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# THE DYNAMICS OF EARLY CLEAVAGE EMBRYO IN TWO OVARIAN STIMULATION PROTOCOLS

Medicine

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## ABSTRACT

**Aim and Objective:** The aim of this study was to evaluate the impact of the embryonic early cleavage status in GnRh agonist long protocol and GnRh antagonist protocol. **Materials and Methods:** A total of 294 patients who underwent Intra Cytoplasmic Sperm Injection (ICSI) were included in the study. They were divided in to two groups. Group I- Embryos which cleaved before 27 hours after injection. Group II- Embryos which cleaved after 27 hours, The effects of GnRh agonist and antagonist on embryo cleavage were compared between the two groups. **Results:** All the 294 patients were analysed. There was no difference in the mean age, duration of ovarian stimulation, number of oocytes retrieved, fertilization, cleavage rates and embryo quality between the three groups. Out of 169 patients in the agonist protocol the early cleavage was observed in 57.99% and late cleavage was observed in 42.01%. Out of 125 patients in the antagonist protocol the early cleavage was observed in 53.60% and late cleavage was observed in 46.40%. There was no significant difference in the stimulation protocol (p=0.454), in both the groups. **Conclusions:** The early cleavage was not affected by ovarian stimulation protocols.

## KEYWORDS

Agonist, antagonist, early cleavage, embryo quality, intracytoplasmic sperm injection, ovarian stimulation.

## INTRODUCTION

Success of IVF depends on retrieving an adequate number of oocytes to create good quality embryos for transfer, without exposing the patient to the risks of excessive ovarian stimulation.[1] In order to attain a maximal rate of implantation and reduce multiple pregnancies, selection of the most viable embryo for transfer has become a high priority in assisted conception treatment.[2] Embryo selection is a challenging task performed on the day of embryo transfer. Usually, the embryo selection is performed by using embryo morphology as the guideline.[3] To develop the embryo selection methods, various advanced technologies (metabolomics, proteomics, timelapse algorithm-based selection (TLM), preimplantation genetic testing for aneuploidy (PGT-A)) have been practiced as tools.[4,5] Recently an embryo respiration measuring system has been developed to assess embryo quality using Scanning Electrochemical Microscopy (SECM), which assays oxygen consumption in the embryo. Based on their respiration activity the embryos are selected and are predicted to have high implantation rates; however, the instruments and skills are needed to analyze respiration activities of the embryos. Monitoring the dynamics of the early cleavage (EC) has proven useful for the selection of best embryos without any specialized equipment.[6] It is of great significance to select the high-quality embryos to enhance the rates of implantation, pregnancy, and live birth and lower the abortion rate.[7] Identification to select the best embryos in human by using EC has been first reported by Edwards et al.[8] Several authors have found that early cleavage of the embryo, which occurs at 25–27 hours post insemination or injection, can be an additional indicator of viable embryos.[7,9-14]

### Ovarian stimulation

The first IVF cycles were carried out in natural unstimulated cycles.[15] Now gonadotrophins are given to induce ovarian stimulation.[16] Today four decades into the history of ART, ovarian stimulation consider as the single most efficient measure to increase the yields—implantation and pregnancy rates of ART.[17] Modern infertility practice provides us with several protocols in controlled ovarian hyper stimulation for the assisted reproductive techniques.[18] The Recent regimens are based on the administration of high doses of either urinary FSH or recombinant FSH (recFSH). The aim is to increase serum FSH levels above the threshold required for follicle development for a longer period, in order to facilitate the growth and maturation of not just single, but the complete cohort of follicles. In addition to FSH, LH may also be given, though LH has been found not to be absolutely essential for follicular development. Stimulation of the growth of several follicles leads to their production of supraphysiological serum oestradiol (OE2) levels, which by means of positive response at the pituitary may cause a premature LH peak and hence premature luteinisation and ovulation occurs. In order to prevent this, the administration of GNRH analogues is usually supplemented with exogenous gonadotrophin treatment.[19]

Majority of the previous studies were only using the gonadotrophin-releasing hormone **GnRH agonist** long protocol for pituitary suppression. Recently, a GnRH **antagonist** protocol has become existing in ART treatment.[20] Prevention of premature LH surge and the recruitment of a larger cohort of follicles are the major advantages of the agonist. The main disadvantages include the growth of ovarian cysts due to the stimulatory effects of the agonist, signs and symptoms of estrogen deficiency, including headaches, hot flushes, and vaginal dryness, and high costs due to increased requirement of gonadotrophin injections and increased need for hormonal and ultrasonographic measurements.

Since the late 1990s GnRH antagonists have been used in clinical practice for IVF. The main advantages of the antagonists include prevention of the adverse effects of agonists, such as ovarian cysts and estrogen deprivation, and potentially a more patient's friendly stimulation protocol.[21] The advantages of GnRH antagonist regimens are associated with reduce the amount of gonadotrophin needed for stimulation, a dramatic reduction in the duration of stimulation, a lower risk of developing severe ovarian hyperstimulation syndrome (OHSS[20], and a lower cancellation rate, especially in poor responders.[3,22]

### Effects of ovarian stimulation on embryology parameters

The goal of a 'good' stimulation is the one that produces a homogeneous cohort of mature oocytes, with the least inconvenience and risk to the patient, and achieve in the birth of a healthy singleton baby.From the Clinical embryologist's perspective, a good stimulation is the retrieval of well-expanded cumulus-oocyte complexes (COC) with a high proportion of metaphase II (MII) oocytes). On the other hand, a poor stimulation, due to sub-optimal decisions regarding timing or stimulation dose, is one that may end in a high rate of abnormal COC morphology, probably resulting in an increased rate of abnormal fertilization and an increased embryo aneuploidy rate. Aggressive ovarian stimulation affects the patient's wellbeing, by increasing the risk of OHSS, as well as on the endometrium and the ovaries. To find out whether there is a stimulation method that could yield a higher number of competent oocytes, first needs to consider the effects of LH and FSH as the principal drivers of ovarian stimulation, and their pharmacodynamics.[23]

The quality of oocytes and embryos is one of the most significant factors determining the success of an ART treatment. In order to

improve the effectiveness of the treatment, either more embryos will be transferred or a well-recognized stimulation protocol and embryo-selection procedure is practiced. In order to reduce the occurrence of multiple gestations, there is a need for transfer of less but more viable embryos. As ovarian stimulation protocol is one of the most eligible factors during an ART treatment, its embryo quality influencing effects are essential to know. The comparison of GnRH agonist vs. GnRH antagonist protocols has been well evaluated in clinical studies since 2000, most of them focused on the clinical outcome of the two protocols only. However the effects of the GnRH analogues on oocyte and embryo-quality and on embryo development are still not known in detail.[16]

In order to compensate for inefficiencies in ART procedures, patients undertake ovarian stimulation using high doses of exogenous gonadotrophins to get more oocytes in a single cycle. Recently it is evident that although ovarian stimulation has significant role in ART, it may also have detrimental effects on oogenesis, embryo quality, endometrial receptivity and perinatal outcomes. In this review, we examined the evidence for these effects and possible underlying mechanisms.[19] The aim of this study was to evaluate the impact of the embryonic early cleavage status in GnRh agonist long protocol and GnRh antagonist protocol.

## MATERIALS AND METHODS

It was a prospective observational study conducted in the Department of Reproductive Medicine & Surgery, at a tertiary care centre from Oct 2010-Dec 2013. A total of 294 patients who underwent ICSI were included in the study in the age group of 21-45 years.

**Inclusion criteria:** All patients enrolled for ICSI during this study period were included in the study. The patient had only early cleavage embryos and the patient had only late cleavage embryos for transfer were included in the study.

**Exclusion criteria:** Patient had both early and late cleavage embryos for transfer was excluded from the study. Patient age <21 and >45 yrs were excluded from the study. Short and ultra short protocols for stimulations were excluded from the study. Embryos beyond Grade III for transfer were excluded from the study. Informed consent was taken before the enrolment of each participant and the Institutional ethical committee of Sri Ramachandra Institute of Higher Education and Research (SRIHER) approval was obtained (IEC/10/JULY/83/29).

Two stimulation protocols were used in this study as per the institutional protocol; they were (i) agonist protocol to the patients with young age and good ovarian reserve and (ii) antagonist protocol to the patients with advanced age, poor ovarian reserve and Poly Cystic Ovarian Syndrome (PCOS).

**The gonadotropin-releasing hormone (GnRH) agonist protocol-** First, pituitary down regulation was done with GnRH agonists. Once the patient was down regulated completely (had menses, E2 <30 pg/ml, endometrium<5mm) gonadotropin injections (recombinant follicle stimulating hormone/human menopausal gonadotropin) were given until the day of h CG administration. The initial dose was individualized based on age and ovarian reserve markers. Subsequent doses were adjusted according to the patient's ovarian response.

**The GnRH antagonist protocol** - In this protocol, gonadotropin injections were administrated daily from the second day of the menstrual cycle till day of hCG when dominant follicle reaches 14 mm in mean diameter, GnRH antagonist was administered subcutaneously at a dose of 0.25 mgs daily until the day of h CG administration.

In both groups, oocyte maturation was induced by the administration of either recombinant hCG or urinary hCG or GnRH agonist, when at least two follicles reached 18 mm in diameter, and oocyte retrieval was performed 34-36 hours later. Oocytes were retrieved under transvaginal ultrasound guidance. Motile sperms were isolated by a swim-up or gradient centrifugation. Ejaculated, testicular sperm aspiration/ testicular sperm extraction, cryopreserved ejaculated and cryopreserved testicular sperm aspiration/testicular sperm extraction specimens. The ICSI was performed 2-3 h after oocyte aspiration with the prepared sperm. Normal fertilization was confirmed by the presence of two pronuclear and two polar bodies 16-20 h (day1) after the procedure. Normally fertilized oocytes (zygotes) were spherical and had two polar bodies and two pronuleus (PNs). PNs had approximately the same size, centrally positioned in the cytoplasm with two distinctly clear, visible membranes. The presence of nucleolar precursor bodies their number and size aligned at the PN junction were assessed. On the same day, early cleavage examination was performed on the zygotes at 27 hours. Embryos displaying two cells before 27 hours were designated as 'early cleavage' and those not yet cleaved to the 2-cell stage were designated as 'late cleavage'. Two or three embryos were transferred depending on the patient's age and embryo quality while the excess embryos were cryopreserved. Micronized progesterone was used for luteal phase support as per the Unit protocol. Pregnancy was confirmed by a serum β human Chorionic Gonodotropin (β h CG) test 14 days post transfer. The clinical pregnancy was confirmed by the presence of an intrauterine gestational sac with fetal cardiac activity by ultrasound examination at 4 weeks after embryo transfer.

## STATISTICAL ANALYSIS

The collected data was analysed with SPSS 16.0 version. To describe about the data, descriptive statistics frequency analysis, percentage analysis, mean and standard deviation were used. To find significant difference in the multivariate analysis, the one way ANOVA with Tukey's Post - Hoc test was used. For skewed data bivariate analysis Mann-Whitney U test was used. For multivariate analysis Kruskal Wallis test was used. To find the significance in categorical data Chi-Square test was used. In all the statistical tools, the probability value ($p<0.05$) was considered as statistically significant.

## RESULTS

Based on the cleavage characteristics the study population were divided into two groups.

Group I Early cleavage (EC) and Group II late cleavage (LC)

**Table 1: Baseline Characteristics**

| Parameters | Group I N=165 (%) | Group II N=129 (%) | p value |
|---|---|---|---|
| Age (Mean±SD) | 30.7±4.8 | 31.9±5.2 | #0.116 |
| Duration of infertility(Yrs) (Mean±SD) | 7.0±3.9 | 7.7±4.7 | #0.222 |
| Primary infertility-N=257 | 144(56.03) | 113(43.97) | #0.934 |
| Secondary infertility-N=37 | 21 (56.76) | 16 (43.24) | |

*p<0.05 is significant #p>0.05 is not significant

This table (table1) shows the base line characteristics and the type of cleavage among the study subjects. There was no significant difference in age (p=0.116), duration of infertility (p=0.222), type of infertility (p=0.934) in both the groups.

**Table 2: Stimulation Protocol**

| Protocol | Group I N=165(%) | Group II N=129(%) | p value |
|---|---|---|---|
| Agonist protocol-169 (%) | 98 (57.99) | 71(42.01) | #0.454 |
| Antagonist protocol-125 (%) | 67 (53.60) | 58 (46.40) | |

*p<0.05 is significant #p>0.05 is not significant

Out of 169 patients in the agonist protocol the early cleavage was observed in 57.99% and late cleavage was observed in 42.01%. Out of 125 patients in the antagonist protocol the early cleavage was observed in 53.60% and late cleavage was observed in 46.40%. There was no significant difference in the stimulation protocol (p=0.454), in both the groups.(table2)

**Table 3: Dose Of Gonodotropins-rFSH**

| Dose of gonodotropin rFSH(IU) | Group I N=165(%) | Group II N=129(%) | p value |
|---|---|---|---|
| <1500 | 1(0.61) | 0(0.00) | #0.716 |
| **1500-2500** | **42(25.45)** | **30(23.25)** | |
| **2501-3500** | **77(46.66)** | **58(44.96)** | |
| **3501-4500** | **40(24.24)** | **32(24.81)** | |
| 4501-5500 | 3(1.82) | 5(3.87) | |
| 5501-6500 | 1(0.61) | 3(2.33) | |
| 6501-7500 | 1(0.61) | 1(0.78) | |
| >7500 | 0(0.00) | 0(0.00) | |

*p<0.05 is significant #p>0.05 is not significant

Even though there was no statistically significant difference in the requirement of rFSH (p=0.716) between the groups, 72.11 % of group I required rFSH in the range of 1500 to 3500 IU whereas 69.77% of group II required rFSH in the range of 2501 to 4500 IU. So EC embryos are more when the dose of gonodotropins is less even though it is not statistically significant (table3).

**Table 4: Number of days of stimulation**

| Parameter | Group I (N=165) | Group II(N=129) | P value |
|---|---|---|---|
| No. of days of stimulation (Mean±SD) | **11.0±1.7** | **11.4±2.0** | #0.124 |

*p<0.05 is significant #p>0.05 is not significant

There was no significant difference in the number of days of stimulation between the two groups. (p=0.124)(table4).

**Table 5: Dose of Gonodotropins- r FSH+HMG**

| Dose of gonodotropin r FSH+HMG | Group I N=101(%) | Group II N=92(%) | p value |
|---|---|---|---|
| <1500 | 0(0.00) | 0(0.00) | #0.308 |
| 1500-2500 | 20(19.80) | 17(18.48) | |
| **2501-3500** | **52(51.48)** | **37(40.22)** | |
| **3501-4500** | **24(23.76)** | **27(29.35)** | |
| 4501-5500 | 3(2.98) | 7(7.61) | |
| 5501-6500 | 1(0.99) | 3(3.26) | |
| 6501-7500 | 1(0.99) | 0(0.00) | |
| >7500 | 0(0.00) | 1(1.08) | |

*p<0.05 is significant #p>0.05 is not significant

There was no significant difference in the dose of both rFSH and HMG required in both the groups (p=0.308). Even though there was no statistically significant difference in the requirement of both rFSH and HMG between the groups, 75.24 % of group I required both rFSH and HMG in the range of 2501 to 4500 IU whereas 69.57% of group II required both rFSH and HMG in the range of 2501 to 4500 IU. Only 4.96% required >4500IU of rFSH and HMG in group I whereas 11.95% in group II even though statistically not significant (table5).

The number of MII oocytes, good quality oocytes, oocytes fertilised and good quality embryos were significantly more with early cleavage group followed by late cleavage group (p<0.05).

**Table 6: Outcome of controlled ovarian stimulation**

| Parameters | | Group I N=165 | Group II N=129 | p value |
|---|---|---|---|---|
| Mature oocytes(Metaphase II) (Mean±SD) | | 13.28±6.5 | 7.98±5.6 | *0.005 |
| Quality of oocytes | Good N=194 | 151(77.84) | 43(22.16) | *0.005 |
| | Fair N=32 | 6(18.75) | 26(81.25) | |
| | Poor N=68 | 8(11.76) | 60(88.24) | |
| Quality of embryos | Grade I (Mean±SD) | 8.19±4.9 | 3.18±3.01 | *0.005 |
| Clinical pregnancy rate- N=171 | | 124/165(75.20) | 47/129(36.40) | *0.005 |

*p<0.05 is significant #p>0.05 is not significant

So good quality oocytes and embryos significantly influence the cleavage of embryos with more early cleavage embryos. 77.84% of good quality oocytes in early cleavage group, 22.16% in late cleavage groups. Good quality embryos in early cleavage group was more when compared to late cleavage group which was statistically significant **(p<0.05). (table6)**

**Table 7: Outcome of treatment**

| Parameter | Group I (N=165) | Group II(N=129) | P value |
|---|---|---|---|
| **Clinical pregnancy rate- N=171** | 124/165(75.20) | 47/129(36.40) | *0.005 |

So study population with early cleavage embryos had significantly better clinical pregnancy rate 75.20% when compared to late cleavage embryos 36.40 % (p<0.05) (table7)

## DISCUSSION

The success of ART treatment is influenced by many factors. Despite recent advances in assisted reproduction, the pregnancy and implantation rate is still remains low. To improve the efficacy of ART treatments, most IVF units transfer more than one embryo which results in increased rates of multiple pregnancies. To achieve a singleton pregnancy without affecting the implantation rate should be the primary goal in assisted reproduction treatment To increase pregnancy rates and reduce multiple pregnancy rates, there is a need for reliable embryo selection method for determining embryo potential.[24] Till now, embryonic morphology has been one of the most valuable tools to achieve this goal. Embryonic early-cleavage observed 25–27 h after insemination or injection has been suggested to be another available parameter for embryo selection.[22]

Stimulation protocols for recruitment of multiple healthy fertilizable oocytes for the purpose of in vitro fertilization (IVF) have been constantly evolving over the last 40 years.[21]

Despite the clinical outcomes of ovarian stimulation with either GnRH-agonist or GnRH-antagonist analogues for ART being well analysed, the effect of analogues on early cleavage embryo and embryo development is still not well known in detail. With this condition, the study was conducted to compare the efficacy of a multiple-dose GnRH antagonist protocol with that of the GnRH agonist long protocol with a view to early cleavage embryo quality, embryo development and ART treatment outcome.

In our study the mean age in early cleavage group was 30.7±4.8 and 31.9±5.2 in late cleavage group. This is similar to the study conducted by Jing Fu in which the average age was 27.0 to 34.2 years.[25] In our study there was no significant difference in duration of infertility in both the groups with the mean duration of infertility being 7.0±3.9 in group I and 7.7±4.7 in group II.This correlates with the study by Edessy et al in which the mean duration was 5.7yrs.[26] In fact, female age-related infertility is the most common cause of infertility today. Hence the ability to conceive naturally decreases from early 30s and women are rarely fertile beyond the age of 45 years.

In our study, out of 294 study population, 57.48% of women underwent agonist protocol and 42.52% were in the antagonist protocol. This was similar to the study conducted at Taiwan by Wen-Jui Yang et al, of the 534 patients; in their studies 62% underwent GnRH agonist long stimulation protocol for ovarian stimulation and GnRH antagonist protocol in 38%.[3]

The half-life of the GnRH antagonist reported by the manufacturer (Serono, Baxter Oncology GmbH, and Halle, Germany) is 30 hours, and the time period between the last dose of the GnRH antagonist to the early cleavage of embryo is about 80 to 90 hours. Subsequently, the GnRH antagonist may still have some effect on zygotes to delay the first mitosis. This could clarify why lower rates of EC were found in women stimulated with GnRH antagonist. Early-cleavage is a reliable in agonist protocols. But, in the following 20 to 40 hours, the potential detrimental effect on the developing embryo may be significantly diminished. This could be the reason for a lack of effect of the GnRH antagonist on pregnancy outcome. In fact, several studies have shown that there are no differences in pregnancy outcome between GnRH antagonist and agonist protocols.[3,27-29] Overall; we believe that by using a GnRH antagonist protocol for ovarian stimulation, there is a delay in the first mitosis of the zygote but later embryonic development is not affected since the short half-life of the GnRH antagonist.

Evaluation was carried out to find out whether the stimulation drugs affect the type of cleavage. We did not find any significant effect between the stimulation drugs and the type of cleavage among the study subjects. Even though there was no statistically significant difference in the requirement of rFSH (p=0.716) between the groups, (table3). In contrast, in a retrospective study by Meng-Ju Lee et al, they found the FSH dosage of 1969.09±1382.4 in the EC group and 2521.57±1261.8 in the LC group which was significant (p<0.05).[30]

There was no significant difference in the dose of both rFSH and HMG required in both the groups (p=0.308).(table5) But in the Laetitia Hesters et al study, they found the mean hMG required was 2617±755 in the EC group and 2656±787 in the LC group.[31] In our study, no of days of stimulation with a mean of 11.0±1.7 in EC group and 11.4±2.0 in LCgroup. There was no significant difference in the number of days

of stimulation between the two groups (p=0.124).(table 4) This correlates with Meng-Ju Lee et al study where they found 11.68±2.1 in EC group and 11.49±1.8 in LC group (p=0.81) which was not statistically significant.[30]

In our study the number of MII oocytes , good quality oocytes, oocytes fertilised and good quality embryos were significantly more with early cleavage group followed by late cleavage group (p <0.05).(table 6). So good quality oocytes and embryos significantly influence the cleavage of embryos. It could be due to an intrinsic factor with in oocyte. and higher metabolic fitness of the embryo. Higher densities of actin and chromatin in early cleavage embryos contribute significantly contributed to more efficient cell division and therefore, greater developmental competence. Factors that contribute to the superiority of early cleavers are still not clear. It has been hypothesized that it may be associated to cytoskeletal ultra structure of the embryos. This is because the cytoskeleton plays vital role in organelle transport, cell division, motility, and signalling.[32]

A finding from our study showed that the transfer of EC embryos resulted in a significantly higher clinical pregnancy rate (75.20%) than those with LC embryos (36.40%) (p<0.05) (table7) This correlates with Fancsovits et al study where they found significantly higher clinical pregnancy rate in EC embryos than with LC embryos (48.3% vs. 27.3%) (p<0.0045).[33] This was also similar to Edessy et al study, they found significantly higher clinical pregnancy rate in EC embryos than with LC embryos (48.30% vs. 21.88%) (p<0.005). [34]

The results of our study showed that, the mean numbers of MII oocytes, good quality oocytes good embryos, and clinical pregnancy rates were all comparable with those in the GnRH agonist group and antagonist protocol. We also observed that embryonic early-cleavage was a good predictor for early embryonic development. In the present study, we found that there was no difference in embryonic early-cleavage rate in using the GnRH antagonist protocol and agonist protocol.

## CONCLUSION

Early cleavage can be a new criterion for selecting embryos for transfer and therefore increasing successful pregnancy rates, excluding nonviable embryos, and reducing the number of transferred embryos. According to the studies available so far, most medications used in ART appear to be safe. The optimal stimulation protocol is one that maximizes the recruitment of fertilizable oocytes and minimizes the risks and is administered and monitored in an acceptable and friendly manner to the patient.

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