6. DISCUSSION

The success of IVF/ICSI treatment is influenced by many factors. Despite recent advances in assisted reproduction, the implantation rate is still relatively low. To increase pregnancy rates and reduce multiple pregnancy rates, there is a need for reliable embryo selection method for determining embryo potential.\textsuperscript{19}

In our study population of 403, the overall pregnancy was 57.82\%, of them 48.07\% had fresh embryo transfer and 51.93\% had frozen embryo transfer. The overall clinical pregnancy and implantation rate for both fresh and frozen embryo transfer was 55.83\% and 27.43 respectively. The study population with EC embryos had significantly better fertilization rate, implantation rate and clinical pregnancy rate (p<0.05). Our study revealed that transfer of EC led to significantly higher pregnancy rates as compared to LC, 78.2\% versus 38.8\%; (p<0.05). This was similar to studies by Tesarik et al., Ludin et al., Salumets et al., Fancsovits et al., Hammoud et al., Brezinova et al.\textsuperscript{139,113,155-157} The possibility that EC embryos might be a highly significant biological indicator of embryo growth potential, may predict IVF outcome was first proposed by Shoukir et al., and Sakkas et al.\textsuperscript{112,133}

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). 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).\textsuperscript{155} 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).\textsuperscript{137} Interestingly, in the study by
Lee et al. (2012), they found the clinical pregnancy rate was significantly higher for the EC group than the non-EC group for the IVF cycles (75 versus 37.5%, \( p<0.05 \)) In contrast it was not statistically higher for the ICSI cycles (68.2 versus 51.4 %, \( p=0.27 \)). This study varied from our study as the clinical pregnancy rate was not statistically significant for the ICSI cycles between the groups.\(^{158}\)

In our study there was a significant difference between the EC and LC groups with respect to implantation rates (\( p<0.05 \)). The implantation rate was 38.57% in the EC group and 16.82% in the LC group. This correlates with Fancsovits study where they found the implantation rate was 26.5% in the EC and 15.1% in the LC group.\(^{155}\) Similar to our study Lee et al. also found that the EC group had significantly higher implantation rates than the non-EC group in both the IVF (42.9 versus 19.7%, \( p<0.05 \)) and ICSI cycles (48.1 versus 24%, \( p<0.05 \)).\(^{158}\)

In our study a total of 5840 oocytes were retrieved, and were at different stages of maturation. Out of them 79.83% were matured M II oocytes. The mean oocytes retrieved per subject were 15\( \pm \)8 and the mean MII oocytes were 11\( \pm \)7. This was similar to the study conducted by Haydar Nadir Çiray et al at Turkey. In their study the mean oocytes retrieved were 14.4\( \pm \)8.1 and mean MII oocytes were 11.8\( \pm \)6.9.\(^{159}\)

In our study the mean number of oocytes injected were 11\( \pm \)7 and the mean number of oocytes fertilized were 9\( \pm \)6. This correlates with Haydar Nadir Çiray et al study in which the mean oocytes injected were 11.8\( \pm \)6.9 and mean fertilized oocytes were 8.6\( \pm \)5.7.\(^{159}\) In our study the fertilization was achieved in 80% of the oocytes injected. But in the Jing fu et al study the fertilization rate was slightly
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lower (67.7%).\textsuperscript{15} Our results showed significantly higher fertilization rates, seems to reflect a good quality oocyte.

In our study, we found early cleavage in 40.94 % in ICSI. This correlates with Meng-Ju Lee et al where they found 36.8 % in ICSI.\textsuperscript{158} Similar findings were studied by Edessy et al with 46.2% early cleavage in ICSI\textsuperscript{44} and Haydar Nadir Çiray et al, with 42.5% early cleavage in ICSI.\textsuperscript{159} But in Isiklar et al study they found slightly lower percentage of early cleavage in ICSI (25.6%).\textsuperscript{19}

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). So good quality oocytes and embryos significantly influence the cleavage of embryos with more number of early cleavage embryos.

However, most of the studies examined transfers in which two or more embryos were transferred, of them at least one embryo had shown early cleavage. This makes it difficult to conclude to which embryo the pregnancy can be attributed.\textsuperscript{137} In our study two or three embryos were transferred either all EC or LC embryos, which makes it possible to determine the relationship between early cleavage and pregnancy arising from one specific embryo category.

Although many factors influence the results of an IVF cycle (e.g. stimulation response, endometrial receptivity, oocyte maturity, culture conditions), embryo quality is regarded as one of the most important factors.\textsuperscript{15}
In our study majority (66.18 %) were grade I embryos. Of this EC group yielded about 76.25% and LC group yielded 23.75% of good quality embryos which was statistically significant (p<0.05). This also correlates with the study conducted by Jing Fu et al where they found that 52.5% had excellent quality embryos in EC group and 28.9% in the LC group (p<0.01). It can be the reason that the early cleaving embryos stem from oocytes in which cytoplasmic and nuclear maturation are better synchronized.

Similar result was observed in the study by Fancsovits et al, where they reported that the number of top quality embryos, regarding the number of cells and the morphology score, was significantly higher in the EC group (35.9%) than in the LC group of embryos (19.7%). It has been demonstrated that EC embryos have better morphology.

In our study most of the patients were in the age group of 26-35 years (67.25 %) and the average duration of infertility was 1-10 years. This is similar to the study conducted by Jing Fu in which the average age was 27.0 to 34.2 years and the duration was 1.6-7 years. In our study there was no significant difference in duration of infertility in both the groups (EC(group I) and LC(group II) with the mean duration of infertility being 7.4 yrs. This correlates with the study by Edessy et al in which the mean duration was 5.7yrs.

Traditionally before the latter part of 20th century, women were conceiving in their teens and twenties, when age-related abnormalities with the oocyte were not evident. However, in our modern era, women are delaying child birth until their thirties and forties, which has lead to the identification of the adverse effect of
advanced maternal age on oocyte function. Female age is important for conception. Increasing infertility rates with aging are well documented and apparent in our society. The real issue is oocyte quality and quantity—which translates in to embryo quality after fertilization. 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.

A finding from our study showed that at any given age group, there was no statistically significant (p>0.05) difference on the type of cleavage observed among the two groups. However, if the study subjects are divided in to two groups with 35 years as cut off, we observed statistically significant difference in the age distribution among these two groups, (p<0.05) with early cleavage rate of 60.68% in patients ≤35 yrs and 38.33% in patients >35yrs. This correlates with Windt et al study where younger age women had more EC embryos 110 and in Edessy et al study, where significantly more EC embryos were observed in younger patients (<30 years). 137

Lundin et al in their prospective study in Sweden confirmed that the EC and female age were shown to have positive correlation with pregnancy, 149 whereas Shoukir et al and Sakkas et al did not observe any correlation with age and EC. 112,133 However our results are consistent with the study by Edessy et al. 137 A possible reason why they did not observe the effect of age on the pregnancy rate was, that all patients were ≤ 35 years.
Logistic regression analysis was performed for early cleavage status and the female age. There were statistically no significant influence of age on early cleavage status (p=0.940) (figure-29).

In our study, primary infertility was the commonest (87.59%). Among them, 56.03% were EC group and 43.97% in LC group. This correlates with Shamila et al study where they observed primary infertility was dominant in south India. Of the secondary infertility, we found 56.76% had EC embryos and 43.24% had LC embryos, whereas in the study conducted at Shanghai by Jing fu et al between January 2006 and December 2008, they included the secondary infertility population only (51.35%) and found 51.5% had EC and 50.4% had LC.

In our study the female factor was the commonest indication (42.67%), followed by male factor (25.33%), both female and male factor (20.09%) and unexplained factor (11.91%). This correlates with a multicentric prospective study by Maria Jos et al where they observed the female factor was 45.7% followed by male factor 28.6% and unknown factor 25.7%. In contrast Laetitia Hesters et al observed the tubal factor (29.3%), male factor infertility (40.8%), endometriosis (9.0%), anovulatory (2.5%), or unexplained infertility (18.4%). This study varied from our study with male factor being the commonest.

Over the last few years it has become increasingly apparent that there are several lifestyle factors that influence fertility, such as stress, being overweight or underweight, and smoking. The increasing availability of high caloric western
diets, cheap processed sugars has driven an increase in obesity and may herald a further increase in incidence of infertility.\textsuperscript{164}

It was found that majority of men who were exposed to high temperature at their work place—welders, dyers, blast furnace workers, and those employed in cement and steel factories were more prone to infertility. This is due to excess environmental heat which increases the temperature of the scrotum, causing a negative effect on sperm production. The exact reason for the decline in semen quality is not clear, but it may be due to environmental, nutritional, socioeconomic or other unknown causes. Aging is an important factor responsible for the decline in semen quality.\textsuperscript{165}

In our study we found the mean FSH was $7.5 \pm 3.2 \text{ mIU/ml}$ and the mean AMH was $4.1 \pm 3.0 \text{ ng/ml}$. This correlates with Jing fu et al study where they also found mean FSH was $7.5 \pm 1.3 \text{ mIU/ml}$.\textsuperscript{15} In our study the mean AFC was $13.7 \pm 7.1$. But in Laetitia Hesters et al study they found the mean AFC was $18.6 \pm 8.6$.\textsuperscript{120} Traditionally, good quality embryos are identified on the basis of morphological findings and it has been suggested that AMH may reflect embryo morphology.\textsuperscript{166} Majumder et al study showed that when serum AMH concentration on the day of HCG administration was higher than $2.7 \text{ ng/mL}$, good quality of oocytes were always present, which might in turn result in higher implantation and pregnancy rates. Serum AMH and AFC on Day 3 have been shown to be correlated with the number of good quality embryos.\textsuperscript{167}

Among numerous factors affecting embryo quality, ovarian stimulation is an eligible and adjustable one. Despite the established clinical impact of different
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stimulation protocols, analysis of ovarian stimulation on quality of oocytes and developing embryos is not well known yet.

In our study, out of 403 study population, 56.33% of women underwent agonist protocol and 43.67% 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%.\textsuperscript{168}

In our study neither the type of infertility nor the stimulation protocol adopted showed any effect on the cleavage. This correlates with the study done at Hungary by Akos Murber et al, they showed that the rate of mature oocytes, rate of multinucleated blastomeres, amount of fragmentation and rate of early-cleaved embryos were similar in the two stimulation protocol groups.\textsuperscript{169} The same finding was reported by Rabinson et al, who demonstrated that the use of GnRH agonists or antagonists had no significant effect on the EC rate of human embryos.\textsuperscript{170} But in contrast, Wen-Jui Yang et al in their study at Australia, observed in women >35 years, the EC embryos were significantly lower in GnRH antagonist group than in agonist group.\textsuperscript{171}

In this study, there was a positive correlation $r=0.039$ which was not statistically significant between female age and early cleavage status ($p=0.701$) (figure-30) in the agonist group. In the antagonist group, there was a negative correlation $r=-0.100$ which was not statistically significant between female age and early cleavage status ($p=0.419$) (figure-31). But Wen-Jui Yang et al in 2015,
examine the relationship between embryonic early cleavage status and age of women, a logistic regression analysis was performed. In the agonist group, they found there was a significant correlation between age and early-cleavage status (p<0.001). They also found a negative correlation was noted between embryonic early-cleavage status and the age of patients with coefficients (R2) of 0.49 receiving a GnRH agonist protocol for controlled ovarian stimulation. In the antagonist group, there was no significant correlation (p=0.61) between embryonic early-cleavage status and the age of patients and showed low coefficients (R2) of 0.01.\(^\text{172}\)

Theoretically, if IGF, EGF, and the MAP kinase pathway are inhibited by GnRH antagonists, first mitosis of the zygote (early cleavage) could be retarded. In the murine model, Raga et al showed the presence of the GnRH receptor mRNA in the developing embryo and preimplantation embryonic development were significantly decreased by GnRH antagonists. This detrimental effect of the GnRH antagonist on preimplantation embryonic development was a specific receptor-mediated effect, rather than a toxic effect.\(^\text{173}\)

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 zygotes 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 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.\textsuperscript{174-176} 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.

In our study 46.15\% of the patients required r FSH dose in the range of 2501-3500 IU and a mean dose of 3120.3±924.2. Also 47.60\% of the patients required both r FSH and HMG dose in the range of 2501-3500 IU and a mean dose of 3257.7±996.2. This correlates with Laetitia Hesters et al study where they found the mean hMG required was 2637±771.\textsuperscript{120} Similar findings were observed by Jing fu et al study where the mean gonadotropin dose required was 2492±772.5.\textsuperscript{15}

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 between the groups, 72.11\% of group I required rFSH in the range of 1500 - 3500 IU whereas 69.77\% of group II required rFSH in the range of 2501 - 4500 IU. So EC embryos are more when the dose of gonadotropins are less even though it is not statistically significant. 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).\textsuperscript{158}

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 2501-4500 IU whereas 69.57% of group II required both rFSH and HMG in the range of 2501-4500 IU. Only 4.96% required >4500IU of rFSH and HMG in group I whereas 11.95% in group II even though statistically not significant. 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.\textsuperscript{120}

In our study, the majority of them required 10-12 days of stimulation with a mean of 11.2±1.9. There was no significant difference in the number of days of stimulation between the two groups (p=0.124). This correlates with Meng-Ju Lee et al study where they found 11.68±2.1 in EC group and 11.49±1.8 (p=0.81) which was not statistically significant.\textsuperscript{158}

In our study there was no significant difference in the ovulation trigger between the two groups. So the drugs used for ovulation trigger did not influence the cleavage of embryos.

The insemination techniques used by Maria Jos et al were IVF in 7.2%, ICSI in 77.8%, and mixed IVF/ICSI in 14.3%.\textsuperscript{163} But ICSI was the only method of insemination technique in our study for uniformity in methodology. Possible reasons why the use of ICSI has increased nowadays is declining sperm quality,
Early cleavage of human embryos to the two-cell stage: A simple, effective indicator of implantation and pregnancy in intracytoplasmic sperm injection. The use of ICSI as primary prevention of fertilization failures in couples without a clear male factor infertility. The embryos derived after ICSI had a higher rate of early cleavage than those derived after conventional IVF. The explanation of this phenomenon might be that the injected spermatozoa bypass many of the fertilization steps, resulting in a shorter fertilization time.

Van Royen et al also showed that routine use of early cleavage rather than assessment of embryos on the day of transfer improves overall pregnancy rates. This implies that pre selection of embryos for transfer at the early 2-cell stage is superior to relying on assessment at the time of transfer.

Timing of the first cleavage can be easily observed by light microscopy. Thus, it becomes the centre of interest in the development of more effective embryo selection protocols in human IVF treatment.

Time-lapse monitoring is another tool to identify the embryo(s) with the highest implantation potential. With this technology, the embryos can be monitored without removing them from the incubator. This type of monitoring allows for the collection of much more information on the timing of the cleavages and the dynamics of the morphologic changes, whereby human handling can be minimized and keep air, temperature and gas compositions are stable, and embryos are not exposed to bench-top light microscopy.

The extensive light exposure in the time-lapse may be detrimental to embryo development, and especially that short wavelength light exposure should be minimized (Oh et al., 2007; Ottosen et al., 2007a,b; Takenaka et al., 2007). If
the time-lapse solution incorporates moving parts or moving of embryos, then heat accumulation due to motion and friction may theoretically be an issue along with sheer stress to the embryos and the presence of lubricants and fumes from lubricants. The complexity of and access to maintenance, risk of pollution with infectious agents and consequences of potential breakdowns must also be considered. Moreover, the continuous presence of electromagnetic fields found in some time-lapse systems can affect embryo development (Cameron et al., 1985; Beraldi et al., 2003). Stability of the culture conditions that can vary between different designs of time-lapse instruments. This is also another important factor that must be considered.\(^{21,136}\)

One advantage of embryo selection based on the timing of first cleavage is that this is a clearly visible event, whereas pronuclear morphology and cleavage embryo morphology may vary during the dynamic process of syngamy. The reason why early cleavage yields better quality embryos and higher pregnancy rates is unknown, but it may be speculated that such zygotes derive from oocytes with adequately synchronized cytoplasmic and nuclear maturation.\(^{111,137}\) In our study, we found that early cleavage assessment is effective in selecting viable embryos for transfer. Both pregnancy and implantation rates significantly increased in relation to the number of early cleaving embryos available for transfer.
6.1 STRENGTHS

- Study design - Prospective observational study with a sample size of 403 patients.
- There are no Indian studies until now.
- It is not time consuming and do not impair the viability of the embryos.
- No special equipment is needed.
- Can be easily observed by light microscopy.
- Clearly visible event
- No inter observer variability.
- Cost effective.
- Easy, simple, and more efficient method of viable embryo selection.
- Minimal risk of technique or selection bias.
- Early cleavage embryo selection is a significant predictor of both pregnancy and implantation.
6.2 LIMITATIONS

• Single centre study: Couples attending the outpatient unit of the Department of Reproductive Medicine and Surgery at Sri Ramachandra University only were included.

• Only Intra Cytoplasmic Sperm Injection (ICSI) was taken as the method of insemination.

• Need multicentric study with more number of patients including both IVF & ICSI methods.

• Large sample size required to extrapolate to the entire Indian population.