Morphokinetic assessment of human embryo development using time-lapse imaging

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2018

MORPHOKINETIC ASSESSMENT OF HUMAN EMBRYO DEVELOPMENT USING TIME-LAPSE IMAGING

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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FACULTY OF BIOLOGICAL SCIENCES

QUAID-I-AZAM UNIVERSITY

ISLAMABAD, PAKISTAN

2018

CERTIFICATE

This dissertation"Morphokinetic assessment of human embryo development using time-lapse imaging" submitted by **Noureen Akhter**is accepted in its present form, by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Doctor of Philosophy in Animal Sciences.

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I dedicate the successful completion of this thesis and all my achievements in the field of Animal Sciences to my honorable Supervisor

Professor Dr. Muhammad Shahab

and my loving family

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ACKNOWLEDGEMENTS

All praise is due to ***Almighty Allah*** (SWT) the creator of all the creations, who is eternal and exists without a place. I praise Him and seek His assistance. I ask for His forgiveness and take refuge in Him from the evil within myself and from the evil of my deeds. I bear witness that there is no god but Allah (SWT) alone, He has no partner. I thank Almighty Allah (SWT) for blessing me with the opportunity to successfully complete this work. Countless salutation upon the ***Holy Prophet*** (peace be upon him), who gave us path to recognize our self and the Creator.

I would like to express my deep and sincere gratitude to my supervisor, ***Professor Muhammad Shahab***, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-I-Azam University. I would like to offer my special thanks to ***Dr. Nasim Ashraf***, Medical Director and ***Dr. Anjum Ara Siddiq***, Scientific Director Islamabad Clinic Serving Infertile couples (ICSI) Islamabad, Pakistan for their insightful comments and suggestions during whole project. Their understanding, encouragement and personal guidance have provided a good basis for the present thesis. Their ideas and concepts have had a remarkable influence on my entire career in the field of assisted reproduction.

I wish to express sincere thanksto ***Dr. Sarwat Jahan***, Chairperson of the Department of Animal Sciences,Faculty of Biological Sciences, Quaid-I-Azam University for her generous support and guidance.

I am thankful to all my lab fellows and friends especially to ***Riffat,Tanzeel, and Hira*** for their support in various ways during my research work. I am especially thankful to my dear friend ***Dr.Saima Jabeen*** for her extreme help and cooperation during thesis compilation. Without the courage and initiative she gave to me, it would really have been a hard task. My sincere wishes are for her family and for success in her life ahead.

I warmly thank the whole staff at ICSI clinic. Their moral support throughout my research work and prayers for my successare noteworthy to be acknowledged.

The cooperation of ***Anwar bhai***and ***sajid bhai***in laboratory of reproductive neuroendocrinology is also worth mentioning.

The financial support of the ***Higher Education Commission*** (HEC), Islamabad, Pakistan is highly acknowledged. I am grateful to HEC for providing me Indigenous PhD fellowship.

I would like to express my immense love for my father (late) who always supported me during my studies and my late sister ***(Yasmeen)*** who encouraged me and always dreamed high for me. May their souls rest in peace (ameen). I would like to extend my love for my dear mother who has always been so loving and caring throughout my career that I am lacking the words to express my immense love, respect and gratitude for her. I am extremely thankful to my sister (***Nazia***) and brothers (***Amjad Rehman***and ***Kamran Ashraf***) for their prayers, loving and emotional support over the years. I am also thankful to my nieces (***Aleena, shiza and Menahil)***and nephews ***(Rohail, Haider and Ayaan)*** for filling my life with their love and care.

Finally, I would like to thank everybody who was important to the successful completion of this thesis, as well as expressing my apology that I could not mention personally their names.

Noureen Akhter

LIST OF ABBREVIATIONS

%	Percentage
˚C	Centigrade
µm	Micrometer
AA	Amino acid
AI	Artificial insemination
aCGH	Array comparative genomic hybridization
AMA	Advanced maternal age
AMH	Anti mullerian hormone
ANOVA	Analysis of variance
AR	Acrosomal reaction
ART	Assisted reproductive technologies
ATP	Adenosine triphosphate
AZH	Assisted zona hatching
BMI	Body mass index
cc2	Second cell cycle
cc3	Third cell cycle
CO_2	Carbon dioxide
COC	Cumulus oocyte complex
COH	Controlled ovarian hyperstimulation
D0	Day zero
D1	Day one
D2	Day two
D3	Day three
D4	Day four
D5	Day five
DC	Direct cleavage
DETs	Double embryo transfers
DFI	DNA fragmentation index
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
e.g.	For example
EB	Expanding blastocyst
EC	Early cleavage
EFI	Endometriosis fertility index
EGA	Embryonic gene activation
ESHRE	European society of human reproduction and embryology
ET	Embryo transfer
FEB	Fully expanded blastocyst
FISH	Fluorescence in situ hybridization
FMR1	Fragile mental retardation 1
FSH	Follicle stimulating hormone

GCs	Granulosa cells
GIFT	Gamete intrafallopian transfer
GnRH	Gonadotropin releasing hormone
GnRH-a	Gonadotropin releasing hormone agonist
GnRH-ant	Gonadotropin releasing hormone antagonist
hCG	Human chorionic gonadotropin
hMG	Human Menopausal gonadotropin
hr.	Hour
HSG	Hysterosalpingography
IAM	Inner acrosomal membrane
ICI	Intracervical insemination
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IL	Interleukein
IMSI	Intracytoplasmic morphologically selected sperm injection
IR	Insulin resistance
IUI	Intrauterine insemination
IVF	In vitro Fertilization
Kg	Kilogram
LFOR	Low follicular ovarian reserves
LH	Luteinizing hormone
LNV	Large nuclear vacuoles
LUF	Luteinized un ruptured follicle syndrome
MATER	Maternal antigen that embryo requires
MESA	Microepididymal sperm aspiration
Min	Minutes
MIS	Mullerian inhibiting substance
Ml	Milliliter
Mm	Millimeter
MSOME	Motile sperm organelle morphology evaluation
N_2	Nitrogen
NOA	Non obstructive azoospermia
NSAIDS	Nonsteroidal anti-inflammatory drug
O_2	Oxygen
OB	Obese
OHSS	Ovarian hyperstimulation syndrome
OPU	Oocyte pick up
OS	Oxidative stress
OW	Over weight
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PESA	Percutaneous epididymal sperm aspiration
PGD	Preimplantation genetic diagnosis
PGS	Pre implantation genetic screening
PID	Pelvic inflammatory disease
PN	Pronuclei

PNB	Pronuclear binding
PNf	Pronuclear fusion
PRs	Pregnancy rates
RBCs	Red blood cells
RC	Reverse cleavage
RCT	Randomized controlled trials
r-hFSH	Recombinant human follicle stimulating hormone
Rpm	Revolution per minute
s2	Synchrony of second cleavage
s3	Synchrony of third cleavage
SB	Start of blastulation
SDF	Sperm DNA fragmentation
SETs	Single embryo transfers
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
T	Testosterone
TE	Trophectoderm
TESE	Testicular sperm aspiration
TLM	Time-lapse monitoring
TLS	Time-lapse system
TMC	Total motile count
TNF	Tumor necrosis factor
UC	Ulcerative colitis
Vs.	Versus
WBCs	White blood cells
WHO	World health organization
ZIFT	Zygote intrafallopian transfer

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General Abstract

GENERAL ABSTRACT

Background: The success rate of IVF (In vitro fertilization) is associated with the methods to select best embryos for uterine transfer thus facilitating the acceptance and adoption of single embryo transfer regime to avoid the risks of multiple pregnancies. Time-Lapse imaging is an emerging tool to evaluate the developmental potential of embryos noninvasively by continuous monitoring. Recent advancements in the Time-Lapse monitoring have provided us with the knowledge of many new morphokinetic markers for embryonic potential. There is limited information available regarding the relationship between morphokinetics of human embryo, its chromosomal composition and the implantation potential hence the specific aims of the present study were to analyze the cleavage data of embryos from our population of infertile couples, visiting the IVF center, using Time-Lapse imaging system established first time in an IVF clinic in Pakistan and to compare this analysis with previously acquired data sets with the help of this novel technique. Moreover this study also aimed to compare the cleavage data of embryos from patients with different factors contributing to their infertility and also to follow their treatment outcome.

Methods: The current study is the retrospective observe of the prospectively acquired data at Islamabad Clinic Serving Infertile Couples (ICSI), Islamabad, Pakistan. A total number of 200 patients undergoing ICSI treatment cycles at the clinic was selected for the study and divided into five age groups (< 26, 26-30, 31-35, 36-40, and > 40 years) for first analysis which focused on effect of female age on embryo morphokinetics. The second analysis comprehensively compared the morphokinetic markers among different pathology groups of patients presenting with the history of infertility. There were eight groups included in this analysis namely the endometriosis, advanced female age, high FSH, idiopathic, low AMH, male factor, PCOS, and tubal factor. The third analysis of the study compared the embryo morphokinetics in four groups of the drugs used for ovarian stimulation i.e. patients treated with Gonal-f, IVF-M, puregon and a combination of IVF-M+puregon. Embryo culture was done at 37°C, 6% CO_2 and 5% oxygen for 5-6 days. Ten kinetic markers were selected for analysis i.e. time for appearance of pronuclear stage (tPNa), time for 2-cell (t2), 3-cell (t3), 4-cell (t4), 5-cell (t5) and 8-cell

(t8) cleavages, time for compaction i.e. morula formation (tM), start of blastulation (tSB), expanding blastocyst (tEB) and fully expanded blastocyst stage (tFEB). The cycle outcome was also compared among different groups in all sets of analyses to see the effect of embryo morphokinetics on embryonic competence.

Results: The first analysis i.e. effect of female age on embryo morphokinetics showed that the number of retrieved, matured, fertilized and cleaved oocytes were significantly different ($P \leq 0.0001$) when compared among different age groups of females. There was no significant difference in average morphokinetic parameters in young vs. old women. Whereas timely cleaved embryos showed significant difference in tPNa i.e. time for pronuclear appearance ($P \leq 0.001$), t4 and t5 i.e. time for 4 and 5-cell cleavage ($P \leq 0.05$) when compared among different age groups. The quality of blastocysts formed and the clinical pregnancy rates showed a decline with increasing age. The comparison among different pathology groups also showed the number of retrieved, matured, fertilized and cleaved oocytes to be significantly different ($P \leq 0.0001$) among groups. Among the average morphokinetic parameters only t3 and tM showed significant difference ($P \leq 0.05$) when compared among different pathologies of patients. There was no significant difference in all other morphokinetic time-points among all study groups. Whereas the timely cleaved embryos showed significant difference in t3 ($P \leq 0.01$), t5 ($P \leq 0.01$) and t8 ($P \leq 0.05$) when compared among different pathology groups. The blastocyst formation rates were comparable among different groups while the clinical pregnancy rates were quite low in fresh cycle transfers in all study groups except PCOS and endometriosis. The frozen-thawed cycle showed comparable pregnancy rates in all groups except the patients with advanced age, high FSH and low AMH whose rates improved but not to an appreciable extent. The cell cycle intervals for second and third cycle i.e. cc2 and cc3 respectively were found to be in normal range for different groups in both analyses. Regarding the analysis of ovarian stimulation groups, the mean cleavage timings for 2-cell (t2) and 8-cell cleavage (t8) showed a significant difference when compared among different groups. The rate of blastocyst formation is lowest in patients using IVF-M for ovarian stimulation. The clinical pregnancy rates in fresh cycle transfers were low in patients using puregon+IVF-M and the ones who used IVF-M only.

Conclusions: Hence it is evident that advanced female age and other different pathological conditions affect the oocyte and embryo quality as well as the clinical outcome during an IVF/ICSI cycle when compared by using Time-Lapse morphokinetic evaluation. Similarly the high doses of ovarian stimulation drugs also affect the development of embryos adversely.

Limitations and wider implication of findings: The current study is based on retrospective analysis of embryos from patients with different factors contributing to clinical indications for infertility so there were certain limitations for exclusion of cleavage data in order to highlight the effect of pathologies on embryonic competence. The prospective study design is although time taking and need more labor work but can overcome this limitation by selective analyses and further subgrouping among embryos showing mild, moderate or severe deviation from normal cleavage patterns. Hence it is suggested that the prospective approach in future can help to add in the already existing information of kinetic markers for selection of best embryo for transfer in patients with various factors contributing to infertility.

Chapter # 1

General Introduction

GENERAL INTRODUCTION

1.1. HUMAN FERTILITY

Reproduction is the mandate of nature but gradually has evolved into an option as human has attempted to control nature. Acquiring reproduction on demand is difficult than preventing the child bearing. Fertility is a complicated phenomenon determined both by physiological and behavioral approaches. The history has shown clearly a dramatic decline in human fertility over last two centuries (Sear *et al.,* 2016). The demographic transition theory which assumes that fertility decline is irreversible has also shown that fertility changes in human beings are difficult as well as crucial to explain because they are influences by both social and environmental factors (Dyson, 2001).

Infertility is defined as inability to conceive after twelve months of unprotected intercourse. Infertility is a prevalent condition participated both by the male and female partners or it can be unexplained resulting into an emotional stress for the couple. The evaluation of infertility starts with the schematic history of both partners. It can include the cervix, the uterus, the endometrium, the semen, the ovarian function, the fallopian tubes and the peritoneum (De Groot*et al*., 2000). The historical evidence is followed by subsequent laboratory tests and physical examination before beginning the treatment. The general prevalence of infertility is documented as 9 to 18 % (Aghajanova, 2016). Since late 1970s when ARTs (Assisted reproductive technologies) became available for infertile couples as an option of treatment, the number of women undergoing fertility treatment has been increasing gradually. It has been documented that there were 190-394 IVF treatment cycles started in year 2014 (SART., 2014).

There are also certain risk factors associated with ART related to pregnancy and neonatal health e.g. preterm delivery, low birth weight, and prenatal mortality (Joshi, 2012). However much less is known about the complications of female associated factors during pregnancy like polycystic ovaries and endometriosis etc. There are number of factors participating in women reproductive health, these could be genetic anomalies,

hormonal imbalance and also the environmental factors which play a role in inducing any disease process. Almost 5 to 10% of infertile women show varying degree of genetic abnormalities (Tarin, 2015).

There is also an increased risk of gynecological complications in infertile women which has been studied by multiple authors (Venn, 1995; Escobedo, 1991; Modan, 1998; Althius, 2005; Silva-Idos, 2009; Lerner-Geva, 2012). Moreover, a very complicated reproductive disease of women i.e. endometriosis has been shown to be linked with higher ratio of malignancies, asthma, autoimmune disease, atopic disease, cardiovascular disease, and ovarian cancer (Kvaskoff, 2015). Similarly PCOS (Polycystic ovarian syndrome) is associated with increased risk of central/ visceral adiposity and insulin resistance (Hart, 2015; Ghaffarzad, 2016). The PCOS patients also show the increase ratio of endometrial cancer, metabolic disorders, cardiovascular disease and obesity (Kvaskoff, 2015; Parikh, 2016; El-Hayek, 2016; Polat, 2016; Zahiri, 2016; Ramezani-Binabaj, 2014). PCOS patients are more likely to develop infertility (El-Hayek, 2016) and diagnosis of disease is difficult due to significant overlapping between PCOS infertility and other poor health conditions. So there is a need to incorporate a healthy life style in women with PCOS to reduce the health risks associated with it.

1.2. INFERTILITY AND SUBFERTILITY

There are multiple definitions of infertility; it is the biological inability of male or female to contribute to conception. It also refers inability of women to carry a pregnancy to full term. However, the reproductive endocrinologist and gynecologist consider a couple infertile if.

- The couple not being able to conceive after 1 year of contraceptive free intercourse keeping in view the woman is under the age of 34.
- The couple has not conceived after 6 months of unprotected intercourse in case the female is over 35 years old.
- Other contributing factors of female due to which she is unable to carry a pregnancy to full term.

- The couple having fecundability rate of 3-5% is stated to be sub fertile. Subfertility refers to unsuccessfully trying to bear a child for a year or more.

1.2.1. Primary vs. Secondary infertility

Primary infertility is defined as never been able to conceive a baby while, secondary infertility is the inability of couples in conceiving after already have bearing a normal pregnancy.

1.3. CAUSES OF INFERTILITY

1.3.1. Male infertility

- **Impaired production or function of sperm**.

Male factor infertility includes multiple causes related to sperm function and hormonal imbalance. The oligozoospermia/azoospermia is the condition of low sperm concentration or no sperm at all in the ejaculate respectively which could be the first cause of male infertility. Secondly the morphological contribution of sperm is very important to acquire the fertility and it has been studied well historically. These defects could be related to the head, mid piece and the tail of sperm and are collectively referred as teratozoospermia i.e. semen sample with morphological scores below normal (Figure 1.1). The slow progression of spermatozoa i.e. asthenozoospermia also make it difficult to acquire fertilization.

Other causes of male factor infertility could be varicocele, undescended testicle and testosterone deficiency. Genetic disorders related to sperm dysfunction e.g. Klinefelter's syndrome are one of the main reason of male infertility. In this case instead of bearing one X and one Y chromosome, male has two X and one Y chromosome which

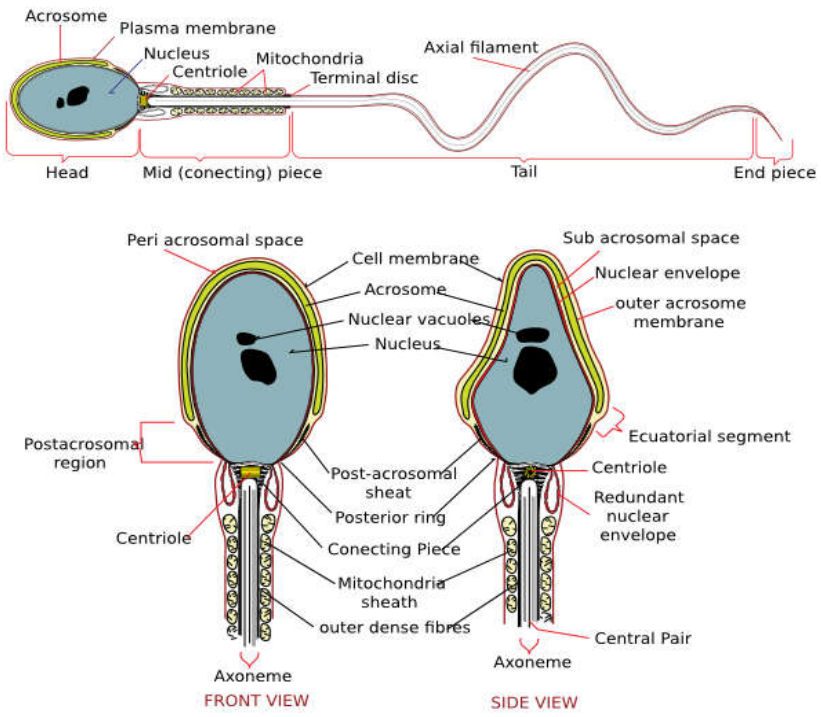


Figure 1.1. Detailed structure of human spermatozoa. (Ishijima *et al.*, 1986)

results in abnormal development of testes, low testosterone and low sperm count. Other than above stated reasons infections such as Chlamydia, gonorrhea and prostatitis also causes severe male factor infertility possibly affecting the sperm concentration and progression.

- **Impaired delivery of sperm.**

Irrespective of normal sperm counts and function, male infertility can be caused by other sexual issues like erectile dysfunction, dyspareunia and psychological issues. The ejaculate volume below normal, blockage of ejaculatory ducts and no semen in ejaculate are other main reasons of impaired delivery of sperm. The genetic factor causing impaired delivery of spermatozoa is observed in cystic fibrosis patients who often have missing or obstructed vas deference (De Jonge and Barratt, 2002).

- **General Health and lifestyle**

The general health and lifestyle of male also adds to the causes of delayed or no conception at all. The emotional stress, malnutrition, obesity, use of excessive alcohol or drugs and smoking are known factors adding to male infertility. In addition to this cancer and its treatment affects the sperm function drastically. Age related decline in male fertility has also been documented.

The ratio of male factor subfertility has been documented as at least 30% of total cases while both male and female factors contribute to another 20% hence collectively 50% of sub fertile couples are affected by both partners (Mecham, 1996) and around 39% of sub fertile men have idiopathic subfertility (WHO. 1987). The commonly used treatment protocol is the use of gonadotropins i.e. FSH and LH that play an important role in enhancing the spermatogenesis. It has been documented earlier that severe male factor subfertility can be treated by systemic administration of FSH. It results in improving the fertilization and pregnancy rates also (Acosta, 1991; Acosta, 1992). In contrast to this, another study reported that the use of recombinant human FSH in men with idiopathic subfertility did not improve the semen parameters (Kamischke, 1998). There are many others studies which have shown an increase in the sperm count in case of oligospermic patients as well as in the sperm count of patients undergoing testicular biopsy (Foresta, 1998; Foresta, 2002). The fertilization rate has also been reported to show a significant increase in the sub fertile males with administration of gonadotropins (Ben-Rafael, 2000). On the other hand Ashkenazi (1999) has reported that there is no effect of such treatment on fertilization of sub fertile male.

1.3.2. Female infertility

Female infertility is contributed by a number of factors which are briefly discussed below (Figure 1.2).

- **Fallopian tube damage or blockage**

Tubal infertility can involve the entire tube dysfunction or proximal or distal ends only and severity of condition also varies in different women (Serafini and Batzofin, 1989). The most common cause of this tubal infertility is pelvic inflammatory disease representing more than 50% of cases and affecting the fallopian tubes adversely (Honore

et al., 1999). The Hull & Rutherford classification (2002) classification of tubal disease signifies three categories namely mild/grade I, moderate/grade II and severe/grade III (Akande *et al*., 2004). Diagnosis is made by hysterosalpingography (HSG) or laparoscopy. Treatment options include noninvasive management, surgical tubal repair and IVF.

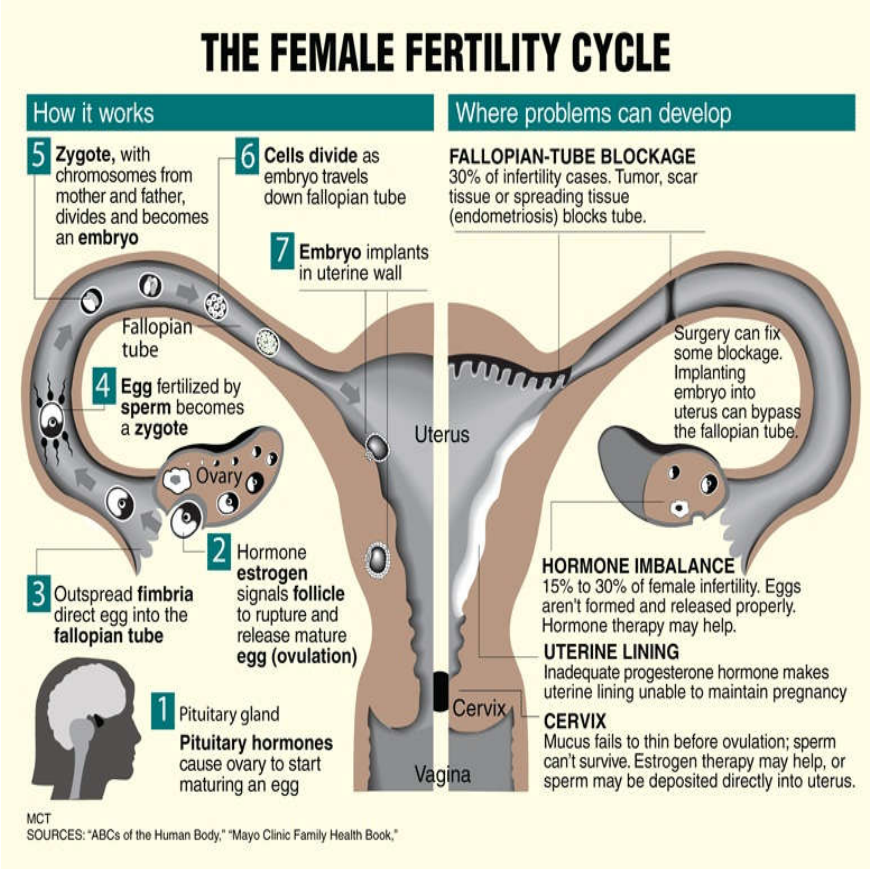


Figure 1.2.A complete view of human female fertility cycle. (Scott, 2009)

- **Endometriosis**

Endometriosis is a chronic inflammatory disease which is resulted by uterine tissue implant growing outside the uterus. These implants continually grow and bleed each month as the respond to hormone cycle thus leading to inflammation **(**Malpani and Malpani, 2001; Parks, 1996).

- **Polycystic ovary syndrome (PCOS)**

Polycystic ovary syndrome (PCOS) is the most common endocrinological disorder affecting 5-10 % of women in the reproductive age (Knochenhauer *et al.,* 1998). This syndrome is related to androgen excess and ovulatory dysfunction (ESHRE, 2004).PCOS is also associated with insulin resistance and obesity.

In vitro fertilization is the main treatment option for women with ovulation disorder. The medications used to regulate or induce ovulation constitute a group of fertility drugs. These medicine works like natural hormones i.e. FSH and LH to trigger ovulation. The surgical treatment for damage of fallopian tube is done laproscopically. The ovulation therapy is used to treat the infertility caused by endometriosis thus stimulating and regulating the ovulation and proceeding for IVF. The oocyte is thus fertilized in the laboratory and resulted embryo is transferred back to the mother's uterus **(**Malpani and Malpani, 2001; Parks, 1996).

1.4. ASSISTED REPRODUCTIVE TECHNOLOGIES

In the past, infertility treatment has been done by treatment of reproductive system in male or female by management of chronic diseases such as diabetes or thyroid diseases (Templeton *et al.,* 1996; Andrews *et al.,* 2003). In the past three decades, infertility treatment has been improved with advances in medical science. Sexually transmitted diseases have decreased as the level of public health has increased. Since 1970, ARTs have widened the perception of infertility treatment and specialist's approach has changed from research to practice hence fertility probability has increased (Gurunath *et al.,* 2011; Leushuis *et al.,* 2009; Nardelli *et al.,* 2014).

There is no consensus on the definition of ART but it is referred as a general term describing the methods used to conceive a child by artificial or partially artificial means. Usually the process of intercourse is bypassed either by artificial insemination (IUI) or by getting the oocyte fertilized in the laboratory environment i.e. IVF. The treatment options are given to patients according to their history and the severity of their problems. These could be in the form of medications only or by manual procedures of artificial

insemination. IVF along with other assisted reproductive techniques is now available all over the worlds however different rules and guidelines regarding the use of techniques have been adopted by different countries due to difference in cultural, religious, and moral preferences related to ART practices (Ory *et al.,* 2014; Ory and Devroey, 2013).

The evolution of assisted reproductive technologies has not only provided a ray of hope to 15 % couples with infertility problems but also the people facing diseases like cancer and heritable genetic disorders. IVF technology at its outset (including > 9% of all ART procedures) was taken as a treatment with great concerns about the health issues of potential offspring (Johnson *et al.,* 2010) but now 1.5 million ART cycles per year worldwide are producing 350,000 babies (ESHRE. 2014). From 1970s onwards these fears and concerns were replaced by the energy and enthusiasm regarding this treatment and IVF emerged as the best treatment option for infertile couples thus resulting in the birth of five million babies (ICMART. 2012). With the passage of time many innovative techniques have been added to improve the efficacy of IVF like intracytoplasmic sperm injection (ICSI), embryo cryopreservation and thawing, and preimplantation genetic diagnosis (PGD).

The fertility medicines are gonadotropins and Gonadotropin Releasing Hormone (GnRH) used to stimulate the follicular development inside the ovary and the couple is guided for well-timed intercourse to have the best chances of conception (Goodman and Gilman, 1984). The manual procedures include all types of ART used for forceful manipulation to induce fertilization in addition to medication too. Generally used ART techniques are IVF and its expansions which include ICSI, Gamete intrafallopian transfer (GIFT), Zygote intrafallopian transfer (ZIFT) and PGD etc. Serious fertility issues like endometriosis, male factor infertility, hormonal imbalance and unexplained infertility are known to be helped by the use of ART procedures. ART has revolutionized the field of gynecology and reproduction by helping the infertile couples in achieving conception. To have the best outcomes of the procedure an ART team should include physicians, embryologists, psychologists, laboratory technicians and nurses. (Malpani and Malpani, 2001)

Artificial insemination (AI) is the basic ART procedure use to achieve conception. In AI either the freshly ejaculated sperm or the semen sample which has been washed with culture media or frozen-thawed sample can be place into the reproductive track of female either in cervix called intracervical insemination (ICI) or in uterus i.e. intrauterine insemination (IUI) by artificial means. The semen sample is preferably washed, as raw sample can cause uterine cramping, expelling semen and causing pain due to contents of prostaglandin. IUI is the most popular type of artificial insemination as it by passes the hostile cervical mucus barrier which resists the sperm to enter. Hence this proves to be of great help for the patients with cervical mucus hostility.

In vitro fertilization is the first major type of ART with invasive management. The procedure involves hormonally controlled ovarian stimulation, then removing the eggs from ovaries with the help of transvaginal needle aspiration and letting the sperm fertilize them in the culture media. The fertilized egg is grown in the laboratory and then transferred back into the mother's uterus with the help of a catheter (Malpani and Malpani, 2001). Intracytoplasmic sperm injection (ICSI) is a recent advancement of IVF which is mostly used to fertilize the ova in case of severe male factor infertility. In ICSI the eggs are retrieved from the women ovary, washed off the cumulus and checked for maturity. Then mature eggs are injected with the morphologically normal looking sperm (one sperm into one egg) with the help of micromanipulators. The success rate of ICSI has proved to be equal or better than IVF fertilization. Major reasons to do ICSI include azoospermia, oligozoospermia, globozoospermia, poor ovarian response and PCOS.

Gamete intrafallopian transfer (GIFT) is another popular form of ART. In GIFT the male or female sex cells i.e. sperm and egg are mixed and injected to the fallopian tubes. After the gametes have been placed in the tubes, fertilization can takes place in the natural way and the embryo can travel to the uterus as it does in natural process. Zygote intrafallopian transfer (ZIFT) is an alteration of GIFT in which the egg and sperm are mixed in the laboratory to allow the sperm to fertilize egg. Once the pronuclear stage is visible, which is a sign of fertilization, the formed zygote is transferred to the fallopian tube during laparoscopy.

One of the major problems associated with all above mentioned procedures of ART is the low pregnancy rate despite the good quality embryos being transferred. It has been reported that one of the major reason of failure to implant is the thick shell of embryo called zona pellucida. Therefore, such embryo need assisted zona hatching (AZH) which is often referred to as zona drilling. This procedure thins out the shell of embryo either manually with the help of needles or laser assisted zona hatching thus improving the chances of implantation. Another issue faced by the infertile couples is the diagnosis of diseased embryos. Pre-implantation genet diagnosis (PGD) is a new technique which enables the physicians to identify the genetic disorders in the embryos before implantation.

1.4.1. ART- risks and complications.

The most commonly faced complication of IVF is ovarian hyper stimulation syndrome (OHSS) caused due to super ovulation. It is mostly observed in PCOS patients in whom the super ovulated ovaries with many follicles get loaded with estrogen. This estrogen rich fluid is from enlarged ovaries directly poured into the abdominal cavity after ovulation. It then coats the lining of peritoneum and makes it permeable. The ovaries become enlarge in size, abdomen get swelled up, resulting in low blood pressure and dizziness because of decreased blood volume. The OHSS if not managed timely and properly can result into severe health hazards and risks (Malpani and Malpani, 2001) (Zhang *et al.,* 2008).

Another risk factor associated with IVF is increased chance of multiple pregnancies as commonly more than one embryo is transferred during an IVF cycle. Some couple opts for a recent treatment option for this issue which is called as selective fetal reduction. But this involves the risk of all fetuses being lost. Hence it is preferred in recent IVF to transfer single best quality embryo by static morphological selection or extended embryo culture technique which naturally permits the best viable embryo to survive (Kurinczuk *et al.,* 2004). The emotional stress level of couples suffering from infertility is known to us. The ART procedures are lengthy as well as costly and create

high hopes but are more likely to fail than to succeed. The unsuccessful couple often suffers frustration leading to depression which could be detrimental for their health and wellbeing.

1.4.2. Recent developments of ART

There are certain recent advancements of ART helping to improve the IVF success rate. In order to evaluate the quality of eggs or determine if the egg is defective or carrying abnormal genetic package a new test is being recommended which is called the spindle view. It helps to detects whether there is no spindle or a spindle with incomplete genetic material which can result into defects like Down's syndrome or cerebral palsy.

Porcu *et al.* (2004) has documented that another recent technique used for embryo cryopreservation called 'vitrification' has improved the freeze-thaw cycle of the embryo noticeably. The vitrification involves the processing the embryo in the specific freezing solutions and then plunged into the liquid nitrogen. The quick temperature drop and high concentration of antifreeze prevents the water in the cells from turning into ice thus avoiding the formation of ice crystals which can damage the embryo. The slow freezing process involves the formation of ice crystals which harm the egg quality and results into poor thawing.

IMSI is a modified form of ICSI (Figure 1.3) in which the spermatozoa is selected at a high magnification than conventional ICSI. This procedure is offered to the patients with severely low sperm count and morphological defects of spermatozoa. It can help to improve the fertilization rate as compare to conventional ICSI **(**Van Voorhis, 2007).

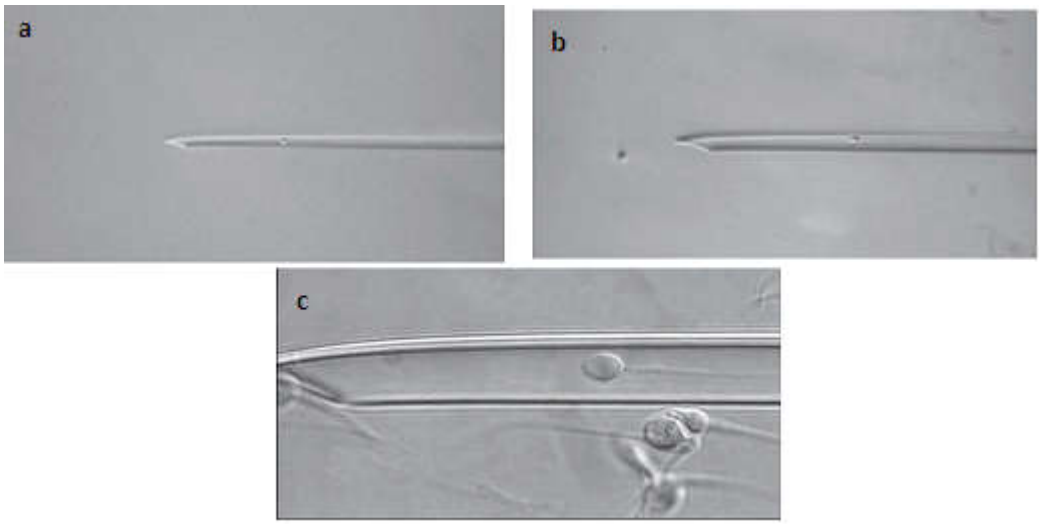


Figure 1.3.Intracytoplasmic morphologically-selected sperm injection (IMSI).

A human spermatozoon shown at different magnifications: morphological appearance in microinjection pipette (**a**) ×200 undetectable, (**b**) ×400 undetectable, (**c**) ×5880 normal morphology.(Antinori1 *et al*., 2007)

Time Lapse embryo culturing is another novel technique which evaluates the development of embryos without removing them from the optimal culture conditions of the incubator. The embryo scope incubator has been found to be safe thus potentially producing more number of healthy blastocysts (Cruz *et al.,* 2011; Meseguer *et al.,* 2012; Kirkegaard *et al.,* 2012a; Barlow *et al.,* 1992).

1.5. FACTORS AFFECTING THE ART OUTCOME

1.5.1. Aging

Aging affects the number of oocytes that can be retrieved with the use of controlled ovarian hyper stimulation (COH). Oxidative stress (OS) increases with age (Kregel and Zhang HJ, 2007), owing in part to lowered endogenous antioxidant defenses generating tissue glutathione levels (Suh *et al.,* 2014). OS is associated with increased granulosa cell (GC) apoptosis, which is in turn associated with reduced embryo quality and less successful births (Dumesic *et al.,* 2015; Tatone and Amicarelli, 2013).

Assisted reproduction treatments such as ovulation induction and IUI are commonly offered to increase the chances of pregnancy. This presumption works with the younger women but the success rate declines in older women even after medical interventions (Reindollar *et al.,* 2010). Similarly the risk of aneuploidy also rises as the women get older. The most common known aneuploidies are due to meiotic spindle abnormalities known to occur in older oocytes, thus resulting chromosomal nondisjunction and abnormal embryos that results in spontaneous abortion (Fragouli *et al.,* 2013; Warburton, 2005). The risk of trisomy rises from 1 in 1,000 to 1 in 30 in women aged 30 years vs. 45 years (Snijders *et al.,* 1999).

As far as the embryo morphokinetic is concerned (Bos-Mikich *et al.,* 2001) it was observed that early cleavage (EC) phenomenon is affected by maternal age, younger women are more commonly observed with early cleaving embryos with highest implantation rates. Many other findings have also documented the effect of maternal age on stimulation protocol to induce follicular development, ovulation induction, embryo quality, gestation and implantation rates (Lass *et al.,* 1998; Schoolcraft *et al.,* 2000; Wittemer *et al.,* 2000). Whereas Gardner and Schoocraft(1999) reported no significant difference among the blastocyst grading of different age groups.

The observed decline in female fertility is most likely due to decrease ovarian reserves and endometrial receptivity with increasing age thus resulting in poor ART outcome (Hassan and Killick, 2003; Battaglia*et al.,* 1996; Frattarelli*et al.,* 2008;

Whitcomb*et al.,* 2011; Tsai *et al.,* 2013). Decreased ovarian reserve and endometrial receptivity resulting from increased age are likely reasons for this observed reduction in fertility (Ishikawa *et al.,* 2009; Ron-El *et al.,* 2000), beginning at the age of 37 years (Ron-El *et al.,* 2000 ; Gougeon *et al.,* 1994; Devroey*et al.,* 1996; Faddy and Gosden, 1996; Hull*et al.,* 1996; Marcus and Brinsden, 1996). It has been reported historically that the patients 40-49 years old showed poor pregnancy rates as compare to those less than 40 years of age (Flamigni, 1993; Borini *et al.,* 1996). Same results have been documented by Devroey*et al.,* (1996) that the delivery rates of women ≥ 40 years were 8.5% and ≤ 40 years were 25.4% per ET. In addition to this the chances to conception has also shown to be low in women older than 41 years (Ron-El *et al.,* 2000).

The success rate of ART declines with increasing age (Van Voorhis, 2007). The ART is unable to overcome the detrimental effect of age on fertility but may proves to be of some benefits like it can increase the chances of pregnancy and shorten the duration of infertility. It is known well that ovarian hyperstimulation results in the production of some non-competent oocytes which do not reach ovulation in natural conditions (Baart *et al.,* 2007). Hence in a randomized controlled trial (RCT) the mild and conventional hyperstimulation protocols were compared and it was found that the number of oocytes retrieved was higher in hyperstimulation protocol than mild one but the chances of pregnancy did not differ (Heijnen *et al.,* 2007). Overall there is insufficient knowledge regarding the relation between the competence of oocytes and hyperstimulation regimen for a definitive conclusion.

1.5.2. FSH and AMH defining the infertility

Age is however very important but not the single predictor of IVF outcomes. Functional ovarian reserves (FOR) is the term indicating the growing follicle pool i.e. egg and embryo numbers (Barad *et al.,* 2007). Abnormally low FOR (LFOR) is defined by increasing FSH and decreasing AMH (Antimullerian hormone) with age (Barad *et al.,* 2011). LFOR is associated with declining embryo quality and pregnancy rate also (Lukaszuk *et al.,* 2013). These two hormones have different degree of significance in defining FOR at different ages of female (Gleicher *et al.,* 2012).

The gonadotropins FSH and LH are produced in the anterior pituitary, whereas hCG is largely produced in the placenta during pregnancy (Arey and Lopez, 2011). The FSH helps in proliferation and maturation of granulosa cells (Leao and Esteves, 2014). It also promotes the development of LH receptors in granulosa cells, which helps in ovulation (Yong *et al.,* 1992; Chappel and Howles, 1991). Several parameters have been suggested as predictor of ovarian reserves e.g. the FSH, inhibin B, 17-β-estradiol and AMH. Ultrasound variables like the ovarian volume, antral follicles count and measurement and ovarian stromal blood flow have also proved to be of predictive value (Broekmans *et al.,* 2006). The best predictive measure have been suggested as basal level of FSH on third day of menstruation and the AMH levels which emerges as a new marker of ovarian reserves (Tinkanen *et al.,* 1999; Hansen *et al.,* 2011). The AMH also called as Mullerian-inhibiting substance (MIS) is a dimeric glycoprotein (Cate *et al.,* 1986) and a member of the transforming growth factor β family (di Clemente *et al.,* 2003). AMH is produced entirely by gonads, in females by granulosa cells of antral follicles (Weenen *et al.,* 2004). It seems to be derived only by ovary hence premenopausal women with bilateral oophorectomy and menopausal women are presented with undetectable AMH levels (La Marca *et al.,* 2009).

The AMH is secreted by the granulosa cells into the blood stream and continues to be expressed in growing follicles till the size of 5-6 mm and a differentiation stage when they become responsive to exogenous FSH and may be selected for dominance (Ledger, 2010; Durlinger *et al.,* 2002). Its expression declines as the follicular maturation starts. (Weenen *et al.,* 2004; Laven *et al.,* 2004). Antral follicles are the first major source of AMH as they contain the large number of granulosa cells (La Marca *et al.,* 2009). AMH also acts in pre-granulosa cells in order to limit the number of recruited oocytes in addition to modulation of follicular recruitment (Visser and Themmen, 2005). This hormone may serve as an effective tool to differentiate the various reasons of secondary oligo-amenorrhea (Li *et al.,* 2011). In addition to these important functions the AMH levels may also be used to detect the extent of ovarian damage after surgery or chemotherapy (Lie *et al.,* 2008; Lutchman *et al.,* 2007) and also for follow up of

granulosa cell tumors (La Marca and Volpe, 2007). Ovarian aging is represented by a decline in quantity and quality of oocytes in the ovary (Loh and Maheshwari, 2011). It is reported that fertility decline 13 years before menopause (Nikolaou and Templeton, 2003). This premature ovarian failure is also associated with low or undetectable serum AMH levels (de Koning *et al.,* 2008; Knauff *et al.,* 2009). According to Broer *et al.* (2011) the age range in which menopause can occur can be calculated using AMH and age.

1.5.3. Polycystic ovary syndrome and obesity

Rates of overweight and obese women (OW/OB) as defines by BMI within the ranges 25–29.9 and .30 kg/m2, respectively show a continuous rise in reproductive age. The obesity has negative affect on reproduction in terms of reduce conception, increase rate of miscarriage (Boots and Stephenson, 2011), and fetal complications (Balen and Anderson, 2007).Obesity is an important factor affecting the IVF outcome. It is a state of high OS (Matsuda and Shimomura, 2013) in follicular fluid (Bausenwein *et al.,* 2010), and is associated with decreased clinical pregnancy (Luke *et al.,* 2011). The effect of obesity in recipients of egg donation in some studies suggests effects on the endometrium as well as the oocyte (Bellver *et al.,* 2013a). The incidence of IVF live birth is reduced even more when obese women have twins (Dickey *et al.,* 2012). Obesity also increases miscarriage (Veleva *et al.,* 2008). All obese women, particularly with a BMI >35 kg/m2, should be advised of these risks and encouraged to lose weight before starting IVF, especially old age women. An exercise program may be the most reasonable strategy to overcome the effects of obesity. In a case-control study the vigorous exercise program was found to be associated with a more than threefold increase of the clinical pregnancy rate in obese women having IVF (Palomba *et al.,* 2014).Moreover, being overweight in pregnancy increases the chance of developing gestational diabetes. It is also shown to be associated with cardiovascular and metabolic diseases in the offspring in later life. (Lawlor *et al.,* 2012; Reynolds *et al.,*2013).

Polycystic ovarian syndrome as defined by the Rotterdam criteria has indications of hyperandrogenism, Oligo- or amenorrhea and presence of at least one polycystic ovary on ultrasound (ESHRE, 2004). Approximately 60% women with PCOS are obese and

40% normal weight women have insulin resistance (IR). The polycystic ovarian syndrome has strong association with infertility, hyperinsulinemia, IR, impaired glucose tolerance, dyslipidemia, and obesity (Carmina and Lobo, 2004; Legro *et al.,* 2004). PCOS is a common endocrine metabolic disorder, affecting 5–10% women in reproductive age (Svendsen *et al.,* 2010). The multiple metabolic endocrine disorders affect the folliculogenesis and maturation of oocytes (Desforges-Bullet *et al.,* 2010). But it was found that PCOS and non PCOS patients do not show any significant difference in pregnancy rates (Heijnen *et al.,* 2006). Obesity shares common features with PCOS regarding insulin resistance and low-grade chronic inflammation.

1.5.4. Endometriosis

Endometriosis is an estrogen dependent chronic inflammatory disease affecting the women in their reproductive age resulting pelvic pain and infertility (Vercellini *et al.,* 2014; Gylfason *et al.,* 2013). The prevalence of this disease is~5% with a peak observed among 25 and 35 years of age. The annual incidence of disease is 0.1% among women aged 15-49 years. The exact cause of the disease is still not known although many theories about the pathophysiology have been proposed (Viganò *et al.,* 2012).

The extent of the disease could be in various forms, ranging from a few implants on pelvic peritoneum to extensive adhesions even outside the pelvis. The pain and sub fertility associated with the disease co-relate with the extent of endometriosis. The American Society for Reproductive Medicines (ASRM) has published the classification of disease in 1979 and revised in 1996 which is based on localization, size and extent of adhesions (ASRM, 1997). The disease has been classified as minimum, mild, moderate and severe endometriosis. This scoring does not consider the depth of lesions hence it has been remained unclear for many years that weather ASRM classification has a prognostic value regarding the women fertility (Guzick *et al.,* 1997). Recently another system of disease classification has proposed which is called Endometriosis Fertility Index (EFI). This system includes the basic classification from ASRM combined with anamnestic and post-surgical information (Adamson and Pasta, 2010). The EFI system proposed 0 to 10 point scoring for the disease and after three years the patients with the score 0 to 3 showed 10% chances of pregnancy while those with 9 to 10 points had 75% success rate of IVF (Tomassetti *et al.,* 2013; Boujenah *et al.,* 2015), the latter study including results from both non-IVF and IVF treatment.

The hormonally responsive endometriotic lesions can develop into chronic inflammation thus leading to pelvic adhesion, pain and infertility (Taylor and Lebovic, 2014). The clinical investigations have shown that in some women with established endometriosis lesions, the pain and subfertility increases as the disease progresses (Koninckx *et al.,* 1991). Hence, there is an association between the severity of disease and infertility although, the strength of this association is variable (Guzick *et al.,* 1997). The rate of conception among the minimum to mild diseased women who conceive

without treatment is around 50% whereas the moderately diseased women show 25% conception (Olive et al. 1985). The rate of pregnancy among women with unexplained infertility and with mild endometriosis has proved to be comparable, thus showing that minimum condition of disease have reduced effect on fertility (Bérubé *et al.,* 1998). Chronic condition of endometriosis can affect the fertility in many ways. An increased concentration of IL1b, IL8, IL10 and TNF alpha in the follicles near to lesions is associated with low ovarian response (Opøien *et al.,* 2013).

In normal fertile women with the LH surge determines follicle ruptures and releases the oocyte within 38 hours. The luteinized unruptured follicle syndrome (LUF) is the condition in which follicle fails to rupture in spite of luteinization hence ovum is not released. The patients with endometriosis show a high prevalence of LUF syndrome than normal women (Dmowski *et al.,* 1980). In addition, non-steroid inflammatory drugs (NSAIDS) which are often prescribed to patience for dysmenorrhea also contribute to the risk of LUF syndrome. NSAIDS inhibit cyclooxygenase with a resulting low prostaglandin production in the ovaries, inhibition of matrix metalloproteinases, and loss of follicle rupture (Smith *et al.,* 1996). The coordinated muscular contractions in uterus help the sperm through its way to fallopian tube where it under goes capacitation and ultimately fertilizes the ovum ampullary part of the tube. In endometriosis uterotubal dysperistalsis may contribute to infertility by disturbed transport of gametes and embryos (Leyendecker *et al.,* 1996).

The prevalence of endometriosis is 0.5 to 5 % in fertile women and 25 to 40 % among infertile (Ozkan *et al.,* 2008). In vitro fertilization is the major treatment options for women with endometriosis. The endometriosis have a strong effect on the success rate of IVF but the mechanism involve is poorly understood (Olivennes, 2003; Barnhart *et al.,* 2002). The most probable reason of endometriosis related infertility is thought to be altered pattern of folliculogenesis and poor egg quality due to increased espouse to a hostile environment of macrophages, cytokines and vasoactive substances in the peritoneal fluid (Pellicer *et al.,* 1998a; Matalliotakis *et al.,* 2007). The distortion of pelvic anatomy due to organ adhesions found in mild to severe conditions of the disease may

affect the oocyte development embryogenesis and implantation (Gupta *et al.,* 2008). The ART being the major treatment option have provided the highest success rate in women suffering from endometriosis (de Ziegler *et al.,* 2010).

1.5.5. Tubal factor

Tubal factor infertility is the most common cause of female infertility. The disease causes the adhesions due to peritoneal pathology (Miller *et al.,* 1999). As pelvic infections increase the risk of infertility rises hence the incidence of infertility is 10% - 12% after one episode, 23% - 35% after two episodes, and 54% - 75% after three episodes (Westrom, 1975; Westrom, 1994). Other reasons of tubal factor infertility include scarring from abdominal and pelvic surgeries. The risk of ectopic pregnancy also gets increases six to seven times after an episode of PID (Westrom *et al.,* 1992). Bilateral tubal ligation is an iatrogenic cause of tubal blockage. A hysterosalpingogram (HSG) after traditional or laparoscopic tubal ligation is performed after the 3 months to document occlusion.

Tubal disease is common in 25 to 35 % of female infertility. The severity of disease can affect the proximal, distal or entire tube. Pelvic inflammatory disease is the cause of 50 % of tubal factor cases (Honore, 1999). The tubal damage can be categorized as mild/grade I, moderate/grade II and severe/grade III (Akande, 2004). Diagnosis is made by hysterosalpingography (HSG) or laparoscopy and the treatment options are majorly the surgical repair and IVF which completely by passes the tubal blockage and offers 18 to 29 % love birth rate per cycle (AIHW 2012; SART 2014).

1.5.6.Male factor

The worldwide population experiencing infertility is over 80 million and cause of 1/3 is male factor infertility. The main cause of the sperm dysfunction is premature acrosomal reaction (AR) and AR failure (Liu *et al.,* 2006). The AR is mainly involved in two key functions. First, the proteolytic enzymes (hyaluronidase and acrosin) in the acrosome play a role in the penetration of sperm in the zona pellucida of the oocyte. In second step the acrosome content reveals a new surface membrane called inner acrosomal membrane (IAM) which is required for fertilization (Baba *et al.,* 1994). The ICSI

procedure have provide the best options for patients with severe oligospermia and azoospermia to get the successful fertilization with the help of Microepididymal sperm aspiration (MESA) and testicular sperm aspiration(TESE) (Bromage *et al.,* 2007).

Approximately 15% couples in Poland suffer infertility problems and 20% are with idiopathic infertility. The most common cause of reproductive failure is genetic integrity of the sperm. Sperm DNA fragmentation (SDF) can be seen in alive, normal looking sperm which can fertilize an oocyte hence it is difficult to detect the SDF using common fertility tests (Bungum *et al.,* 2011; Aktan *et al.,* 2013) thus SDF has been shown to affect the embryo growth, pregnancy rate as well as implantation and pregnancy outcome (Tamburrino *et al.,* 2012).

The paternal influence is not only observed in oocyte and embryo quality but also on early embryonic development (Tesarik *et al.,* 2004). The lack of sperm-specific activating protein and centrosome defects can affect the early cell divisions but cannot be detected until 8-cell stage when sperm-derived genes start to express themselves. This late paternal affect is associated with DNA fragmentation and disorganization of sperm chromatin (Knez *et al.,* 2013). The introduction of a new technology called motile sperm organelle morphology examination (MSOME) helps to evaluate the nuclear morphology of spermatozoa at a high magnification, thus making it easy to select spermatozoa without the nuclear vacuoles in the head (Bartoov *et al.,* 2001). Literature shows that large nuclear vacuoles (LNV) in the sperm head indicate nuclear dysfunction, failure of chromatin condensation and packaging (Franco *et al.,* 2012; Utsuno *et al.,* 2014). The LNV can be detected after the onset of EGA (Embryonic Gene Activation) and has been reported to associate with decreased blastocysts formation, pregnancy rates and increase miscarriage rates (Setti *et al.,* 2014; Cassuto *et al.,* 2014).

The treatment options for couples suffering from male factor infertility depend upon the sperm parameters. If the total motile count (TMC) of unprocessed semen sample is 13106-33106, which is an indicative of mild to moderate oligo-astheno-

teratozoospermia, IUI can be offered as a first option of treatment but only when at least 0.83106 motile sperm can be recovered after the processing of semen samples. The IUI is although cost effective treatment but has been reported that if the post processing TMC is 13106, IUI has a very low chances of conception. Instead of IUI the IVF/ICSI or combination of both may be a better option for a couple presenting with severe male factor infertility (Goverde *et al.,* 2000; Van Weert *et al.,* 2004). The gonadotropins (hormone that stimulate sperm production) can be used for male idiopathic subfertility i.e. subfertility with an unknown origin (Attia *et al.,* 2013).

1.5.7. Unexplained infertility

The clinical investigation for infertility is usually advised after trying unsuccessfully to conceive a year. Unexplained infertility is a subject of discussion among practitioners. It usually refers to lack of diagnosis in spite of all standard investigations i.e. test of ovulation, tubal patency and semen analysis and all being in normal range (Smith *et al.,* 2003). Unexplained infertility is found among 30 to 40 % infertile couples. The probable reasons for unexplained infertility may be hormonal imbalance, immunology, genetic and reproductive physiology (Pellicer *et al.,* 1998b). However, when there is no explanation for infertility it proves to be frustrating for the couple when duration exceeds three year and female is above 35 years of age. Hence, the treatment is usually advised when duration is more than two years and female is above 35 years age (Collins *et al.,* 1995). The prevalence of unexplained infertility is still debate able because there are chances of misdiagnosis due to lack of specific tests. According to ASRM (1992, 2006) the standard evaluation for unexplained infertility should include a semen analysis, post coital test, ovulation test and check of tubal potency.

1.6. ART and the stimulation Protocols

The aim of ART is to help the infertile couples by providing them with a chance of healthy and normal pregnancy which is directly related to the choice of the best embryo for implantation. Embryo quality is an important predictor of successful

implantation and healthy pregnancy. The use of morphological evaluation for selection of best embryo for implantation has been in widespread use in the history of ART and still this strategy is considered to be the best criteria for selection of competent embryos. (Gardner and Schoolcraft, 1999; Gardner and Sakkas, 2003).

The choice of controlled ovarian stimulation (COS) protocol is very important in predicting of the outcome of the ART cycles and it mainly depends upon the clinical indications of the couple (Figure. 1.4). The most commonly used protocols are GnRH agonist and antagonist protocol (Mun˜oz *et al.,* 2013). It has been demonstrated previously that the efficiency of ART cycles increases with the increase in number of oocyte up to a certain threshold value. The efficiency of ART cycles decline if the number of oocyte increases more than 15 per cycle (Sunkara *et al*., 2011). The boundary criteria for prediction of hyper stimulation are considered to be the presence of 14 antral follicles at the time hCG administration on final maturation of oocytes. (Kwee *et al*., 2007).

The TL imaging has provided us with the non-invasive strategy for embryo scoring and selection. The first model presented for the embryo evaluation based on time laps imaging was presented by Meseguer *et al*., (2011). This model include the morphological evaluation in the absence of any exclusion criteria and up till division to 5 cell stage, duration of second cell cycle and synchrony of division from 2-4 cell. It produced ten categories of embryos with respect to implantation potential (Fréour *et al*., 2015). Recently another study has presented the model based on timings of start of blastulation and the time of full blastocyst stage in order to select the chromosomally normal blastocyst with high implantation potential (Campbell *et al*., 2013a, b). The same criteria were applied by two more groups but they found no significant difference in the proportion of euploid or aneuploid embryos while comparing the kinetic data (Kramer *et al*., 2014; Rienzi *et al*., 2015). In contrary to time laps imaging PGD is an invasive method of embryo selection. Initially it was applied for investigation of inheritable sex-linked diseases but later on it also include the comprehensive chromosomal screening to know the genetic status of the embryos i.e. preimplantation genetic screening (PGS)

(Greco *et al*., 2013 ; Handyside *et al*., 1990). This technique has provided enormous help to the categories of patients with history of genetic diseases, in women with advance maternal age and with history of recurrent miscarriages (Kahraman *et al*., 2000; Rubio *et*

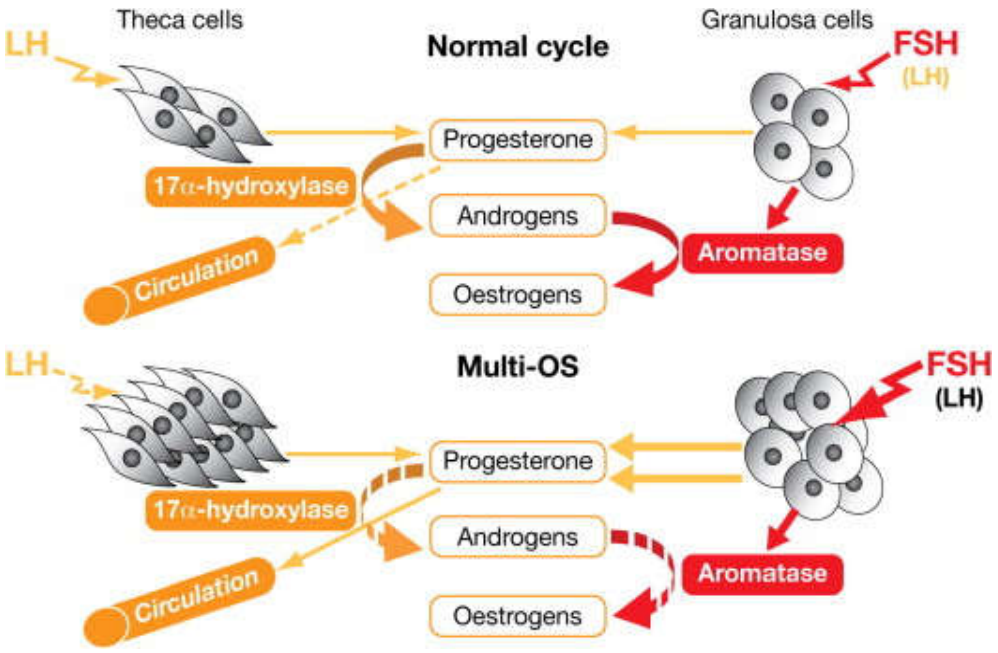


Figure 1.4. Steroidogenesis during normal follicular phase and following the ovarian Stimulation (Fleming and Jenkins, 2010).

al., 2003; Greco *et al*., 2014). More recently the embryo biopsy at blastocyst stage has proved to give more accurate for diagnosis the genetic disorders (Scott *et al*., 2013a; Minasi and Greco, 2014).

It is a well-known fact that fertility declines with advanced maternal age and female age is a very important factor determining the success rate of IVF cycle (Faddy *et al*., 1992). The ART can help the younger patients undergoing treatment to get pregnant but it does not promise to help out the age dependent decline in female fertility (Malizia *et al*., 2009). The increasing female age results in low number of oocytes, poor oocyte and embryo quality (Malizia *et al*., 2009) and low implantation (Spandorfer *et al*., 2000),

pregnancy and live birth rates. The increased aneuploidy rate results in increased miscarriages in women above 40 years of age (Andersen *et al*., 2000). The management of IVF cycle in case of poor responders is always has been a challenge as these patients lie among the cases with worst prognosis. Their treatment often results in low fertility and poor clinical outcomes (Jovanovic *et al*., 2011).

1.7. Time-Lapse imaging and ART

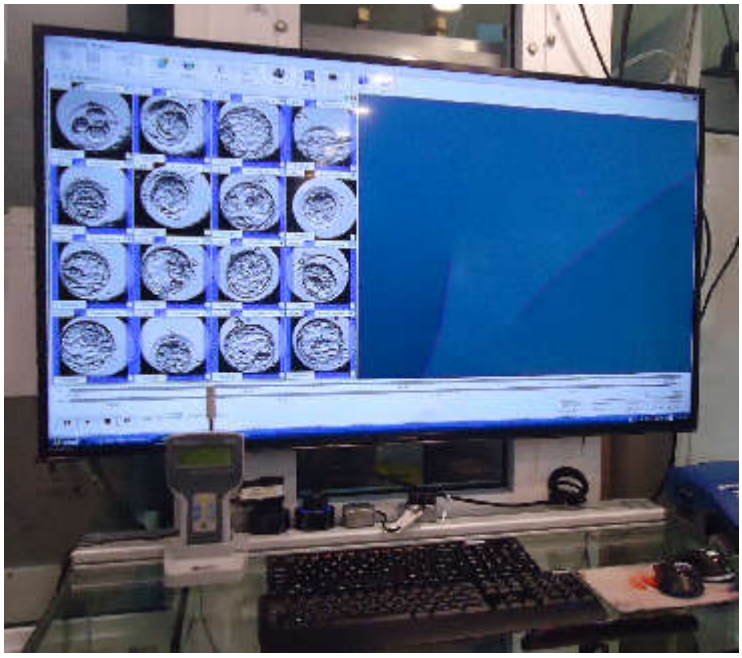
ART has resulted in more than five million babies born worldwide. There are enormous improvements in this field but still certain problems exist. First is that clinical pregnancy rates (PRs) are around 30% i.e. still low and secondly the multiple pregnancy rates are high i.e. 20% as high number of embryos are being transferred (Kupka *et al.,* 2014). Multiple pregnancies not only add to medical complications to the mother and the baby (hypertension, low birth weight and neurological damage), but also they induce social and financial problems. Hence there is a need to improve the embryo selection techniques. It is here where genetic screening (improving selection based on genetic and chromosomal viability) and time-lapse embryo culture (selection based on morphokinetic markers) may play a major role. The currently used embryo selection criteria in most IVF laboratories are the daily assessment at defined hours of the day-2 or day-3 of embryonic development. But the nonspecific and subjective selection may not help to select the best embryo for transfer. Extended embryo culture and transfer at blastocyst stage (Day 5 or 6) is another method to select the best quality embryos and minimize the risk of multiple pregnancies (Strauss *et al.,* 2008).

The Time-Lapse system (TLS) combines three basic elements: an incubator, an optical microscope, and a software program (Figure 1.5 a, b). These three elements when used combined with optimal culture conditions can help to select the best embryos qualitatively after analyzing the developmental stages with respect to the cleavage hours (Wong *et al.,* 2010; Azzarello *et al.,* 2012). The kinetic markers identified by TLS are still under consideration for their clinical value in embryo selection

1.7.1. Time-Lapse microscopy and kinetic markers of embryonic development

The Time Lapse System has been used for many years now, especially in research (Cole 1967; Massip and Mulnard, 1980; Wale and Gardner 2010). In 1997, Payne`s group (Payne *et al.,* 1997) was the first one to describe the events from 17-20 hours after ICSI i.e. first polar body extrusion and appearance of pronuclei . Some years later, Mio and Maeda described the kinetics of events until blastocyst stage (Mio and Maeda 2008) including the fertilization and the developmentof the embryo from 2- cell stage to a hatched blastocyst. A few years later, Pribenszky *et al.* (2010b) reported the first live birth after Time-Lapse assessment of a five embryo cohort up to blastocyst stage. Nowadays, this technology is becoming more suitable for clinical use due to automation and advancement in the field of bioinformatics.

(a)



(b)

Figure 1.5. (a) The conventional IVF incubator showing the Time-Lapse setting. **(b)** Time-Lapse imaging displayed on monitor.

The TLS is not only helpful in determining the exact timings of cell divisions but also for morphological evaluation of the embryo. The detection of formation and resorption of fragments, irregular divisions, compaction and start of blastocoel cavity are very important phenomena to evaluate embryo quality which can be analyzed easily by using TL imaging (Pribenszky *et al.,* 2010a; Hardarson *et al.,* 2002; Van Blerkom *et al.,* 2001) (Figure 1.6). It has been documented that embryos cleaving within 25 hours after ICSI or IVF show better pregnancy and implantation rates as compared to those cleaving late. (Stensen *et al.,* 2015; Shoukir *et al.*, 1997). The same results have been reported by Sakkas *et al.* (1998) for ICSI also. Hence the early cleavage pattern of the embryo is found to affect its quality and pregnancy outcome.

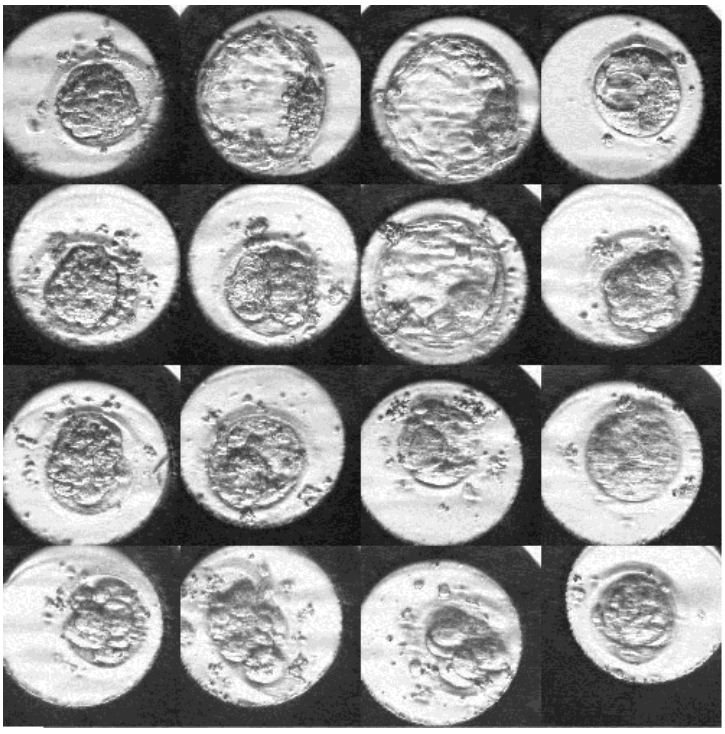


Figure 1.6. Normal developmental events of human embryo shown with respective time-points.

The recent publications have shown that the conventional embryo culturing does not indicate the anomalous development patterns like abnormal syngamy, first cytokinesis and as reverse cleavage (RC) as shown in Figure 1.7 (Athayde Wirka *et al.,* 2014). It has also been shown that embryos reaching 8 cell stage earlier form batter quality blastocysts and show batter implantation rates. (Dal Canto *et al.* (2012). Although it has been accepted that TLS is an effective tool for embryo analysis but it does not take into account the other variables of IVF treatment such as culture conditions, ovarian stimulation protocols used and the patient's characteristics (Racowsky *et al.,* 2015).

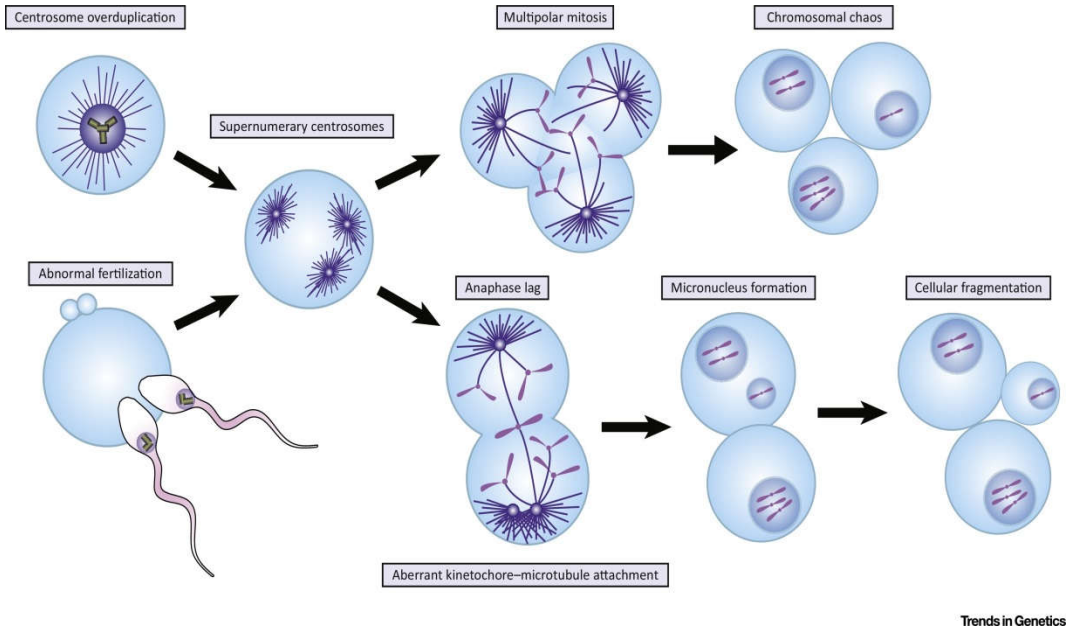


Figure 1.7. Abnormal pattern of human embryo cleavage. (Rajiv, 2017)

1.7.2. Aneuploidy and morphokinetic analysis

Aneuploidy affects the outcome of ICSI/IVF by increasing the miscarriage rate and lowering the live birth rates **(**Franasiak *et al.* 2014). To help this PGS has been used as an embryo selection method, although it is an expensive procedure and readily

available at every laboratory. Taking this into consideration some research groups have focused on morphokinetic analysis of embryos to select the chromosomally normal and abnormal embryos in order to help the couples who do not want to choose PGD/PGS due to economical or ethical reasons**.** Magli *et al.* (2007) observed that slow and arrested embryos as well as those with accelerated cleavages show a high rate of abnormalities.

In the same way Campbell *et al.* (2013a), showed by TLS that embruos with multiple aneuploidies show a delayed growth and blastocysts formation. Chawla *et al.,* 2015 reported a significant difference for mean time of PNf (pronuclear fusion) t2, t5, t5-t3 and t5-t2 among normal and abnormal embryos. Similar to this Basile *et al.* (2014) also showed that t5 - t2 and t5-t3 are the most relevant kinetic variables related to normal chromosomal content.

Rienzi *et al.* (2015) on the other hand found that morphokinetic parameters have no correlation with embryo aneuploidy. This may happen due to inter laboratory variations, differeny culture conditions, different population studies and female age. Nevertheless, the use of this technology is relevant because it allows us to analyze and compare the development of an abnormal embryo versus a normal one.

1.7.4. Time-Lapse imaging and future modifications

In spite of the intensive research in the recent fields of human reproduction, the static morphological assessment of human embryos is based on blastomeres count, symmetry, fragmentation, appearance of blastocoel cavity, ICM and TE quality. The TLS is also providing information about the growth and quality of embryo but additionally due to this semi-automated system it has become easy to collect and analyze the data from different IVF centers. Secondly the incubation of embryonic development can be shared through internet from different centers. Similarly through TLS system we get the more precise knowledge of fertilization and different cleavage events (ESHRE 2011). Hence the embryo selection process has become more precise and improved (Azzarello *et al.,* 2012).

Aims of the study

The specific aims and objectives of the current study were as follows.

1. To assess the morphokinetic parameters of embryonic development among infertile couples of our population and correlate them with their pathological conditions.

2. To correlate the clinical outcome of IVF/ICSI treatment cycles with embryonic developmental potential taking into consideration the Time-Lapse evaluation.

3. To compare the important morphokinetic markers of our population with the known values studied previously in different populations.

Chapter # 2

Materials and Methods

GENERAL MATERIALS AND METHODS

Ethical approval of the study

The data used in this study were obtained from Islamabad Clinic Serving Infertile Couples (ICSI), Islamabad, Pakistan from January 2013 to December 2015 after an informed consent of patients in accordance with ethical guidelines set by the clinic for IVF cycles.

Study Design

This study was a retrospective observe of prospectively obtained data of time-lapse imaging of human embryos during in vitro development. The procedures and protocols were approved and practiced by the Institution. The number of oocytes observed in this study was 4080 resulting into 2266 cleaved embryos in 200 ICSI (Intracytoplasmic sperm injection) treatment cycles. A total number of 1704 embryos were included in time-lapse monitoring due to some split cycles for conventional culturing as a quality control measure at the outset of time-lapse system in the clinic. For first analysis the patients were divided into five age groups to investigate the effect of female age on human embryo morphokinetics. The second analysis comprised of eight pathology groups to study the variation in time-lapse parameters of human embryo development in different pathological conditions of both male and female partners and also to evaluate the effect of age and pathologies on ART outcomes in the study population. The third analysis included three groups based on the ovarian stimulation protocols. Most of the embryos were obtained after fertilization by ICSI while very few patients had ICSI+IVF cycles. Embryos were evaluated for time-lapse morphokinetics by measuring precise timings of development. Ten important time-points for embryo development were compared. Embryo transfer data of both fresh and cryopreserved cycles of patients was analyzed. Implantation was confirmed by the βhCG levels in the blood sample taken after 10 days of embryo transfer and later on at an ultrasound scanning for fetal heart after 7 weeks of pregnancy.

The time-points (hours post ICSI) analyzed along with the abbreviations used to indicate the corresponding developmental stages and also the reference range used for each time-point (Herrero *et al.,* 2013) is as follows :

a. Two pronuclear stage (tPNa) = 17±1 hrs.
b. 2-cell stage (t2) = 26±1 hrs.
c. 4-cell stage (t4) = 44±1 hrs.
d. 8-cell stage (t8) = 68±1 hrs.
e. Compaction (tM) = 92±2 hrs.
f. Fully Expanded blastocyst (tB) = 116±2 hrs.

In addition to these, the embryos were analyzed for four more time-points i.e. at 3-cell cleavage (t3), 5-cell cleavage (t5), start of blastulation (tSB) and at expanding blastocyst stage (tEB) in order to assess the developmental events more critically. The reference range for these four time-points was taken from the average of optimally growing embryos at the study set up. Thus time range for t3 was taken as (31-42 hrs.) for t5 (45-54 hrs.) for tSB (98-105 hrs.) and for tEB (107-114 hrs.) respectively.

TIME-LAPSE SYSTEM

The Primo Vision Time-Lapse System (Primo Vision^{TM} V4.4; Frolunda, Sweden) consists of 1-6 Microscopes Units, an external Controlling unit that runs the installed Capture and Analyzer software, and microwell culture dishes for in vitro culture of the observed embryos. USB and power cables are also included. A specially designed compact, airtight, digital inverted microscope (Primo Vision; Cryo-Innovation, Budapest, Hungary) was placed inside a common multi gas incubator (Sanyo, Japan) (Figure 2.1 a,b). Primo vision culture dishes were placed on the top of the glass window, into the sample holder of the microscope and the objective was mechanically focused. The culture dish enabled all the embryos to be positioned in the field of view. The computer screen displayed the actual developmental stages of the embryos, while all the images recorded earlier were saved to be analyzed later with software. The embryos were not moved or disturbed in any way for the whole period (5-6 days) of development, completely eliminating sheer stress.

(a)

(b)

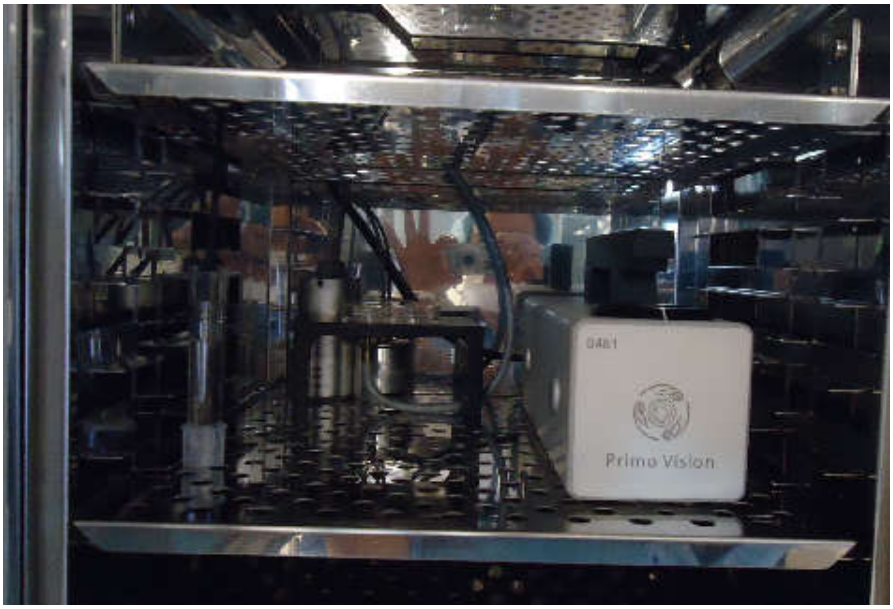


Figure 2.1. (a) The display of Primo Vision time-lapse embryo monitoring system. **(b)** Setting of Primo Vision microscope in a conventional IVF incubator.

DOWN REGULATION AND OVARIAN STIMULATION

For pituitary desensitization both long and short protocols were used. Women were given a subcutaneous administration of GnRH-agonist (gonadotropin releasing hormone) depot preparation (Decapeptyl 3.75 mg) or Buserelin acetate daily injections (suprefact 0.1 mg) until follicles mature. The doses of FSH were adjusted according to the age of patient, AMH levels and AFC. The subsequent doses were adjusted according to follicular growth monitored by transvaginal ultrasound (7.5 MHz probe; Aloka 500, Tokyo, Japan) three to four days after commencement of the ovarian stimulation.

The stimulation protocols used were either GnRH- agonist or GnRH-antagonist. In the GnRH agonist group, 0.1 mg of Buserelin acetate (suprefact) or Decapeptyl (0.1mg or 3.75 mg) was administered daily, commencing on day 21 of the previous menstrual cycle. Recombinant follicle-stimulating hormone (Follitrope; LG Life Sciences, Korea or puregon; Organon, Ireland) and human menopausal gonadotropin (IVF-M; LG Life Sciences, Korea) were administered from the third day of menstruation until the day of human chorionic gonadotropin (hCG) administration. In the GnRH antagonist group, recombinant FSH was administered daily from the third day of the menstrual cycle. The doses were also adjusted according to each patient's individual ovarian response. From the same day 0.25 mg cetrorelix acetate (Cetrotide; Merck, Germany) or Orgalutron (Organon; Ireland) was administered subcutaneously daily until the day of hCG administration. In both groups, final maturation was induced using recombinant hCG (IVF-C; LG Life Sciences, Korea) when at least 2 leading follicles reached 18 mm in diameter. The oocyte retrieval was performed 36–37 h later.

OOCYTE RETRIEVAL, ICSI AND EMBRYO CULTURE

Protocols for ICSI and IVF procedures

Daily checks for the laboratory

- The HEPA towers for recovery area, laboratory and theatre were switched ON and kept running for at least one hour daily.

- The Coda Towers (gen X; Lab IVF, Singapore) in recovery and laboratory area were switched ON to clean the air.
- All the HEPA filters above the work stations were switched ON and kept running.
- The room temperature of the laboratory was checked which should not be less than 25^{o}C and not more than 28^{o}C.
- All thermal plates (Tokai Hit Co; Shizuoka-ken, Japan) under the stereomicroscopes, thermal plates for table heating and heater for inverted microscope were switched ON.
- Before starting the work, using surface thermometer (RI; San Jose, CA, USA), the temperature of table thermal plates and those under the stereo-microscopes, and central heater for inverted microscope were checked that are all at 38-39^{o}C (allow 2^{o} degrees for heat loss). *It is recommended to heat all the surfaces of work stations at 38 – 40^{o}C, to ensure the temperature inside the fluid drop at 37^{o}C, verify using thermometer.*
- All the tables (work stations) were wiped with distilled water.
- The incubators (MCO-5M; Sanyo, Osaka, Japan) for embryo culture and oocyte collection were checked for indicating a temperature of 37^{o}C and 6%CO_2 and 5% oxygen on the display panel.
- The internal temperature of incubators was confirmed from thermometer inside the incubators and inner chamber CO_2 and oxygen using Geotech gas analyzer (G100; Geotech, Warwickshire, UK)
- CO_2 and nitrogen gas cylinders were checked and the gauge display was noted for gas volume (>500) and gas supply to the incubators (should be between 10 – 15psi and not more than 20psi)
- The toxins level in air of all important places in laboratory and theatre was checked with VOC meter (RI; RAE, San Jose, CA, USA).
- The level of liquid N_2 in all dewars (Taylor Wharton; Theodore, Alabama-AL, US) i.e. embryo and sperm storage as well as the surplus N2 storage dewars was checked.
- The display of CO_2 and oxygen gases, temperature and pH of all incubators, temperature of all dewars and refrigerators of the laboratory and theatre was also

checked by OCTAX Log & Guard online monitoring system (OCTAX, Microscience GmbH, Germany) either by logging in or on the display screen of the system.

- All the information was entered in their respective sheets or books and confirm with signature.
- All UPS were checked for indicating green light i.e. fully charged, if red that indicate insufficient battery charging.

Note: it is very important to wash hands thoroughly with HEPES scrub before starting any work in the lab. No disinfectant can be used on hands as their vapors can be cytotoxic to the sperm, eggs or embryos, therefore the contamination in the lab can be only avoided by the cleanliness.

Preparation before egg collection

Preparation of culture dishes 18 – 20hrs prior to egg collection

- Daily embryology sheet was made for all the preparations and procedures.

Materials required

- Culture dishes 60mm 3
  (Oosafe OOPW-ST10; SparMED Aps, Stenlose, Denmark)
- Culture dishes 60mm (BD Falcon 353653; San Jose, CA, USA) 1
- Culture dish 90mm (Greiner) 1
- Glass Pasteur pipettes (Hunter Sci.
- PPB-230-100; Essex, UK) 5
- Rubber teats 1
- Culture tube 14ml (BD Falcon 352001) 1
- Culture tube 5ml (BD Falcon 352003) 1
- Spirit lamp 1
- Lighter 1

Culture Media (Vitrolife; Frolunda, Sweden)

- G-IVF^{TM} PLUS cat#10136
- G-GAMETE^{TM} PLUS cat#10126
- G-1^{TM} PLUS cat#10128

- G-TL™ cat#10145
- OVOIL™ cat#10029

- HEPA filters above the work station were switched ON.
- The surface of table was wiped with distilled water.
- The name and registration no. of the patient was printed using Casio printer (LABELIT) and label the 90mm culture dish.
- All the dishes were labeled with patient's name and registration no. under culture dishes using Lab Expert labeling system (Lab Expert; Brady, Milwaukee, WI, US). All the above requirements for preparation were placed on the table i.e. culture media, sterile glass pasture pipettes, rubber teat etc.

For ICSI procedure

- One culture dish (Oosafe; 60mm or BD Falcon 60mm, 353652) was made by putting three large drops (0.3ml each) and the other single well dish (BD Falcon 60mm, 353653) by putting approximately 1.0 ml of G-IVF™ PLUS both overlaid with OVOIL™.
- Two culture dish (BD Falcon 60mm, 353652) were madeby putting 6 to7 micro-drops of 40-50µl each of G-GAMETE™ PLUS and G-1™ PLUS respectively and overlaid with OVOIL™.
- These dishes were stacked in a 90mm culture dish labeled with patient's name and registration number and incubated overnight at 37°C, 6% CO_2 and 5% oxygen, before use.
- Approximately 3.0ml G-IVF™ PLUS and 3ml of G-GAMATE™ PLUS was poured in 5ml culture tube keeping the cap loose and incubated overnight 37°C, 6% CO_2 and 5% oxygen.
- The ICSI™ holding medium was incubated in dry oven at 37°C without gas (Incucell MMM).
- The OVOIL™ was also incubated at 37°C in dry oven.

For IVF procedure

- Depending upon the expected number of oocytes of the patient one or two extra 60mm single well culture dishes (BD Falcon, 353653) labeled with patient's name and registration number were made by putting in 1.0 ml of G-IVF™ PLUS

overlaid with OVOIL™ and incubated at 37°C, 6% CO_2 and 5% oxygen overnight with other dishes.

- There is no need to made G-GAMATE™ PLUS and G-1™ PLUS dishes for IVF.
- Approximately 3ml of G-IVF™ PLUS in 5ml culture tube was incubated for sperm preparation in incubator at 37°C, 6% CO_2 and 5% oxygen.
- OVOIL™ was also incubated at 37°C in dry oven without gas (Incucell MMM).

Egg collection or oocyte pickup (OPU) - D0

The aim of egg collection procedure is to collect maximum number of eggs from the follicles as quickly as possible and to minimize the exposure of eggs to non-physiological conditions. Important parameters are temperature, osmolality and pH. Any deviation from these physiologic conditions may have deleterious effects on oocytes, fertilization, cleavage and finally the implantation of embryos and success rate of the procedure. Egg collection is done transvaginally under ultrasound guidance 36 – 37 hr. after hCG injection (IVF-C 10,000 iu) using follicle aspiration needle (cat# 14114, vitrolife).

- On D0 2-3 ml of sperm preparation medium (SPERM RINSE™) was placed in 5ml culture tube at 37°C, 6% CO_2 and 5% oxygen.
- The patient's name, husband's name, registration no. and time of hCG injection was confirmed from the patient and on the embryology sheet before egg collection and the sheet was signed for this confirmation.
- Following items were kept ready on the table before the egg collection:
    - 6–8 culture dishes 60mm (BD Falcon 353652), spread them on heated plate so that the dishes become warm.
    - sterile rubber teat
    - sterile glass Pasteur pipettes
    - A container to discard the follicular aspirate after picking out the egg from the dish.
- Powder-free nontoxic Maxitex gloves (TERANG NUSA, Malaysia) and surgical mask (3M Healthcare; Neuss, Germany) were used for egg collection to protect from the direct contact with the follicular fluid to avoid any infection.

- Just before the egg collection starts, two pre-incubated dishes of G-IVF^{TM}PLUS were taken out of multiple gas incubator (MCO18; Sanyo, Osaka, Japan) and placed in the MINC^{TM} Benchtop Incubator (K-MINC-1000; Cook Medical; Bloomington, USA)
- A sterile glass Pasteur pipette was attached with the teat, and place it in a holder or stand.
- The focus of stereomicroscope (Figure 2.2) was adjusted in the dish and on the video monitor, so that the image is sharp and clearly visible to all in the theatre too.
- The surgeon was informed to start the OPU.

Figure 2.2. The stereomicroscope used for oocyte pick up (Leica).

- As soon as the follicular aspirate arrived in the laboratory, it was poured it into a 60mm culture dish and observed under phase contrast, x10 magnification of stereo- microscope. The egg itself is about 120 - 125µm. It can not be seen by naked eye, however, at the time of egg collection, egg is almost always surrounded by a layer of corona radiata that appears as black circle around the egg

and thick layers of cumulus (Figure 2.3). On average egg & cumulus together form a size of about 2 – 3mm. Cumulus is whitish in color and can be gelatinous in appearance which is recognizable even with the naked eye. *Therefore while pouring the aspirate into the culture dish, cumulus, if seen, immediately focused and observed under the microscope. It has a high probability of having an egg.* It is easy to recognize egg with cumulus if the follicular aspirate is straw colored but if the aspirate contains lots of blood and it is red in color, then first look for any shiny white cumulus tissue, if not then scan the dish using variable magnification and focusing planes. In case the egg is naked or with very thin cumulus then try to look at the base of the dish, as it settles down.

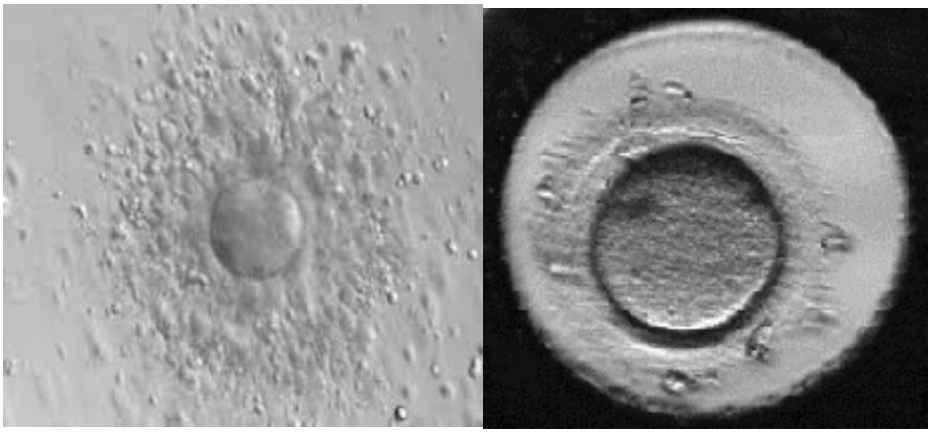


Figure 2.3. The human oocyte with cumulus (left) (Mtango, 2008) and washed off cumulus showing the polar body (right).

- The surgeon was informed immediately about the retrieval of egg or not so that he can flush the follicle until egg is retrieved
- If the egg was found it was picked up using glass Pasteur pipette applying gentle pressure and was washed in multiple drops of G-IVF^{TM} PLUS and then placed in one drop.
- All eggs were collected in the same manner. When 4-5 eggs were retrieved, they were washed and transferred to the G-IVF^{TM} PLUS dish. This helps to prevent trauma to the egg due to a change in pH, gas and temperature. Finally all the

collected eggs were transferred in the same dish, washed in other two drops and finally transferred into pre-equilibrated single well G-IVF^{TM}PLUS dish lying in the incubator.

- At the end of procedure the patient's husband or any laboratory colleague was asked to confirm the name of the patient labeled under the dish from the video monitor and on the embryology notes with their signature.
- The culture dish was moved to the incubator at 37^{o}C, 6% CO_2 and 5% oxygen.
- All important notes regarding the OPU were mentioned in the embryology sheet of the patient along with the number of oocytes retrieved.

SPERM PREPARATIONS

Protocol for semen sample collection

- Ideally the semen sample should be collected in a room near the laboratory so that it is handed over immediately to the laboratory.

- The sample should be obtained by masturbation and ejaculated into a clean, sterile wide mouthed container made of nontoxic glass or plastic.

- When the patient is unable to collect semen by masturbation, then he should be provided with special nontoxic condoms available for semen collection. Ordinary latex condoms must not be used as they interfere with the sperm viability.

- Coitus interruptus is not acceptable as means of collection as the first portion of ejaculate which is lost has the highest concentration of spermatozoa. Moreover, the cellular and bacteriological contaminants and the acidic pH of the vaginal fluids adversely affect the sperm motility.

- The patient was given an appointment and he was asked to come for semen analysis and its preparation for ICSI or IVF with an abstinence period of 3-5 days.
- The request form was filled in and he was provided with the sterile container labeled with the name and registration number of the couple and the room for semen collection.

- He was given detailed instruction about the DO NOT'S.
- The laboratory personal preferably the seminologist collected the sample along with the request form after getting the sign of confirmation of details on the form and the container from the patient. The seminologist also signed the form to confirm the delivery of the specimen to the seminology laboratory.
- On receipt of the sample to the laboratory the name of the couple and their registration number was confirmed on the container as well as on request form.
- The sample was assigned a lab ID.

Semen Analysis

It was made sure to analyze one sample at a time on one working station and all glassware used was labeled with the registration number of that patient/couple. Semen samples are potentially infectious and should be handled with extreme care as for biohazard. It should be kept in mind to take aseptic measure for semen analysis and preparations.

- For semen analysis and preparation the powder free nontoxic gloves (Maxitex; TERANG NUSA, Malaysia) were used.
- Sterile transfer and glass pasteur pipettes were used for analysis and preparations.
- It was checked that HEPA filters are ON over the working stations.

Materials Required

Following items are required for semen analysis and preparation:

- Makler Chamber (Hunter Sci. PPB-230-100; Essex, UK) (Figure 2.4)
- Glass slides and cover slips (Sail Brand; Main land, China) 2
- Sterile transfer pipettes (BD Falcon; San Jose, CA,USA) 2
- Sterile glass pasteur pipettes (Hunter Sci. PPB-230-100; Essex, UK) 1
- Cell counter (NET surgical shop; Narang Medical Limited, New Dehli, India)
- Rubber teats 1
- Sterile gloves (Maxitex; TERANG NUSA, Malaysia)
- Papanicolaou staining (abcam; Cambridge, MA, USA)

- phase contrast microscope (Olympus scientific solutions; Waltham, America or Leica Microsystems GmbH Ernst-leitz-Strasse; Wetzlar, Germany) (Figure 2.5)
- Conical tubes 15 ml (BD Falcon; San Jose, CA,USA) 2
- 5 ml culture tube (BD Falcon; San Jose, CA, USA) 1

Figure 2.4. The Makler Chamber used for semen analysis.

Figure 2.5. The phase contrast microscope (Olympus).

- Centrifuge (Labofuge 300, Heraeus, Thermo Fisher Scietific; Waltham, MA,USA)
- Pipette Aid (Bibby Jet Pro with recharger)
- Mobile Nest test tube warmer (SS-FX-3036; LabIVF, Singapore)

Culture Media

- Sperm Grad™ (cat# 10102,Vitrolife, Frolunda, Sweden)
- Sperm Rinse™ (cat# 10101, Vitrolife, Frolunda, Sweden)
- G-IVF™ (cat# 10136,Vitrolife, Frolunda, Sweden)

- One sample was analyzed and processed at a time at one working station.
- All glassware to be used was labeled with registration number of the patient/couple.
- The physical appearance of the sample was observed first. Normally the semen depicts pale color but if it is more yellow then it shows presence of increased pus cells and brownish or red color indicates presence of RBCs.
- The consistency of the semen was observed which is viscous at the time of production. It becomes watery and thinner after completion of liquefaction time which normally takes 20-30 minutes.
- The pH of semen lies between 7.2-8.2. The seminal fluid is a powerful buffer in maintaining the pH of the ejaculate and therefore has important role in maintenance of sperm motility.
- The volume of ejaculate was measured with the sterile transfer pipette. The normal volume ranges from 1.5-5.0 ml.
- Sperm concentration was checked after the sample has liquefied completely i.e. converted to watery consistency.
- A drop of 10-15 µl was placed in the center of the Makler chamber and covered with the glass lid avoiding any bubbles. The chamber was placed under phase contrast microscope at 200x magnification.
- The number of total and motile spermatozoa was counted in 10 squares of the chamber. At least three observations were taken and the average of total and

motile number of spermatozoa was calculated. This gives the number of spermatozoa x 10^6/ml. the percentage motility was calculated using the formula as following.

% motility = average number of motile sperm count/average number of total sperm count x 100

- The progression or motility grading of the semen sample was done by placing a 5-10 ul drop of liquefied sample on a labeled glass slide (1"x 3", 1.0-1.2mm thick) and placing a cover slip (22x22 mm) avoiding the air bubbles. The progression scoring was done as an average of three fields under 400x magnification using phase contrast microscope. The scoring is as follows.
  I. **0/4** dead spermatozoa
  II. **1/4** non motile with non-progressive movement, twitching movement either at head or tail.
  III. **2/4** sluggish progressive motility laterally non directional.
  IV. **3/4** sluggish to normal forward progression.
  V. **4/4** good to excellent forward progression.
- The sperm morphology was assessed on Strict Kruger's Criteria (Kruger *et al*.,1986,1988)

Various criteria for assessment of morphological normality of sperm have been applied so far. According to strict Kruger's criteria the most normal and fertile semen has □14% sperm with normal morphology. Recent multicenter studies by Kruger and coworkers have shown that the most normal and fertile semen has a cut off value of □5% normality of sperm. Papanicolaou staining was done to make the permanent slides to study the morphological defects of sperm. These include the head, mid piece and tail defects.

- In addition to this there are number of other cells which are normally seen in the seminal plasma. These are number of immature germ cells, blood cells (WBCs, RBCs) and epithelial cell, bacteria, speramine crystals and debris were also observed in the semen sample.

Sperm preparation for ICSI

After detailed semen analysis the sample was processed for preparation to be used in IVF or ICSI.

- The Sperm Grad (1.0 ml) was poured into conical tube and placed in the dry oven or the mobile nest almost 1-2 hours before the start of sperm preparation so that its temperature is set at 37C°. Different dilutions of the gradient can also be used depending upon the concentration and progression of the semen sample.
- The gradient tube was layered with 1-3 ml of the liquefied semen sample using sterile plastic pasteur pipette and centrifuged at 1700 rpm for 10 minutes. If the semen sample is not fully liquefied the some volume of the culture media for sperm preparation can be added in it to make it less viscous.
- The pellet formed was aspirated gently with sterile glass pasteur pipette and transferred to a clean labeled conical test tube already having 1-2 ml of the Sperm Rinse™ at 37C° in it.
- The pellet was mixed well in the culture media and centrifuged at 900 rpm for 5 minutes.
- The supernatant was discarded and pre incubated 1 ml of G-IVF™ PLUS was added to the pellet and centrifuged again at 900 rpm for 5 minutes.
- After the second wash the supernatant was discarded and pellet was mixed well.
- The volume of culture media used for washing the sperm and the speed and time for centrifugation can be adjusted according the concentration and motility of the semen sample to get the maximum concentration of progressive spermatozoa.

Sperm preparation for IVF

- For IVF the same protocol is followed for semen preparation as that is for ICSI but for IVF we need the sperm concentration to be not more than 0.7×10^6 /ml hence the pellet formed was reconstituted in pre-incubated G-IVF™ PLUS until it was diluted till the desired concentration.
- The final concentration was verified by taking multiple observations of the sperm count on Makler chamber after every dilution.

INSEMINATION PROCEDURES

IVF Insemination

- Confirmation of the name of patient scratched under the dish for oocytes and on test tube containing sperm preparation was done by any other operator and signed in the embryology notes for confirmation
- Pre-incubated 60mm single well culture dish with G-IVF^{TM}PLUS was taken out of the incubator and almost 0.6 ml of the culture media was aspirated out and refilled with the sperm preparation for IVF and incubated for 1-2 hr. before insemination (37^{o}C, 6% CO_2 and 5% oxygen).
- After 1-2 hours of incubation transfer the eggs with minimum possible volume of the culture media from 60mm single well culture dish to the other single well dish containing sperm preparation (not more than 10eggs in one dish, if the egg number is more, which can be assessed from patient's follicular tracking file before preparation of the dishes, then prepare more than one dish as required).

- Insemination should be done within 3 – 4hr after egg collection.
- Incubate the culture dish overnight at 37^{o}C, 6% CO_2 and 5% oxygen.
- The time of insemination was noted in embryology notes.

Intracytoplasmic sperm injection (ICSI)

Materials required

- culture dishes 50x9mm (BD Falcon 351006) 3 – 5
- culture dishes 60mm (BD Falcon 353652) 1 – 2
- Glass Pasteur pipettes (Hunter Sci.) 6 – 8

Draw 2 pipette jets of approximately 250 - 300 µm and 3 pipette jets of 130 - 150µm using spirit lamp.

- EZ Strips (denuders) 145µm and 290µm (RI Ltd., Cornwall, UK) 1 – 2

- Holding pipette RI 30^{o} (RI Ltd., Cornwall, UK) 1
- Injecting pipette RI 30^{o} (RI Ltd., Cornwall, UK) 1
- Injecting pipette MIC-SI-30^{o} (Origio; Charlottesville, USA) 1

Culture Media

- Hyase^{TM} 10X (cat# 10017,vitrolife) 1vial (0.1ml) dilute
- it by adding 2.0ml of G-GAMETE^{TM} PLUS
- ICSI^{TM} (cat# 10111, vitrolife) 1vial (0.1ml)
- G-GAMETE^{TM} PLUS (cat# 10126,vitrolife) 5ml
- Ovoil (cat# 10029,vitrolife)

- All the above mentioned culture media was incubated at 37C° in dry oven, at least 2hrs before use except G-GAMETE^{TM} PLUS which was incubated overnight at 37C°, 6%CO2 and 5% oxygen. Preferably incubate ovoil overnight.

The process of intra-cytoplasmic sperm injection involves two continual major steps.

1. Denudation of oocytes for removal of cumulus and selection of mature oocytes for microinjection.
2. Intra-cytoplasmic sperm injection into the mature oocytes.

Preparation of culture dishes for denudation

For denudation of oocytes, prepare 60mm culture dishes (BD Falcon 353652) as under:

- The culture dishes for denudation were prepared by putting a large central drop (100µl) of pre-incubated G-GAMETE^{TM} PLUS and 5 – 6 drops(40µl) in a circle around it overlaid the with ovoil.
- The large central drop of G-GAMETE^{TM} PLUS was aspirated and replaced with diluted Hyase.
- The dishes were incubated at 37C°, 6% CO2 and 5% oxygen until use.

Preparation of culture dishes for microinjection

- The culture dishes for microinjection were prepared either in 50x9mm (BD Falcon 351006) or in the lid of 60 mm culture dishes (BD Falcon 353652). The dished were labeled with the patient's name and registration number.
- Pre-incubated G-GAMETE^{TM} PLUS was used to make 6-8 micro drops in the center of the dishes parallel in two rows.
- An oval small drop of ICSI^{TM} was also made on the left of microdrops.
- The dishes were overlaid with the ovoil and moved inside the incubator at 37C°, 6% CO2 and 5% oxygen.

Oocyte Denudation

Before starting denudation of oocytes, it was made sure that:

- The inverted microscope (Leica DMRBE) is switched on and its heaters are at 38-39^{o}C.
- Micro-injection pipettes are aligned properly on manipulators (Integra 3 for IX51/IX71 with 2 x SAS-SE).
- The injection pipettes are charged by dipping in the ovoil in charging dish.
- The culture dishes for microinjection (ICSI dishes) in which the eggs and sperm are placed are ready, the sperm preparation has been added to the sperm pool drop and placed in the dry oven at 37^{o}C.
- One glass pipette with teat and 2 glass pipettes drawn to 300µm and 2 glass pipettes with 130 - 150µm diameter are also placed on the table
- Mouth pipette is in your pocket i.e. easily approachable.

Procedure

- The culture dishes with Hyase^{TM} and the dish with oocytes collected were taken out of the incubator and focused well under the stereomicroscope.
- With the help of glass pasteur pipette a little of Hyase solution was aspirated and then oocytes were picked up and transferred to the Hyase drop.
- The oocytes were aspirated in and out 2-3 times with the glass pipette.

- The denuder (EZ Strip 290µm) was used to move the oocytes from Hyase drop to next 2 drops of G-GAMETE^{TM}PLUS moving then in and out and flushing in fresh drops of the culture media.
- After this the denuder (EZ Strip 145 µm) was used to wash the oocytes in fresh culture media and removing the remnant cumulus.
- The oocytes were counted, assessed for maturity and transferred to pre-incubated dish of G-GAMETE^{TM}PLUS using mouth pipette and drawn pipette of 130um-150 µm diameter till the microinjection.
- After giving the rest time of approximately1 hour to oocytes from trauma, the mature oocytes were transferred to the pre-incubated dish of G-GMATE^{TM} PLUS prepared for microinjection. There should be one oocyte per micro drop of the culture media.
- Approximately 3-5 µl of semen sample preparation (the stock) was also transferred to the ICSI^{TM} drop of the same dish after confirmation of the name and registration number of patient by another operator/colleague both on the dish with oocytes and the culture tube with semen preparation.
- The dish was then moved inside the incubator till the microinjection starts.
- The microinjection was done following the standard procedures by selecting the morphologically normal looking spermatozoa with normal size and dimensions and least vacuoles in the sperm head area (Figure 2.6 a, b)
- The microinjected eggs were then shifted to pre-incubated 60mm culture dish of G-1^{TM}PLUS overnight at 37C°, 6% CO2 and 5% oxygen.

EVALUATION OF EMBRYO DEVELOPMENT

Examination for fertilization / pronuclei – D1

Preparation of culture dishes for 2PN checks on D0

Material Required

- Culture dish 60mm (BD Falcon 353652) 1

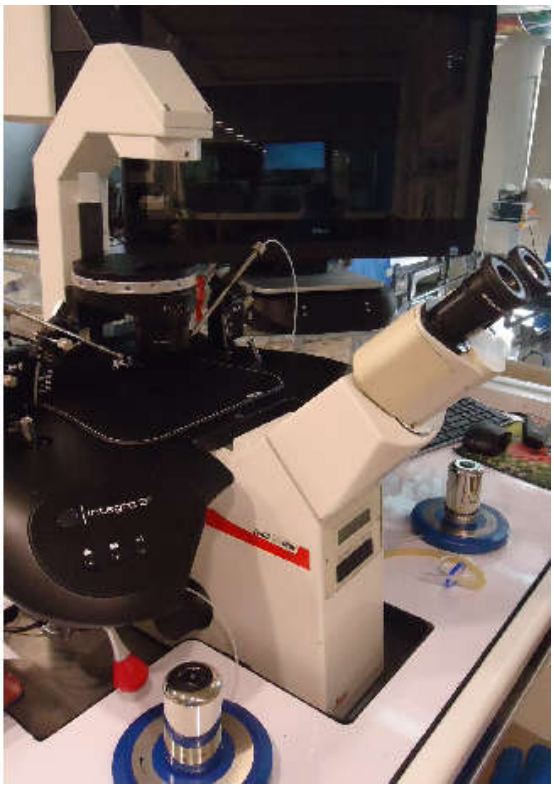
- 9 or 16 micro well group culture dish (Vitrolife; Frolunda, Sweden)1
- Sterile glass pasteur pipette (hunter Sci.) 2-3
- EZ Strip (denuder) 290um 1

Culture Media

- G-TL™ (cat#10145,Vitrolife; Frolunda, Sweden)
- Ovoil™ (cat# 10029,Vitrolife; Frolunda, Sweden)

- Both the 60mm culture dish and group culture dish were labeled with patient's name and registration number.
- 6-7 drops of 40-50µl of G-TL™ were made in the 60mm culture dish and overlaid with ovoil.
- The micro wells of group culture dish were filled with G-TL™ using drawn glass pipette of appropriate diameter avoiding any bubbles and overlaid with ovoil.
- Incubate both dishes overnight at 37C°, 6% CO2 and 5% oxygen.

(a)



(b)

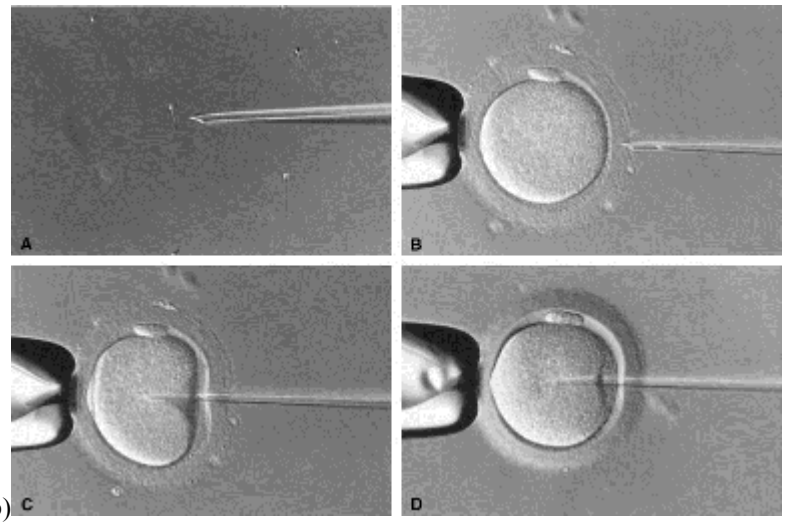


Figure 2.6. (a) Intracytoplasmic sperm injection (ICSI) procedure with manipulators. **(b)** Injection of sperm into the oocyte shown in four steps.
(Uehara and Yanagimach, 1976).

Fertilization check (15-18 hours post insemination)

As the sperm comes in contact with the egg, it results in acrosomal reaction, releasing hyaluronidase that helps sperm to dissolve cumulus and penetrate into the egg. When it fuses with the vitalline membrane, the second meiotic division is triggered resulting in division of chromosome and release of second polar body. The other set of chromosomes form the female pronucleus. The nuclear material of sperm head also decondenses and migrates into the egg cytoplasm, forming male pronucleus. Both pronuclei are visible approximately 16-18hr hpi as dense round structures 'crater-like' in appearance and confirm fertilization. This is called 2 pronuclear stage (2PN) - an evidence of fertilization.

- Before the fertilization check the mouth pipette was made ready along with glass Pasteur pipettes jets of approximately 150 - 200μm diameter and bent at an angle of 90˚. For every patient new glass pipette was used.
- For each fertilization check 1- 2 extra pipettes (both straight and bent) were made just in case the tip or diameter of a pipette is not appropriate.

Fertilization check in case of IVF

- The name and registration number of the patient was confirmed on embryology notes and daily embryology sheet.
- Pre-incubated G-IVF^{TM}-PLUS dish with inseminated eggs and G-TL^{TM}PLUS dish for fresh transfer were taken out of the incubator.
- With the help of mouth pipette and 290um denuder all the eggs from G-IVF dish were washed, removed off the cumulus one by one and placed on the side of same drop.
- Glass pasteur pipette jet was used to shift the eggs from G-IVF to G-TL 60mm dish and given several washes to clean the remaining cumulus.
- The 2PN eggs were selected by rolling and rotating to confirm from different angles as sometimes polar bodies or attached cumulus cells at some angles can give an impression of pronucleus and may lead to confusion.
- The eggs with clear 2PN were transferred to the micro wells of group culture dish (Vitrolife) using the sterile bent drawn glass pipettes. Remaining eggs with no

evidence of 2PN (unfertilized), with faint 2PN, 1PN or 3PN should be transferred in separate microdrops of the 60mm G-TL culture dish.

- The group culture dish was placed on the PrimoVision™ microscope (Vitrolife; Frolunda, Sweden) placed inside the incubator (Sanyo) and attached to the the PrimoVision™ software for time-lapse monitoring of embryonic development displayed on the monitors attached.
- The 60mm culture dish with remaining eggs was placed inside the metal dish holder labeled with the patient name and registration number and shifted next to the primo vision microscope of the same patient.
- The observations were recorded immediately on daily embryology sheet and then in the patient's embryology notes and computer record data sheet.

Fertilization check in case of ICSI

- The name and registration number of the patient was confirmed on embryology notes and daily embryology sheet.
- Pre-incubated G-1™-PLUS dish with microinjected eggs and G-TL™PLUS dish for fresh transfer were taken out of the incubator.
- Glass pasteur pipette jet of diameter 150-200µm with mouth pipette was used to shift the eggs from G-1 to G-TL 60mm culture dish and given several washes to clean the oocytes.
- The 2PN eggs were selected by rolling and rotating to confirm from different angles as sometimes polar bodies or attached cumulus cells at some angles can give an impression of pronucleus and may lead to confusion.
- The eggs with clear 2PN were transferred to the micro wells of group culture dish (Vitrolife) using the sterile bent drawn glass pipettes. Remaining eggs with no evidence of 2PN (unfertilized), with faint 2PN, 1PN or 3PN should be transferred in separate microdrops of the 60mm G-TL culture dish.
- The group culture dish was placed on the PrimoVision™ microscope (Vitrolife; Frolunda, Sweden) placed inside the incubator (Sanyo) and attached to the the PrimoVision™ software for time-lapse monitoring of embryonic development displayed on the monitors attached.

- The 60mm culture dish with remaining eggs was placed inside the metal dish holder labeled with the patient name and registration number and shifted next to the primo vision microscope of the same patient.
- For the purpose of fertilization check in time-lapse monitoring system the microinjected oocytes were transferred to the primo vision group culture dish immediately after ICSI on D0.
- After 16-18 hours on D1 the time-lapse video created was run to evaluate the time for appearance of 2PN.
- The observations were recorded immediately on daily embryology sheet and then in the patient's embryology notes and computer record data sheet.

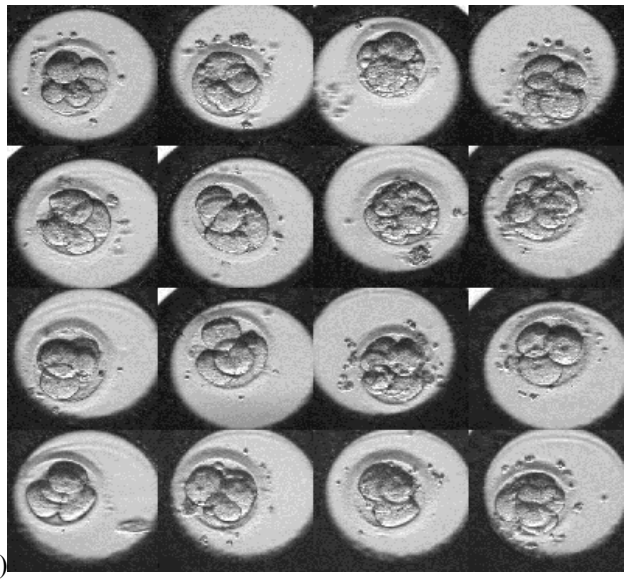
Cleavage stages and embryo selection - D2

Cleavage of fertilized eggs starts 25 – 29 hrs post insemination. However embryos are observed for cleavage stage 20 – 24hrs after 2PN stage. One day post fertilization, mostly the embryo are seen between 2 – 4 cell stage and two days post fertilization they are at 4 – 8 cell stage. Grading of embryo morphology is based on the following factors:

1. cell stage
2. shape of zona pellucida
3. regularity of blastomere shape and size
4. evenness of cleavage
5. fragmentation

Cell stages

The embryos with more advanced stages of growth are used for ET, when quality of embryos is the same, e.g. from grade 1 embryos, on D2 those at 4 cell stage will be preferred over 2 cell stage or on D3, the embryos at 8-cell stage will be preferred over 4 – 6 cell stage. However, those 2 cell stages on D2 or 4-6cell stage at D3 may equally have good chances for implantation as they are only few hours behind the others. But it is also possible that 2 – 4 cell stage embryos have arrested growth and may not proceed further (Figure 2.7 a, b).

(a) 

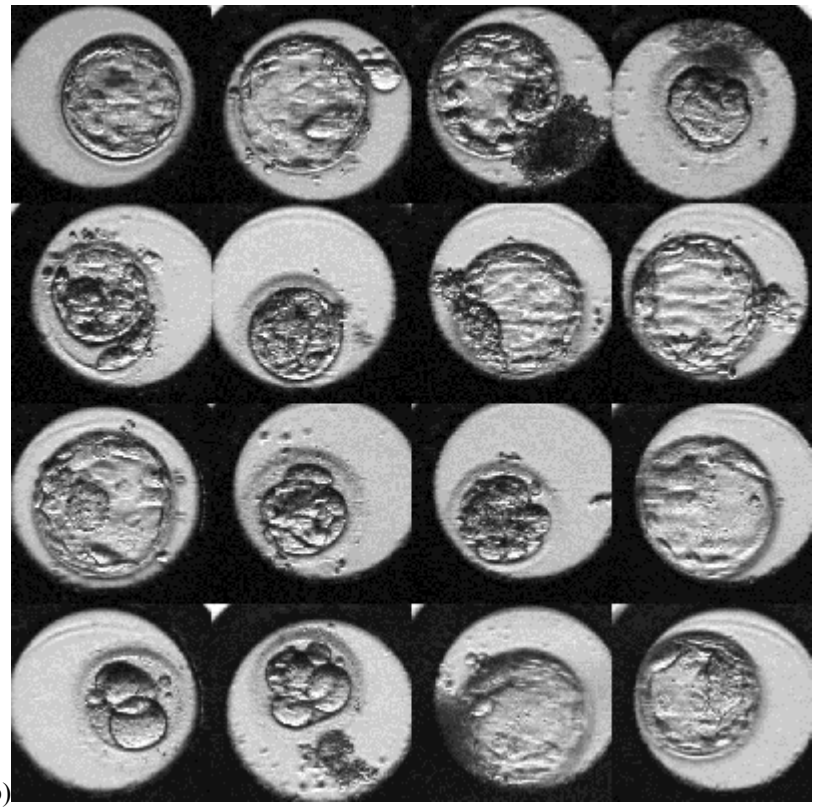
(b) 

Figure 2.7.(a) The human embryo development (4-8 cell stage) in time-lapse imaging.

(b) Human embryo showing compaction and blastulation in time-lapse imaging.

Zona pellucida

Zona is circular in shape, of uniform thickness. At times it may appear deformed, elongated or oval due to some damage and as a result the embryo will also be oval and elongated in shape and may not grow properly.

Blastomeres

The circumference of the blastomeres should be circular and smooth. Their size should be equal and they are slightly flattened where they are in contact with other blastomeres.

Evenness

During early cell division, usually the cells are of equal size therefore embryos having uniform cell size are preferred for ET. At times odd cell number is seen such as 3, 5 etc. in such cases one odd numbered cell may be large undergoing late cleavage but the other must be of same size.

Fragmentation

Sometimes cytoplasmic fragments are seen in embryo. Fragmentation may vary from one or two fragments to extreme condition of whole cell fragmentation where embryo even at 2 – 4 cell stage, appear as multicellular containing unequal sized cells. Such embryo degenerate quickly and should not be used for ET. Those embryos with few fragments should be followed for further growth as they may repair themselves and grow normally.

Embryo Grading

The embryos are graded from 1 –4, based on the above mentioned criteria.

- **Grade 1:** Normal, blastomeres of equal size, even circular zona pellucida, with no cytoplasmic fragmentation
- **Grade 2:** Normal, blastomeres of equal size, even circular zona pellucida, with upto 10%- 25% fragmentation, (sometimes embryos of grade 1 with large peri-

vitalline space or with slightly irregular or oval shaped embryos are graded in this category)

- **Grade 3:** Irregular or oval shaped embryos, fragmentation 25 – 50% unequal blastomere size (Figure 2.8)
- **Grade 4:** Irregular shaped, unequal sized blastomeres, fragmentation more than 50%

Embryo cleavage check

- In the morning of D2 of embryo growth (after approximately 25-29 hpi) the time-lapse video created was run to evaluate the time for appearance of the first cleavage event i.e. 2-4 cell.
- The other observations related to the cleavage e.g. unevenness of blastomeres, fragmentation, embryo grading and uncleaved embryos were also recorded.
- The observations were recorded on daily embryology sheet and then in the patient's embryology notes and computer record data sheet.

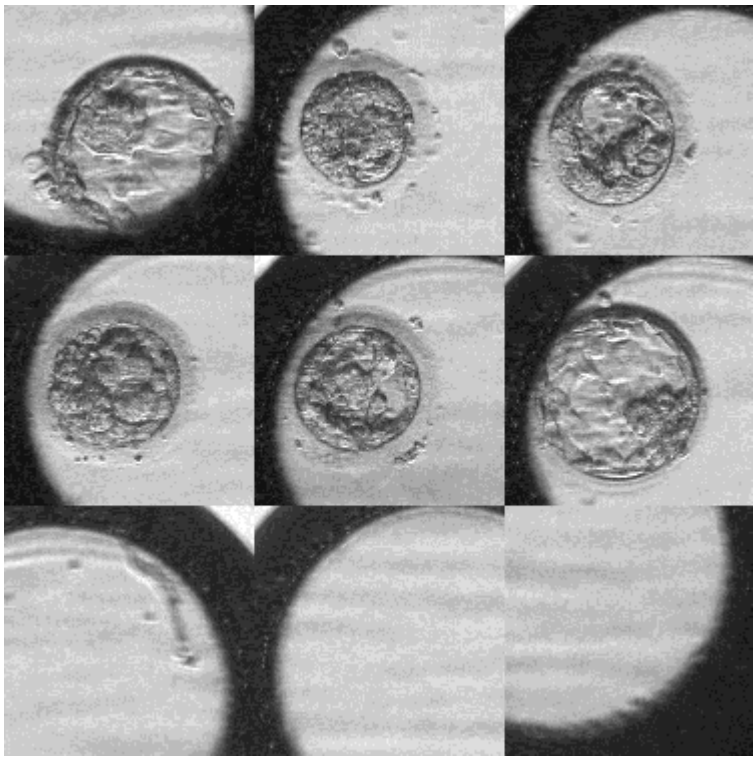


Figure 2.8. Human embryo showing good quality blastocysts as well as the fragmented and arrested embryos in time-lapse imaging

Cleavage stages and embryo selection – D3

Embryo cleavage check

- In the morning of D3 of embryo growth (after approximately 66-68 hpi) the time-lapse video created was evaluated for the time for appearance of 8-cell stage.
- The other observations related to the cleavage e.g. unevenness of blastomeres, vacuolation, fragmentation and embryo grading were also recorded.
- The observations were recorded on daily embryology sheet and then in the patient's embryology notes and computer record data sheet.

Cleavage stages and embryo selection – D4

Embryo cleavage check

- In the morning of D4 of embryo growth (after approximately 92-94 hpi) the time-lapse video created was evaluated for the time for appearance of compaction stage.
- The other observations related to the embryo quality e.g. vacuolation, fragmentation and early cavitation signs were also recorded.
- The observations were recorded on daily embryology sheet and then in the patient's embryology notes and computer record data sheet.

Cleavage stages and embryo selection – D5

Embryo cleavage check

- In the morning of D5 of embryo growth (after approximately 116-118 hpi) the time-lapse video created was evaluated for the time for appearance cavitation in the morulae and also the extent of expansion of cavity till morning of D5.
- The other observations related to the blastocyst quality may also be recorded e.g. vacuolation, granularity, inner cell mass and trophectoderm quality. The hatching of blastocyst or the collapsed cavity was also noted.
- On the basis of time-lapse development of the embryos and all the qualitative observations made, the most suitable embryos were marked for the transfer.

- The observations were recorded on daily embryology sheet and then in the patient's embryology notes and computer record data sheet.
- Time for embryo transfer was decided depending upon the blastocyst expansion.

Embryo assessment and Selection using Time-laps system

Daily observations of the embryo development time point were taken using the capture software. Time of micro injection was considered as (t0). The subsequent timings were taken as hour's post-insemination (hpi). Normally zygotes were placed in the time-lapse system at 17-18 hpi but some of them were also placed at 1-2 hpi for monitoring the appearance of 2 pronuclei. The evaluation of time points were done according to standards set for each cleavage stage. Fragmentation, symmetry of blastomeres and vaculation was also assessed (Desai*et al*., 2000; Machtinger and Racowsky, 2013). The quality of blastocyst was assessed on the bases of its timings, ICM (inner cell mass) development and cellular arrangement of TE (trophectoderm) (Desai*et al*., 1997). Blastocoel volume and development were used to gradify blastocysts as: SB = start of blastulation; EB = expanded blastocyst with more than half volume with cavitation; and FEB = fully expended blastocyst. The quality of inner cell mass was determined on the bases of either being absent or invisible, loosely arranged and well defined. Trophectoderm quality was evaluated either being organized well with high cell number, loosely arranges with low cell number and stretched with ill-defined cellular arrangement. Poor quality blastocysts show degenerative cells with low cell counts. For embryo transfer optimally growing blastocysts evaluated by morphological assessment through conventional method as well as by time-lapse imaging were selected at 114-116 hpi. For cryopreservation morulae with start of cavitation and blastocysts with good quality ICM and TE were selected. Time-lapse videos were reviewed in detail once conventional grading had been completed.

ASSISTED HATCHING

- The assisted hatching is a procedure to weaken the zona pellucida of the embryo either mechanically or chemically in order to help the embryo hatch out of its shell.
- This allows the embryo to leave the protected environment of the zona pellucida and commence implantation in the uterus.
- Currently the laser assisted hatching is done to thin out the zona of thick shelled blastocysts to facilitate the implantation.

The embryos selected for assisted hatching were separated and hatching was done following the protocols.

EMBRYO TRANSFER

Decision for number of embryos to be transferred depends upon

I. the embryo quality
II. total number of embryos
III. the age of the female,
IV. male or female factors for which ICSI treatment was opted

The embryo transfer at 4 – 8 cell stage and blastocyst follow the same protocol. Eggs and embryos should be observed closely for zona thickness and its granularity. Usually female patients suffering from PCOS, endometriosis and high FSH, have eggs with thick granular zona pellucida and require assisted hatching before embryo transfer. Cryo-preservation of embryos also lead to zona hardening and assisted hatching helps them in implantation.

Embryo transfer procedure was carried out when patient's bladder was full. The position of uterus was checked on ultrasound using abdominal probe, and when it seemed almost at 160 – 180^{o} angle, patient was prepared for ET by bringing her to lithotomy position.

Materials required

- 2 piece syringe 1
- Embryo transfer catheter (Vitrolife) 1

Various types of ET catheter such as Embryo Genesis, EchoCath, and hard ET catheter should be kept in approach. Suitable catheter can be used according to the position of the uterus (A/V or R/V) and cervical length

- Stylet 1
- Artificial insemination Catheter (Wallace) 1
- 5cc Disposable Syringe 1
- Gloves (Maxitex 6-7) 1 pair

Culture Media

- EMBRYO GLUE™ (cat# 10085,vitrolife) 1 ml in 5ml culture tube incubated overnight at 37°C, 6% CO_2 and 5% oxygen.
- G-RINSE™ (cat# 10069,vitrolife) 3ml in 5ml culture tube incubated overnight at 37°C, 6% CO_2 and 5% oxygen.

- Before ET, the tube containing G-RINSE medium was placed in the warm block, an ET catheter, 2 piece syringe on the table.
- The selected embryos were shifted into embryo transfer medium (EMBRYO GLUE) and focus them at x10 magnification under the microscope.
- The hands were scrubbed properly with HEPES under running water, dried using sterile towel and sterile powder free gloves were used to do the embryo transfer.
- 0.5ml of G-RINSE from culture tube was drawn into the syringe and ET catheter was fixed on it. Removing the air bubbles from syringe and catheter the medium was flushed out until 0.1ml graduation on the syringe.
- All embryos to be transferred were picked up in approximately 10-20µl of medium (Embryoglue) and the catheter was handed over to the gynecologist who passed the catheter through cervical canal into the uterus and then the media

containing embryos was injected into the uterus till the graduation the embryos were picked up.

- In order to be assured that all embryos were transferred to the uterus the catheter was checked under the microscope after the embryo transfer by flushing out the remaining medium into a clean culture dish and the gynecologist was informed about the completion of the embryo transfer process.
- Malleable stylet is needed if uterine cavity is not approachable due to an acute angle of uterus or cervical canal.

Luteal Support

Luteal support was provided to the patients as in all IVF protocols. They were given progesterone either intravaginaly (Cycologest 400mg; Actavis, UK) or Gestone injections when needed. After 10 days of embryo transfer beta-hCG levels were measured and on the establishment of pregnancy this luteal support continues for another twelve weeks.

Chapter # 3

The effect of female age on human embryo developmental potential: A time-lapse study

ABSTRACT

Time-lapse imaging technique has provided embryologists with a unique chance of studying the embryo morphokinetics to select the most viable embryos for implantation in the field of IVF (In vitro fertilization). Conventional method of morphological evaluations has proved that maternal age affects the human embryo quality. This retrospective study carried out at Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan mainly focuses on the effect of female age on human embryo morphokinetics. A total number of 200 patients undergoing ICSI treatment cycles at the clinic were selected for the study and divided into five age groups (< 26, 26-30, 31-35, 36-40, and > 40 years). Embryo culture was done at 37°C, 6% CO_2 and 5% oxygen for 5-6 days. Ten time-points were selected for kinetic analysis. The number of retrieved, matured, fertilized and cleaved oocytes showed highly significant difference ($P \leq 0.0001$) when compared among different age groups. There was no significant difference among average morphokinetic time-points in young and old women. Whereas timely cleaved embryos showed significant difference in tPNa i.e. time for pronuclear appearance ($P \leq 0.001$), t4 and t5 i.e. time for 4 and 5-cell cleavage ($P \leq 0.05$) in different age groups.The cell cycle intervals i.e. cc2 and cc3 were found to be in normal range for different age groups. The clinical pregnancy rates showed a decline with increasing age. These results indicate the effect of female age on time-lapse embryo morphokinetics. In future the addition of time-lapse analysis in routine IVF can help to improve the success rate by selecting the most viable embryos for uterine transfers.

INTRODUCTION

In early 1980s, the definition of pregnant elderly women in medical dictionaries was over the age of 35. (MMD, 2009). These patients were considered to be at high risk of obstetric problems (Buehler *et al.,* 1986, 2015). In old age patients the pregnancy out comes and risk associated with maternal health have been studied well (Usta and Nassar,

2008). The obstetric complication associated with older gravidity could be chronic illnesses, particularly obesity, hypertension, and diabetes mellitus (Jacobsson *et al.,* 2004).

The oocyte development is supported by endocrine and paracrine interaction with its environment, most importantly the surrounding granulose cells. (Li and Albertini, 2013; Dumesic *et al.,* 2015). Controlled ovarian hyperstimulation (COH) results in growth of multiple follicles, however only 7 % oocyte reached up to term delivery (Patrizio and Sakkas, 2009), and still out of fertilized oocytes which are found to be euploid only two-thirds result in a viable birth. During the course of oocyte maturation specifically its cytoplasmic maturation i.e. increase in quantity and quality of cytoplasm determines its ability to form a viable embryo. Aging has a major impact on oocyte maturation, now it has been established that oocyte mitochondrial maturation have a critical impact on its development which ultimately results in success of in vitro fertilization. MATER (maternal antigen that embryos require) is expressed in growing oocytes and encodes cytoplasmic proteins (Tong *et al.,* 2004; Ohsugi *et al.,* 2008). It was found to be linked with age dependent oocyte quality decline. Mater-null females were found to have severe centromere cohesion weakening resulting in high aneuploidy rate. Hence mutation of MATER can cause human reproductive decline with maternal aging (Docherty *et al.,* 2015).

Another important effect of advancing female age on ovarian reserve and cytoplasmic quality is the impact of decrease androgen levels (Meldrum *et al.,* 2013). As age increases the serum level of adrenal androgen DHEA (dehydroepiandrosterone) and circulating and free T (Testosterone) decreases (Davison *et al.,* 2005). Basal T levels correlate with reduced ovarian response when controlled for age (Barbieri *et al.,* 2005). As circulating T increases the count of antral follicle and GC proliferation increases. Oppositely GC apoptosis decreases which results in more competent oocyte yield (Vendola *et al.,* 1998).

Cytoplasmic components of growing oocytes are considered to be the determinant factor for ability of the embryo to pass through the transition from maternal to zygotic control during maturation (Goddard and Pratt, 1983). The functional status of oocyte mitochondria contributes to oocyte quality (Santos *et al.,* 2006). Inappropriate mitochondrial activity contributes to early developmental arrest (Van Blerkom *et al.,* 2001). With increasing female age mitochondrial activity declines (Ramalho-Santos *et al.,* 2009; Keefe *et al.,* 1995). Further more direct correlation exists among the oocyte mitochondrial number and its competence to get fertilized and grow (May-Panloup *et al.,* 2005). Compromised mitochondrial function results in failed IVF attempts in women with increasing age (Santos *et al.,* 2006).

The embryo expresses its own genome and potential genetic abnormalities after third day of culture (Alfarawati *et al.,* 2011; Fragouli *et al.,* 2014). Hence mostly the euploid embryos can form the blastocyst. However maternal age is not considered to be the likely causal factor of preblastulation delays in embryo growth but the aneuploidy itself (Ottolini *et al.* (2013). Moreover (Kroener *et al.* (2012) suggested that delayed blastulation is not related to embryo aneuplody rates however this study did not use the time-lapse monitoring for precise assessment of cleavage timings and blastomere biopsy was done for ploidy assessment which itself is demonstrated to delay the compaction (Kirkegaard *et al.,* 2012a). With increased maternal age oocyte and embryo aneuploidy effect the implantation of more than half embryos and result in increased abortion rate in IVF cycles. It was studied that in euploid group the female age was younger the aneuploidy and aneuploidy rate increases by 10% per year of female age (Macklon *et al.,* 2002; Fragouli and Wells, 2012; Fragouli *et al.,* 2013).

Assisted reproduction treatments (ARTs) have provided tremendous help to infertile couples. Now days the latest advancements in the field of IVF have made it easy to observe the human embryonic development more closely. It is critical to identify good quality viable embryos with the highest implantation potential. Hence many criteria for selection of best quality embryos have been suggested so far emphasizing the morphological assessment of embryos. But the conventional method of cell count and

morphology are not the only points to be taken into consideration while interpreting the quality of embryos and their implantation potential. Presently embryos are assessed once a day at defined hours post microinjection or insemination to avoid any undesired change in embryo culture environment i.e. temperature and pH etc. (Mio, 2006; Ebner *et al.,* 2000; Scott and Smith, 1998; Van Montfoort *et al.,* 2004). In order to improve the effectiveness of IVF treatment, there is a need to define noninvasive embryo markers to enhance the implantation rates (ICWEA, 2011).

The dynamic nature of embryo development is known to all of us and therefore a fixed time-point evaluation of the morphological features can mislead to differentiate between their potential at different cell stages. This developmental kinetics of embryos has become more evident by use of time-lapse imaging in the clinical IVF set ups. It has been shown by the previous information that the accurate timing of particular events, for example, pronuclear arrangement, syngamy, early cleavage, cell cycle intervals, synchronicity of cell division and start of blastulation are important markers of embryonic development. A constant assessment of embryos for these markers may therefore aid in selecting best embryos for implantation. The previous data show that morphokinetic observations can help enormously in the critical evaluation of embryonic development (Conaghan *et al.,* 2013; Kirkegaard *et al.,* 2013b).

The Primo Vision^{TM} Time-Lapse System (VitroLife, Frolunda, Sweden), has been in widespread practice in IVF laboratories and gained huge interest in recent years. This embryo monitoring system includes primo vision microscopes, software and culture dishes. Using this system, embryonic development can be monitored at all times without eliminating embryos from the incubator. So we can conveniently asses their developmental potential without any risks of temperature changes, light exposure, high oxygen exposure, and pH changes in the culture medium (Kirkegaard *et al.,* 2012a; Kirkegaard *et al.,* 2012b).

It must also be noted that the growth of embryos is not consistent and regular event and may be influenced by external factors including culture conditions as well as patient factors. One of the most important parameter is the age of the patients, when

blastocyst score is assessed in IVF treatment (Ahlström *et al.,* 2011, 2013). In a previous study it is shown that the embryos with early cleavage occurred more commonly in younger than older women and showed high implantation and pregnancy rates (Bos-Mikich *et al.,* 2001). Moreover maternal age does affect the stimulation regime, embryo viability, quality as well as the conception rates (Schoolcraft *et al.,* 2000; Wittemer *et al.,* 2000). In contrary to this another finding showed no difference in the blastocyst quality comparing different age groups of patients. However, the mean age of patients with good quality blastocyst was quite young around 33 years of age (Gardner and Schoocraft, 1999).

The success of single embryo transfer (SET) is associated with selection of best quality embryo for transfer and blastocyst culturing automatically provides this natural selection (Gardner *et al.,* 1999). The blastocyst scoring system proposed by Gardner *et al,* (2000), describes three scores for each embryo its inner cell mass (ICM) and trophectoderm (TE) quality and, finally, the blastocyst expansion/hatching (EH) status. The embryo showing the top score grading exhibit the best implantation and live birth rates. However, it has been recently investigated that TE quality was the single best predictor of successful pregnancy (Ahlström *et al.,* 2011). There are number of studies highlighting the prognostic effect of embryo morphokinetics with conflicting results (Kaser and Racowsky, 2014). Azzarello *et al.* (2012), found that euploid embryos have a significantly shorter time for initiation of blastocysts formation, expansion and hatching as compare to aneuploid ones. Similarly Campbell and coauthors (2013a, b) also found delay in start of compaction and blastulation in aneuploid embryos as compare to euploid ones.

Most of the patients seeking Assisted Reproduction (AR) treatments present with the female age more than 35 years, thus it is important to investigate the effect of maternal age on blastocysts quality and viability in order to counsel them properly for pregnancy probabilities with extended embryo culture.

We had following aims to carry out this study:

1) To focus on early cleavage pattern of embryos analyzed from female patients of different age groups and to assess their potential of blastocyst formation, quality of blastocyst formed and success rates using time-lapse imaging.

2) To evaluate any differences in morphokinetic parameters between good quality and poor quality embryos and to compare them with previous data.

MATERIALS AND METHODS

Study Design

This study was a retrospective observe of prospectively obtained data of time-lapse imaging of human embryos during in vitro development. The data used in this study were obtained from Islamabad Clinic Serving Infertile Couples (ICSI), Islamabad, Pakistan. The procedures and protocols were approved and practiced by the Institution. The number of oocytes observed in this study was 4080 producing 2266 embryos in 200 ICSI (Intracytoplasmic sperm injection) treatment cycles. A total number of 1704 embryos were included in time-lapse monitoring due to some split cycles for conventional culturing as a quality control measure at the outset of time-lapse system in the clinic. The patients were divided into five age groups (<26, 26-30, 31-35, 36-40 and 40 years old). Females showed a mean age of 31.8±0.3 years. Most of the embryos were obtained after fertilization by ICSI while very few patients had ICSI+IVF cycles. Embryos were evaluated for time-lapse morphokinetics by measuring precise timings of development. Ten important time-points for embryo development were compared. Embryo transfer data of both fresh and cryopreserved cycles of patients was analyzed. Implantation was confirmed by the βhCG levels in the blood sample taken after 10 days of embryo transfer and later on at an ultrasound scanning for fetal heart after 7 weeks of pregnancy.

The time-points (hours post ICSI) analyzed along with the abbreviations used to indicate the corresponding developmental stages and also the reference range used for each time-point (Herrero *et al.,* 2013) is as follows :

g. Two pronuclear stage (tPNa) = 17±1 hrs.

h. 2-cell stage (t2) = 26±1 hrs.

i. 4-cell stage (t4) = 44±1 hrs.

j. 8-cell stage (t8) = 68±1 hrs.

k. Compaction (tM) = 92±2 hrs.

l. Fully Expanded blastocyst (tB) = 116±2 hrs.

In addition to these, the embryos were analyzed for four more time-points i.e. at 3-cell cleavage (t3), 5-cell cleavage (t5), start of blastulation (tSB) and at expanding blastocyst stage (tEB) in order to assess the developmental events more critically. The reference range for these four time-points was taken from the average of optimally growing embryos at the study set up. Thus time range for t3 was taken as (31-42 hrs.) for t5 (45-54 hrs.) for tSB (98-105 hrs.) and for tEB (107-114 hrs.) respectively.

The exclusions criteria for this study were as follows.

a) Unfertilized oocytes were excluded from data.

b) All embryos whose time-lapse image acquisition were started later than 20 hours and ended before 60 hours after ICSI were not included.

c) Extreme outliers were excluded that may bias the analysis (as extremes may distort the mean of the regular divisions).

The images of embryo development by time lapse analyses was re-evaluated either the lab director or one of the senior embryologists. At the end a collective consensus was made by reviewing the videos and setting the guidelines.

Down regulation, ovarian stimulation, Oocyte retrieval, ICSI and embryo culture in Time-Lapse System

For pituitary desensitization both long and short protocols were used. Ovarian stimulation is started with the administration of FSH injection by adjusting the starting dosage based on the age of patients, serum AMH hormones levels, the number of antral follicles and the response of patients to any previous cycles. Final maturation was induced using recombinant hCG when at least 2 leading follicles reached 18 mm in diameter. The oocyte retrieval was performed 36–37 h later. The denudation of oocytes was done 2-3 hours after the retrieval and ICSI was performed on mature oocytes. The fertilization check was done 16-18 hours post ICSI. Zygotes were moved to pre-equilibrated primo vision group-culture dish and cultured for 5-6 days at 37°C with 6% CO_2 and 5% oxygen in the Primo-Vision Time-Lapse embryo monitoring system to monitor their daily growth. The embryo transfer and cryopreservation were decided on day-5 or day-6 of the embryonic development. The detailed methodology has been explained in the second chapter.

Statistical Analysis

The statistical analysis was done using analysis of variance (ANOVA) followed by Tuckey's test to check the difference of means among different age groups for different clinical and kinetic parameters. The correlation statistics was applied to find out the correlation among female age and basic embryology parameters as well as kinetic markers. The continuous variables are presented as mean±SEM and categorical variables are expressed as numbers (%). $P \leq 0.05$ was considered as indicative of a statistical significance. All results were obtained using statistical software GraphPad Prism 5 (GraphPad Prism Software, Inc. CA, USA).

RESULTS

The demographic and cycle characteristics of five female age groups are shown in table 3.1. The patients were divided into five age groups (<26, 26-30, 31-35, 36-40 and 40 years old). Mean BMI was statistically significant when compared among the age groups (P≤0.05). The rate of primary infertility was higher than secondary infertility in all age groups except the females aged above 40 years. The rate of 1st attempt of the cycle was also higher than 2nd attempt in all age groups. Similarly the number of ICSI cycles was higher than ICSI+IVF cycles in all groups studied. The number of retrieved, matured, fertilized and cleaved oocytes showed highly significant difference (P≤ 0.0001) when compared among different age groups.

Time-lapse analysis (Table 3.2) showed mean of all kinetic parameters found to have no significant difference among all age groups when compared by ANOVA. The timely and untimely cleaved embryos of each age group were segregated and ANOVA was performed among timely cleaved embryos for all morphokinetic parameters to check the significance of differences. The mean time for fertilization (tPNa) was found to be significantly higher in 26 years when compared to 26-30 years (P≤0.05) and 36-40 years age group (P≤0.0001). The tPNa was also significantly higher in 31-35 years age group as compared to 36-40 years (P≤0.05)(Figure 3.1). There was no significant difference in time for 2-cell (t2) and 3-cell (t3) cleavages among all age groups but the time for 4-cell cleavage (t4) turned out to be significantly higher in 40 years female as compared to 26-30 years age group (P≤0.05) (Figure 3.2). Similarly time for 5-cell cleavage (t5) also found to be significantly higher (P≤0.05) in 40 years females when compared to 26-30, 31-35 and 36-40 years age groups (Figure 3.3).Time for 8-cell cleavage was also found to have no significant difference among the study groups. All events for compaction and start of cavitation i.e. tM, tSB, tEB, tFEB, were found to have no significant difference when compared among timely cleaved embryos of all age groups. Out of non-significant cleavage stages among timely and untimely cleaved embryos only t2 (Figure 3.4) and tSB (Figure 3.5) are shown.

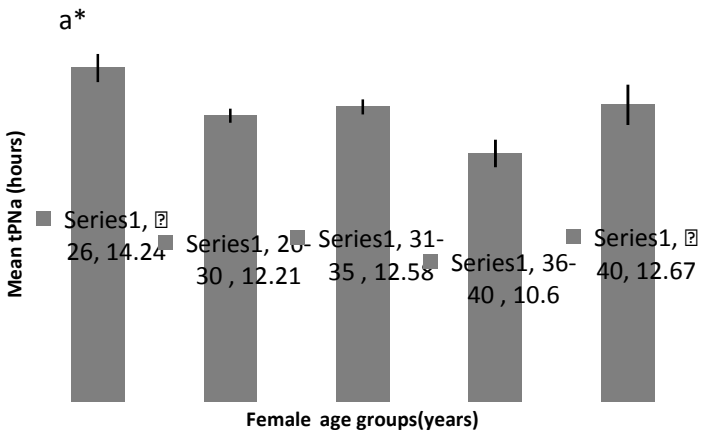


Figure 3.1. Comparison oftime for pronuclear appearance (tPNa) among timely-cleaved embryos of different female age groups.

a* P≤0.05 vs. 26-30 yrs. age group (ANOVA followed by Tukey's test)

b* P≤0.001 vs. 36-40 yrs. age group (ANOVA followed by Tukey's test)**

c* P≤0.05 vs. 36-40 yrs. age group (ANOVA followed by Tukey's test)

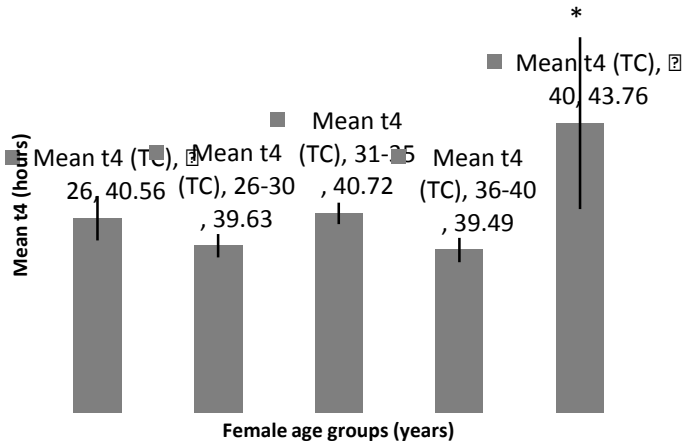


Figure 3.2. Comparison of time for 4-cell cleavage (t4) among timely cleaved embryos of different female age groups.

*** P≤0.05 vs. 26-30 yrs. age group (ANOVA followed by Tukey's test)**

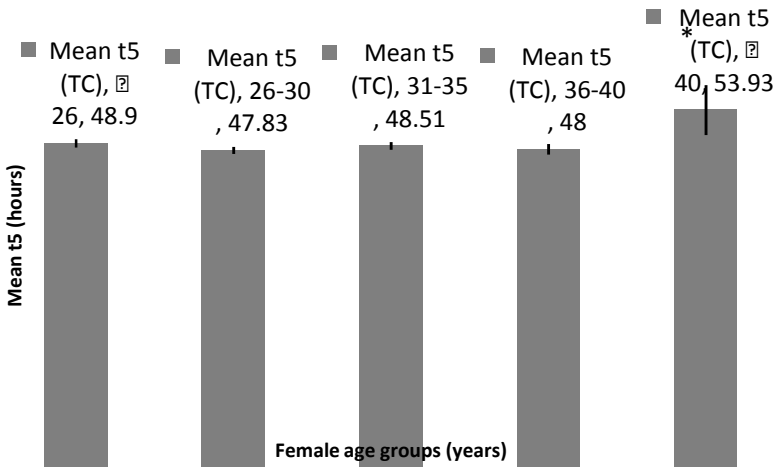


Figure 3.3. Comparison oftime for 5-cell cleavage (t5) among timely-cleaved embryos of different female age groups.

*** P≤0.05 vs. 26-30, 31-35 & 36-40 yrs. age groups (ANOVA followed by Tukey's test)**

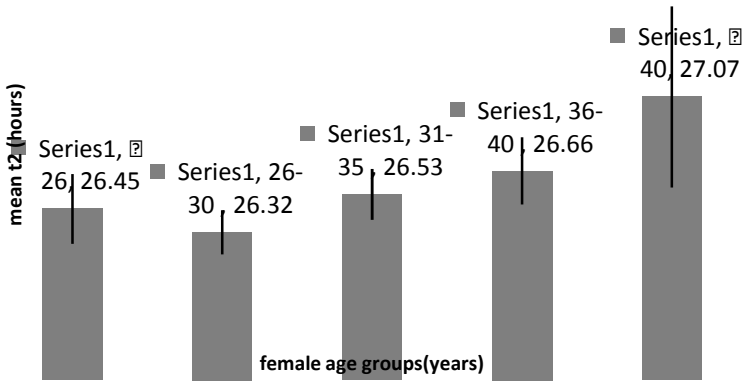


Fig 3.4. Comparison oftime for 2-cell cleavage (t2) among timely-cleaved embryos of different female age groups.

P>0.05 (ANOVA followed by Tukey's test)

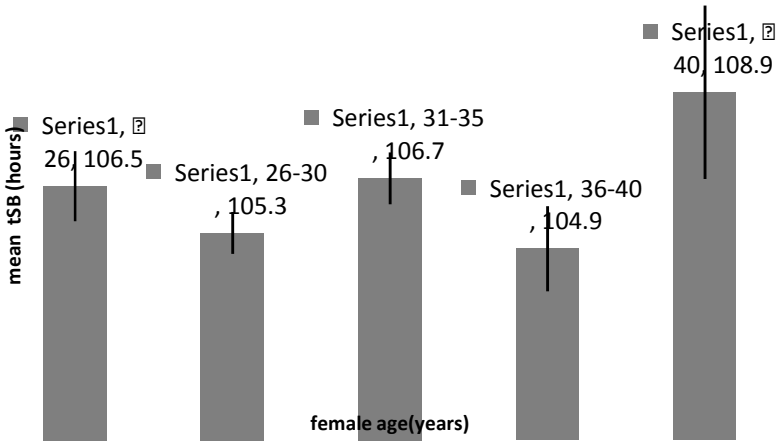


Figure 3.5. Comparison oftime for start of blastulation (tSB) among timely-cleaved embryos of different female age groups.

P>0.05 (ANOVA followed by Tukey's test)

The female age was found to have a strong negative correlation with number of retrieved, matured, fertilized and cleaved oocytes (P≤ 0.0001) (Figure 3.6 to 3.9) whereas none of time-lapse parameters of embryo development showed the correlation with female age (3.10 to 3.12).

The table 3.2 also shows the durations of the second cycle (cc2; t3-t2), third cell cycle (cc3; t5-t3) as well as finally the kinetic parameters looked at were the time to complete synchronous divisions s2 (t4-t3) and s3 (t8-t5). The range of duration of second cell cycle (cc2) is 6.8±1.4 to 8.4±1.0 and for duration of third cell cycle (cc3) ranges from 14.1±1.3 to 15.8±1.7 hpi for different age groups. The range of s2 is 5.1±1.1 to 5.8±1.3 and for s3 ranged from 15.5±1.8 to 18.6±2.1. All these parameters were calculated from mean cleavage timings of all embryos in five age groups irrespective timely or untimely dividing embryos.

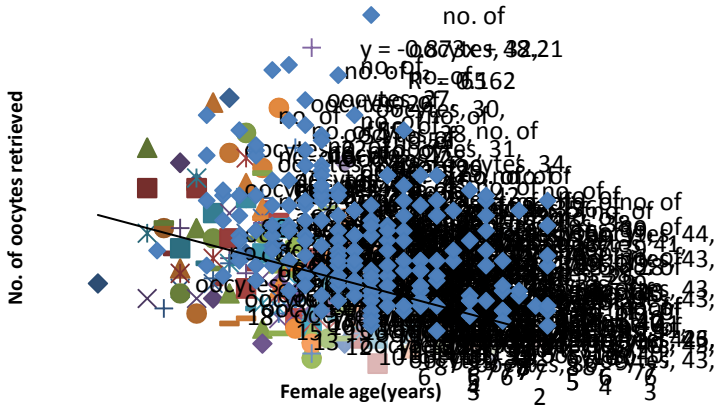


Figure 3.6. Correlation between female age and number of oocytes retrieved in an IVF/ICSI cycle.

***** P≤0.001**

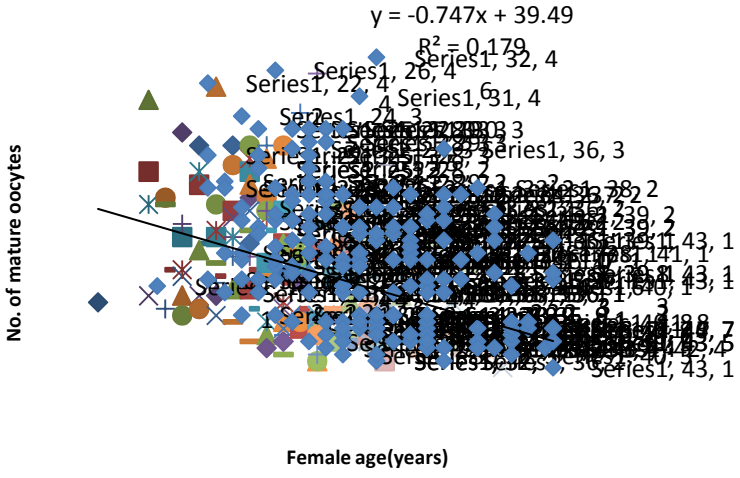


Figure 3.7. Correlation between female age and number of mature oocytes.

*** P≤0.001

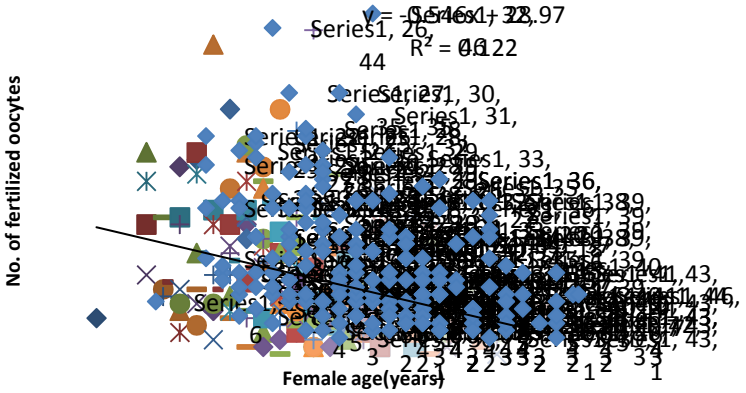


Figure 3.8. Correlation between female age and number of oocytes fertilize in an IVF/ICSI cycle.

*** P≤0.001

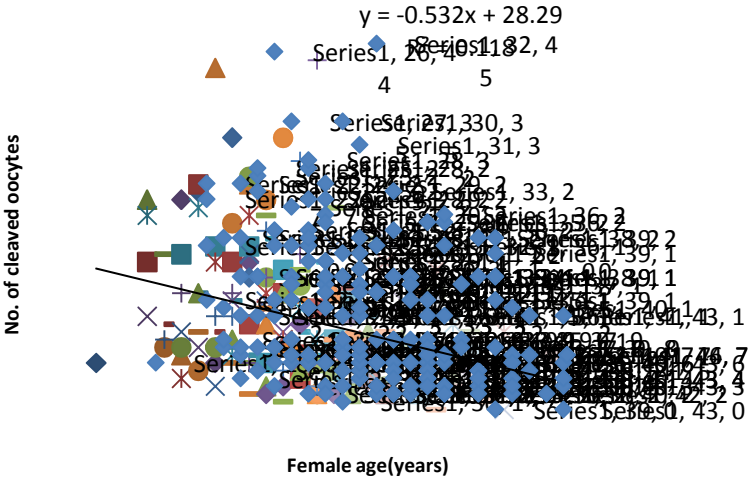


Figure 3.9. Correlation between female age and number of cleaved oocytes.

*** P≤0.001

Figure 3.10. Correlation between female age and time for pronuclear appearance (tPNa)

P>0.05

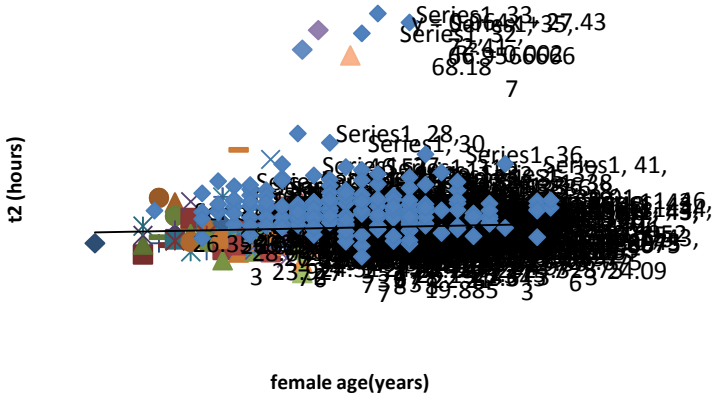


Figure 3.11. Correlation between female age and time for 2-cell cleavage (t2).

P>0.05

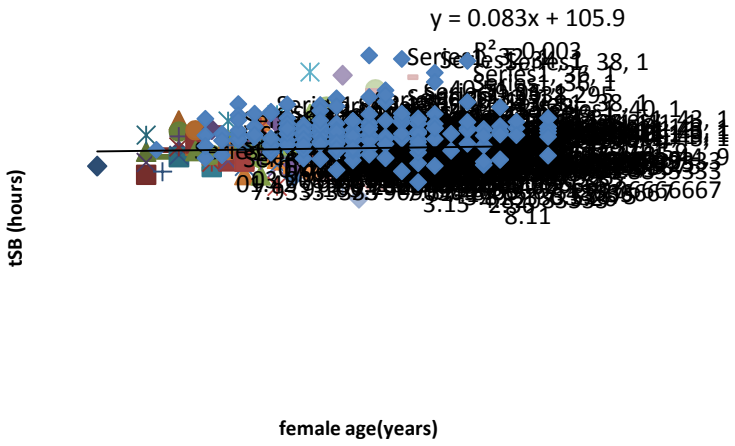


Figure 3.12. Correlation between female age and time for start of blastulation (tSB).

P>0.05

In table 3.3 the timely cleaved and untimely cleaved embryos were separated for each age group. The number of timely cleaved embryos was greater than untimely cleaved in all age groups except the above 40 years group. For 2-cell cleavage the rate of timely cleaving embryos was higher than untimely dividing in all age groups except the above 40 years in which more 2-cell cleavage was out of timeline. Among other early cleavage parameters i.e. for 4 and 5-cell stage the untimely cleaving embryos showed a higher rate than timely cleaving in all age groups whereas for 8-cell cleavage the rate of embryos dividing untimely was higher than timely cleavage for all other age groups except 36-40 years in which timely dividing 8-cell embryo rate was higher than untimely dividing but with a minor difference in rate. On the other hand the rate of embryos cleaving timely for start of blastulation (tSB) was far lower than those cleaving untimely in all age groups.

As far as the developmental potential and qualitative parameters of embryonic development are concerned (Table 3.4) the rate of embryo cleavage did not vary much among all age groups. The rate of blastocysts formation showed a decline with increasing age of females. The rate of good quality blastocysts was higher than poor quality in all age groups but both did not show any remarkable change with increasing age of females

as shown in the table. The rate of embryos with slow early cleavage was higher than abnormally cleaving embryos in all age groups. The percentage of embryos failed to form blastocyst showed an increase with increasing age of females.

Table 3.5 shows the pregnancy outcome of patients. The mean number of embryo transferred in fresh cycle had no significant difference among all age groups while the mean number of frozen-thawed embryos transferred showed significant differences when compared among the age groups ($P \leq 0.01$). The percentage of frozen-thawed embryo transfers was higher than fresh cycle transfers in all age groups but each of them did not vary much when compared individually with increasing age of females however the rate of frozen transfers was comparatively low in younger (<26 years) patients. The clinical pregnancy rate of both fresh and frozen transfer showed a decline with increasing age of females. The implantation rates were higher than abortion rates in both fresh and frozen-thawed cycles but did not vary much with increasing age. The single pregnancy rates came out to be higher than multiple pregnancy rates both for fresh and frozen transfers as shown in the table.

Table 3.1. Patient demographic and cycle characteristics of females of different age groups.

Patient's Characteristics	□26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	□ 40 yrs.	P Value
No. of patients (n)	**24**	**62**	**68**	**34**	**12**	
Age (yrs.) (mean±SEM)	23.6±0.2	28.2±0.2	33.0±0.2	37.9±0.2	42.7±0.4	**P≤0.001**
BMI (Kg/m^2) (mean±SEM)	25.0±0.8	24.9±0.4	26.2±0.4	27.3±0.5	26.3±1.2	**P≤0.05**
Primary infertility (%)	23/24 **(95.8%)**	50/62**(80.6%)**	48/68 **(70.5%)**	23/34 **(67.6%)**	5/12 **(41.6%)**	
Secondary infertility (%)	1/24 **(4.1%)**	12/62**(19.3%)**	20/68 **(29.4%)**	11/34 **(32.3%)**	7/12 **(58.3%)**	
1st icsi attempt (%)	21/24 **(87.5%)**	54/62**(87.0%)**	55/68 **(80.8%)**	26/34 **(76.4%)**	9/12 **(75%)**	
2nd icsi attempt (%)	3/24 **(12.5%)**	8/62 **(12.9%)**	13/68 **(19.1%)**	8/34 **(23.5%)**	3/12 **(25%)**	
ICSI cycles (n)	23	55	64	33	10	
ICSI+IVF cycles (n)	1	7	4	1	2	
No. of oocytes retrieved (mean±SEM)	26.5±2.0	25.5±1.4	17.0±1.3	15.9±1.5	13.1±2.4	**P≤0.001**
No. of mature oocytes (mean±SEM)	21.6±1.9	19.3±1.2	12.9±0.9	12.7±1.3	8.5±1.5	**P≤0.001**
No. of oocytes fertilized (mean±SEM)	15.5±1.7	14.3±1.1	9.8±0.9	9.4±1.1	5.5±0.8	**P≤0.001**
No. of oocytes cleaved (mean±SEM)	15.1±1.6	14.0±1.1	9.6±0.9	9.2±1.1	5.4±0.8	**P≤0.001**

*** P≤0.05 , *** P≤0.001 (ANOVA followed by Tuckey's test)**

Table 3.2.Morphokinetic parameters of embryos in females of different age groups from day-1 to day-5 of embryonicdevelopment.

Morphokinetic parameters	□ 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	□ 40 yrs.	P Value
tPNa (mean±SEM)	18.9±4.9	13.4±0.4	14.9±1.4	13.8±1.8	13.9±1.1	**ns**
t2(mean±SEM)	28.9±0.6	28.8±0.5	30.3±1.1	28.9±0.6	30.5±1.4	**ns**
t3(mean±SEM)	37.3±0.8	36.9±0.7	37.1±0.6	36.8±0.7	38.1±1.5	**ns**
t4(mean±SEM)	42.4±0.8	42.2±0.6	42.6±0.6	42.6±1.0	43.4±1.7	**ns**
t5(mean±SEM)	52.0±1.3	51.3±0.9	51.2±1.0	52.6±1.4	53.3±1.8	**ns**
t8(mean±SEM)	67.5±1.3	68.8±0.9	69.0±1.1	71.2±1.6	71.5±1.8	**ns**
tM (mean±SEM)	100.4±1.2	99.4±1.2	100.4±1.1	100.6±1.7	99.9±2.3	**ns**
tSB (mean±SEM)	108.4±1.2	108.0±0.7	108.6±1.2	110.0±1.9	109.0±1.4	**ns**
tEB (mean±SEM)	112±0.9	112.9±0.8	112.1±1.0	112.0±1.6	112.8±2.3	**ns**
tFEB (mean±SEM)	114.1±0.8	115.5±0.3	115.4±0.9	115.5±0.6	116.8±1.5	**ns**
CC2 (t3-t2)	8.4±1.0	8.1±0.9	6.8±1.4	7.9±0.9	7.6±2.0	
CC3 (t5-t3)	14.7±1.5	14.4±1.2	14.1±1.3	15.8±1.7	15.2±2.4	
S2 (t4-t3)	5.1±1.1	5.3±1.0	5.5±0.9	5.8±1.3	5.3±2.3	
S3 (t8-t5)	15.5±1.8	17.5±1.3	17.8±1.5	18.6±2.1	18.2±2.3	

P>0.05 (ns = non-significant)

Table 3.3. Percentages of embryos with timely and untimely cleavage for important kinetics parameters in females of different age groups.

	Timely cleaved embryos					Untimely cleaved embryos				
Age (Yrs)	**□ 26 yrs.**	**26-30 yrs.**	**31-35 yrs.**	**36-40 yrs.**	**□ 40 yrs.**	**□ 26 yrs.**	**26-30 yrs.**	**31-35 yrs.**	**36-40 yrs.**	**□ 40 yrs.**
(n)	**133**	**346**	**271**	**135**	**24**	**112**	**271**	**259**	**116**	**37**
t2	133/245 **(54.3%)**	332/617 **(53.8%)**	270/530 **(50.9%)**	135/251 **(53.8%)**	24/61 **(39.3%)**	112/245 **(45.7%)**	285/617 **(46.1%)**	260/530 **(49.0%)**	116/251 **(46.2%)**	37/61 **(60.6%)**
t4	120/245 **(48.9%)**	278/617 **(45.0%)**	222/530 **(41.8%)**	116/251 **(46.2%)**	21/61 **(34.4%)**	125/245 **(51.0%)**	339/617 **(54.9%)**	308/530 **(58.1%)**	135/251 **(53.7%)**	40/60 **(66.6%)**
t5	92/245 **(37.5%)**	216/617 **(35.0%)**	171/530 **(32.2%)**	**97/251 (38.6%)**	18/61 **(29.5%)**	153/245 **(62.4)**	401/617 **(64.9%)**	359/530 **(67.7%)**	154/251 **(61.6%)**	43/60 **(71.6%)**
t8	121/245 **(49.4%)**	306/617 **(49.6%)**	240/530 **(45.3%)**	126/251 **(50.1%)**	21/61 **(34.4%)**	124/245 **(50.6%)**	311/617 **(50.4%)**	290/530 **(54.7%)**	125/251 **(49.8%)**	40/61 **(65.5%)**
tSB	73/245 **(29.8%)**	239/617 **(38.7%)**	182/530 **(34.3%)**	88/251 **(35.0%)**	15/61 **(24.9%)**	172/245 **(70.2%)**	378/617 **(61.2%)**	348/530 **(65.6%)**	163/251 **(64.9%)**	46/61 **(75.4%)**

Table 3.4. Developmental potential and embryos quality in females of different age groups.

Embryonic development	□ 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	□ 40 yrs.
Total embryos analyzed (n)	**372**	**890**	**670**	**319**	**67**
Cleavage rate (%)	364/372 **(97.8%)**	869/890 **(97.6%)**	655/670 **(97.7%)**	313/319 **(98.1%)**	65/67 **(97%)**
Blastocyst formed (%)	215/364 **(59%)**	475/869 **(54.6%)**	310/655 **(47.3%)**	137/313 **(43.7%)**	32/65 **(49.2%)**
Good quality blastocysts (%)	163/215 **(75.8%)**	367/475 **(77.2%)**	238/310 **(76.7%)**	96/137 **(70%)**	23/32 **(71.8%)**
Poor quality blastocysts (%)	52/215 **(24.1%)**	108/475 **(22.7%)**	72/310 **(23.2%)**	41/137 **(29.9%)**	9/32 **(28.1%)**
Blastocyst not formed (%)	149/364 **(40.9%)**	394/869 **(45.3%)**	345/655 **(52.6%)**	176/313 **(56.2%)**	33/65 **(50.7%)**
Slow early cleavage (%)	128/364 **(35.1%)**	293/869 **(33.7%)**	249/655 **(38%)**	131/313 **(41.8%)**	28/65 **(43%)**
Abnormal cleavage (%)	21/364 **(5.7%)**	101/869 **(11.6%)**	96/655 **(14.6%)**	45/313 **(14.3%)**	5/65 **(7.6%)**
Not cleaved (%)	8/372 **(2.1%)**	21/890 **(2.3%)**	15/670 **(2.2%)**	6/319 **(1.8%)**	2/67 **(2.9%)**

Table 3.5. Clinical data outcome in females of different age groups after culture in Time-Lapse System.

Clinical Outcome	≤ 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	≥ 40 yrs.	P Value
No. of embryos transferred Fresh cycle (mean±SEM)	2±0.2	1.8±0.1	2.1±0.1	2.0±0.2	1.6±0.2	**ns**
No. of embryos transferred Frozen-thawed cycle (mean±SEM)	2.3±0.1	2.1±0.1	1.8±0.1	1.8±0.1	2.4±0.3	**P≤0.01**
Fresh cycle transfers (%)	11/24 **(45.8%)**	19/62**(30.6%)**	31/68 **(45.5%)**	14/34**(41.1%)**	5/12 **(41.6%)**	
Clinical pregnancies Fresh cycle (%)	5/11 **(45.4%)**	7/19 **(36.8%)**	11/31 **(35.4%)**	4/14**(28.5%)**	1/5 **(20%)**	
Implantations Fresh cycle (%)	4/5 **(80%)**	6/7 **(85.7%)**	9/11 **(81.8%)**	3/4**(75%)**	1/1 **(100%)**	
Abortions Fresh cycle (%)	1/5 **(20%)**	1/7 **(14.2%)**	2/11**(18.1%)**	1/4**(25%)**	Nil	
Singleton pregnancies Fresh cycle (%)	3/5 **(60%)**	5/7 **(71.4%)**	10/11**(90.9%)**	3/4**(75%)**	1/1 **(100%)**	
Multiple pregnancies Fresh cycle (%)	2/5 **(40%)**	2/7 **(28.5%)**	1/11 **(9.0%)**	1/4**(25%)**	Nil	
Frozen-thawed transfers (%)	18/24 **(54.1%)**	50/62 **(80.6%)**	46/68 **(67.6%)**	26/34**(76.4%)**	9/12 **(75%)**	
Clinical pregnancies Frozen-thawed (%)	14/18 **(77.7%)**	41/50 **(82%)**	25/46 **(54.3%)**	13/26**(50%)**	2/9 **(22.2%)**	
Implantations Frozen-thawed (%)	14/14 **(100%)**	37/41 **(90.2%)**	23/25 **(92%)**	12/13**(92.3%)**	2/2 **(100%)**	
Abortions Frozen-thawed (%)	Nil	4/41 **(9.7%)**	2/25 **(8%)**	1/13**(7.6%)**	Nil	
Singleton pregnancies Frozen-thawed (%)	10/14 **(71.4%)**	38/41 **(92.6%)**	24/25 **(96%)**	13/13**(100%)**	1/2 **(50%)**	
Multiple pregnancies Frozen-thawed (%)	4/14 **(28.5%)**	3/41 **(7.3%)**	1/25 **(4%)**	Nil	1/2 **(50%)**	

**** P≤0.01, P>0.05 (ns= non-significant)**

DISCUSSION

It is known that female age is one of the essential factors influencing effectiveness of IVF cycles and pregnancy outcomes (Malizia *et al.,* 2009). Female with advanced age showed decreased numbers of retrieved oocytes (Ziebe *et al.,* 2001) and reduced fertilization and implantation rates (Szamatowicz and Grochowski , 1998; Pantos *et al*., 1999) thus increased spontaneous miscarriage rate and congenital anomalies (Ventura *et al*., 2000). To our knowledge, there are limited studies that consider female age and morphokinetics. Therefore, in our study we stratified patients by age into five groups (< 26 years, 26–30 years, 31–35 years, 36–40 years and > 40 years) for a clear comparison of young and old ages for embryonic development (De Croo *et al.,* 2000). A number of previous studies have compared IVF outcomes in women aged 40 years and above versus those younger than 40 years. Women over 37 years of age showed reduced fertility and in the age of 40 years, the likelihood of success for ART declines sharply with a low chance of a successful pregnancy in women aged ≥41 years (Schieve *et al.,* 1999).

In current study the embryo quality parameters assessed primarily i.e. the number of oocytes retrieved, matured, fertilized, cleaved and the blastocyst formation rate showed a decline with increasing age and BMI. It has been shown that more than 15 oocytes per cycle affect the efficiency of ART cycle negatively (Sunkara *et al*., 2011). Similarly the presence of 14 antral follicles at triggering of final maturing of oocytes is a boundary criterion for prediction of the ovarian hyperstimulation syndrome (OHSS) (Kwee *et al.,* 2007). The maternal age was found to be negatively correlated with oocyte yield and their quality. The deterioration of mitochondrial function with increasing female age is the result of empty DNA mutation and deletions. The continuous exposure to ROS generated mitochondrial metabolism oftenly results in cumulative DNA damage (Harman, 1956; Shigenaga *et al*., 1994). Resting follicles have a low level of mitochondrial oxidative phosphorylation. The continuous exposure of empty DNA to ROS produced as a byproduct of OXPHOS system, as well as lacking the protection of histone protein and lack of non-coding introns makes empty DNA more susceptible to functional mutations (Parsons *et al*., 1997). The recent publications have suggested that

the high rate of mutations in empty DNA can be a result of non-affective empty DNA repair mechanism of the enzymes responsible to maintain the nuclear genome (Krishnan *et al*., 2008). One of the most frequent empty DNA deletions is that of 4977 base pairs, almost a third of the whole of the empty DNA genome. This deletion is common in unfertilized eggs from older patients (Keefe *et al*., 1995).

The present study showed that the average time-points of all kinetic parameters of embryo development were found to be non-significantly different among the study groups. It has been documented earlier that the average values of morphokinetic parameters from oocytes of females with advanced age were found to be in normal range as of younger women (Gryshchenko et al., 2014). The aneuploidy rate and genetic abnormalities in oocytes of aged patients affects the IVF outcome badly (Yan *et al.,* 2012; Fragouli *et al*., 2011). Maternal age is associated with genetic impairment of oocytes due to aging mitochondrial apparatuses and fragmented DNA (Eichenlaub-Ritter *et al.,* 2004). It has been studied previously that both aneuploid and euploid embryos grow similarly till 8-cell stage but before blastulation the aneuploidy embryos show significant developmental delay as compared to euploid embryos (Campbell *et al.,* 2012). Using conventional microscopy with static imaging, Alfarawati *et al*. (2011) showed a weak association between blastocyst morphology and aneuploidy and reported aneuploid embryos showing slower progression to the most advanced blastocyst stages. While the present study showed that with increasing female age not only there is a delayed embryo growth observed but also the blastocyst formation rate decline along with the raise in percentage of poor quality blastocysts formed. The rate of embryos showing slow cleavage pattern increases as women get older.

The two most common mechanisms responsible for mitotic aneuploidies in human embryos are anaphase lagging and non-disjunction. Anaphase lag describes the delayed movement of one chromatid during anaphase hence it fails to connect to spindle apparatus and is not incorporated in either nucleus of daughter cells. The lagging chromosome is lost giving rise to monosomy in one of the two daughter cells. Similarly non-disjunction is the failure of sister chromatids to separate during mitosis hence

resulting in a cell with a loss and other cell with the gain of a chromosome (Daphnis *et al*., 2008; Coonen *et al.,* 2004). Other mechanisms leading to aneuploidies are premature cell division, chromosome demolition, errors in cytokinesis and cell fusion. Errors in cytokinesis like failed or a symmetric cytokinesis results in binucleated cells, tetra poloidy and spindle pole abnormalities thus ending up in chromosomal chaos (Chatzimeletiou *et al*., 2005). The human embryos at two to four cell stage shows multinucleation in 17% of cases with the likely hood of peak incidence during third cleavage division i.e. 65% (Hardy *et al*., 1993).

Defective maternal messenger RNA and protein pool can be responsible for the failure of correct cell divisions as many of the proteins regulating chromosomal segregation during early cleavage are contributed by the oocyte (Braude *et al*., 1998). The deteriorated oocyte protein pool can be a result of accumulated free radicals, exposure to radiation of chemicals or poor vascularization of antral follicles. This leads to defective microtubule kinetics, DNA repair proteins, shortening of telomere, chromosomal cohesion and mitochondrial function (Baumann *et al.,* 2010). The data suggest that mitotic non-disjunction results in increased mosaicism rate in human embryos (Rius *et al*., 2011). Although overall mosaicism does not increase with maternal age (katz-Jaffe *et al*., 2005).

In the present study the comparison between timely and untimely cleaving kinetic parameters indicated that time for 2 pronuclei appearance and early cleavage time-points (t4 and t5) showed significant difference among the five age groups whereas late kinetic parameters did not show any significance difference among different ages of female. The time for pronuclear appearance found to be significantly delayed in < 26 years as compared to other study groups whereas the t4 and t5 were found to be delayed in > 40 years as compare to young patients. In another study data were analyzed in younger versus older subjects (20-30, 30-40, and >40). The early morphokinetic parameters i.e. the times from insemination to tPnf, t2, t3, t4 were significantly shorter in the younger females than older ones ($p < 0.05$) (Akarsu *et al.,* 2017). In this study the percentages

of optimal embryos, according to t5 did not differ statistically between the groups while the current study showed that rate of timely cleaving embryos for t5 cleavage were far lower than untimely dividing among all age groups as shown in table 3. It has been shown previously that abnormally and untimely cleaving embryo are predominant than timely cleavages (Hlinka *et al.,* 2012). This is evident from present study that percentages of embryos showing delayed growth rises as developmental stages progress towards the start of blastulation. Another important observation shown in the current study is that the embryos rate for t5 is found to be closely associated with the embryos starting blastocoel cavity. This is in accordance with the findings of the study (Herrero *et al.,* 2013) which showed that t5 and t8 cleavages were more predictive of blastulation as compare to t2, t3, t4. Moreover same results declared by another finding have shown that t5 as one of the most important variables predicting implantation (Meseguer *et al.,* 2011). The same study has shown the optimal range for second cell cycle (cc2) as ≤ 11.9 h. The present work also revealed that cc2 was within this optimal range for all age groups. Whereas (Chamayou *et al.,* 2013) has shown that optimal range for duration of third cell cycle (cc3) to be from 9.7-21 h and the current study also found cc3 to be within the optimal range for all age groups. Herrero *et al.,* 2013 documented the timings for s2 and s3 for general population to be 2.7 and 9.9 hours respectively but significant difference was found in s2 and s3 among viable embryos with optimal morphology and those without optimal morphology whereas present study found s2 to be in range of 5.1±1.1 to 5.8±1.3 hpi and s3 to be in range of 15.5±1.8 to 18.6±2.1 hpi however the grouping was on the basis of female age irrespective of optimal morphology of embryos. Future work regarding the effect of female age on embryo morphokinetics can be more refined by selecting the prospective study design with limited number of embryos focusing on their pregnancy out comes.

In previous studies, significantly worse pregnancy results were achieved in subjects aged above 40 than those with age below 40 years (Flamigni, 1993; Borini *et al.,* 1996). Consistent with this, the present study also showed low conception rates in embryo transferred in fresh and frozen-thawed cycles in above 40 years age group (table 4) but implantation and abortion rate did not show any marked difference with increasing

age. Contrary to this, it has been reported that aging can result in high miscarriage rate and congenital abnormalities (Dain *et al.,* 2011).

The present study revealed that mean number of embryo transferred in fresh cycle did not show any difference with increasing female age but in cryopreserved cycle there is a significant rise in number of embryos transferred in old age patients. This is in consistence with the findings of (Dew *et al.,* 1998) who suggested to select the best quality embryo as well as increase the number of embryos transferred to improve the pregnancy outcome in old age women. However in present study the mean number of embryo transferred in patients >40 years of age was 2.4±0.3. Similar to current findings Altay *et al*. (2002) found no significant difference between the fertilization and cleavage rates of younger versus older women. There are some limitations of the present study worth to be mentioned. First, embryo scoring is a subjective assessment, which may include inter-observer variations, when the same embryo is analyzed by different embryologists. Secondly the number of patients in groups aged above 40 years is considerably smaller than in the younger groups, when pregnancy and implantation rates after SETs and DETs were analyzed. Last, the number of patients was considerably less in the older group, when blastocyst scores were related to female age. The current study also showed the multiple pregnancy rates in fresh cycle ranging from 9 to 40% and in frozen cycle between 4 to 50% for all age groups.

In view of the increasing usage of time-lapse imaging further information is also needed in order to elucidate if the time values underlying the model can be affected by certain variables and in particular, if maternal age has an influence too. The present study illustrates the effect of female age on embryo morphokinetics and the importance of time-lapse imaging to study the embryonic developmental pattern more precisely. More over the study emphasizes the early cleavage as being the most crucial factor for viable embryo development.

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Chapter # 4

A time-lapse evaluation of embryonic development among different pathological groups of infertile couples

ABSTRACT

The conventional static method of morphological evaluations has proved that human embryo quality is affected both by the patient and treatment related factors. Time-lapse imaging has revolutionized the study of human embryo morphological events by adding the concept of morphokinetics to select the most viable embryos for implantation in the field of IVF (In vitro fertilization). This retrospective study carried out at Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan aimed at investigating the morphokinetic parameters in different pathological conditions of patients visiting the IVF centers. A total number of 200 patients undergoing ICSI treatment cycles at the clinic were divided into eight pathology groups i.e. endometriosis, female age, high FSH, idiopathic, low AMH, male factor, PCOS, and tubal factor. Embryo culture was done at 37°C, 6% CO_2 and 5% oxygen for 5-6 days. Ten kinetic markers were analyzed and the cell cycle intervals were calculated too. The number of retrieved, matured, fertilized and cleaved oocytes showed highly significant difference ($P \leq 0.0001$) when compared among different pathologies. The eight pathological conditions showed significant difference ($P \leq 0.05$) for average t3 and tM only. There was no significant difference in all other morphokinetic time-points among all study groups. Whereas the timely cleaved embryos showed significant difference in t3 ($P \leq 0.01$), t5 ($P \leq 0.01$) and t8 ($P \leq 0.05$) when compared among different pathology groups. The cell cycle intervals i.e. cc2 and cc3 were found to be in normal range for different groups.The blastocyst formation rates were comparable among different groups while the clinical pregnancy rates were quite low in fresh cycle transfers in all study groups except PCOS and endometriosis. The frozen-thawed cycle showed comparable pregnancy rates in all groups except the patients with advanced age, high FSH and low AMH whose rates improved but not to an appreciable extent. Hence it is evident that different pathological conditions affect the oocyte and embryo quality as well as the clinical outcome during an IVF cycle when compared by using time-lapse morphokinetic evaluation.

INTRODUCTION

To address the problem of embryo selection with highest implantation potential many innovative approaches have been made. These include metabolic characteristics, transcription profile and genetic competence of embryo (Sfontouris *et al.,* 2013; Reich *et al.,* 2011; Rubio *et al.,* 2013). Recently time laps monitoring of morphokinetic have made it evident that embryo cleavage rate in influenced by different factors that include the patient factors, culture media, culturing condition s as well as some other factors (Ciray *et al.,* 2012; Zaninovic *et al.,* 2013).

Assisted reproduction technology (ART) has provided the patients with the number of advancements such as ICSI with improved culture media usage and PGD for the selection of healthy embryos (Van Steirteghem *et al.,* 1994; Palermo *et al.,* 1995). Still to introduce the concept of SET in clinical embryology, the time laps monitoring of embryo development has played a tremendous role (Handyside *et al.,* 1989). The time laps morphokinetic studies have also helped to overcome problems associated with static observations of embryo development, thus introducing new kinetic markers associated with higher implantation potential (Baxter Bendus *et al.,* 2006; Scott , 2003; Meseguer *et al.,* 2011). With the start of morphokinetic era in IVF imprecision of SET was observed when done only with conventional morphology observing 44.9% aneuploidy rate for good quality blastocysts (Yang *et al.,* 2012).

Many aspects of sperm can influence the infertility. Men with a low sperm count can have children and those whose sperm counts are normal can be infertile (Krausz, 2011). The defects in spermatozoa can be divided into three major categories i. sperm morphological defects ii. DNA and chromosomal damage; and iii. Biochemical/acrosome problems. These categories can be influence by life style, environment and physiological factors (Zini and Al-Hathal, 2011; Liu and Baker, 2002). For example smoking effect the sperm count and motility, both showing a significant decrease (Vine, 1996). Available studies shown 10% of male factor infertility is exogenous and reversible (NHIS 2008).

Complete fertilization failure is an unwanted fact which is reported to be as high as 50 % (Molloy *et al.,* 1991; Coates *et al.,* 1992), which may be contributed by the egg, sperm and laboratory factors (Liu *et al.,* 1995). It has been documented well that absolute indication for ICSI include surgically retrieve sperm (azoospermia), use of immotile but viable spermatozoa, globozoospermia (Nagy *et al.,* 1995; Lahteenmaki *et al.,* 1995),and necrozoospermia (Mobberley , 2010). Globozoospermia is although an uncommon cause of male infertility but is affecting 0.1 % of all patients. It is the result of disturbed spermiogenesis resulting in round headed sperm with acrosomal aplasia and is associated with very poor ICSI outcome (Holstein *et al.,* 1973; Dam *et al.,* 2007; Liu *et al.,* 2010). As compare to other structural abnormalities of sperm the effect of teratozoospermia on ICSI outcome remains limited (Hotaling *et al.,* 2010) however showing low implantation rate (de Vos *et al.,* 2003).

Most of the studies showing ICSI out come with surgically retrieve sperm are based on conventional static embryo quality assessment (Ben-Ami *et al.,* 2013; Desai *et al.,* 2009; Ishikawa *et al.,* 2009). The recent advancement of TLM system have given us a chance of accurate cleavage timing measurement hence providing a batter comparison of ICSI outcome of azoospermic patients with conventional morphology as well as time laps morphokinetic (Herrero and Meseguer , 2013; Sundvall *et al.,* 2013). Time-laps imaging has a great advantage of indicating the cell anomaly at different stages that may result into genetic abnormality of embryo such as mosaicism (Athayde *et al.,* 2014). Embryo cleavage abnormalities are result of various factors which could be inherited by both partners. Campbell *et al.* (2013) studied the correlation between aneuploidy and morphokinetic markers and established that sperm drive factors could influence the early embryo cleavage. This is because of the fact that centrosome organizing the cleavage patron is inherited paternally. Furthermore the abnormalities in the spindle formation can lead to missegregation of genetic material during mitosis and contributes to cleavage abnormalities (Chatzimeletiou *et al.,* 2005). During the last decade a new technique has been introduced which involves the observation of unstained spermatozoa with real-time, magnified view named ‘motile sperm organelle morphology examination (MSOME). (Bartoov *et al.,* 2003).

Obesity has been proved to affect the fertility involving multiple complications during pregnancy, changes in menstrual patron, anovulation and delayed conception negatively (Pasquali *et al.,* 2007; Lash and Armstrong, 2009). Obesity not only effect the natural conception but also the fertility treatment out comes (Sebire *et al.,* 2001). There are some studies which illustrate that BMI does not affect the assisted reproductive outcome (Fedorcsak *et al.,* 2004). This controversy may elaborate the effect of obesity on other female related disorders like oocyte quality (Martinuzzi *et al.,* 2008). Nutritionally induced hypercholesterolemic conditions has been shown to affect the oocyte developmental potential and embryo quality in terms of cell number and gene expression patron and may result in growth retardation (Leroy *et al.,* 2010; Picone *et al.,*2011).

Female age has been studies well as a first line marker of ovarian reserves and it is related to decline in reproductive capacity (Templeton *et al.,* 1996). Ovarian aging is associated with decline in both quantity and quality of oocytes in ovaries. Younger patients have proved to be better responders to IVF treatment than older women (35 years) (Loh and Maheshwari , 2011; Busso and Pellicer , 2011). Moreover the embryo selection through time-lapse imaging is a noninvasive tool to select chromosomally normal embryos for implantation not only in age women with high risk of embryo aneuploidies but also among young good-prognosis patients (Munne , 2006; Yang *et al.,* 2012). Ovarian reserves is the one of main issues in fertility treatments (Visser and Themmen , 2005) which allows us to know the patient's possible response in the management of stimulation protocols with appropriate gonadotrophin dosages (Fleming *et al.,* 2013). Approximately 2–30% of all women undergoing controlled ovarian stimulation experience poor response (Hendriks *et al.,* 2005). AMH has been found to be related with oocyte yield (La Marca *et al.,* 2012) and embryo quality (Ebner *et al.,* 2006) after ovarian stimulation hence capable of predicting the ovarian response.

There are a number of markers to predict the ovarian response such as basal FSH, estradiol, inhibin B and antral follicle count (AFC), and AMH but each with scope from modest to poor (Robertson *et al.,* 2008; Broer *et al.,* 2010). The decline of AMH with age is already documented well and capacity of AMH as a predictor marker of ovarian reserves is significantly higher than other hormonal parameters (Fréour *et al.,* 2007;

Fanchin *et al.,* 2005). Both the Follicle stimulating hormone (FSH) and anti-Müllerian hormone (AMH) represent FOR (follicular ovarian reserves) (Gleicher *et al.,* 2012) and both are reported to have different significance at different ages in defining FOR and hence the oocyte yield (Fusco *et al.,* 2011). The result is the decline in number and quality of the oocytes. Anti-mullerian hormone has been diagnosed as a prognostic factor in reproductive cycles and its levels are a better predictor of ovarian response to gonadotrophic stimulation in IVF cycles then FSH levels or the female age (Satwik *et al.,* 2012). A few studies have shown that AMH is a good prognostic factor for IVF pregnancy outcome (Kamel *et al.,* 2014) and blastocyst formation (Lin *et al.,* 2013; Sills *et al.,* 2011).

Polycystic ovary syndrome (PCOS) is the most common endocrinological disorder affecting 5–10 % of women in the reproductive age (Knochenhauer *et al.,* 1998). This syndrome is related to androgen excess and ovulatory dysfunction (ESHRE, 2004). The treatment choice for these women is IVF. PCOS patients are found to produce an increased number of oocytes but of poor quality leading to low maturity ad fertilization rate (Sengoku *et al.,* 1997; Urman *et al.,* 1992). More over these patients are at high risk of early abortion and fetal birth (Sahu *et al.,* 2008; Roos *et al.,* 2011). Causes for these poor pregnancy out comes are not clear but most probably the inherited oocyte factors are involved. The pregnancy rates of these patients are reported to be satisfactory as compare to other infertile patients with normal ovaries. A precautionary measure has to be taken by developing the controlled ovarian stimulation regime for these patients to avoid ovarian hyper stimulation syndrome (Kim *et al.,* 2010; Vause *et al.,* 2010).

Most of the studies to evaluate embryo quality in PCOS patients have been based on conventional static assessments which indicate the week correlation with embryo viability (Lane and Gardner, 1997). On the other hand the time laps monitoring have suggested that the timing of development is critically related to clinical outcome in IVF patients (VerMilyea *et al.,* 2014; Kirkegaard *et al.,* 2015). It has been documented that the embryo from hyper androgenic PCOS patients showed significantly delayed early cleavage as compare to non PCOS women. Another very important factor affecting the

female fertility is tubal disease which involves 25% to 35% of infertility cases (Wissing *et al.,* 2013).

Tubal infertility can involve the entire tube dysfunction or proximal and distal ends and severity of condition also varies in different women (Serafini and Batzofin, 1989). The most common cause of this tubal infertility is pelvic inflammatory disease representing more than 50% of cases and affecting the fallopian tubes (Honore *et al.,* 1999). The (The Hull & Rutherford classification (2002) classification of tubal disease signifies three categories namely mild/grade I, moderate/grade II and severe/grade III (Akande *et al.,* 2004). Diagnosis is made by hysterosalpingography (HSG) or laparoscopy. Treatment options include non-invasive management, surgical tubal repair and IVF.

Endometriosis is a chronic inflammatory disease in women causing infertility and pain. Laparoscopy is done for diagnosis specifically of the suspected lesion are and the histological verification of the condition. The population base studies indicate the overall prevalence of endometriosis from 0.8% to 6% (Abbas *et al.,* 2012; Fuldeore and Soliman , 2016) however the subfertile population have a considerably higher prevalence i.e. 20 to 50% (Meuleman *et al.,* 2009). There is significant variation in the severity of condition based on age of patients and the time period of disease. In another study it was shown that infertility raises two folds in women below 35 years with endometriosis as compare to women without endometriosis (Prescott *et al.,* 2016). Endometriosis can impair fertility either by itself or in combination with other factors. Endometriosis affect the female fertility both by endocrine dysfunction and peritoneal inflammation which results in ovarian malfunctioning and ultimately reduce the oocyte competence. Surgical resection of endometriomas and deep infiltrating lesions has been found to improve the fertility rate (Hamdan *et al.,* 2015).

The unexplained infertility is defined as the infertility or subfertility in the absence of any obvious barrier to conception (Evers, 2002; Practice Committee of the American Society for Reproductive Medicine, 2006). Despite the advancements in the

field of reproductive medicines, one quarter of cases of infertility remains unexplained which needs a multidimensional approach to investigate the causes behind it (Practice Committee of the American Society for Reproductive Medicine, 2015).

All the above mentioned condition comprises the pathological barriers of reproduction. Assisted reproductive technology (ART) offers several treatment options to bypass these barriers ART can be mainly divided into in vivo or in vitro procedures depending upon whether or not the oocytes are extracted from the ovaries, fertilized, cultured and transferred back to uterus. One of very important in vivo procedure is intrauterine insemination (IUI) with or without follicle stimulation, (Jeon *et al.,* 2013) followed by gamete intrafallopian transfer (GIFT). The most commonly used in vitro procedures include IVF for couples with normal sperm count in which the embryo is cultured in the laboratory and transferred back to uterus. With the history of previous failure of fertilization with IVF the patients are referred for intracytoplasmic sperm injection (ICSI). ICSI is the most frequently used treatment option for the male factor infertility i.e. low sperm counts, slow progression of spermatozoa and poor morphological parameters of sperm.

Objective of the study:

The current study had following aim and objectives:

1) To study the early cleavage pattern of embryos from patients with different pathologies, their potential to form blastocysts and quality of blastocysts formed using time-lapse imaging and success rates of the treatment.

2) To investigate whether there are any differences in morphokinetic parameters between good quality and poor quality embryos of different pathology groups and to compare the embryo morphokinetic data with previously evaluated important kinetic markers for embryonic development.

MATERIALS AND METHODS

Study Design

The current retrospective study was derived prospectively acquired data of time-lapse imaging of human embryos during in vitro development. The data used in this study were obtained from Islamabad Clinic Serving Infertile Couples (ICSI), Islamabad, Pakistan. The procedures and protocols were approved and practiced by the Institution. The number of oocytes observed in this study was 4080 producing 2266 embryos in 200 ICSI (Intracytoplasmic sperm injection) treatment cycles. A total number of 1704 embryos were analyzed for time-lapse monitoring due to split cycles for conventional culturing as a quality control measure at the outset of time-lapse system in the clinic. The patients were segregated on the basis of major indication for infertility hence divided into eight pathological groups i.e. endometriosis, female age, high FSH, idiopathic, low AMH, male factor, PCOS, and tubal factor. Females showed a mean age of 31.8±0.3 years. Most of the embryos were obtained after fertilization by ICSI with a very few ICSI+IVF cycles. Ten kinetic parameters were selected for evaluation. Embryo transfer data of both fresh and cryopreserved cycles of patients was analyzed. The βhCG level in the blood sample taken 10 days after embryo transfer confirmed the pregnancy and later on at an ultrasound scanning for fetal heart was done after 7 weeks of pregnancy. The kinetic time-points used for embryo evaluation at different stages of development were measured as hours post icsi (hpi) and corresponding reference ranges are the same as described in chapter 2.

The exclusions criteria:

d) Unfertilized oocytes were excluded from data.

e) All embryos whose time-lapse image acquisition were started later than 20 hours and ended before 60 hours after ICSI were not included.

f) Extreme outliers were excluded that may bias the analysis (as extremes may distort the mean of the regular divisions).

g) However the inclusion criteria for this specific study was based on the severity of pathological condition of the patient to be included into a specific group as very few patients presented with exclusively single indication of infertility.

h) A collective consensus was made by reviewing the videos and images of time-lapse monitoring of patients by one of the senior embryologist and the lab director and the guidelines were set.

Down regulation, ovarian stimulation, Oocyte retrieval, ICSI and embryo culture in Time-Lapse System

The pituitary desensitization was done using both long and short protocols. Ovarian stimulation is started with the administration of FSH injection by adjusting the starting dosage depending upon the age of patients, serum AMH hormones levels, the number of antral follicles and the response of patients to any previous cycles. Final maturation was induced using recombinant hCG when at least 2 leading follicles reached 18 mm in diameter. The oocyte retrieval was performed 36–37 h later. The denudation of oocytes was done 2-3 hours after the retrieval and ICSI was performed on mature oocytes. The fertilization check was done 16-18 hours post ICSI. Zygotes were shifted to pre-equilibrated primo vision group-culture dish and cultured for 5-6 days at 37°C with 6% CO_2 and 5% oxygen in the Primo-Vision Time-Lapse embryo monitoring system to monitor their daily growth (Figure 4.1 and 4.2). The embryo transfer and cryopreservation were decided on day-5 or day-6 of the embryonic development. The second chapter contains the detailed methodology of the above mentioned procedures.

Figure 4.1. Using the Primo Vision group culture dish for time-lapse evaluation of
embryos.

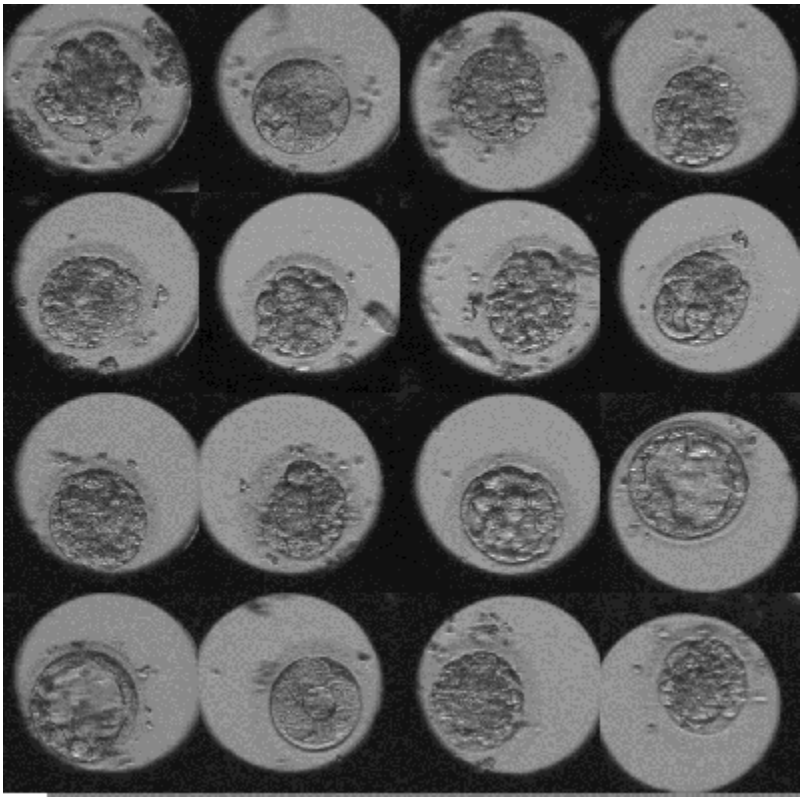

Figure 4.2. Humanembryos showing high fragmentation and poor blastocyst quality.

Statistical Analysis

The analysis of variance (ANOVA) followed by Tuckey's test was done to check the difference of means for different clinical and kinetic parameters among different pathology groups. The least significant difference (LSD) analysis was also done to investigate the specific differences in different groups regarding the important kinetic markers. Data are presented as mean±SEM. $P \leq 0.05$ was considered as indicative of a statistical significance. All results were obtained using statistical software GraphPad Prism 5 (GraphPad Prism Software, Inc. CA, USA).

RESULTS

We have analyzed in the present study the embryology outcome of couples visiting the IVF center with different pathological conditions. The table 4.1 showed that most common pathologies observed among patients are male factor infertility, PCOS and tubal factor whereas patients visiting IVF centers for other causes of infertility were considerably lower in number. The age of patients was found to be significantly different ($P \leq 0.001$) when compared among all groups whereas BMI has no significant difference among the different pathological groups. The rate of primary infertility was highest in patients with endometriosis, male factor infertility, high FSH and PCOS, as compared to other groups. Similarly the rate of secondary infertility was highest in patients of advanced age, idiopathic infertility and patients with low AMH as compare to other pathologies. The patients appearing for 1st attempt of ICSI were considerably high in number in all groups as compared to repeaters while the rate of 2nd attempt was still high in patients of increased age, high FSH, and with severe male factor infertility as compared to other groups. The number of eggs retrieved, matured, fertilized and cleaved was significantly different among the eight groups of pathologies ($P \leq 0.001$).

In the table 4.2 the mean time-lapse morphokinetics parameters for all pathology groups are shown. The cell cycle intervals cc2 and cc3 along with the synchronous divisions s2 and s3 are also given in the table. The mean time for 3-cell stage (t3) and compacted morula (tM) showed a significant difference ($P \leq 0.05$) when compared among different pathology groups while all other parameters were non-significantly different (Figure 4.3, 4.4). The LSD (least significance difference) when calculated for the mean time for 3-cell cleavage among different pathological groups showed that the patients with endometriosis and high FSH showed significantly higher t3 as compared to all other groups except the high female age group and. Similarly the mean time for morula formation (tM) when compared among different pathology groups applying LSD showed that the patients with low AMH levels had significantly higher tM as compared to all other pathologies except the high FSH group which showed significantly higher tM when

Table 4.1: Patient demographic and cycle characteristics in eight pathology groups.

Patient's Characteristics	Endometriosis	Female age Factor	High FSH	Idiopathic	Low AMH	Male Factor	PC
No. of patients (n)	**11**	**19**	**10**	**10**	**13**	**66**	**34**
Age (yrs.) (mean±SEM)	30.3±0.8	41.7±0.4	30.8±1.3	30.1±1.0	33.7±0.8	30.3±0.6	28.6±
BMI (Kg/m^2) (mean±SEM)	24.5±0.7	26.4±1.0	24.6±1.3	27.6±1.3	25.6±1.5	26.1±0.4	24.8±
Primary infertility (%)	9/11 **(81.8%)**	9/19 **(47.4%)**	8/10 **(80%)**	6/10 **(60%)**	8/13 **(61.5%)**	57/66 **(86.4%)**	27/ **(79.4**
Secondary infertility (%)	2/11 **(18.2%)**	10/19 **(52.6%)**	2/10 **(20%)**	4/10 **(40%)**	5/13 **(38.5%)**	9/66 **(13.6%)**	7/3 **(20.6**
1st icsi attempt (%)	10/11 **(90.9%)**	15/19 **(78.9%)**	7/10 **(70%)**	10/10 **(100%)**	12/13 **(92.3%)**	47/66 **(71.2%)**	31/ **(91.2**
2nd icsi attempt (%)	1/11 **(9.1%)**	4/19 **(21.1%)**	3/10 **(30%)**	Nil	1/13 **(7.7%)**	19/66 **(28.8%)**	3/3 **(8.8**
ICSI cycles (n)	7	16	10	10	12	66	28
ICSI+IVF cycles (n)	4	3	Nil	Nil	1	Nil	6
No. of oocytes retrieved (mean±SEM)	20.2±2.4	11.6±1.6	15.1±2.7	18.4±1.7	9.7±1.7	20.3±1.2	33.6±
No. of mature oocytes (mean±SEM)	14.4±1.6	7.8±1.0	10.2±1.9	14.3±1.5	6.6±1.2	15.8±1.0	26.4±
No. of oocytes fertilized (mean±SEM)	12.4±1.7	5.5±0.6	6.4±1.4	11.6±1.1	5.5±1.3	10.5±0.8	20.6±
No. of oocytes cleaved (mean±SEM)	12.4±1.7	5.4±0.6	6.1±1.3	11.4±1.2	5.2±1.2	10.1±0.8	20.3±

***** P≤0.001 (ANOVA followed by Tuckey's test)**

P>0.05, ns- Non-significant (ANOVA followed by Tuckey's test)

Table 4.2 : Embryo morphokinetic parameters of eight study groups from day-1 to day-5 of development.

Morphokinetic parameters	Endometriosis	Female age Factor	High FSH	Idiopathic	Low AMH	Male Factor	PCOS	Tubal Factor	P Value
tPNa (mean±SEM)	13.3±0.6	12.9±0.9	23.8±7.7	12.9±0.9	13.1±1.0	14.1±1.0	13.7±0.9	14.5±1.7	**ns**
t2(mean±SEM)	31.6±1.7	29.5±0.8	32.4±4.4	27.7±0.7	30.7±1.5	28.7±0.7	28.6±0.4	30.3±1.3	**ns**
t3(mean±SEM)	41.1±2.6	37.9±1.0	41.1±2.4	36.2±1.4	37.1±1.5	36.1±0.6	37.2±0.6	36.2±1.0	**P≤0.05**
t4(mean±SEM)	45.1±1.5	42.3±1.1	45.2±3.1	41.0±1.1	43.1±2.0	41.8±0.6	42.1±0.6	43.0±0.9	**ns**
t5(mean±SEM)	53.8±1.3	53.1±1.6	49.8±3.9	49.6±1.5	51.1±2.9	51.5±0.8	51.0±1.2	52.6±1.6	**ns**
t8(mean±SEM)	72.4±2.1	69.6±1.4	68.7±2.3	65.4±1.2	75.7±3.8	68.5±0.9	68.2±1.2	69.6±1.5	**ns**
tM (mean±SEM)	97.1±2.3	98.1±1.9	103.8±2.3	96.2±2.3	106.5±2.6	101.3±1.2	99.9±1.4	98.2±1.4	**P≤0.05**
tSB (mean±SEM)	106.0±1.8	108.2±1.4	109.6±1.3	105.1±2.2	114.5±3.5	110.1±1.2	108.2±1.3	106.5±1.1	**ns**
tEB (mean±SEM)	111±1.4	112.2±1.5	113.7±1.4	108.0±1.2	113.6±1.0	112.9±0.9	113.7±1.3	111.6±1.3	**ns**
tFEB (mean±SEM)	115.1±0.9	115.7±1.0	116.2±0.9	114.7±0.7	115.0±2.2	114.4±0.4	114.8±0.6	117.0±1.3	**ns**
CC2 (t3-t2)	9.5±3.0	8.4±1.3	8.7±5.5	8.5±1.5	6.4±2.2	7.4±1.0	8.6±0.7	5.9±1.7	
CC3 (t5-t3)	12.7±2.7	15.2±2.0	8.7±4.6	13.4±2.0	14.0±3.5	15.4±1.0	13.8±1.4	16.4±2.0	
S2 (t4-t3)	4.0±2.8	4.4±1.5	4.1±3.9	12.8±1.8	6.0±2.7	5.7±0.8	4.9±0.9	6.8±1.4	
S3 (t8-t5)	18.6±2.5	16.5±2.1	18.9±4.3	15.8±1.9	24.6±4.8	17.0±1.3	17.2±1.7	17.0±2.2	

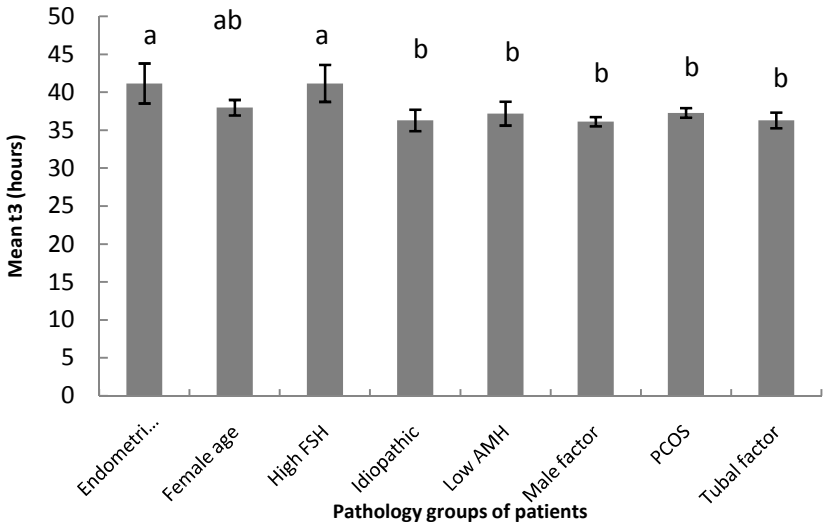

Figure 4.3. Comparison of mean time for 3-cell cleavage (t3) among patients with different pathologies.

ANOVA (P≤0.05) confirmed by LSD (The superscript above the bars indicate specific differences among the groups)

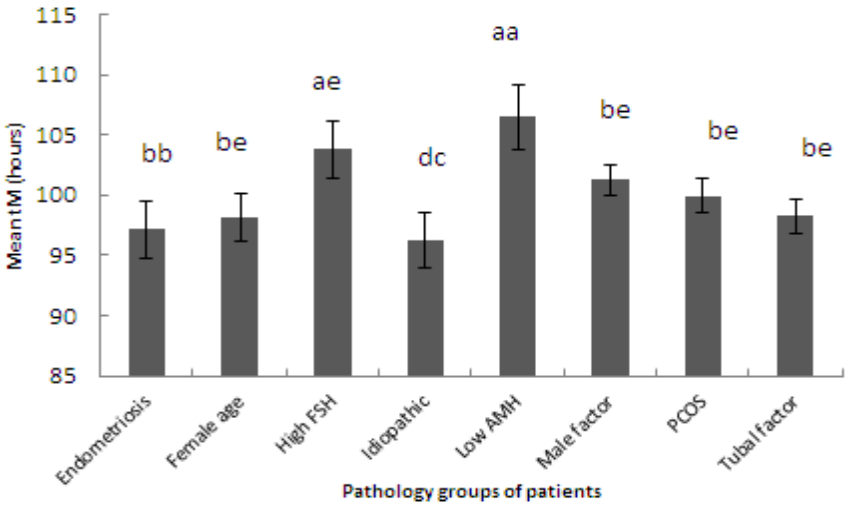

Figure 4.4. Comparison of mean time for morula formation (tM) among patients with different pathologies.

ANOVA (P≤0.05) confirmed by LSD (The superscript above the bars indicate specific differences among the groups)

compared to endometriosis and idiopathic groups. Similarly the patients with male factor infertility showed significantly higher tM as compared to idiopathic group only. Hence overall the patients with high FSH and low AMH levels showed delayed morula formation as compared to other pathologies.The cell cycle interval for second cell cycle (cc2) ranged from 6.4±2.2 to 9.5±3.0 for all study groups whereas the third cell cycle showed a range from 8.7±4.6 to 16.4±2.0. Similarly the range of s2 was 4.0±2.8 to 12.8±1.8 and of s3 was 15.8±1.9 to 24.6±4.8. The embryos were segregated for each pathology group on the basis of timely and untimely early cleavage.

The ANOVA performed on the data of timely cleaving embryos of all pathology groups and the result showed significant difference in timings of 3-cell, 5-cell and 8-cell stage i.e. t3, t5 and t8 in all study groups. The specific differences were as follows.The (Figure 4.5) shows that t3 came out to significantly high in patients with endometriosis as compared to idiopathic and high FSH group ($P \leq 0.05$), the male factor and PCOS groups ($P \leq 0.01$) and the tubal factor group ($P \leq 0.001$). Similarly t5 was also significantly high (Figure 4.6) in patient with endometriosis as compared to the idiopathic, high FSH, male factor and PCOS groups ($P \leq 0.01$). As far as t8 is concerned it was shown (Figure 4.7) to be significantly high ($P \leq 0.05$) in endometriosis patients as compared to idiopathic and PCOS group. All remaining time-lapse parameters were found to be non-significantly different among all study groups.

As the table 4.3a and 4.3b shows clearly that number of timely cleaving embryos was higher than untimely cleaving in all pathologies except the endometriosis and low AMH groups. Regarding the timely cleaved embryos there was a decline in rate of timely cleavage as the development progressed from t2 to tSB and a harmony in rates of t5 and tSB was observed i.e. the rate of embryos cleaving for 5-cell stage and for start of blastulation (tSB) were in close proximity.

The rate of untimely cleavage was observed to be dominant over timely cleavage as the development of embryos proceeds hence there was an increase in rate of untimely cleaving embryo from t2 to tSB stage. It was observed here too that rate of t5 was very close to tSB as compared to other kinetic markers.

In table 4.4 the quality and developmental potential of embryos in eight pathological conditions is elaborated. As it is evident from the table that the cleavage rate of embryos in eight groups did not vary much whereas the rate of embryos not cleaved was very low in all groups but still among them highest rates were observed in patients with low AMH and high FSH. The blastocyst formation rate is highest in PCOS group and lowest in patient with low AMH levels. The rate of good quality blastocysts also varied a little in groups but came out to be much higher than rate of poor quality blastocyst in all groups. The poor quality blastocysts are most frequently observed in patients with tubal factor.

Similarly the rate of embryos which failed to form the blastocyst is highest among the patients with low AMH levels. The patients with endometriosis, low AMH and male factor infertility showed the most of embryos failing to reach the blastocyst stage as compared to other groups. The embryos showing slow and arrested growth were common among patients with high female age, low AMH and endometriosis as compared to other pathologies. The rate of abnormal cleavage was high in patients with low AMH, high FSH, male factor, tubal factor and also in idiopathic group.

In the table 4.5 the clinical outcomes of patients from all pathological groups is shown. There was no significant difference in number of embryos transferred in fresh and frozen-thawed cycles of patients from all pathology groups. The frozen-thawed embryo transfers were considerably high than fresh cycle transfers in all groups studied except the patients with high FSH levels in which the fresh transfer rate was higher than frozen-thawed. The clinical pregnancy rate was high in frozen thawed cycles of patients with idiopathic infertility, low AMH levels, male factor infertility, PCOS and tubal factor while the patients with increased female age, high FSH levels and endometriosis show high pregnancy rate in fresh cycle as compared to frozen-thawed. The clinical pregnancies were found to be highest among patients with endometriosis and no pregnancy occurred in low AMH group in fresh cycle transfers whereas for frozen-thawed transfers the pregnancy rate was highest in patients with idiopathic infertility and considerably low in patients of increased age. The implantation rates were appreciably higher than abortion rates in both fresh and frozen-thawed cycles of all study groups as shown in

the table. While comparing among the study groups the implantations from fresh cycle were 100% in patients with increased female age, high FSH, idiopathic infertility and PCOS whereas the patients with endometriosis, male factor and tubal factor showed low implantation rates. The patients with low AMH showed no pregnancies in fresh cycle while in frozen-thawed cycle the pregnancy and implantation rate were 55.6% and 80% respectively. The frozen-thawed cycle of all study groups showed good implantation rates with little variation among them. The abortion rates in fresh cycle transfers were highest in patients with diagnosis of endometriosis while in frozen-thawed transfers the low AMH group showed the maximum i.e. 20% abortions. The single pregnancy rate was remarkably higher than multiple pregnancies in both the fresh and frozen-thawed cycles.

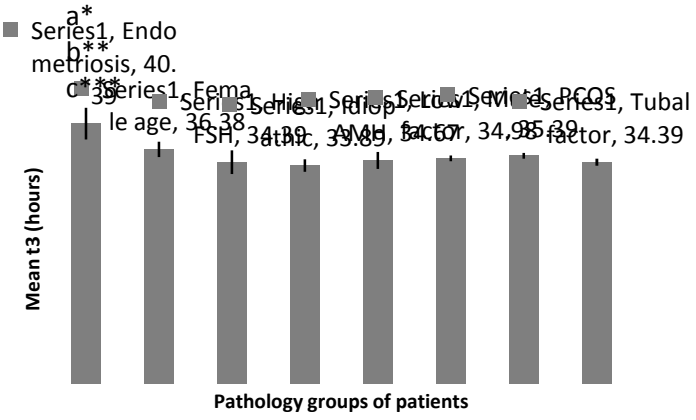

Figure 4.5. Comparison of mean time for 3-cell cleavage (t3) among timely-cleaved embryos of patients with different pathologies.

a* P≤0.05 vs. high FSH and idiopathic group (ANOVA followed by Tukey's test)
b P≤0.001 vs. male factor and PCOS group (ANOVA followed by Tukey's test)**
c* P≤0.05 vs. tubal factor group (ANOVA followed by Tukey's test)**

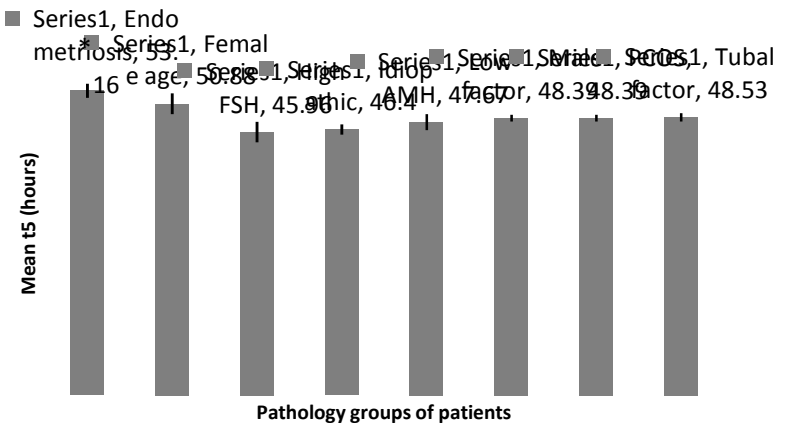

Figure 4.6. Comparison of mean time for 5-cell cleavage (t5) among timely-cleaved embryos of patients with different pathologies.

P≤0.05 vs. high FSH, idiopathic group, male factor and PCOS groups (ANOVA followed by Tukey's test)

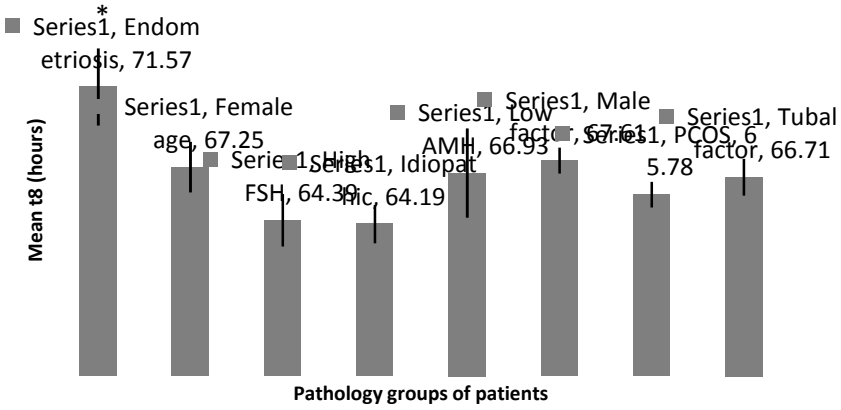

Figure 4.7. Comparison of mean time for 8-cell cleavage (t8) among timely-cleaved embryos of patients with different pathologies.

*** P≤0.05 vs. idiopathic and PCOS group (ANOVA followed by Tukey's test)**

Table 4.3a. Percentage of embryos with timely cleavage for important kinetics parameters in infertile couples with different pathologies.

Timely cleaved embryos								
Pt. pathology	**Endo-metriosis**	**Female age Factor**	**High FSH**	**Idiopathic**	**Low AMH**	**Male Factor**	**PCOS**	**Tubal Factor**
(n)	**52**	**57**	**26**	**59**	**25**	**286**	**249**	**183**
t2	38/105 **(36.1%)**	47/99 **(47.4%)**	25/50 **(50%)**	54/96 **(56.2%)**	25/61 **(40.9%)**	278/522 **(53.2%)**	244/446 **(54.7%)**	182/325 **(56%)**
t4	46/105 **(43.8%)**	50/99 **(50.5%)**	22/50 **(44%)**	46/96 **(47.9%)**	20/61 **(33.3%)**	240/522 **(45.9%)**	208/446 **(46.6%)**	147/325 **(45.2%)**
t5	34/105 **(32.3%)**	42/99 **(42.4%)**	20/50 **(40%)**	29/96 **(30.2%)**	20/61 **(33.3%)**	188/522 **(36.0%)**	165/446 **(36.9%)**	117/325 **(36%)**
t8	47/105 **(44.7%)**	53/99 **(53.5%)**	26/50 **(52%)**	56/96 **(58.3%)**	25/61 **(40.9%)**	255/522 **(48.8%)**	218/446 **(48.8%)**	168/325 **(51.6%)**
tSB	38/105 **(36.1%)**	35/99 **(35.3%)**	20/50 **(40%)**	43/96 **(44.7%)**	21/61 **(34.4%)**	161/522 **(30.8%)**	176/446 **(39.4%)**	127/325 **(39.1%)**

Table 4.3b. Percentage of embryos with untimely cleavage for important kinetics parameters in infertile couples with different pathologies.

Untimely cleaved embryos								
Pt. pathology	**Endo-metriosis**	**Female age Factor**	**High FSH**	**Idiopathic**	**Low AMH**	**Male Factor**	**PCOS**	**Tubal Factor**
(n)	**53**	**42**	**24**	**37**	**36**	**236**	**197**	**142**
t2	67/105 **(63.8%)**	52/99 **(52.5%)**	25/50 **(50%)**	42/96 **(43.7%)**	36/61 **(59.0%)**	244/522 **(46.7%)**	202/446 **(45.2%)**	143/325 **(44%)**
t4	59/105 **(56.1%)**	49/99 **(49.4%)**	28/50 **(56%)**	50/96 **(52.1%)**	41/61 **(67.2%)**	282/522 **(54.0%)**	238/446 **(53.3%)**	178/325 **(54.7%)**
t5	71/105 **(67.6%)**	57/99 **(57.5%)**	30/50 **(60%)**	67/96 **(69.7%)**	41/61 **(67.2%)**	334/522 **(63.9%)**	281/446 **(63%)**	208/325 **(64%)**
t8	58/105 **(55.2%)**	46/99 **(46.4%)**	24/50 **(48%)**	40/96 **(41.6%)**	36/61 **(59.0%)**	267/522 **(51.1%)**	228/446 **(51.1%)**	157/325 **(48.3%)**
tSB	67/105 **(63.8%)**	64/99 **(64.6%)**	30/50 **(60%)**	53/96 **(55.2%)**	40/61 **(65.5%)**	361/522 **(69.1%)**	270/446 **(60.5%)**	198/325 **(60.9%)**

Table 4.4: Developmental potential and embryos quality in eight pathology groups.

yonic development	Endometriosis	Female age Factor	High FSH	Idiopathic	Low AMH	Male Factor	PCOS
embryos analyzed (n)	**137**	**103**	**64**	**116**	**72**	**694**	**692**
eavage rate (%)	137/137 **(100%)**	102/103 **(99.0%)**	61/64 **(95.3%)**	114/116 **(98.2%)**	68/72 **(94.4%)**	670/694 **(96.5%)**	682/692 **(98.5%)**
tocyst formed (%)	66/137 **(48.1%)**	53/102 **(51.9%)**	32/61 **(52.4%)**	60/114 **(52.6%)**	30/68 **(44.1%)**	310/670 **(46.2%)**	390/682 **(57.1%)**
quality blastocysts (%)	52/66 **(78.7%)**	40/53 **(75.4%)**	25/32 **(78.1%)**	46/60 **(76.6%)**	24/30 **(80.0%)**	232/310 **(74.8%)**	298/390 **(76.4%)**
quality blastocysts (%)	14/66 **(21.2%)**	13/53 **(24.5%)**	7/32 **(21.8%)**	14/60 **(23.3%)**	6/30 **(20.0%)**	78/310 **(25.1%)**	92/390 **(23.5%)**
tocyst not formed (%)	71/137 **(51.8%)**	49/102 **(48.0%)**	29/61 **(47.5%)**	54/114 **(47.3%)**	38/68 **(55.8%)**	360/670 **(53.7%)**	292/682 **(42.8%)**
early cleavage (%)	59/137 **(43.0%)**	42/102 **(41.1%)**	20/61 **(32.7%)**	40/114 **(35.0%)**	29/68 **(42.6%)**	264/670 **(39.4%)**	230/682 **(33.7%)**
rmal cleavage (%)	12/137 **(8.7%)**	7/102 **(6.8%)**	9/61 **(14.7%)**	14/114 **(12.2%)**	9/68 **(13.2%)**	96/670 **(14.3%)**	62/682 **(9.0%)**
ot cleaved (%)	0/137 (**0%)**	1/103 **(01%)**	3/64 **(4.6%)**	2/116 **(1.7%)**	4/72 **(5.5%)**	24/694 **(3.4%)**	10/692 **(1.4%)**

Table 4.5. Clinical data outcome in eight pathology groups after culture in Time-Lapse System.

linical Outcome	Endometriosis	Female age Factor	High FSH	Idiopathic	Low AMH	Male Factor	PCOS	Tubal Facto
No. of embryos transferred cycle (mean±SEM)	2.0±0.0	1.7±0.1	2.1±0.4	1.8±0.1	2.5±0.5	2.0±0.1	1.8±0.2	2.1±0.1
No. of embryos transferred zen-thawed cycle (mean±SEM)	1.8±0.1	2.1±0.2	2.0±0.4	2.0±0.0	1.6±0.2	2.0±0.1	2.2±0.1	2.1±0.1
sh cycle transfers (%)	4/11 **(36.6%)**	8/19**(42.1%)**	7/10 **(70%)**	6/10**(60%)**	2/13 **(15.4%)**	24/66**(36.3%)**	7/34**(20.6%)**	22/37**(59.5%**
nical pregnancies Fresh cycle (%)	3/4 **(75%)**	2/8 **(25%)**	2/7 **(28.6%)**	2/6**(33.3%)**	Nil	7/24**(29.2%)**	4/7**(57.1%)**	8/22**(36.3%**
Implantations Fresh cycle (%)	2/3 **(66.7%)**	2/2 **(100%)**	2/2 **(100%)**	2/2**(100%)**	Nil	5/7**(71.4%)**	4/4**(100%)**	6/8**(75%)**
Abortions Fresh cycle (%)	1/3 **(33.3%)**	Nil	Nil	Nil	Nil	2/7**(28.6%)**	Nil	2/8**(25%)**
leton pregnancies Fresh cycle (%)	2/3 **(66.7%)**	2/2 **(100%)**	1/2**(50%)**	2/2 **(100%)**	Nil	5/7**(71.4%)**	4/4**(100%)**	6/8**(75%)**
ltiple pregnancies Fresh cycle (%)	1/3 **(33.3%)**	Nil	1/2 **(50%)**	Nil	Nil	2/7**(28.6%)**	Nil	2/8**(25%)**
n-thawed transfers (%)	9/11 **(81.8%)**	15/19 **(78.9%)**	4/10 **(40%)**	7/10**(70%)**	9/13 **(69.2%)**	51/66**(77.3%)**	29/34**(85.3%)**	25/37**(67.6%**
nical pregnancies ozen-thawed (%)	7/9 **(77.8%)**	3/15 **(20%)**	2/4 **(50%)**	6/7**(85.7%)**	5/9 **(55.6%)**	31/51**(60.8%)**	24/29**(82.8%)**	17/25**(68%**
Implantations ozen-thawed (%)	6/7 **(85.7%)**	3/3 **(100%)**	2/2 **(100%)**	5/6**(83.3%)**	4/5 **(80%)**	31/31**(100%)**	24/24**(100%)**	15/17**(88.2%**
Abortions ozen-thawed (%)	1/7**(14.3%)**	Nil	Nil	1/6**(16.7%)**	1/5**(20%)**	Nil	Nil	2/17**(11.7%**
leton pregnancies ozen-thawed (%)	6/7 **(85.7%)**	2/3 **(66.7%)**	1/2 **(50%)**	6/6**(100%)**	4/5 **(80%)**	29/31**(93.5%)**	21/24**(87.5%)**	16/17**(94.1%**
ltiple pregnancies ozen-thawed (%)	1/7**(14.3%)**	1/3 **(33.3%)**	1/2 **(50%)**	Nil	1/5**(20%)**	2/31**(6.5%)**	3/24**(12.5%)**	1/17**(5.9%)**

DISCUSSION

Time laps microscopy has generated a great hope in the field of ART for selection of best quality embryos taking into account several kinetic key points. Time-lapse development of embryos has a strong correlation with distinct morphokinetic parameters and blastocysts formation (Conaghan *et al.,* 2013; Kirkegaard *et al.,* 2013b; Kirkegaard *et al.,* 2015). Although this technique has provided us a unique chance to study the human embryo development closely but there are still many other factors contributing to the developmental potential of embryo. Kirkegaard *et al.* (2016) demonstrated that 31% variations in timings of kinetic events are due to factors related to the embryo origin i.e. the patient-related and treatment-related factors.

The present study also showed that 40.9% of all male factor infertility were patients whose sperm were extracted either by percutaneous epididymal sperm aspiration (PESA) or testicular sperm extraction (TESE) and remaining 59.1% also presented with severely low sperm count and poor sperm morphology with very low chance of finding the normal sperm for ICSI. The numbers of fertilized and cleaved oocytes in these patients were found to significantly lower ($P \leq 0.001$) than PCOS group but the cleavage rate was found to have no marked difference from other study groups. The clinical pregnancy rates of azoospermic or severe oligozoospermic patients were found to be considerably lower than PCOS, tubal factor, idiopathic and endometriosis group in both fresh (29.2%) and frozen-thawed cycles (60.8%) but higher than patients with high FSH, low AMH and increased age. Similarly the implantation rates of these patients were lower than all other pathologies except the low AMH and endometriosis group in fresh cycle but in frozen-thawed cycle no remarkable difference was observed in implantation rates of all pathology groups. It has been studied well that one of the main reasons of male infertility is non-obstructive azoospermia (NOA). These patients should be counseled for low sperm recovery rate and decreased fertilization and implantation rate as

compare to normal spermatogenesis (Vernaeve *et al.,* 2003). Osmanagaoglu *et al.* (2003) reported that chances of conception with NOA after three cycle attempts was 17% for

ICSI. The congenital abnormalities of still birth are comparable while using epididymal sperm for ICSI and using ejaculated sperm (Belva *et al.,* 2011). ICSI derived embryos from NOA patients also showed increase aneuploidy and mosaicism (Silber *et al.,* 2003). This is evident from the karyotypes of miscarriages after TESE–ICSI which showed higher aneuploidy rates (Bettio *et al.,* 2008).

As far as the kinetic parameters are concerned the current study found that only the time for 3-cell cleavage (t3) and time for compacted morula (tM) showed significant difference among different pathologies of patients. The patients with male factor infertility were observed to be third group to have a delayed initiation of compaction (101.3±1.2) after patients with low AMH and high FSH. The blastocyst formation rate in these patients was also considerably lower (46.2%) than all other study group except patients low AMH. However the quality of blastocyst formed was comparable to other groups. The embryos with slow cleavage were also quite high in number among the male factor infertility i.e. 39.4% as compared to patient with tubal factor, PCOS, high FSH and idiopathic. The rate of abnormal cleavage was 14.3% which was remarkably high than all other groups except tubal factor and high FSH groups. It has been shown previously that embryos of patients with severe male infertility are observed to have three quarters of all embryos not full filling the standard development pattern. These patients showed 36.3 % embryos reaching the blastocysts stage and still 29.8 % of these were good quality blastocysts (Belva *et al.,* 2011; Vernaeve *et al.,* 2003).

It was observed in the current study that the pregnancy rate as well as implantation rate was improved in frozen-thawed cycle as compared to fresh transfers among patients with use of compromised parameter sperm for ICSI. Although there is little literature available related to paternal effect on embryo morphokinetic. It has also been observed that embryos with compromised morphological morphokinetic criteria can develop into a blastocysts and lead to birth of healthy baby (Stecher *et al.,* 2014). These observation are supported by the fact that warming of fair to poor quality vitrified

blastocyst can result in high pregnancy rate (Shaw-Jackson *et al.,* 2013; Kaartinen *et al.,* 2015). Furthermore the sperm dysfunction may not only be related to zygote and early cleavage stage abnormalities but also to implantation failure due to disorganization of chromatin material and sperm DNA fragmentation (Simon *et al.,* 2014). Contrary to this another study has documented that ICSI cycles with higher and lower content of normal spermatozoa in ejaculate resulted in no significant differences in embryo growth parameters. Average values of all parameters were with in normal range (Ugajin *et al.,* 2010; Maettner *et al.,* 2014). It was suggested that the sperm source can contribute to embryo morphokinetic most importantly the early cleavages after embryonic genome activation (EGA) (Braga *et al.,* 2013).

The present study findings support this as the delayed kinetic parameters were more frequently observed at later stages of embryonic development when embryonic gene activation occurs. Ben-Ami *et al*. (2013) compared ICSI outcome in patients with severe oligozoospermia after use of either ejaculated or testicular sperm. The morphological scoring of embryos from both groups did not have any significant difference, whereas significantly lower cleavage rate was observed with ejaculated sperm as compare to TESE. The clinical pregnancy rate was significantly higher with surgical sperm them with ejaculated sperm. Hence TESE was recommended in patients with oligozoospermia for batter ICSI outcome.

The current study showed the idiopathic group of patients with highest BMI (27.6±1.3) which is not in the range of obesity (30 or greater) but do comes in overweight category exhibited quite low pregnancy rate in fresh cycle transfers (33.3%) although the blastocyst formation rate was 52.6% out of which 76.6% were of good quality. The embryos showing slow and abnormal cleavage pattern were 35% and 12.2% respectively. It is a well-known fact that oocyte quality is affected by its micro environment which may determine its final maturation and competency of the oocyte (Revelli *et al.,* 2009; Leroy *et al.,* 2012). Bellver *et al.* (2012) stated that there was no significant difference in embryo quality parameter among women with increase BMI but a significant reduction in implantation, pregnancy and live birth rates was observed. Epigenetic modifications of

the embryo genome by female obesity are responsible for poor implantation, miscarriage, congenital malformations, intrauterine death (Jungheim and Moley, 2010).

The present study although has the limitation of low number of advanced female age patients as the population visiting the infertility centers mostly include the patients of below 40 years of age. But these patients were most common among those repeatedly trying for conception through IVF or ICSI. They have a markedly increases rate of secondary infertility (52.6%) as compared to other groups. Their BMI was also more towards the overweight category (26.4±1.0). The number of oocytes retrieved, matured, fertilized and cleaved were also significantly lower ($P \leq 0.001$) in these patients than other study groups. The morphokinetic parameters t3 and tM were found to be significantly different ($P \leq 0.05$) when compared among different pathology groups. The patients with high FSH and low AMH levels who were mostly the old age women, showed delayed t3 and tM. The clinical pregnancy rates of these patients were remarkably low in both fresh and frozen-thawed cycle i.e. 25% and 20% respectively as compared to patients with PCOS, tubal factor, endometriosis, male factor and endometriosis. The blastocyst formation rate of the old age patients is comparable to other groups but the rate of embryos with slow cleavage is considerably higher (41.1%) than all study groups except the endometriosis and low AMH group. Yarde *et al.* (2013) found that there is a significant correlation between AMH and chance of natural conception in women with elevated serum FSH. There is biological evidence that not the older age of female but the residual amount of ovarian reserve can affect the oocyte quality and impair oocyte competence (Thum *et al.,* 2008).

Female age is known to be one of the essential factors influencing effectiveness of IVF cycles (Yan *et al.,* 2012). This influence is due to increased risk of genetic abnormalities and aneuploidy rate with increasing age (Fragouli *et al.,* 2011). The advanced female age is also associated with malfunctioning of mitochondria and inability of oocyte to repair the fragmented DNA. It has also been documented that the increased female age did not affect the embryo morphokinetic parameters when compared among younger and older patients. The mean values of morphokinetic parameters in both groups corresponded to normal ranges (Eichenlaub-Ritter *et al.,* 2004).

The present study showed significantly lower (P≤0.001) number of oocytes retrieved, matured, fertilized and cleaved in low AMH group than other study groups. Not only the ovarian response was found to be poor but also the kinetic marker for compaction i.e. tM was appreciably high (106.5±2.6) in this group as compared to other pathology groups. The blastocyst formation rate of these poor responders was found to be the lowest (44.1%) among all study groups. In addition to this the rate of slow cleavage (42.6%) was also higher in this group as compared to other pathologies except the endometriosis and the rate of abnormal cleavage was found to be 13.2%. No clinical pregnancies were observed in fresh cycle in this group while in frozen-thawed cycle the pregnancy rate was 55.6% which is still lower than all study groups except the group with high FSH and advanced female age. The low AMH and high FSH levels were found to be strongly correlated with female age (Jayaprakasan *et al.,* 2010). It was found that patients with high AMH levels and high gonadotropin dose during ovulation induction have considerably high blastocyst rate, more oocyte retrieve and more mature oocytes. This is evident from the results of present study too in which the number of oocytes produced, matured, fertilized and cleaved were significantly higher (P≤0.001) in PCOS patients as compare to other study groups. Furthermore it was found that as a result of erratic recruitment of follicles in ovulation induction there is a probability of more follicles produced with likely hood of poor quality oocytes with incomplete or abnormal cytoplasmic maturation. This could result in high aneuploidy rates among women with high level of AMH, hence higher AMH serum levels presented with poor blastulation and pregnancy outcomes. Thus a standard of ovulation induction need to be set for such patients to recruit the sufficient number of good quality eggs avoiding the risk of hyper stimulation (Lie *et al.,* 2008; Broekmans *et al.,* 2006). Similarly it was seen that with the increase in oocyte number there is a rise in live birth rate until 11 to 15 oocytes but not above this number (Steward *et al.,* 2014). About 2-30% of women populations are poor responders to ovarian stimulation (Klinkert *et al.,* 2005). Their AMH levels can predict the ovarian response (La Marca *et al.,* 2009). It was found that patients with AMH concentrations below 1.0 pmol/l can be expected to have no change of response at all (Nelson *et al.,* 2009).

Time-lapse technology in human IVF programs is mainly used to select the most suitable embryo for uterine transfer and many studies focused on finding the best predictive morphokinetic parameter for implantation but the question is whether morphokinetic markers can predict the outcome of a cycle in poor responders where embryo selection is not possible (Basile *et al.,* 2015; Wong *et al.,* 2010). The early cleavage abnormalities affect the embryo viability more than those at later stages of development. As the blastocysts are pluripotent (Geens *et al.,* 2009) they can repair any aberrant division but the early stage anomalies have no repairing potential. In 50.9% of all cycles, we transferred embryos with one of the major cleavage abnormalities detected with time-lapse microscopy, mainly in the first cell cycle (Wirka *et al.,* 2014). Hence the implantation rates in poor responders cannot be compared to normal responders in whom we have freedom of selecting the best quality embryo from a cohort. The reduced pregnancy rate in poor responders is because of transferring embryo with frequent cleavage abnormalities due to unavailability of better ones (Kovacic *et al.,* 2004; Kovacic *et al.,* 2002). The possible reason for these cleavage abnormalities could be chromosomal missegregation in mitotic divisions during early human embryogenesis (Vanneste *et al.,* 2009). Mitotic errors can be a major cause of cleavage stage embryo aneuploidy (Chow *et al.,* 2014).

It has been documented that the embryos derived from PCOS patients showed a delay in development i.e. a slower development from ICSI to 2PN breakdown (Wissing *et al.,* 2014). In contrary to this it was observed in present study that all morphokinetic parameters were within normal range of cleavage. Cleavage timings are important prognostic criteria for embryo quality (Dal Canto *et al.,* 2012). The PCOS patients in the present study showed the normal range of kinetics parameters except the t2 which was seen to be slightly delayed (28.6±0.4) when compared to the standard hours of cleavage mentioned earlier. Moreover the s2 for PCOS in the present study is elevated (4.9±0.9) the most probable reason for this could be that the average of present data included all the timely and untimely cleaving embryos. Early cleavage has been related to better embryo quality and cycle outcome previously (Lemmen *et al.,* 2008; Van Montfoort *et al.,* 2004). Although the delays observed in development of embryos from PCOS patients did not

affect the pregnancy and implantation rates but this does confirm the effect of maternal hormonal dysfunction on early embryo kinetics (Bellver *et al.* (2013). The delayed cleavage kinetics from fertilization up to the 8-cell stage in embryos from PCOS women can be a result of aberrant pool of maternal mRNA transcripts in the early embryo (Wood *et al.,* 2007). The current study showed that the blastocyst formation rate of PCOS patients were the highest (57.1%) of all groups out of which 76.4% were of good quality.

There are some studies suggesting that PCOS oocytes do have molecular abnormalities resulting in reduced fertility. With the discovery of a number of genes whose transcript abundance is altered in PCOS oocytes are having nuclear receptive binding sites, it has been suggested that pharmacological or life style changes can correct the molecular defects in PCOS oocytes to improve pregnancy outcomes (Wood *et al*., 2007). It has been studied that time to reach compaction and morula stage was significantly shorter in PCOS embryos compared to non-PCOS embryos. There was no difference in proportion of oocytes with normal fertilization, cleavage and expansion of blastocyst per treatment cycle between the two groups. Moreover the selection of embryos on basis of morphological scoring (Gardner criteria) showed a non-significantly higher pregnancy rate in PCOS than in non PCOS women (Sundvall *et al.,* 2015). Supporting these findings the present study also showed that the clinical pregnancy rate in fresh cycle of PCOS group was 57.1% which was highest of all groups except patients with endometriosis. The frozen-thawed cycle had the pregnancy of 82.8% which was comparable to other groups. The implantation rates in both fresh and frozen-thawed cycles were 100%. The increased risk of miscarriages in PCOS patients might be due to the fact that multinucleated embryos are at high risk of being chromosomally abnormal hence reducing the implantation rates (Saldeen and Sundstrom, 2005). De Vincentiis *et al*. (2013) recently reported that multinucleation is associated with oocyte immaturity.

The patients with endometriosis have not been studied well so far with respect to embryo morphokinetics using time-lapse imaging. The current study found that the morphokinetic parameters for patients of endometriosis and were in normal ranges for cleavage points. The blastocyst formation rate is 48.1% out of which 78.7% is the rate of good quality blastocyst which is comparable to other study groups. The rate of slow

cleaving embryos (43%) was highest of all pathology groups. The pregnancy rate of this group was comparable to other study groups while the implantation rate was lowest (66.7%) and abortion rate was highest (33.3%) in fresh cycle transfers when compared to other groups. The frozen-thawed pregnancy, implantation and abortion rated did not show any marked difference from other study groups. The mechanism by which the endometriosis can affect the IVF outcome is poorly understood yet and the disease has been found to have a low prevalence of 0.8% to 6% (Abbas *et al*., 2012; Fuldeore and Soliman , 2016). The mild form of endometriosis has been reported to have the pregnancy rate comparable to that of unexplained infertility (Opøien *et al.,* 2013) but chronic form of the disease may impair in several ways. The increased concentration of IL1b, IL8, IL10 and TNF alpha in follicle near to endometriomas is shown to affect that ovarian response (Opøien *et al.,* 2013).

Recent studies have shown that apoptosis of cumulus cells leads to poor oocyte quality and maturation defects (Russel and Robker, 2007). Oocyte morphological characters like extracytoplasmic and cytoplasmic defects are good indicator to show the potential of an oocyte to produce an embryo. Among the extracytoplasmin defects the first polar body extrusion and large perivitelline space seems to impair fertilization rate (Rienzi *et al.,* 2008). Cytoplasmic defects like granularity and presence of vacuoles also play a role to impair fertilization. The mechanism involve in apoptosis of embryo associated with endometriotic lesions is probably due to increased concentration of inflammatory cytokines or reactive oxygen species (Agic *et al*., 2006; Jana *et al.,* 2010). Similarly ROS can result in DNA strand breaks and ultimately mitochondrial damage (Lao *et al.,* 2009).

The current study included the morphokinetic parameters for tubal factor patients which were found to be within the normal range except the time for full expansion of balstocyst i.e. tFEB (117±1.3) which was a little elevated than normal. The blastocyst formation and good quality blastocyst rates were comparable to other study groups whereas the rate of abnormal cell cleavage was the highest of all groups i.e. 16.2% while the slow cleavage rate was 34.4%. The clinical pregnancy, implantation and abortion rate

were comparable to other pathology groups both in fresh and frozen-thawed cycles. The tubal factor infertility affects the 25-30% of women in reproductive age involving the entire tube or only the proximal or distal end. The effect can be transient or permanent. Pelvic inflammatory (PID) is the most common reason of the disease (Honore *et al*., 1999). IVF-ET is the treatment option which bypasses the tubal factor and offers almost 30% delivery rate per cycle (ASRM. 2002). The success rate however decreases from 50% to 28% as female age increases from below 30 years to 35-38 years. The pregnancy rate has been documented to be 70% either the tubal factor exclusively or combined with other infertility factors (Benadiva *et al*., 1995).

In the present study the cell cycle interval for second cell cycle (cc2) ranged from 6.4±2.2 to 9.5±3.0 for all study groups whereas the third cell cycle (cc3) showed a range from 8.7±4.6 to 16.4±2.0 thus corresponding to normal ranges for both kinetic parameters but the range of s2 was 4.0±2.8 to 12.8±1.8 and of s3 was 15.8±1.9 to 24.6±4.8 for different pathology groups which is elevated than normal values documented (Meseguer *et al*., 2011; Wong *et al*., 2010; Chamayou *et al*., 2013). The probable reason for these elevated levels could be the sorting of embryos on the basis of timely cleavages and implantations in these studies whereas the present study focused on the mean timings of embryo cleavages excluding the extreme outliers only.

As far as the timely and untimely cleavages are concerned, it was observed that the rate of timely cleaving embryos declines as the embryonic development proceeds while the untimely cleaving cohort showed an increase in the rate of untimely cleavage as the cleavage stages advanced to start of blastulation (tSB) but in both groups t5 remained the best predictor of blastulation which is in accordance with the findings of Meseguer *et al*., 2011 who reported that t5,cc2 and s2 to be positively associated with implantation potential of embryos.

Our data indicated the normal morphokinetic parameters for idiopathic group of patients with blastocyst formation comparable to other groups as well as the pregnancy and implantation rates. When fertility work fails to detect any obvious reasons for not conceiving then it becomes important to discriminate between unexplained and age-

related infertility. In such case the medical history of patients, history of recent pregnancy or miscarriages can provide critical information. Unexplained infertility is diagnosed if fertility works failed to identify any obvious reason for infertility or it shows mild abnormalities that are not enough to hinder the natural conception. It has been suggested that unexplained infertility diagnosis should include some other factors like mild-male factor, single patent tube and endometriosis (Kersten *et al.,* 2015).

It has been found in the current study that different pathologies of patients visiting the infertility centers have a remarkable effect on the embryo morphokinetics. Moreover the pathological conditions affecting the developmental kinetics of the embryos the most seems to be the advanced female age, low AMH, high FSH and endometriosis. However the number of patients presenting with PCOS, tubal factor and male factor infertility is appreciably higher than other pathologies but they are shown to have a greater chance of selection of best embryos for implantation as compared to aged patients or with low AMH and high FSH hence show the better IVF/ICSI outcome. In addition to this the early cleavage kinetics like t3, t5 and t8 seems to be better predictor of blastocyst formation.

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Chapter # 5

Comparison of ovarian stimulation Drugs and embryonic competence using Time-Lapse system.

ABSTRACT

The TL system is an emerging technology that helps us to identify the kinetic parameters which non-invasively predict the embryonic potential by continuous monitoring. The important kinetic markers of the embryonic development have been found to be associated with blastocyst formation, its quality and the implantation potential hence helping to select the most viable embryos. Conventional static method of morphological evaluations has shown that human embryo quality is affected both by the patient as well as treatment related factors. The present retrospective study was carried out at Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan and the aim was to differentiate between different kinetic markers among women using various stimulation drugs and dosages. A total number of 200 patients undergoing ICSI treatment cycles at the clinic were divided into four stimulation groups i.e. Gonal-f, puregon (normal/high responders) IVF-M and puregon+IVF-M (poor responders). The number of retrieved, matured, fertilized and cleaved oocytes showed highly significant difference ($P \leq 0.0001$) when compared among women with different stimulation drugs and dosages. The patients stimulated with IVF-M showed poor oocyte yield. The groups showed significant difference for average t2 ($P \leq 0.001$) and t8 ($P \leq 0.05$) only. There was no significant difference in all other morphokinetic time-points among all study groups. The blastocyst formation rates and the clinical pregnancy rates were quite low in patients stimulated with IVF-M when compared to other groups. The frozen-thawed cycles showed comparable pregnancy rates in all groups but still lower in IVF-M group while implantation and abortion rates did not vary much among all groups. Hence the poor responders treated with high doses of gonadotropins showed poor the oocyte and embryo quality as well as the clinical outcome during an IVF cycle when compared to normal responders using time-lapse morphokinetic evaluation.

INTRODUCTION

The aim of ART is to help the infertile couples by providing them with a chance of healthy and normal pregnancy which is directly related to the choice of the best embryo for implantation. Embryo quality is an important predictor of successful implantation and healthy pregnancy. The use of morphological evaluation for selection of best embryo for implantation has been in widespread use in the history of ART and still this strategy is considered to be the best criteria for selection of competent embryos (Gardner and Schoolcraft, 1999; Gardner and Sakkas, 2003). In addition to this the effect of each individual morphological parameter on embryonic development need to be investigated more which has some limitations. Although the recent studies of the ALPHA and ESHRE scientists in reproductive medicines have suggested the common criteria for embryo selection and their grading terminologies, still it is considered to be a subjective assessment (ALPHA and ESHRE, 2011a, b). The second limitation is the static embryo evaluation which is conventionally being done at only few time points, thus skipping the important developmental events in between these time points (Meseguer *et al*., 2011; Cruz *et al*., 2012). The third limitation is the reality of dynamic developmental events of the cells, thus introducing high variability in embryo scoring (Gardner *et al*., 2015).

The introduction of time laps monitoring in the fields of ART has been very help full to overcome many of these limitations. The TL imaging has provided us with the noninvasive strategy for embryo scoring and selection. The time laps technology works by integrating frequent images captured at predefined timings without disturbing the culture conditions. Hence it allows more accurate embryo morphological evaluating as well as the developmental kinetic analysis through the software. Despite the enormous study on the time laps imaging, there is conflicts in the data reported about the importance of certain morphokinetic markers for prediction of blastocysts formation, ploidy status of embryos and their implantation (Kaser and Racowsky, 2014; Gardner *et al*., 2015). In addition to this it has been studied that developmental kinetics are affected by many factors including the culture media, (Ciray *et al*., 2012), stimulation protocols (Muñoz *et al*., 2013), insemination method (Lemmen *et al*., 2008; Dal Canto *et al*.,

2012), and clinical conditions of the patients (Bellver *et al*., 2013;: Fréour *et al*., 2013; Kirkegaard *et al*., 2013). Therefore it is suggested that every infertility clinic should develop its own analysis parameters for morphokinetic evaluation depending upon their protocols and procedures (Campbell *et al*., 2013; Kirkegaard *et al*., 2014).

The success rate of IVF treatment is known to be effected by a number of patient factors and culture conditions. The outcome of IVF can be optimized by focusing on individualized patients directed approach during the course of whole treatment (Fréour *et al*., 2015). The most important and crucial step during the treatment cycle is the selection of a suitable COH protocol and gonadotropin dosage. The close monitoring of follicular growth, serum E2 levels and subsequent adjustment of the gonadotropin dosage to avoid ovarian hyperstimulation plays an equally important role. At the same time the individualized timing of human chorionic gonadotropin (hCG) injection is also very crucial during the monitoring. It is believed that a close COH monitoring can add to the oocyte and embryo quality as well as improves the pregnancy and implantation rates minimizing the risk of OHSS.

The selection of an appropriate stimulation protocol plays a significant role in the success of IVF treatment cycle and has proven to be very challenging while dealing with variability between women responding differently to the gonadotropin dosages. It is an important question while starting the stimulation that whether a woman will show a good or poor response to exogenous gonadotropin. So it is critical to have assessment of the ovarian reserve and a complete history of patient characteristics before starting the stimulation protocol. The characteristics may include age, parity, reproductive history, body mass index, and prior response to ART. Various endocrine markers have proven to be the bench mark of ovarian reserve like follicle stimulating hormone (FSH), oestradiol (E2), inhibin B, and, more recently, anti-Müllerian hormone (AMH) levels (Cameron *et al*., 1988; Toner *et al*., 1991; Smotrich *et al*., 1995; Licciardi *et al*., 1995; Broekmans *et al*., 2006; Broer *et al*., 2009; Nelson *et al*., 2009). In addition to this the ultrasound assessment i.e. antral follicle count (AFC), ovarian volume, and ovarian blood flow are also helpful in distinguishing the good and poor responders (Tomas *et al*., 1997). More

over several tests related to dynamic evaluation of ovarian reserve are available like clomiphene citrate challenge testing, GnRH agonist stimulation testing, and exogenous FSH ovarian test but they are proven to be of limited predictive values (Navot *et al*., 1987; Fanchin *et al*., 1994; Winslow *et al*., 1991). Hence Anti-Müllerian hormone and AFC seem to be more reliable ovarian response predictors (Broer *et al*., 2009; Broer *et al.,* 2011) because of showing the consistent serum levels during the menstrual cycle and less cycle to cycle availability, which is an advantage over AMH and AFC (Fanchin *et al*., 2005).

Among the normally responding women there are certain characters serving as prognostic factors which are young age, normal BMI, the AFC count between 6 to 10, normal basal FSH and E2 levels, shorter sub fertility duration or history of previous live birth /IVF treatment (van Loendersloot *et al*., 2010). This group shows adequate to good response to COH protocols including GnRH agonist down regulation or 'long' protocol (Barbieri and Hornstein, 1999) and the GnRH antagonist 'short' protocol (Devroey *et al*., 2009).

The high responders are the women who are at a high risk of developing an exaggerated response to stimulation dosages. An important consideration during management of such patients is to avoid the risk of hyper stimulation. The PCOS women even in the absence of other clinical factors are at high risk of developing OHSS (MacDougall *et al*., 1993; Engmann *et al*., 1999). The incidence of hyper stimulation is as high as 30% in the PCOS women (Navot *et al*., 1992; Enskog *et al*., 1999; Whelan and Vlahos, 2000). The other risk factors of PCOS patients include young age, high gonadotropin dosage and high E2 levels (Huang and Rosenwaks, 2010). The strategies for prevention of ovarian hyperstimulation include firstly to identify the patients at high risk, secondly to individualize the COH protocol and the judicious use of gonadotropins. Thus ideally the aim is to decrease the ovarian response approximately 5-15 follicles while maintaining the E2 levels less than 3000 pg/ml. Two effective COH protocols for high responders are the oral contraceptive pill GnRH agonist dual suppression protocol (Damario *et al*., 1997) and the GnRH antagonist protocol in combination with GnRH

agonist ovulatory trigger (Felberbaum *et al*., 1995; Itskovitz-Eldor *et al*., 2000; Ludwig *et al*., 2000).

The prevalence of poor ovarian responders ranges from 10-25% among women undergoing the IVF treatment (Keay *et al*., 1997). Mostly the definition of poor responders is based on the arbitrary levels of peak serum E2 and the oocyte yield hence the variation in the prevalence rate can be due to the lack of universally accepted definition of poor responders. The poor responders mostly include the patients above 40 years of age (Karande *et al*., 1997), raised basal serum FSH levels (over 10 to 15 mIU/L) (Karande *et al*., 1997; Droesch *et al*., 1989; Feldberg *et al*., 1994; Faber *et al*., 1998), previous cycle cancellations, bas response to clomiphene citrate challenge testing (Navot *et al*., 1987), prolonged duration of COH (Toth *et al*., 1996), increased daily dosage of gonadotropins (Faber *et al*., 1998; Shaker *et al*., 1992), and ultimately resulting in less than 3-5 oocyte retrieved per cycle (Surrey *et al*., 1998). Recently a group of scientists from European Society of Human Reproduction and Embryology consensus defined the poor ovarian responders on the basis of three important features: 1) advanced female age (above 40 years) or any other factor contributing to diminished ovarian reserves; 2) previous history of poor response i.e. less than 3 oocytes retrieved with a conventional COH protocol; and 3) abnormal ovarian reserves as indicated by AFC less than 5-7 follicles or AMH more than 0.5-1.1 ng/ml (Ferraretti *et al*., 2011).

There are a number of ovarian stimulation protocols being followed for the poor responders. The most widely used are commonly used COH protocols include Luteal E2 patch and GnRH antagonist protocol (Dragisic *et al*., 2005)co-flare and micro-flare protocols (Garcia *et al*., 1990; Scott and Navot, 1994;Manzi *et al*., 1994)clomid and letrozole protocols (D'Amato *et al*., 2004;Schoolcraft *et al*., 2008; Jovanovic *et al*., 2011)and modified natural cycles (Pelinck *et al*., 2005). The optimum strategy is the IVF treatment (Pandian *et al*., 2010). The timings of hCG administration should also be individualized on the basis of diameter of the leading follicle, estrogen level, previous cycle response, oocyte quality and the type of stimulation protocol used. It is recommended that every clinic should set its own criteria for hCG administration based

on their methods of ultrasound. In the case of polyspermic fertilization after IVF the ovulatory trigger at smaller size of follicles can help to prevent it.

Objective of the study:

The current study had following aim and objectives:

1) To evaluate the early cleavage pattern of embryos from patients using various ovarian stimulation protocols, their potential to form blastocysts and quality of blastocysts formed using time-lapse imaging and also to correlate the success rates of the treatment.

2) To investigate whether there are any differences in morphokinetic parameters between good quality and poor quality embryos from women treated with optimal and high doses of gonadotropins and to compare them with previously evaluated important kinetic markers for embryonic development.

MATERIALS AND METHODS

Study Design

The current section of the study focused on retrospective data analysis of time-lapse imaging of human embryos during in vitro development. The data used in this study were obtained from Islamabad Clinic Serving Infertile Couples (ICSI), Islamabad, Pakistan. The procedures and protocols were approved and practiced by the Institution. The number of oocytes observed in this study was 4080 producing 2266 embryos in 200 ICSI (Intracytoplasmic sperm injection) treatment cycles. A total number of 1704 embryos were analyzed for time-lapse monitoring due to split cycles for conventional culturing as a quality control measure at the outset of time-lapse system in the clinic. This part of the study focused on the grouping of patients visiting the IVF center on basis of ovarian stimulation drugs and dosages. There were four groups who were treated with Gonal-f, IVF-M, puregon and a combination of IVF-M+puregon for ovarian stimulation. Most of the embryos were obtained after fertilization by ICSI with a very few ICSI+IVF cycles. Ten kinetic parameters were selected for evaluation. Embryo transfer data of both fresh and cryopreserved cycles of patients was analyzed. The βhCG level in the blood sample taken 10 days after embryo transfer confirmed the pregnancy and later on at an ultrasound scanning for fetal heart was done after 7 weeks of pregnancy. The kinetic time-points used for embryo evaluation at different stages of development were measured as hours post icsi (hpi) and corresponding reference ranges are the same as described in chapter 2.

The exclusions criteria:

j) Unfertilized oocytes were excluded from data.

k) All embryos whose time-lapse image acquisition were started later than 20 hours and ended before 60 hours after ICSI were not included.

l) Extreme outliers were excluded that may bias the analysis (as extremes may distort the mean of the regular divisions).

m) However the inclusion criteria for this part of the study was based on the selection of drugs used for ovarian stimulation and also the minimum and maximum doses of gonadotropins were also focused to evaluate patients as normal or poor responders.

n) A collective consensus was made by reviewing the videos and images of time-lapse monitoring of patients by one of the senior embryologist and the lab director and the guidelines were set.

Down regulation, ovarian stimulation, Oocyte retrieval, ICSI and embryo culture in Time-Lapse System

Both long and short protocols were used for pituitary desensitization. Ovarian stimulation is started with the administration of FSH injection by adjusting the starting dosage keeping in view the age of patients, serum AMH hormones levels, the number of antral follicles and the response of patients to any previous cycles. Recombinant hCG was used to induce the final maturation when at least 2 leading follicles reached 18 mm in diameter. The oocyte retrieval was done 36–37 h later. The denudation of oocytes was done 2-3 hours after the retrieval and ICSI was performed on mature oocytes. The fertilization check was done 16-18 hours post ICSI. Zygotes were moved to pre-equilibrated primo vision group-culture dish and cultured for 5-6 days at 37°C with 6% CO_2 and 5% oxygen in the Primo-Vision Time-Lapse embryo monitoring system to monitor their daily growth (Figure 5.1, 5.2 and 5.3). The embryo transfer and cryopreservation were decided on day-5 or day-6 of the embryonic development. The detailed methodology of the procedures has been explained in the second chapter.

Figure 5.1. The conventional IVF incubators equipped with Time-Lapse system.

Figure 5.2. The Primo Vision group cultured dish placed on Time-Lapse microscope
in an IVF incubator.

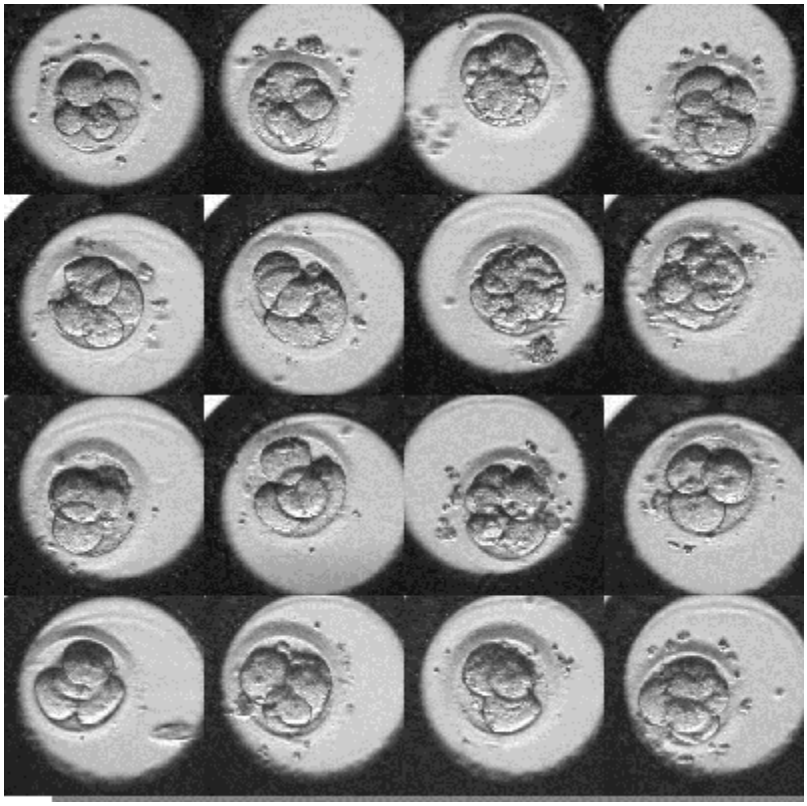

Figure 5.3. Embryo cleavage (4-6 cell stage) at day-2 of culture.

Statistical Analysis

The analysis of variance (ANOVA) followed by Tuckey's test was done to check the difference of means for different clinical and kinetic parameters among different pathology groups. Data are presented as mean±SEM. $P \leq 0.05$ was considered as indicative of a statistical significance. All results were obtained using statistical software GraphPad Prism 5 (GraphPad Prism Software, Inc. CA, USA).

RESULTS

In this section of the study the morphokinetic of embryos was compared for different groups of ovarian stimulation drugs commonly used in the IVF treatment cycle. The table 5.1 shows the demographic and cycle characteristics of the patients. The mean age of patients is significantly higher ($P \leq 0.001$) in patients who used IVF-M as compared to all other groups of stimulation drugs. The BMI did not vary among these groups. The majority of patients underwent the ICSI cycle only but few also had ICSI+IVF treatment. All groups showed most of patients with primary infertility and they appeared for first treatment cycle but the percentage of patients with secondary infertility and repeated attempt was higher in IVF-M group of ovarian stimulation. Similarly the mean number of oocytes retrieved, matured, fertilized and cleaved was also found to be significantly different ($P \leq 0.001$) among all groups except the patients using puregon and a combination of puregon+IVF-M. These groups showed the poor oocyte yield as compared to other groups.

The kinetic parameters were compared among all groups of ovarian stimulation drugs and the mean cleavage hours are displayed in the table 5.2. The mean cleavage timings for 2-cell stage (t2) and 8-cell cleavage (t8) showed a significant difference when compared among different stimulation groups whereas other kinetic markers were found to show no significant difference out of which only the mean timings for 5-cell cleavage is shown in the figure 5.4. The mean t2 was found to significantly higher ($P \leq 0.01$) in patients using puregon+IVF-M for stimulation as compared to all other groups (Figure 5.4).

As far as the developmental potential and embryo quality is concerned the cleavage rate is found to be comparable among all groups. The rate of blastocyst formation is lowest in patients using IVF-M for ovarian stimulation whereas the percentages of good and poor quality blastocyst are comparable among all stimulation groups. The rate of slow and abnormal early cleavage is also found to be highest in IVF-

Table 5.1

Patient's Characteristics	Gonal-f	IVF-M	Puregon	Puregon+IVF-M	P Value
No. of patients (n)	**20**	**71**	**90**	**21**	
Age (yrs.) (mean±SEM)	28.5±1.0	35.2±0.5	30.2±0.5	30.4±0.9	**P≤0.001**
BMI (Kg/m^2) (mean±SEM)	25.5±0.9	26.3±0.5	26.0±0.4	24.7±0.7	**ns**
Primary infertility (%)	15/20 **(75%)**	47/71**(62.8%)**	72/90 **(80%)**	17/21 **(80.9%)**	
Secondary infertility (%)	5/20 **(25%)**	24/71**(33.8%)**	18/90 **(20%)**	4/21 **(19%)**	
1st icsi attempt (%)	18/20**(90%)**	57/71**(80.2%)**	74/90 **(82.2%)**	18/21 **(85.7%)**	
2nd icsi attempt (%)	2/20 **(10%)**	14/71 **(19.7%)**	16/90 **(17.7%)**	3/21 **(14.3%)**	
ICSI cycles (n)	18	67	84	19	
ICSI+IVF cycles (n)	2	4	6	2	
No. of oocytes retrieved (mean±SEM)	30.7±2.6	12.4±0.8	24.7±1.2	18.3±1.6	**P≤0.001**
No. of mature oocytes (mean±SEM)	24.2±1.8	9.1±0.6	19.5±0.9	13.5±1.4	**P≤0.001**
No. of oocytes fertilized (mean±SEM)	20.6±1.8	6.3±0.5	14.2±0.8	9.6±1.2	**P≤0.001**
No. of oocytes cleaved (mean±SEM)	20.5±1.8	6.2±0.5	13.8±0.8	9.2±1.2	**P≤0.001**

Table 5.2

Morphokinetic parameters	Gonal-f	IVF-M	Puregon	Puregon+IVF-M	P Value
tPNa (mean±SEM)	11.4±0.9	15.1±1.3	13.4±0.4	18.9±4.9	**ns**
t2(mean±SEM)	27.5±0.5	29.4±0.7	28.8±0.4	33.9±2.9	**P≤0.01**
t3(mean±SEM)	36.1±0.7	37.1±0.7	37.3±0.5	36.9±1.3	**ns**
t4(mean±SEM)	44.6±0.7	42.9±0.8	42.5±0.5	43.0±1.2	**ns**
t5(mean±SEM)	49.0±0.9	51.5±1.1	52.3±0.7	52.9±1.8	**ns**
t8(mean±SEM)	66.2±1.1	71.3±1.1	68.3±0.8	69.5±1.5	**P≤0.05**
tM (mean±SEM)	96.2±1.1	101.3±1.4	100.1±0.7	98.8±1.9	**ns**
tSB (mean±SEM)	107.0±0.7	109.5±1.4	108.5±0.8	107.9±1.5	**ns**
tEB (mean±SEM)	111.3±0.8	112.2±1.1	112.8±0.8	111.8±1.1	**ns**
tFEB (mean±SEM)	115.1±0.6	115.4±0.6	115.4±0.7	114.8±0.8	**ns**

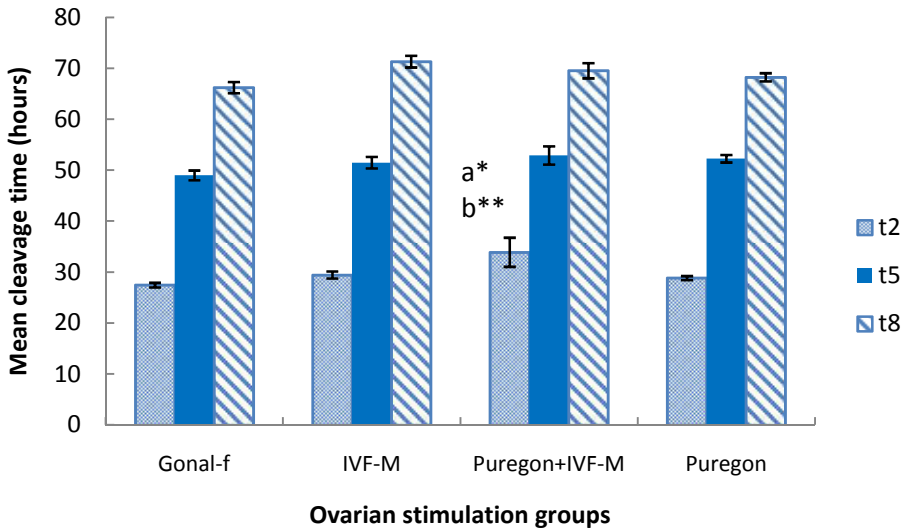

Figure 5.4. Mean time for 2-cell (t2) 5-cell (t5) and 8-cell (t8) cleavage stage embryos of patients using different stimulation drugs.

a* P≤0.05 vs. IVF-M group (ANOVA followed by Tukey's test)

b P≤0.001 vs. Gonal-f and Puregon groups (ANOVA followed by Tukey's test)**

*** P≤0.05 (ANOVA for t8)**

P≥0.005 – non-significant difference among stimulation groups for t5.

-M group while the rate of uncleaved oocytes was highest in the group on treatment of puregon+IVF-M for ovarian stimulation (Table 5.3).

The clinical outcome is shown in table 5.4. The embryo transfer was done both for fresh cycle and frozen-thawed cycle of patients. The average number of embryos transferred was

higher in frozen-thawed cycles as compared to fresh cycle transfers and was found to be significantly different (P≤0.01) among groups also. Similarly the rate of frozen thawed transfers was considerably higher in all groups as compared to fresh transfers. The clinical pregnancy rate in fresh cycle transfers showed a drastic fall in patients using puregon+IVF-M and the ones who used IVF-M only. The implantation and abortion rate were comparable among different groups and single pregnancy rate was found to be higher than multiple pregnancy rate. As far as the frozen thawed embryo transfers are concerned the IVF-M group showed the lowest pregnancy rate and highest abortion rate as compared to all other groups in which the implantation and abortion rates were found to be comparable. The single pregnancy rate was considerably higher than multiple pregnancy rate in frozen-thawed cycle also.

Table 5.3.Developmental potential and embryos quality in females using different ovarian stimulation drugs.

Embryonic development	Gonal-f	IVF-M	Puregon	Puregon+IVF-M
Total embryos analyzed (n)	**412**	**446**	**1232**	**203**
Cleavage rate (%)	410/412**(99.5%)**	438/446 **(98.2%)**	1200/1232 **(97.4%)**	196/203 **(96.6%)**
Blastocyst formed (%)	265/410 **(64.3%)**	189/438 **(43.2%)**	604/1200 **(50.3%)**	93/196 **(47.5%)**
Good quality blastocysts (%)	206/265 **(77.7%)**	145/189 **(76.7%)**	452/604 **(74.8%)**	48/93 **(73.1%)**
Poor quality blastocysts (%)	59/265 **(22.3%)**	44/189 **(23.3%)**	152/604 **(25.2%)**	25/93 **(26.9%)**
Blastocyst not formed (%)	145/410 **(35.4%)**	249/438 **(56.8%)**	596/1200 **(49.7%)**	103/196 **(52.6%)**
Slow early cleavage (%)	105/410 **(25.6%)**	176/438 **(40.2%)**	461/1200 **(38.4%)**	72/196 **(36.7%)**
Abnormal cleavage (%)	40/410 **(9.8%)**	73/438 **(16.7%)**	135/1200 **(11.2%)**	31/196 **(15.8%)**
Not cleaved (%)	2/412**(0.5%)**	8/446 **(1.8%)**	32/1232 **(2.6%)**	7/203 **(3.5%)**

Table 5.4. Clinical data outcome in various groups of ovarian stimulation drugs after culture in Time-Lapse System.

Clinical Outcome	Gonal-f	IVF-M	Puregon	Puregon+IVF-M	P Value
No. of embryos transferred Fresh cycle (mean±SEM)	1.8±0.2	2.2±0.2	1.9±0.1	1.8±0.2	**ns**
No. of embryos transferred Frozen-thawed cycle (mean±SEM)	2.4±0.1	1.8±0.1	2.1±0.1	2.2±0.1	**P≤0.01**
Fresh cycle transfers(%)	5/20 **(25%)**	29/71**(40.8%)**	36/90 **(40%)**	10/21**(47.6%)**	
Clinical pregnancies Fresh cycle (%)	4/5 **(80%)**	5/29 **(14.2%)**	18/36 **(50%)**	1/10 **(10%)**	
Implantations Fresh cycle (%)	3/4 **(75%)**	4/5 **(80%)**	15/18 **(83.3%)**	1/1**(100%)**	
Abortions Fresh cycle (%)	1/4 **(25%)**	1/5 **(20%)**	3/18 **(16.6%)**	Nil	
Singleton pregnancies Fresh cycle (%)	3/4 **(75%)**	3/5 **(60%)**	15/18**(83.3%)**	1/1**(100%)**	
Multiple pregnancies Fresh cycle (%)	1/4 **(25%)**	2/5 **(40%)**	3/18 **(16.6%)**	Nil	
Frozen-thawed transfers (%)	16/20 **(80%)**	51/71 **(71.8%)**	70/90 **(77.8%)**	14/21**(66.6%)**	
Clinical pregnancies Frozen-thawed (%)	14/16 **(87.5%)**	20/51 **(39.2%)**	52/70 **(74.2%)**	11/14 **(78.5%)**	
Implantations Frozen-thawed (%)	14/14 **(100%)**	17/20 **(85%)**	50/52 **(96.2%)**	11/11**(100%)**	
Abortions Frozen-thawed (%)	Nil	3/20 **(15%)**	2/52 **(3.8%)**	Nil	
Singleton pregnancies Frozen-thawed (%)	11/14 **(78.5%)**	18/20 **(90%)**	49/52 **(94.2%)**	9/11**(81.8%)**	
Multiple pregnancies Frozen-thawed (%)	3/14 **(21.4%)**	2/20 **(10%)**	3/52 **(5.8%)**	2/11 **(18.2%)**	

DISCUSSION

The dynamics of embryo development and IVF outcomes are conditioned by many factors. We evaluated the influence of gonadotropins on the timing of embryo development with use the time-lapse monitoring. In the present study four groups of different gonadotropins were used for ovarian stimulation and the cycle characteristics, kinetic markers, blastocyst quality and the cycle outcome was compared.

The present study revealed that average age of patients varied significantly among different stimulation groups. The increasing female age results in low number of oocytes, poor oocyte and embryo quality (Malizia *et al*., 2009) and low implantation (Spandorfer *et al*., 2000), pregnancy and live birth rates. Our study also showed that the females with advanced age were stimulated with high doses of gonadotropins hence the groups with the treatment of high doses of either IVF-M alone or a combination of puregon+IVF-M were actually the patients with increased age. These patients proved to be poor responders to ovarian stimulation and produced significantly lower number of oocytes ($P \leq 0.001$) also showed considerably low pregnancy rates and less number of embryos approaching the blastocyst stage as well as compromised blastocyst quality.

It was shown earlier, that application of total FSH dose above 2200 IU during COS was associated with reduction in pregnancy rate (Yilmaz *et al*., 2013). The present study assumption was that the type of gonadotropin used and the total dosage may affect the embryo cleavage. It was observed that the range of gonadotropin dosage in two groups considered being normal responders i.e. Gonal-f and Puregon was 1650 iu - 4950 iu and 1100 iu – 7800 iu respectively. Whereas the poor responders i.e. patients in the groups on treatment with Puregon + IVF-M and the IVF-M alone consumed 4550 iu – 12800 iu and 2025 iu – 18000 iu respectively. Furthermore it has been stated earlier that the FSH dose of 1400 to 1800 IU when administered to the patients the resulting embryos did not show significant difference for important kinetic markers. Whereas the high dose of 2500 IU showed a significant difference in the morphokinetic parameters of resulting embryos. The difference between groups was about 3 h on t5, t4, t3 parameters and about 2 h on t2 time. Hence it was concluded that FSH more than 2500 IU resulted in delayed

cleavage hours for different kinetic parameters (Mykola *et al*., 2014). These results corresponds to the present study in which the poor responder groups with high dose administration showed significantly ($P \leq 0.01$) delayed time for early cleavage i.e. at 2-cell stage (t2) when compared to the normal responders. Similarly the time for 8-cell cleavage (t8) was also found to be significantly different ($P \leq 0.05$) among the stimulation groups. All other kinetic markers did not show any significant difference among the groups. On the other hand, as the doses of FSH depend upon the specific clinical indications of the patients, so it is hard to exclude the patients with decreased rate of embryonic development with increased dose of gonadotropins.

When evaluating the impact of gonadotropins on embryos growth the present study revealed better embryo development and cycle outcome after stimulation with rFSH than after hFSH administration. Similar results have been documented by Muñoz *et al.* (2013) that after stimulation with recombinant FSH the embryos show optimal development timings as compare to stimulation with hFSH or a combination of both. However, these differences were not significant at early cleavage hours i.e. t2 (Muñoz *et al*., 2012).

In another study 243 patients aged<38 years with at least eight harvested oocytes were included. A total of 1,507 embryos were monitored for 6 days in a time-lapse incubator. The authors concluded that 16%–31% of the observed variation in timing of embryo development was due to patient- and treatment related factors. In general, no single patient–or ovarian stimulation– related factor was reported to elicit a systematic influence on the overall timing from the cleavage to the blastocyst stage. However, female age, number of previous cycles, and cumulative FSH dose had an influence on timing of blastocyst development (Kirkegaard *et al*., 2016). Similarly in our study, we did not note a systematic influence of patient–and ovarian stimulation–related factors on TLM parameters. However, we noted that the total FSH dose, duration of infertility, number of previous cycles, antral follicle count, and number of retrieved oocytes had a significant impact on some TLM parameters.

In the present study we also noticed the possible influence of oocyte number retrieved on morphokinetic parameters of embryos. It has been observed that the patients who are poor responders and produced less number of oocytes per cycle, showed delayed development of their embryos with poor quality blastocysts produced comparatively greater in number. Their pregnancy rates were also lower both in fresh and frozen-thawed cycles than normal responders who produced adequate number of oocytes per cycle. Whereas another study reported that there was no significant difference among the morphokinetic parameters of the embryos obtained in the cycles where the number of oocytes was more than 15 or less. The important kinetic markers were found to be in normal ranges (Kwee *et al*., 2007).

The clinical pregnancy rates in frozen-thawed cycle were comparable but still higher in normal responders than poor responders whereas in fresh cycle transfers poor responders had considerably low pregnancy rates. Our fresh cycle transfers showed 50% - 80% while frozen-thawed cycles showed 66.6% - 80% pregnancies in normal responders. This ratio is even higher than results obtained by other authors e.g. Kirkegaard *et al*. (31 %) and Polanski *et al*. (35 %) (Kirkegaard *et al*., 2013; Polanski *et al*., 2014). Taking into consideration the kind of gonadotropin used, we noted that the pregnancy rate was slightly higher in women stimulated with rFSH than in women stimulated with hFSH. In contrast to this the observations made by Flicori *et al*. revealed that women treated with hFSH had higher frequency of pregnancies than in women treated with rFSH, but their results also turn out to be statistically insignificant (Filicori *et al*., 2003). In turn, Kilani *et al*. reported 35% pregnancy rate both for women stimulated with hFSH and rFSH. However they observed that stimulation with hFSH showed more efficient response than rFSH (Kilani *et al*., 2003). As far as the implantation rates are concerned the current study found them to be comparable among all stimulation groups and the abortion rate of poor responders i.e. the patients on treatment with high dose gonadotropins (IVF-M) was found to be highest in frozen-thawed transfers.

In summary, all gonadotropins dose-dependently effectively enhanced oocyte maturation either used alone or in any combination. However, high concentrations of

gonadotropins may result in maturation arrest. These results clearly show that when comparing a gonadotrophin containing FSH activity as well as LH-like activity in a 1:1 ratio (HMG) or a mixture of both (FSH + HMG), embryo cleavages could not be seen to favor any of the preparations. The association between total dose of gonadotropins and steroid hormones may define optimal ranges, reflected in embryos with a high implantation potential. Taking into account that this was a retrospective analysis, a future prospective randomized study will be needed to confirm the hypothesis presented here. The results of the present study could provide guidance as to the optimal gonadotropin stimulation protocols for both IVF and non-IVF cycles, and potentially help improve IVF success rates.

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Chapter # 6

General Discussion

GENERAL DISCUSSION

Since 1978, when the first IVF baby was born, the in vitro fertilization has become a major treatment option for the couples seeking infertility treatment. Although the IVF has shown major developments in past few years but the live birth rate remained only about 30-40%. The emotional and financial cost of the procedure remained as a stress for infertile couple as well as the additional health risks. The success rate of IVF can be increased by multiple embryos transferred but this may result in rise of multiple pregnancy rates which can add to complications of mother and baby. Therefore the need of single embryo transfer is crucial hence, determining the importance of embryo selection methods (Gerris, 2009). Some of important embryo assessment strategies are discussed below.

The morphological assessment of embryo has been traditionally used on the bases of visual evaluations. It is a popular method of embryo selection at different development stages providing enough information about the embryo quality (Ebner *et al.,* 2003). Embryos can be graded according to the morphology of their pronuclei on day 1 after fertilization (Montag and van der., 2001), the number and shape of blastomeres and degree of fragmentation on day 2 or 3 (Van Royen *et al.,* 1999; Hardarson *et al.,* 2001), or by the morphology of the blastocyst at day 5 or 6 (Richter *et al.,* 2001; Balaban *et al.,* 2000).

Depending on the procedure, embryos are evaluated at one or several developmental stages. Although the static morphological assessment is easy to implement and inexpensive in a clinical setting, but it has certain drawbacks. Firstly, the visual embryo grading is subjective and requires significant expertise. It has been noticed that even when such expertise is available this evaluation cannot be always accurate as some times the low graded poor quality embryos results into healthy full term pregnancy. Thus there has always been a need to adopt alternative method for embryo selection based on the quantitative and objective information of embryo status.

Preimplantation genetic diagnosis (PGD) is another invasive method to detect the genetic disorders. It mainly involves the PCR based techniques to detect specific gene

mutations (Spits and Sermon, 2009). The fluorescence in situ hybridization (FISH) is also used to diagnose chromosomal anomalies and the sex of embryos for patients carrying X-chromosome-linked diseases (Griffin *et al.,* 1994). The PGD can be used to detect the abnormalities at different stages of embryonic development i.e. zygote, cleavage stage and blastocysts. The preimplantation genetic screening (PGS) can be used to improve IVF success rate in advanced age females presenting with implantation failures and sever male factor infertility (Harper and Harton, 2010). Both of these approaches allow the whole embryonic genome to be analyzed and, as for preimplantation genetic diagnosis, used at different embryonic stages.

Another criterion of embryo selection is based on the analysis of embryo metabolism. The energy metabolism of the embryo can be portrayed by changes in pyruvate or glucose concentration in the culture medium although their benefit as a tool to predict the embryo's quality is not clear (Gardner *et al.,* 2001; Jones *et al.,* 2001). On the other hand, oxygen consumption (Magnusson *et al.,* 1986; Lopes *et al.,* 2007) and amino-acid turnover (Seli *et al.,* 2008) have proved to be more reliable indicators of an embryo's viability. Advance studies shows that the analysis of single metabolites can now be replaced by metabolomics which is a border approach (Nel-Themaat and Nagy, 2011). It provides a detail picture of metabolic status of embryo and its gene expression patrons which is directly related to the viability of the embryo. Thus metabolomics can add to enhance the potential value of IVF treatment but the limitation being its implementation in the clinical setup because the secreted protein analysis involves the spectrometric and chromatographic techniques which involves skilled staff as well as the financial issues (Nel-Themaat and Nagy, 2011).

The human embryo development can be monitored by static morphological assessment but it can be done at the pre-defined time points only thus provide the limited information about the growth pattern of embryos. The Time-Lapse system has improved the effectiveness of the IVF treatment by providing the continuous monitoring of embryonic development and selection of embryos with maximum implantation potential (Basile *et al.,* 2015) hence reducing the risk of multiple pregnancies. This recent

technology has helped to monitor the embryo growth without removing the from incubator thus avoiding the stress the embryo can suffer outside the optimal culture conditions. It has provided us with more detailed information of some very important parameters of embryonic development by recording the precise time points (Aparicio *et al.,*2013; Athayde *et al.,* 2014).

The Time-Lapse system represents a state-of-the-art on embryonic development. But analysis of a huge data is very laborious and time taking hence demands proper training. Moreover even the trained users cannot perform the regular analysis of all embryo images before the embryo transfer due to routine work load in IVF clinics and it demands extra time on behalf of laboratory staff. Finally, potential inter-observer and intra-observer variability may affect time-lapse marker interpretation, similarly to what has been found with manual embryo morphology grading (Chen *et al.,* 2013).

The recently evolved noninvasive time laps imaging studied in mouse embryos has established a strong correlation between the dynamics of the developmental events and the embryonic competence (Bischoff *et al.,* 2008). Many researchers have studied the embryo potential with respect to the important kinetic markers. A team at Stanford University reported that the time and synchrony of first two embryonic divisions can be used to predict embryonic potential (Wong *et al.,* 2010). Their study revealed that the embryos with prolong first cytokinesis and short interval between the first and second divisions failed to reach the blastocysts stages (Fenwick *et al.,* 2002; Lemmen *et al.,* 2008). In a completely different approach it has been shown the fertilization of mouse eggs triggers repetitive cytoplasmic movements that correlated with Ca2+ oscillations and depend upon the functionality of cytoskeleton. These cytoplasmic flows are predictive of embryonic competence also. It was observed that embryos with frequent cytoplasmic movements at low speed were not successful to develop into pups than embryos with average of these parameters. These methods if tested in a clinical environment and controlled randomized trials can prove to be more reliable for evaluating the embryonic quality (Ajduk *et al.,* 2011).

Our findings mainly focused on the effect of female age and other factors of both male and female partners contributing to infertility as a couple, on the embryonic developmental potential monitored in a time-lapse set up. The other pathologies included the male factor or sperm factor, endometriosis, tubal factor, high FSH and low AMH of female, PCOS and idiopathic infertility.

The present study has supported the fact that the oocyte yield and quality is found to be negatively correlated with female age. The maternal age has been studied less with respect to kinetic parameters. This study also showed no significant difference among various age groups studied regarding the average kinetic parameters. In another study also the average values of morphokinetic parameters of embryos obtained in younger and older women were found to be in normal ranges (Gryshchenko *et al.,* 2014). The increased maternal age shows drastic decline in the reproductive competence. The human females aged above 35 years present with decreased reproductive ability (Hawkes and Smith, 2010). The primary reason of this decline in reproductive competence is considered to be the declining oocyte quality with age as compared to the uterine functions (Navot *et al.,* 1991; Hunt and Hassold, 2008). It is a known fact that cytoplasmic abnormalities add to the poor quality of oocytes but primarily the aneuploidies during meiosis I are the reason of poor oocyte yield and quality with maternal ageing (Hunt and Hassold, 2008; Nagaoka *et al.,* 2012). The aneuploidies due to high age add to the defective meiotic recombination, failure of spindle assembly (Battaglia *et al.,* 1996; Steuerwald *et al.,* 2001; Hassold *et al.,* 2007; Yun *et al.,* 2014; Sakakibara *et al.,* 2015), kinetochore microtubule formation (Shomper *et al.,* 2014) and loss of centromeric cohesion (Vogt *et al.,* 2008; Chiang *et al.,* 2010; Lister *et al.,* 2010; Duncan *et al.,* 2012; Merriman *et al.,* 2013). Moreover it is observed that poor IVF cycles of aged patients are result of increase aneuploidy and genetic abnormalities in their oocytes (Yan *et al.,* 2012). The aging oocytes are a result of aging mitochondrial apparatus and reduced ability of oocyte to repair fragmented DNA thus resulting poor cleavage rate of embryso (Fragouli *et al.,* 2011; Eichenlaub-Ritter *et al.,* 2004).

The current study also showed that with increasing female age not only there is a delayed embryo growth observed but also the blastocyst formation rate decline along with the raise in percentage of poor quality blastocysts formed. The rate of embryos showing slow cleavage pattern increases as women get older. The early cleavage of an embryo is defined as division of the zygote into 2 blastomere after 25-27 hpi (Edwards *et al.*1984). And it has been now established from several observational studies that early cleavage is a good indicator of an embryo's growth potential (Ciray *et al.,* 2005; Lundin *et al.,* 2001; Sakkas *et al.,* 1998; Salumets *et al.,* 2003; Shoukir *et al.,* 1997). However in these studies the transfer of 2 or 3 embryos with mixed pattern of growth did not allow to associate the cleavage pattern with pregnancy outcome. Meseguer *et al.* (2011) conducted a study focusing the early cleavage of transferred embryos only where implantation was 0% or 100% and concluded that there exists an optimal range for all cell division and also that too early cleavage (completed before 24.3 h) can also lead to lower implantation rates.

This is evident from present study that percentages of embryos showing delayed growth rises as the age progressed and delay is more evident in late developmental stages i.e. towards the start of blastulation thus an indication of increasing chances of aneuploidy too with increasing female age. It has been declared by another finding that t5 as one of the most important variables predicting implantation (Meseguer *et al.,* 2011) which is in accordance with the present study too. We have observed that the cell cycle intervals i.e. cc2 and cc3 were in normal range in all age groups. Moreover the synchrony of second cell division divisions i.e. s2 although elevated but corresponded to normal range whereas the s3 (synchrony of third cell division) showed remarkably elevated range. The most probable reason of this elevation could be that our data was sorted according to female age and we excluded the embryos extremely outlying the normal divisions that could bias the mean values but embryos with delayed growth were included hence these data sorting was not on the basis of only best quality, timely cleaving and optimum morphology embryos but on the basis of age of female. It has also been reported that aneuploid and euploid embryos show similar growth pattern until embryonic genome activation at around the 8-cell, day-3 stage, but during the blastulation phase, aneuploid

embryos are significantly delayed in development compared with euploid embryos (Campbell *et al.,* 2012). It has also been shown previously that abnormally and untimely cleaving embryo are predominant than timely cleavages (Hlinka *et al.,* 2012).

Our data also showed low conception rates in embryo transferred in fresh and frozen-thawed cycles in above 40 years age group, but implantation and abortion rate did not show any marked difference with increasing age. Whereas it has been shown previously that maternal aging is associated with increased spontaneous miscarriage rate, pregnancy complications, congenital anomalies, and higher perinatal mortality (Dain *et al.,* 2011). The poor oocyte quality contributes to high miscarriage rate in older women (Meldrum, 1993). Similarly delivery rates also decline by 50% for women aged 38 to 40 years and by another 50% for women above 40 years (Ventura *et al.,* 2000).

The time-lapse imaging system has provided us a unique chance to study the human embryo development closely but still there are some limitations when we implement in clinical environment. The specific timing of kinetic events are very important indicator of blastocyst formation but these markers cannot be used universally as there are many other factors contributing to the developmental potential of embryo. Kirkegaard *et al.* (2016) demonstrated that 31% variation in timings of kinetic events is due to factors related to the embryo origin i.e. the patient-related and treatment-related factors.

Our data has shown a marked elevation in tM of patients with severe male factor infertility. These patients also showed blastocyst forming capacity lower than other pathologies. They showed better pregnancy and implantation rates in frozen-thawed cycle as compare to fresh cycle. As studied earlier one of the main reasons of male factor infertility is non-obstructive azoospermia (NOA). These patients present with low sperm recovery rate and decreased fertilization and implantation rate as compare to normal spermatogenesis (Vernaeve *et al.,* 2003). Although there is little literature available related to paternal effect on embryo morphokinetic Knez and colleagues reported that

blastocyst development in quality is affected by the origin of sperm used for ICSI. They found that blastocysts produced from morphologically normal looking spermatozoa free of vacuoles take shorter mean time for developmental events as compare to those originating from poor quality spermatozoa (Knez *et al.,* 2013). Furthermore the sperm dysfunction may not only be related to zygote and early cleavage stage abnormalities but also to implantation failure due to disorganization of chromatin material and sperm DNA fragmentation (Simon *et al.,* 2014). Contrary to this another study has documented that ICSI cycles with higher and lower content of normal spermatozoa in ejaculate resulted in no significant differences in embryo growth parameters. Average values of all parameters were with in normal range (Ugajin *et al.,* 2010; Maettner *et al.,* 2014).

The current data suggested that time for initiation of compaction was remarkably high among patients with low AMH as compared to other groups. These patients showed low pregnancy rate compared to other patients even in frozen-thawed cycle although improved as compare to fresh cycle. Yarde *et al.* (2013) found that there is a significant correlation between AMH and chance of natural conception in women with elevated serum FSH. There is biological evidence that not the older age of female but the residual amount of ovarian reserve can affect the oocyte quality and impair oocyte competence (Lie Fong *et al.,* 2008; Thum *et al.,* 2008). The serum FSH and AMH levels are useful biomarkers for age related infertility. It has been studied that women with AMH levels below 1.0 pmol/L can be expected to have no response to ovarian stimulation (Nelson *et al.,* 2009). The AMH has proven to be a better predictor of ovarian response than female age, BMI, basal FSH or inhibin B. It was found that AMH level above 15 pmol/L shows the signs of OHSS (Nelson *et al*. (2007).

The current data showed all the kinetic parameters to be within normal range of development but t2 was found to be slightly delayed. Moreover the s2 was found to be slightly elevated most probably due to inclusion of delayed embryos in the analysis as our grouping focused more towards patient pathologies than classifying them on basis of best morphology. The pregnancy rates of PCOS group were highest among all pathologies and implantation rates were also found to be 100% in both fresh and frozen-thawed

cycles. It has been documented previously that the embryos derived from PCOS patients showed a delay in development i.e. from the sign of fertilization (appearance of 2pn) till the cleavage into 3-4 cell stage as compared to non PCOS women (Wissing *et al.,* 2014). The time to reach compaction i.e. morula stage of embryonic development was significantly shorter in PCOS embryos than non-PCOS. There was no difference in proportion of oocytes with normal fertilization, cleavage and expansion of blastocyst per treatment cycle between the two groups. Moreover the selection of embryos on basis of morphological scoring (Gardner criteria) indicated a higher ratio of biochemical pregnancies in PCOS than in non-PCOS group. Live birth rates were comparable among the two groups (Sundvall *et al.,* 2015).

The present findings showed the average kinetic markers for patients with endometriosis to be within normal range. The blastocyst formation in these patients was also comparable to other groups. The pregnancy rate was comparable whereas the implantation rate was lowest and abortion rate was highest when compared to other groups in fresh cycle. The frozen-thawed cycle did not show any remarkable difference regarding the pregnancy and implantation rate. The patients with endometriosis and tubal factor infertility have not been studied so far with respect to morphokinetic parameters. The mild form of endometriosis has been reported to have the pregnancy rate comparable to that of unexplained infertility (Opøien *et al.,* 2013) but chronic form of the disease may impair in several ways. The oxidative stress, prostaglandins and cytokines not only impair the oocyte-sperm interaction but also hinder the normal embryo development, and implantation (Pellicer *et al.,* 1995).

Our study has also included the morphokinetic parameters for tubal factor patients which were found to be within the normal range except the time for full expansion of balstocyt i.e. tFEB (117±1.3) which was a little elevated than normal. The blastocyst formation and good quality blastocyst rates were comparable to other study groups. The clinical pregnancy, implantation and abortion rate were comparable to other pathology groups both in fresh and frozen-thawed cycles. The PID has been diagnosed as most important cause of tubal factor infertility. The severity of disease increases as the severity

and number of pelvic adhesions increase. The prevalence of infertility is 10% -12% after one episode, 23%-35% after two episodes, and 54%-75% after three episodes (Westrom, 1975; Westrom, 1994).

When data was segregated according to timely and untimely cleavages, it was observed that the rate of timely cleaving embryos declines as the embryonic development proceeds while there is an increase in the rate of untimely cleavages as the developmental stages advanced to start of blastulation (tSB) but in both groups t5 remained the best predictor of blastulation which is in accordance with the findings of Meseguer *et al.,* 2011 who reported that t5,cc2 and s2 to be positively associated with implantation potential of embryos.

Our data indicated the normal morphokinetic parameters for idiopathic group of patients with blastocyst formation comparable to other groups as well as the pregnancy and implantation rates. Unexplained infertility is a subject of discussion among practitioners. It usually refers to lack of diagnosis in spite of all standard investigations i.e. test of ovulation, tubal patency and semen analysis and all being in normal range (Smith *et al.,* 2003). This has been suggested that diagnosis of unexplained infertility should include the chances of mild male factor infertility, single patent tube, endometriosis stages I and II and cervical factors also (Kersten *et al.,* 2015).

It was observed among all pathologies that cell cycle intervals i.e. cc2 and cc3 were in normal range in all age groups. Moreover the synchrony of second cell division divisions i.e. s2 although elevated but corresponded to normal range whereas the s3 (synchrony of third cell division) showed remarkably elevated range. The reason for these elevated values is most likely that the grouping based on factors contributing to infertility instead of embryo quality.

The pituitary gonadotropins LH and FSH are important for regulation of gonadal function and reproduction in human. The gonadotropins are used widely in ovarian stimulation during IVF/ICSI procedures and are crucial for the cases of anovulation

disorders, such as hypo gonadotropic hypogonadism and hypothalamic hypophyseal dysfunction. The concept of individualization of IVF treatment protocol has helped the clinicians to maximize the pregnancy rates, reduce the risks of OHSS and also to minimize the cycle cancellation rates in case of poor responders to the ovarian stimulation. The response of an individual patient to her stimulation protocol is really useful in selecting the type and dosage of gonadotropins used which is likely to be effective as well as safe. The decision making regarding the selection of protocol is usually empirical and based on the clinician's preference, if no previous cycle has been performed (La Marca and Sunkara, 2014).

The AFC is also a potential marker of ovarian response (La Marca *et al.,* 2010; Broer *et al.,* 2013). It may involve the limitations of time frame to evaluate AFC and the expertise of clinician performing it. Another group has found that the AMH level is a better option to be used to individualize the treatment strategies, minimize the clinical risk and improve the pregnancy rates in the field of IVF (Nelson *et al.,* 2009).

The present study also found that the poor responders who were categorized on the basis of basal FSH, AMH and AFC, can be helped by increasing the gonadotropin dosage in terms of yield of oocytes but they showed delayed early embryonic cleavage, poor blastocyst quality and low pregnancy rates. A key factor determining the outcome of COS is the selection of the starting dose of gonadotropins. The need to individualize the gonadotropins dosage is related to the fact that ovarian reserves and pool of recruitable follicle vary widely among patients and a standard dose cannot be fixed for all (La Marca and Sunkara, 2014). Lee et al (Lee *et al.,* 2011) also reported that women above 40 years of age with AMH levels in middle third range (0.49~1.22 ng/mL) or upper third range (_ 1.23 ng/mL) showed high number of oocytes retrieved, number of embryos available for ET and low cycle cancellation rates than the women with AMH in lower third range (_ 0.48 ng/mL). Hence it showed that increased gonadotropin starting dosage used for the patients with lower and middle third range of AMH concentration has resulted in increased pregnancy rates as compare to upper third range.

The embryo competence is related to the oocyte quality which is affected by a number of factors including the treatment protocol (Rubio *et al.,* 2010; Baart *et al.,* 2009). As the exact effect of dosage and drugs used for stimulation on the oocyte quality cannot be judged during an IVF treatment so the natural cycle and mild stimulation protocols are favored to increase the embryo quality and reduce the aneuploidy rates (Rubio *et al.,* 2010). To trigger the ovulation is the last important step of ovulation induction and hCG has been used to trigger the oocyte maturation because of its homology with LH and prolonged half-life.

Time-lapse imaging however involves the periodic light exposure of embryos for recording the growth patterns continuously but still it is suggested to be noninvasive to the embryo, as it does not involve any physical disturbance or chemical exposure as compared to traditional morphological scoring method routinely being used in IVF clinics for decades (Nakahara *et al.,* 2010).

The findings of current study can be concluded as follows:

With increasing female age there is a delay observed in the early cleavage pattern of embryos hence slow cleavage is commonly observed in advanced age. The rate of blastocysts formation as well as clinical pregnancy also declines.

The rate of untimely cleaving embryos dominates the timely cleaving ones as the developmental stages proceeds but t5 seems to be the best predictor of blastocysts formation with respect to age as well as patient pathologies.

Endometriosis, low AMH, high FSH and advanced female age are the conditions which affect the embryo morphokinetics, as well as the clinical pregnancy rates more adversely than PCOS, male factor, tubal factor and idiopathic infertility.

The poor responders to ovarian stimulation and those treated with high doses of gonadotropins show poor oocyte yield, delayed early cleavage pattern, low blastocyst formation rate and poor pregnancy outcome.

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