Original Article

A Study to Find Optimal Intra-cytoplasmic Sperm Injection Timing of Oocytes Matured from Germinal Vesicle in *in Vitro* Maturation Cycles Using a Time Lapse System

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Background: The use of *in vitro* maturation (IVM) has allowed patients with polycystic ovary syndrome (PCOS) to have a positive fertility outcome, as it allows utilisation of immature oocytes to mature *in vitro*. **Aim:** The aim of the study is to establish an optimum intra-cytoplasmic sperm injection (ICSI) timing for IVM oocytes (germinal vesicles [GV] →, metaphase I [MI]→ and metaphase II [MII]) using time lapse system (TLS) for patients with PCOS. **Setting and Design:** Patients included in this study were diagnosed with PCOS, ≤35 years of age, anti-Müllerian hormone levels >6 ng/ml and antral follicle counts <40. Furthermore, we included only GV oocytes at the time of denudation in our study. **Materials and Methods:** Patients were minimally stimulated and their oocytes were retrieved. *In vitro* maturated oocytes were monitored using TLS to a maximum of 30 h. MII oocytes were further cultured and injected at five different time intervals (1–2 h, 3–4 h, 5–6 h, 7–8 h and >8 h) to observe for fertilisation, cleavage and utilisation rate. **Statistical Analysis:** Chi-square test was applied to compared the treatment groups **Results:** Amongst 328 oocytes retrieved from 27 female patients, 162 oocytes were in the time-monitored cohort and 162 oocytes were grouped as the control cohort. Maturation rate between GV→ MII was highest at 18 h in the time-monitored cohort MII (n = 57). Utilisation rate was highest when ICSI was performed between 5 and 6 h after the first polar body extrusion, n = 17 (63%). **Conclusion:** This study provides valuable insight into the optimal maturation timing using a TLS to yield the good number of oocytes. In addition, optimising ICSI timing is important to provide the best utilisation rate in an IVM cycle to achieve synchrony between nuclear and cytoplasmic maturation.

Keywords: *Cleavage, fertilisation, mitosis, polycystic ovary syndrome, utilisation*

Introduction

Intra-cytoplasmic sperm injection (ICSI) is a procedure that allows for the direct entry of spermatozoa into the cytoplasm of a mature oocyte.[1] Using ICSI, the likelihood of fertilisation of an egg increases up to 80%. This treatment is recommended for patients with known male infertility such as oligozoospermia, teratozoospermia and asthenozoospermia.[2,3] ICSI, however, requires a streamlined establishment of timed insemination to achieve a positive fertility outcome. From previous literatures, it was recommended that ICSI or *in vitro* fertilisation (IVF) should be performed within a window of 37 to 41 h post-ovulation trigger.[4,5]

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In vitro maturation (IVM) is the process of culturing immature oocytes, germinal vesicles (GV) and metaphase I (MI) to metaphase II (MII) stage in a laboratory setting. IVM as a treatment is recommended for patients suffering from PCOS, cancer and patients with previous history of complete oocyte maturation arrest (meiotic maturation resistant oocytes).[6-8] During the IVM cycle, extended culture of these oocytes will result in the thickening of the zona pellucida,[9,10] thereby hindering sperm entry if techniques such as IVF are used. Hence. ICSI is a viable fertility treatment that can be offered to females with the use of IVM cycles. ICSI has been reported to yield a fertilisation rate of around 70–80%.[8,11]

Furthermore, IVM cycles are largely time sensitive from maturation to insemination. Hence standardising the timing of ICSI becomes essential. However, recent studies reported that some MII oocytes may not complete cytoplasmic maturation even with the first polar body (PB) being released. Cytoplasmic maturation is also thought to be asynchronous with nuclear maturity.[12] Cytoplasmic maturity is completed when the spindle fibers adequately align with the first PB.[13] Therefore, appropriate incubation timing would be required to achieve synchrony in maturation between the nucleus and cytoplasm that could eventually increase fertilisation and improve pregnancy rates.[9,10,13] Asynchrony between the maturation of the cytoplasm and nucleus can hinder the ability of fertilisation in an IVM cycle. Balakier *et al*. reported that human oocytes progressively develop to enable complete activation and normal development during the MII stage.[14]

Time lapse system (TLS) allows for the visualisation and imaging of embryo/oocyte growth whilst in incubation. It provides uninterrupted culture conditions, which includes temperature and gas exchange in order to attain optimal embryo/oocyte development.[15,16] Hence, it allows for the selection of embryos/oocytes based on developmental milestones compared to conventional incubators where embryos are usually selected through morphological assessment.[17,18] The technology of TLS is particularly advantageous as it provides real-time imaging in relation to time interval to detect oocyte maturation accurately.[19]

The aim of the study was to analyse optimal ICSI timing for *in vitro* matured oocytes after the first PB extrusion and their outcomes using a TLS.

Materials and Methods

Study population

This study was conducted in fertility centre in fertility center in Bangalore, India. The study uses a retrospective design, and patient's records were reviewed from January 2013 to May 2016. This study was approved by Gunasheela institutional ethics committee (IEC/0004/2019). Patients were recruited based on them signing an informed consent form outlining the procedure and outcomes. This study adheres to the ethical principles for medical research involving human subjects under the Helsinki Declaration. There were 27 patients diagnosed with polycystic ovary syndrome (PCOS) who attended the centre for infertility treatment by undergoing minimal stimulation and human chorionic gonadotropin (HCG) primed for IVM cycles. Patients were included in this study if they were ≤35 years of age, anti-Müllerian hormone levels were >6 ng/ml, antral follicle counts were >40 follicles in both ovaries combined and with no known male infertility. We included patients who yielded only GV on the day of pickup after denudation. Amongst the 27 patients recruited for this study, 328 GV oocytes were collected and underwent IVM.

Ovarian stimulation and oocyte retrieval

Patients started ovarian stimulation on day 3 of their menstrual cycle with low-dose gonadotrophins (375 IU, Gonal-F, Merck) (260IU, HMG, Ferring). Once the follicles reached 1–1.1 cm (identified by transvaginal ultrasound), rHCG trigger (250 µg, Ovitrelle, Merck) was administered 38 h before ovum pickup. Oocyte collection was performed under spinal anaesthesia using a transvaginal guided ultrasound. A 16 gauge single lumen needle (Cooks Medical®, Ovum Aspiration Single Lumen Needle) was used to aspirate follicles from the ovaries with the suction pressure maintained between 80 to 100 mmHg. The aspirated fluid was collected into a pre-warmed (37°C) 14 ml round-bottom tubes (Falcon, USA) containing 1 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Vitromed, Germany) medium. The fluid was later screened for cumulus-oocyte complexes (COCs) under stereomicroscope (SZ61TR, Olympus Corporation, Japan). The fluid was later filtered through a 70 µm nylon mesh cell strainer (Falcon, USA). The filtrate was rewashed using HEPES media and was rescreened for COCs.

Oocytes preparation and *in vitro* maturation

All the retrieved COCs were cultured in an oosafe four-well dish containing 0.5 ml IVM media (Sage, CooperSurgicals, USA) supplemented with 75 IU/ml follicle-stimulating hormone, luteinising hormone and epidermal growth factor in each well and were incubated at 37°C, with tri-gas (6% CO_2, 5% O_2, 89% N_2) in a benchtop incubator (Origio, Planer). Following 2–3 h from oocyte pickup, partial denudation (30%–40%

COCs were stripped) of oocytes for better visualisation of maturation occurred in two steps: (i) enzymatic treatment; the cumulus corona cells were removed by exposure to HEPES buffered human tubal fluid media (80 IU/ml hyaluronidase enzyme for 30 s) and (ii) mechanical treatment; further removal and wash of cumulus cells using 0.5 ml of HEPES media (Vitromed, Germany) were performed by using flexipets (Inner diameter 175 µm and 150 µm consecutively (Vitromed, Germany). Extra precaution (mechanical denudation) was taken to limit the complete removal of cumulus cells. Previous studies have shown that conservation of some COCs will aid in the maturation and growth of the oocytes.[20,21]

Oocyte culture under time lapse system

Oocyte maturation was detected using a TLS (Embryoscope™, Unisense FertiliTech, Aarhus, Denmark). The oocytes after the denudation process were transferred to a TLS slide (Embryoscope™ culture dish, Vitrolife, Denmark) which contained 25 µl IVM media in each well (Sage, CooperSurgical, USA) overlaid with 2 ml of paraffin oil (Vitromed, Germany), to prevent evaporation of the culture medium. The TLS slides were then transferred into the TLS incubator at 0 h post denudation. The TLS used in this study is a tri-gas incubator which can accommodate six slides (12 wells in each slide), with built-in microscope and camera to automatically acquire images of up to 72 individual embryos/oocytes during development. Morphological changes were monitored between maturation stages (GV→MI→MII) for 30 h. However, in the research group, we categorised the maturation timing into six-hourly intervals. The control cohort did not undergo time monitoring for maturation as they were not cultured in the TLS.

Intra-cytoplasmic sperm injection procedure

To account for the availability of embryologists to perform ICSI and to limit exposure time of oocytes, ICSI was planned during normal business h (9 am–6 pm) starting from the first observation of PB extrusion (MII oocytes). To investigate optimal ICSI timing post maturation, oocytes were injected at five different time intervals 1–2 h, 3–4 h, 5–6 h, 7–8 h and >8 h. ICSI was performed with a micromanipulator under an inverted microscope (Narishige™, Olympus IX71, Japan). Semen samples used in this procedure were normozoospermic according to the World Health Organisation 2010 guideline for normal semen parameters[22] and were processed using a density gradient method, following wash (sperm wash media, Vitromed) and swim up. Single sperm of good quality was immobilised and then injected (injecting and holding needles, TPC, CooperSurgical, USA) into the oocyte. The injected oocytes were later transferred back into the TLS, further cultured using the one-step culture media in a fresh culture slide (Vitromed, Germany).

Embryo culture

Monitoring continued for fertilisation and cleavage following the same time interval as the injection timing. Fertilisation was assessed 17–19 h post insemination by identification of two distinct pronuclei (PNs) and two PBs. Embryonic development was assessed daily according to the regularity (blastomere size and number), fragmentation and all dysmorphic characteristics of the blastomeres. Good embryos were considered for utilisation (either transfer or freezing) depending on the embryo grading according to Istanbul and Gardner consensus. Day 3 embryos were graded to be good when they contained more than six evenly sized blastomeres with <10% fragmentation (anucleated).[23,24] On day 5, blastocysts were graded based on the morphology of the inner cell mass, trophectoderm cells and the volume of expansion of the blastocoele cavity.

Power calculation

Power analysis was calculated using the following assumptions: alpha 0.05, 80% power, background fertilisation rate for the chosen group of patients is 81% to estimate the difference of 10% between each group, a total sample of 100 oocytes in each group have been recruited.

Statistical analysis

The data were entered into Microsoft Excel. Treatment groups were compared using a Chi-square test (or Fisher's exact test) as appropriate. A two-sided statistical test with a 5% level of significance ($P < 0.05$) was used for all analysis. All analysis were carried out in R-Software Version 4.0.2

Results

There were 328 immature (GV) oocytes retrieved from 27 female patients, of which 162 oocytes were incubated in the time lapse group and 162 oocytes were taken as control group. Four oocytes were discarded as they had a fractured zona pellucida.

Figure 1 and Table 1 describe the maturation stage and rate of oocytes from GV→MI→MII over a time interval of 6 h up to 30 h. We observed that there were only 82 oocytes which had matured from GV to MI between 0 and 6 h time interval. However, there were no oocytes that matured to MII stage in this time interval. At 12 h, it was observed that maturation of GV to MI oocytes reached its peak ($n = 102$) compared to other time intervals. In addition, there were five oocytes that had

also matured into MII stage within 12 h. Furthermore, at 18 h, there were 50 MI oocytes. It was also noted that thereafter, 57 oocytes matured from MI to MII stage, indicating the highest maturation point for this study. At 24 h, a decline in maturation in MI (n = 17) and MII (n = 36) oocytes was observed. Finally, at 30 h 25 oocytes remained arrested at the GV stage and 37 oocytes had arrested at MI stage. Hence, only two oocytes matured to MII stage.

In the control group amongst the 162 GV oocytes, only 110 oocytes had matured to MII stage up to 30 h, with 20 oocytes arresting at GV stage and 32 oocytes arresting at MI stage.

Table 2 presents the rate of fertilisation, cleavage and utilisation in relation to the timing to ICSI of MII oocytes. In the time lapse monitored group, ICSI was performed at various time intervals, namely 1–2 h, 3–4 h, 5–6 h, 7–8 h and >8 h of first PB release, whereas in the control group, ICSI was performed post 30 h of culture. There were 10 oocytes injected between 1 and 2 h time interval of which 4 (4%) oocytes fertilised and cleaved. However, none of these embryos were of good quality; hence, they were discarded. Thirty-three oocytes were injected between 3 and 4 h of maturation, out of which 27 (82%) were fertilised, 26 (96%) cleaved and 11 embryos (41%) were used. At 5–6 h, 32 oocytes were injected which yielded 27 (85%) fertilised zygotes, 25 (93%) cleaved embryo and 17 (63%) embryos were utilised. In addition, at 7–8 h, amongst the 16 injected oocytes, 11 (69%) fertilised and all cleaved (n = 11, 100%); however, only 6 (55%) were used. >8 h of maturation, 9 MII oocytes were injected, which accounted for 6 (67%) fertilised zygotes following 6 (100%) cleaved embryos, resulting in them all being utilised (n = 6, 100%). Statistical significance was observed between ICSI timing post maturation (hours) and total fertilised zygotes (P = 0.049). Furthermore, a statistical correlation was also observed between ICSI timing post maturation (hours) and embryo utilisation rate (P = 0.030), although no statistical significance was detected between ICSI timing post maturation (hours) and total embryos cleaved (P = 0.984).

Amongst the control group, 110 oocytes were injected post 30 h following IVM. We observed 89 (81%) oocytes that were fertilised, 86 cleaved (97%) resulted in 38 (43%) embryos, which were used [Table 2]. However, no statistical difference was observed for the control group (non-timed group) for fertilisation, cleavage and utilisation rate.

Table 1: Maturation rate of germinal vesicle post-denudation until 30 h, control group shows the final maturation post-30 h from ovum pickup in a cohort of polycystic ovary syndrome patients

Oocytes	Nuclear maturation timing (h)	Total number of oocytes		
		GV (n)	MI (n)	MII (n)
GV→ PB	0	162	0	0
	6	80	82	0
	12	55	102	5
	18	50	50	57
	24	47	17	36
	30	25	37	2
Control GV→ PB	30	20	32	110

GV=Germinal vesicle, PB=Polar body, MII=Metaphase II

Discussion

The results produced from this study indicate that nuclear maturation timing is best at 18 hours, yielding the highest numbers of MII oocytes. Son *et al*.[9,25] reported on maturation timing of IVM oocytes and suggested that

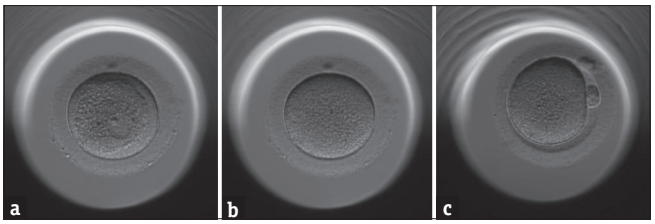

Figure 1: Stages of oocyte maturation during *in vitro* culture. (a) Germinal vesicle (GV); (b) Metaphase (M) I; (c) Metaphase (M) II

Table 2: Fertilisation and utilisation rate post-timed and control (30 h) *in vitro* maturation intra-cytoplasmic sperm injection (metaphase II) from germinal vesicle to the first polar body in a cohort of polycystic ovary syndrome patients

Oocytes	ICSI timing post-maturation (h)	Total MII oocytes (n)	Total fertilised, n (%) (P=0.049)	Total embryo cleaved, n (%) (P=0.984)	Utilisation rate, n (%)* (P=0.030)
GV→ PB	1-2	10	4 (40)	4 (100)	0
	3-4	33	27 (82)	26 (96)	11 (41)
	5-6	32	27 (84)	25 (93)	17 (63)
	7-8	16	11 (69)	11 (100)	6 (55)
	>8	9	6 (67)	6 (100)	6 (100)
Total		100	75 (75)	72 (96)	40 (53)
Control GV→ PB at 30 h		110	89 (81)	86 (97)	38 (43)

*P<0.05. GV=Germinal vesicle, PB=Polar body, ICSI=Intra-cytoplasmic sperm injection, MII=Metaphase II

amongst the 929 oocytes that matured, over a period of 48 h, 627 developed into MII oocytes on day 1 (within 24 h). The authors further showed a substantial decrease in MII oocytes (163 MII on day 2 within 48 h) following oocyte collection.[9,25] This is consistent with findings reported in this study, where the highest maturation yield occurred within 24 h. However, with the use of a TLS to monitor the time intervals in relation to the maturation process, we were able to provide specific time window (6 hourly intervals) for optimal maturation number. Our research group did not observe any MII maturation after 30 h of culture. We believe that a meiotic arrest had occurred at both prophase I (GV) and MI stages. The importance of establishing a narrow time window for maturation process is critical, as it assists in avoiding oocyte ageing. Aged oocytes have been reported to result in poor utilisation rates as they are more likely to be arrested for a prolonged period at MII stage and are morphologically identified with large peri-vitelline space, fragmented PB.[26] Fertilisation of aged oocytes can lead to a large pro nucleus (PN) with abnormal number of nucleolar precursor bodies, which can adversely affect embryo growth.[27,28]

Time-monitored ICSI, after first PB extrusion, allows for an assessment of the optimal time to inject a mature oocyte with sperm in order to produce a positive pregnancy outcome. When ICSI was performed at 5–6 h post PB extrusion, it yielded the highest utilisation rate of embryos. These results were confirmed by Son *et al.,*[9] where the authors also reported that ICSI should be conducted, after first PB extrusion of MI at 4–8 h in order to yield the best utilisation rate of 39% compared to 33% at <1 h. The literature also suggests that meiotic spindles do not arrange themselves to the PB at 1–2 h post-MII maturation, as the cytoplasm is not completely prepared for fertilisation.[9] Montag *et al*. further reported that meiotic spindle formation usually occurs after 2 h (~1.50h–2.30h) post-PB as this was shown amongst a cohort of 104 MII oocytes which were collected.[29] Due to our small sample size in the ICSI-timed group, we were unable to provide a rationale as to the observation of low fertilisation, cleavage and utilisation rates at this time interval. Interestingly, in our timed ICSI group at more than 8 h, highest cleavage and utilisation rates were (100%) observed compared to the other groups. Similar studies have suggested that prolonged incubation of MII oocytes can lead to aging, hence causing a reduction in embryo development and poorer fertility outcomes. Khazaei and Aghaz stated that prolonged oocyte culture without fertilisation can alter production of M-phase promoting factor and mitogen-activated protein kinase that lead to a decrease in calcium ion concentration and production of reactive oxygen species resulting in poor quality oocytes.[30]

Previous literature has suggested that cumulus cells help in the oocyte maturation and embryo development by allowing for nourishment through gap junctions.[31,32] Therefore, conservation of cumulus cells has been reported to be critical in oocyte maturation, as supported by Zhou *et al.* where the authors demonstrated that the conservation of cumulus cells in a mouse model increased oocyte maturation and fertilisation.[31] Similarly, Goud *et al.* also concluded that nuclear maturation and cleavage rates were also higher in the cumulus co-cultured group. Conversely, this study was unable to show a difference in partially denuded (time-monitored cohort) and cumulus conserved oocytes (control cohort) during maturation and fertilisation.[33] According to the authors, Zhang *et al*., Johnson *et al.* and Kim *et al*., oocytes cultured without cumulus cells were not found to alter maturation rates between MI and MII. We suspect a variation in study outcomes may be the result of period of *in vitro* culture, denudation timing, ICSI timing, laboratory protocols and stimulation protocols.[34-36] This study also recognises that IVM medium provides adequate supplements for sufficient nourishment as it contains essential reproductive hormones and growth factors for oocyte maturation and embryo development.[34-36]

Finding an optimal time window in order to perform ICSI for IVM oocytes is beneficial in order to achieve a positive fertility outcome (clinical pregnancy). Patients with PCOS and specific types of cancers (such as estrogen receptor-positive breast cancers) will require specialised stimulation protocols (minimal stimulation) so as to yield higher number of oocytes.[37] IVM would be an appropriate technique to be used for these patients. Furthermore, following the maturation process post-IVM, conventional *in vitro* fertilisation is not an option as these IVM oocytes undergo extended culture which will harden the zona pellucida.[9,38] ICSI is the only viable option for fertilisation of these oocytes. Currently, there is no published literature around specific timing of ICSI for *in vitro* matured oocytes. These findings may help embryologists to time their ICSI procedure in *in vitro* matured oocytes.

Strengths and limitations

The key strength of this study was the use of a TLS for monitoring the time of maturation of oocytes. This allowed for continuous and undisturbed culture conditions, which reduced exposure to time, leading to better outcomes. Several studies in this field have used conventional incubation techniques that may have led to greater exposure time, causing sub-optimal growth conditions.

Further, limitations included the inability to identify a key meiotic status under a conventional light microscope, which made it challenging to detect the complete maturation process of an oocyte. Hence, the use of a PolScope (computer-assisted polarisation microscopy system) would be considered the ideal technique to visualise spindle location and alignment of chromosomes.[9] It has been suggested in the literature that the success rate of ICSI would be enhanced with a precise injection site, which would avoid damage to spindle fibres.[39] In addition, the availability of an embryologist at our centre was limited to business hours (9 am–6 pm); therefore, round the clock monitoring of maturation with ICSI was not possible, causing any oocytes to be matured outside business hours to be injected later.

Conclusion

Timed ICSI post maturation is beneficial in increasing utilisation rate of embryos and potentially improving fertility outcomes (pregnancy rates). Our study shows that the best timing for maturation would be at 18 hours post denudation and the best timing for ICSI post-PB extrusion occurs at 5–6 h, yielding a higher utilisation rate. Many embryology labs do not have access to TLS as they are expensive to acquire and maintain. We believe further comparing our data through TLS to the conventional incubation process will help in standardising guidelines for maturation and ICSI timing in an IVM cycle. Future research would be required at a molecular level to fully explore oocyte maturation dynamics.

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Data availability statement

All data pertaining to this study are contained and presented in this article.

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Conflicts of interest

Devika Gunasheela is a member of the National Advisory Board of the Journal of Human Reproductive Sciences. She has had no role in the reveiw process or the editorial decisions. Other authors have no conflicts of interest.

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