

Optimal Timing of Oocyte Preincubation for Intra Cytoplasmic Sperm Injection (ICSI) in IVF Treatment.

shubhangi Kakade¹, amit Kamat², savita Datar³

¹Embryologist, Niramya IVF Center, Phd Student OfTilak Maharashtra Vidyapeeth,

²Director, Kamat Hospital,

³Head, Department Of Zoology, S.P. College.

-----ABSTRACT-----

IN Vitro Fertilization (IVF) i.e. fertilizing an oocyte with the sperm under in vitro condition is the most convincing option for treating infertility in the couples in which conception is not possible with conventional treatments. It is achieved either by co-culturing oocyte with sperms (conventional IVF) or by injecting single sperm in the cytoplasm of oocyte (Intra Cytoplasmic Sperm Injection - ICSI). The cultured embryos are then transferred from day2 to 4 (cleavage stage) or day 5 (blastocyst stage) in the uterus of the woman under treatment for implantation. The benefit of in vitro oocyte culture prior to insemination during conventional IVF has been demonstrated; however there are discrepancies about its advantage during ICSI procedure. We undertook this work to examine the effect of duration of pre-incubation on the rate of fertilization after ICSI. This work was carried out by making the retrospective analysis of data regarding oocyte pre incubation accumulated at Niramaya IVF Center during June 2010 to December 2015. ICSI cycles were categorized in to 5 different groups according to the duration of oocyte incubation period prior to ICSI as : Group I - oocytes not incubated, Group II - oocytes incubated between 1-3 hours, Group III- oocytes incubated between 3-5 hours, Group IV - oocytes incubated between 5-7 hours and Group V - oocytes incubated formore than 7 hours. It was observed that rate of fertilization varies with the duration of pre-incubation of oocyte prior to ICSI. We concluded that in vitro culture of oocyte for short duration prior to ICSI has beneficial impact on fertilization.

Keywords: Oocyte, Pre- incubation, IVF, ICSI.

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I. INTRODUCTION

Oocyte is a female gamete produced by process of oogenesis. Quality and developmental competence is acquired by this female gamete during folliculogenesis- i.e. growth and development of primordial follicle to Graafian follicle. As an oocyte grows and matures it acquires the ability to resume meiosis which was arrested at diplotene stage of meiosis I. In natural cycle it undergoes a process of nuclear maturation from Germinal Vesicle (GV) to Metaphase I (M I) and arrests in Metaphase II (M II) after a pre ovulatory surge of Luteinizing Hormone (LH), whereas, in the stimulated cycle, ovulation is achieved by Human Chorionic Gonadotropin (HCG) administration. In general, the oocyte reaches MI at 20h and M II at 35h after HCG injection. Oocytes with Complete nuclear and cytoplasmic maturation at MII stage are ready to receive spermatozoa and proceed for completion of meiosis, successfully undergo fertilization process and initiate the persistent embryonic development. Incapability of completion of any of these processes leads to infertility.

In Vitro Fertilization is a procedure designed to enhance likelihood of conception in couples for whom other fertility therapies have been unsuccessful or are not possible. It is a process by which an egg is fertilized by sperm outside the body. The process includes retrieval of oocytes from hormonally stimulated woman's ovary, fertilizing them individually under in vitro conditions with the sperms. Fertilized oocytes are then cultivated at standard culture conditions up to certain stage of the embryonic development. Embryos are then transferred in to the uterus for successful implantation.

After the birth of world's first in vitro fertilized baby, IVF accomplished a status of a principle option to treat most of the infertility causes and has led to a considerable amount of research and development in this field. Efforts have been taken to optimize each and every aspect of this treatment option. To achieve a desired result of IVF and ICSI mature oocytes are obtained by retrieving them at the time which is as close as possible to ovulation. However considerable variations have been observed in the time of ovulation over an interval of 32-40 hrs. after HCG injection. Hence in IVF treatment oocyte retrieval is usually scheduled 34-36 h after hCG injection[1].

In conventional IVF small aliquot of sperm suspension is placed close to oocyte in a small drop of culture medium. The sperm penetrates through the surrounding cumulus cells and binds to the zonapellucida. Concepts of in vitro culture of oocyte prior to insemination have been introduced by Trounson et. al. in 1982 during

conventional IVF [2]. Its positive effect on fertilization has been subsequently confirmed by other studies [3,4]. Sometimes fertilization may not occur even the gametes are placed in close vicinity due to various reasons e.g. low sperm count or poor sperm motility. In 1992 Palermo introduced the revolutionary technique called ICSI which enabled the use of sperm with poor motility as well as surgically obtained sperms to achieve fertilization [5, 6, 7, 8,9]. Over the period of time most of ICSI procedures have been standardized and there is uniformity in the protocols employed. Thus it has been optimized that to avoid spindle damage the oocyte injection site should be at 3 O'clock position when polar body is at 6 O'clock or 12 O'clock position. It is also now accepted that, along with the insertion of injection pipette through zonapellucida the slight suction of ooplasm is essential for successful ICSI[10]. Literature survey also shows agreement in opinion that only live spermatozoa are essential irrespective of sperm count, motility or morphology for successful fertilization during ICSI. [11-13]. However, the conflicting opinions do exist about the need and duration of oocyte in vitro incubation prior to ICSI for successful fertilization [14-22].The purpose of this study was to analyze retrospectively the impact of oocyte incubation prior to microinjection on the rate of fertilization after ICSI. Understanding of the optimal time of oocyte pre-incubation will be useful to carry out corrective measures to improve the results.

II. MATERIALS AND METHODS

Patients:

We carried out a systematic retrospective analysis of data generated at Niramaya IVF Center, Chinchwad, Pune, Maharashtra, India. ICSI cycles performed between January 2010 and December 2015 only, were included in this retrospective study. ICSI cycles with oocytes retrieved from female patients with age < 36 years and freshly ejaculated sperms only were included in the study. Cycles with female infertility due to (i) premature ovarian failure (FSH at Day 3 \geq 10IU/mL), (ii) poor responder i.e. retrieval of <4 oocytes, (iii) grade III or IV endometriosis or (iv) polycystic ovaries syndrome (ESHRE2003) were excluded. ICSI performed with surgically retrieved spermatozoa were also excluded.

ICSI cycles were categorized in to 5 different groups according to the duration of oocyte incubation period prior to ICSI as : Group I - oocytes not incubated, Group II - oocytes incubated between 1-3 hours, Group III- oocytes incubated between 3-5 hours, Group IV - oocytes incubated between 5-7 hours and Group V - oocytes incubated for more than 7 hours.

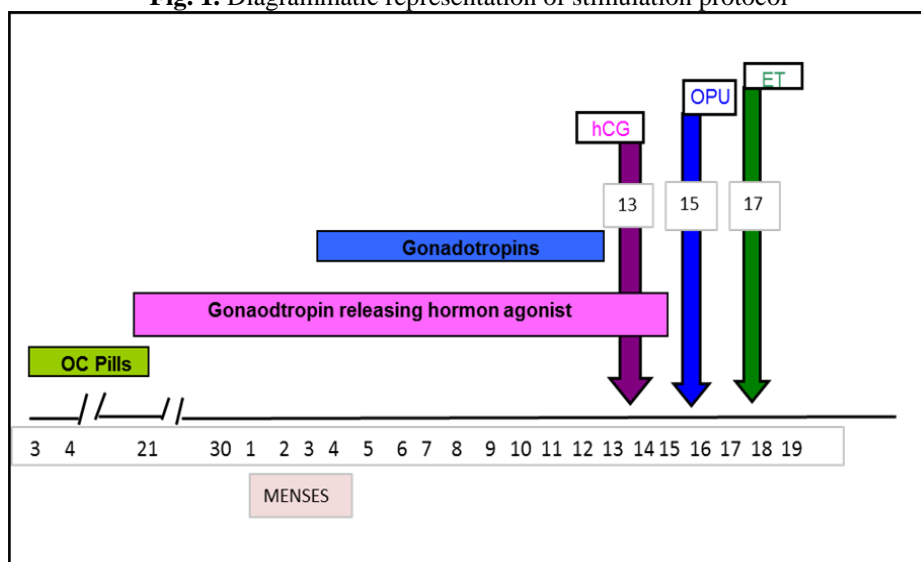
Ovarian stimulation

In all cycles, ovarian stimulation was carried out by previously established down regulation protocol using gonadotropin releasing hormone analogue (GnRHa) followed by injection of human menopausal gonadotropin. The patient received 0.5 mg of GnRHa - Buerelin acetate (Intas Pharmaceutical) by daily subcutaneous injection starting at the mid-luteal phase of the previous cycle. Reduction of GnRHa dosage to 0.1 mg daily and 2-3 ampoules of gonadotropin (IVF M, LG life science or Humog HP, Bharat serum) daily were started on Day 3 of the menstrual cycle. Gonadotropin dosage was adjusted accordingly by monitoring the follicles using trans-vaginal ultrasound. Ovulation was triggered by using recombinant hCG, Ovitrell 250 μ g (MerkSerono) when two leading follicles reached a mean diameter of 18 mm. Oocyte retrieval was performed by vaginal ultrasound 35 h after hCG administration. The OCC were recovered in Quinn's HEPES buffered medium supplemented with 5% human serum albumin(Fig. 1).

Oocyte preparation

The cumulus- oocyte complexes were pooled and incubated in Quinn's protein plus fertilization medium under oil at 37°C and an atmosphere containing 5% CO₂ until injection (Heraeus HERA cell). Oocytes denudation was performed using denuding pipette after enzymatic digestion of cumulus cells with 80 IU/mL Quinn's hyaluronidase. Denuded oocytes were transferred to the micro drop of Quinn's protein plus fertilization medium covered with oil. Maturation stage of denuded oocytes was checked (Fig.2). Only morphologically normal-appearing mature oocytes with a visible first polar body by the time of ICSI procedure were microinjected. During all the time of the current study, the ICSI conditions were identical (equipments, media and culture conditions).

Fig. 1. Diagrammatic representation of stimulation protocol



Semen Preparation

Semen samples were collected at the time of oocyte retrieval after 2–5 days of abstinence. The semen parameters were evaluated according to World Health Organization (1992) criteria for sperm concentration and motility by counting with a Makler chamber [23]. The sample was processed by swim up technique [24]. Briefly i) semen sample was mixed by double volume of Quinn's sperm wash medium in 5 ml test tube ii) centrifuged for 15 minutes at 1800 rpm iii) supernatant was discarded and pellet was overlaid with 0.5 ml of Quinn's sperm wash medium iv) tubes were incubated at 37°C in slanting position for 30 minutes. Hyper activated sperm separated from seminal plasma were counted using Makler Chamber and loaded in the central drop of Quinn's 7% PVP covered with oil in ICSI dish. The timing between the end of sperm preparation and the beginning of ICSI was between 30 minutes and 2 hours.

ICSI

Only MII oocytes and motile sperm with normal morphology were selected for ICSI. A micromanipulator and an inverted microscope (Olympus IX 70) with Hoffman modulation were used to perform ICSI by previously established method at 400x magnification. Briefly, oocytes to be injected were placed in Quinn's HEPES medium supplemented with human serum albumin. Sperm cell to be used for ICSI was immobilized by touching tail and aspirated by injection pipette. Sperm cell was injected in the oocyte cytoplasm at 3 O'clock position relative to polar body. After injection oocyte were placed in the 20 µl drop of Quinn's protein plus cleavage medium covered with oil in the culture dish and further cultivated at standard culture conditions.

Assessment of Fertilization

Injected oocytes were assessed at 17-19 hours after ICSI for fertilization check by observing under inverted phase contrast microscope (Olympus IX 70) at 400x magnification. Fertilization was confirmed by observing the formation of two pronuclei and two polar bodies. The Fertilization Rate (FR) was defined as the ratio between the number of diploid zygotes formed and the number of mature oocytes injected. A single, same operator participated to the gamete and embryo manipulations during this study.

Statistical Analysis.

Quantitative variables are reported as mean-plus or-minus standard deviation, while qualitative variables are reported as frequencies and percentages. To study the impact of the duration of preincubation on rate of fertilization, logistic regression model was used. Impact was considered significant if the corresponding P values were less than 0.05.

III. RESULTS

1. In every woman receiving ovarian stimulation multiple mature follicles were formed which were retrieved as close as possible to the time of ovulation. The retrieved oocyte cumulus complexes were transferred to the Petri dish containing drop of culture medium. Hyaluronidase digested the cumulus cells and denuded oocytes were formed. These oocytes were observed under the phase contrast microscope for maturity. Oocytes at MII stage were confirmed by the presence of first polar body (Fig.2).

2. A total of 100 ICSI cycles performed during December 2010 and June 2015 were included in this study. A total of 1030 oocytes cumulus complexes were obtained (10 ± 3.0 oocytes per retrieval) and were incubated for different durations prior to ICSI during these five years.

3. Total 639 MII Oocytes from five categories of ICSI cycles injected with sperms and incubated under standard conditions were observed under the phase contrast microscope for assessment of fertilization. Fertilized oocytes showing two pronuclear and two polar bodies were counted. Percentage of fertilization was calculated from the number of fertilized oocytes and unfertilized oocytes in the cohort. The FR of the oocytes incubated for different durations prior to ICSI was variable as shown in the Table 1.

4. Figure 3 graphically represents the co relation between duration of incubation time of oocyte prior to ICSI and the rate of fertilization. Group III oocytes, i.e.preincubationbetween 3 to 5 hours isan optimal duration for obtaining maximum fertilized oocytes.

Figure 2

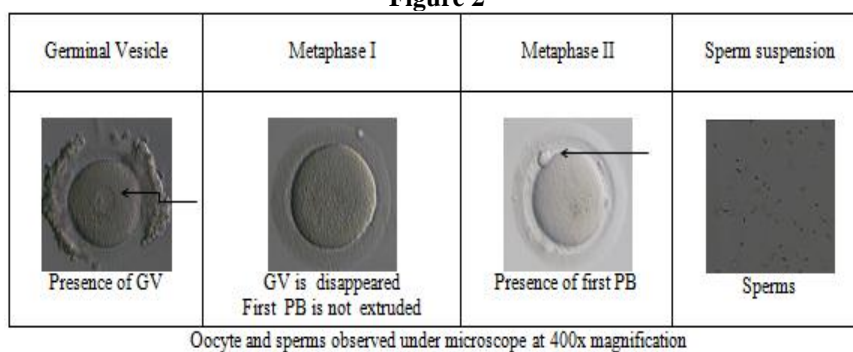
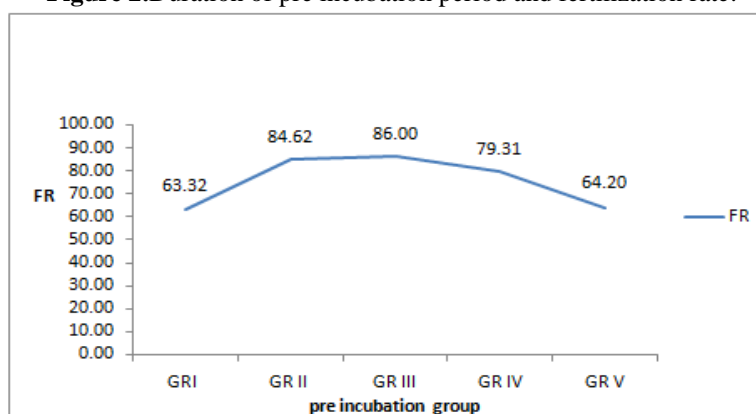


Table 1. Results of fertilization in ICSI cycles

	Pre incubation period	No of MII oocyte	No. of fertilized oocyte	Fertilization Rate
Group I	0-1hr	349	221	63%
Group II	1-3 hr	91	77	84%
Group III	3-5hr	100	86	86%
Group IV	5-7hr	58	46	79.31%
Group V	> 7	41	26	64.19%
Total		639	439	68.70%

Impact of pre incubation of oocyte on the rate of fertilization was analyzed. To study the impact of the duration of pre-incubation on rate of fertilization, logistic regression model was used. Impact was considered significant if the corresponding P values were less than 0.05.

Figure 2.Duration of pre incubation period and fertilization rate.



Total 100 ICSI cycles met the inclusion criterion. There was no statistical difference among groups regarding mean age of the women, doses of stimulation and number of oocyte retrieved. The overall fertilization rate is 69.79%. Group I the immediate injection group and group V in which injection was delayed by 7 hr had poor fertilization rate as compared to other 3 groups. Group III with pre-incubation of 3-5 h showed higher FR of 86%. Thus results demonstrate that irrespective of the maturity of oocyte the fertilization rate increases with incubation period up to 5hr and then gradually decreases beyond 5 hrs.

IV. DISCUSSION

For IVF treatment multiple follicles are recruited by exogenous hormonal stimulation. Follicular growth is monitored by ultrasound and the trigger shot of HCG is administered when at least two follicle reach to 18mm diameter. The follicles are aspirated for oocyte retrieval approximately at 35- 37 hr after HCG administration. As oocyte retrieval is scheduled prior to ovulation and follicles of variable sizes are aspirated at the same time, there is possibility that all oocytes may not be at optimum maturity. Oocyte maturity can be grossly estimated according to the appearance of CCC which may not be always precise. To compensate with this situation it is routine practice to delay the insemination several hours from 2-9h after collection of the oocyte in most IVF centers. This pre incubation time provides the time for maturation of slightly immature oocyte.

However for ICSI procedureremoval of surrounding CCC is essential to perform microinjection. This denudation enables to ascertain nuclear maturity which is characterized by extrusion of first polar body. The conflict about need of pre incubation arises during ICSI as only oocytes exhibiting first polar body are injected with spermatozoa.

It indicates that factors other than nuclear maturation appear to affect the fertilization ability of oocyte in ICSI. Nuclear maturation is estimated by appearance of first polar body however the cytoplasmic maturity cannot be evaluated by microscopic observations.

As cytoplasmic and nuclear maturation accrue independently (26-27), and may not be synchronous, all oocytes exhibiting polar body may not have mature cytoplasm. Such oocyte with immature cytoplasm requires additional time to mature completely and accomplish maximum potential of fertilization.

Pre incubation of oocytes prior to ICSI may induce cytoplasmic maturation that eventually increases fertilization and PR. However over a period of time stimulation protocol has been improved drastically. Also the advances in biotechnology especially in the field of pharmaceutical industries enabled availability of quality drugs. Patients who were treated with Controlled ovarian hyper stimulation protocol generate more oocyte. Additionally the HCG induced Oocytes are more mature and synchronous than those collected after spontaneous LH surge. Due to which Long Pre incubation time remained doubtful.

Prolonged incubation may result in i) in vitro oocyte aging ii) subsequent instability iii) Scattering of chromosomes iv) An accumulation of oxidative stress which induces the decrease in intracellular ATP, alters the glutathione/glutathione disulphidratio, damages the mitochondrial DNA causing disorder of cytoskeletal fiber. This affects fertilization and embryo development (28-30).

In spite of greater understanding of the nutritional and environmental requirement of oocytes and embryos, Impact of culture conditions on in vitro maturation process is not well understood. Therefore In vitro gametes and embryo handling is crucial step during IVF treatment. It is essential to optimize In vitro culture condition enough that it affects quality and developmental competence of gametes and embryos as little as possible. The short pre incubation time of 1-5h after oocyte retrieval seemed advantageous over immediate injection as well delayed injection for all sorts of pre-ovulatory oocytes. It provides time for mature oocyte to acclimatize with In Vitro Culture condition as well as for maturation of slightly immature oocytes. The increased fertilization rate enables the availability of multiple embryos for selection and transfer in IVF treatment. The originality of our study design was to separate the ICSI cycles on the basis of duration of pre-incubation of oocytes. When duration of pre-incubation was correlated with rate of fertilization, we could demonstrate that 3 to 5 hours is the optimal duration of pre-incubation which results in the improvement in the rate of fertilization. Nevertheless, standardization of exact time for pre-incubation of this optimal time between 3 to 5 hours at the respective IVF centers is essential to improve the rate of fertilization and eventually to decrease the need of multiple transfers for increasing the success rate of IVF treatment. Analysis of the same data for studies regarding implantation rate, pregnancy rate and rate of take home baby is being undertaken at Niramay IVF center.

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BIBLIOGRAPHY

Mrs. Shubhangi Kakade

Qualification : M.Sc. Bio-Technology,

Embryologist - Niramaya IVF center, Chinchwad, Pune, Maharashtra, India.

Aditya Birla Memorial Hospital, Pune, Maharashtra, India.

Pursuing PhD. from Tilak Maharashtra Vidyapeeth Pune, Maharashtra, India.

Mob- 9766983938.