignificant increase in clinical pregnancy and live birth rates was observed. Another question that rises at this juncture is whether we should incubate denuded oocytes before ICSI for some time. Zhang *et al.*[12] have shown that ICSI done within 4 hours after denuding yields higher clinical pregnancy and implantation rates ($P < 0.05$) than done beyond 4 hours after denuding. These authors have shown that timing from HCG to ICSI and timing from denuding to ICSI affect fertilization and clinical pregnancy rates more than other timings. The usable embryos rate is higher when the interval between denuding and ICSI is within 2 hours after denuding. It may be beneficial to perform ICSI within a short interval as much as possible after denuding. This is in alignment with Pujol *et al.*[4] where the authors showed that every additional hour between OPU and ICSI decreased the chance of biochemical pregnancies by 7.3% (95% CI: 0.7–13.5%) and clinical pregnancies by 7.7% (95% CI:0.8–14.1%).

The same principle may be applied when oocyte freezing and ICSI of frozen–thawed oocytes are carried out. Incubation of oocytes at least for an hour between denuding and vitrification and between warming and ICSI yields better survival rate of oocytes.[13] On extrapolation of this time interval and those mentioned before, it may be beneficial when denuding and vitrification of oocytes are done around 38 hours post-ovulation trigger and ICSI is done at 2 to 3 hours post-warming. On the contrary, a recent study has shown no correlation of time interval between HCG and oocyte vitrification with post-warming survival of oocytes in regular cycles.[14] However, this study analyzed, retrospectively, a small sample size of 390 oocytes from 66 patients.

Time line for checking fertilization and cleavage

Extrusion of the second polar body after ICSI is completed in 98% of the oocytes by 18 hours of post-ICSI.[15] These authors have also shown that the formation of 2 PN is completed in 96% of the oocytes at 16 hours post-ICSI and in 100% of the oocytes at 18 hours post-ICSI. This is consistent with the Istanbul Consensus Workshop's recommendation on the timing of checking fertilization.[16] The Istanbul Consensus Workshop advises to perform a fertilization check at 17 ± 1 hours post-ICSI or insemination. The same workshop advises time of assessment of embryos for Day 2 at 44 ± 1 hours, Day 3 at 68 ± 1 hours, Day 4 at 92 ± 2 hours and Day 5 at 116 ± 2 hours post-ICSI or insemination. However, such assessments are observation of single snap shot of the development. A continuous observation may aid in better assessment and that is where time lapse could help. Time taken to the onset of blastulation post-insemination is significantly shorter ($P < 0.0001$) in euploid embryos, particularly those euploid embryos that resulted in fetal heart beat (around 94 hours) than aneuploid embryos (around 97 hours).[17] Similarly, time taken to reach blastocyst stage is significantly shorter in excellent (104.04 ± 8.53 hours) and good quality (104.10 ± 10.72 hours) blastocysts than average and poor quality (106.86 ± 8.44 hours) blastocysts.[18] It is obvious from these reports that embryos reaching the blastocyst stage at a faster rate may yield a higher implantation rate.

Time line in resting after embryo transfer

There is always a query lingering in the mind of patients on resting period after embryo transfer. This may not be a proper time line in alignment with the topic of this article. Bust addressing the issue at this juncture may aid readers in counseling their patients whether rest is mandatory after an embryo transfer procedure. An RCT on mobilization of patients after IUI has established that mobilizing patients immediately after insemination does not affect ongoing pregnancy (40.0% vs. 32.2%; $P < 0.97$), live birth (37.6% vs. 30.9%: $P < 0.13$), miscarriage rates (12.2% vs. 10.2%: $P < 0.47$) compared to patients resting for 15 minutes at supine immobilization.[19] Ultrasound study on the change of position of air bubbles introduced at the embryo transfer procedure (the embryos are sandwiched between two air bubbles in the catheter) clearly demonstrated that the bubbles moved from the fundus in a lesser proportion in patient without bedrest than in patients with 15 minutes bedrest (15% vs. 22%).[20] This can be correlated to another prospective study that compared resting for an hour

after ET with immediate mobilization and reported no difference in pregnancy rate between the groups.[21] A systematic meta-analysis[22] done on five RCTs has evidently shown no significant difference in implantation rate [RR 0.90, 95% CI (0.72–1.13), P = 0.38], ongoing pregnancy rate [RR 0.84, 95% CI (0.60–1.20), P = 0.34], miscarriage rate [RR 1.08, 95% CI (0.46–2.57), P = 0.86], and live birth rate [RR 0.93, 95% CI (0.51–1.69), P= 0.81]. Another systematic review and meta-analysis,[23] of four papers, also shown no difference in clinical pregnancy rates and live birth rates whether the patients rested after ET or ambulated immediately. The clinical pregnancy rate was 18.7% for patients with rest and 25.3% for patients with no rest after the embryo transfer procedure (OR 0.75; 95% CI 0.7–0.9). The lower pregnancy rate in patients with rest was significantly (P < 0.001) different from that of patients without rest. The live birth rate was lower (43.6%) in patients with rest after the embryo transfer procedure compared to patients without rest (52.5%), but not statistically significant. However, both reviews had small sample sizes of five RCTs,[22] three papers for clinical pregnancy, and two papers for live birth rates.[23] Some of these papers are retrospective analyses.

It is evident from all these reports that bedrest after ET or IUI is not mandatory.

RECOMMENDATIONS

This article recommends the following after analysis of reports mentioned above.

(1) OPU should be scheduled at 36 hours post-ovulation trigger.
(2) All lab procedures should be related to trigger time.
(3) OCC may be inseminated at 4 to 5 hours after OPU (40–41 hours post-trigger).
(4) Denuding of OCC for ICSI may be done at 39 to 40 hours post-trigger.
(5) ICSI may be performed within 2 hours after denuding.
(6) Checking fertilization and embryo assessment may be performed following Istanbul consensus.[3]
(7) Embryos reaching blastulation around 94 hours and becoming blastocyst around 104 hours post-insemination may yield better implantation rate.
(8) Bedrest after ET is not mandatory.

CONCLUSION

The laboratory procedures must follow the time line in relation to post-ovulation trigger timing. Adhering to such time line does not need to be accurate to minutes and seconds as the intervals have a range of hour.

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Conflicts of interest

There are no conflicts of interest.

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