2. REVIEW OF LITERATURE

2.1 ASSISTED REPRODUCTIVE TECHNOLOGY

WHO defines Assisted Reproductive Technology as infertility treatment handling both eggs (oocytes) and sperms to achieve a live birth. In conventional ART procedure, the oocytes and sperms are combined in a laboratory in vitro and the resultant embryo is transferred in to the uterus of the female.

ART includes IVF and ICSI. Originally, IVF was developed as a medical technology to overcome female infertility. In 1992, the introduction of ICSI significantly helped men with very low sperm counts, who could not be treated previously by standard IVF procedures.20

IVF is one of the fastest developing fields in medicine. The first birth following successful treatment was reported in 1978.21,22 According to a report in 2014, over one million treatment cycles have been performed worldwide in 2005, and, more than 250,000 children born were conceived by IVF.23 As of now, it has been estimated that more than 5 million children have been born as a result of IVF.21

2.1.1 IVF

IVF is the original ‘test-tube baby’ technique, and probably the most commonly practiced assisted-conception procedure in the world. In simple terms, IVF includes retrieval of several oocytes from the ovary, fertilizing them in the
laboratory with sperms from the male partner and transferring a few resulting embryos into the uterus for implantation and pregnancy.

2.1.2 ICSI

ICSI is the most effective assisted reproductive procedure achieving fertilization in severe forms of male factor indications and sperm dysfunction. Even men with a zero sperm count may produce sperms in their testes that can be retrieved and used in ICSI.

Figure 1: Intra Cytoplasmic Sperm Injection (ICSI)

The ICSI procedure is based on micromanipulation of oocytes and spermatozoa. Initially, Partial Zona Dissection (PZD) was established to facilitate sperm penetration. The barrier to fertilization represented by the zona pellucida was disrupted mechanically to allow the inseminated sperm cells, a direct access to
the perivitelline space of the oocyte. Sub zonal Insemination (SUZI) was the next step in micromanipulation techniques. SUZI enabled the immediate delivery of motile sperm cells into the perivitelline space by means of an injection pipette. ICSI is even more invasive because a single spermatozoon is directly injected into the ooplasm, thereby crossing not only the zona pellucida but also the oolemma.

The first human pregnancy and birth resulting from this novel assisted fertilization procedure was reported in 1992 by Palermo and colleagues. Thereafter, ICSI was found to be superior in terms of oocyte fertilization rate, number of embryos produced, and embryo implantation rate in male factor infertility. As a result, ICSI has been used successfully worldwide to treat infertility due to severe oligoasthenoteratozoospermia or azoospermia caused by impaired testicular function or obstructed ejaculatory ducts.  

2.1.3 Factors affecting the success rate in IVF/ICSI

1. Age of the partner
2. Oocyte quality and quantity
3. Sperm quality
4. Embryo developmental rates, embryo quality,
5. Endometrial receptivity

Age of the female partner of more than 45 is a contra indication for ART. Sometimes in young women, we do find low ovarian reserve due to certain pathology. Oocyte quality and quantity does affect the outcome. Similarly, the sperm quality is assessed and the couple is counselled regarding their chances of
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success in conception. Age, ovarian reserve, oocyte quality, sperm quality, underlying pathology are factors which cannot be completely standardized. Skills of an embryologist and IVF lab quality can be stringently standardized.

2.1.4 Follicle aspiration and oocyte handling

Transvaginal ultrasound guided oocyte retrieval is performed 34-36 hours after hCG injection, when the oocytes are fully mature just prior to rupture of the follicle. The procedure of oocyte retrieval is performed under short general anesthesia and ultrasound guidance. The follicular fluid is carefully screened for oocyte-cumulus complexes (OCCs) under low power magnification using stereo zoom microscope.

Figure 2: Follicle aspiration

![Figure 2: Follicle aspiration](image)
2.2 ASSESSMENT OF OOCYTE

The oocyte is the largest cell of our body measuring up to 120 – 160 µm. The oocyte comprises of double layered zona pellucida (ZP) which is an acellular matrix composed of sulfated glycoproteins. It has been demonstrated that the ZP plays a distinct role during fertilization and embryo development. The main function of ZP is however to prevent polyspermy and to protect the integrity of the embryo.24

2.2.1 Oocyte maturation

Oocyte quality is characterized by interrelated factors and usually classified as nuclear and cytoplasmic competence. Nuclear competency determines the quality of oocyte chromatin and spindle, which are essentially governed by the level of metaphase promoting factor (MPF) activity and mitogen activated protein (MAP) kinase. Cytoplasmic changes occur to make sure ideal fertilization and to support embryonic development.27, 28 Oocyte maturation is a complex process including changes in nuclear, cytoplasmic, structural, biochemical and functional states.29-31

2.2.2 Nuclear maturation

The primary oocyte, inside the Graafian follicle and developing under the effect of FSH, progresses from the first meiotic arrest (Prophase I) under the influence of Luteinizing Hormone (LH) to the second meiotic division to be arrested at metaphase II, which is finally overcome by fertilization.32
Primary oocytes arrested at the first metaphase stage are known as Metaphase I Oocyte (M I oocytes). Within 2-2.5 hours following prophase, the first meiotic division is completed with anaphase and telophase and the first polar body is extruded to form the secondary oocytes or Metaphase II Oocyte (M II oocyte). The MII oocytes are inseminated or used for ICSI in ART cycles. After fertilization (16-18 hours of insemination or ICSI), the secondary oocyte completes the second meiotic division and the second polar body is extruded.\textsuperscript{33}

2.2.3 Cytoplasmic maturation

The cytoplasmic changes for e.g. synthesis and migration of cortical granules towards the oolemma, is a crucial step in the chemical hardening of the zona to prevent polyspermy or fertilization by multiple sperm.\textsuperscript{28,33,34} One of the changes during cytoplasmic maturation is the reorganization of cell organelles. This cytoplasmic maturation facilitates the decondensation of sperm chromatin following the sperm penetration.

2.2.4 Oocyte maturity and type according to oocyte-cumulus complex

Metaphase II

Pre-Ovulatory

It is the best stage for fertilization, the cumulus is expanded and radiating, corona cells are still opposed to the oocyte.

- Very mature

The cumulus is very profuse but still present, few coronal cells are still visible but dissociated from the oocyte and the oocyte is pale.
• **Luteinized**

There is no cumulus and the oocytes are pale and difficult to visualise. These oocytes show low fertilization rate.

• **Atretic**

The granulosa cells are fragmented and the oocyte is very dark and difficult to visualise. These oocytes show no fertilization.

**Metaphase I (M I) (Germinal vesicle breakdown)**

There is tight cumulus measuring about five times the oocyte diameter and a tight layer of corona cells surround the oocyte. *In vitro* maturation (IVM) can be applied to these oocytes before fertilizing them.

**Germinal vesicle (GV)**

It is a very immature oocyte with highly tight surrounding cumulus. IVM can be applied to these oocytes before fertilizing them.  

**Figure 3: Oocytes maturity and type according to oocyte-cumulus complex**

- An expanded Cumulus Corona cell Complex surrounds the oocyte
- Tight cumulus measuring about five times the egg diameter is seen, and tight layer of corona cells surrounds the oocyte.
- A very immature oocyte with highly tight surrounding cumulus
2.2.5 Oocyte maturity and grade after denuding

Following oocyte denudation, oocyte assessment is more accurate and is based on the nuclear maturation status, the morphology of the cytoplasm, and the appearance of the extracytoplasmic structures.

**Germinal Vesicle oocyte** is characterized by the presence of a large nucleus in its cytoplasm.

**Metaphase I oocyte** is characterized by the breakdown of the GV and its absence in the cytoplasm. Absence of the polar body indicates that the oocyte is M I stage.

**Metaphase II oocyte** is characterized by the presence of the first polar body (PBI), which contains half the number of oocyte chromosomes.\(^\text{35}\)

**Figure 4: Oocyte maturity and grade after denuding**

![Metaphase II oocyte](image1)

![Metaphase I oocyte](image2)

![Germinal Vesicle oocyte](image3)
2.2.6 Oocyte morphology

A mature, healthy metaphase II (MII) oocyte is spherical having a diameter of about 120 µm. Rarely giant oocytes of more than 200 µm diameter with multiple polar bodies (PBs) are observed which are generally associated with reduced viability. Most of the giant oocytes are reported to be associated with aneuploidy.36

**Figure 5: Oocyte morphology**

Oocyte before denudation

Denuded MII oocyte; an intact PBI is clearly visible in the PVS

The polar body

Human chorionic gonadotropin or the luteinizing hormone (LH) surge resumes meiosis, resulting in extrusion of the first PB. The second PB separates only after fertilization or activation. A healthy oocyte has spherical or oval PB. Occasionally, large PBs are observed and if inseminated increases the risk
of aneuploidy. The presence of fragmented PB indicates post maturation of the oocyte.

**Zona pellucida**

The zona pellucida (ZP) is a matrix of three intermediate filament proteins-ZP1, ZP2 and ZP3, laid down during folliculogenesis. The ZP is a dynamic multifunctional structure at different stages of embryogenesis. At oocyte stage, it prevents polyspermy; at cleavage stage, it promotes selective permeability to maintain optimum concentration of nutrients, and at blastocyst stage it thins out to facilitate hatching. A healthy zona is about 16-18µm in width with a smooth, spongy and flexible texture.\(^\text{37}\)

**Cytoplasm and cell organelles**

A healthy oocyte displays translucent cytoplasm with evenly distributed fine granularity. It contains mitochondria, peroxisomes, endoplasmic reticulum, Golgi complex, vacuoles and other organelles.

**Meiotic spindle**

It is not possible to view meiotic spindle with commonly used contrasting system like Hofman modulation or Differential Interference Contrast (DIC). However, they can be visualized using polarized microscopy due to special property of macromolecular tubules of spindle called anisotropy. These spindles are generally placed near the first PB.\(^\text{38}\)
Perivitelline space

It is the space between the oolemma and zona pellucida. Normally the gap is almost invisible throughout the contact area, except near polar body (PB). The oocyte quality is influenced by the stimulation protocol, e.g; exposure to high dose of human menopausal gonodotropin has been shown to be associated with granularity of the perivitelline space.\textsuperscript{39}

2.2.7 Oocyte dysmorphism

Around 3\% of the oocytes retrieved even though are mature, may display certain variations from the normal morphology. The oocytes with absent or ruptured zonae, empty zonae or severely vacuolated should be discarded.

Following are a few oocyte dysmorphism that are seen within the population of the oocytes retrieved.

Zona pellucida anomaly

This includes distorted, elliptical, thinner or thicker ZP than usual.\textsuperscript{40-44}

First polar body anomaly

First PB anomalies include fragmented, elongated, flattened, very large or small size PB.\textsuperscript{45-48}

Ooplasm anomalies

They are non uniform granularity, aggregation of smooth endoplasmic reticulum, centrally dark, refractile bodies, vacuoles, retracted ooplasm.\textsuperscript{43, 44, 49-51}
The distorted oocytes have been associated with higher incidence of aneuploidy\textsuperscript{52, 53} and also indicate reduction in the fertilization and implantation rates.\textsuperscript{54}

**Vacuoles**

Some of the oocytes may display circular cytoplasm inclusions either single or clusters. These large vacuoles (>6\(\mu\)m diameter) are associated with fertilization failure.\textsuperscript{55}

**Refractile bodies**

These are dense, insoluble protein bodies that are produced within the cells presumably as a result of accumulation of lipofuscin. They are called refractile bodies because their greater density (than the rest of the cells’s body mass) causes light to be refracted (bent) when it is passed through them causing appearance of very bright and dark areas around them which are visible under a microscope. It is observed that refractile bodies are the single largest cause of failed fertilization even after ICSI.

**Smooth endoplasmic reticulum**

Smooth endoplasmic reticulum (SER) is an interrelated network of membranes without ribosome. SER is seen only in MII oocytes and is generally associated with high estradiol (E2) levels. Smooth endoplasmic reticulum aggregation may be associated with early fetal demise and in newborns, with certain imprinting disorders (e.g. Beckwith-Wiedemann Syndrome).\textsuperscript{56}
Oocytes are one of the most essential entities in any ART program as they hold key factors for normal development of the embryo. Their limited number, sensitivity to the temperature fluctuations as well as the adverse effects on their morphology correlating to the advanced maternal age has made their culture, challenging aspects to embryologist around the world.
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Figure 6: Oocyte dysmorphism

(A) Diffuse cytoplasmic granularity,
(B) Centrally located cytoplasmic granular area,
(C) Smooth endoplasmic reticulum clusters,
(D) Vacuoles,
(E) Abnormal zona pellucida shape,
(F) Large perivitelline space with fragments.

(G) Large polar body
(H) Giant polar body
(I) Large refractile body
(J) Fragmented polar body
(K) Dark egg
(L) Thick zona
2.2.8 Oocyte grading

After retrieval, oocytes are graded as follows:

**Grade I**

These oocytes have not undergone germinal vesicle breakdown, i.e. these are still diploid. The Germinal vesicle is distinctly visible following denudation. A very immature oocyte with highly tight corona. These oocytes are generally retrieved from small follicles of less than 10 mm size and they need to be matured in vitro before insemination.

**Grade II**

These oocytes reveal germinal vesicle breakdown but the polar body is yet to be extruded. The corona and the cumulus cells are still tightly packed. Such oocytes may show absent or delayed fertilization if inseminated immediately.

**Grade III**

These oocytes are mature oocytes; the polar body is already extruded. The oocyte is arrested in metaphase II. The corona cells are less tightly packed.

**Grade IV**

These oocytes are the ovulatory oocytes. These are arrested at metaphase II. The corona cells appear as “sun burst” while the cumulus cells are fully expanded and abundant.

**Grade V**

Cumulus is gelatinous and mucified and the corona cells have begun to degenerate.\(^{24}\)
2.2.9 Oocyte grading on a Good-Fair-Poor scale

**Figure 7: Oocyte grading on a Good-Fair-Poor scale**

<table>
<thead>
<tr>
<th>Oocyte grading on a good-fair-poor scale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Good</strong></td>
</tr>
<tr>
<td>• Clear cytoplasm/normal shape</td>
</tr>
<tr>
<td>• Single distinct polar body</td>
</tr>
<tr>
<td>• Clear/thin zona pellucida</td>
</tr>
<tr>
<td><strong>Fair</strong></td>
</tr>
<tr>
<td>• Slightly grainy cytoplasm/misshapen</td>
</tr>
<tr>
<td>• Fragmented/abnormal polar body</td>
</tr>
<tr>
<td>• Slightly pigmented/amorphous zona</td>
</tr>
<tr>
<td>• Cytoplasmic bodies</td>
</tr>
<tr>
<td>• PV debris</td>
</tr>
<tr>
<td><strong>Poor</strong></td>
</tr>
<tr>
<td>• Dark/grainy cytoplasm/misshapen</td>
</tr>
<tr>
<td>• &gt;1 polar body structure</td>
</tr>
<tr>
<td>• Pigmented/thickened zona</td>
</tr>
<tr>
<td>• Vacuoles</td>
</tr>
<tr>
<td>• PV debris</td>
</tr>
</tbody>
</table>
2.3 ASSESSMENT OF SPERM

Conventionally the assessment of sperm quality is based upon the semen profile, according to World Health Organisation (WHO) guidelines (WHO, 2010). Conventional semen analysis includes the volume of the ejaculate, the concentration, motility and morphology of the spermatozoa, and the presence of anti-sperm antibodies.

Neubauer and Makler counting chamber can be used for concentration. The lower reference limit for sperm concentration is $15 \times 10^6$ spermatozoa per ml. (WHO 2010). It is recommended that Motility should be categorized as:

- Progressive motility (PR): Spermatozoa moving actively, either linearly or in a large circle, regardless of speed.
- Non-Progressive motility (NP): All other patterns of motility with an absence of progression, e.g., swimming in small circles, the flagellar force hardly displaying the head, or when only a flagellar beat can be observed.
- Immotile (IM): No movement

The lower reference limit for total motility (PR+NP) is 40%. The lower reference limit for PR is 32% (WHO 2010).

Sperm morphology is regarded as possibly the most consistent sperm variable that appears to be related to IVF success. Hence this observation has a very important clinical and diagnostic role to play in the management of infertile couple. Normal sperm morphology has been related to the fertilizing potential of sperm. It can be assessed in several ways, the most common classification system...
being, the WHO standard and the Kruger strict criterion. According to the WHO manual 2010, the cut off is 4% for normal sperm morphology.60

**Figure 8: Sperm morphology**

![Image of sperm morphology]

Whilst conventional semen analysis maintains a central role in assessment of male fertility, a definitive decision of fertilising ability of sperm often cannot be made by basic semen analysis.

Therefore a number of different additional sperm functional tests have been developed, trying to get more accurate diagnostic tools for sperm quality. These new tests have focused on the sperm chromatin integrity, including both chromatin packaging anomalies and deoxyribonucleic acid (DNA) strand damage.62,63
Figure 9: Normal and Abnormal sperm morphology.64


2.3.1 Semen preparation

Motile sperms are isolated by a standard swim-up technique. In cases of oligo- and asthenozoospermia, gradient centrifugation is the method of choice. Ejaculated, testicular biopsy, cryopreserved ejaculated and cryopreserved testicular biopsy specimens are also used.

Figure 10: Sperm preparation-swim-up technique

![Figure 10: Sperm preparation-swim-up technique](image)

Figure 11: Sperm preparation-Density gradient technique

![Figure 11: Sperm preparation-Density gradient technique](image)
2.3.2 Insemination and injection

Oocytes are pre-incubated further for 1 - 4 hours to reach the required maturation. In the conventional IVF method, oocytes are co-incubated with the appropriate number of motile sperms for 16-18 hours. ICSI procedure is performed on MII oocytes using conventional techniques. Oocytes are first enzymatically treated to remove the cumulus cells. After denudation, a single sperm is injected into the cytoplasm of the oocyte.
2.4 ASSESSMENT OF FERTILIZATION

Human fertilization is defined as a process by which the two human gametes, the spermatozoon from the male and the oocyte from the female, unite to give rise to a new cell entity, the zygote. The nuclear envelope of the fertilizing spermatozoon lyses after the sperm and oocyte fusion and the nuclear material from both sperm and oocyte lies in the ooplasm, under the metabolic regulation of oocyte cytoplasmic proteins.

For successful fertilization to occur, several events must follow in a disciplined manner:

- Sperm penetration through cumulus oophorus
- Binding of the sperm to zona
- Sperm – oocyte fusion
- Oocyte activation
- Sperm nucleus decondensation forming male pronucleus
- Development and migration of male and female pronuclei and
- Association of the parental chromosomes on spindle of first cleavage division.

2.4.1 Fertilization in Assisted reproductive technology

As contrast in natural fertilization, in ART less fertile and less viable sperm have a chance of fertilizing the oocyte. While conventional in vitro fertilization (IVF) is more or less similar to natural fertilization, ICSI is comparatively an invasive procedure circumventing some of the major steps in the process of oocyte
activation and fertilization. In ICSI, fertilization has to be assessed approximately 2 hours earlier (16-18 hours) than in IVF (18-20 hours). Normal fertilisation and embryo development have been achieved even with acrosomeless round-headed\textsuperscript{70} and immotile spermatozoa.\textsuperscript{71} The only factor that appears to influence fertilisation in ICSI is sperm deoxyribonucleic acid (DNA) strand integrity.\textsuperscript{72}

2.4.2 Zygote morphology

Formation and differentiation of pronuclei are the most prominent morphological events taking place in the early post fertilization period.

The important parameters included in the zygote scoring system used include:

- Pronuclear morphology
- Pronuclear alignment
- Cytoplasmic appearance and halo formation
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- Timing of nuclear membrane breakdown
- Nucleoli or Nuclei precursor bodies (NPBs) number and distribution in each pronuclei (PN)$^{73}$
- Orientation of the polar bodies with respect to the pronuclei.

Morphologically, an optimally fertilized oocyte displays two spherical PBs and two centrally located, juxtaposed pronuclei that are even sized with distinct membranes. The pronuclei containing nucleolar precursor bodies equivalent in number and size, equatorially aligned at the region of membrane juxtaposition are called polarized pronuclei. When pronuclei contain uneven number or uneven distribution of precursor bodies, they are known as non-polarized pronuclei.$^{68, 69, 74, 75}$

Oocytes with two pronuclei are normally fertilized. Oocytes with single pronuclei, multiple pronuclei, or fragmented pronuclei are abnormally fertilized.$^{76}$

2.4.3 Abnormal fertilization

Majority of fertilization abnormalities are caused by the deficiencies of sperm-derived oocyte-activating factor or of the oocyte cytoplasmic systems that have to generate an adequate response to the sperm factor.$^{77}$

Single pronucleate zygotes (1PN)

It is seen in two to five percent of IVF and ICSI fertilizations. Two mechanisms could be responsible for this observation, asynchronous appearance or fusion of both nuclei.
Three pronucleate zygotes (3PN)

This is most commonly seen anomaly in IVF and ICSI fertilizations. This is mostly caused by dispermy (3 pronuclei, 2 polar bodies), and majority of corresponding embryos will cleave but stop development at later stages.\(^7\)

Digynic triploidy

It is seen in four percent of ICSI fertilizations. A single sperm is present in the oocyte, but the second polar body is not extruded.

No pronuclei

It is seen in four percent of ICSI fertilizations. The reasons could be an abnormal developmental speed and/or inaccurate timing of fertilization control.

**Figure 13: Abnormal fertilization**

![Abnormal fertilization](image)

2 PN 1 PN 3 PN

2.4.4 Embryo culture

In ART, embryo culture is one of the cornerstones of the success of assisted reproductive procedures. Oocytes and embryos are cultured in the medium providing physical and chemical conditions imitating the *in vivo* environment. The different stages of cells have varying nutritional and physical needs and these should be taken into consideration before we subject them for *in vitro* culture.\(^6\)
2.5 HUMAN PREIMPLANTATION EMBRYO DEVELOPMENT

Following fertilisation, the zygote divides to form the morula and subsequently progresses to the blastocyst stage. By day 5 the blastocyst reaches the uterus, expands and hatches from the ZP. The blastocyst attaches to the uterine wall between day 7-9 and implants itself in the endometrium. In IVF, the embryos are transferred to the uterus usually on days 2-3 or day 5.

2.5.1 Cleavage

Embryos fertilized after IVF or ICSI will undergo cleavage. Cleavage is the division of cells in the early embryo. The zygotes of several species undergo rapid cell cycles with no significant growth, producing a cluster of cells. The different cells derived from cleavage are called blastomeres and form a compact mass called the morula. This cleavage ends with the formation of the blastula.79

2.5.2 Zygote cleavage

Several authors have investigated the timing of PN breakdown and cleavage of zygotes at normal IVF80–82 and ICSI.83 The development of the human zygote to the two-cell stage can occur as early as 20 hours after insemination, although most of the zygotes start dividing 25–27 hours post-insemination.82 Before the first cleavage, sperm centrosome divides and forms the two centers of the first division spindle. In humans, the PN do not fuse, and the combination of parental genomes (syngamy) occurs only after the pronuclear breakdown when maternal and paternal chromosomes intermingle and align on the metaphase plate of the first division.
Early cleavage

After fertilization check, on the same day early cleavage examination is performed at 27 hours after post injection. An embryo displaying two cells at 27 hours are designated as 'early cleavage'.\textsuperscript{19}

**Figure 14: Types of cleavage**

### 14.1: Early cleavage

![Early cleavage](image1)

### 14.2: Late cleavage

![Late cleavage](image2)

Late cleavage

The embryos that have not cleaved to the 2-cell stage are designated as late cleavage.\textsuperscript{19}
2.5.3 Fertilisation and First Cleavage

Normal fertilisation is indicated by the presence of two pronuclei within the cytoplasm and the extrusion of the second polar body (PB) to the perivitelline space. In the conventional method, the fertilisation process takes place in a similar way to natural fertilisation, where as in ICSI, several steps of sperm-oocyte communication do not take place. Following sperm penetration, the oocyte metabolism intensifies, second meiotic division is completed, and a second PB is extruded, which denotes the beginning of the G1-phase of the first cell cycle (figure-15).

Most of the zygotes enter the G1-phase, 3 hours after sperm penetration. Formation of male and female pronuclei begins in this phase as well. The decondensed nucleus of a sperm forms the male pronucleus while the nucleus of the matured oocyte forms the female pronucleus. Male and female pronuclei generally have the same size and they form simultaneously. These pronuclei can be seen 7-8 hours after insemination by light microscopy. Formation of pronuclei is usually completed 8-14 hours after insemination. The male pronucleus evolves near the place of sperm penetration while the female pronucleus forms next to the first polar body.

The G1-phase terminates 8-14 hours after fertilisation and is followed by the S-phase during which the chromosomes replicate. DNA synthesis in the pronuclei begins 8-14 hours post-insemination and terminates 14-24 hours after fertilisation. Apposition of pronuclei in the centre of the cytoplasm is directed by the cytoskeleton of the oocyte. Nucleoli appear and align into the equatorial plane.
Early cleavage of human embryos to the two-cell stage: A simple, effective indicator of implantation and pregnancy in intra cytoplasmic sperm injection.

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of the two pronuclei. The DNA synthesis cannot be observed any more during the 5-6 hours long G2-phase. The M-phase of the first cell cycle begins with the disappearance of pronuclei (pronuclear breakdown, PNBD) and lasts until completion of the first cell division.\textsuperscript{13}

The duration of the M-phase is 3-4 hours which is relatively constant. During this phase, the membranes of the male and female pronuclei dissolve, chromosomes become free and integrate in the central region of the cytoplasm (syngamia), which is followed by the first meiotic cell division of the zygote. Contrary to some mammalian species, fusion of pronuclei is not preceded by the assimilation of chromatin in the human zygote.\textsuperscript{65, 81-83, 85, 86}

Pronuclear breakdown in some oocytes can be observed 17 hours after conventional IVF using light microscopy. However, majority of the oocytes enter the M-phase 24-30 hours after fertilisation. Cleavage can be finished after 20-33 hours, but in most of the oocytes this phase lasts until 27-30 hours post-insemination.\textsuperscript{65, 87}

\textbf{Figure15: First cell cycle of a human zygote}\textsuperscript{13}
2.6 ASSESSMENT OF THE FIRST CLEAVAGE AND ITS IMPORTANCE

The method of fertilisation influences the duration of time elapsed between fertilisation and first cleavage. The sperm penetration through zona pellucida and oolemma to the cytoplasm takes a few hours during conventional IVF treatment. During ICSI treatment, however, several steps of sperm-oocyte interaction do not occur. Subsequently, oocytes fertilised by ICSI undergo pronuclei break down (PNBD) and the first cleavage division approximately 2-4 hours earlier than oocytes derived from conventional IVF.65

Table: 1 Timing of observation88
2.6.1 Causes of early cleavage

It could be due to an intrinsic factor within the oocyte, and higher metabolic fitness of the embryo.

**Paternal factor**

- Centrioles introduced by the spermatozoon
- Differences in individual sperm to stimulate calcium transients
- DNA status of the spermatozoa

**Maternal factor**

- Oocyte maturity (cytoplasmic and nuclear)
- Correct spatial arrangement of the oocyte
- Oocyte’s ability to respond to the calcium transients stimuli
- DNA replication (shorter S-phase)
- Chromosomal abnormalities
Higher densities of actin and chromatin in early cleavage embryos contribute significantly 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.\textsuperscript{89}

There are three subclasses of cytoskeleton – microfilaments including actin, intermediate filaments, and microtubules, including tubulin.\textsuperscript{89} Actin and tubulin have been demonstrated to play an important role in embryo cleavage. Tubulin assists migration of pronuclei during the fertilization process. It is involved in mitosis and chromosomal spindle formation and movement of organelles such as mitochondria.\textsuperscript{90} Actin is also engaged in many important cellular processes, such as cell motility, cytokinesis and division, organelle movement, spindle migration, distribution of mitochondria,\textsuperscript{91,92} polarization of embryos, pronuclear apposition, cell signalling and maintenance of cell shape.\textsuperscript{93-95}

It has been demonstrated that oocytes with a “high metabolic fitness” cleave earlier due to the availability and competence of ATP, mRNA, mitochondria, etc. The first mitotic division distributes the cellular components that are laid down in the oocyte during development to two blastomeres and disruption of the cytoplasmic replacement after fertilization may result in unequal and incorrect distribution of certain gene products or cytoplasmic components to the cells, which will be perpetuated during development.\textsuperscript{96}
2.7 ASSESSMENT OF EMBRYO

An accurate embryo quality assessment is of paramount importance to maintain a successful IVF program. In most of the IVF clinics around the world, this quality assessment relies mostly on the morphological evaluation of cleavage stage embryos. So the embryologists should be able to associate the features observed at the optical microscope with the implantation potential of each embryo.  

To achieve this goal, many scoring systems based on the morphological characteristics of the dividing embryo have been developed. These embryo classification systems are based on the assessment of the number of blastomeres, degree of fragmentation, symmetry of the blastomeres, presence of multinucleation and the compaction status. It is very important that the features related to implantation potential are assessed accurately and similarly.

Good quality embryos must display appropriate kinetics and synchrony of division. In normal-developing embryos, cell division occurs every 18–20 h. Embryos dividing either too slow or too fast may have metabolic and/or chromosomal defects. The number of blastomeres is used as the main characteristic with the highest predictive value. The blastomeres divides at regular intervals from day2.

2.7.1 Assessment of embryo on Day2

At 44 ± 1 h post insemination, a healthy embryo displays four equal sized mono nucleated blastomeres in a three dimensional tetrahedral arrangement, with preferably nil or less than 10 percent fragmentation.
2.7.2 Assessment of embryo on Day 3

At 68 ± 1 h post insemination, a healthy embryo displays 6 to 8 equal sized mono nucleated blastomeres with nil or less than 10 percent fragmentation and a sign of beginning of compaction. This is an important stage from embryogenesis point of view, as paternal genome is activated at this point that decides effect of genetic filiation on embryo development. Embryos with greater than 15 percent fragmentation are categorized as Grade II. Those with greater than 50 percent fragments are termed as Grade III.

2.7.3 Assessment of embryo on Day 4

At 92 ± 2 h post insemination, one can observe a structure called “morula”, where it is not possible to count number of blastomeres that range from 8 - 32.
Immediately after morula within 2 to 4 h, healthy embryo displays stage of compaction where intercellular junctions of blastomeres fuse to form an uneven, irregular ball-like structure. Such embryo almost always results in a blastocyst.

2.7.4 Assessment of embryo on Day5

At 106 to 108 h post insemination, a healthy embryo turns into a blastocyst. However, not all blastocysts are capable of implantation. The timing of blastocyst formation and its morphology is crucial for its viable status. An expanded blastocyst with thin zona, blastocoel filling almost entire area, clear sickle-shaped trophectoderm and tightly packed inner cell mass (ICM) comprising about 25 percent of blastocoel are markers to certify a blastocyst as Grade I.35

Figure 21: Human pre implantation embryo development
2.7.5 Grading of embryos

Criteria for grading embryos

The following criteria are commonly used for the grading of embryos:

- The number of cells (blastomeres)
- The size and uniformity of blastomeres
- The level and type of fragmentation
- Presence of multinucleation in the blastomeres
- Rate of development of the embryos.  

The number of cells (blastomeres)

About 2 - 4 cells should be present in the embryos of Day 2; likewise 6 - 8 on Day 3 and the blastocysts on Day 5. If the number of cells is lower in number then it is indicative of poor development status.

Size and uniformity of blastomeres

A “good” embryo is considered to be the one where all blastomeres are of identical size.

Fragmentation

The percentage and location of fragmentation is reflective of the quality of the embryo. Minor fragmentation is often detected in embryos and this has not been found to be detrimental to the implantation of the embryos.
2.7.6 Cleavage stage embryo grading

**Figure 22: Cleavage stage embryo grading**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
</table>
| 1     | Good   | <10% fragmentation  
Stage specific cell size  
No multinucleation |
| 2     | Fair   | 10-25% fragmentation  
Stage specific cell size for majority of cells  
No evidence of multinucleation |
| 3     | Poor   | Severe fragmentation (>25%)  
Cell size not stage specific  
Evidence of multinucleation |
2.7.7 Embryo grading on day 4

Table 2: Day 4 embryo grading

<table>
<thead>
<tr>
<th>Grade</th>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Good</td>
<td>Entered a fourth round of cleavage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Evidence of compaction that involves virtually all the embryo volume</td>
</tr>
<tr>
<td>2</td>
<td>Fair</td>
<td>Entered a fourth round of cleavage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Compaction that involves the majority of the embryo volume</td>
</tr>
<tr>
<td>3</td>
<td>Poor</td>
<td>Disproportionate compaction involving less than half the embryo, with two or three cells remaining as discrete blastomeres</td>
</tr>
</tbody>
</table>

2.7.8 Embryo grading on day 5

Table 3: Day 5 embryo grading

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Grade</th>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early</td>
<td>Blot-cyst</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Expanded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Hatched</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner cell mass</td>
<td>1</td>
<td>Good</td>
<td>Prominent, easily discernible with many cells that are compacted and tightly adherent together</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Fair</td>
<td>Easily discernible with many cells that are loosely grouped together</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Poor</td>
<td>Difficult to discern, with few cells</td>
</tr>
<tr>
<td>Trophoderm</td>
<td>1</td>
<td>Good</td>
<td>Many cells forming a cohesive epithelium</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Fair</td>
<td>Few cells forming a loose epithelium</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Poor</td>
<td>Very few cells</td>
</tr>
</tbody>
</table>
2.7.9 SART embryo grading system

Standardization of morphological assessment for embryo grading system was developed and is being implemented by the Society for Assisted Reproductive Technology (SART) in 2010.\textsuperscript{107}

Table 4: SART embryo grading system\textsuperscript{107, 108}

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Overall grade</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleavage</td>
<td>Good, Fair, Poor</td>
<td>Cell #: 1 through &gt;8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fragmentation: 0%, &lt;10%, 11–25%, &gt;25%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Symmetry: Perfect, Moderately Asymmetric, Severely Asymmetric</td>
</tr>
<tr>
<td>Monula</td>
<td>Good, Fair, Poor</td>
<td>Compaction: Complete, Incomplete</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fragmentation: 0%, &lt;10%, 11–25%, &gt;25%</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>Good, Fair, Poor</td>
<td>Expansion: Early, Expanding, Expanded, Hatched</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inner Cell Mass: Good, Fair, Poor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trophoderm: Good, Fair, Poor</td>
</tr>
</tbody>
</table>

2.7.10 Embryo Transfer

Embryo transfer (ET) requires no sedation or anesthesia under ultrasound guidance. The ultrasound is performed using an abdominal probe and a full bladder is needed at the time of transfer. Usually 2 or 3 embryos are placed into the uterus with a small catheter through the cervix. It is relatively painless procedure. The whole procedure just takes 5 minutes to complete. Excess embryos may be frozen for future use.\textsuperscript{76}
2.7.11 Luteal support and pregnancy evaluation

The luteal phase is supported by vaginal supplementation of micronized natural progesterone the day after the egg retrieval. After 14 days of embryo transfer serum beta hCG is measured. Traditionally the IVF outcome has been expressed in terms of biochemical or clinical pregnancies and total live births.
2.8 EMBRYO SELECTION METHODS

It becomes very important to develop a method of choosing the embryos that possess the greatest potential to implant. Many different methods have been proposed to be effective in selecting good embryos for transfer. Routinely the selection of embryos for transfer is based on embryo morphology and developmental stage. Despite the fact, sometimes implantation may not occur after transferring good quality embryos to a receptive endometrium.

Other methods of selection of embryos include pronuclear morphology, (Payne et al., 1997) oocyte and pronuclear polarity, blastomere symmetry (Edwards et al 1997), and blastocyst culture (Gardner et al., 1998). Pronuclear zygote morphology may vary during the dynamic process of syngamy. According to Cummins et al., 1986 study, selection of embryos on the basis of cell number and quality at the time of transfer is of more significant benefit.

Other morphological features such as variation in zona thickness (Cohen et al., 1989) and the presence of multinucleated blastomeres (Pelinck et al., 1998) have also been shown to affect the implantation and pregnancy. Gardner et al found and shown that the blastocyst score to select the high quality blastocyst on the basis of inner cell mass and trophectodermal cells leads to higher pregnancy and implantation rates.
Nevertheless, blastocyst transfer may have some drawbacks such as cancellation of embryo transfer because of not having a blastocyst to transfer after five days culture, a lower embryo cryopreservation rate and increased laboratory workload.\textsuperscript{115} This practice, however, adds to the cost associated with laboratory procedures and may be associated with adverse effects due to epigenetic changes, though the data are sparse to support such an effect.\textsuperscript{116,117} The morphological characteristics of oocytes (e.g. appearance of cytoplasm, morphological features of the first polar body, size of the perivitelline space) are closely correlated to the embryo viability.\textsuperscript{118,119}

Several other approaches to identify the best embryos for embryo transfer are cleavage speed, whether too slow or too fast are considered to have a negative effect on implantation rate.\textsuperscript{120-124} Cellular cleavages, when uneven, seems to negatively affect the developmental capability of the embryo.\textsuperscript{122, 123, 125} The extent of fragmentation has been reported to have a predictive value on embryo potential implantation rate by several investigators.\textsuperscript{122-128} Improvement of the embryo culture systems and laboratory conditions in recent years rendered a better environment for embryo survival.

The ability of various screening technologies (preimplantation genetic screening, metabolomics, and proteomics) to identify embryo(s) with the highest implantation potential has been investigated in recent years. Some of these methods require complicated technology, elective embryo cryopreservation and are
associated with significant treatment expenses. In addition, most of the randomized trials do not support their use.\textsuperscript{129-132}

Several biochemical methods have been used to assess the human embryo quality, such as oxygen (O\textsubscript{2}) consumption, uptake of pyruvate and glucose, lactate production and secretion of platelet-activating factor or amino acid turnover.\textsuperscript{133} Nonetheless, these procedures are all more complex and time-consuming and it is very difficult to follow in routine practice.

Over the past 5 years a number of different Time-Lapse Systems (TLS) have been introduced to fertility laboratories with the aim of identifying better quality embryos that would ultimately improve the success rates of IVF.\textsuperscript{134,135} An important issue to consider before implementing time-lapse analysis in a clinical setting is the safety of the instrument. Time-lapse imaging necessitates periodical exposure to light.\textsuperscript{21,136}

Time-lapse technology is just one of the methods that is currently being evaluated for embryo selection. None of these technologies are perfect, and rather than looking at them as competing technologies, we should evaluate how they could complete each other and further improve embryo selection during IVF.\textsuperscript{21}

More effort have been put to refine the existing embryo scoring systems and to find additional easy, simple, non-invasive technique and more efficient method of viable embryo selection procedure.\textsuperscript{137-144}
The timing of first cell division in humans has been reported to be between 20 and 22 h (Balakier et al., 1993) and 25 h (Capmany et al., 1996). Higher percentage (64%) of cycles in which embryos cleaved to two cell was observed in 25 h post-insemination, in comparison with others in which a proportion of early-cleavage positive cycles was included between 9.5 and 59% at the same time-point. (Shoukir et al., 1997; Sakkas et al., 1998a, 2001; Bos-Mikich et al., 2001; Lundin et al., 2001; Petersen et al., 2001; Fenwick et al., 2002; Brezinova et al., 2003, 2004; Salumets et al., 2003; Cyray et al., 2004; Van Montfoort et al., 2004). The reason for this difference could be the differences in embryo culture conditions existing between IVF laboratories (Gardner et al., 2005), in sperm characteristics (Sathanantan et al., 1991, 1998; Palermo et al., 1997) and women’s age (Lundin et al., 2001; Ciray et al., 2004), all factors that can influence embryo cleavage rate.145

The kinetics of fertilization and cleavage to the two-cell stage after ICSI have been previously documented in a study by Nagy et al. In their study, cleavage to the two-cell stage was initially observed 20 h after ICSI and found 11% had cleaved to the two-cell stage. Again the fertilized oocytes were examined at 27 h after ICSI and at this stage a 30.5% had cleaved to the two-cell stage. Sakkas and colleagues (1998) reported that transfer of EC embryos observed at 27 hours after ICSI results in a higher pregnancy rate in ICSI treatment as well.112

Study conducted by Jing fu et al found that transfer of embryos with an early cleavage led to significantly higher pregnancy and implantation rate.15
Edessy et al found the transfer of EC embryos resulted in two times higher clinical pregnancy rate (43.30% versus 21.88%; p< 0.005) and more than double implantation rate (25.58% versus 11.35%; P = 0.000) when compared to LC embryo transfers. They found that the EC embryos had significantly higher proportion of good quality embryos when compared to LC embryos (p< 0.000).\textsuperscript{137}

Studies have showed that the assessment of the time of cleavage to the two cell stage was a reliable parameter for the selection of embryos with the highest capability of implantation and successful pregnancy after transfer.\textsuperscript{146-154}

Considering these advantages, the aim of the present study was to evaluate the impact of early cleavage over late cleavage in assessing pregnancy outcome using Intra Cytoplasmic Sperm Injection (ICSI).
2.9 LACUNAE IN KNOWLEDGE AND NEED FOR THE STUDY

- From reviewing the literature, it is clear that there is lack of reliable and simple marker to judge the quality of embryos to be transferred.

- Most of the ART centres select embryos for transfer on only morphological criteria. Even after selecting morphologically good embryos there is no improvement in implantation rate and pregnancy rate.

- Selection of time-lapse imaging which is expensive. It has been shown that extensive light exposure and the continuous presence of electromagnetic fields found in some time-lapse systems may be detrimental to embryo development, inaccessible to many IVF centres in developing countries may not be feasible.

- Few studies in literatures suggest selection of EC embryos may improve the outcome. There are no Indian studies to suggest the importance of EC embryos.

- Most of these criteria will be fulfilled by selection of early cleavage embryos. Hence this study was conducted.
2.10 RESEARCH HYPOTHESIS

NULL HYPOTHESIS

1. Early cleavage (EC) is not an additional parameter for selecting the embryo with the highest implantation potential.

2. Transfer of early cleavage embryos cannot lead to a higher pregnancy and implantation rate compared to the transfer of late cleavage embryos.

ALTERNATE HYPOTHESIS

1. Early cleavage (EC) is an additional parameter for selecting the embryo with the highest implantation potential.

2. Transfer of early cleavage embryos leads to a higher pregnancy and implantation rate compared to the transfer of late cleavage embryos.