THE PREDICTORS FOR HIGH-QUALITY EMBRYOS FOR ADVANCED AGE WOMEN UNDERGOING INTRACYTOPLASMIC SPERM INJECTION CYCLES IN HOSPITAL USM FERTILITY UNIT

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by

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"In the name of Allah, Most Gracious, Most Merciful"

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LIST OF ABBREVIATIONS

AACP	: Agonist/Antagonist Conversion Protocol
AMA	: Advanced maternal age
AMH	: Anti-Muellerian hormone
ART	: Assisted Reproductive Techniques
ASA	: Anti-sperm antibody
bhCG	: beta hCG
BMI	: Body mass index
CBR	: Crude birth rate
CLCG	: Centrally Located Granulation of the Cytoplasm
DNA	: DeoxyriboNucleic Acid
ESHRE	: European Society of Human Reproductive and Embryology
FSH	: Follicle-stimulating hormone
GnRHa	: Gonadotropin releasing hormone agonist
GnRH-anta	: GnRH antagonist
hCG	: human Chorionic Gonadotropin
ICSI	: Intracytoplasmic Sperm Injection
IU	: International unit
IVF	: In vitro Fertilisation
LH	: Luteinizing hormone
mtDNA	: mitochondrial DNA
PB	: Polar bodies
PCOS	: Polycystic ovarian syndrome
PVS	: Perivitelline space
SART	: Society for Assisted Reproductive Technology
SD	: Standard of Deviation
TFR	: Total Fertility Rate
EA	: Embryonic Arrest

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PREDIKTOR UNTUK EMBRIO BERKUALITI TINGGI DI KALANGAN WANITA BERUSIA MATANG YANG MENJALANI KITARAN SUNTIKAN SPERMA INTRACYTOPLASMIC DI UNIT KESUBURAN HOSPITAL USM

ABSTRAK

Penurunan tahap kesuburan wanita yang berusia matang bukan sahaja disebabkan oleh ovari yang mempunyai kuantiti oosit yang semakin berkurangan, malahan ia juga melibatkan faktor penurunan kualiti oosit dan janin yang terhasil. Oleh itu, adalah penting untuk kita mengenalpasti faktor-faktor yang mempengaruhi kualiti oosit dan janin di kalangan wanita berusia matang ini untuk meningkatkan kejayaan perawatan '*Intracytoplasmic Sperm Injection'* di kalangan kumpulan ini. Kajian ini dijalankan dengan tujuan untuk melihat kualiti oosit dan janin yang terhasil di kalangan wanita-wanita berusia matang yang menjalani rawatan ICSI di Unit Rawatan Masalah Kesuburan Hospital USM. Faktor peramal oosit dan janin berkualiti baik juga dikenalpasti. Kajian prospektif ini telah dijalankan di Unit Rawatan Masalah Kesuburan Hospital USM selama 24 bulan. Sebanyak 941 oosit telah diperolehi daripada 124 orang wanita berusia matang. Kualiti oosit dan janin telah ditentukan melalui ciri-ciri morfologinya. Kualiti janin dikelaskan berdasarkan kriteria SARTCORS. Kadar persenyawaan dan kadar pembahagian sel dinyatakan sebagai peratus untuk setiap kitaran, manakala kadar kehamilan dinyatakan sebagai peratus untuk setiap pemindahan janin ke dalam rahim. Faktor-faktor peramal oosit dan janin berkualiti baik ditentukan menggunakan analisa linear regresi tunggal dan analisa linear regresi berganda, dengan mengambilkira $p<0.05$ sebagai kadar signifikan. Daripada 941 oosit yang diperolehi, sebanyak 291 adalah oosit gred 1 (39.64%, purata 2.35 dengan sisihan piawai 1.79 bagi setiap kitaran), manakala oosit gred 2 adalah sebanyak 272 (37.06%, purata 2.19 dengan sisihan piawai 1.27 setiap kitaran) dan selebihnya adalah oosit gred 3 (23.30% purata 1.38 oosit dengan sisihan piawai 1.22 setiap kitaran). Daripada 586 janin yang terhasil, 500 (85.32%) merupakan janin berkualiti tinggi (purata 4.03 dengan sisihan piawai 3.22 setiap kitaran). Kadar persenyawaan dan kadar pembahagian sel adalah 81.6% dan 97.8% setiap kitaran. Sebanyak 76 pemindahan janin ke dalam rahim telah dilakukan. Daripada jumlah tersebut, 17 orang pesakit berjaya mengandung, dan menjadikan kadar kehamilan setiap pemindahan sebanyak 22.37% bagi setiap pemindahan embrio. Tiada keguguran didapati di kalangan mereka. This study has proved that increasing the number of collecting mature oocytes and cleavage rate could increase the chance of obtaining high-grade embryos. Kajian ini mengesahkan dengan meningkatkan bilangan oosit yang matang dan kadar pembahagian sel dapat meningkatkan peluang untuk mendapatkan janin berkualiti tinggi dan seterusnya meningkatkan peluang untuk mengandung di kalangan wanita-wanita berusia matang. Oleh itu, usaha untuk meningkatkan kuantiti dan kualiti oosit di kalangan wanita berusia matang haruslah ditingkatkan.

THE PREDICTORS FOR HIGH-QUALITY EMBRYOS FOR ADVANCED AGE WOMEN UNDERGOING INTRACYTOPLASMIC SPERM INJECTION CYCLES IN HOSPITAL USM FERTILITY UNIT

ABSTRACT

Advanced age women associated with fertility decline as a result of ovarian reserve depletion and declining oocyte and embryo quality. Determining the predictor factor of oocytes and embryo quality may assist in stimulation target and cycle prediction. The study is to determine the oocyte and embryo quality among advanced age woman receiving Intracytoplasmic Sperm Injection cycles in Hospital USM Fertility Unit. Predictors for high-quality oocytes and embryos were identified. This prospective observational study was performed over a period of 24 months in Hospital USM Fertility Unit, on 941 oocytes retrieved from 124 women of advanced age receiving ICSI. Oocytes grading by means of its morphological appearances and the SARTCORS grading system for embryo grading were used to determine their qualities. Fertilization rate and cleavage rate were expressed as percentage per cycle, while pregnancy rate was expressed as percentage per transfer. Possible predictors of good-quality oocytes and high-quality embryos were evaluated using single and multiple regression tests, with $p < 0.05$ considered as significant. From 941 retrieved oocytes, 291 oocytes (39.64%, mean 2.35 SD=1.79 per cycle) were Grade 1 oocytes, while 272 oocytes (37.06%, mean 2.19 SD=1.27) were Grade 2 oocytes and the rest (n=171, 23.30% mean 1.38 SD=1.22) were Grade 3 oocytes. Among 586 available embryos, 500 (85.32%, mean 4.03 SD=3.22) high-quality embryos were obtained. The fertilization and cleavage rates were 81.6% and 97.8%, per cycle respectively. Seventy-six embryo transfers were conducted, with 17 successful conceptions (pregnancy rate = 22.37% per

transfer). There were no miscarriages among the pregnancies. This study has proved that increasing the number of collecting mature oocytes and cleavage rate could increase the chance of obtaining high-grade embryos. This could increase the success of ICSI among women of advanced age. Therefore, efforts to find ways to increase oocytes quantity and quality among advanced aged women must be made.

CHAPTER 1

INTRODUCTION

1.1 Kelantan population and the total fertility rate

Kelantan, as seen in Figure 1.1, is located in the north-eastern of Malaysia. This state is an agricultural green paddy field with rustic fishing villages and casuarina-lined beaches.

Figure 1.1: The map of Peninsular Malaysia, indicating the location of Kelantan state

The population in Kelantan are mostly Malays (94%), and under the Malaysian Constitution, almost all Malays are Muslim's religion. In other words, Islam the most influential religion. Being true Muslims, Assisted Reproductive Techniques (ART) as part of infertility management is accepted.

According to the Demographics of Malaysia data, the Total Fertility Rate (TFR) in Kelantan is the highest among all the states in Malaysia. However, the rate of population replacement is decreasing. The TFR of Kelantan was 3.77 in 2011 as compared to 3.07 in 2015. The decrement in the TFR involves the whole of Malaysia. The TFR of Malaysian women has declined from 4.9 babies (1970) to 1.9 babies (2017) between the age of 15 to 49 years old (Department of Statistics Malaysia Official Portal). The mean age for childbearing for Malaysian women has increased from 29.41 years in 1970 to 30.75 years in 2017. The increment of age-related fertility could be attributed to the changes in social lifestyle and life achievement priority of modern women. Modern women tend to postpone their marriage and childbirth to be successful in their careers, and it has become their priority, rather than building up their families.

Unfortunately, the postponement of family development is inversely correlated to female fertility. It is statistically correlated between female fecundity declines as women age with the decrease in ovarian follicles (Vollenhoven and Hunt, 2018). This fact also has a remarkable impact on the ART successful outcomes. Despite in advancement of ART, the successful outcome has not been shown to improve because of the advanced reproductive age. The failure in ART treatment may cause many disappointments and economic wastage among the advanced age women (Gleicher, Weghofer and Barad, 2007). When female-age fertility and the United States of America reported on age-based IVF outcome, the curves are compared, and they are identical. It suggested a decline at an early age. It accelerated at 37-38 years old, where approximately only 25,000 follicles are left in the ovaries from million at the foetus stage. These declining curves have also been reported for intrauterine insemination and inversely for miscarriages (Of and Aging, 2002).

1.2 Problem statement and rationale of the study

Hospital Universiti Sains Malaysia (HUSM) has provided ART service since Jun 2014, being the first and only one in Kelantan. As to date, all patients receiving ART in HUSM underwent Intracytoplasmic Sperm Injection (ICSI) rather than In vitro fertilisation (IVF). The first ICSI baby of HUSM was born on 10th August 2015. According to the HUSM Registry, more than 90% of women who came to the HUSM Fertility Unit seeking fertility treatment were more than 35 years of age. It also reported that the pregnancy outcome in HUSM was 26.67% per cycle in 2017 (Annual Report of Hospital USM Fertility Unit, 2017).

The success of any ART among women aged 35 years and above, termed advanced maternal age (AMA), is low. Reproductive ageing is not just due to the quantity depletion of oocytes in the women's' ovaries. Still, it may also involve the declining quality of the oocyte, embryo and endometrial receptivity, which cause the low ART outcomes and high-cost treatment.

The high cost of treatment also leads to the need to assess the predictors of success in ART cycles. Therefore, managing women in their late reproductive age may cause a major challenge. Determining the factors that could predict the outcome of ART treatment will help counsel the women, which may remove the stress emotion before treatment and may further improve the treatment outcome for these women. Previous studies have looked into the factors as a separate entity. This study will look into the possibility of a combination of factors that may influence the ART successful outcome in advanced age women. Determining the predictors for success with ART for the advanced age group can provide better management and care for them.

CHAPTER 2

LITERATURE REVIEW

2.1 An overview of infertility

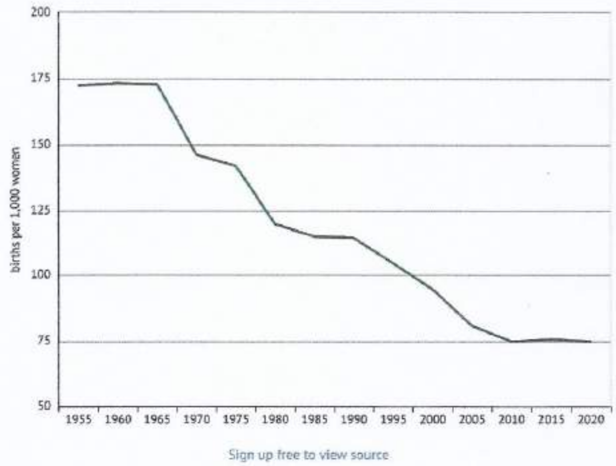
Infertility is defined as women in reproductive age group that fails to conceive in one year of trial without using any contraceptive device in women aged less than 35 years and in women aged over 35 years between 6 months of trial (Zegers-Hochschild et al., 2017). Infertility may be divided into primary and secondary infertility problems. A couple who has never conceived is characterized as primary infertility, while a couple who have conceived before, regardless of its outcome refer to secondary infertility. Secondary infertility carries a better prognosis than primary infertility (Benksim et al., 2018). In addition to types of infertility, the prognosis of ART is also been affected by age of the patient, duration of infertility, body mass index (BMI), their baseline follicle stimulating hormone (FSH), luteinizing hormone (LH), total dosage of gonadotrophins used for ovarian stimulation, the level of progesterone and endometrial thickness on human chorionic gonadotrophin (hCG) injection day in ovarian stimulation protocol, as well as the duration of ovarian stimulation.

There was a progressive decline in birth rates throughout Malaysia. The Malaysian recorded 487,957 in 2019 in live births, which decreased about 2.8% compared to 2018, 501,945 with a live birth rate. The Crude Birth Rate (CBR) decreased from 15.5 births per thousand in 2018 to 15.0 births per thousand in 2019. The decrement involved all states in Malaysia except for Wilayah Persekutuan Kuala Lumpur, which showed an increment in CBR from 13.5 births per thousand population to 15.2 births per thousand population. The CBR for Kelantan was 20.6 births per thousand in 2018, which decreased to 19.5 births per thousand in 2019 (LabourforceSurvey, 2015) (Department of Statistics Malaysia, 2020).

Malaysia has seen a declining CBR and its population fertility, as measured by the TFR. It recorded a dramatic decline in Malaysian's TFR from 4.9 babies (1970) children to 1.8 (2019). Therefore, since 2013, the national TFR was below the replacement level, which is 2.1, and it indicates insufficient replacement to replace the community population.

Demographic and socioeconomic factors are the main determinants in Malaysia's declining trend in TFR. Improvements in providing a good health care service and strategies to reduce infant mortality rates may decrease the fertility rates. While in socioeconomic factors, increases in expenditure, education achievements and female employment may reduce the fertility rates in Malaysia. Subramaniam and Salleh concluded that younger women with higher education levels, higher income, and living in the city areas are more likely to have fewer children (Subramaniam and Mohd Saleh, 2016). Therefore, the Malaysian government must design particular policies by restructuring the tax for households with relatively large family sizes. The policy may achieve by offering or helping the employed women with the child benefits and providing childbearing and childrearing assistance. Other factors that can be considered about fertility rates are the impact of miscarriage, increasing the mean age of marriage among women, reducing contraceptive devices, abolishing poverty, and controlling urbanization (Awad and Yussof, 2017).

The trend of delaying pregnancy has contributed to a significant impact on Malaysian's fertility rate. As a result, the fertility rate at age 35 to 39 years of Malaysia has gradually fallen from 141.64 births per thousand women in 1975 to 74.84 births per thousand women in 2020 (Figure 2.1). As female age is a significant factor that affects the prognosis of any fertility treatment, this trend has caused many challenges to the medical personnel who give fertility services.



DATE	VALUE	CHANGE, %
2020	74.84	-1.16 %
2015	75.72	1.11 %
2010	74.89	-7.43 %
2005	80.90	-14.38 %
2000	94.49	-9.57 %
1995	104.50	-8.47 %
1990	114.16	-0.49 %
1985	114.73	-4.09 %
1980	119.63	-15.54 %
1975	141.64	-2.94 %
1970	145.93	-15.45 %

Figure 2.1: Malaysia's fertility rates at age 35 to 39 years

Source: Department of Statistics Malaysia (2020)

2.2 Aetiology of Infertility

The human reproductive process is complex. It involves the following mechanisms where;

- The sperm needs to be near the cervix at ovulation time, which ascends into the fallopian tubes and fertilizes the oocyte.
- An ideal ovulation time must occur at a regular and predictable women period cycle with a mature and good quality oocyte.
- The function of the cervix must capture, filter, nurture, and release the sperm into the uterine cavity and fallopian tube.

- In order to capture ovulated matured oocytes and effectively transfer an embryo, the fallopian tubes must be able to function.
- The function of the uterus must be receptive to embryo implantation and capable of supporting its development.

In general, infertility can cause by both female and male-factors. Therefore, before proceeding with any investigations for infertility, the major cause of infertility and its basic component for evaluation should be outlined. According to ESHER Press Information 2016, between 20% to 30% of cases are contributed by male factors. Female factors contribute about 20 to 35% of cases. The combination infertility cases are contributed about 25 to 40% of male and female factors. 10 to 20% falls under unexplained infertility (Information, 2016).

Among the female-factors, ovulatory dysfunction is the most typical cause. Usually, it is associated with polycystic ovarian syndrome (PCOS). PCOS will impair the normal ovulation cycle, which may cause by hormonal imbalance control. Another reason for anovulation includes excessive weight or high body mass index (BMI). Obesity may affect the egg and sperm quality; therefore, they must strive to have a normal BMI because of the accumulation of fat in the abdomen, which causes estrogen and progesterone imbalances and finally leads to anovulation. Primary ovarian insufficiency or premature ovarian failure may interfere with the ovulation cycle before age 40. Hyperprolactinemia also affects the ovulation function as well as hyperthyroidism and hypothyroidism.

Tubal and peritoneal factors are among the commonest infertility aetiology after ovarian dysfunction. Other factors may involve pelvic inflammatory disease, septicmiscarriage, ruptured in an appendix apparatus, tubal surgery, or ectopic pregnancy may cause the possibility of tubal damage. In endometriosis, the problems occur when the tissue lining in the uterus may start to grow in other places such as at the Pouch of Douglas, fallopian tubes, abdomen, pelvis or ovaries. Endometriosis may block the function of fallopian tubes, disrupt the implantation potential, cause severe inflammation, and negatively impact oocyte quality via its endogenous production of various cytokines. It may cause irritation and the development of scar tissue.

Uterine abnormalities are not common problems in infertility but need to be considered. The abnormalities in the uterine's anatomic include congenital malformations, leiomyomas formation, and intrauterine adhesions development. The presence of endometrial polyps may also affect fertility, probably by interfering with implantation. Endometrial receptivity is one of the major issues which is under consideration at present. It involves a variety of intrinsic endometrial dysfunction, which impairs or prevent implantation. Another important factor that is associated with infertility is stress one of psychological factor. Couples in urban areas often lead stressful lives due to high-cost of living that’s may be affects a change in heart rate and cortisol which are the predictive of a decreased probability of achievement a viable pregnancy. Fatigue also may lead to ‘sleepy’ ovaries and It may affects the hormonal imbalances, which lead to irregular menstrual cycles (Cwikel, Gidron and Sheiner, 2004).

2.3 Fertility in advanced maternal age (AMA)

A woman’s fecundity gradually decreased with advancing age and became significant starting from 32 years and more rapid after 37. Decreasing fecundity is a condition where the quantity and quality of oocytes decrease (Schwartz D et al.,1982).

Atresia is a process of oocyte degradation in the ovaries by natural processes, and it may decrease progressively. There is a maximum of 6 to 7 million oocytes in the ovaries of a fetus (at 20 weeks of gestation). It decreases in quantity approximately to one or two million at birth and about 300,000 to 500,000 at puberty. At the age of 37 years, the left-over of oocytes is about 25,000 and 1,000 oocytes at 51 years, which is the average menopausal age (Faddy et al., 1992). The decrement in the number of oocytes (also termed as ovarian reserve) is reflected by a low Anti-Mullerian Hormone (AMH) level and a decrease in inhibin B concentrations. Female age has been proven to be a strong indicator of female fecundity. Naturally, there is an age-related decline in female fecundity. The decrement usually starts at the age of 32-years and drastically reduces after 37-years-old. Spelt differently, the monthly natural fecundity rate decreases by 25% between 20 and 30-year-old and becomes below 10% at the age of 37-years (Figure 2.2).

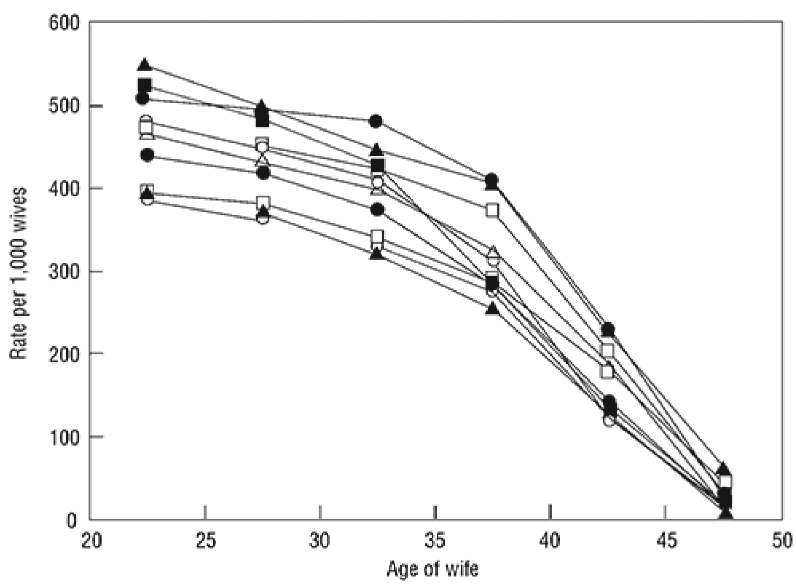


Figure 2.2: Marital fertility rates according to female age (Age and Infertility Fertility Associates of Memphis.

Source: https://www.fertilitymemphis.com/female-infertility/age-and-infertility/

A similar trend in pregnancy rates was observed among patients receiving intrauterine insemination cycle, in which successful cycles were 74% in women 31-years and below, 62% among women 31 to 35-years and only 54% for women 35-year-old above (Schwartz D et al., 1982). The Centers for Disease Control and Prevention reported IVF success rate in women aged 35–37 years was 31.9%, while 22.1% in women aged 38–40 years. The women aged 41-42, it was 12.4% followed by women aged 43–44 years with 5%, and lastly, women older than 44 years it was recorded about 1% (Centers for Disease Control and Prevention,2010) (Committee on Obstetric Practice, 2002). However, these contrasts when the center uses donor eggs for their patients (Committee on Obstetric Practice, 2002). Regardless of the recipient's age, the oocytes from healthy, young donors may give 51% of fresh transfers resulting in higher rates in a live birth (Committee on Obstetric Practice, 2002). This figure proved that female age does affect not only ovarian reserve but also the quality of oocytes.

As age increases, leiomyomas, tubal disease, and endometriosis always affect the fertility potential. The size of follicular pool and declining fertility potential increase with women suffering from a history of ovarian surgery, chemoradiation therapy, endometriosis, smoking, under drugs treatments, pelvic infection diseases, or any strong family history of early menopause (Battaglia *et al.*, 1996)(Pellestor *et al.*, 2003).

Usually, fertility decline by age-related is caused by significant aneuploidy increment rates and may give higher spontaneous abortion (Balasch and Gratacós, 2011). Meiotic spindle predisposes to nondisjunction resulting in the most finding in the case of autosomal trisomy. (Committee on Obstetric Practice, 2002). The prevalence of aneuploidy embryos is high in AMA, even the normal morphology of embryo transferred (Committee on Obstetric Practice, 2002).

As age increases, the prevalence of fetal loss is recorded with 9.9% in women aged less than 33 years who conceive with a fresh embryo transfer. The pregnancy loss with fetal heart activity was observed after seven weeks of gestation (Committee on Obstetric Practice, 2002). The miscarriage rates increased progressively for women aged 33 to 34 years from 11.4% to 13.7% for women aged 35–37 years. For women aged 38 to 40 years the miscarriage rate increase to 19.8%, for women aged 41 to 42 years with 29.9%, and women older than 42 years with 36.6% (Farr, Schieve and Jamieson, 2007). It also has been reported that these data are similar to the increased miscarriage rates nationally with IVF. The miscarriage rate, from 13% in women less than 35 years to 54% in women aged 44 years and older, increased progressively with age (Centers for Disease Control and Prevention, 2010) (Committee on Obstetric Practice, 2002). In other words, declining fertility may cause by the incidence of impaired infertility disorders and higher pregnancy loss in women over 35 years old (Committee on Obstetric Practice, 2002) who should receive an expedited evaluation and undergo treatment after six months of failed attempts to conceive or earlier if clinically indicated. Women aged 40-years and above warrant immediate fertility evaluation and treatment.

The number of AMA women seeking fertility treatment is increasing in Kelantan. Despite explanation on the prognosis of any fertility treatment among AMA, there were many inquiries on the cause of failed fertility treatment, especially from those who received ART cycles—because of this, determining the factors that could improve ART's success among AMA is required.

2.4 Assisted Reproductive Technology (ART)

ART involves the treatments or procedures that include *in vitro* handling of both human oocytes and sperm or embryos, for the purpose of establishing a pregnancy. It includes IVF, ICSI and embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation and gestational surrogacy. IVF/ICSI procedures comprises ovarian stimulation, oocyte retrieval, ICSI procedure, fertilization and embryo transfer. In IVF cycle, eggs and sperms are collected and placed together in a dish to fertilize. The embryos which derived from the successful fertilization will be chosen and transferred into a woman's uterus. The potential surplus embryos can be kept frozen for future use. ICSI is a procedure where a single sperm is injected directly into the cytoplasm of each egg. ICSI is often used if male partner has a very low sperm count, low motility or poor sperm quality. Once the fertilization occurs, embryo transfer and freezing would follow next as it goes with IVF.

2.4.1 Evaluation of oocyte quality by morphological grading

There is variation in morphological of Metaphase II (MII) oocytes retrieved from patients after ovarian stimulation. Morphological variation may affect the competence of development and the potential of embryo implantation. Oocyte morphological grading depends on some criteria. The criteria may involve the pattern of zona pellucida (ZP), the presentable of polar body (PB), the absences of vacuoles or refractile bodies, the shape of an oocyte, the darken cytoplasm or diffusion of granulation, the area size of perivitelline space (PVS), the position of cytoplasmic granulation, the condensed pattern of cumulus-oocyte complex and the viscosity of cytoplasm within the oolemma.

Good quality oocyte should have perfect spherical shape, small PVS, regular ZP, intact 1st PB, translucent homogenously colored cytoplasm without inclusions. According to Veeck et al, 1999 the grading based on morphological abnormalities, three groups of oocyte grades had been established based on the anomalies. Grade I is based on without any anomalies, which is in great and perfect morphology. Grade II with one anomaly, while Grade III are the combination of at least two anomalies. The anomalies of oocyte morphology were observed under two different subgroups: the abnormalities of extracytoplasmic and intracytoplasmic.

2.4.1.(a) Cytoplasmic abnormalities

Centrally Located Granulation of the Cytoplasm (CLCG)

The granulated cytoplasm may be accompanied by clear transparent border-line results from condensed at the central cytoplasm. Condensed cytoplasm showed a prominent darker colour appearance than normal finely diffused cytoplasm granulation easily distinguishable by phase contrast microscopes. This strange morphological feature can be seen by an extensive, dark, granulation look like spongy area in the cytoplasm with severe diameter and depth of the granular area. Various studies have shown its detrimental effect on clinical outcomes, with a high risk of aneuploid embryos and high abortion rates. This poor grade of oocytes might significantly lower cryo-survival rate, decreasing achievement in blastocyst formation with poor quality and poor-hatching deficiencies. Coarse granulation of cytoplasm may derive from ‘organelle clustering’, where the repetitive dysmorphism happened and can be seen as a sign of severe intrinsic pathology associated with decreasing implantation and pregnancy rates.

Refractile bodies (RB)

RB are the inclusions in the cytoplasmic composition with the appearance of darkening incorporations. RB may be associated with a higher degree of blastomeres fragments, coarse and dense cytoplasm granulation with lipids droplets and lipofuscin. A study by Veeck et al. reported that the average diameter under bright-field microscopy is about ten µm (Veeck, 1991). Published studies show controversial results for the effect of RB in the cytoplasm on clinical outcomes. The presence of RB is controversial because the effect is still unknown until nowadays, but confounding factors reported the differing in diameters could affect the outcome. The clinical outcome is detrimentally affected if the diameter is over five µm (Otsuki, Nagai and Chiba, 2007).

Vacuoles

Vacuoles structures may be derived from uncontrolled endocytosis of smooth endoplasmic reticulum and Golgi apparatus and finally produce vesicles fusion (Ebner et al., 2005). The presence of vacuoles in mature oocytes varies from 3.9% of oocytes collection, of which 66% had single, 21.3% had double, and 12.7% had multiple vacuoles (Ebner et al., 2005). Ebner et al. suggested a cut-off value of 14 µm for vacuole diameter for fertilization outcome. The detrimental effect of oocytes increases as the vacuoles increase in size or the presence of multiple vacuoles. The larger size of multiple vacuoles is associated with the functionless of the oocyte cytoskeleton (e.g., microtubules) (Ebner et al., 2005). Besides the negative effect on the fertilization outcomes, it may affect the blastocyst formation competence, the quality and capability of hatching, and the percentage of euploid embryo rates, which can be impaired after ICSI of vacuolated oocytes. The cryo-survival rates and the resuming of embryonic development from cryopreserved embryos decreased.

Smooth Endoplasmic Reticulum Clusters (sERCs)

Vacuoles can easily distinguish from smooth endoplasmic reticulum clusters (sERCs). Vacuoles are separated in cytoplasm by a clear membrane-bound while sERCs is seen as translucent membrane-bound. The presence of sERCs is correlated in the functional and structural alteration of the sERCs during the oocyte maturation process, even though the mechanism of sERCs is unknown. The incidence of affected cycles with such oocytes varied from 5% to 10%. Some studies reported the detrimental effect in implantation potential with sERCs from the cohort of oocytes with clusters of at least one oocyte. The first report of transferring an affected embryo with sERCs indicated Beckwith Wiedemann syndrome (Otsuki *et al.*, 2004). Sat et al. reported a lower take-home baby rate and a higher miscarriage rate with two unexplained neonatal deaths. (Sá *et al.*, 2011). Following evidence-based data from Special Group and Scientists suggested precisely examined and discarded for this such features of the oocyte because according to the reported the baby might develop multiple malformations and ventricular septal defects (Special, Group and Scientists, 2017).

2.4.1(b) Extracytoplasmic abnormalities

Dysmorphic zona pellucida (ZP)

The composition or patterning of ZP may be altered and interrupted. The changes in ZP structure can be seen in the three-dimensional structure under light microscope. In conventional IVF, the ZP thicker in diameter (e.g., >20 μm) are usually associated with decreasing fertilization outcomes. Despite ICSI, a thicker zona may not interfere with subsequent fertilization outcomes or implantation potential since assisted hatching can be applied.

Discoloration

Under light microscopes, the ZP may appear in a dark or brownish color. Previous literature reported a common phenomenon with the presence of a discolored ZP (e.g., 9.5% to 25.7%). Must know that not completely dark or brown zonae/oocytes occur for the same reasons. Nowadays, these oocytes are termed 'brown eggs' because of the thick and dark ZP. This brownish ZP is accompanied by small PVS where sometimes filled with debris from granular cytoplasm. Studies showed similar fertilization, embryo quality, implantation, and clinical pregnancy rates both for IVF and for ICSI cycles outcome between brown oocytes and the normal appearance of oocytes.

Shape abnormality

The vast majority of ovoid ova are the result of anomaly matured oocytes within the follicles. The ovoid shape is not quantified in the grading of oocytes because of the fertilization capacity, and a rise in healthy babies has been reported. If an objective measurement of the shape abnormality of the MII oocyte and ZP were to be performed, it would be shown that the degree of anomaly shape is neither correlated to fertilization nor embryo quality. Still, interestingly, the observation had been made between two ovoid oocytes. On day 2 of cleavage, the morphology patterns of cleavage were observed. Ovoid oocytes may cleave normally like a tetrahedron. If the ovoid zona fails to cleave in good morphology positions, it may develop in a flat array of four or eight blastomeres. This abnormal pattern may reduce the contactless from cell to cell, making the compaction and blastulation performed delayed.

Perivitelline Space (PVS)

The maturation phase of oocytes may closely relate to the size of PVS. The maximum PVS size may be reached only at the completion of maturation (MII). 50% resulting in a lower fertilization rate with a large PVS size. Female age had not been reported in influencing the PVS performances even though the ratio of estradiol to testosterone (and to progesterone) had a significant influence. Large PVS may describe as over-mature oocytes when data collected from in vitro and in vivo matured oocytes had shrunk in relation to the ZP. During 1st PB formation, a larger portion of cytoplasm may be extruded through oolemma together with haploid chromosome set and develop a larger PVS area. Therefore, there are two explanations in describing the origin of debris in the PVS. First, the debris may derive from ultra-structural components in the cytoplasm or maybe from the extracellular matrix indicating granules and filaments in the PVS area. Studies support the latter theory because it matched the cells from cumulus cells and the corona radiata and had a close relationship between the frequency of PVS granularity and maturation. Some studies relate the PVS debris with gonadotrophin dose-dependent because it is significantly increasing for high-dose patients. The implantation and pregnancy rate decreases with the presence of debris in PVS even though the fertilization rate, cleavage rate, and high-embryo quality are unaffected.

First Polar Body Morphology (1st PB)

Various groups of earlier reports suggest a good system in grading the oocytes based on the anomalies of morphological appearance of the 1st PB. Even though the efficiency of scoring 1st PB morphology, mainly based on the fragmentation level, is debatable, the appearance of a large 1st PBs should be noted. The use of such MII oocytes should be prevented because it may have a high risk of abnormality composition of chromosome. Therefore, 1st PB morphology may indicate as a tool in assessing the high-quality embryo and implantation potential can have limited predictive value due to the dynamic formation of this structure, which is highly changeable over the hours of culture.

Some prominent morphological anomalies at the oocyte stage are shown in Figure 2.3.

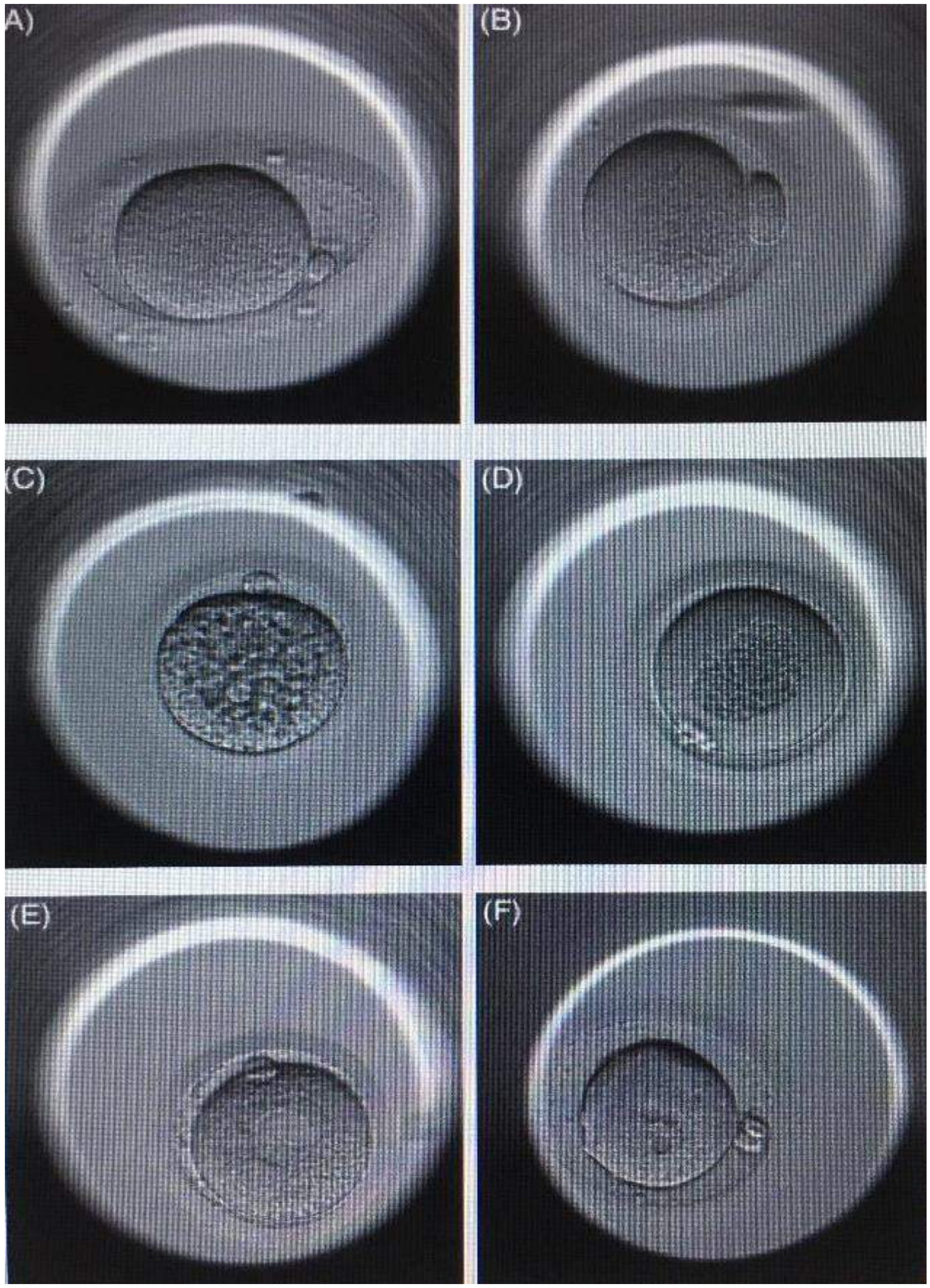


Figure 2.3: Overview of oocyte morphological anomalies: (A) zona anomaly; (B) large polar body; (C) diffuse granulation; (D) dense granulation; (E) smooth endoplasmic reticulum clusters and (F) vacuoles.

Source: Balaban, B. (n.d.). Oocyte Grading by Morphological Evaluation. Principles of IVF Laboratory Practice, 132–137. doi:10.1017/9781316569238.021

2.4.2 Morphological evaluation of embryo quality/grading

Embryo grading or cleavage assessment is done 48 to 72 hours later depending on the time elapsed post-insemination on embryos in the G-TL micro-droplet culture dish based on their morphology on the first cleavage. The number of blastomeres, the frequencies of fragmentation, the quality of symmetry in the blastomere, and the absence of multiple nucleation (multinucleation) are the criteria that need to be included in the embryo grading scores. Usually, if the embryo may cleave slowly or even faster than the time range should be achieved, it is expected to have metabolic and chromosomal defects and reduce implantation potential (Van Royen et al., 1999). The embryo's development is monitored and graded according to SART CORS grading system with Grade A, Grade B or Grade C (Racowsky *et al.*, 2010). Only Grade A and B are transferred at the Day 3 development.

The consensus of ALPHA Scientists in Reproduction Medicine and ESHRE Special Interest Group of Embryology 2011 agreed with some of the findings ('The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting.', 2011). The embryos with cleaved blastomere more slowly or faster than expected have a decreasing rate in implantation potential. The first early cleavage failure 24 to 28 hours after fertilization is related to lower implantation rates even in embryos with good morphology on transfer day (Balaban B, 2002). According to Edwards et al., appropriate kinetics and synchrony of division must be ensured to exhibit a high-quality embryo. If the embryos have metabolic or chromosomal defects, it may divide too slowly or too fast (Edwards et al., 1980). It is important to know that the specific laboratory environment influences development kinetics, such as media culture and temperature. Therefore, the consensus expected the observation for embryo development is four cells on Day 2 and cleavage eight cells on Day 3, depending on the time elapsed post-insemination. Therefore, blastomeres quality may be used as the high predictive value (Van Royen et al., 1999).

The consensus agreed that the fragmentation was defined as an extracellular membrane-bound cytoplasmic structure or anucleate structures of blastomeres origin. The fragmentation should be <45um in diameter in Day 2 embryo and <40um diameter in Day 3 embryo. The relative degrees of fragmentation were defined as: mild (<10%), moderate (10-25%) and severe (>25%). The per cent values are based on the cell equivalents, so for a 4-cell or 8-cell embryo, the 25% of fragmentation would equate to one blastomere in volume. The implantation and pregnancy rates negatively correlated with the degree of fragmentation (Racowsky et al., 2000) because the impact of fragmentation is localization due to a dynamic phenomenon, i.e., the fragments can move within the embryo. Finally, the degree of fragmentation may be included in almost all embryo scoring systems.

Each blastomere should have a single nucleus. If multiple nuclei are found in each blastomere, it may decrease implantation potential due to the chromosome abnormality (Kligman et al., 1996). This may cause impairment in cleavage rates and implantation potential, and, as a consequence, the risk of spontaneous miscarriages may be increased (Kligman et al., 1996). The consensus was agreed to monitor the multinucleation on Day 2, and the presence of multinucleation in one blastomere is sufficient for the embryo to be considered multinucleated.

According to the consensus also, it was agreed that the cell number should be even in size. If the cleavage phase has not been completed, one would expect a size difference in the cells for all other cell stages. Uneven blastomere cleavage is associated with uneven cytoplasmic molecules distribution, such as proteins and mRNAs. It also reported an increased rate of aneuploidy and multinucleation (Hardarson et al., 2001).

The other morphological abnormalities vary on different embryos and different patients. The cytoplasm of cleaving embryos is usually pale, clear and finely granular appearance (Hartshorne, 2000). Veeck reported anomalies with coarse granularity and darkened at the centre of the cytoplasm. A cortical halo is present when all the cytoplasmic organelles retract toward the centre of the blastomere, which may impair the implantation potential and be destined for degeneration (Veeck, 1999). Presenting pitting, vacuoles, smooth endoplasmic reticulum clusters, and refractile bodies should record the presence. It may associate with loss of gestational sacs (Ebner et al., 2005). These abnormalities have to be graded as poor grade embryos.

The embryo assessment was introduced by Veeck in 1999, including cell number, fragmentation, cell size and multinucleation and agreed by Alpha and ESHRE, 2011. Each laboratory was encouraged to develop owned descriptions as the culture medium and culture system also significantly impact embryo morphology. In conclusion, the cleavage-embryo rate and the rate of development are extremely important indicators in predicting the high-quality embryo.

Because of the numerous systems used to grade the embryos, a standardization of morphological assessment for the embryo grading system was developed and implemented by the Society for Assisted Reproductive Technology (SART). The committee realized that the embryo grading system must be simple, comprised of fields that have a basis in scientific inquiry with some proven predictive value, and easily adopted in laboratories not routinely capturing these parameters. For these reasons, the SART CORS embryo grading criteria, as shown in Table 2.1, was developed (Racowsky *et al.*, 2010). This grading system was used in this study.

Society for Assisted Reproductive Technology (SART) develops a standard embryo assessment according to their morphological grading. In that time, there were numerous systems used to grade the embryos. The committee agrees that embryo grading must be simple and comprised a scientific inquiry with proven predictive value, and important that this is easily adopted in any laboratory. For these reasons, the SART CORS embryo grading criteria, as shown in Table 2.1, was developed. This grading system was used in this study.

Table 2.1: The SARTCORS embryo grading system

Growth phase	Overall grade	Stage
Cleavage	Good, fair, and poor	Cell no.: 1 through >8
		Fragmentation: 0, <10%, 11%–25%, and >25%
		Symmetry: perfect, moderately asymmetric, and severely Asymmetric
Morula	Good, fair, and poor	Compaction: complete and incomplete
		Fragmentation: 0, <10%, 11–25%, and >25%
Blastocyst	Good, fair, and poor	Expansion: early, expanding, expanded, and hatched
		Inner cell mass: good, fair, and poor
		Trophectoderm: good, fair, and poor

2.5 Oocyte and embryo quality among AMA

AMA correlates with their oocyte age. The quality of an oocyte is defined as the competence of an oocyte to complete the meiosis process and undergo fertilization in producing healthy embryos that can go to the blastocysts stage and produce healthy offspring. AMA were suffering from decreasing in ovarian reserve and the competence