The evaluation of different embryo markers and their subsequent effect on embryo development, implantation and pregnancy outcome in an in-vitro fertilization program

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DECLARATION

I, Dirk Jacobus Kotze, the undersigned, hereby declare that the work contained in this dissertation, is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature **Date**

..... **.....**

Prediker 11:5 *(1983) "Net so min as wat jy kan verstaan hoe gees en liggaam by mekaar uitkom in die skoot van 'n swanger vrou, net so min kan jy die werk van God verstaan. Hy doen dit alles."*

Ecclesiastes 11:5 *"As you do not know the path of the wind, or how the body is formed in a mother's womb, so you cannot understand the work of God, the Maker of all things."*

✍ *"Having performed embryology over the last 25 years I have experienced God's amazing and* ***impressive*** *science every day"*

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In this chapter the aim is to outline the different chapters under section A.

Against this background, we will conduct a literature review of relevant studies performed, and evaluate their comments regarding identifying embryo markers which can be utilized to improve overall ART outcome. We will evaluate the embryo marker sHLA-G in detail, using a prospective randomized study as well as a retrospective multi-centre study. The role of the morphology and genetic profile of an oocyte, zygote and embryo and subsequent blastocyst formation will be evaluated with the help of WGA/CGH. The work will then be summarized and conclusions will be made as well as possible suggestions for future directions will be indicated. In section B the methodology of the studies explaining the role of the candidate is illustrated.

CHAPTER 2

In this chapter the impact of the oocyte/zygote and the embryo on implantation/pregnancy rate was discussed. The morphologic characteristics of the oocyte, the cumulus–oocyte-complex (COC), the zona pellucida, the perivitelline space, cytoplasm and meiotic spindle and the polar body and its appearance were discussed in detail. The morphologic characteristics of embryo fragmentation and its effect on embryo development, ploidy and blastocyst formation were also studied. Embryo markers to predict pregnancy outcome were researched based on the international literature. The pronuclear morphology and early cleavage were highlighted as non-invasive embryo markers to predict outcome.

A non-invasive biochemical marker, soluble Human Leucocyte-Antigen-G (sHLA-G), that is expressed by developing embryos was researched. The value of blastocyst transfer and the improved ongoing pregnancy rate compared to

cleavage stage embryos were highlighted based on a recent meta-analysis. A detailed discussion on sHLA-G as well as Array-CGH and the future of these tests followed.

CHAPTER 3

In this chapter the aim was to compare pregnancy and implantation rates when embryos are selected based on a single Day 3 (D 3) morphology score vs. a GES score plus sHLA-G expression. This was a prospective randomized study (n = 214) undergoing fresh ICSI cycles. Embryos were selected for transfer based on either Day 3 morphology score (Group A) or GES-scoring plus sHLA-G expression (Group B). The following results were reported: Clinical [35/107 (33%) vs. 52/107 (49%)] and ongoing pregnancy [20/107 (19%) vs. 52/107 (49%)] rates were significantly different between Group A and Group B ($p < 0.05$). Implantation rates were not significantly different between Group A [52/353 (15%)] and Group B [73/417 (18%)] ($p < 0.05$). The number of pregnancies lost during the first trimester was nearly 12 times higher in Group A [25/52 (48%)]. It was concluded that the miscarriage rate was significantly lower in Group B than Group A and the pregnancy results were superior when embryos were selected based on GES plus sHLA-G expression.

CHAPTER 4

Several studies have reported an association between the presence of soluble human leukocyte antigen-G (sHLA-G) in human embryo culture supernatants (ES) with implantation and pregnancy outcome in vitro. However, the actual presence role during implantation and effect on implantation and pregnancy outcome are still controversial. A retrospective multi-centre study was performed on 2040 ICSI patients in six different centers. All embryos were individually cultured and a chemiluminescence enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of sHLA-G in culture medium surrounding embryos. In all centers, a positive sHLA-G result was associated with an increase in odds of multiple clinical implantations (OR: 1.48, 95% CI: 1.07 to

2.05, p-value: 0.0170), and an increased odds of multiple on-going pregnancies (OR: 1.66, 95% CI: 1.10, 2.51, p-value: 0.0170). Data from this multi-centre study emphasize that sHLA-G expression is a valuable non-invasive embryo marker to assist in improving pregnancy outcome with the theoretical potential to reduce multiple pregnancies. A combination of sHLA-G expression and extended embryo culture to the blastocyst stage might provide future tools by which to select single embryos for transfer and reduce the risk of multiple gestational, without compromising their pregnancy rates.

CHAPTER 5

In this chapter the ploidy status of first and second polar bodies and Day 3 blastomere, embryo morphology and biochemical (sHLA-G) characteristics were correlated with blastocyst development and subsequent pregnancy outcome. All oocytes/zygotes and embryos were individually cultured to the blastocyst stage. PB-I, PB-II and blastomeres underwent whole genome amplification (WGA) and comparative genome hybridization (CGH) and complete karyotyping. Each embryo's culture medium supernatant was collected and analyzed for sHLA-G expression on Day 2. The following results were reported: Fifty seven mature (MII) donor oocytes were obtained, 33/57 (57.9%) were aneuploid, 21/57 (36.8%) were euploid and 3/57 (5%) were "inconclusive". No correlation was found between CGH status of PB-I, PB-II and the GES-score. Furthermore, no correlation was established between PB-I CGH results and blastocyst morphology grade. There was a significant correlation between PB-I CGH and blastomere CGH results. Euploid and aneuploid PB-I developed into 58% and 67% blastocysts, respectively. Kappa statistics (>0.7) revealed a positive correlation between the ploidy of PB-I, PB-II and the blastomeres. It was concluded that following ICSI and sequential genetic karyotyping of the oocyte/zygote and subsequent blastomeres, the majority of oocytes fertilized and subsequent zygotes developed into blastocysts, despite their ploidy status. We therefore conclude that blastocyst development is not associated with ploidy.

CHAPTER 6

Identifying a developmentally competent embryo to transfer that has the highest probability to develop into a live baby has been an issue of debate and continues research. The aim of this chapter is to discuss the morphological, biochemical and genetic features of an embryo that has been shown to be predictive of implantation and pregnancy outcome in ART using most current evidence.

A literature search was performed looking at the correlation between pronuclear morphology, early cleavage, cleavage stage embryos, blastocyst development, the presence of sHLA-G, CGH, embryo development and implantation/pregnancy rates in ART. Based on the available literature, a combination of observations could assist the scientist with embryo selection. The pronuclear stage morphology, the early embryo division, cleavage embryo stage and quality of the day 3 embryos provides limited guidance. However, choosing a blastocyst with a positive sHLA-G result on Day 5 is the optimal combination to make the final selection before embryo transfer or freezing. This non-invasive approach should improve pregnancy outcome and reduce multiple pregnancy rates. As far as the use of the more invasive technology such as aCGH is concerned, more research on pregnancy outcome is needed.

CHAPTER 7

A combination of observations for embryo selection, starting with oocyte grading, pronuclear stage morphology, early zygote cleaving and cleavage-stage embryo morphology/quality on Day-3, however, ultimately using extended embryo culture and choosing a blastocyst on Day 5 with positive sHLA-G values available, will assist the scientist in making the final decision before selecting an embryo for transfer or cryopreservation. The use of aCGH (for chromosomal analysis) is invasive and is still considered experimental.

Finally we conclude that despite all the above mentioned parameters to select an embryo for transfer that will develop into a live baby, more extensive research

and international corroboration is needed in order to improve and standardize embryo selection criteria.

OPSOMMING VAN DIE TESIS

HOOFSTUK 1

Die doel in hierdie hoofstuk is om die verskillende hoofstukke onder Afdeling A uiteen te sit. Daar word beplan om ‘n literatuur oorsig te doen van toepaslike studies rakend embriomerkers wat swangerskap-uitkoms in *in vitro* bevrugting kan verbeter. Verder sal die embriomerker sHLA-G deeglike bestudeer word met behulp van ‘n prospektiewe gerandomiseerde studie, asook ‘n retrospektiewe multisentrum studie. Die rol van embrio morfologie en die genetiese profiel van die ovum, sigoot asook die embrio en die daaropvolgende blastosist vorming sal geëvalueer word met behulp van WGA/CGH. Alle bevindings sal daarna opgesom word, gevolg deur ‘n sinvolle gevolgtrekking en laastens sal voorstelle gemaak word vir toekomstige navorsing op die gebied.

In Afdeling B sal die metodiek van die studies verduidelik word, asook ‘n beskrywing gegee word van die kandidaat se rol gedurende die navorsings projekte in hierdie tesis.

HOOFSTUK 2

In hierdie hoofstuk word die impak van die oösiet en die embrio op die inplanting/swangerskap-koers bespreek. Die morfologiese eienskappe van die oösiet, die kumulus-oösiet kompleks, die sona pellucida, die perivitelline spasie, sitoplasma en meiotiese spoel, die poolliggaam en die se voorkoms word breedvoerig bespreek.

Die morfologiese eienskappe van die embrio, fragmentasie en die invloed daarvan op die embrio, ploïdie, en blastosistvorming word bespreek. Embriomerkers om swangerskapsuitkoms te voorspel, gebaseer op

internasionale literatuur, is ook nagevors. Die pronukleêre morfologie en vroeë deling word as nie-indringende embriomerkers uitgelig om swangerskapsuitkoms te voorspel.

'n Biochemiese, nie-indringende merker wat deur ontwikkelende embrios uitgedruk word, oplosbare menslike leukosiet antigeen-G (sHLA-G), word bespreek. Die waarde van blastosist oordrag en die verbeterde koers van voortgaande swangerskappe in vergelyking met verdelende embrios, is ook uitgelig, gebaseer op 'n onlangse metanalise. 'n Breedvoerige bespreking van sHLA-G asook "Array-CGH" en die toekoms van hierdie toetse word behandel.

HOOFSTUK 3

Die doel van hierdie hoofstuk is om swangerskap en inplantingskoerse te vergelyk wanneer embrios geselekteer word op 'n enkel Dag 3 (D 3) morfologie beoordeling, teenoor 'n kumulatiewe GES-telling plus sHLA-G uitdrukking. Hierdie was 'n prospektiewe ewekansige studie (n=214) waar pasiënte ICSI-siklusse ondergaan het. Embrios is geselekteer vir terugplasing gebaseer op óf Dag 3 morfologie telling (Groep A), óf 'n kumulatiewe GES-telling plus sHLA-G uitdrukking (Groep B).

Die volgende resultate is gerapporteer: kliniese swangerskappe [35/107 (33%) vs 52/107 (49%)] en voortgaande swangerskappe [20/107 (19%) vs. 52/107 (49%)] se sukses koerse is beduidend verskillend tussen Groep A en Groep B ($p<0.05$). Inplantingskoerse is nie beduidend verskillend tussen Groep A [52/353 (15%)] en Groep B [73/417 (18%)] ($p<0.05$) nie.

Die aantal swangerskappe wat tot niet gegaan het tydens die eerste trimester was bykans 12 keer hoër in Groep A [25/52 (48%)]. Die slotsom was dat die miskraamsyfer beduidend laer in Groep B as in Groep A is en die swangerskap syfer betekenisvol beter was wanneer die selektering van embrios op GES plus sHLA-G gebaseer is.

HOOFSTUK 4

Verskeie studies het ‘n assosiasie getoon tussen die teenwoordigheid van oplosbare menslike leukosiet antigeen-G (sHLA-G) in menslike embrio kultuur en swangerskaps uitkoms in vitro. ‘n Retrospektiewe studie is op 2040 ICSI pasiënte by 6 verskillende sentra gedoen om die effek van s-HLAG verder te bestudeer. Alle embrios is individueel gekweek om die teenwoordigheid van sHLA-G in ‘n kultuurmedium rondom die embrios te identifiseer. In alle sentra is ‘n positiewe sHLA-G uitslag met ‘n toename in die waarskynlikheid van veelvuldige inplantings geassosieer (OR: 1.48, 95% CI: 1.07 tot 2.05, p-waarde: 0.0170), asook ‘n toename in waarskynlikheid van meervoudige swangerskappe wat voortduur (OR: 1.66, 95% CI: 1.10, 2.51, p-waarde: 0.0170).

Data uit die multisentriese studie beklemtoon dat sHLA-G uitdrukking ‘n waardevolle nie-indringende embriomerker is om by te dra tot die verbetering van swangerskapsuitkoms, asook die teoretiese potensiaal om meervoudige swangerskappe te verminder.

‘n Kombinasie van sHLA-G uitdrukking en verlengde embrio kultuur tot die blastosist stadium mag moontlik ‘n toekomstige hulpmiddel wees waardeur enkele embrios vir terugplasing geselekteer kan word. Daardeur kan die risiko van meervoudige swangerskappe beperk word sonder om die swangerskapkoerse in gevaar te stel.

HOOFSTUK 5

In dié hoofstuk word die ploïdie status van die eerste en tweede poolliggaampies en Dag 3 blastomere, embrio morfologie en biochemiese (sHLA-G) eienskappe gekorrelleer met blastosist ontwikkelling en uiteindelike swangerskapsuitkoms. Alle oösiete/sigote en embrios is individueel tot die blastosist stadium gevolg.

PB-I, PB-II en blastomere het “volledige kariotipering ondergaan deur gebruik te maak van die toets “comparative genome hybridization (CGH)”. Elke embrio se kultuurmedium supernatant is versamel en ontleed vir sHLA-G uitdrukking op

Dag 2. Die volgende uitslae is gerapporteer: Sewe-en-vyftig mature (MII) donor oösiete is verkry; 33/57 (57.9%) is aneuploïd, 21/57 (36.8%) is euploïd en 3/57 (5%) is onbeslis. Geen verwantskap is gevind tussen CGH status van PB-I, PB-II en die GES-telling. Geen verwantskap is gevind tussen CGH status van sHLA-G. Verder was daar geen verwantskap gevind tussen PB-I CGH uitslae en blastosist morfologie graad nie. Daar was ‘n beduidende korrelasie tussen PB-I CGH en blastomeer CGH uitslae.

Euploïde en aneuploïde PB-I het onderskeidelik in 58% en 67% blastosiste ontwikkel. Daar is ‘n positiewe verwantskap tussen die ploïdie van PB-I, PB-II en die blastomere aangetoon [Kappa (>0.7)]. Dit is afgelei dat na ICSI en sekwensiële genetiese kariotipering van die oösiet/sigoot en daaropvolgende blastomere, die meerderheid oösiete bevrug is en die daaropvolgende sigote ontwikkel het tot blastosiste, ongeag hul ploïdie status. Ons afleiding is dus dat blastosist ontwikkelling nie aan ploïdie verwant is nie.

HOOFSTUK 6

In hierdie hoofstuk bespreek ons waarnemings wat betref seleksie kriteria om die beste embrios te kies vir terugplasing wat uiteindelik tot ‘n suksesvolle swangeskap sal lei. Morfologiese, biochemiese en genetiese faktore is ondersoek. ‘n Onderskeiding is gemaak tussen nie-indringende (mikroskopiese en biochemiese) en indringende (embrio biopsie, aCGH) tegnieke.

'n Kombinasie van nie-indringende observasies, wat insluit pronukliere mofologie, vroee sigoot verdeling en vroeë embrio morfologie/kwalitieit op Dag-3 het beperkte inligting verskaf wat betref swangerskapkans. Verlengde embrio kweking tot die blastosist stadium (Dag-5) plus ‘n positiewe sHLA-G resultaat gee egter veel meer voordelige inligting aan die embrioloog met die embrio seleksie proses, voor embrio terugplasing of bevriesing. Laasgenoemde inligting sal die swangerskap syfer bevoordeel en die meervoudige swangerskap kans verlaag. Wat die indringende tegniek (aCGH) betref, word veel meer data

benodig rakend die potensiele voor- en nadele wat betref swangerskap uitkoms, voordat ‘n sinvolle gevolgtrekking gemaak kan word.

HOOFSTUK 7

‘n Volledige literatuur oorsig dui daarop dat alle beskikbare riglyne om embrios te kies vir terugplasing, ingespan moet word. In die studie is daar gekyk na ‘n kombinasie van hierdie voorstelle. Daar is begin met die morfologie van die pronukliere stadium, gevolg deur vroeë sigoot-verdeling, asook beoordeling van embrios se morfologie/kwaliteit op Dag-3 van ontwikkeling. Daar word voorgestel dat die keuse van ‘n blastosist op Dag 5, gekombineerd met ‘n positiewe oplosbare menslike leukosiet antigeen G (shla-G) die embrioloog van hulp kan wees om die beste embrio te kies vir terugplasing of bevriesing. Hierdie nie-indringende riglyn behoort swangerskap-uitkoms te verbeter asook meervoudige swangerskappe te verminder. Indringende tegnieke soos ACGH benodig verdere in diepte navorsing en data verkryging om die waarde van hierdie toets te kan beoordeel.

CHAPTER 1

Chapter 1

The evaluation of different embryo markers and their subsequent effect on embryo development, implantation and pregnancy outcome in an in-vitro fertilization program.

ABSTRACT

In this chapter the aim is to outline the different chapters under section A.

Against this background, we will conduct a literature review of relevant studies performed, and evaluate their comments regarding identifying embryo markers which can be utilized to improve overall ART outcome. We will evaluate the embryo marker sHLA-G in detail, using a prospective randomized study as well as a retrospective multi-centre study. The role of the morphology and genetic profile of an oocyte, zygote and embryo and subsequent blastocyst formation will be evaluated with the help of WGA/CGH. The work will then be summarized and conclusions will be made as well as possible suggestions for future directions will be indicated. In section B the methodology of the studies explaining the role of the candidate is illustrated.

INTRODUCTION:

There are unique challenges to having successful healthy pregnancies at the age > 37 in life. In general, one out of every 6 women in the reproductive age experiences problems with infertility (CDC Statistics in 2001). In this group, only 25% of women under the age of 40 conceive after proper treatment and deliver a healthy baby (CDC Statistics in 2001). Although there have been substantial improvements in ART over the last two decades the reported success rate with IVF has not improved significantly. In this group, only 25% of women under the age of 40 conceive after proper treatment and deliver a healthy baby (CDC Statistics in 2001). Although there have been substantial improvements in ART over the last two decades the reported success rate with IVF has not improved significantly.

Controlled ovarian hyperstimulation (COH) was introduced.

Transfer of multiple embryos to the uterus (instead of one) was introduced to increase the likelihood of pregnancy, hoping that at least one embryo would implant and result into a baby. However, unfortunately this approach also lead to high order multiple (triplets or greater) pregnancies, which is followed by the risk of premature-related peri-natal and neonatal complications.

Originally the indication to perform IVF was irreparable fallopian tubular disease. Subsequently, unexplained infertility, infertility caused by endometriosis, and recurrent failure with intrauterine insemination (IUI) were added to the list of indications for IVF.

In 1990, researchers at the Jones Institute in Virginia (USA), were first to describe successful male and female pro-nuclear development following intracytoplasmic sperm injection (ICSI). In 1992, researchers at the Free University of Brussels refined micromanipulation techniques, protocols and procedures and reported successful oocyte fertilization, embryo transfer and established pregnancies, following ICSI. There have been numerous modifications of ICSI procedures as well as multiple indications for its performance. (Nagy *et al.*, 1993; Van Steirteghem *et al.*, 1993; Van Steirteghem *et al.*, 1994; Palermo *et al.*, 1992) .Although ICSI was initially reserved for cases associated with severe male infertility (Palermo *et al.*, 1995), the technique was subsequently applied on patients with immunologic disorders, AMA (advanced maternal age), ASA (anti-sperm anti-bodies as well as unexplained infertility (Devroey *et al.*, 1994).

In the past, most approaches aimed at identifying the “best” embryos for transfer have focused on: (i) morphological assessment of embryos prior to transfer, (ii) pre-implantation genetic testing of biopsied polar bodies (derived from mature oocytes) and blastomeres (derived from 3-day old embryos) and (iii) prolonged embryo culture for 5-6 days post fertilization; and then transferring only embryos that, by having reached the blastocyst stage of development, have improved implantation potential. Morphological evaluations apparently furnish clues that enhance proficiency at choosing the best pre-embryos for transfer; however, these systems are severely limited in their ability to provide reliable evidence for subsequent normal development.

Recently, different criteria for selecting embryos for transfer have been introduced and influenced on the success rates of IVF clinics throughout the world (Alikani *et al.*, 1999;

Sakkas *et al.*, 1999; Van Royen *et al.*, 1999; Scott *et al.*, 2000). How to select "the best embryo" for transfer has become a very important topic of interest. The initial selection of embryos for transfer was based on morphology on the day of embryo transfer (Ng et.al., 1999). However, a higher IVF success rate has been reported using various different selection criteria, which have been used during the course of embryo development to day three. (Alikani *et al.*, 1999; Sakkas *et al.*, 1999; Van Royen *et al.*, 1999). Implantation of an embryo with a high embryonic grade significantly increases the chances that it will successfully develop into a healthy fetus and subsequent normal baby. The graduated embryo scoring (GES) system has recently been introduced, in which an embryo is separately cultured in its own drop, allowing for sequential microscopic assessment of developmental criteria (Fisch *et al.*, 2001) (See Chapter 3 Table 1). A score is then assigned to each embryo on Day 3, 72 hours post-oocyte retrieval. It was possible to demonstrate that embryos that score ≥70 out of a possible 100 allotted points have the greatest potential to implant after being transferred to the uterus and/or survive to the blastocyst stage if maintained in extended embryo culture for 2-3 additional days.

Currently, most IVF programs select the best quality embryos for transfer on Day 3 and the remaining embryos are kept in culture until Day 6 after insemination; therefore, such embryos are allowed to progress to the blastocyst stage in vitro before freezing (Racowsky *et al.*, 2000). In most IVF settings, embryos that have been defined as being of lesser morphologic quality are frequently cultured to Day 5/6 to evaluate their developmental potential to the blastocyst stage.

Which scoring or evaluation system to use on Day 3 to achieve the acceptable pregnancy rate comparable to embryos that are transferred at the blastocyst stage is a subject for controversy (Coskun *et al.*, 2000 and Kovacic *et al.*, 2002). The transfer of selected embryos on day 3 should yield a pregnancy rate equivalent to that which could be achieved had the transfer been done on Day 5/6 (Scholtes *et al.*, 1996 and Blake *et al.*, 2002).

Several researchers have set individual criteria to use in the process of selecting which embryos to transfer. Scott et al., (2000) suggested that embryos should be cultured in micro drops and pro-nuclei be evaluated on Day 1 of culture, in order to optimize assessment of an embryo's implantation potential.

Alikani *et al.* (1999) looked at embryo fragmentation and its influence on pregnancy rates and concluded that fragmented embryos resulted in lower pregnancy rates. Sakkas *et a.,.* (1999) evaluated early cleavage of embryos and concluded that early cleavage could be important criteria for selecting day 3 embryos for the transfer. Strict morphologic grading/evaluation were established for embryos on Day 3, by Van Royen *et al.,* (1999) and for blastocyst by Gardner *et al.,* (2000). Both groups stated that these findings are central to the attainment of optimal implantation and pregnancy rates on Day 3 and 5, respectively.

The detection of soluble Human Leukocyte Antigen-G (sHLA-G) in the culture medium of early cleaving embryos at 44-46 hr after insemination provided evidence that embryos could be selected by their maternal genomic function since there was a correlation between sHLAG expression by an embryo and the positive predictability of pregnancy (Sher *et al.*, 2004).

Karyotypic studies of aborted fetuses have been used to draw the inference that the proportion of concepti with chromosome abnormalities is very high. Whole genome amplification (WGA) and comparative genomic hybridization (CGH) has previously been used to identify the presence of genomic imbalance in embryonic cells during pre-implantation genetic screening (PGS). CGH has been used to the study of numerical and structural abnormalities of single blastomeres from disaggregated 3-day-old human embryos. (Voullaire *et al.* 2000). PGS has been used to identify the gender and embryos with genetic abnormalities. Although PGS provided some evidences for unexplained early abortions (Rubio et al., 2003), and cured for various chromosomal translocation cases (Scriven *et al.*, 2001), it fundamentally failed what it was supposed to provide, improved pregnancy rates. In best case scenario, the pregnancy rate after PGD was 30%, and currently it could offer information for only 7 autosomal chromosomes (13,15, 16, 18, 21, and 22) and 2 sex chromosomes (X and Y) (Ziebe et. al., 2003) . High-order multiple pregnancies (Jones *et al.*, 2001) and monozygotic twin pregnancies (Tarlatizis *et al.*, 2002; Sheiner *et al.*, 2001) are additional issues that are associated with the transfer of multiple embryos. There is an urgent need to develop a reliable system to identify embryos in culture with high potential to develop into a viable pregnancy. Embryologists/physicians

will be able to selectively limit transfer to one or two embryos, by using a very specific set of criteria, in order to virtually eliminate the risk of high order multiple gestations.

Currently several tools are available to evaluate embryos for transfer, as indicated above, but we are still faced with unknown factors, such as oocyte and embryo dimorphism.

It is known that in patients < 39 years of age 40-60 % and in patient's › 39 years of age up to 80% of their embryos is genetically abnormal (Kahraman *et al.*, 2000) despite all the tools that are used to separate the "best" embryo. The current grading is soley based on morphology and biochemistry (s-HLA-G); however, even a top quality embryo on Day 3 or blastocyst can be genetically abnormal.

Wind *et al*., (2001) illustrated that there are specific differences in the intra structural organelles of normal and abnormal oocytes when transmission electron microscopy (TEM) is used for such evaluation.

If we are heading in the direction of identifying a normal embryo for transfer, this study might provide some insight to achieve this goal. Starting observations at the follicular level, then grading the COC and oocyte by performing light microscopy, then first and second polar body (PB) biopsy/ whole genome amplification (WGA), comparative genome hybridization (CGH) analysis, soluble human leukocyte antigen-G (sHLA-G) expression, Day 3 morphology, blastomere biopsy/ whole genome amplification (WGA), comparative genome hybridization (CGH), using a Graduated score (GES) and subsequent blastocyst formation.

The overall objective of this thesis is to study different embryo markers and their subsequent effect on embryo development, implantation and pregnancy outcome in an in-vitro fertilization program.

Section A

Chapter 2

Against this background, we conduct a literature review of relevant studies performed, along with their comments regarding identifying embryo markers which can be utilized to improve overall ART outcome.

Chapter 3

A prospective randomized controlled study was performed to compare pregnancy and implantation rates when embryos are selected based on a single Day 3 (D 3) morphology score vs. a GES score plus sHLA-G expression.

Chapter 4

A retrospective multi-centre study was performed on 2040 ICSI patients in six different centers, to determine if the presence of sHLA-G have an effect on implantation and pregnancy outcome in vitro.

Chapter 5

To correlate first and second polar bodies and Day 3 blastomere ploidy, embryo morphology and biochemical (sHLA-G) characteristics with blastocyst development and pregnancy outcome in vitro.

Chapter 6

The aim of this chapter is to discuss the morphological, biochemical and genetic features of an embryo that has been shown to be predictive of implantation and pregnancy outcome in ART using most current evidence.

Chapter 7

Summary and conclusion

Section B

Methodology of the studies and explaining the role of the candidate.

CHAPTER 3

MATERIALS AND METHODS

Patients

Stimulation (Performed by MD)

Patients were pre-treated with oral contraceptives received Lupron (TAP Pharmaceuticals, Lake Forest, IL) in a long protocol after pre-treatment with oral contraceptive (OC) birth control pills for 1–3 weeks and were treated by a human derived gonadotropin (Bravelle; Ferring Pharmaceuticals Inc, Suffern, NY) to activate ovarian follicular stimulation. Ovulation was triggered when at least two follicles were 18 mm and half the remaining were ≥15 mm. Oocytes were harvested transvaginally using ultrasound guidance 35 h post hCG. All patients underwent controlled ovarian hyper-stimulation (COH) by the same physician.

Embryo culture (Performed by Dirk Kotze)

All metaphase II (MII) embryos were injected by ICSI 3–4 hours post retrieval. All zygotes were cultured individually in 50 µl droplets of P-1 medium, supplemented with 10% Synthetic Serum Substitute (SSS) (Irvine Scientific, Santa Ana, CA). After 44–46 h embryos were moved to Complete Blastocyst Medium (Irvine Scientific, Santa Ana, CA). At this point, ±35 µl from the remaining P-1 culture media drops were collected in 200 µl micro-centrifuge tubes and immediately frozen. All samples were shipped to a central location and tested for sHLA-G, using an enzyme-linked immunosorbent sandwich (ELISA) assay. All embryos were transferred on day 3.

Embryos scoring (Performed by Dirk Kotze)

The GES-score (Dirk Kotze applied GES-score to all embryos)

To apply the GES-score, all mature oocytes underwent ICSI with a single sperm and were individually cultured. In order to apply the GES-score, oocytes were evaluated at 16–18 h post ICSI when the presence of pronuclear as well as nucleoli alignment along the pronuclear axis was evident; a score of 20 was allotted where nucleoli alignment was prevalent. The second observation took place at 25–27 h, at which time early cleavage was noted. A score of 30 was allotted when cleavage was observed. Furthermore, at this time a score was given based on the presence/absence of fragmentation. When fragmentation was absent a score of 30 was given, <20% fragmentation received a score of 25 and >20% received a score of zero (0). The third and final score was given 64–67 h after ICSI and involved the number of blastomeres and embryo grade. Example: six cell grade one—6(I),7(I), 8(I), 8(II), 9(I) scored 20 points, seven cell, grade II (7(II)), 9(II), 10(I), 11(I) and compacting(I) scored ten points. The maximum GES-score totaled 100 points
(See Chapter 3 Table 1 and Chapter 3 Figure 3: GES-score illustration)

The Day 3 score (performed by Dirk Kotze)

The Day 3 score that was applied in this study was a modification Veeck's criteria for Day 3 embryos combined with our laboratory's criteria (unpublished data DK). Embryos were scored based on their blastomere number, size and symmetry as well as the percentage of fragmentation that was present. Example: a grade one embryo: 8–11 cell with even sized blastomeres (+8 points) and without fragmentation (+2 points) scored a max of ten points, a grade two embryo: 8–11 cell with even sized blastomeres (+8 points) and with <10% fragmentation (0 points) scored a max of eight points, a grade three embryo 8–11 cell (+8 points) with uneven blastomere (−1 points) and >10% fragmentation (−3 points) scored a max of four points.
The same embryologist (Dirk Kotze) preformed all embryology and embryo scoring in this study.

Soluble HLA-G assay (Shipped to SIRM Las Vegas for analysis - Dirk Kotze competant to perform sHLA-G assay)

A monoclonal antibody (mAb; MEM-G9 MCA 2044; Serotec, Raleigh, NC) against sHLA-G was used to coat a 96-well Nunc-Immunoplate (Fisher Scientific, Chino CA) using a concentration of 2 µg/ml in 0.1 mol/l carbonate buffer at pH 9.5 for 1 h at 37°C. The plate was then refrigerated overnight at 4°C. On the following day, the plate was thoroughly washed using 100 µl of phosphate-buffered saline (PBS) and 0.05% Tween-20. The wash was repeated twice using 100 µl PBS and 5% bovine serum albumin (BSA) for 15 min each. A 50 µl aliquot of PBS and 5% BSA was added to each well before adding the sample of 50 µl of embryo supernatant. Amniotic fluid (AF) was used as a positive control. AF (50 µL) and 50 µl of pure Complete Blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) Samples were incubated for a period of 1 h at 37°C. After incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin conjugated mAB (w6/32 MCA81B; Serotec) at a 1:1000 dilution in PBS and 1% BSA for 45 min at 37°C and then washed five times with PBS. Streptavidin alkaline phosphatase conjugated (BD Bioscience Pharmigen, San Diego, CA) at a concentration of 1:1000 in carbonate buffer was incubated for 30 min at 37°C and washed five times with PBS, after which phosphatase substrate was added at a concentration of 1 mg/ml in 10% diethanolamine at pH 9.8 for 30 min. The colorimetric reaction was then stopped by the addition of 50 µl of 3 mol/l NaOH. The sHLA-G concentration was determined by absorbance at 405 nm on the EL800 Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT).

Embryo selection and transfer (Dirk Kotze performed embryo selection and assisted the physician with transfer for all patients in this study)

Individual embryos were defined as having positive sHLA-G expression if their surrounding media expressed sHLA-G with an optical density inside the range of 0.184–0.196. Those outside the above mentioned range were designated as sHLA-G negative. In Group A, one hundred and seven (107) patients received embryos for transfer and all of these embryos were selected by using a Day 3 score only. In Group B, one hundred and seven (107) patients received embryos for transfer by first selecting any embryos that had a positive sHLA-G expression, and correlating such with the highest available GES score. Furthermore, in Group B the patients received embryos containing at least one sHLA-G positive in the cohort for transfer. In this study no more than four (4) embryos were recommended for transfer. However, the final decision regarding the number of embryos

for transfer was left to the patient after an informed consent that was based on American Society for Reproductive Medicine guidelines. All embryos were transferred on day 3 of culture, using ultra sound guidance and an echogenic catheter (Wallace, Smith Medical, UK). Serum β-hCG levels were measured 11 days and 13 days after the transfer (Dirk Kotze performed phlebotomy on patients and used an Immulite immunoassay analyzer to run β-hCG as well as Progesterone assays.) The patient was considered positive for biochemical pregnancy when the first value was >5.0 IU and the next value 2 days later was double the first. Clinical pregnancy rates were based on a six (6) week ultrasound with a gestational sac containing a fetal heartbeat. Ongoing pregnancy rates were based on a ten to twelve (10–12) week ultrasound with gestational sac containing one or more growing fetuses with appropriate heart rates.

Statistical analysis

Basic statistics such as the number and percentage (%) of categorical data as well as the mean and standard deviation (SD) of continuous data were calculated (by Dirk Kotze using Excel). Detailed statistical analysis was carried out using SPSS version 15 (Statistical Package for the Social Science). Comparison between mean values of continuous variables was calculated using the Students *t*-test for parametric and the Mann–Whitney-*U* test for non-parametric data, while the Chi-square was used for categorical data and the Odds ratio and 95% confidence calculated. Significance value was set at $p < 0.05$ (Performed by Dr Carl Lombard).

Miscarriage rates between treatment groups were calculated as a percentage of pregnancy loss between biochemical, clinical and ongoing rates. Fetal loss between treatment groups were calculated between clinical and ongoing pregnancies. Retrospective data were calculated as a percentage. (Calculated by Dirk Kotze using Excel)

CHAPTER 4

MATERIALS AND METHODS

Patients

Consenting ART patients between July 2003 and Dec 2010 who underwent intracytoplasmic sperm injection (ICSI), the majority used a specific ELISA test 46 hours post-ICSI to determine sHLA-G expression, however, some embryos were transferred as "untested".

The Study design (Dirk Kotze designed this multi-center study and compiled all raw data into organized tables for each site)

Data was retrospectively gathered from six fertility clinics (Los Angeles (LA), Las Vegas (LV), New Jersey (NJ), New York (NY), FISCH, and Sacramento (SAC) that performed a sHLA-G assay/test on the majority of their consenting ART patients between July 2003 and Dec 2010. Since protocols at all clinics were standardized, the procedural variabilities were limited. We retrospectively compared IVF outcome in all patients regardless of age since the goal was to compare ART outcome for sHLA-G positive, sHLA-G negative and untested cohorts. The majority of clinics transferred embryos on Day 3, however, data for embryos transferred on Day 5/6 (blastocyst) as well as single embryo transfer were also recorded.

For each group, the data consists of the number of chemical pregnancies observed, number of clinical implantations observed together with whether these were single implantations, twins, triplets or quadruplets, and the number of on-going pregnancies together with whether these were single pregnancies, twins, triplets or quadruplets. In addition, we have the mean age and standard deviation of the women in each group, and the number of embryos returned per group together with the average number of embryos returned per individual per group. We are interested in the existence of an association between the outcome of the sHLA-G test and the different pregnancy outcomes (chemical pregnancy, clinical implantation, and on-going pregnancy).

Ovarian Stimulation ***(*Performed by MD)***

Patients were stimulated using similar protocols at all sites. All patients received Lupron (TAP, Pharmaceuticals) in a long protocol after pretreatment with oral contraceptive pills for one to three weeks. Ovarian follicular development was stimulated with recombinant-FSH at doses of 225-450IU a day. Ovulation was triggered when at least 2 follicles were 18 mm and half the remainder was ≥15mm. Oocytes were recovered transvaginally under ultrasound guidance 34.5 hours later. All monitoring of controlled ovarian hyper stimulation (COH) as well as egg retrievals (ER's) and embryo transfers (ET's) were performed by the same physician at each center.

Embryo Culture **(Dirk Kotze performed all aspects of embryology involved and applied GES-score to all embryos at Sacramento site)**

All metaphase II (MII) oocytes were fertilised using ICSI 4-6 hours after retrieval. All embryos were cultured individually in 35µl droplets of P1 (Irvine Scientific) supplemented with 10% SSS (Irvine Scientific) using Nunc 60x15mm dishes, since 2007 embryos were cultured individually in 35µl droplets of GLOBAL (LifeGlobal) supplemented with 10% SSS (Irvine Scientific) using Embryo Corral (SunIVF) dishes under oil at 37°C in a 6% $C0_2$, 5% O_2, 89% N_2 environment. All embryos were sequentially microscopically evaluated over a period of 72 hours following ICSI and graded by Graduated Embryo Scoring (GES) (Table 1). Embryos were transferred into extended culture medium 44 - 46 hours post-ICSI, which can improved embryo development, as reported previously (Keskintepe). Initially individual embryos whose surrounding media expressed sHLA-G within an optic density (OD) range of 0.190 ± 0.006 (the geometric mean) were defined as having positive sHLA-G expression while those outside this range were designated as sHLA-G negative.
Each centers applied different criteria for "negative" to "positive" sHLA-G ranges, (See Chapter 4 Table 2).

The original droplets of culture medium (35µl) were collected in 0.5 ml micro-centrifuge tubes with attached cap (VWR-Scientific) – frozen immediately at -20 °C and shipped on ice for sHLA-G expression testing – to a central location- were identical sHLA-G Assay were used (west of Mississippi to Las Vegas (LV) –east of Mississippi to New York (NY) - using the same specific enzyme-linked immunosorbent (ELISA) assay. Furthermore, all embryos were graded by applying the GES – score, GES≥70 scoring embryos combined with positive sHLA-G expression were selected for transfer (Kotze *et al*., 2010).

Soluble HLA-G Assay (Sacramento is west of Mississippi we shipped to Las Vegas (LV))

sHLA-G Assay monoclonal antibody (mAb) (MEM-G9 MCA2044; Serotec, Raleigh, NC, USA) against sHLA-G was used to coat a 96-well Nunc-Immunoplate (Fisher Scientific, Chino, CA, USA) using a concentration of 2 µg/ml in 0.1 mol/l carbonate buffer pH 9.5 for 1 h at 37 °C. The plate was then refrigerated at 4 °C overnight. On the following day, the plate was thoroughly washed twice using 100 µl phosphate-buffered-saline (PBS) plus 0.05% Tween-20. The next wash was repeated twice using 100 µl of PBS+5% bovine serum albumin (BSA) for 15 min each. A 50 µl aliquot of PBS+5% BSA was added to each well prior to adding the sample of 50 µl embryo supernatant. JEG-3 cell line (which secretes HLA-G) supernatant was used as a positive control (Bamberger *et al.*, 2000). Fifty microlitres of JEG-3 supernatant and 50 µl of pure blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) were incubated for a period of 1 h at 37 °C.

Following incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin-conjugated mAb (w6/32 MCA81B; Serotec) at a 1:1000 dilution in PBS+1% BSA for 45 min at 37 °C and then washed five times with PBS. Streptavidin–alkaline phosphatase conjugate (BD Bioscience PharMigen, San Diego, CA, USA) at a concentration of 1:1000 in carbonate buffer was incubated for 30 min at 37 °C and washed five times with PBS, after which phosphatase substrate was added at a concentration of 1 mg/ml in 10% diethanolamine pH 9.8 for 30 min. The colorimetric reaction was then stopped by the addition of 50 µl of 3 mol/l NaOH. The relative concentration of sHLA-G was estimated from absorbance measured at 405 nm on an EL800-ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). A standard supernatant of sHLA-G/.221 transfectant served as reference in standard calibration curves.

Embryo transfer **(Dirk Kotze performed embryo selection and assisted the phycisian with transfer for all Sacramento patients in this multi-center study)**

Embryo transfers were performed on Day 3 (70-72 hours post ICSI) or blastocyts on Day5/6 (84-96 hours post ICSI) - depending on each center's preference. All centers used a Wallace Trail, followed by a Wallace Sure View catheter, both under direct ultrasound guidance.

Pregnancy testing

A pregnancy test was performed 10 days after embryo transfer (ET) and an ultrasound performed at 7 weeks pregnancy duration. Serum β-hCG levels were measures 11 and 13 days after the date of the egg retrieval. (Dirk Kotze performed phlebotomy on patients and used an Immulite immunoassay analyzer to perform β-hCG as well as Progesterone assays for Sacramento patients). An initial value of >5.0 IU followed by a doubling of this number was considered positive. Pregnancies were defined as chemical (β-hCG positive), clinical (6-7 week OBUS including a sac FHB)) and ongoing (10-12 week OBUS including a sac FHB)

STATISTICAL METHODS (Data was compiled by Dirk Kotze and organized into tables in Excel - detailed stats was performed by Dr Carl Lombard).

Chapter 5

MATERIALS AND METHODS

The candidate was involved in the following steps. The candidate shared PB-1, PB-2 and blastomere biopsies and vitrification of embryos with Dr Levent Keskintepe.

Patients

Oocyte donors for this study were five healthy, young (≤28 years of age), consenting patients without reproductive problems. Sperm from proven donors (with a record of successfully fathering a pregnancy) were obtained from Cryogam bank (Cryogam, CO). All donors were appropriately screened for infectious and sexually transmitted diseases and for auto-immune parameters that might adversely affect embryo implantation.

Embryo Culture: (Dirk Kotze performed with Dr Levent Keskintepe) Embryology data collection: All oocytes collected were kept in chronological sequence and were individually

cultured in 100 µl modified human tubal fluid (mHTF) + 10% HSA**,** under oil at 37 °C. All oocytes were denuded immediately after retrieval and graded (modification of Xia *et al.*, 1997) (Table 1). PB-1 was micro-surgical removed and underwent whole genome amplification (WGA), followed by complete karyotyping using metaphase comparative genome hybridization (CGH). All mature oocytes (M II) were fertilized by intracytoplasmic sperm injection (ICSI) and sequentially cultured in 50 µl droplets of Quinn's Advantage Protein Plus Cleavage medium (In-vitro fertilization, Inc., Trumbull, CT) under oil at 6% CO_2, 5% O_2 and 90% N_2, 37°C in a humidified environment. On Day 1 fertilization was assessed 16-18 hours post-ICSI, at which time alignment of the nucleoli was documented, and subsequently signs of cleavage at 26 hours post-ICSI. At this time PB-II was micro-surgically removed from normally fertilized (2PN) oocytes and underwent WGA/CGH karyotyping. On Day 2 (at 46 hours post-ICSI the embryos were moved into 50 µl droplets of Multi Blastocyst medium (Global one, IVF Online) + 10% SSS (Irvine Scientific, Santa Ana, CA). The original droplets of culture medium surrounding each individual embryo were collected and tested for sHLA-G expression, using a specific enzyme linked immunosorbent assay (ELISA). Embryos with an optical density (O/D) range of 0.190±0.006 were considered "positive' for the expression of sHLA-G. Each individually cultured embryo was evaluated at 72 hours post-ICSI – applying a graduated embryo Score (GES) described by Fisch *et al.* (2001 /2003) Then, a single mono-nucleated blastomere was micro-surgically removed from each cleaved embryo and underwent WGA/CGH karyotyping. All embryos were cultured to the blastocyst stage and graded, using a modified version of Dokras *et al.*'s (1993) procedure. (See Chapter 5 Table 3)
See Figure A –Sequence of events pictures

Blastocyst Vitrification and Warming

Vitrification: was done by modifying the method previously described by Mukaida *et al.* (2003). This modified technique involved the use of an ICSI needle as follows: About 10 minutes prior to vitrification, expanded blastocysts were placed in 50 µl drops of mHTF with 10% SSS, vol/vol (Irvine Scientific). Each blastocyst had its blastocoel artificially collapsed through assertive needle aspiration. The inner cell mass (ICM) was positioned at the 6 or 12 o'clock position using a holding pipette connected to a micromanipulator. A 30° ICSI needle was then introduced via the cellular junction of the trophectoderm into the blastocoel cavity and the fluid was aspirated until the blastocyst had completely collapsed

and no blastocoel fluid remained, (Mukaida *et al.*, (2001), (2003). Blastocysts were then placed in 0.5 ml of mHTF (Base solution; IVF Online) supplemented with 10% (vol/vol) SSS for 2) minutes at 37°C. Thereafter, blastocysts were placed in base solution + 0.96 mol/l DMSO (D 5879, Sigma, St. Louis, MO) + 1.2 mol/l ethylene glycol (EG; P 3265, Sigma) for 90 seconds. Finally, blastocysts were placed in base solution + 1.9 mol/l DMSO + 2.4 mol/l EG + 1 mol/l sucrose (S 7903, Sigma) + 0.1 mol/l Ficoll (F 8636, Sigma) for 30 seconds. Then, each blastocyst was individually placed onto a cryo-loop (Hampton Research, CA) using 1-2 µl cryo-solution and plunged into liquid nitrogen. Blastocyst grading applied: (See Chapter 4 Table 3)

Warming: The cryo-loop was removed from its vial and immersed into base solution + 0.34 mol/l sucrose for 2 minutes at 37°C. The embryo was then moved to base solution + 0.17 mol/l sucrose for 3 minutes and finally placed in base solution for 5 minutes. Intact embryos were cultured in 50 µl of Global One medium at 37°C in 6% CO_2 for a minimum of 2 hours to evaluate blastocoel re-expansion (i.e. survival).

Recipient Hormonal Treatment and Embryo Transfer: Following the onset of birth control pill-induced menstruation, oestradiol valerate IM (4-8mg) was administered every 3 days for a period of 8-12 days, until endometrial thickness had reached > 8 mm in sagital diameter and the plasma [E2] had stabilized at 1300-3000 Pmol/l. At that point, daily IM injections of 100 mg progesterone in oil (PIO) were initiated. On the 6th day of PIO, subject to patient choice and availability, two (2) thawed/warmed blastocysts were cryo-transferred, using ultrasound guidance and an echogenic catheter (Wallace, Smith Medical, UK). Serum beta human chorionic gonadotropin (β-hCG) levels were measured 8-10 days after the transfer. The patient was considered biochemically pregnant when the first value was >5.0 IU and the next value 2 days later was double the first. Clinical pregnancy rates were based on a 6-week ultrasound scan detecting a gestational sac containing a fetal heartbeat. In the event of an ultrasound-confirmed pregnancy, an estrogen/progesterone supplementation regimen was continued. Ongoing pregnancy rates were based on a 10–12-week ultrasound examination. In all cases where pregnancy did not occur or did not survive, hormonal treatment was immediately stopped.

Each blastocyst was referenced back to its oocyte, zygote and embryo of origin. All clinical procedures were performed by the same physician and laboratory procedures were performed by the same embryologist.

Stimulation (Performed by MD)

Patients received Lupron (TAP Pharmaceuticals, Lake Forest, IL) in a long protocol after pre-treatment with oral contraceptive (OC) birth control pills for 1-3 weeks and were treated with a human-derived gonadotropin (Bravelle; Ferring Pharmaceuticals Inc, Suffern, NY) in order to stimulate follicular development in the ovaries. Ovulation was triggered when at least two follicles were 18 mm and half of the remaining were 15 mm. Oocytes were retrieved transvaginally using ultrasound guidance at 35 hours post hCG.

(All the steps below for WGA/CGH were analyzed at SIRM's genetics referrence lab on site in Las Vegas)

Extraction and Amplification of Genomic DNA

Genomic DNA was obtained by laser dissection and needle aspiration from three sources: the oocyte's first polar body (PB-I), zygote's second polar body (PB-II), and subsequent day 3 embryo's blastomere. The cellular material was aspirated into a 200 µl thin-walled PCR tube (VWR catalog, 82006-602) for lyses and amplification using the Qiagen Repli-g kit (catalog, 59045). Ten ng of Repli-g control genomic DNA was used as a positive control and 0.5 µl of nuclease-free water as a negative control (Eppendorf catalog, 955155017). The reactions were set up following the Repli-g protocol and incubated for 8 hours at 30°C for amplification, followed by 10 minutes at 70°C for enzyme inactivation. Both incubation steps were performed using an Applied Biosystems 2720 Thermal cycler. Amplified DNA was placed at either 4°C for short-term storage or at -20°C for long-term storage.

Genomic DNA Confirmation and Quantification

For confirmation of WGA products, 5 µl reaction aliquots were mixed with 1 µl 6X Blue-Orange Loading Dye (Promega # G1881) and electrophoresed through a 1.0% agarose gel containing 0.1% ethidium bromide in TBE buffer (90mM Tris-HCl, 90 mM boric acid

and 2 mM EDTA, pH 8.0). For quantification of the genomic DNA, 5 µl reaction aliquots were diluted with 95 µl of molecular biology-grade, nuclease-free water and analyzed with an Eppendorf BioPhotometer.

Nick Translation of Genomic DNA

The WGA products were fluorescently labeled using the Vysis Nick Translation Kit (catalog, 32-801024). One µg genomic DNA for each sample was labeled with SpectrumGreen™ direct-labeled dUTP by nick translation, following the Vysis protocol. Translation reactions were carried out using an Applied Biosystems 2720 Thermal cycler at 15°C for 2 hours and 70°C for 10 minutes to stop the reaction. The size of the resulting SpectrumGreen™ labeled probes was assessed by electrophoresis of 5 µl aliquots of 1.0% agarose gel containing 0.1% ethidium bromide in TBE buffer (90 mM Tris-HCl, 90 mM boric acid and 2 mM EDTA, pH 8.0). Probes of usable quality were in the range of 300 - 3000 bp, as per the manufacturer's recommendation.

Probe Preparation and Comparative Genomic Hybridization

The SpectrumGreen™ labeled probes were purified by ethanol precipitation. Briefly, 10 µl/200 ng nick-translated reference DNA, 1 µl/100 ng SpectrumRed reference DNA (Vysis # 32-80423 or 32-804024), and 10 µl/ 10 µg Cot-1 DNA (Vysis # 32-800028) used to suppress repetitive sequences and prevent non-specific hybridization, were added to a 1.5 ml micro-centrifuge tube.

For precipitation of the hybridization mix, 2.1 µl (0.1 volume) 3M sodium acetate, followed by 52.5 µl (2.5 volumes) of 100% EtOH was added to the DNA, vortexed briefly and placed on dry ice for 15 minutes, then centrifuged at 12 000 rpm for 30 minutes at 4°C to pellet the DNA. The supernatant was removed and the pellet dried for 10–15 minutes under a vacuum at ambient temperature. The pellet was resuspended in 3 µl molecular biology grade water and 7 µl CGH hybridization buffer (Vysis # 30670003) and placed in the dark during slide preparation. CGH-normal metaphase slides (Vysis # 30-806010) were prepared following the manufacturer's protocol. Briefly, the slides were denatured in 70% formamide, 10% SSC pH 5.3, and 20% molecular biology grade water for 5 minutes at 73°C for 5 minutes followed by an ethanol dehydration series of a minute each (70%, 85%,

and 100%). The slides were dried by touching the bottom edge to a blotter and wiping the underside with a paper towel, then placed on a 45–50°C slide warmer to allow remaining EtOH to evaporate. The probe mix was denatured by heating for 5 minutes in a 73°C water bath. Ten µl of the probe mix was applied to the slides. The coverslip was placed over the slide and sealed with diluted rubber cement. The hybridizations were carried out with Vysis Hybrite chambers for a minimum of 48 hours and a maximum hybridization time of 72 hours to clear background. Following hybridization, the cover slips were carefully removed along with any remaining rubber cement residue, taking care not to damage the slide surface. The slides were then agitated in 0.4X SSC/0.3% NP-40 wash solution at 74+1°C for 1-3 seconds, then let stand for 2 minutes, then agitated in 2X SSC/0.1% NP-40 at ambient temperature for 1-3 seconds, then let stand for 1 minute. The slides were allowed to dry vertically at ambient temperature in the dark. Once completely dry, 10 µl DAPI II counter stain (Vysis 30804931) was added to each side, followed by a cover slip, and immediately sealed with clear non-fluorescing fingernail polish. Keskintepe *et al.*, (2007) collected and validated results from first, second PB's and two blastomeres and reported a positive/negative occurrence of less that 10%.

Image Capture and Analysis

The following optical filters visualized the fluorochromes used in the hybridization: a filter set specific for DAPI, Texas Red® (Cat. No. 30-150491) and FITC (fluorescein isothiocyanate) (Cat. No. 30-150291) to view the counter stain, SpectrumRed or SpectrumGreen DNA, respectively, a triple band pass filter set designed to simultaneously excite and emit light specific for DAPI.

Soluble HLA-G Assay (Performed at SIRM Las Vegas for analysis)

A monoclonal antibody (mAb; MEM-G9 MCA 2044; Serotec, Raleigh, NC) against sHLA-G was used to coat a 96-well Nunc-Immunoplate (Fisher Scientific, Chino CA) using a concentration of 2 µg/ml in 0.1 mol/l carbonate buffer at pH 9.5 for 1 hour at 37ºC. The plate was then refrigerated overnight at 4ºC. On the following day, the plate was thoroughly washed using 100 µl of phosphate-buffered saline (PBS) and 0.05% Tween 20. The wash was repeated twice using 100 µl PBS and 5% bovine serum albumin (BSA) for 15 minutes each. A 50 µl aliquot of PBS and 5% BSA was added to each well before

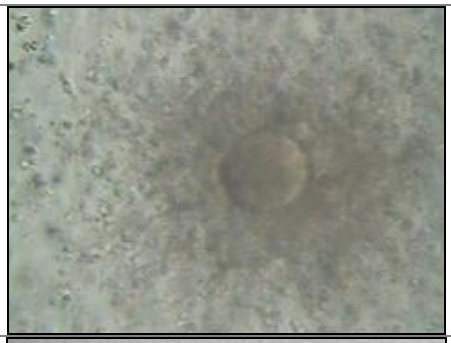
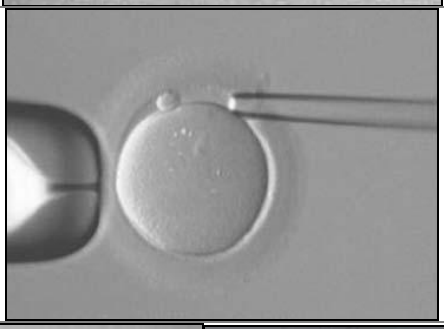
adding the sample of 50 µl of embryo supernatant. Amniotic fluid (AF) was used as a positive control. AF (50 µl) and 50 µl of pure blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) were incubated for a period of 1 hour at 37ºC. After incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin conjugated mAB (w6/32 MCA81B; Serotec) at a 1:1000 dilution in PBS and 1% BSA for 45 minutes at 37ºC and then washed five times with PBS. Streptavidin alkaline phosphatase conjugated (BD Bioscience Pharmigen, San Diego, CA) at a concentration of 1:1000 in carbonate buffer was incubated for 30 minutes at 37ºC and washed five times with PBS, after which phosphatase substrate was added at a concentration of 1 mg/ml in 10% diethanolamine at pH 9.8 for 30 minutes. The colorimetric reaction was then stopped by the addition of 50 µl of 3 mol/l NaOH. The sHLA-G concentration was determined by absorbance at 405 nm on the EL800 Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT).

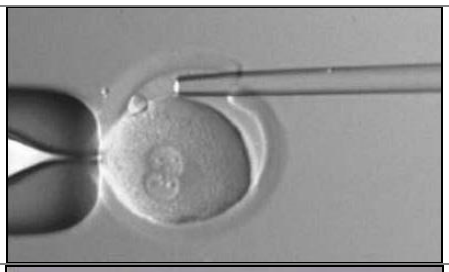
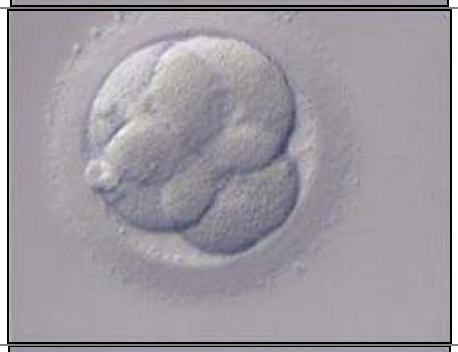
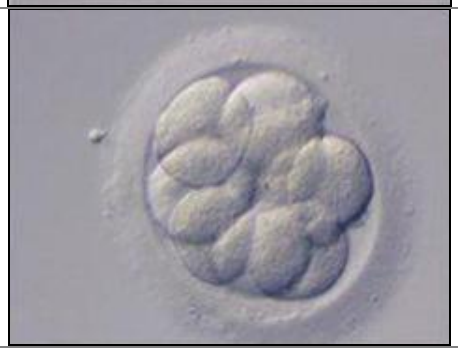
Statistical analysis (Performed by Dr Carl Lombard)

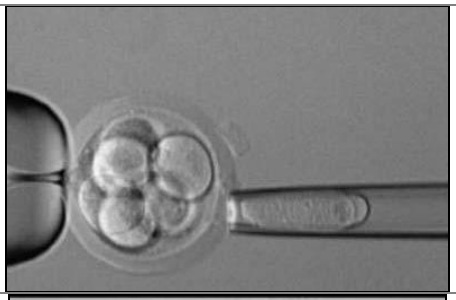
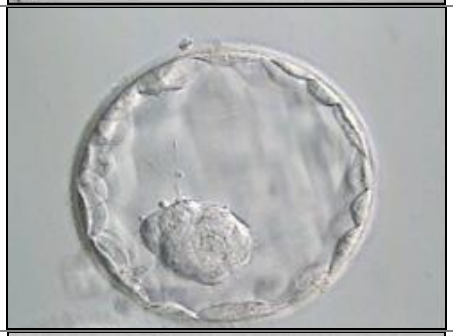
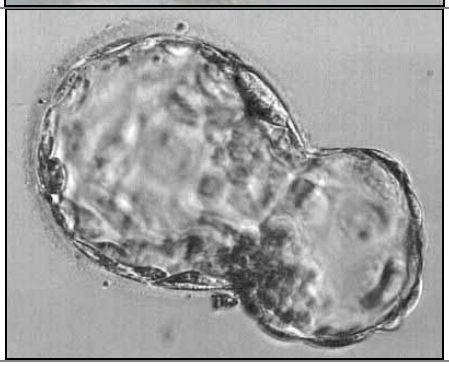
Fisher's exact test was used to test for association between two categorical studied variables. The small marginal totals for some levels of the categories variables necessitate the use of this test. The agreement between the PB-I and PB-II and blastocyst was evaluated through the kappa statistic. The median levels of expression of sHLA-G for the categories of oocyte grade, PB-I, PB-II and blastocyst grade were calculated together with the interquartile range (IQR). To compare the median levels of sHLA-G between the levels of these variables two statistical methods were used. Since oocyte grade has a strict ordinal structure the estimation and testing of the Spearman correlation coefficient between sHLA-G and oocyte grade is an optimal test for the hypothesis of no difference in median sHLA-G levels across the categories of oocyte grade. For the remaining variables a Kruskal-Wallis test (non-parametric analysis of variance) was performed for the comparison of the median levels of soluble sHLA-G.

Figure A: Sequence of events

Picture presentation of procedures that occurred during thesis data gathering

1) Egg Retrieval (COC)	
2) Denuding COC in hyalironidase to determine maturity (MII oocyte)	
3) PB-1 biopsy	
4) ICSI	

5) Fert Check and PB-2 biopsy (2PN-zygote)		
6) Early Cleavage (2-cell)		
7) Day-2 (4-cell)		
8) Day-3 (8-cell)		

9)	Day-3 blastomere biopsy	
10)	Day 5 (Early Blastocyst)	
11)	Day 5 (Expanded Blastocyst)	
12)	Day 5/6 (Hatching Blastocyst)	

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CHAPTER 2

CHAPTER 2

GAMETE FACTORS AFFECTING EMBRYO QUALITY AND PREGNANCY OUTCOME IN ASSISTED REPRODUCTION TECHNOLOGY (ART)

ABSTRACT

In this chapter the impact of the oocyte/zygote and the embryo on implantation/pregnancy rate was discussed. The morphologic characteristics of the oocyte, the cumulus–oocyte-complex (COC), the zona pellucida, the perivitelline space, cytoplasm and meiotic spindle and the polar body and its appearance were discussed in detail. The morphologic characteristics of the embryo-fragmentation and its effect on embryo development, ploidy and subsequent blastocyst formation were also studied. Embryo markers to predict pregnancy outcome were researched based on the international literature. The pronuclear morphology and early cleavage were highlighted as non-invasive embryo markers to predict outcome.

A non-invasive biochemical marker, soluble Human Leucocyte-Antigen-G (sHLA-G), that is expressed by developing embryos was researched. The value of blastocyst transfer and the improved ongoing pregnancy rate compared to cleavage stage embryos were highlighted based on a recent meta-analysis. A detailed discussion on sHLA-G as well as Array-CGH and the future of these tests followed.

INTRODUCTION

In vitro fertilization (IVF) was initially performed during natural fertility cycles, and involved removal of a single oocyte from its natural environment. In 1978, a mature oocyte was recovered through the use of laparoscopy, fertilized outside the body, and the cleaved embryo was transferred back into the patient's uterus. This resulted in the first successful IVF-conceived pregnancy by Steptoe & Edwards 1978.

Over the last three decades, major adjustments to this basic theme have been introduced with the goal of increasing the efficiency and the success rates. One addition was the introduction of COH (Controlled Ovarian Hyper-stimulation) by Sher *et al.* (1987). The goal of COH was to increase the number of mature oocytes available for fertilization. In 1988 Lazendorf *et al.*, were the first to describe successful male and female pro-nuclear development following intracytoplasmic sperm injection (ICSI). In 1992 Palermo *et al.*, researchers at the Free University of Brussels refined micromanipulation techniques, protocols and procedures and reported successful oocyte fertilization, embryo transfer (ET), and established pregnancies following ICSI. Since then there have been numerous modifications of ICSI procedures as well as expanded indications for its applications.

There has been growing interest in strategies to reduce the multiple birthrates without compromising the success of IVF pregnancies. Most approaches aimed at identifying the "best" embryos for transfer have focused on: (i) morphological assessment of embryos prior to transfer (Ng *et al.*, 1999) and (ii) prolonged embryo culture for 5-6 days post-fertilization, with the goal of then transferring only embryos that have reached the blastocyst stage of development. Blastocysts have improved implantation potential (Gardner *et al.,* 1998; Schoolcraft, 1999), but require specialized culture systems. During extended embryo culture *in vitro* – from the cleavage stage to blastocyst stage, some embryos with inferior developmental potential did not progress into blastocysts. In order to select competent embryo(s) for transfer strict morphologic grading/evaluation criteria for day 3 embryos were established by Van Royen *et al.,* (1999a) and for blastocysts by Gardner *et al.* (2000a). Both groups stated that these criteria were central to the attainment of optimal implantation and pregnancy rates on Days 3 and 5, respectively. Although morphological evaluations apparently furnish clues that enhance proficiency in selecting embryos for transfer, these systems are severely limited in their ability to provide reliable evidence for subsequent implantation and normal development.

Karyotypic studies of aborted fetuses have revealed that the proportion of concepti with chromosome abnormalities is very high. Early studies using pre-implantation genetic screening with fluorescent in situ hybridization (PGS FISH) have demonstrated that ≥50% of embryos considered for transfer with IVF are in fact aneuploid (Munne *et al.*, 1993). FISH has been the most widely used method for analyzing the polar body and blastomere chromosomal complements. A primary limitation of this technique is the limited number of chromosomes able to be tested. Wells *et al.* (2002) reported the first use of comparative genome hybridization (CGH) on polar bodies and reported an improved IVF outcome when transferring embryos with a completely normal genome. Since then several researchers have reported application of CGH techniques (Le Caignec *et al.*, 2006; Sher *et al.*, 2007; Treff *et al.,* 2010), as genetic screening tools on human embryos.

An additional technique for assessing embryo competence is to look for markers of healthy metabolism. For instance, researchers reported the detection of soluble human leukocyte antigen (sHLA), a non-classical major histocompatibility complex (MHC), in culture medium surrounding clusters of developing embryos (Jurisicova *et al.*, 1996a/b; Menicucci *et al.*, 1999), provided clues for better quality embryos. sHLA may be a biochemical marker to assist in the identification of embryos with the highest implantation potential for transfer. The presence of sHLA-G improved pregnancy (Noci *et al.*, 2005), implantation potential (Fuzzi *et al.*, 2002), embryo selection and pregnancy outcome (Rebmann *et al.*, 2007). Despite reports for its positive influence as far as ART outcome, there have also been questions regarding sHLA-G delectability and true value (Sageshima *et al.*, 2007; Sargent *et al.*, 2007; Vercammen *et al.*, 2008).

Despite the above-mentioned “improvements”, less than one in four women under the age of 40 achieve a live birth following IVF treatment, and approximately 5% of fresh oocytes collected produce a viable baby. Furthermore, birthrates vary from single digits to over 50% depending on the number of embryos transferred. Over the last decade, the reported success rate with IVF has not significantly changed. Against this background, we conduct a literature review of relevant studies performed, along with their comments regarding identifying embryo markers which can be utilized to improve overall ART outcome (SART data, 2010).

CUMULUS-OOCYTE-COMPLEX (COC)

The background information was based on non-human and human studies. Prior to ovulation oocytes are surrounded with tightly layered cumulus granulose cells. These cells are interconnected to each other and the oocyte by a complex membrane-cellular process and gap junctions (Albertini et al., 1994). Cumulus cells are dependent on this process to provide a continuous supply of glucose and pyruvate as energy source (Gardner *et al.*, 1996; Preis *et al.*, 2005). Cumulus cells have many FSH-receptors (Robker & Richards, 1998) and are exposed to growth factors expressed by the oocyte itself (Erickson & Shimasaki, 2000). Gregory *et al.* (1998) reported a correlation between proliferation of cumulus cells and implantation potential. The combined presence of both growth factors (epidermal growth factor [EGF] and insulin-like growth factor I [IGF-I]) resulted in maximum rates of cumulus expansion and nuclear maturation (Lorenzo *et al.*, 1994).

The luteinizing hormone (LH) - surge causes the cumulus layers to expand and plays an important role in final maturation and subsequent fertilization (Somfai *et al.*, 2004; Yang *et al.*, 2005 [human]). Rattannachaiyanont *et al.* (1999) reported no correlation between COC-morphology and fertilization, cleavage and pregnancy rates. Ebner *et al., (*2008) drew the same conclusion; furthermore, decreased maturation was reported when oocytes were surrounded by dense layers of corona radiate cells. Applying their criteria to evaluate COC quality, Ng *et al.* (1999) reported an association between COC quality and both fertilization and pregnancy. The cumulus matrix and cumulus cells also participate in fertilization by influencing spermatozoa binding and penetration of the COC in humans (Chung *et al.*, 2009; Hong *et al.*, 2009). A study by Lin *et al.,* (2003) using a 5-scale cumulus-corona-radiate cell evaluation found a correlation between embryo development potential and blastocyst quality. In a review study about the mechanism involved during ovulation by Russel & Robker (2007), they concluded that cumulus cells surrounding oocytes in the follicle play a very important part in an oocyte's development in vitro, maturation and ovulation. Cumulus expansion is a direct result of endocrine exposure and growth factor released by the oocyte to the surrounding cumulus layers. These factors synthesized, processed and secreted by oocytes within the cumulus impacts the developmental potential of zygotes (Hussein *et al.*, 2006). Finally they hypothesized that these factors facilitate detachment and expulsion of the COC mass from the follicle.

ZONA PELLUCIDA

The oocytes and embryos of all mammals are surrounded by a “protective barrier” of glycol-proteins, known as the zona pellucida (Herrler *et al.*, 2000; Sinowatz et al., 2001; Lefievre *et al.*, 2004). These proteins are expressed by the oocyte as well as the granulose cells and might play an important role in granulose cell differentiation and folliculogenesis. Prasad *et al.*, (2000) described the structure and function of mammalian zona proteins. These proteins might play an important role during the fertilization process: attaching and binding of capacitated sperm, inducing the acrosome reaction (Ganguly *et al.*, 2010); penetration of zona pellucida (ZP) (Gardner & Evans, 2006); and a mechanism to prevent polyspermia (Soupart & Strong, 1975). During folliculogenesis, both the oocyte and the granulose cells contribute to the protein expression (Sinowatz *et al.*, 2001). The ultra-structure of the zona has also been described (Wassarman *et al.*, 1999; Oehninger *et al.*, 2003; Wassarman *et al.,* 2004). Using conventional scanning electron microscopy (SEM) to determine the structure of the ZP of in vitro mature (MII) human oocytes revealed a large spongy multilayered network; however, in immature (germinal vesicle (GV) and atretic oocytes the surface of the ZP was compact and smooth (Familiari *et al.,* 2008). Continued communication between the developing oocyte and the surrounding environment is essential (Albertini *et al.*, 1994) and should this communication be disrupted between the oocyte and its surrounding cells – ZP – essential messages will not reach the oocyte and could jeopardize meiotic arrest, oocyte maturation and subsequent embryo development (Rankin *et al.*, 1999).

Methods to assess the morphology of the zona have been described – video-cinematography with Hoffman Modulation optics (Cohen *et al.*, 1989), digital imaging system (Wright *et al.*, 1990), PolScope real time (Shen *et al.*, 2005) and SEM (Familiari *et al.*, 2008).

Characteristics

The following aspects of the ZP will be discussed:

- Zona thickness
- Birefringence
- Zona darkness and thickness

- *Zona thickness*: as early as 22 years ago Cohen *et al.* 1989 reported areas of "thinning" in the zona of cleaved embryos. Wright *et al.* (1990) associated thinning with implantation and pregnancy potential. Bertrand *et al.* (1995) concluded that ZP thickness influences sperm penetration, even when the spermatozoa are considered normal. ZP thickness appears to be an additional factor that should be taken into account when interpreting the fertilization rate and could be an indicator for the use of micro-injection procedure (ICSI). The morphological appearance of the ZP could function as an indicator of folliculogenesis and subsequent oocyte maturation. A correlation was found between overall ZP thickness and its variation during pre-implantation development and pregnancy outcome, (Cohen *et al.*, 1989; Palmstiema *et al.*, 1998; and Gabrielsen *et al.*, 2001). The inner zona layer can be evaluated by either the radial orientation of glycoproteins and its angular deviation (Frattarelli *et al.*, 2007) or the birefringence of the inner layer (Montag *et al.*, 2007; Montag *et al.,* 2008). Light retardation of the ZP, in particular of the inner layer, was correlated with conception by Shen *et al.,* (2005). Furthermore, Rama Raju *et al.,* (2007) also found an association between the retardance of the inner layer of the zona and blastocyst development. Montag *et al., (*2007, 2008) - where German strict IVF regulations apply - used zona imaging on mature (MII) oocytes as primary selection criteria for ET, they concluded that ZP features are a powerful predictor of pregnancy outcome.

  Changes in zona thickness correlated with the number of blastomeres, grade, % fragmentation, patient age and were more evident in embryos transferred from cycles resulting in successful pregnancies (Garside *et al.*, 1997). Rankin *et al., (*2000) concluded that the ZP itself plays an important role in vivo during fertilization and implantation. Thickness and organization of ZPs of human eggs and embryos varies considerably and can be quantitatively imaged with the PolScope (Pelletier *et al.*, 2004). Zona thickness could be used to predict implantation potential (Cohen *et al.*, 1989). Bertrand *et al.* (1995) reported higher fertilization rates in oocytes surrounded by "thinner" ZPs. On the other hand, Host *et al.* (2002) reported improved blastocyst formation and improved embryo quality when the inner zona layer was "thicker". Rama Raja *et al., (*2007) reported that an increased thickness of the inner layer of the zona correlated with increased blastocyst formation. Shen *et al.*, (2005) reported a correlation between increased zona layer thickness and improved embryo development and clinical pregnancy rates. No correlation was

found between zona thickness and fertilization, pronuclear morphology, embryo development and clinical pregnancies (Esfandiari *et al.*, 2006). Blastocyst formation is associated with thinning ZP (Garside *et al.*, 1997; Check *et al.*, 1998; Mantoudis *et al.*, 2001; Balaban *et al.*, 2000). Zona thickness was associated with embryo quality, embryo development and pregnancy rates by Gabrielson *et al.* (2000). Other studies, however (Garside *et al.,* 1997; Palmstierna *et al.*, 1998; Gabrielson *et al.*, 2001; Host *et al.*, 2002), reported that ZP thickness was not associated with improved pregnancy rates. Kultu *et al.* (2010) concluded that laser-assisted zona thinning of Day 3 embryos has no beneficial effect on clinical pregnancy and implantation outcomes. Furthermore, Cohen (2010) commented on the detrimental effect of artificially thinning of the ZP by inexperienced technicians or the "over exposure" to laser energy during assisted hatching.

- *Zona birefringence*: the birefringence of the inner zona layer could affect the fertilization rate and embryo development (Shen *et al.*, 2005; Rama Raju *et al.*, 2007; Montag *et al.*, 2008). In contrast to the above-mentioned, Madaschi *et al.* 2009 reported no correlation between variations in birefringence of the zona's inner layer on fertilization rates and embryo quality. However, when oocytes with high birefringence were used, clinical pregnancy rates were improved (Shen *et al.*, 2005; Rama Raju *et al.*, 2007; Montag *et al.*, 2008; Madaschi *et al.*, 2009), whereas oocytes with a low birefringence were associated with increased miscarriage rates (Madaschi *et al.*, 2009). Loutradis *et al.* (1999) considered multiple morphological features of the zona and their subsequent effect on oocyte development and concluded that only dramatic damage to the zona (broken or empty) has an effect on ICSI outcome.

  The inner zona layer (using a birefringence score) was a strong predictor of blastocyst formation but not of embryo quality or pregnancy (Ebner *et al.*, 2010). In another study De Almeida Ferreira Braga *et al.*, (2010) suggested that ZP birefringence (ZPB) could be a useful tool to predict embryo development for MII oocytes, but did not report any association between ZPB and oocyte maturity. Using polscopy – the ZP characteristics can be used as a positive predictor of embryonic development in vitro (Rama Raju *et al.*, 2007). In contrast, Cheng *et al.* (2010) reported no differences in ZPB scores between normally fertilized (2PN) and abnormally fertilized (3PN) oocytes with conventional IVF. Furthermore, Day 3

embryo quality and clinical pregnancy outcome could not be predicted by ZPB-scoring.

- *Zona darkness and thickness*: there was no association between zona darkness and fertilization, pronuclear morphology, embryo development and clinical pregnancies (De Sutter *et al.*, 1996; Balaban *et al.*, 1998; Ten *et al.*, 2007). Rienzi *et al.*, (2008) reported no correlation between both zona darkness and thickness, subsequent fertilization, pronuclear morphology, embryo development and clinical pregnancy rates.

PERIVITELLINE SPACE

Background

The perivitelline space (PV-space) is the space between the inner layer of the ZP and the cell membrane of an oocyte (oolemma). The PV-space of unfertilized human oocytes contains a hyaluronan-rich extracellular matrix. Granules and filaments are present and appear similar to the matrix between cumulus and corona radiate cells (Dandekar *et al.*, 1992). The PV-space is relevant because it is where the polar body is released after meiosis.

Post fertilization cortical granules released from the ovum are deposited in the PV-space to form a cortical granule envelope (containing proteins). Polysaccharides released from the granules cause the space to swell, pushing the ZP farther away from the oolemma. This envelope could play a role in the polyspermic block (Talbot *et al.,* 2003). In a study by Ueno *et al.*, (2008) the PV-space size in mice was significantly greater for in vivo matured oocytes vs in vitro matured oocytes; however, no difference in fertilization rates was reported between the two groups. Furthermore, the incidence of polyspermy for in vivo matured oocytes was significantly lower than for those matured in vitro. These findings suggest that the PV-space might be related to the increased incidence of polyspermia in mouse oocytes.

In a study by Danfour *et al.*, (2010), evaluating the behaviour of the oolemma during ICSI identified preimplantation embryos with good potential to form blastocysts, and consequently on implant result in a pregnancy. Hassan *et al.*, (1998) and Ten *et al.,*

(2007) reported no correlation between debris present inside the PV-space and both in vivo and in vitro embryo development. In contrast Farhi *et al.*, (2002) reported that the presence of coarse granules in the PV-space was associated with lower implantation and pregnancy rates. The size of the PV-space and presence of cortical granules was associated with embryo quality, but not with implantation and pregnancy rates (Chamayou *et al.*, 2006). Ten *et al.*, (2007) associated embryo quality with an increased PV-space. However, no correlation was reported between increased PV-space and subsequent embryo development by De Sutter *et al.*, (1996) and Balaban *et al.,* (1998 and 2008). Large PV-space correlated with low fertilization, but had no effect on further embryo development (Rienzi *et al.*, 2008).

POLAR BODIES

Background

Early oocytes are also classified as immature (GV or metaphase I (MI) stage). The breakdown of the GV initiated by human chorionic gonadotropin (hCG) in vitro promotes the resumption of meiosis 1. At this time the oocyte (diploid cell) divides into two haploid (23 chromosomes) daughter cells in which each chromosome contains two chromatids. One daughter cell becomes the secondary oocyte, the other cell forms the first polar body (*PB-I*), contains the excess DNA from the reductive division and is an indication that meiosis 1 has been completed successfully.

The secondary oocyte then activates meiosis 2 resulting in metaphase II (MII) arrest which will not continue until fertilization occurs. At this time meiosis 2 will be completed, extruding the second polar body (*PB-II*).

Morphology/shape/appearance:

Xia (1997), reported a significant correlation between normal PB-I morphology and fertilization rate as well as embryo quality after ICSI. In a study by Younis *et al.*, (2009) oocytes with normal round intact polar bodies were compared with abnormal fragmented polar bodies. They concluded that the morphology was related to oocyte maturity and significantly correlated to clinical implantation and pregnancy rates in ICSI cycles. Using morphologic features such as fragmented, intact, rough-surfaced and enlarged PB-I, Ebner *et al.*, (2000) reported a strong correlation between these criteria and fertilization

rates as well as embryo quality. In contrast, Ten *et al.*, (2007) concluded that PB-I morphology shape (intact/fragmented or irregular) did not affect fertilization rates and embryo quality. Investigating PB-I size, fragmentation and surface appearance, Chamayou *et al.*, (2006) found no influence on implantation and pregnancy rates, but reported a “slight” influence on embryo quality. Studies involving ICSI looked at polar body "quality" and concluded that *PB-I* morphology is related to mature oocyte viability and has the potential to predict oocyte fertilization, implantation rates and pregnancy potential (Younis *et al.*, 2009; Ebner *et al.*, 2000). Verlinsky *et al.*, (2003) found no relation between fragmented or uneven-shaped *PB-I*s and embryo quality, blastocyst developmement, implantation rates and aneuploidies. Ciotti *et al.*, (2004) used similar criteria but added the size of the polar body and reported no effect on fertilization, embryo cleavage, embryo quality, implantation rates and pregnancy rates. Fertilization rates, embryo quality and subsequent blastocyst formation were not associated with PB-I fragmentation and surface appearance (De Santis *et al.*, 2005). Large and degenerated polar bodies were associated with decreased fertilization rates or embryo quality; however, fragmentation had no influence any of these outcomes (Rienzi *et al.*, 2008). In a contrasting study by Fancsovits *et al.*, (2006) they reported that degeneration/fragmentation of the *PB-I* was related to improved fertilization and improved embryo quality (less fragmentation). However, PB-I with increased size was associated with lower fertilization and poorer embryo quality.

Oocytes were thus classified using characteristics for extracytoplasmic abnormalities or cytoplasmic abnormalities.

- *Extracytoplasmic abnormalities included***:** ZP abnormalities (thick and/or dark), abnormal oocyte shape (oval oocytes), size of PV-space, *PB-I* normal (intact) or abnormal (fragmented, large or degenerative), as mentioned above.

- *Cytoplasmic abnormalities included***:** granular cytoplasm, centrally located granular area, vacuoles, smooth endoplasmatic reticulum (SER) clusters, and refractile bodies. A “normal good quality” metaphase II oocyte has clear, moderately granular cytoplasm, a relatively small PV-space, surrounded by a clear/colourless ZP (Veeck, 1988). Morphological variations most frequently observed in oocytes are intra-cytoplasmic and include changes in colour, granularity and homogeneity of the cytoplasm, and cytoplasmic inclusions. Variations from normal PV-space, ZP colour and oocyte shape were considered extra-cytoplasmic (Van Blerkom, 1990). Oocyte

grading based on *PB-I*, size of PV-space and cytoplasmic inclusions had a significant effect on fertilization rate and embryo quality after ICSI (Xia *et al.*, 1997). In a study by Balaban *et al.*, (1998), when patients underwent ICSI, abnormal oocyte morphology was not associated with decreased fertilization rate or poor embryo quality. However, clinical pregnancy and implantation rates when transferred were similar between embryos derived from abnormal versus normal oocytes. Oocytes undergoing in vitro maturation (IVM) without anomalies produced improved quality embryos and cleavage rates, compared to those with anomalies (Mikkelson & Lindenberg, 2001).

Based on the above-mentioned studies regarding oocyte morphology, only severe deviations from cytoplasmic normality should be considered as abnormal and should be taken into consideration for the selection of the viable oocyte that would result in an embryo with a higher implantation potential (Balaban, 2006). However, whether these abnormalities could be phenotypical or genotypical cannot be predicted without sufficient genetic screening of the whole embryo's genome.

Ovally shaped ooplasma and/or ZP were associated with delayed developmental progress in vitro. The higher the degree of dysmorphism, the more likely the chance was that the cleaving pattern of the corresponding zygote was not a tetrahedron. Consequently, this abnormal cleavage was associated with delayed compaction and blastocyst formation, but subsequent blastocyst quality was not affected (Ebner *et al.*, 2008).

- *Cytoplasmic infarctions*: Rienzi *et al.*, (2011) published an excellent review in which they defined cytoplasmic inclusions (refractile bodies, dark incorporations, fragments, spots, dense granules, lipid drops, lipofusion and vacuoles (saccules, smooth endoplasmic reticulum clusters) and their subsequent effect on ART outcome. Studies by De Sutter *et al.*, (1996) and Balaban *et al.*, (1998) reported no correlation between cyotoplasmic inclusions and fertilization, implantation and embryo quality rates. However, Xia *et al.*,(1997) and Otsuki *et al.,* (2007) reported a decrease in fertilization rate and subsequent embryo development. Furthermore, the presence of vacuoles in an oocyte's cytoplasm had a negative effect on embryo development potential (Balaban *et al.*, 2008), but did not have an effect on fertilization rates and embryo quality (Ten *et al.,* 2007). Embryos transferred from vacuolated oocytes resulted in increased biochemical pregnancy rates, but were

followed by a decrease in clinical pregnancy rates (Otsuki *et al.* 2004). It can thus be concluded that such embryos contribute to an increase in early pregnancy loss. Loutradis *et al.* 1999 reported decreased pregnancy rates when both inclusions and vacuoles were present in the original oocytes.

Meiotic spindle

Definition: Birefringence has been mentioned earlier and was associated with the presence of the meiotic spindle as reported in studies by Wang *et al.,* (2001), Moon *et al.,* (2003), Cohen *et al.,* (2004), Konc *et al.,* (2004), Fang *et al.,* (2007), Rama Raju *et al.,* (2007), Braga *et al.,* (2008) and Madashi *et al.,* (2008). Furthermore, the presence of the meiotic spindle was associated with improved fertilization rates. In studies by Moon *et al.,* (2003), Rama Raju *et al.,* (2007) and Madashi *et al.,* (2008) a positive correlation was reported between the presence of the meiotic spindle and early embryo development. However, these findings were contradicted by Cohen at al., (2004) who reported no association. Higher pregnancy rates were reported when embryos originated from oocytes when the spindle was present (Konc *et al.*, 2004; Madashi *et al.*, 2008; Madashi *et al.*, 2009). If the spindle was positioned close to the polar body, an improved fertilization and embryo cleavage rate was reported by Fang *et al.*, (2007). Furthermore, improved early embryo development and subsequent embryo development was associated with spindle position by Cooke at al., (2003). In contrast, Moon *et al.*, (2003) reported no association between spindle position and fertilization and embryo cleavage rates or embryo quality. Rienzi *et al.*, (2003) associated abnormal fertilization with “misalignment” between the meiotic spindle and polar body. However, embryo development was not affected if the fertilization was “normal” despite misalignment. PolScope imaging of oocyte spindle retardance is an optical property of organized macromolecular structures that can be observed in living cells without fixation or staining. The meiotic spindles during the initial stages of oocyte activation showed a dynamic increase in meiotic spindle retardance, particularly of the mid-region, before spindle rotation and *PB-II* extrusion. The pronounced increase in spindle retardance was quantified for the first time in living oocytes by Liu *et al.,* (2000). Furthemore, post *PB-II* extrusion and spindle retardance of fertilized oocytes (zygotes) were significantly higher than that of MII oocytes. These results establish that increased spindle retardance precedes polar body extrusion and pronuclear formation. Therefore, increased birefringence of the spindle provides an early indicator of oocyte activation. This noninvasive quantitative imaging of oocytes might provide information

regarding an oocytes/zygotes developmental potential in vitro. Chen *et al.*, (2006) associated higher retardance with a higher pregnancy rate and Kilani *et al., (*2009) found a strong relationship when the meiotic spindle was “normal” (complete barrel-shaped with strong birefringence) and retardance with subsequent pregnancy rates. Rama Raju *et al.,* (2007) reported improved blastocyst formation with higher retardence. However, De Santis *et al.*, (2006) found no correlation between spindle retardance and embryo quality.

PRONUCLEAR MORPHOLOGY

In a study by Lundqvist *et al.*, (2001) zygotes were evaluated for alignment and polarization of nucleolar precursor bodies (NPB) as well as early cleavage and their effect on implantation and pregnancy rates were reported. They found pregnancy rates were significantly higher when transferring one or two embryos when polarization and/or early cleavage occurred. They concluded that a cohort of morphologically good embryos, assessed for alignment/polarization of NPB and/or early cleavage, together with conventional morphological criteria, were produced, and suggested this noninvasive method for identifying embryos with high implantation potential. Scott (2003a/b) reported that the pattern of the NPB or Z-score and the presence/absence of a halo had a significant effect on the rate of embryo development as well as overall embryo morphology score. Poor Z-score resulted in delayed development, poor blastocyst development and low subsequent morphology scores. Furthermore, the absence of a halo also resulted in delayed and poor development, resulting in poor morphology, increased fragmentation and increased numbers of poor Z-scored embryos. They concluded that using the pronuclear (PN) scoring to predict embryos with poor developmental potential might also be a tool by which to evaluate oocyte health. Chen *et al.,* (2003) reported that a high probability of subsequent normal diploid embryos resulted when good zygote pronuclear morphology (Z1) was used as a selection criterion.

In a study by Balaban *et al.*, (2004) (applying Tesarik and Greco’s PN score), they reported that both embryo cleavage characteristics and chromosome constitution were related to PN morphology. Embryos originating from zygotes with the normal PN pattern (pattern 0) cleaved faster and developed into embryos with better morphology compared to zygotes with abnormal PN patterns. Furthermore, aneuploidy rates of embryos derived from zygotes with the normal PN pattern were more than three times less compared to zygotes with multiple PN anomalies. Lastly, embryos of normal PN pattern progressed to the blastocyst stage twice as rapidly as embryos with multiple PN anomalies. The same

relationship applied to chromosomally abnormal embryos. They concluded that PN morphology predicts both the risk of embryo developmental arrest and that of chromosomal abnormalities. Payne *et al.*, (2005), using the Z-score, concluded that Z1 and Z3 scoring embryos resulted in similar morphology and pregnancy rates. Furthermore, decisions based on the Z-score did not improve embryo morphology and did not predict pregnancy outcome. Borges *et al.*, (2005) found that about half fertilized oocytes resulted in zygotes with good pronuclear-nuleoli morphology, which mostly developed into good-quality Day 3 embryos. Furthermore, when good-quality Day 3 embryos (originating from good pronuclear-nucleoli zygotes), underwent biopsy and pre-implantation genetic screening (PGS) lower chromosomal abnormalities were reported. They concluded that zygote pronuclear-nucleoli morphology seems to be correlated with PGD results and therefore could be a useful embryo selection criterion for patients who refuse PGD testing.

Chen *et al.*, (2006) reported no significant difference between PN morphology and early cleavage rate in terms of implantation and pregnancy outcome. However, they concluded that PN morphology is a better criterion than early cleavage to assist in embryo selection on Day 3. Their theory is that zygotes with early cleavage ability can be identified from their PN morphology and therefore observation for early cleavage at 24-26 hours post fertilization in addition to PN morphology scoring is unnecessary when selecting embryos on day 3 for transfer. Shen *et al.*, (2006) used noninvasive polarization microscopy (PolScope to assess spindle integrity compared with PN score after fertilization. They reported that oocytes with mean retardance of light by birefringence developing into zygotes with a good PN score after ICSI and was significantly higher compared with oocytes developing into a lower PN score zygotes. When transfer cohorts included oocytes with high retardance and at least one good PN score embryo, conception resulted more frequently compared to transfer cohorts consisting of oocytes with a lower mean light retardance of the spindle and lower PN score embryos. This study suggested that quantitative evaluation of mean retardance of light by the oocyte spindle might reveal the oocyte's health which could be related to PN score of the embryo and its subsequent potential.

In a study by Nicoli *et al.*, (2007), they confirmed other findings that Z-score did not correlate with implantation and pregnancy rates when the "best" scores were compared to the "worst". They concluded that PN morphology alone cannot be considered superior to standard morphologic criteria applied as a tool for identifying/selecting embryos that have

a better chance of resulting in an ongoing pregnancy. Depa-Martynow *et. al*., (2007) reported that when polarization of NPB and pronuclei exist, and they are similar in size (Z1and a cytoplasmic halo is present, embryo quality and development were improved. The Z-score (Scott-modified) and halo score, combined with Day 3 embryo morphology, are useful criteria to identify embryos for transfer to achieve optimal pregnancy rates and minimize high-order multiple pregnancies.

This study affirmed findings in previous studies about a positive correlation between the appearance of a cytoplasmic halo and embryo quality (Van Blerkom *et al.*, 2002; Ebner *et al.*, 2003; Scott, 2003). Furthermore, Payne *et al.*, (1997) reported that the cytoplasmic halo effect attributes to cytoplasmic rotation/movement and/or to the differential distribution of mitochondria.

Alvarez *et al.*, (2008) associated both nucleolar pattern 0 (modified Tesarik and Greco scoring system) and equal size pronuclei with improved embryo quality. Furthermore, when at least 1 embryo classified as pattern 0 was included in the cohort of embryos transferred, they reported higher pregnancy rates. In contrast to other studies, Arroyo *et al.*, (2010) reported that when PN patterns (according to the classifications of Tesarik and Scott) were assessed together with embryo quality and chromosome constitution, no correlation between PN pattern and embryo quality was found. Interestingly, no significant differences were reported when comparing the distribution of chromosomally normal and abnormal embryos in respect of embryo quality. However, differences were observed when distinguishing between normal, aneuploid and polyploid embryos. This study's results show that Tesarik's and Scott's PN classification systems are not related to the embryo developmental potential or its chromosome constitution and cannot replace PGS to predict an embryo's chromosomal status. Pronuclei positioning within the ooplasm, their size, nucleoli distribution, and their orientation in respect of the polar bodies were highly predictive of complex chromosomal abnormalities in subsequent embryos. Zygotes displaying juxtaposed pronuclei, oversized-size nucleoli, and small angles between pronuclei and polar bodies were associated with the highest rates of euploidy as confirmed by embryos generated from PGD cycles (Gianaroli *et al.*, 2003). Reichman *et al.*, (2010) reported that abnormally fertilized 1PN and 3PN zygotes tend to develop into poorer-quality embryos when compared to normally fertilized 2PN control zygotes. They furthermore suggested proper counselling regarding the very low likelihood of viable pregnancy after transfer of abnormally developing zygotes.

EMBRYO FRAGMENTATION

Introduction to embryo fragmentation

The completion of meiosis 1 (extrusion of the *PB-I*), fertilization with IVF/ICSI, and completion of meiosis 2 (extrusion of the *PB-II* and appearance of the male and female pronucleus) which is considered a "normally fertilized oocyte and is usually checked between 16 and 20 hours post insemination. The subsequent zygote next undergoes mitosis, resulting in multiple blastomeres [Day 2 (2-5) and Day 3 (6-10)]. The goal is even-sized, mono-nucleated blastomeres, without fragmentation, on Day 4 blastomeres compaction starts (morula), and on Day 5 early cavitation and the beginning of cell separation, dividing into trophectoderm (TE) and inner cell mass (ICM) respectively. Many embryos contain some fragments surrounding blastomeres of various sizes, some nucleated and others non-nucleated. Fragmented embryos can also contain multi-nucleated blastomeres, blastomeres of variable sizes and uneven shapes, "loosely floating" blastomeres (poor inter-cell connections) and possibly thick zonas.

The cause of fragmentation, its influence on embryo competency and its effect on ART outcome are ongoing topics of interest. Embryo fragmentation was mentioned as early as 1954 by Hertig *et al.*, as well as by Edwards *et al.*, (1970), Ortiz *et al., (*1979) and Alikani *et al.*, (2001). Fragmented embryos have been associated with low embryo viability and decreased ability to develop into a viable pregnancy (Puissant *et al.*, 1987).

Several researchers have speculated about the origin of fragments:

- Poor follicular conditions (Van Blerkom *et al.*, 1997)
- Poor culture conditions (Pickering *et al.*, 1990)
- Poor culture medium – reactive oxygen species (Yang *et al.*, 1998)
- Apoptotic and necrotic processes (Juriscova *et al.*, 1996c)
- Chromosomal anomalies (aneuploidy) (Pellestor *et al.*, 1994)

Classification of different fragment types

Cleavage stage embryo grading criteria are based on the number of blastomeres, their evenness (size) and percentage of fragmentation present. Fragmentation should be measured/quantified as a percentage of the whole embryo's volume. Alikani *et al.*, (1999) concluded that anucleated blastomeres resulted in a higher percentage of embryos containing fragments.

- *Classification I* (Alikani *et al.*, 1999)
  Alikani *et al.*, (1999) used fragmentation size and position of blastomeres (nucleated cells) versus the distribution of fragments (anucleated cells) to classify fragmentation:

  Type I: one blastomere surrounded by few small fragments.

  Type II: one/more blastomere/s surrounded by many small and localized fragments.

  Type III: small fragments scattered across the all blastomeres.

  Type IV: uneven-sized blastomeres surrounded by larger fragments scattered across the embryo.

  Type V: fragments that appear like granularity in the cytoplasm and surrounding all blastomeres – this type of fragmentation does not allow clear visualization of blastomeres and is considered a non-distinct pattern.

  They reported that Type III was the most common and Type V the least common. Furthermore, they found that maternal age was not associated with any pattern of fragmentation, but interestingly the percentage of fragmentation actually decreased with an increase of maternal age. Fragment Types II and IV have been associated with a reduced number of blastomeres, which could be due to losing a blastomere to fragments and/or retarded cell-division due to loss of cytoplasm space**,** being filled up by fragments.

- *Classification II* (Antczak & Van Blerkom, 1999)
  Antczak & Van Blerkom (1999) also developed a fragmentation classification:

Type I: mono layer carpet of small fragments, coating only a modest portion of the blastomere surface with little or no reduction of blastomere size.

Type II: multiple layer of fragments, covering a considerable portion of blastomere surface and a significant reduction of blastomere size.

Type III: embryo with a blastomere showing complete fragmentation which had no fragmentation the previous day.

Type IV: occasional fragments of variable size scattered over blastomeres developmentally in otherwise normally progressing embryos.

More on embryo fragmentation:

The presence of anucleated cells surrounding blastomeres during the early stages of an embryo's development is an important indicator by which to evaluate its subsequent implantation potential was reported (Edwards *et al.* 1984, Trounson *et al.* 1984, Cummins *et al.* 1986 and Puissant *et al.* 1987).

The morphological evaluation has been integral to embryo selection criteria since the study of embryology began – evaluating and grading of embryos have been part of a subjective evaluation of an embryo based on the number of blastomeres, symmetry (size and shape), cleaveage rate and fragmentation percentage. Scoring of Day 2 embryos was reported as early as 1986 by Cummins *et al.*, and subsequently by Pruissant *et al.* (1987), Steer *et al.*, (1992), Visser and Fourie (1993), Giorgetti *et al.,* (1995) and Roseboom *et al., (*1995).

Scott and Smith (1998) introduced a corrected embryo score – based on combination of pronuclear morphology and early embryo cleavage. Desai *et al.,* (2000) were the first to report on a day3 embryo scoring system, followed by (Fisch *et al.,* 2001, 2003, De Placido *et al.,* 2002, Lan *et al.,* 2003, Nagy *et al.,* 2003, Ciray *et al.,* 2005, Chen *et al.,* 2006). The combination of day 3 morphology with embryo scoring has also shown correlations with implantation (Steer *et al.*, 1992; Rijnders and Jansen; 1998; Van Royen *et al.*, 1999; Desai *et al.*, 2000; Van Royen *et al.*, 2001; Rienzi *et al.*, 2002). During embryo cleavage (mitosis), the shape or symmetry of blastomere division (Roux *et al.*, 1995a/b; Hardarson

et al., 2001), the fragmentation patterns (Antczak & Van Blerkom, 1999; Alikani *et al.*, 2000; Van Blerkom *et al.*, 2001) and the embryo's developmental rate (Racowsky *et al.*, 2000; Shapiro *et al.*, 2000; Check *et al.*, 2001) had an effect on its implantation potential. The effect of fragmentation on embryo viability was reported by several researchers – (Erenus *et al.,* 1991, Giorgetti *et al.,* 1995 , Ziebe *et al.,* 1997, Alikani *et al.,* 1999, Antczak *et al.,* 1999, Racowsky *et al.,* 2003 and Ciray *et al.,* 2005) – they found that when embryos with no fragmentation were transferred the outcome was associated with higher pregnancy rates compared to fragmented embryos; however, Hoover *et al.,* (1995) controversially claimed that fragmentation had no influence on implantation/pregnancy rates.

In a large study Alikani *et al.,* (1999) reported that embryos transferred from the same category (degree or percentage of fragmentation, type of fragmentation ie.Type I (0-5% fragmentation), resulted in higher implantation and pregnancy rates established a clear relationship between a percentage of fragmentation and embryo viability. In this cohort more blastomeres per embryo were present compared to other groups. The presence of all other types of fragments resulted in a decrease in implantation and pregnancy rates and Type IV (the highest percentage fragmentation) had the lowest. Racowsky *et al.,* (2003) reported that fragmentation higher than 25% of the number of blastomeres and asymmetric blastomeres were associated with lower embryo viability and very low implantation rates, concluding that the optimal day 3 embryos therefore must contain more than seven evenly sized blastomeres with less than 20% fragmentation. It had been reported that pregnancies could derive from highly fragmented embryos (Scott *et al.*, 1991; Staessen *et al.*, 1992; Alikani *et al.*, 1999; Ebner *et al.*, 2001).

An inverse relationship between embryo fragmentation and maternal age was reported by Alikani *et al.,* (1999) in contrast to Ziebe *et al.,* (2001) Stone *et al.,* (2005) and Keltz *et al.,* (2006) that reported and increase in fragmentation with increased maternal age; however Giorgetti *et al.,* (1995) and Stensen *et al.,* (2010) found no correlation between maternal age and embryo fragmentation.

Embryo fragmentation and embryo ploidy

Munne and Cohen (1998) reported the following to be associated with a high percentage of aneuploidy when embryos were screened by FISH: embryos that were arrested or retarded in their development; contained uneven pro-nuclei, multi-nucleated blastomeres

or fragmentation; giant eggs or dominant blastomere embryos. In another study, Ebner *et al.,* (2001) reported that transferral of highly fragmented embryos (≥ 25%) resulted in pregnancies with significantly higher fetal abnormalities compared to embryos with less than 25% fragmentation. However, aneuploidy was not related to an embryo's morphology but to the maternal age and led to increased pregnancy loss when highly fragmented embryos were transferred. In contrast, Plachot *et al.,* (1987), Pellestor *et al.,* (1994) and Munne and Cohen (1998) reported that highly fragmented embryos were associated with a higher rate of chromosome abnormalities, in particular mosaicism.

A correlation between embryo development and chromosomal complement makes the incidence of chromosomal abnormalities significantly higher in embryos dividing according to a time frame and a symmetry plan which is different from expected. The type of fragmentation is also related to chromosomal status, which explains why the extrusion of fragments might severely affect embryo viability. Magli *et al.,* (2007) applying FISH screening (13,15,16,18,21,22 and XY) found that embryos undergoing retarded or excelled division and with an uneven number of blastomeres as well as the presence of fragments inside the PV-space, were associated with a significantly higher incidence of chromosomal abnormalities; this possibly explains why these anomalies profoundly affect an embryo's viability.

Interestingly Gianaroli *et al.,* (2005) and Munné *et al.,* (2006) reported that up to 50% of embryos were genetically abnormal, despite the fact that they were graded on Day 3 as eight cells without fragmentation - optimal morphology according to Racowsky *et al.,* (2003).

The effect of embryo fragmentation and blastocyst formation

Alikani *et al., (*2000) reported that largely scattered fragments plus uneven blastomeres caused decrease in blastocyst formation. Hardy *et al.,* (2003) reported that embryos with severe fragmentation lead to lower blastocyst formation as well as poor ICM formation and low numbers of TE cells. Moderate fragmentation, however, leads to a lower TE cell count but normal ICM formation. Furthermore, the fragmentation pattern influenced the distribution of cells to the ICM or TE during blastocyst formation.

Using embryo compaction criteria to determine blastocyst development has been researched. Nikas *et al.,* (1996) suggested that compaction on Day 4 could reveal an embryo's ability to develop into a normal blastocyst. Complete compaction is more successful than partial compaction, which excludes some cells/fragments. These findings were confirmed by Alikani *et al.,* (2000) who reported 50% of completely compacted, 33% of partially compacted and 10% of non-compacted embryos on Day 4 developed into blastocysts, respectively and concluded that compaction could be used as a prognostic tool for normal blastocyst formation.

Antzak & Van Blerkom (1999) applied their fragmentation grading criteria and reported that the presence of Types 2 and 3 fragments on Day 2 did continue to develop past the 4-cells stage; of these about 50% advanced to blastocysts, however, delayed by 12-24 hours. They also reported that fragmentation present at the 8-cells stage did not hinder development to the blastocyst, concluding that the influence of fragmentation might have a more detrimental effect on developmental potential at an earlier stage. Alikani *et al.,* (1999) reported that no fragmentation and Type 1 fragmentation led to the highest blastocyst development. In another study by Stone *et al.*, (2005), they reported similar findings, that no fragments and Type I fragments had the highest number of blastocysts, whereas Types 2 and 3 (moderately fragmented) resulted in fewer blastocysts. Type 4 (severely fragmented) resulted in no normal blastocyst development.

Van Blerkom *et al.*, (2001) and Hardarson *et al.*, (2001) both suggested that reabsorption of fragments occurs during embryo development. Furthermore, Van Blerkom *et al., (*2001) speculated that the disappearance of fragments could be due to lyzing of small fragments and larger fragments might inflate and burst. It is assumed that, to a certain degree, affected blastomeres can be entirely excluded from further development by selective fragmentation (Warner *et al.*, 1998). Cellular fragmentation is likely to be related to programmed cell death (Jurisicova *et al.*, 1996c; Warner *et al.*, 1998).

Yang *et al.*, (1998) reported that the majority of fragments do not persist during the embryo's developmental term but are separated at the compaction stage and pushed aside or they might be present in the blastocoel cavity at the blastocyst stage. Hnida *et al.,* (2004) reported that the blastomere size will decrease when more than 50% of fragmentation is present, which can lead to the arrest of these small "blastomeres". Alikani *et al.*, (1999) classified cells without a nucleus, either to be fragmented or abnormally

formed blastomeres and further speculated that degenerating material could be excreting toxicities that could potentially negatively affect healthy blastomeres. Antzak & Van Blerkom (1999) suggested that certain fragmentation patterns could decrease regulatory proteins and subsequently starve the surrounding blastomeres of essential nutrients. In light of the potential side-effects of fragmentation, removal might be beneficial and improve pregnancy outcome, as suggested by Rienzy *et al.*, (2002) and Nagy *et al., (*2005). Interestingly Alikani *et al.*, (2002, 2005) and Keltz *et al.*, (2006) conducted experiments to evaluate the effects of fragments on surrounding blastomeres, where blastomeres were removed and cultured separately or by "transplanting" the blastomeres into surrogate zonas. In the first group, Type IV fragmented embryos underwent biopsy to remove blastomeres and were cultured individually; 40% arrested, 60% continued to divide. In the second group, highly fragmented embryos were biopsied and the removed blastomeres were transplanted into surrogate zonas; one third developed into viable blastocysts with normal ICM and TE and the majority was diploid, however, chaotic mosaicism was the most common chromosomal abnormalitiy present in these embryos. Poor development of fragmented embryos might be due to the effect of the fragments combined with abnormally anucleated blastomeres.

How does fragmentation affect blastomere to blastomere intra/inter communication? All blastomeres are connected by microbules and tight bindings – fragmentations might cause separation by wedging in between blastomeres and therefore interfere with communications between blastomeres.

Communication and exchange of growth factors might be influenced by the spatial separation as suggested by Alikani *et al.*, (1999). Van Blerkom *et al.*, (2001) speculated that fragments between blastomeres obstructed the contact point between adjacent blastomeres. Alikani *et al.*, (2000) reported that even though the mechanism by which fragmentation affects embryo development is unknown, it is clear that fragmented embryos result in less compaction and that at this time the embryo can also extrude some fragments/cells.

The presence of E-cadherin protein plays a critical role in the formation of a blastocyst. The protein is transported from the cytoplasm of each blastomere to the contact point between adjacent blastomeres. This relocation is critical in the formation of the TE cell line surrounding each blastocyst. This distribution of the E-cadherin protein might be negatively

affected by fragments between blastomeres and interfere with its expression, location/distribution and subsequently causing failed blastocyst formation. All these adverse effects on blastocyst development could be related to the disruption of critically needed inter-cell communication mediated by E-cadherin (Johnson *et al.*, 1985; Vestweber *et al.*, 1987; Alikani *et al.*, 2005).

Conclusion

An embryo containing fragmentation is capable – even though at a lesser rate – of developing into a viable pregnancy. Fragmentation should be evaluated and graded in order to predict what its potential effect might be on embryo viability. The effect that transfer of highly fragmented embryos has on pregnancy outcome in this review, suggests that an increase in pregnancy loss as well as higher fetal anomalies might be the result of transferring such an embryo population. This fact is of concern and needs to be studied by multiple centres and in large numbers. To establish a correlation between a standard fragmentaion grading systems and establish its subsequent effect on an embryo's ploidy and viability could be of great interest to ART programs worldwide.

BLASTOCYST MORPHOLOGY AND CULTURE

Introduction

The ultimate goal of any ART clinic is to find specific embryo selection criteria in order to optimize implantation and pregnancy rates and simultaneously avoid/reduce high order multiple pregnancies. Several scoring systems evaluating pronuclei, early cleavage and cleavage stage embryo morphology have markedly improved ART outcome (Cummins *et al.*, 1986; Balakier *et al.*, 1997; Alikani *et al.*, 1999; Van Rooyen *et al.*, 1999; Ebner *et al.*, 2003; Fisch *et al.*, 2003; Scott *et al.*, 2003b; Rienzi *et al.*, 2005; Sakkas *et al.*, 2005; Windt *et al.*, 2004). Improved culture conditions, systems and media over the last decade have further improved the ability and effectiveness of extended culture of embryos to the blastocyst stage (Gardner *et al.*, 1997/1998a/b/2000, Menezo *et al.*, 1998; Pool *et al.*, 2002).

Factors associated with blastocyst transfer:

- A reduction in number of cleavage stage embryos that will develop/convert to blastocyst stage in vitro and leaving fewer embryos for transfer and/or cryopreservation – the worst scenario is in some cases no embryos might be available for the latter purposes. However, it has been suggested that this reduction of available embryos in most cases leads to fewer, but improved embryo quality, by weaning out the embryos of which the embryonic genome was not capable of supporting successful conversion to the blastocyst stage (extended culture might eliminate non-viable embryos).
- Sex-ratio imbalances have been associated with blastocyst transfer as reported by Luna *et al.*, (2007).
- Blastocyst transfer might decrease ectopic pregnancies, because the larger diameter of an expanded blastocyst might help prevent retroflux into the fallopian tubes as suggested by Schoolcraft *et al.*, (2001).
- Fachin *et al.*, (2001) reported that uterine contractility decreased by Day 7 or 8 post hCG when embryos are at the blastocyst stage versus Day 3 embryos.
- There might be additional cost added for extending culture to Day 5/6.
- Need to distinguish between embryos with a poor or high developmental potential (Tesarik at al., 1994; Van Blerkom, 1994).
- The uterine environment is more synchronized or receptive to a blastocyst stage embryo by reduction of cellular stress (Croxatte *et al.*, 1978; Gardner *et al.*, 2000).
- Reduces the embryo's exposure to possible detrimental effect of hyperstimulation of the uterine lining (Simon *et al.*, 1995; Pellicier *et al.*, 1996; Ertzeid *et al.*, 2001; Kelley *et al.*, 2006).
- Less uterine contraction prevents expelling the embryo (Lesny *et al.*, 1998; Fachin *et al.*, 2001).
- A blastomere biopsy can be performed on Day 3, the biopsied embryos remain in culture until the completion of the genetic screening and a fresh blastocyst transfer can be performed (Munné *et al.*, 2002).
- Embryos convert from maternal genome to embryonic genome control between the 4- and 9-cell stages; the health of the embryo's genome will determine its viability and ability to grow into a blastocyst (Taylor *et al.*, 1997; Braude *et al.*, 1988).

- Improved implantation rates with a reduced number of embryos needed for transfer and subsequently a reduction of multiple pregnancies (Gardner *et al.*, 1998 and 2003/2004).
- Blastocysts have a higher potential to be viable post cryopreservation (Veeck *et al.*, 2003).
- Gardner *et al.*, (2004) suggested that transferring embryos at the blastocyst stage might eliminate some chromosomally abnormal embryos for transfer.

Some studies have speculated that extended embryo culture results in higher quality embryos and more chromosomally normal embryos compared to cleavage stage embryos, leading to increased implantation. However, many early embryos with a ”normal” morphology on Day 3 are actually chromosomally abnormal due to mosaicism, as reported by Magli *et al*., (2000). However, once the oocyte is aneuploid the genetic status of an embryo is not influenced by extended culture --- many chromosomally abnormal Day 3 embryos develop into top quality chromosomally abnormal blastocysts (Kotze *et al.,* 2012 Gynecol Obstet Invest DOI: 10.1159/000339632 Accepted (Chapter 4). Furthermore, although extended embryo culture does provide information about an embryo’s viablity in vitro, therefore, it should not be directly associated with pregnancy outcome. Reduction in the number of embryos to transfer might reduce multiple pregnancy outcomes; however, the transfer of a single blastocyst could result in monozygotic twins.

Blastocyst morphology

Early in the evolution of extended embryo culture to the blastocyst stage, non-invasive morphological and biochemical markers for blastocyst selection prior to transfer were suggested (Renard *et al.,* 1980, Gardner *et al.,* 1987 and Lane *et al.,* 1996). Blastocyst grading systems were developed by Cohen *et al.*, (1985), Dokras *et al.*, (1993), Gardner and Schoolcraft (1999), Richter *et al.*, (2001) and Kovacic *et al.*, (2004). These morphological grading systems take into account expansion of the blastocoel cavity, characteristics of the ICM and the TE. Balaban *et al.*, (2000) reported that the transfer of good quality blastocysts was associated with improved implantation rates compared to that of poor quality blastocysts. In another study, Gardner *et al.*, (2000) reported that applying their blastocyst grading system resulted in improved ART outcome.

Theoretically, after 5-6 days of *in-vitro* embryo culture, a healthy human embryo should be at the blastocyst stage, comprised of some 25 to 50 cells of the ICM and 55 to 110 cells of TE. The blastocyst could start hatching on Day 5 and should be completely hatched from the ZP on Day 6 and ready to undergo implantation into the uterine mucosa. Blastocyst grading should accommodate a measure of the three parameters: blastocoel expansion (ZP thinning), ICM and TE-layer.

Earlier, mouse and bovine embryos were the only sources of information regarding the morphology and rate of development of the blastocyst. At that time it was presumed that human embryos/blastocysts produced in vivo develop more rapidly than those produced in vitro and furthermore, it was assumed that extended culture of the human embryo could be a detrimental approach; therefore the reason for transferring early cleaved embryos on Day 2-3, (Buster *et al.*, 1985). More recently, with improved culture medium and conditions, IVF laboratories seem to be able to apply extended embryo culture to cleavage stage embryos with a consistent rate of blastocyst formation. Some clinics moved all ETs to the blastocyst stage (Marek *et al.*, 1999), although some centres still report no major benefits gained by extended culture (Huisman *et al.*, 2000). Embryo morphology on Day 3 predicted approximately 50% of embryos that will eventually develop into blastocysts (Rijnders & Jansen, 1998).

Graham *et al.*, (2000) reported that blastocyst scoring was used as a tool that contributed to discrimination against poor viability and earlier morphological scoring (Scott *et al.,* 2000), when evolving from routine Day 3 transfers to the blastocyst stage approach. Individually cultured embryos graded as good quality on Day 3 can be tracked and are associated with ability to convert to blastocyst and their quality on Day 5-6. Improved pregnancy rate after blastocyst transfer is solely related to improved selection of embryos and by no means through intrinsic improvement of embryonic quality through extended culture.

In 2000, Racowsky *et al.* suggested that extended culture of at least two, but preferably three or more high quality eight-cell embryos on Day 3, should result in sufficient blastocyst development for transfer and/or cryopreservation. Papanikolalaou *et al.*, (2005), similarly suggested that four good quality Day 3 embryos should undergo extended culture and be transferred at the blasocyst stage, with a better chance of achieving a live

birth compared to cleavage stage ET. Shapiro *et al.*, (2001) reported that blastocysts were twice more likely on Day 5 to implant, compared to delayed blastocyst transferred on Day 6.

At present the main drive to move to blastocyst transfer is to reduce the number of embryos transferred routinely, in an attempt to reduce multiple implantations after IVF-ET therapy. A blastocyst's quality is very much determined by the quality of the gametes from which it came (Janny & Menezo, 1994, 1996), tempered by the in vitro environment in which it was cultured. It is doubtful that we can currently actually improve embryo quality beyond that which might be provided in vivo; consequently the only potential way for embryo quality to go is down! Thus, we as embryologists have a very real responsibility to "do no harm" while nurturing the pre-implantation human embryo; conversely, extended culture is a real privilege that allows us access to study the complete pre-implantation period of development in the human.

Is morphological assessment of blastocysts a measure of quality? Dokras *et al.*, (1993) (Table 1) proposed a fairly simple approach to blastocyst grading which incorporated both morphology and rate of development. Its simplicity to some extent, however, compromises its usefulness, as it does not allow sufficient flexibility of description. Gardner & Lane (2000), on the other hand, have suggested an approach to grading that is more comprehensive; it also incorporates assessment of rate of development and independent comment on the ICM and TE (Table 2). The best morphological assessments are based on photographic images. While numeric and alphabetic scores can be ascribed, a photo-library for embryo grading leaves little to the imagination (Menezo *et al.*, 1999).

In a study by Richter *et al.*, (2001), they added additional parameters to the “regular” blastocyst grading criteria: blastocyst diameter (measured from outer zona to outer zona); ICM - measured across the widest and longest length (calculated as square micrometers); an ICM roundness index (RI - length divided by width; example - an ICM with an RI of 1 would be perfectly round, whereas a larger RI-score would indicate progressively more elongated ICM); and recording the number of TE cells in a cross-sectional circumference. Blake *et al.*, (2004) revealed a significant increase in implantation rate when blastocysts were transferred compared with cleavage stage embryos. Does this benefit outweigh the fact that some patients might have no blastocyst to transfer as mentioned earlier? These contradictory findings can be used to advise clinics that consider an “all blastocyst

approach", since scientific evidence does not support it. Every case should be evaluated on its own merits before deciding the optimal stage at which to transfer embryo(s) for each patient. Furthermore, they reported no significant difference in live birth rate and no reduction in multiple births between cleavage stage and blastocyst transfers. Finally this review did not report on monozygotic twinning. In a follow up review, Blake *et al.*, (2005), confirmed previous findings, but additionally concluded that the implantation potential of blastocysts was more obvious when extended culture took place in sequential culture media compared to a single medium. In 2007 Blake *et al.*, in a continuation of their series about cleavage stage versus blastocyst stage embryo transfer (ET), concluded that in "good prognosis" category patients, pregnancy and live birth rates were significantly higher in the blastocyst transfer compared to the cleavage stage group. Based on these findings, they suggest extended culture and single blastocyst transfer for a selected patient population, and thereby support findings by: The Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology 2008.

Controversially compared to other findings, Levron *et al.*, (2002) reported a significant decrease in implantation rates associated with blastocyst transfer and a negative effect on clinical pregnancy rates. Schwarzler *et al.*, (2004) evaluated pregnancy rates when embryos were transferred at the cleavage stage or morula/blastocyst stage and reported improved pregnancy rates in the morula/blastocyst group compared to the cleavage stage embryos. The birth rate in the morula/blastocyst stage was significnaly higher. However, the multiple pregnancy rates were significantly higher for morula/blastocyst stage transfer. They concluded that morula/blastocyst transfer may lead to a higher pregnancy rate with an overall better live birth rate but potentially at a higher multiple gestations rate. In 2005, Barrenetxea *et al.* reported on a patient population that had previous failed IVF outcomes when their embryos were transferred at the cleavage stage. In subsequent cycles the same patients received blastocyst transfers and interestingly they found that when the transfer was on Day 5, implantation rates were nearly five times higher and pregnancy rates were nearly three times higher compared to Day 6, respectively. They concluded that blastocyst transfer on Day 5 after retrieval successfully improved pregnancy outcome in patients with prior multiple failed IVF attempts and limited the risk of multiple pregnancies because fewer embryos would be transferred.

Reports from a few prospective randomized studies

Van der Auwera *et al.*, (2002) concluded in their randomized study that a significantly higher clinical pregnancy rate was achievable when blastocysts compared with Day 2 embryos were transferred. In 2005 Papanikolaou *et al.*, reported that blastocyst-stage transfer resulted in a significantly higher ongoing pregnancy rate and live birth rates compared with Day 3 ET. However, a high twin birth rate was observed in both groups. Furthermore a minimum of four good embryos on Day 3 followed by extended embryo culture to the blastocyst stage will have a better chance of a live delivery compared to cleavage-stage ET. In another study by Papanikolaou *et al.*, (2008) they performed a meta-analysis to compare studies where equal numbers of embryos in both the cleavage-stage and the blastocyst-stage were transferred, and compared clinical pregnancy rate, multiple pregnancy rate and live birth rates. They found that clinical pregnancy rates were significantly higher in patients with a blastocyst-stage ET as compared to patients in whom a cleavage-stage ET was performed. Live birth rate per randomized patient was significantly higher in patients who had a blastocyst-stage transfer compared to patients with cleavage-stage ETs. They concluded that the probability of live birth is significantly higher after blastocyst-stage as compared to cleavage-stage ET when equal numbers of embryos are transferred in each group. Levitas *et al.*, (2004) compare blastocyst-stage ETs with Day 2-3 ETs in patients who failed to conceive in three or more Day 2-3 IVF-ET cycles in a prospective, randomized study. They reported higher clinical pregnancy rates per blastocyst versus Day 2-3 transfers. Furthermore, a significantly higher implantation rate for blastocyst embryos as compared with Day 2-3 was reported. However, the multiple pregnancy rates was not significantly different between the two groups. They concluded blastocyst-stage embryos had a significantly higher implantation and higher pregnancy rate, even though statistical significance was not reached because of a small patient population.

A few studies reported no difference between blastocyst and cleavage stage ETs. Utsunomiya *et al.*, (2004) concluded that the pregnancy rate and implantation rate of ET with hatching stage blastocysts had no advantage compared with the conventional Day 3 ET. In a prospective, randomized study by Bungum *et al.*, (2003) the implantation and pregnancy potential of embryos transferred on Day 3 or Day 5 were compared. Equal numbers of embryos (two) were replaced in each group. No statistically significant difference between chemical, clinical pregnancy, implantation rates, twinning rates and

early pregnancy loss rates were found between Day 3 or Day 5 groups. Implantation rates were similar when equal numbers of embryos were transferred. They concluded that ETs at the blastocyst stage have no advantage over Day 3 transfers. In a prospective randomized study by Hreinsson *et al.*, (2004) they compared the implantation and pregnancy rates after cleavage stage and blastocyst-stage (days 5-6) embryos were transferred. They found no significant difference in implantation rates and clinical pregnancy rates between the two groups; however, they concluded that blastocyst transfer might be a good alternative for couples with many good quality embryos on Day 2.

Finally a few studies suggested that transferring of a single blastocyst could effectively reduce multiple pregnancies without compromising the live birth rates, as reported by the Criniti *et al.*, (2005), Henman *et al.*, (2005) American Society for Reproductive Medicine 62nd Annual Meeting, 2006 and Zech *et al., (* 2007).

Conclusion

Overall, when looking at the meta-analysis (Papanikolaou *et al.,* 2008) blastocyst transfer increased implantation rates and live birth rates compared to cleavage stage embryos. In a population of good prognosis patients, transferring fewer blastocysts could be considered to reduce the rate of multiple births without compromising live birth rates. In all cases the potential risks associated with prolonged culture should be considered. Lastly, successful blastocyst development depends on optimal culture conditions and selection of a specific patient population.

TABLE 1

Dokras Blastocyst grading:

Blastocyst grading according to Dokras *et al.* 1993 was performed as follows:

Grade 1 blastocysts were characterized by early cavitation resulting in the formation of an eccentric and then expanded cavity lined by a distinct inner cell mass region and trophectoderm layer.

Grade 2 blastocysts exhibited a transitional phase where single or multiple vacuoles were seen which over subsequent days developed into the typical blastocyst appearance of grade 1 blastocysts.

Grade 3 blastocysts were defined as blastocysts with several degenerative foci in the inner cell mass with cells appearing dark and necrotic

TABLE 2

Gardner blastocyst grading/developmental criteria/explanation (Gardner & Schoolcraft, 1999)

Expansion grade

Blastocyst expansion grade	Description of blastocyst stage/development criteria
Grade 1	Early blastocyst: the blastocoel is less than half the volume of the embryo
Grade 2	Blastocyst: the blastocoel is greater than or equal to half of the volume of the embryo
Grade 3	Full blastocyst: the blastocoel completely fills the embryo
Grade 4	Expanded blastocyst: the blastocoel volume is larger than that of the early embryo and the zona pellucida is thinning.
Grade 5	Hatching blastocyst: the trophectoderm has started to herniate through the zona pellucida
Grade 6	Hatched blastocyst: the blastocyst has completely escaped from the zona pellucida

Inner cell mass (ICM) Grade	Description of ICM quality criteria
Grade A	Tightly packed, many cells
Grade B	Loosely grouped, several cells
Grade C	Very few cells

Trophectoderm (TE) Grade	Description of TE quality criteria
Grade A	Many cells forming a tightly knit epithelium
Grade B	Few cells
Grade C	Very few cells forming a loose epithelium

GENOMICS, TRANSCRIPTOMICS, PROTEOMICS, AND METABOLOMICS

Introduction

Over the last 30 years assisted reproductive technology (ART) has been lacking an international standard by which to select an embryo with optimal potential to develop into a live baby. Multiple embryos have been transferred in an effort to optimize pregnancy rates. Adamson et al., (2004) concluded that a lack of knowledge about the reproductive potential of embryos exist, resulting into failed ART cycles and/or multiple pregnancies.
With the introduction of extended embryo culture to the blasotcyst stage, fewer embryos are being transferred; however, the ideal of a single-embryo transfer without compromising pregnancy rates has still not been attained as reported by Van Montfoort et al., (2006). Early stage embryo morphology, cleavage stage embryos and blastocyst embryos currently provides insufficient information to the embryologist in order to accurately select a single embryo for transfer.

Lately, advances in genomics, transcriptomics, proteomics, and metabolomics might be a helpful addition to current embryo selection criteria.

Proteomics - (non-invasive) - describes the changes in all proteins expressed and translated from a single genome. Currently little is known about either the genome or proteome of human gametes or the pre-implantation embryo. The dialogue between the developing embryo and the maternal endometrial environment needs a much better understanding. Proteomics technology might be a futuristic tool to select competent embryos for transfer. Proteomics alone involves several sophisticated techniques including imaging, mass spectrometry and bioinformatics to identify, quantify and characterize a proteome. Continuous research can lead to profiling the proteome of individual human oocytes and embryos, as well as the proteins produced by the embryo into the surrounding medium (the secretome). Furthermore, the identification of proteins that are involved in oocyte maturation, embryo development and implantation could lead to further improvements in assisted reproduction techniques as well as the development of new diagnostic tests. Finally, proteomics may contribute in the design of a non-invasive viability assay to assist in the selection of embryos for transfer in human assisted reproduction.

Transcriptomic - (non-invasive) - studies that evaluate cumulus masses surrounding oocytes for the presence of specific messenger RNA (mRNA). Katz-Jaffe et al., (2008) reported that when these embryos were transferred, live births resulted compared with those that failed to deliver. Assou et al., (2010) in a review study reported on the cumulus cells (CCs) transcriptomic profiling that predict oocyte and embryo competence. Using RT–PCR or DNA microarrays, evidence of genes expressed in CCs might present potential biomarkers to predict embryo quality and pregnancy outcomes.

Proteomics - (non-invasive) - is the measurement of amino acids or proteins in spent culture media. Brison et al., (2004) found that changes in the levels of some amino acids are associated with implantation rates. Sturmey et al., (2008) reviewed amino acid profiling as possible predictor early embryo viability. Amino acid supplementation of embryo culture media and the role of amino acids in early embryo development were reviewed as well as methods to quantify amino acid depletion and production by single embryos. They concluded that improved metabolic assay methods and provided great potential to improve the selection of single embryos for transfer in vitro.

Metabolomics – (non-invasive) - evaluates how the embryo alters its surrounding microenvironment and is evaluated in spent culture medium surrounding embryos. Scott et al., (2008) used a Raman spectroscope to determine if varying spectral patterns predict ongoing pregnancies. Initial studies to measure metabolomic changes in the culture medium of embryos and oocytes have demonstrated that different types of spectrophotometric tests, including Raman and near-infrared (NIR) techniques, are similarly well capable of detecting specific changes of the 'secretome' It has been demonstrated that metabolomic measurements correlate well with embryo development and morphology assessment. The viability index on oocytes/embryos established by metabolomic tests may be a stronger predictor for implantation potential than traditional morphological assessment. Gardner et al., (2011) reported that glucose consumption by embryos which resulted in a pregnancy was significantly higher at the morula and blastocyst stage compared to those that failed to develop into a pregnancy. Interestingly, female embryos consumed 28% more glucose compared to male embryos. They concluded that a human embryos glucose metabolism could be used as selection criteria and hypothesized that male and female human embryos differ in their physiology and glucose consumption.

Genomics - (invasive) - was used as early as 1989 by Handyside et al.,–when they introduced PGD. FISH has been applied to identify aneuploidy and known single-gene disorders and using genomics to prevent the transmission genetic disorders. However, the latter did not improve pregnancy rates among infertile couples as reported by Mastenbroek et al., (2007). A few years later Kallioniemi *et al.*, (1992) developed comparative genome hybridization (CGH) to screen the whole genome's DNA in tumors. This technique was modified to study the DNA of single cells (like blastomeres) by Voullaire *et al.*, (1999) and Wells *et al.*, (1999). Lately, a more rapid technology has been developed, allowing a more rapid and more detailed analysis of PB-I, PB-II, Day 3 blastomeres and trophectoderm cells, namely array-CGH (Hu *et al.*, 2004; Wells *et al.*, 2004; Le Caignec *et al.*, 2006; Treff *et al.*, 2010, 2011). More recently Treff *et al.*, 2012 introduced real-time qPCR .

We conclude that although the results of these initial investigations are promising, further prospective studies are required to define the potential benefits of this novel non-invasive technology.

SOLUBLE HUMAN LEUKOCYTE ANTIGEN –G (sHLA-G)

Background

The search for a non-invasive embryo marker that can identify embryos with high implantation potential is continuing.

Human leukocyte antigen-G (HLA-G) is a non-classic type I human leukocyte antigen produced by the embryo (Neumann *et al.*, 1994; Menicucci *et al.*, 1999; Salihu *et al.*, 2003) and by thymus cells (Mallet *et al.*, 1999). It probably plays a pivotal role in the development of pregnancy. Soluble HLA-G (sHLA-G) has been identified in the media surrounding groups of embryos in culture for several days, where its concentration has been found to be related both to cleavage rate (Menicucci *et al.*, 1999) and implantation potential (Fuzzi *et al.*, 2002).

The detection of sHLA-G (a major histocompatibility complex [MHC]) in culture medium surrounding embryos was first reported by Jurisicova *et al.*, (1996a/b), Menicucci *et al.* (1999) and Fuzzi *et al.*, (2002). sHLA-G was expressed during preimplantation human embryo development and was detected in culture medium surrounding groups of embryos. Sher *et al.*, (2004) was the first to detect sHLA-G in the culture medium of

individually cultured early cleaving embryos (as early as 44–46 h after insemination). This early expression of sHLA-G provided evidence that embryos could be selected based on a healthy maternal genomic function. sHLA-G expression by an embryo has been associated with an embryo's potential to reach a pregnancy.

The greater the sHLA-G expression, the more superior the embryo competency, as evidenced by implantation and pregnancy rates (Roussev *et al.*, 2003). Early studies suggested that HLA-G was expressed and secreted by the extravillous trophoblast, and by thymus cells (Ellis *et al.*, 1990; Chu *et al.*, 1998). In later studies evidence indicated that the syncytiotrophoblast also secretes this compound (Chu *et al.*, 1998). HLA-G is believed to play a pivotal role in immunoprotection of the semi-allogenic embryo (Jurisicova *et al.*, 1996a; Fernandez *et al.*, 1999). Its role is to prevent allo-recognition by maternal cytotoxic lymphocytes and to protect against natural killer (NK) cell-mediated lysis of target cells (Choudhury & Knapp, 2001; Kanai *et al.*, 2001). HLA-G has been found to cause a polarizing shift of two subtypes of T helper cells (Th1 and Th2), in the embryo-decidual interphase (Jurisicova *et al.*, 1996b). Th1 predominates in the non-pregnant state and expresses interferon (IFN) and tumor necrosis factor (TNF) beta, the cytokines predominantly involved in cellular immunity, delayed hypersensitivity, tissue injury in infection and autoimmune disease. Th2 cells secrete interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10 and IL-13, cytokines that are involved in antibody production. Th2 response down-regulates the Th1 response and *vice versa* (Choudhury & Knapp, 2001). The balance between Th1 and Th2 cytokines largely determines whether an immune response is protective or pathological. The HLA-G-induced shift toward Th2 during pregnancy establishes and perhaps coordinates a cytokine network that protects the developing embryo from the maternal immune system (Makhseed *et al.*, 1999 and Hunt et al., 2005).

(Figure 1a/b: sHLA-G mechanism schematics)

The implantation mechanism has been a very poorly understood aspect of reproduction. It is a highly complex dialogue (cross-talk) between the embryo and the endometrium, (Simón *et al.*, 1995a/b; Hill. 2001; Viganò *et al.*, 2003; Shimomura *et al.*, 2006). Implantation requires the successful suppression of the maternal immune system. The human body will identify and discriminate against foreign tissue by MHC, HLA originated from a group of genes located on the short arm of chromosome six (6). The MHC evokes T-cell intervention to non-self antigens expressed by an individual of the same species. The human fetus is considered a non-self tissue by the maternal uterus because of

paternal MHC antigens; however, during pregnancy the immune system develops fetal tolerance (Saito *et al.*, 2007). HLA-G produced by the extra-villous cytotrophoblast (the only fetal contact with maternal uterine cells), confers immuno-tolerance through interaction with maternal uterine membrane lymphocytes. This scenario would suggest that HLA-G protects the fetus from maternal immune response attack. HLA-G is expressed by the placenta throughout gestation and is also present in amniotic fluid. sHLA-G (a spliced iso-form of membrane-bound HLA-G) is in suspension and can be detected in culture medium. sHLA-G, a non-classic type I HLA, was first identified in the media surrounding groups of embryos and blastocysts in culture by Jurisicova *et al.*, (1996). Menicucci *et al.*, (1999) first identified, sHLA-G in the culture media surrounding a group of Day 3 embryos. Fuzzi *et al.*, (2002) showed that the presence of sHLA-G in the culture media harbouring groups of 3-day-old cleaved embryos correlated both with their cleavage rate and with their overall subsequent implantation potential. Hviid *et al,* (2004) postulated that the presence of sHLA-G protected the conceptus from destruction by the maternal immune response. Several studies regarding sHLA-G and its effect on pregnancy outcome have been reported since (Sher *et al.*, 2004, Criscuoli *et al.*, 2005; Noci *et al.*, 2005; Sher *et al.*, 2005a; Sher *et al.*, 2005b; Yie *et al.*, 2005; Desai *et al.*, 2006; Fisch *et al.,* 2007; Rebmann *et al.*, 2007; Kotze *et al.*, 2010). In review studies by Sargent *et al.*, (2007) and Vercammen *et al.*, (2008) they thoroughly evaluated and discussed previous studies regarding sHLA-G and ART outcome. Furthermore, Rebmann *et al.,* *(*2004) addressed specific issues dealing with sHLA-G enzyme-linked immunosorbent assay (ELISA) protocols.

There has been some criticism of use of the optical density (OD) value as our sHLA-G unit value; no previous reported studies (Fournel *et al.*, 2000; Criscuoli *et al.*, 2005; Noci *et al.*, 2005; Yie at al., 2005; Rebmann *et al.*, 2004; Desai *et al.*, 2006; Rebmann *et al.*, 2007; Sargent *et al.*, 2007; Vercammen *et al.*, 2008) performing sHLA-G analysis have reached consensus on the appropriate way to report these results. In the only prospective randomised controlled study, Kotze *et al.*, (2010) emphasized the benefit of transferring sHLA-G positive embryos and predicting pregnancy outcome in ART. Kotze's data from a multi-centre sHLA-G study furthermore suggested that the presence of sHLA-G is a valuable non-invasive embryo marker to assist in improving pregnancy outcome with the theoretical potential to reduce multiple pregnancies. A combination of sHLA-G expression and extended embryo culture to the blastocyst stage might provide future tools by which to

select single embryos for transfer and reduce the risk of multiple gestation, without compromising pregnancy rates.

EMBRYO PRE-IMPLANTATION GENETIC SCREENING (PGS)

Aneuploidy screening in embryos is applied to patients at risk ie advanced maternal age (AMA) recurrent pregnancy loss, repeat IVF failure or severe male factor infertility (Baruch *et al*., 2008) However, according to 2009 European Society for Human Reproduction and Embryology PGD consortium data collection revealed that after PGS embryos were transferred, only 14.3% 2433/16975 resulted into fetal heart rates (Goossens *et al*., 2009. In the past the most common technique used for PGS was fluorescence in situ-hybridization (FISH) – a fast and relatively easy procedure to perform. Recently the validity of FISH PGS was scrutinized by Staessens *et al.*, 2008 since this procedure did not improve implantation and delivery rates in women <36. Furthermore, it caused a decrease in women with advanced maternal age (AMA) as reported by Hardarson *et al.*, 2008. Was that cleavage stage embyos are affected by mosaicism that affected the accuracy of PGS. (Munne *et al.*, 1994, Bielanska *et al.*, 2002 and Li *et al.*, 2005) Finally another reason why FISH PGS does not improve pregnancy/implantation rates is because the complete genome (22 pairs of outosomal and the two sex chromosomes) are not all screened – leaving the potential to transfer aneuploid embryos, which could potentially influence embryo development and implantation. Initially, Kallioneni *et al.*, 1992 developed comparative genome hybridization (CGH) to screen the whole genome's DNA in tumors. This technique was adopted to study the DNA of single cells – (like blastomeres) by Voullaire *et al.*, 1999 and Wells *et al.*, 1999. The CGH technique is superior to FISH since it screens the whole genome for anomalies such as chromosome imbalances due to aberrant segregation and structural imbalances (gains/losses) larger than 10-20 Mb as reported by Griffin *et al.*, 1998 and Malmgren *et al.*, 2002. A disadvantage of CGH is that it is very time/labor intensive – it takes up to 4 days to obtain results after hybridization and amplification of the DNA and comparing the test DNA to to that of a normal template of chromosomes. The approach of PB-1 and PB-II screening and evaluating the oocyte and zygote's genome (in order to perform of fresh transfer) has been reported by Wells *et al.*, 2002, Sher *et al.*, 2007, Obrados *et al.*, 2008 and 2009 and Fragouli *et al.*, 2009 Day 3 blastomere was evaluated to detect the influence of patenternal contribution as reported by Wilton *et al.*, 2001, Voullaire *et al.*, 2002 Sher *et al.*, 2007 and 2009 and Kotze *et al.*, 2012. Kotze *et al.*, 2012 (GOI accepted for publication) reported no association between

the chromosomal status of PB-1, PB-II and Day 3 blastomeres and subsequent blastocyst formation. However, they reported respectable live birth outcome when sequential screening was applied and normal embryos developed into blastocyst and were transferred.

Lately, the removal of several trophectoderm cells at the blastocyst stage has been reported by Fragouli *et al.*, 2009 and Schoolcraft *et al.*, 2010. Day 3 and blastocyst embryo biopsy required embryo cryopreservation allowing time to complete CGH analysis. In a follow-up FET cycle (frozen embryo transfer) euploid embryos can be thawed and transferred. The potential detrimental effects of cryopreservation post PGD has been reported by Joris *et al.*, 1999, Edgar *et al.*, 2000 and Zheng *et al.*, 2005, resulting in a 30-40 % reduction in implantation potential.

However, recently the introduction of vitrification of blastocyst by Mukaida *et al.*, 2003 and Zhang *et al.*, 2009 has significantly improved the survival of warmed embryos. Kotze *et al.*, 2012 in press, reported 50% live birth rates after the transfer of PGS vitrified/warmed blastocyst. This ideal scenario for embryos undergoing PGS, would be to have results on the analysis in sufficient time for a fresh transfer and avoid cryopreservation.

However, a faster technology emerged, allowing a more rapid and more detailed analysis of PB-I, PB-II, Day 3 blastomeres and trophectoderm cells, namely array-CGH (Hu etal., 2004, Wells *et al.*, 2004, Le Caignec *et al.*, 2006, Treff *et al.*, 2010 and 2011). Different approaches to screen the whole genome compliment within 16 hours, by modifying earlier procedures – to potentially avoid embryo cryopreservation of PGS embryos has been suggested by Gutierrez-Mateo *et al.*, (2004a) and Landwehr *et al.*, (2008).

FISH based technology has been superseded by technology that is able to screen all 22 pairs of autosomal chromosomes and 2 pairs of sex chromomes. Screening the whole genome has indicated that anomalies could be present in any of an embryo's chromosomes. In the study by Koze *et al.*, 2012 (GOI accepted for publication) and Traversa *et al.*, 2011 it has been shown that aneuploid embryos have the potential to develop to a blastocyst. These findings contradict the belief that aneuploidy of the larger chromosomes (1-12) are less likely to develop into a blastocyst. FISH indentifies a single point on a chromosome while CGH-probes cover the entire length of a chromosome, therefore structural anomalies can be detected such as partial duplications and deletions.

Translocation errors can be detected by the ability of CGH to determine a loss/gain of parts of chromosomes. Traversa *et al.*, 2010 has shown that FISH suffers from high false positive abnormality rates. Improved PCR technologies (array-CGH) have been reported by Traversa *et al.*, 2010 to improve translocation screening. Furthermore, CGH can detect translocation in cases where the fragments are large enough – balanced translocation can thus be determined as well as the ploidy status of all other chromosomes. FISH analyzes each cell individually for the specific probe(s) that are applied

Traversa *et al.*, 2010 demonstrated the reliability and feasibility of CGH to detect aneuploidy in blastocysts. Kotze et al 2012 used sequential CGH screening of PBI, PBII and blastomere to identify aneuploid embryos, subsequent blastocysts were vitrified. Blstocyst**s** that were classified as euploid were warmed and transferred, resulting in improved implantation and pregnancy outcomes.

Recently a more rapid method to perform cyto-genetic screening has been introduced – array CGH. Currently there are two approaches that are being applied – single nucleotide polymorphism array (SNP –array) and CGH-array.

- SNP-array:- Common polymorphic DNA sequences found throughout the genome is used to detect any chromosome imbalances and genome wide linkage analysis. Handyside *et al.*, 2009 Vanneste *et al.*, 2009 and Treff *et al.*, 2010/2011.
- CGH-array:- test and reference DNA is hybridized to DNA probes fixed to a slide. Several reports on the successful use of array-CGH Hu et al 2004. Wells *et al.*, 2004 and Le Caignec *et al.*, 2006. Furthermore, some clinical applications have been reported by Hellani *et al.*, 2008, Fishel *et al.*, 2009 and Rius *et al.*, 2010.

The latest studies to improve the efficiency of PGS is the use of BAC microarrays in order to detect aneuploidy in all chromosomes as reported by Fragouli *et al.*, 2011, Guiterrez-Mateo *et al.*, 2011 as well as segmental errors during chromosomal rearrangement as reported by Alfarawati *et al.*, 2011 and Fiorentino *et al.*, 2011.

Array-CGH technology can be applied to improve the identification process of aneuploidy/anomalies of the whole genome's chromosomes. The transfer of embryos deemed euploid should improve pregnancy outcome in patients where aneuploidy contributed to their IVF failure.

In this chapter a literature review was performed to research the impact of the oocyte/zygote and the embryo on implantation/pregnancy rates. The morphologic characteristics of the oocyte;- cumulus –oocyte-complex (COC), zona pellucida, perivitelline space, cytoplasm, meiotic spindle and the polar body and its appearance was studied. The morphologic characteristics of the zygote;- pronuclei orientation, the embryo and its effect on embryo development, ploidy and blastocyst formation and its effect on implantation and pregnancy outcome was also reviewed. The pronuclear morphology, early cleavage and a biochemical marker that is expressed by developing embryos (soluble Human Leucocyte-Antigen-G) and its role in predicting pregnancy outcome predict outcome was discussed as non-invasive markers. The value of blastocyst transfer and the improved ongoing pregnancy rate compared to cleavage stage embryos was also highlighted based on a recent meta-analysis. Genetic testing using FISH, CGH and array-CGH and the future of genomics was discussed as invasive markers.

We conclude that despite all the above mentioned parameters to select an embryo for transfer that will develop into a live baby, extensive research and international corroboration is needed in order to improve and standardize such criteria.

Figure 1a/b: sHLA-G mechanism schematics (Hunt *et al*., (2005)

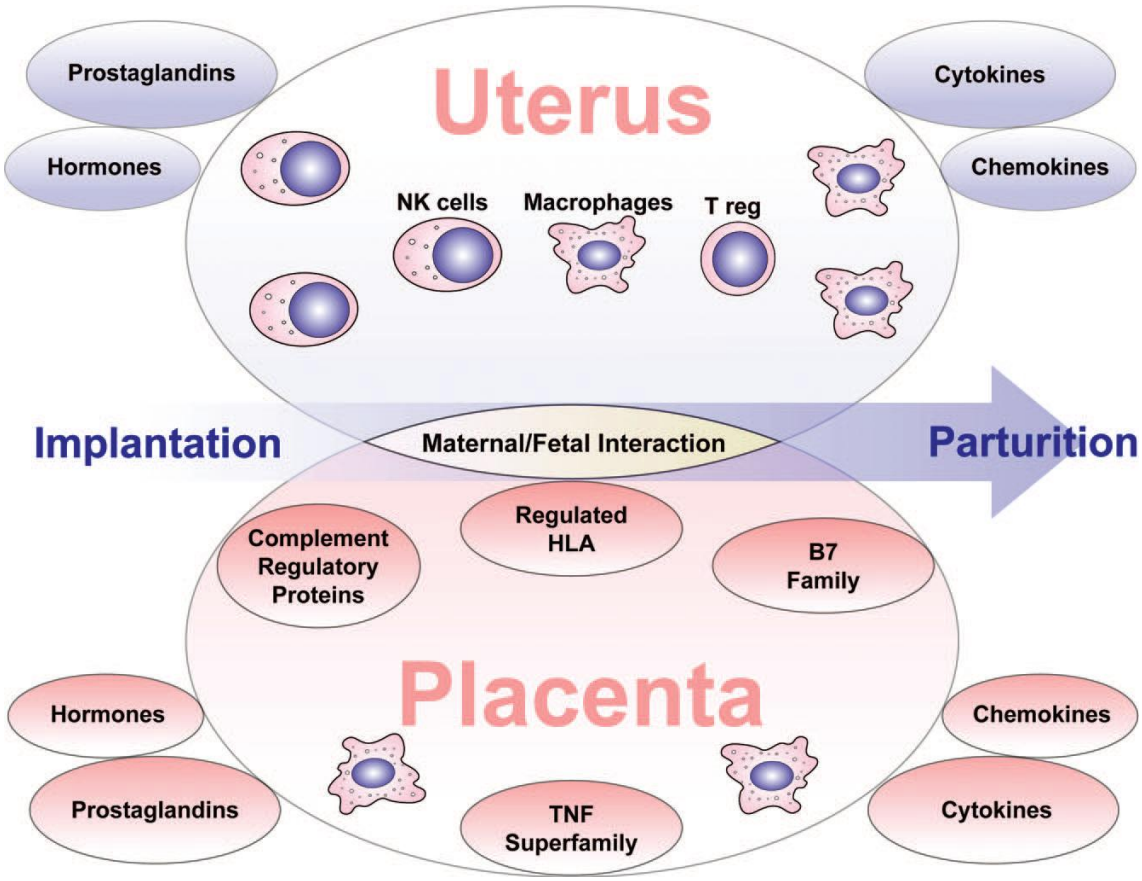

Figure 1a. Multiple mechanisms underlie maternal tolerance of the fetus. Mothers, via changes that occur in the uterus, and embryo/fetuses, via special adaptations of the placenta, contribute to the establishment of an immune privileged environment within which the semiallogeneic fetus resides safely until termination. NK cells, natural killer cells; Treg, CD4_ regulatory T cells; TNF superfamily, tumor necrosis factor superfamily.

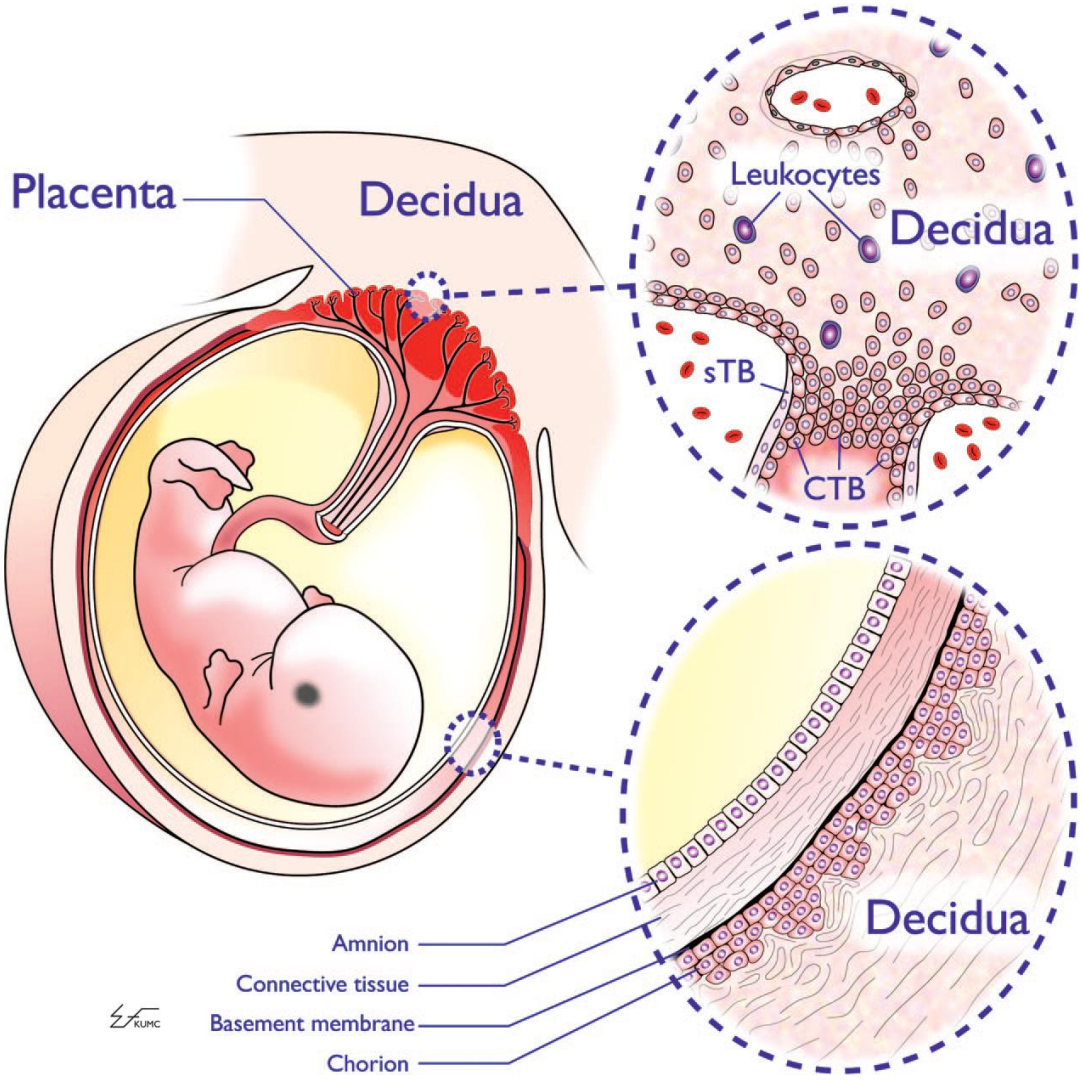

Figure 1b. A schematic illustration of the human fetus, placenta and extraplacental membranes, and modified endometrium known as decidua. The fetus developing within the amniotic sac is surrounded and encased by trophoblast cells in the placenta and chorion membrane (red). Upper insert: Cytotrophoblast (CTB) cells within the placental villi serve as the progenitors for all differentiated trophoblast cell subpopulations, including the syncytiotrophoblast (sTB) layer, which is continuously exposed to maternal blood. This single cell layer is responsible for transfer of maternal-fetal nutrients and wastes, synthesis of placental hormones, and providing a physical barrier to maternal cell traffic into the fetus. CTB cells proliferate and migrate into the decidua, attaching the placenta to the mother and facilitating certain crucial physiological events required for successful pregnancy. Lower insert: The amnion membrane comprised of a single layer of epithelial cells is a strong sac holding the fetus in amnionic fluid. The chorion membrane CTB cells, derived from the migrating extravillous CTB cells, interface directly with maternal decidual cells. (Hunt *et al*., (2005)

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CHAPTER 3

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ASSISTED REPRODUCTION

Embryo selection criteria based on morphology VERSUS the expression of a biochemical marker (sHLA-G) and a graduated embryo score: prediction of pregnancy outcome

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Abstract
Purpose To compare pregnancy and implantation rates when embryos are selected based on a single Day 3 (D 3) morphology score vs. a GES score plus sHLA-G expression.
Methods A prospective randomized study ($n=214$) undergoing fresh ICSI cycles. Embryos were selected for transfer based on either Day 3 morphology score (Group A) or GES-scoring plus sHLA-G expression (Group B).
Results Clinical [35/107 (33%) vs. 52/107 (49%)] and ongoing pregnancy [20/107 (19%) vs. 52/107 (49%)] rates were significantly different between Group A and Group B ($p<0.05$). Implantation rates were not significantly different between Group A [52/353 (15%)] and Group B [73/417 (18%)] ($p<0.05$). The number of pregnancies lost during the first trimester was nearly 12 times higher in Group A [25/52 (48%)].
Conclusion The miscarriage rate was significantly lower in Group B than Group A and the pregnancy results were superior when embryos were selected based on GES plus sHLA-G expression.

Keywords Pregnancy · Implantation · Miscarriage · sHLA-G

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Introduction

Generating criteria to select "competent embryos" for transfer has become of utmost importance. Historically, approaches to identifying the "competent" embryos prior to transfer focused mainly on morphological assessment of embryos. Evidently, morphological evaluations furnish clues that enhance ability in choosing the best pre-embryos for transfer. However, it is severely limited to provide reliable evidence for predicting subsequent normal embryo implantation [1–7]. Desai et al. [8] reported the first scoring system for day 3 embryos, whereas all previous scoring systems were based on the second day of culture (Day 2). Later on the graduated embryo scoring (GES) system was introduced by Fisch et al. [9] in which each embryo was individually cultured, allowing for sequential microscopic assessment of developmental stages starting on day 1 through day 3 of embryo culture.

Determining which morphologic evaluation system [10–13] to use for transferring embryos on Day 2/3, and to achieve pregnancy rates similar to embryos that are transferred at the blastocyst stage, is a controversial subject [14, 15]. Some researchers claimed that transferring selected embryos on day

CHAPTER 3

Embryo selection criteria based on morphology VERSUS the expression of a biochemical marker (sHLA-G) and a graduated embryo score: prediction of pregnancy outcome

ABSTRACT

Purpose

To compare pregnancy and implantation rates when embryos are selected based on a single Day 3 (D 3) morphology score vs. a GES score plus sHLA-G expression.

Methods

A prospective randomized study ($n = 214$) undergoing fresh ICSI cycles. Embryos were selected for transfer based on either Day 3 morphology score (Group A) or GES-scoring plus sHLA-G expression (Group B).

Results

Clinical [35/107 (33%) vs. 52/107 (49%)] and ongoing pregnancy [20/107 (19%) vs. 52/107 (49%)] rates were significantly different between Group A and Group B ($p < 0.05$). Implantation rates were not significantly different between Group A [52/353 (15%)] and Group B [73/417 (18%)] ($p < 0.05$). The number of pregnancies lost during the first trimester was nearly 12 times higher in Group A [25/52 (48%)].

Conclusion

The miscarriage rate was significantly lower in Group B than Group A and the pregnancy results were superior when embryos were selected based on GES plus sHLA-G expression.

INTRODUCTION

Generating criteria to select “competent embryos” for transfer has become of utmost importance. Historically, approaches to identifying the “competent” embryos prior to transfer focused mainly on morphological assessment of embryos. Evidently, morphological evaluations furnish clues that enhance ability in choosing the best pre-embryos for transfer. However, it is severely limited to provide reliable evidence for predicting subsequent normal embryo implantation (Cummins *et al.*, 1986; Puissant *et al.*, 1987; Staessens *et al.*, 1992; Steer *et al.*, 1992; Roseboom *et al.*, 1995; Ziebe *et al.*, 1997; Visser *et al.*, 1993). Desai *et al.* 2000 reported the first scoring system for day 3 embryos, whereas all previous scoring systems were based on the second day of culture (Day 2). Later on the graduated embryo scoring (GES) system was introduced by Fisch *et al.* 2001 in which each embryo was individually cultured, allowing for sequential microscopic assessment of developmental stages starting on day 1 through day 3 of embryo culture.

Determining which morphologic evaluation system (Alikani *et al.*, 1999; Sakkas *et al.*, 1999; Royen *et al.*, 1999; Scott *et al.*, 2000) to use for transferring embryos on Day 2/3, and to achieve pregnancy rates similar to embryos that are transferred at the blastocyst stage, is a controversial subject (Coskun *et al.*, 2000; Kovacic *et al.*, 2002). Some researchers claimed that transferring selected embryos on day 3 yielded a pregnancy rate equivalent to embryos transferred on day 5 or 6 (Scholtes *et al.*, 1996; Blake *et al.*, 2002).

Jurisicova *et al.* 1996 reported that HLA-G expression was present during preimplantation human embryo development. The presence of soluble Human Leukocyte Antigen-G (sHLA-G) mRNA was detected in culture medium surrounding grouped embryos (Fuzzi *et al.*, 2002). The detection of sHLA-G in the culture medium of individually cultured early cleaving embryos (as early as 44–46 h after insemination) provided evidence that embryos could be selected by their maternal genomic function. This is due to the correlation between sHLA-G expression by an embryo and its potential to reach a pregnancy (Sher *et al.*, 2004).

In our study sHLA-G expression within a range of 0.184–0.196 was considered positive. This original optimal range was determined by Keskintepe (unpublished data) and was slightly different from other investigators (Sher *et al.*, 2004; Fisch *et al.*, 2007).

While several tools are available for the evaluation of embryos for transfer, other unknown factors such as oocyte quality and embryo dismorphism, still remain unanswered. The purpose of this study was to compare pregnancy and implantation rates when embryos are selected based on a single Day 3 (D 3) score vs. sHLA-G expression plus a graduated embryo scoring (GES).

MATERIALS AND METHODS

Patients

An initial pilot study was performed on 58 patients to compare pregnancy outcome when using a single D 3 morphology versus the GES-scoring system. We reported no statistical differences between these two treatment groups. Based on the findings of our pilot study, a subsequent study was designed. In Group A, the most common way (morphology and developmental rate) by which embryo selection is performed, was compared to Group B, a combination of non-invasive criteria (the expression of soluble HLA-G plus the GES-score).

A Statistical program (Epi-info, UMassAmherst) was used to perform a power calculation for this study. Using the given parameters—confidence (95%) and the power (80%) and the ratio of cases to controls—it was evident that using one hundred and seven patients per treatment group would be sufficient. Two hundred and ninety seven (297) patients were assessed for eligibility to participate in this study (Fig. 1). Two hundred and thirty three (233) patients underwent randomization prior to egg retrieval. At this time, patients drew a single tab from a prepared envelope to determine their study group and whether their embryos will be transferred based on a single Day 3 score (Group A) or an sHLA-G expression plus a GES-score (Group B). (Furthermore, in Group A, the culture medium also underwent sHLA-G testing but the embryologist was blinded for the sHLA-G results of these embryos prior to transfer). Patient diagnosis included endometriosis, tubal factor, PCOS, unknown and male factor. All patients were <39 years of age and had a normal uterine cavity and normal endometrial thickness (≥ 9 mm) at the time of hCG administration).

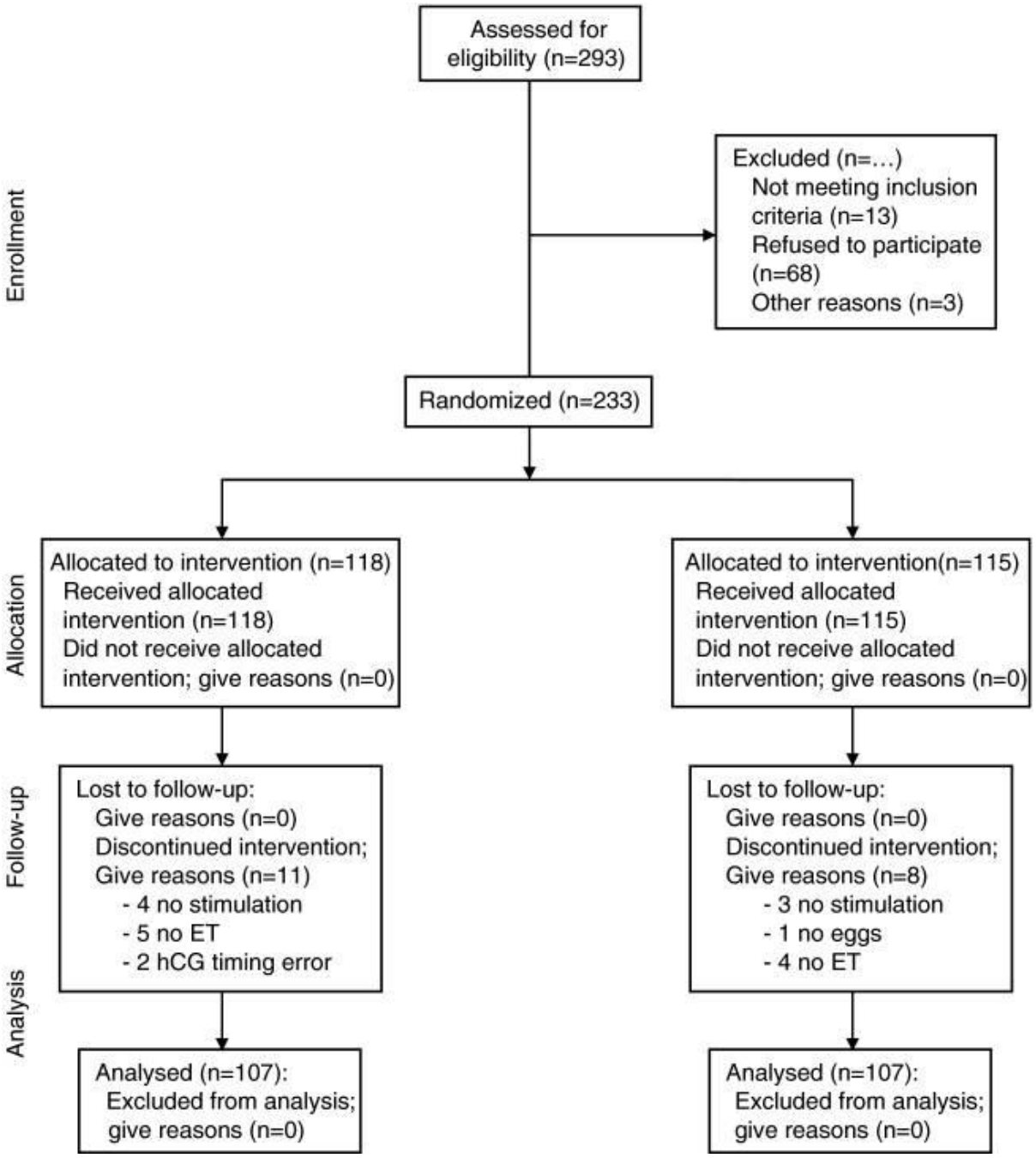


Fig. 1

CONSORT statement flow diagram

Individually cultured embryos whose surrounding media expressed sHLA-G with an OD = 0.190 ± 0.006 (the geometric mean) were defined as positive for sHLA-G expression, whereas those outside the range (0.184–0.196) were designated as negative (Table 2).

TABLE 1

Graduated embryo scoring (GES) of cleavage stage embryos

Evaluation	Hours after insemination	Developmental milestone	Score
1	16–18	Nucleoli aligned along pronuclear axis	20
		Cleavage regular and symmetrical Fragmentation[a]	30
2	25–27	Absent	30
		<20%	25
		>20%	0
3	64–67	Cell number and grade[b] 7CI, 8CI, 8CII, 9CI	20
		7CII, 9CII, 10CI, 11CI, Compacting I	10
Total score			100

Fisch. Graduated embryo score (GES). Fertil Steril 2003

[a]If the embryo was not cleaved at 25–27 h, grading of fragmentation should occur at the 64–67 h evaluation if the embryo reached the seven-cell stage and had <20% fragmentation

[b]Grade I = symmetrical blastomeres and absent fragmentation. Grade II = slightly uneven blastomeres and <20% fragmentation. Grade III = uneven blastomeres and >20% fragmentation. Grade A embryos are seven or more cells with <20% fragmentation.

(Figure 3 sHLA-G Illustration p.126)

Table 2

Comparative analysis data analysis between day 3 score and GES-Score Plus sHLA-G[a]

Parameter	D.3 Score ($n = 107$)	GES score plus sHLA-G expression ($n = 107$)	*P*-value
Age (years)	35.2 ± 4.0[a]	35.1 ± 4.0[a]	0.58
Total no. oocytes	9.7 ± 6.2[a]	11.4 ± 6.2[a]	0.20
Mature oocytes	7.3 ± 5.0[a]	9.2 ± 4.2[a]	0.30
No. Oo[a]cytes fertilized	7.1 ± 4.4[a]	8.5 ± 4.7[a]	0.36
Total no. embryos	6.8 ± 3.3[a]	8.0 ± 3.9[a]	0.32
No. embryo transferred	3.3 ± 0.8[a]	3.9 ± 0.8[a]	0.15
Biochemical pregnancies (%)	42 (39)[b]	60 (56)[b]	0.05*
Clinical pregnancies (%)	35 (33)[b]	52 (49)[b]	0.05*
Ongoing pregnancies (%)	20 (19)[b]	52 (49)[b]	0.05*
Implantation rates (%)	(15)[b]	(18)[b]	0.15

[a]Data expressed as means±SD

[b]Data expressed as (%)

*Significance $p < 0.05$

Stimulation

Patients were pre-treated with oral contraceptives received Lupron (TAP Pharmaceuticals, Lake Forest, IL) in a long protocol after pre-treatment with oral contraceptive (OC) birth control pills for 1–3 weeks and were treated by a human derived gonadotropin (Bravelle; Ferring Pharmaceuticals Inc, Suffern, NY) to activate ovarian follicular stimulation. Ovulation was triggered when at least two follicles were 18 mm and half the remaining were ≥15 mm. Oocytes were harvested transvaginally using ultrasound guidance 35 h post hCG. All patients underwent controlled ovarian hyper-stimulation (COH) by the same physician.

Embryo culture

All metaphase II (MII) embryos were injected by ICSI 3–4 hours post retrieval. All zygotes were cultured individually in 50 µl droplets of P-1 medium, supplemented with 10% Synthetic Serum Substitute (SSS) (Irvine Scientific, Santa Ana, CA). After 44–46 h embryos were moved to Complete Blastocyst Medium (Irvine Scientific, Santa Ana, CA). At this point, ±35 µl from the remaining P-1 culture media drops were collected in 200 µl micro-centrifuge tubes and immediately frozen. All samples were shipped to a central location and tested for sHLA-G, using an enzyme-linked immunosorbent sandwich (ELISA) assay. All embryos were transferred on day 3.

Embryos scoring

The GES-score

To apply the GES-score, all mature oocytes underwent ICSI with a single sperm and were individually cultured. In order to apply the GES-score, oocytes were evaluated at 16–18 h post ICSI when the presence of pronuclear as well as nucleoli alignment along the pronuclear axis was evident, a score of 20 was allotted where nucleoli alignment was prevalent. The second observation took place at 25–27 h, at which time early cleavage was noted. A score of 30 was allotted when cleavage was observed. Furthermore, at this time a score was given based on the presence/absence of fragmentation. When fragmentation was absent a score of 30 was given, <20% fragmentation received a score of 25 and >20% received a score of zero (0). The third and final score was given 64–67 h

after ICSI and involved the number of blastomeres and embryo grade. Example: six cell grade one—6(I),7(I), 8(I), 8(II), 9(I) scored 20 points, seven cell, grade II (7(II)), 9(II), 10(I), 11(I) and compacting(I) scored ten points. The maximum GES-score totaled 100 points

The Day 3 score

The Day 3 score that was applied in this study was a modification of Veeck's [21] criteria for Day 3 embryos combined with our laboratory's criteria (unpublished data DK). Embryos were scored based on their blastomere number, size and symmetry as well as the percentage of fragmentation that was present. Example: a grade one embryo: 8–11 cell with even sized blastomeres (+8 points) and without fragmentation (+2 points) scored a max of ten points, a grade two embryo: 8–11 cell with even sized blastomeres (+8 points) and with <10% fragmentation (0 points) scored a max of eight points, a grade three embryo 8–11 cell (+8 points) with uneven blastomere (−1 points) and >10% fragmentation (−3 points) scored a max of four points.

The same embryologist preformed all embryology and embryo scoring in this study.

Soluble HLA-G assay

A monoclonal antibody (mAb; MEM-G9 MCA 2044; Serotec, Raleigh, NC) against sHLA-G was used to coat a 96-well Nunc-Immunoplate (Fisher Scientific, Chino CA) using a concentration of 2 μg/ml in 0.1 mol/l carbonate buffer at pH 9.5 for 1 h at 37°C. The plate was then refrigerated overnight at 4°C. On the following day, the plate was thoroughly washed using 100 μl of phosphate-buffered saline (PBS) and 0.05% Tween-20. The wash was repeated twice using 100 μl PBS and 5% bovine serum albumin (BSA) for 15 min each. A 50 μl aliquot of PBS and 5% BSA was added to each well before adding the sample of 50 μl of embryo supernatant. Amniotic fluid (AF) was used as a positive control. AF (50 μL) and 50 μl of pure Complete Blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) Samples were incubated for a period of 1 h at 37°C. After incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin conjugated mAB (w6/32 MCA81B; Serotec) at a 1:1000 dilution in PBS and 1% BSA for 45 min at 37°C and then washed five times with PBS. Streptavidin alkaline phosphatase conjugated (BD Bioscience Pharmigen, San Diego, CA) at a concentration of 1:1000 in carbonate buffer was incubated for 30 min at 37°C and

washed five times with PBS, after which phosphatase substrate was added at a concentration of 1 mg/ml in 10% diethanolamine at pH 9.8 for 30 min. The colorimetric reaction was then stopped by the addition of 50 µl of 3 mol/l NaOH. The sHLA-G concentration was determined by absorbance at 405 nm on the EL800 Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT).

Embryo selection and transfer

Individual embryos were defined as having positive sHLA-G expression if their surrounding media expressed sHLA-G with an optical density inside the range of 0.184–0.196. Those outside the above mentioned range were designated as sHLA-G negative. In Group A, one hundred and seven (107) patients received embryos for transfer and all of these embryos were selected by using a Day 3 score only. In Group B, one hundred and seven (107) patients received embryos for transfer by first selecting any embryos that had a positive sHLA-G expression, and correlating such with the highest available GES score. Furthermore, in Group B the patients received embryos containing at least one sHLA-G positive in the cohort for transfer. In this study no more than four (4) embryos were recommended for transfer. However, the final decision regarding the number of embryos for transfer was left to the patient after an informed consent that was based on American Society for Reproductive Medicine guidelines. All embryos were transferred on day 3 of culture, using ultra sound guidance and an echogenic catheter (Wallace, Smith Medical, UK). Serum β-hCG levels were measured 11 days and 13 days after the transfer. The patient was considered positive for biochemical pregnancy when the first value was >5.0 IU and the next value 2 days later was double the first. Clinical pregnancy rates were based on a six (6) week ultrasound with a gestational sac containing a fetal heartbeat. Ongoing pregnancy rates were based on a ten to twelve (10–12) week ultrasound with gestational sac containing one or more growing fetuses with appropriate heart rates.

Statistical analysis

Statistical analysis was carried out using SPSS version 15 (Statistical Package for the Social Science). The number and percentage (%) of categorical data as well as the mean and standard deviation (SD) of continuous data were calculated. Comparison between mean values of continuous variables was calculated using the Students *t*-test for parametric and the Mann–Whitney-*U* test for non-parametric data, while the Chi-square

was used for categorical data and the Odds ratio and 95% confidence calculated. Significance value was set at $p < 0.05$.

Miscarriage rates between treatment groups were calculated as a percentage of pregnancy loss between biochemical, clinical and ongoing rates. Fetal loss between treatment groups were calculated between clinical and ongoing pregnancies. Retrospective data were calculated as a percentage.

Institutional review

Since January 2005, all embryos at SIRM underwent routine sHLA-G assay to determine the expression of this potential biochemical marker in the culture medium surrounding embryos. All patients were counseled regarding the risks, benefits, and alternatives to sHLA-G testing; however, because it was not considered an experimental component of the treatment, a specific institutional review board was not sought. However, for the purpose of this PhD study, ethical approval was obtained from the Ethical committee of the University of Stellenbosch (N06/07/119). Furthermore, all clinical research conducted was in full compliance with guidelines of the American Society of Reproductive Medicine and met ethical principles involving human subjects as defined by the Declaration of Helsinki in 1964.

RESULTS

In the prospective data analysis, no statistical significance was found between the two treatment groups in regards to age, total number of oocytes, mature oocytes, number of oocytes fertilized, number of embryos, or number of embryos transferred and implantation rates. However, significance was reached for biochemical (39.3% vs. 56.1%), clinical (32.7% vs. 48.6%), and ongoing pregnancy (18.7% vs. 48.6%) rates between Group A and Group B, respectively (Table 2). In this study all embryos underwent sHLA-G testing. However, in Group A, sHLA-G results were blinded (therefore, embryos were selected without knowing the sHLA-G results prior to transfer).

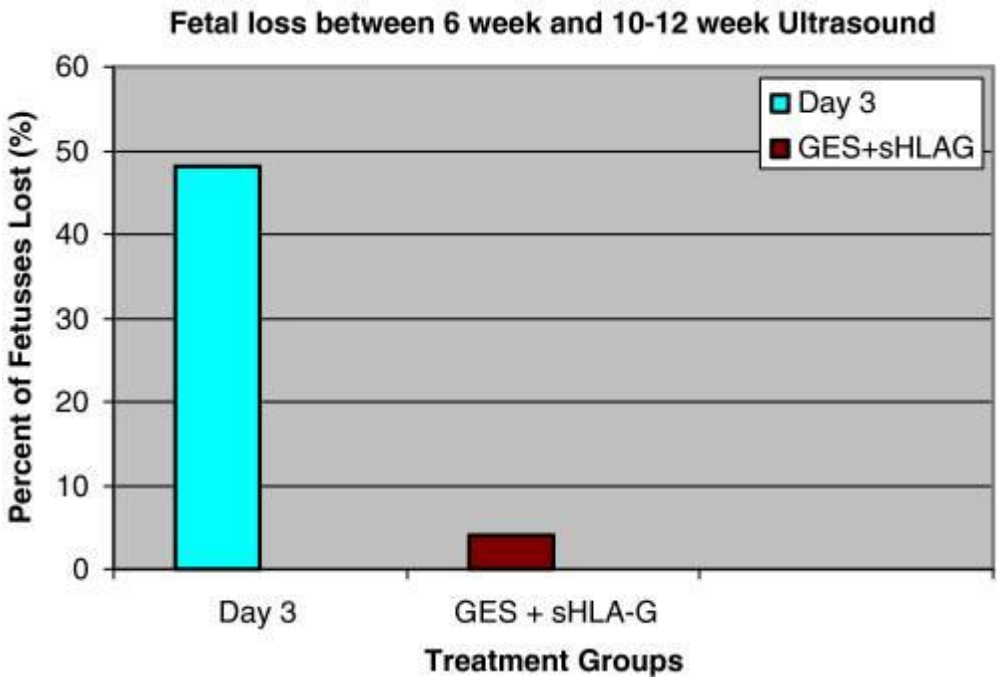
A total of 770 embryos were transferred in both groups:

In Group A, 353 embryos were transferred into 107 patients, 42/107 (39.2%) positive β-hCG resulted. The 6 week ultrasound revealed that 35/42 (83.3%) pregnancies continued since β-hCG testing was performed. The breakdown included 23 singletons, eight sets of twins, three sets of triplets and one set of quadruplets, resulting into 52 fetuses with heartbeats confirming an implantation rate of 14.7%. Furthermore, the 10–12 week ultrasound revealed that 20/35 (57.1%) pregnancies continued since 6 week ultrasound was performed. The breakdown included 13 singletons, seven sets of twins, zero triplets zero quadruplets, resulting into 27 fetuses with heartbeats. The miscarriage rate (fetal loss) between biochemical and clinical pregnancies were 7/42 (16.7%) and between clinical and ongoing pregnancies were 15/35 (42.9%). Furthermore, in Group A the total pregnancy loss between biochemical and ongoing pregnancies were 20/42 (total number of pregnancy losses)/(initial number of biochemical pregnancies) (47.6%). Furthermore, there was a loss of 25 fetuses between 6 week and the 10–12 week ultrasound.

In Group B, 417 embryos were transferred into 107 patients, 60/107 (56.1%) patients had positive β-hCG results. The 6 week ultrasound revealed that 52/60 (86.7%) pregnancies continued since β-hCG testing was performed. The breakdown included 36 singletons, 12 sets of twins, three sets of triplets and one set of quadruplets, resulting into 73 fetuses with heartbeats confirming an implantation rate of 17.5%. Furthermore, the 10–12 week ultrasound revealed that 52/60 (86.7%) pregnancies continued since 6 week ultrasound was performed. The breakdown included 38 singletons, ten sets of twins, four triplets zero quadruplets, resulting into 70 fetuses with heartbeats. The miscarriage rate between biochemical and clinical pregnancies were 8/60 (13%) and between clinical and ongoing pregnancies were 0/52 (0%). Furthermore, in Group B the total pregnancy loss between biochemical and ongoing pregnancies were 8/60 (total number of pregnancy losses)/(initial number of biochemical pregnancies) (13.3%). Furthermore, there was a loss of three fetuses between 6 week and the 10–12 week ultrasound.

Implantation rates per embryo transferred were not significantly different at 52/353 (14.7%) and 73/417 (17.5%) for Group A and Group B, respectively ($p > 0.05$). However, the pregnancy loss rates between biochemical and ongoing pregnancies were significantly different at 22/42 (52.3%) and 8/60 (13.3%) for Group A and Group B, respectively ($p < 0.05$). Fetal loss between treatment groups were 25/52 (48.1%) and 3/73 (4.3%) for Group A and Group B, respectively and was statistically significant ($p < 0.05$) (Fig. 2).

Fig. 2 Fetal loss rates



Prospective data for Group B indicated that ninety three out of one hundred and seven (93/107) patients (86.9%) received a cohort of embryos for transfer that contained at least one sHLA-G positive embryo combined with the highest available GES-score. This “sHLA-G positive sub-group” resulted into 55/93 (59.1%) (biochemical), 50/93 (53.8%) (clinical) and 50/93 (53.8%) (ongoing pregnancy), respectively. Furthermore, fourteen out of one hundred and seven (14/107) patients (13.1%) received embryos for transfer that contained ALL sHLA-G negative embryo combined with the highest available GES-score. This “sHLA-G negative sub-group” resulted into 5/14 (35.7%) (biochemical), 2/14 (14.2%) clinical) and 2/14 (14.2%) (ongoing pregnancy), respectively.

Retrospective data for Group A and un-blinding of sHLA-G results revealed that twenty seven out of one hundred and seven (27/107) patients (25.2%) received a cohort of embryos for transfer that contained at least one sHLA-G positive embryo combined with the highest available GES-score. This “sHLA-G positive sub-group” resulted into 17/27 (63.0%) (biochemical), 14/27 (51.9%) (clinical) and 14/27 (51.9%) (ongoing pregnancy), respectively. Furthermore, eighty out of one hundred and seven (80/107) patients (74.8%) received embryos for transfer that contained ALL sHLA-G negative embryo combined with the highest available GES-score. This “sHLA-G negative sub-group” resulted into 25/80 (31.2%) (biochemical), 21/80 (26.3%) clinical) and 6/80 (7.5%) (ongoing pregnancy), respectively.

Combined data regarding the s-HLA-G status from group A and B revealed that 120/214 (56.1%) of patients received a cohort that included at least one sHLA-G-positive embryo. This subgroup resulted in 72/120 (60.0%) biochemical, 64/120 (53.3%) clinical and 64/120 (53.3%) ongoing pregnancies, respectively. Furthermore, 94/214 (43.9%) of patients received a cohort that included ALL sHLA-G-negative embryos. This subgroup resulted in 30/94 (31.9%) biochemical, 23/94 (24.5%) clinical and 8/94 (8.5%) ongoing pregnancies.

Summarizing: pregnancy outcome for patients that received sHLA-G-positive cohorts for transfer versus those that received sHLA-G negative cohorts for transfer were 60.0% vs. 31.9% (biochemical), 53.3% vs. 24.5% (clinical) and 53.3% vs. 8.5%, respectively and was significant ($p < 0.05$).

DISCUSSION

The aim of this prospective randomized study was to compare a traditional embryo selection method (Day 3 morphology) with a novel GES-score plus sHLA-G expression and its impact on implantation, pregnancy and miscarriage rates. To find non-invasive techniques to improve pregnancy outcome and also reduce high order multiple has been an ongoing challenge since the start of IVF in 1988. Earlier studies have outlined different criteria for embryo selection, focusing on a variety of parameters. Pronuclear morphology such as orientation, surrounding halo, number of nucleoli and its arrangement at the juxta-junction and the role of precursor bodies, early cleavage, and Day 3 blastomere morphology including number of blastomeres, their shape and size, and the percentage of fragmentation provided better understanding of embryo development pattern. Whether to transfer embryos at the cleaved or the blastocyst stage is still an ongoing argument.

All the above approaches has been based on morphology, however, the detection of sHLA-G in culture medium surrounding clusters of developing embryos (Jurisicova *et al.*, 1996; Fuzzi *et al.*, 2002; Menicucci *et al.*, 1999) sparked a new interest in the search for non invasive markers, or so-called proteomics, to assist in the identification of the “competent” embryos for transfer. In the study presented here, a traditional Day 3 morphology compared to the sHLA-G expression plus a GES-score in order to identify embryos for transfer resulting into favorable clinical and ongoing pregnancy rates. These findings are supported by a number of researchers (Sher *et al.*, 2004; Noci *et al.*, 2005;

Fisch *et al.*, 2007; Yie *et al.*, 2005; Desai *et al.*, 2006). Furthermore, we reported a decrease of fetal loss, in the sHLA-G positive group, between 6 week and 12 week ultrasound, a finding supported by Rebmann *et al.* 2007.

The exact mechanism by which sHLA-G improved the ongoing pregnancy outcome is unknown; however, whether it is due to good communication (cross-talk) (Viganò *et al.*, 2003), or the suppression/masking of the natural immune response (Gal *et al.*, 1999; Navarro *et al.*, 1999), the presence of sHLA-G has been postulated to protect the conceptus from destruction by the maternal immune response (Hviid *et al.*, 2004). The first two cell cycles of human embryogenesis (that is embryonic divisions up to the 4-cell stage) are regulated by the maternal genome (Tesarik *et al.*, 1986; Braude *et al.*, 1988). Activation of the embryonic genome takes place between the 4-cell and 8-cell stages post fertilization in humans and is essential for zygote protein synthesis and subsequent embryo development. With these assumptions, we suggest that collecting the culture medium surrounding individually cultured embryos at 44–46 h post ICSI and subsequently analyzing it for the presence of sHLA-G will help to identify embryos with a “healthy” maternal genome, which should convert into a properly activated embryonic genome, resulting into a competent developing embryo.

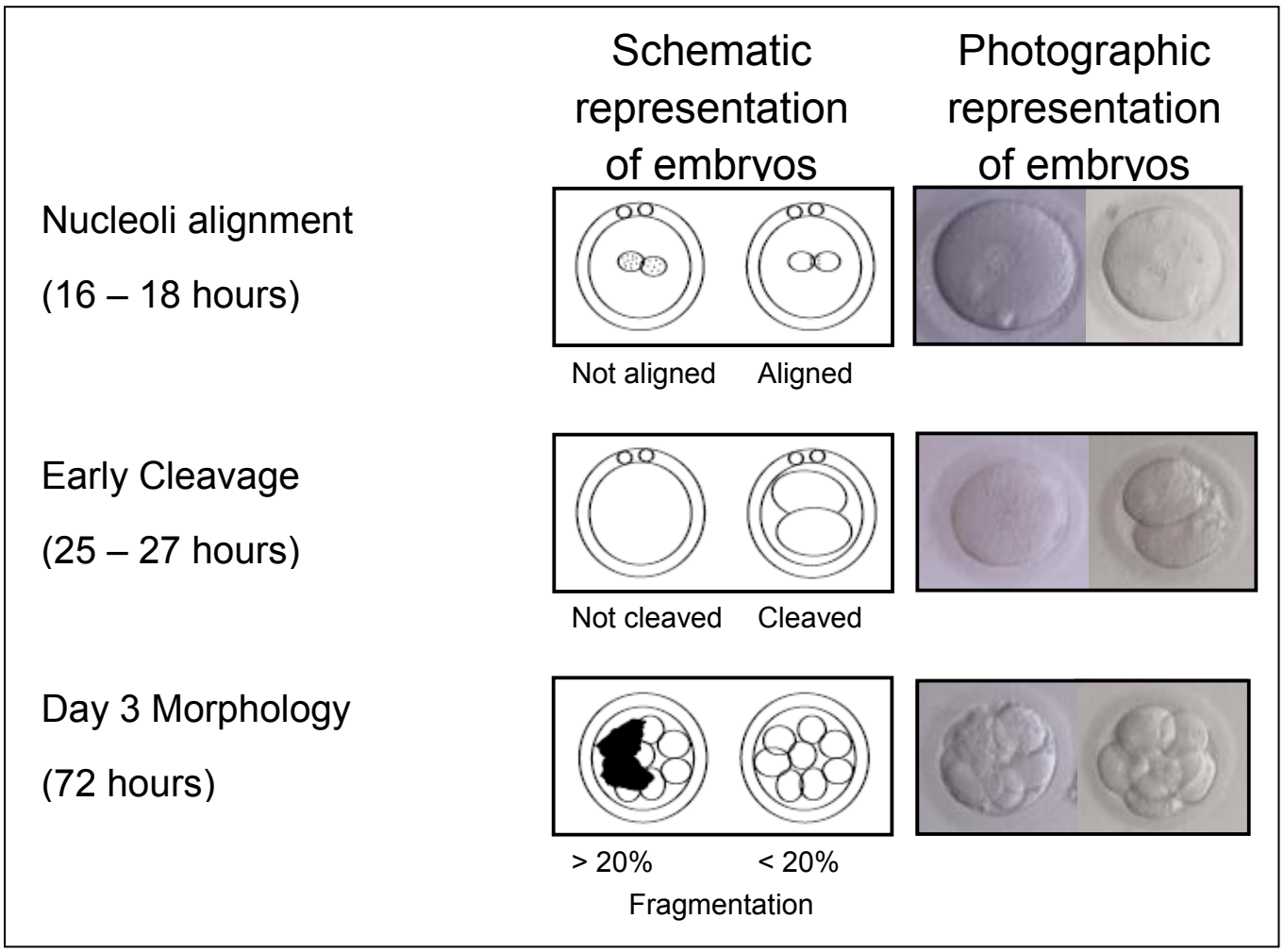
In a recent meta-analysis it was concluded that the presence of sHLA-G in the supernatants of cultured embryos is moderately helpful to predict the ability to achieve pregnancy in women undergoing IVF treatment (Vercammen *et al.*, 2008). Additionally they suggested it to be more beneficial if the embryos are of good quality. In our study we used both the expression of sHLA-G and applied a novel, cumulative embryo score (GES), providing detailed information regarding each embryo’s morphology and biochemical competency The implantation rates in the groups studied, did not differ significantly but the fetal loss rate was significantly lower in the sHLA-G-positive group. Furthermore, data revealed a significant increase in the biochemical, clinical and ongoing pregnancies in patients that received cohorts containing at least one sHLA-G-positive embryo vs. cohorts containing all sHLA-G-negative embryos for transfer. This finding highlights a further benefit in performing sHLA-G in an IVF program. This knowledge prior to embryo transfer will assist in selecting the better embryo.

This study is a first prospective randomized study comparing the traditional way of embryo evaluation versus sHLA-G expression plus a cumulative GES-score on day 3 as selection

criteria. It was performed by the same physician, using the same stimulation drug, the same embryologist performed the embryology on all the patients and sHLA-G ELISA was performed by the same lab in order to minimize as many variables as possible.

We conclude that by combining a positive sHLA-G expression with the highest GES-score on Day 3 resulted into significantly improved ART outcome. Furthermore, that the power of sHLA-G status is a very important criterion to identify prior to embryo transfer.

Figure 3 - GES – SCORE - ILLUSTRATION



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CHAPTER 4

To be submitted: J Assist Reprod Genet

CHAPTER 4

The prediction of pregnancy outcome using a biochemical marker (sHLA-G) – a multi centre study.

ABSTRACT

Introduction

Several studies have reported an association between the presence of soluble human leukocyte antigen G (sHLA-G) in human embryo culture supernatants (ES) with implantation and pregnancy outcome in vitro. However, the actual presence, role during implantation and effect on implantation and pregnancy outcome is still controversial at best.

Aim

A retrospective multi-centre study was performed on 2040 ICSI patients in six different centers, to determine if the presence of sHLA-G have an effect on implantation and pregnancy outcome in vitro.

Methods

All embryos were individually cultured and a chemiluminescence enzyme-linked immunosorbent assay was used to detect the presence sHLA-G in culture medium surrounding embryos. A pregnancy test was performed 10 days after embryo transfer (ET) and an ultrasound performed at 7 weeks pregnancy duration.

Results

In all centers, a positive sHLA-G result was associated with an increase in odds of ongoing pregnancies (OR: 3.27, 95% CI: 2.67 to 3.99, p-value: <0.0001), and an increased odds of multiple on-going pregnancies (OR: 1.66, 95% CI: 1.10, 2.51, p-value: 0.0170).

Discussion

Data from this multi centre study emphasize that sHLA-G expression is a valuable non invasive embryo marker to assist in improving pregnancy outcome with the theoretical potential to reduce multiple pregnancies. A combination of sHLA-G expression and extended embryo culture to the blastocyst stage might provide future tools by which to

select single embryos for transfer and reduce the risk of multiple gestational, without compromising their pregnancy rates.

BACKGROUND

Despite improvement of in vitro embryo culture conditions, stimulation and transfer techniques no significant improvement in implantation rates have been reported over the last decade. Early studies using non-invasive criteria by which to select embryos for transfer have been reported, morphologically assessing of embryos (Ng *et al.*, 1999, Richter *et al.*, 2001, Fisch et al, 2001), extended embryo culture to the blastocyst stage (Schoolcraft and Gardner, 2000) and the detection of soluble HLA-G in culture medium surrounding embryos, (Jurisicova *et al.*, 1997, Menicucci *et al.*, 1999 and Fuzzi *et al.*, 2002). Furthermore, invasive advances in pre-implantation chromosomal screening of embryos and aneuploidy have been reported (Gianaroli *et al.*, 1997a/b, Munne S., 2003a/b, Pehlivan *et al.*, 2003). However, currently no individual criterion is capable of accurately identifying an embryo's potential to develop into a live birth. In an effort to improve IVF success rates, multiple embryos had been transferred, subsequently resulting in a risk of high order multiple pregnancies. In order to limit such risk, the search for an accurate and precise "tool" to identify embryos that has an improved chance to develop into a live baby, is essential.

The implantation mechanism is a very poorly understood aspect of reproduction.It is a highly complex dialog (cross-talk) between the embryo and the endometrium, (Simón *et al.*, 1995, Hill 2001, Viganò *et al.*, 2003 and Shimomura *et al.*, 2006). Implantation requires the successful suppression of the maternal immune system. The human body will identify and discriminate against foreign tissue by major histocompatability complex (MHC), human leukocyte antigen (HLA) originated from a group of genes located on the short arm of chromosome six (6). The MHC evokes T-cell intervention to non-self antigens expressed by an individual of the same specie. The human fetus is a considered a non-self tissue to the maternal uterus due to paternal MHC antigens, however, during pregnancy the immune system develops fetal tolerance (Siato *et al.*, 2007). HLA-G produced by the extra-villous cytotrophoblast (the only fetal contact with maternal uterine cells), confers immuno-tolerance through interaction with maternal uterine membrane lymphocytes. This scenario would suggest that HLA-G protects the fetus from maternal immune response attack. HLA-G is expressed by the placenta throughout gestation and is

also present in amniotic fluid. Soluble-HLA-G (a spliced iso-form of membrane bound HLA-G) is in suspension and can be detected in culture medium. sHLA-G, a non-classic type I human leukocyte antigen, was first identified in the media surrounding groups of embryos and blastocysts in culture by Jurisicova *et al.*, 1996. Menicucci et al 1999, first identified, sHLA-G (a non-classic type I human leukocyte antigen), in the culture media surrounding a group of day 3 embryos. Fuzzi et al, 2002 has shown that the presence of sHLA-G in the culture media harboring groups of three day old cleaved embryos, correlated both with their cleavage rate and with their overall subsequent implantation potential. Hviid *et al.*, 2004 postulated that the presence of sHLA-G protected the conceptus from destruction by the maternal immune response. Several studies regarding sHLA-G and its effect on pregnancy outcome have been reported since, (Sher *et al.*, 2004, 2005a,b; Criscuoli *et al.*, 2005, Noci *et al.*, 2005, Yie at al., 2005, Desai *et al.*, 2006, Fisch *et al.* 2007 Rebmann *et al.*, 2007 and Kotze *et al.*, 2010). In a review studies by Sargent *et al.*, 2007 and Vercammen *et al.*, 2008 they thoroughly evaluated and discussed previous studies regarding sHLA-G and ART outcome. Furthermore, Rebman *et al.*, 2005 addressed specific issues dealing with sHLA-G ELISA protocols.

The goal of this multi-center retrospective study was to evaluate how the presence or absence of sHLA-G expression and untested culture medium surrounding embryos affected IVF outcome.

STATISTICAL METHODS

The data was analyzed, using STATA, through a series of mixed effects logistic regression models in which site was included as a random effect. A mixed effects logistic regression model was fitted for each of the three outcomes; chemical pregnancy, clinical implantation, and on-going pregnancy. Each outcome was regressed against the sHLA-G test result with mean age, mean number of embryos utilized per group, and day of transfer included as covariates in the analysis. We report each parameter estimate with its 95% confidence interval and associated p-value, for each model fitted, as well as the odds ratio for each parameter and its corresponding 95% confidence interval.

Embryos were transferred on either the Day 3 or the Day 5/6 (blastocyst) post-fertilization. For each clinic, the patients were divided in separate groups based on day of transfer (as mentioned above) and sHLA-G results noted (positive, negative, and untested). For each

group, the data consists of the number of chemical pregnancies, clinical implantations and ongoing pregnancies, respectively. The number of on-going pregnancies was also recorded and reported as singleton, twins, triplets or quadruplets pregnancies. In addition, we calculated the mean age and standard deviation of the women in each group, as well as the average number of embryos utilized per group.

MATERIALS AND METHODS

Patients

Consenting ART patients between July 2003 and Dec 2010 who underwent intracytoplasmic sperm injection (ICSI), the majority used a specific ELISA 46 hours post-ICSI to determine sHLA-G expression, however, some embryos were transferred as "untested".

The Study design

Data was retrospectively gathered from six fertility clinics (Los Angeles (LA), Las Vegas (LV), New Jersey (NJ), New York (NY), FISCH, and Sacramento (SAC) that performed a sHLA-G assay/test on the majority of their consenting ART patients between July 2003 and Dec 2010. Since protocols at all clinics were standardized, the procedural variabilities were limited. We retrospectively compared IVF outcome in all patients regardless of age since the goal was to compare ART outcome for sHLA-G positive, sHLA-G negative and untested cohorts. The majority of clinics transferred embryos on Day 3, however, data for embryos transferred on Day 5/6 (blastocyst) as well as single embryo transfer were also recorded.

Ovarian Stimulation

Patients were stimulated using similar protocols at all sites. All patients received Lupron (TAP, Pharmaceuticals) in a long protocol after pretreatment with oral contraceptive pills for one to three weeks. Ovarian follicular development was stimulated with recombinant-FSH at doses of 225-450IU a day. Ovulation was triggered when at least 2 follicles were 18 mm and half the remainder was ≥15mm. Oocytes were recovered transvaginally under ultrasound guidance 34.5 hours later. All monitoring of controlled ovarian hyper stimulation (COH) as well as egg retrievals (ER's) and embryo transfers (ET's) were performed by the same physician at each center.

Embryo Culture

All metaphase II (MII) oocytes were fertilised using ICSI 4-6 hours after retrieval. All embryos were cultured individually in 35µl droplets of P1 (Irvine Scientific) supplemented with 10% SSS (Irvine Scientific) using Nunc 60x15mm dishes, since 2007 embryos were cultured individually in 35µl droplets of GLOBAL (LifeGlobal) supplemented with 10% SSS (Irvine Scientific) using Embryo Corral (SunIVF) dishes under oil at 37°C in a 6% $C0_2$, 5% O_2, 89% N_2 environment. All embryos were sequentially microscopically evaluated over a period of 72 hours following ICSI and graded by Graduated Embryo Scoring (GES) (See Chapter 3 Table 1). Embryos were transferred into extended culture medium 44 - 46 hours post-ICSI, which can improved embryo development, as reported previously (Keskintepe). Initially individual embryos whose surrounding media expressed sHLA-G within an optic density (OD) range of 0.190 ± 0.006 (the geometric mean) were defined as having positive sHLA-G expression while those outside this range were designated as sHLA-G negative. Each centers applied different criteria for "negative" to "positive" sHLA-G ranges, (Table 2).

The original droplets of culture medium (35µl) were collected in 0.5 ml micro-centrifuge tubes with attached cap (VWR-Scientific) – frozen immediately at -20 °C and shipped on ice for sHLA-G expression testing – to a central location- were identical sHLA-G Assay were used (west of Mississippi to Las Vegas (LV) –east of Mississippi to New York (NY) - using the same specific enzyme-linked immunosorbent (ELISA) assay. Furthermore, all embryos were graded by applying the GES –score, GES≥70 scoring embryos combined with positive sHLA-G expression were selected for transfer (See Chapter 3 Table 1).

Soluble HLA-G Assay

sHLA-G Assay monoclonal antibody (mAb) (MEM-G9 MCA2044; Serotec, Raleigh, NC, USA) against sHLA-G was used to coat a 96-well Nunc-Immunoplate (Fisher Scientific, Chino, CA, USA) using a concentration of 2 µg/ml in 0.1 mol/l carbonate buffer pH 9.5 for 1 h at 37 °C. The plate was then refrigerated at 4 °C overnight. On the following day, the plate was thoroughly washed twice using 100 µl phosphate-buffered-saline (PBS) plus 0.05% Tween-20. The next wash was repeated twice using 100 µl of PBS+5% bovine serum albumin (BSA) for 15 min each. A 50 µl aliquot of PBS+5% BSA was added to each well prior to adding the sample of 50 µl embryo supernatant. JEG-3 cell line (which secretes HLA-G) supernatant was used as a positive control (Bamberger *et al.*, 2000).

Fifty microlitres of JEG-3 supernatant and 50 µl of pure blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) were incubated for a period of 1 h at 37 °C.

Following incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin-conjugated mAb (w6/32 MCA81B; Serotec) at a 1:1000 dilution in PBS+1% BSA for 45 min at 37 °C and then washed five times with PBS. Streptavidin–alkaline phosphatase conjugate (BD Bioscience PharMigen, San Diego, CA, USA) at a concentration of 1:1000 in carbonate buffer was incubated for 30 min at 37 °C and washed five times with PBS, after which phosphatase substrate was added at a concentration of 1 mg/ml in 10% diethanolamine pH 9.8 for 30 min. The colorimetric reaction was then stopped by the addition of 50 µl of 3 mol/l NaOH. The relative concentration of sHLA-G was estimated from absorbance measured at 405 nm on an EL800-ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). A standard supernatant of sHLA-G/.221 transfectant served as reference in standard calibration curves.

Embryo transfer

Embryo transfers were performed on Day 3 (70-72 hours post ICSI) or blastocyts on Day5/6 (84-96 hours post ICSI) - depending on each center's preference. All centers used a Wallace Trail, followed by a Wallace Sure View catheter, both under direct ultrasound guidance.

Institutional Review

Since January 2005, all embryos at SIRM clinics underwent routine sHLA-G assay/testing to determine the expression of this potential biochemical marker in the culture medium surrounding embryos. All patients were counseled regarding the risks, benefits, and alternatives to sHAL-G testing; For the purpose of this PhD study, ethical approval was obtained from the Ethical committee of the University of Stellenbosch (N06/07/119). Furthermore, all clinical research conducted was in full compliance with guidelines of the American Society of Reproductive Medicine and met ethical principles involving human subjects as defined by the Declaration of Helsinki in 1964.

RESULTS

The descriptive tabulations: Out of the 3036 women observed, the statistical analyses only include the 2040 women with a sHLA-G assay result. Table 3 provides a breakdown of the number of patients with a sHLA-G assay result by site. Table 4 provides their mean age, mean number of embryos, number of chemical pregnancies, clinical implantations, and on-going pregnancies by site, day of transfer and sHLA-G asay result. As stated in the last row of Table 4, the mean age of patients included in the analysis was 35.8±1.46 years and the mean number of embryos utilized per patient, (across sites), were 2.7 ± 0.81 embryos.

A mixed effects logistic regression model was fitted for each of the three outcomes; chemical pregnancy, clinical implantation, and on-going pregnancy. Each outcome was regressed against the sHLA-G assay result with mean age, mean number of embryos utilized per group, and day of transfer included as covariates in the analysis. Site was included as a random effect. Table 5 and Table 6 provide the results of these model-fittings.

All three models indicated that the sHLA-G assay results were associated with the outcome of a chemical pregnancy, clinical implantation, and on-going pregnancy. A positive sHLA-G result is associated with an increase in odds of a chemical pregnancy (OR: 2.47, 95% CI: 2.04 to 3.00, p-value: <0.0001), an increase in odds of a clinical implantation (OR: 2.60, 95% CI: 2.14 to 3.15, p-value: <0.0001), and an increased odds of an on-going pregnancy (OR: 3.27, 95% CI: 2.67 to 3.99, p-value: <0.0001).

Two additional mixed effects logistic regression models were fitted for the dichotomous outcomes; multiple clinical implantations, and multiple on-going pregnancy subgroups. Each outcome was regressed against the sHLA-g assay result with mean age, mean number of embryos utilized per group, and day of transfer included as covariates in the analysis; site was included as a random effect. The multiple clinical implantations model only includes the 1027 patients for which a clinical implantation was reported (Table 7). The multiple on-going pregnancies model only includes the 924 women for which an on-going pregnancy was recorded. (Table 8) The two models indicate that the sHLA-G assay result is associated with the outcome of multiple clinical implantations and multiple on-going pregnancies. A positive sHLA-G result is associated with an increase in odds of multiple clinical implantations (OR: 1.48, 95% CI: 1.07 to 2.05, p-value: 0.0170), and an

increased odds of multiple on-going pregnancies (OR: 1.66, 95% CI: 1.10, 2.51, p-value: 0.0170).

DISCUSSION

The statistical analysis of the data from 2040 patients in this retrospective multi-centre study indicated that a positive sHLA-G test result was statistically associated with an increase in chemical pregnancy, clinical implantations, and on-going pregnancy outcomes after adjusting for mean age, mean number of embryos utilized and day of transfer. Additionally, the positive sHLA-G test result was statistically associated with higher multiple clinical implantations and multiple on-going pregnancies. A positive sHLA-G increased the odds of each of these mentioned outcomes. This current study confirms the observations of a prospective randomised controlled study by Kotze *et al.*, 2010 emphesizing the benefit of transferring sHLA-G positive embryos and thus predicting and increase pregnancy outcome in ART.

The study has a few limitations. Firstly, this was a retrospective study. Secondly, the majority of centres transferred embryos on Day 3, however, there were two of the six sites (FISCH and New Jersey (NJ) that transferred embryos on Day 5/6, therefore day of embryo transfer could not be differentiated in all sites. Thirdly, all centres used the same sHLA-G assay- however, each clinic applied their own range and threshold values (Table 2).

There has been some criticism of using the optical density (OD) value as our sHLA-G unit value; no previous reported studies (Fournel *et al.*, 2000, Criscuoli *et al.*, 2005, Noci *et al.*, 2005, Yie at al., 2005, Desai *et al.*, 2006, Rebmann *et al.*, 2005, 2007, Sargent *et al.*, 2007 and Vercammen *et al.*, 2008), performing sHLA-G analysis have reached consensus on the appropriate way to report these results. Even though we would like to report our results as an internationally agreed upon unit, no such consensus exists. Other obstacles include a lack of commercially available and universally accepted standards in order to generate standard curves. Initially, we performed extensive studies and generated our own value range of OD=0.170 ± 0.210. With additional data, we expanded the range of positive expression to the range used in this study (OD=0.190 ± 0.006) to 0.210 OD. While the lower limit had high sensitivity, but the increased range improved the specificity of the test. This adjustment allowed for the addition to the “positive” value group, some embryos that

would have been considered “negative” but still resulted in an ongoing gestation. Furthermore, we have conducted several unpublished in house studies using commercially available kits and found that our mean value corresponded to approximately 4.6 ng, or 1 “Unit” using the standard curve generated by the standards supplied in the commercial kit. In future studies, agreements and co-operation between established groups need to be reached in order to standardize the use of the sHLA-G assay successfully. Lastly, to assure accuracy daily controls were run using amniotic fluid (AF) from a pregnant woman as the sHLA-G positive control and culture medium as a negative control.

It is important to note that in case of a positive sHLA-G, the number of embryos selected for transfer must be carefully considered since the data indicated an increase in odds of multiple clinical implantations. A positive sHLA-G result is associated with an increase in odds of multiple clinical implantations (OR: 1.48, 95% CI: 1.07 to 2.05, p-value: 0.0170) and an increased odds of multiple on-going pregnancies (OR: 1.66, 95% CI: 1.10, 2.51, p-value: 0.0170). This is an important finding and in theory, transferring fewer embryos, selected based on sHLA-G results combined with morphologic evaluation can be used to reduce the risk of higher order pregnancies, without compromizing over all pregnancy rates.

Additionally, the results of this retrospective study indicated that transferring embryos on Day 3 as opposed to Day 5/6 post-fertilization reduced the odds of a clinical implantation and on-going pregnancy. Since not all centers used the same stage at which to transfer their embryos, one can therefor not draw any final conclusion reagarding this observation. However, it is interesting to look at previosuly reported studies reporting on Day 4 vs Day5/6 embryos transfers. In 2000 Coskun reported on data form a randomised trial, comparing Day 3 vs Day 5 embryo transfer outcome, they concluded that Day 3 and Day 5 transfer had similar pregnancy, implantation and twinning rates and therfore Day 5 transfers have no advantages over Day 3 transfers. Bungum *et al.*, 2003 reported similar findings in a prospecive randomized study. In a COCHRANE review, Blake *et al.*, 2007 concluded that blastocyst transfer were significantly improving pregnancy rates compared to that of cleavage stage embryos and similar findings were also reported by Papanikolaou *et al.* 2008.

This multi centre study indicates the benefit in the use of sHLA-G but the threshold values must be standardised. Clinics should consider this assay as a valuable non invasive

embryo marker to assist in improving pregnancy outcome and the theoretical potential to reduce multiple pregnancies. A combination of sHLA-G expression and extended embryo culture to the blastocyst stage might provide future tools by which to select single embryos for transfer and reduce the risk of multiple pregnancies, without compromising the pregnancy rates.

TABLE 1: **GRADUATED EMBRYO SCORING (GES)OF CLEAVAGE STAGE EMBRYOS**

Evaluation	Hours after insemination	Developmental milestone	Score
1	16–18	Nucleoli aligned along pronuclear axis	20
2	25–27	Cleavage regular and symmetrical Fragmentation[a]	30
		Absent	30
		<20%	25
		>20%	0
3	64–67	Cell number and grade[b] 7CI, 8CI, 8CII, 9CI	20
		7CII, 9CII, 10CI, 11CI, Compacting I	10
Total score			100

Fisch *et al.,* Graduated embryo score (GES). Fertil Steril 2003.

[a] If the embryo was not cleaved at 25–27 hours, grading of fragmentation should occur at the 64–67 hour evaluation if the embryo reached the seven-cell stage and had <20% fragmentation.

[b] Grade I = symmetrical blastomeres and absent fragmentation. Grade II = slightly uneven blastomeres and <20% fragmentation. Grade III = uneven blastomeres and >20% fragmentation. Grade A embryos are seven or more cells with <20% fragmentation.

TABLE 2: SHLA-G RANGES USED BY EACH CLINIC

CLINC	sHLA-G range	Testing Laboratory
New Jersey	0.190 – 0.210	SIRM-NY
Las Vegas (Fisch)	0.148 - 0.210	SIRM-LV
Las Vegas (Sher)	0.150 – 0.210	SIRM-LV
New York	0.190 - 0.210	SIRM-NY
LA- Glenndale	NEG ≤ 0.99 ≤ POS	SIRM-LV
Sacramento	0.184 - 0.196	SIRM-LV

TABLE 3: COUNTS OF INDIVIDUALS WITH A SHLAG TEST RESULT AT EACH SITE

Site	sHLAg		Total
	Negative	Positive	
FISCH	85	259	**344**
LA	65	93	**158**
LV	150	237	**387**
NJ	134	214	**348**
NY	303	286	**589**
SAC	94	120	**214**
Total	**831**	**1209**	**2040**

TABLE 4: MEAN AGE, MEAN NUMBER OF EMBRYOS, NUMBER OF CHEMICAL PREGNANCIES; CLINICAL PREGNANCIES AND ON-GOING PREGNANCIES BY SITE, DAY, AND SHLAG TEST RESULT

Site	Day	sHLAg	Mean Age	Mean number of Embryos	Chemical Pregnancy†	Clinical Implantation†	Multiple Clinical†	On-going Pregnancy†	Multiple On-going†	Total (n)
			35.1	1.7	277 (80.5)	249 (72.4)	104 (41.8)	247 (71.8)	101 (40.9)	344
			34.7	1.0	29 (87.9)	27 (81.8)	1 (3.7)	27 (81.8)	0 (0.0)	33
	Fifth	Negative	35.5	1.0	5 (71.4)	4 (57.1)	0 (0.0)	4 (57.1)	0 (0.0)	7
FISCH		Positive	34.5	1.0	24 (92.3)	23 (88.5)	1 (4.3)	23 (88.5)	0 (0.0)	26
			35.1	1.8	248 (79.7)	222 (71.4)	103 (46.4)	220 (70.7)	101 (45.9)	311
	Third	Negative	34.7	2.0	55 (70.5)	46 (59.0)	22 (47.8)	44 (56.4)	22 (50.0)	78
		Positive	35.3	1.7	193 (82.8)	176 (75.5)	81 (46.0)	176 (75.5)	79 (44.9)	233
			34.6	3.7	87 (55.1)	80 (50.6)	47 (58.8)	72 (45.6)	38 (52.8)	158
LA	Third	Negative	33.9	3.8	33 (50.8)	31 (47.7)	15 (48.4)	27 (41.5)	9 (33.3)	65
		Positive	35.1	3.6	54 (58.1)	49 (52.7)	32 (65.3)	45 (48.4)	29 (64.4)	93
			33.7	3.0	238 (61.5)	225 (58.1)	57 (25.3)	202 (52.2)	44 (21.8)	387
LV	Third	Negative	33.1	3.1	57 (38.0)	49 (32.7)	14 (28.6)	44 (29.3)	7 (15.9)	150
		Positive	34.0	2.9	181 (76.4)	176 (74.3)	43 (24.4)	158 (66.7)	37 (23.4)	237
			36.8	1.7	143 (41.1)	136 (39.1)	55 (40.4)	125 (35.9)	48 (38.4)	348
			36.9	1.6	90 (42.9)	88 (41.9)	34 (38.6)	78 (37.1)	29 (37.2)	210
	Fifth	Negative	37.2	2.0	37 (37.0)	35 (35.0)	13 (37.1)	32 (32.0)	10 (31.3)	100
NJ		Positive	36.7	1.3	53 (48.2)	53 (48.2)	21 (39.6)	46 (41.8)	19 (41.3)	110
			36.6	1.7	53 (38.4)	48 (34.8)	21 (43.8)	47 (34.1)	19 (40.4)	138
	Third	Negative	39.0	2.2	8 (23.5)	8 (23.5)	1 (12.5)	7 (20.6)	0 (0.0)	34
		Positive	35.8	1.6	45 (43.3)	40 (38.5)	20 (50.0)	40 (38.5)	19 (47.5)	104
			37.5	3.3	282 (47.9)	248 (42.1)	53 (21.4)	206 (35.0)	26 (12.6)	589
NY	Third	Negative	38.0	3.0	119 (39.3)	101 (33.3)	13 (12.9)	65 (21.5)	4 (6.2)	303
		Positive	37.0	3.6	163 (57.0)	147 (51.4)	40 (27.2)	141 (49.3)	22 (15.6)	286
			35.2	3.6	102 (47.7)	88 (41.1)	31 (35.2)	72 (33.6)	26 (36.1)	214
SAC	Third	Negative	35.1	3.7	30 (31.9)	23 (24.5)	5 (21.7)	8 (8.5)	3 (37.5)	94
		Positive	35.2	3.5	72 (60.0)	65 (54.2)	26 (40.0)	64 (53.3)	23 (35.9)	120
Grand Total			**35.8**	**2.7**	**1129 (55.3)**	**1026 (50.3)**	**347 (33.8)**	**924 (45.3)**	**283 (30.6)**	**2040**

† count (percentage of total)

TABLE 5: MIXED EFFECTS LOGISTIC REGRESSION FOR THE OUTCOMES: CHEMICAL PREGNANCY, CLINICAL IMPLANTATION, ON-GOING PREGNANCY

	Chemical Pregnancies				Clinical Implantations				On-going Pregnancies			
Fixed Effects $(\hat{\beta})$	**Estimate**	**Lower CI**	**Upper CI**	**P-value**	**Estimate**	**Lower CI**	**Upper CI**	**P-value**	**Estimate**	**Lower CI**	**Upper CI**	**P-value**
Mean Embryo	-0.09	-0.44	0.26	0.6100	-0.06	-0.40	0.28	0.7160	0.13	-0.27	0.53	0.5200
Mean Age	0.05	-0.13	0.22	0.6000	0.06	-0.12	0.25	0.4890	-0.03	-0.21	0.16	0.7760
Day (3rd)	-0.33	-0.75	0.09	0.1270	-0.43	-0.85	-0.02	0.0420	-0.47	-0.90	-0.04	0.0340
sHLAg (Positive)	0.91	0.71	1.10	<0.0001	0.95	0.76	1.15	<0.0001	1.18	0.98	1.38	<0.0001
Intercept	-1.41	-8.06	5.23	0.6770	-2.27	-9.08	4.55	0.5150	0.10	-7.07	7.27	0.9780
Random Effects $(\hat{\sigma}^2)$	**Estimate**	**Lower CI**	**Upper CI**		**Estimate**	**Lower CI**	**Upper CI**		**Estimate**	**Lower CI**	**Upper CI**	
Site	0.36	0.00	0.81		0.29	0.00	0.69		0.32	0.00	0.78	

TABLE 6: MIXED EFFECTS LOGISTIC REGRESSION FOR THE OUTCOMES: CHEMICAL PREGNANCY, CLINICAL IMPLANTATION, ON-GOING PREGNANCY

	Chemical Pregnancies			Clinical Implantations			On-going Pregnancies		
Fixed Effects $(\hat{\beta})$	**Odds Ratio**	**Lower CI**	**Upper CI**	**Odds Ratio**	**Lower CI**	**Upper CI**	**Odds Ratio**	**Lower CI**	**Upper CI**
Mean Embryo	0.91	0.65	1.29	0.94	0.67	1.32	1.14	0.76	1.70
Mean Age	1.05	0.88	1.25	1.07	0.89	1.28	0.97	0.81	1.17
Day (3rd)	0.72	0.47	1.10	0.65	0.43	0.98	0.63	0.41	0.96
sHLAg (Positive)	2.47	2.04	3.00	2.60	2.14	3.15	3.27	2.67	3.99
Random Effects $(\hat{\sigma}^2)$	**Median Odds Ratio**	**Lower CI**	**Upper CI**	**Median Odds Ratio**	**Lower CI**	**Upper CI**	**Median Odds Ratio**	**Lower CI**	**Upper CI**
Site	1.77	1.00	2.36	1.68	1.00	2.21	1.72	1.00	2.32

TABLE 7: MIXED EFFECTS LOGISTIC REGRESSION FOR THE OUTCOMES: MULTIPLE CLINICAL IMPLANTATIONS, AND MULTIPLE ON-GOING PREGNANCIES

	Multiple Clinical Implantations				Multiple On-going Pregnancies			
Fixed Effects $(\hat{\beta})$	**Estimate**	**Lower CI**	**Upper CI**	**P-value**	**Estimate**	**Lower CI**	**Upper CI**	**P-value**
Mean Embryo	0.41	-0.25	1.08	0.2250	0.35	-0.50	1.19	0.4210
Mean Age	-0.12	-0.37	0.14	0.3600	-0.05	-0.34	0.24	0.7360
Day (3rd)	0.63	0.02	1.25	0.0430	0.72	0.04	1.39	0.0370
sHLAg (Positive)	0.40	0.07	0.72	0.0170	0.51	0.09	0.92	0.0170
Intercept	1.66	-7.78	11.09	0.7300	-0.98	-11.60	9.64	0.8560
Random Effects $(\hat{\sigma}^2)$	**Estimate**	**Lower CI**	**Upper CI**		**Estimate**	**Lower CI**	**Upper CI**	
Site	0.56	0.00	1.43		0.74	0.00	1.91	

TABLE 8: MIXED EFFECTS LOGISTIC REGRESSION FOR THE OUTCOMES: MULTIPLE CLINICAL IMPLANTATIONS, AND MULTIPLE ON-GOING PREGNANCIES

	Multiple Clinical Implantations			Multiple On-going Pregnancies		
Fixed Effects $(\hat{\beta})$	**Odds Ratio**	**Lower CI**	**Upper CI**	**Odds Ratio**	**Lower CI**	**Upper CI**
Mean Embryo	1.51	0.78	2.95	1.41	0.61	3.27
Mean Age	0.89	0.69	1.15	0.95	0.71	1.27
Day (3rd)	1.88	1.02	3.48	2.05	1.05	4.01
sHLAg (Positive)	1.48	1.07	2.05	1.66	1.10	2.51
Random Effects $(\hat{\sigma}^2)$	**Median Odds Ratio**	**Lower CI**	**Upper CI**	**Median Odds Ratio**	**Lower CI**	**Upper CI**
Site	2.04	1.00	3.12	2.27	1.00	3.74

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CHAPTER 5

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A Linear Karyotypic Association between PB-I, PB-II and Blastomere Using Sequentially Performed Comparative Genome Hybridization with No Association Established between Karyotype, Morphologic, Biochemical (sHLA-G Expression) Characteristics, Blastocyst Formation and Subsequent Pregnancy Outcome

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Key Words
Polar body • Blastomere • Whole genome amplification • Comparative genome hybridization • Aneuploidy

Abstract
Background: The importance of oocyte/embryo ploidy to achieve implantation and a subsequent pregnancy. ***Aim:*** To correlate first and second polar bodies and day-3 blastomere ploidy, embryo morphology and biochemical (sHLA-G) characteristics with blastocyst development and in vitro pregnancy outcome. ***Materials and Methods:*** All oocytes/zygotes and embryos were individually cultured to the blastocyst stage. PB-I, PB-II and blastomeres underwent complete karyotyping and sHLA-G expression was measured on day 2. ***Results:*** 57 mature (MII) donor oocytes were obtained, 33/57 (57.9%) were aneuploid, 21/57 (36.8%) were euploid, and 3/57 (5%) were 'inconclusive'. No correlation was found between comparative genomic hybridization (CGH) status of PB-I, PB-II and the graduated embryo score. Furthermore, no correlation was established between PB-I CGH results and blastocyst morphology grade. There was a significant correlation between PB-I CGH and blastomere CGH results. Euploid and aneuploid PB-I developed into 58 and 67% blastocysts, respectively. κ statistics (>0.7) revealed a positive correlation between the ploidy of PB-I, PB-II and the blastomeres. ***Conclusion:*** Following ICSI and sequential genetic karyotyping of the oocyte/zygote and subsequent blastomeres, the majority of oocytes fertilized and subsequent zygotes developed into blastocysts, despite their ploidy status. We therefore conclude that blastocyst development is not associated with ploidy.

Introduction

Several factors are involved in achieving successful embryo implantation and a subsequent pregnancy. Poor embryo quality [1] and transfer techniques [2], endouterine abnormalities and immunologic dysfunction [3] can lead to implantation failure. Noninvasive methods such as embryo morphology/scoring and extended embryo culture to the blastocyst stage have been proposed to se-

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CHAPTER 5:

A linear karyotypic association between PB-I, PB-II and blastomere using sequentially performed comparative genome hybridisation (CGH) with no association established between karyotype, morphologic, biochemical (sHLA-G-expression) characteristics, blastocyst formation and subsequent pregnancy outcome

ABSTRACT

Background

The importance of oocyte/embryo ploidy to achieve implantation and a subsequent pregnancy.

Aim

To correlate first and second polar bodies and Day 3 blastomere ploidy, embryo morphology and biochemical (sHLA-G) characteristics with blastocyst development and in vitro pregnancy outcome.

Materials and Methods

All oocytes/zygotes and embryos were individually cultured to the blastocyst stage. PB-I, PB-II and blastomeres underwent complete karyotyping and sHLA-G expression was measured on day 2.

Results

Fifty seven mature (MII) donor oocytes were obtained, 33/57 (57.9%) were aneuploid, 21/57 (36.8%) were euploid and 3/57 (5%) were “inconclusive”. No correlation was found between CGH status of PB-I, PB-II and the GES-score. Furthermore, no correlation was established between PB-I CGH results and blastocyst morphology grade. There was a significant correlation between PB-I CGH and blastomere CGH results. Euploid and aneuploid PB-I developed into 58% and 67% blastocysts, respectively. Kappa statistics (>0.7) revealed a positive correlation between the ploidy of PB-I, PB-II and the blastomeres.

INTRODUCTION

Several factors are involved in achieving successful embryo implantation and a subsequent pregnancy. Poor embryo quality (Alikani *et al.*, 1999) and transfer techniques (Schoolcraft *et al.*, 2001), endo-uterine abnormalities and immunologic dysfunction (Sher *et al.*, 2000) can lead to implantation failure. Non-invasive methods such as embryo morphology/scoring and extended embryo culture to the blastocyst stage have been proposed to select embryos for transfer that will implant and result in a live pregnancy. Furthermore, a biochemical marker – soluble human leukocyte-antigen-G expression (sHLA-G) - major histocompatability complex - by embryos in culture has been suggested by several researchers to improve assisted reproductive technology (ART) outcome.

However, all the above-mentioned criteria provide no information about the chromosomal status (ploidy) of an embryo, which is of utmost importance because certain chromosomal aberrations in the embryo might contribute to in vitro fertilization (IVF) failure, (Pehlivan *et al.*, 2003). Early studies using preimplantation genetic screening with fluorescent in situ hybridization (PGS-FISH) have demonstrated that ≥50% of embryos considered for transfer during IVF were aneuploid (Munné *et al.*, 1993). In the past, FISH has been the most common method used for analyzing polar body (PB) and blastomere chromosomal complements. Initially it was suggested that the transfer of “chromosomally normal” embryos after PGS-FISH would lead to improved implantation and pregnancy rates. However, recent publications (Harton *et al.*, 2011), ESHRE PGD Consortium Steering Committee-2010) have concluded otherwise. One of the shortfalls associated with FISH in a PGS setting is the limited number of chromosomes that can be analyzed simultaneously; this is due to the number of fluorochromes available. Therefore, since FISH only screens up to 12 chromosomes, it limits the screening of the entire human genome, consisting of 23 chromosome pairs of which 22 chromosomes are autosomal chromosomes and the remaining pair is sex-determining (X, Y). Other potential problems like mosaicism is still an issue when performing Day 3 biopsies, (although mosaicism has been detected at later developmental stages), as well as the removal of more than one blastomere, leaving less of the remaining embryo. Studies have been reported where PGS-FISH was performed on polar bodies using a limited number of probes (Verlinsky *et al.*, 1990; Kuliev *et al.*, 2003). Comparative genomic hybridization (CGH) is another method for identifying oocyte/zygote/embryo chromosomal aberrations. The first studies using PB-CGH reported by Wells *et al.*, 2002; Gutierrez-Mateo *et al.*, 2004 and Fragouli *et al.*, 2006 failed to

demonstrate any promise of practical clinical utility. It was not until Sher *et al.* 2007 reported the first clinical application of CGH performed on both PB-I and PB-II. The same research group reported on the positive correlation between the transfer of euploid embryos (based on CGH performed on Day 3 blastomeres) and IVF outcome (Sher *et al.*, 2009). The advantage of CGH is that the entire chromosomal complement/genome is analyzed. However, a downside is that polyploidy and balanced translocations cannot be detected reliably using mCGH as described by Kallioniemi *et al.*, 1992, and Kirchoff *et al.*, 2001. Furthermore, an initial disadvantage of CGH was that the procedure took about 72 hours to perform. Originally it was applied to polar bodies, leaving 5 days for analysis and allowing for a fresh embryo transfer, without having to cryopreserve the embryos prior to transfer. When blastomere biopsies were performed on Day 3 embryos (6-9 cell), the resulting blastocysts were individually cryopreserved/vitrified and transferred in a later cycle if identified as normal by Sher *et al.* 2009.

The aim of this study is to correlate PB-I, PB-II and Day 3 blastomere ploidy, morphologic and biochemical (sHLA-G expression) characteristics with blastocyst development in vitro and pregnancy outcome.

MATERIALS AND METHODS

Patients

Oocyte donors for this study were five healthy, young (≤28 years of age), consenting patients without reproductive problems. Sperm from proven donors (with a record of successfully fathering a pregnancy) were obtained from Cryogam bank (Cryogam, CO). All donors were appropriately screened for infectious and sexually transmitted diseases and for auto-immune parameters that might adversely affect embryo implantation.

Embryo Culture: Embryology data collection: All oocytes collected were kept in chronological sequence and were individually cultured in 100 µl modified human tubal fluid (mHTF) + 10% HSA**,** under oil at 37 °C. All oocytes were denuded immediately after retrieval and graded (modification of Xia *et al.*, 1997) (Table 1). PB-1 was micro-surgical removed and underwent whole genome amplification (WGA), followed by complete karyotyping using metaphase comparative genome hybridization (CGH). All mature oocytes (M II) were fertilized by intracytoplasmic sperm injection (ICSI) and sequentially cultured in 50 µl droplets of Quinn's Advantage Protein Plus Cleavage medium (In-vitro

fertilization, Inc., Trumbull, CT) under oil at 6% CO_2, 5% O_2 and 90% N_2, 37°C in a humidified environment. On day 1 fertilization was assessed 16-18 hours post-ICSI, at which time alignment of the nucleoli was documented, and subsequently signs of cleavage at 26 hours post-ICSI. At this time PB-II was micro-surgically removed from normally fertilized (2PN) oocytes and underwent WGA/CGH karyotyping. On day 2 (at 46 hours post-ICSI the embryos were moved into 50 µl droplets of Multi Blastocyst medium (Global one, IVF Online) + 10% SSS (Irvine Scientific, Santa Ana, CA). The original droplets of culture medium surrounding each individual embryo were collected and tested for sHLA-G expression, using a specific enzyme linked immunosorbent assay (ELISA). Embryos with an optical density (O/D) range of 0.190±0.006 were considered "positive' for the expression of sHLA-G. Each individually cultured embryo was evaluated at 72 hours post-ICSI – applying a graduated embryo Score (GES) described by Fisch *et al.* 2001 /2003 (GES-score Table 2). Then, a single mono-nucleated blastomere was micro-surgically removed from each cleaved embryo and underwent WGA/CGH karyotyping. All embryos were cultured to the blastocyst stage and graded, using a modified version of Dokras *et al.*'s 1993 procedure, (Table 3), and vitrified.

Blastocyst Vitrification and Warming

Vitrification: was done by modifying the method previously described by Mukaida *et al.* 2003. This modified technique involved the use of an ICSI needle as follows: About 10 minutes prior to vitrification, expanded blastocysts were placed in 50 µl drops of mHTF with 10% SSS, vol/vol (Irvine Scientific). Each blastocyst had its blastocoels artificially collapsed through assertive needle aspiration. The inner cell mass (ICM) was positioned at the 6 or 12 o'clock position using a holding pipette connected to a micromanipulator. A 30° ICSI needle was then introduced via the cellular junction of the trophectoderm into the blastocoel cavity and the fluid was aspirated until the blastocyst had completely collapsed and no blastocoel fluid remained (Mukaida *et al.*, 2001, 2003). Blastocysts were then placed in 0.5 ml of mHTF (Base solution; IVF Online) supplemented with 10% (vol/vol) SSS for 2) minutes at 37°C. Thereafter, blastocysts were placed in base solution + 0.96 mol/l DMSO (D 5879, Sigma, St. Louis, MO) + 1.2 mol/l ethylene glycol (EG; P 3265, Sigma) for 90 seconds. Finally, blastocysts were placed in base solution + 1.9 mol/l DMSO + 2.4 mol/l EG + 1 mol/l sucrose (S 7903, Sigma) + 0.1 mol/l Ficoll (F 8636, Sigma) for 30 seconds. Then, each blastocyst was individually placed onto a cryo-loop (Hampton

Research, CA) using 1-2 µl cryo-solution and plunged into liquid nitrogen. Blastocyst grading applied: see Table 3 (Blastocyst grading)

Warming: The cryo-loop was removed from its vial and immersed into base solution + 0.34 mol/l sucrose for 2 minutes at 37°C. The embryo was then moved to base solution + 0.17 mol/l sucrose for 3 minutes and finally placed in base solution for 5 minutes. Intact embryos were cultured in 50 µl of Global One medium at 37°C in 6% CO_2 for a minimum of 2 hours to evaluate blastocoel re-expansion (i.e. survival).

Recipient Hormonal Treatment and Embryo Transfer: Following the onset of birth control pill-induced menstruation, oestradiol valerate IM (4-8mg) was administered every 3 days for a period of 8-12 days, until endometrial thickness had reached > 8 mm in sagital diameter and the plasma [E2] had stabilized at 1300-3000 Pmol/l. At that point, daily IM injections of 100 mg progesterone in oil (PIO) were initiated. On the 6th day of PIO, subject to patient choice and availability, two (2) thawed/warmed blastocysts were cryo-transferred, using ultrasound guidance and an echogenic catheter (Wallace, Smith Medical, UK). Serum beta human chorionic gonadotropin (β-hCG) levels were measured 8-10 days after the transfer. The patient was considered biochemically pregnant when the first value was >5.0 IU and the next value 2 days later was double the first. Clinical pregnancy rates were based on a 6-week ultrasound scan detecting a gestational sac containing a fetal heartbeat. In the event of an ultrasound-confirmed pregnancy, an estrogen/progesterone supplementation regimen was continued. Ongoing pregnancy rates were based on a 10–12-week ultrasound examination. In all cases where pregnancy did not occur or did not survive, hormonal treatment was immediately stopped.

Each blastocyst was referenced back to its oocyte, zygote and embryo of origin. All clinical procedures were performed by the same physician and laboratory procedures were performed by the same embryologist.

Stimulation

Patients received Lupron (TAP Pharmaceuticals, Lake Forest, IL) in a long protocol after pre-treatment with oral contraceptive (OC) birth control pills for 1-3 weeks and were treated with a human-derived gonadotropin (Bravelle; Ferring Pharmaceuticals Inc, Suffern, NY) in order to stimulate follicular development in the ovaries. Ovulation was

triggered when at least two follicles were 18 mm and half of the remaining were 15 mm. Oocytes were retrieved transvaginally using ultrasound guidance at 35 hours post hCG.

Extraction and Amplification of Genomic DNA

Genomic DNA was obtained by laser dissection and needle aspiration from three sources: the oocyte's first polar body (PB-I), zygote's second polar body (PB-II), and subsequent Day 3 embryo's blastomere. The cellular material was aspirated into a 200 µl thin-walled PCR tube (VWR catalog, 82006-602) for lyses and amplification using the Qiagen Repli-g kit (catalog, 59045). Ten ng of Repli-g control genomic DNA was used as a positive control and 0.5 µl of nuclease-free water as a negative control (Eppendorf catalog, 955155017). The reactions were set up following the Repli-g protocol and incubated for 8 hours at 30°C for amplification, followed by 10 minutes at 70°C for enzyme inactivation. Both incubation steps were performed using an Applied Biosystems 2720 Thermal cycler. Amplified DNA was placed at either 4°C for short-term storage or at -20°C for long-term storage.

Genomic DNA Confirmation and Quantification

For confirmation of WGA products, 5 µl reaction aliquots were mixed with 1 µl 6X Blue-Orange Loading Dye (Promega # G1881) and electrophoresed through a 1.0% agarose gel containing 0.1% ethidium bromide in TBE buffer (90mM Tris-HCl, 90 mM boric acid and 2 mM EDTA, pH 8.0). For quantification of the genomic DNA, 5 µl reaction aliquots were diluted with 95 µl of molecular biology-grade, nuclease-free water and analyzed with an Eppendorf BioPhotometer.

Nick Translation of Genomic DNA

The WGA products were fluorescently labeled using the Vysis Nick Translation Kit (catalog, 32-801024). One µg genomic DNA for each sample was labeled with SpectrumGreen™ direct-labeled dUTP by nick translation, following the Vysis protocol. Translation reactions were carried out using an Applied Biosystems 2720 Thermal cycler at 15°C for 2 hours and 70°C for 10 minutes to stop the reaction. The size of the resulting SpectrumGreen™ labeled probes was assessed by electrophoresis of 5 µl aliquots of 1.0% agarose gel containing 0.1% ethidium bromide in TBE buffer (90 mM Tris-HCl, 90

mM boric acid and 2 mM EDTA, pH 8.0). Probes of usable quality were in the range of 300 - 3000 bp, as per the manufacturer's recommendation.

Probe Preparation and Comparative Genomic Hybridization

The SpectrumGreen™ labeled probes were purified by ethanol precipitation. Briefly, 10 µl/200 ng nick-translated reference DNA, 1 µl/100 ng SpectrumRed reference DNA (Vysis # 32-80423 or 32-804024), and 10 µl/ 10 µg Cot-1 DNA (Vysis # 32-800028) used to suppress repetitive sequences and prevent non-specific hybridization, were added to a 1.5 ml micro-centrifuge tube.

For precipitation of the hybridization mix, 2.1 µl (0.1 volume) 3M sodium acetate, followed by 52.5 µl (2.5 volumes) of 100% EtOH was added to the DNA, vortexed briefly and placed on dry ice for 15 minutes, then centrifuged at 12 000 rpm for 30 minutes at 4°C to pellet the DNA. The supernatant was removed and the pellet dried for 10–15 minutes under a vacuum at ambient temperature. The pellet was resuspended in 3 µl molecular biology grade water and 7 µl CGH hybridization buffer (Vysis # 30670003) and placed in the dark during slide preparation. CGH-normal metaphase slides (Vysis # 30-806010) were prepared following the manufacturer's protocol. Briefly, the slides were denatured in 70% formamide, 10% SSC pH 5.3, and 20% molecular biology grade water for 5 minutes at 73°C for 5 minutes followed by an ethanol dehydration series of a minute each (70%, 85%, and 100%). The slides were dried by touching the bottom edge to a blotter and wiping the underside with a paper towel, then placed on a 45–50°C slide warmer to allow remaining EtOH to evaporate. The probe mix was denatured by heating for 5 minutes in a 73°C water bath. Ten µl of the probe mix was applied to the slides. The coverslip was placed over the slide and sealed with diluted rubber cement. The hybridizations were carried out with Vysis Hybrite chambers for a minimum of 48 hours and a maximum hybridization time of 72 hours to clear background. Following hybridization, the cover slips were carefully removed along with any remaining rubber cement residue, taking care not to damage the slide surface. The slides were then agitated in 0.4X SSC/0.3% NP-40 wash solution at 74+1°C for 1-3 seconds, then let stand for 2 minutes, then agitated in 2X SSC/0.1% NP-40 at ambient temperature for 1-3 seconds, then let stand for 1 minute. The slides were allowed to dry vertically at ambient temperature in the dark. Once completely dry, 10 µl DAPI II counter stain (Vysis 30804931) was added to each side, followed by a cover slip, and immediately sealed with clear non-fluorescing fingernail polish. Keskintepe *et al.*, 2007

collected and validated results from first, second PB's and two blastomeres and reported a positive/negative occurrence of less that 10%.

Image Capture and Analysis

The following optical filters visualized the fluorochromes used in the hybridization: a filter set specific for DAPI, Texas Red® (Cat. No. 30-150491) and FITC (fluorescein isothiocyanate) (Cat. No. 30-150291) to view the counter stain, SpectrumRed or SpectrumGreen DNA, respectively, a triple band pass filter set designed to simultaneously excite and emit light specific for DAPI.

Soluble HLA-G Assay

A monoclonal antibody (mAb; MEM-G9 MCA 2044; Serotec, Raleigh, NC against sHLA-G was used to coat a 96-well Nunc-Immunoplate (Fisher Scientific, Chino CA) using a concentration of 2 µg/ml in 0.1 mol/l carbonate buffer at pH 9.5 for 1 hour at 37ºC. The plate was then refrigerated overnight at 4ºC. On the following day, the plate was thoroughly washed using 100 µl of phosphate-buffered saline (PBS) and 0.05% Tween 20. The wash was repeated twice using 100 µl PBS and 5% bovine serum albumin (BSA) for 15 minutes each. A 50 µl aliquot of PBS and 5% BSA was added to each well before adding the sample of 50 µl of embryo supernatant. Amniotic fluid (AF) was used as a positive control. AF (50 µl) and 50 µl of pure blastocyst culture media (Irvine Scientific) in which no embryos had been cultured (the negative control) were incubated for a period of 1 hour at 37ºC. After incubation, the plates were washed three times with PBS, followed by incubation with a specific biotin conjugated mAB (w6/32 MCA81B; Serotec) at a 1:1000 dilution in PBS and 1% BSA for 45 minutes at 37ºC and then washed five times with PBS. Streptavidin alkaline phosphatase conjugated (BD Bioscience Pharmigen, San Diego, CA) at a concentration of 1:1000 in carbonate buffer was incubated for 30 minutes at 37ºC and washed five times with PBS, after which phosphatase substrate was added at a concentration of 1 mg/ml in 10% diethanolamine at pH 9.8 for 30 minutes. The colorimetric reaction was then stopped by the addition of 50 µl of 3 mol/l NaOH. The sHLA-G concentration was determined by absorbance at 405 nm on the EL800 Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT).

Statistical analysis

Fisher's exact test was used to test for association between two categorical studied variables. The small marginal totals for some levels of the categories variables necessitate the use of this test. The agreement between the PB-I and PB-II and blastocyst was evaluated through the kappa statistic. The median levels of expression of sHLA-G for the categories of oocyte grade, PB-I, PB-II and blastocyst grade were calculated together with the interquartile range (IQR). To compare the median levels of sHLA-G between the levels of these variables two statistical methods were used. Since oocyte grade has a strict ordinal structure the estimation and testing of the Spearman correlation coefficient between sHLA-G and oocyte grade is an optimal test for the hypothesis of no difference in median sHLA-G levels across the categories of oocyte grade. For the remaining variables a Kruskal-Wallis test (non-parametric analysis of variance) was performed for the comparison of the median levels of soluble sHLA-G.

RESULTS

Fifty-seven (57) oocytes from donors (≤28 years of age) were studied. The indication was that the majority of oocytes were aneuploid - CGH results for meiosis I of PB-I tested revealed that 21/57 (36.8%) were euploid, 33/57 (57.9%) aneuploid and 3/57 (5.2%) inconclusive (INC). Furthermore, CGH results for meiosis II of the PB-II tested revealed that 25/57 (44.0%) were euploid, 27/57 (47.4%) were aneuploid and 5/57 (9.0%) were inconclusive (INC). All Day 3 embryos underwent biopsy – removing a mono-nucleated blastomere. Forty-five of 57 embryos had ≥ 7 blastomeres and were categorized grade 1 or 2 (78.9%). CGH results for mitosis (Day 3 mono-nucleated blastomeres) revealed that 23/57 (40.4%) were normal, 31/57 (54.4%) were abnormal and 3/57 (5.3%) were inconclusive (INC): The results were reported as inconclusive as a result of no cell, no amplification or no clear fluorescent staining/ no interpretation.

Metaphase II oocytes were also graded, using light microscopy, the best quality was grade 1 and poorest quality was grade 3 (Table 1). There were 33 aneuploid oocytes – 18/54 (54.5%) originated from grade 1 (best quality) oocytes, the remaining 15 (45.5%) originated from grade 2/3 (poorer quality) oocytes. Furthermore, there were 21 euploid oocytes – 8/21 originated from grade 1 (best quality) oocytes (38.1%) and the remaining 13 (61.9%) originated from grade 2/3 (poorer quality) oocytes. No association was found

between the best oocytes - grade 1 (highest quality) - and euploid chromosomal compliment of PB-I (Fisher's exact: p=0.758). Furthermore, there was no association between oocyte grade and euploid chromosomal compliment of PB-II (the same as for PB-I). The majority of embryos were abnormal across all initial grades (Fisher's exact: p= 0.548).

There was no association between an oocyte's grade/quality and subsequent blastomere biopsy's CGH results. The majority of blastomeres were aneuploid. Sixty one percent (61%) of the embryos that originated from the so called "best" quality oocytes were aneuploid and 58% of the embryos that originated from average/poor (grade 2/3) oocytes were euploid as revealed by the CGH results from the blastomere biopsies (Fisher's exact: p = 0.233). However, our data showed there was a significant agreement between the CGH results of PB-I and PB-II, respectively, Kappa=0.72 (se=0.11, p<0.0001), indicating that PB-I and PB-II provided nearly the same information regarding their ploidy status. Furthermore, there was a significant agreement between PB-I and the blastomere biopsy CGH results. These two parameters also provide nearly the same information regarding their ploidy status, with Kappa=0.84 (se=0.12, p<.0001). Interestingly, no association was observed between the CGH results of PB-I and subsequent blastocyst grade. The agreement between PB-II and the blastomere biopsy was similar to that of BP-I with Kappa=0.75 (se=0.11, p<.0001). There was no association between PB-II and subsequent blastocyst development. In both PB-II categories, euploid or aneuploid oocytes developed into about the same ratio of blastocysts. Interestingly, in cases where PB-I was euploid, 58% reached the blastocyst stage, and where PB-I was aneuploid, 67% reached the blastocyst stage. Furthermore, in cases where blastomeres were euploid, 62% reached the blastocyst stage, and where blastomeres were aneuploid, 58% reached the blastocyst stage, indicating a lack of association (Fisher's exact p=0.923). The findings were similar when the blastomere CGH results were correlated with blastocyst formation (Fisher's exact p= 0.770).

There was no difference in the mean expression of sHLA-G between the categorical levels of PB-I, PB-II and blastocyst grade. However, oocyte grade and sHLA-G expression was negatively correlated, with Spearman's correlation coefficient = -0.27 (p=0.0432). This is a weak correlation but reflects that the poorest oocyte grade had the highest expression (mean sHLA-G OD = 0.170 ± 0.04) compared to best oocyte grade with lower expression (mean sHLA-G OD=0.150 ± 0.03). The data for all 57 oocytes and their chromsomal

status of PB-I, PB-II, Day 3 blastomere's and sHLA-G respectively are presented in Table 4. Implantation/pregnancy outcome of vitrified/warmed blastocyst were 50% implantation, 80% pregancy, three set of singletons and one set of twins
(Table 5).

DISCUSSION

Identifying euploid embryos prior to transfer in order to reduce the number of embryos for transfer, without sacrificing pregnancy rate and at the same time reducing high order multiple gestations have been of great interest to researchers. In our study we attempted to correlate the chromosomal status for PBI, PBII and blastomere, oocyte grading, embryo morphology score (GES) and sHLA-G (a biochemical embryo marker) in order to find any characteristic that could be used to assist in identifying/estimating of an embryo's viability and potential to develop into a live baby We used 57 Mature (MII) oocytes obtained from oocyte donors and fertilized with proven sperm donors to study their chromosomal status by CGH, and resulting "normal" blastocysts were followed to determine if a live birth was obtained. The sequential chromosomal status of PBI, PBII and blastomere, respectively, was screened for by metaphase CGH. The rate of aneuploidy in PB I and II was 53% in all oocytes and 54% in subsequent Day 3 embryos. A further breakdown indicated that the aneuploidy rates for PB I (meiosis I) and PB II (meiosis II) were 58% and 47%, respectively. Any difference in ploidy between an oocyte's PB I and PB II could possibly be due to a rescue/correction mechanism, mosaicism, and malsegregation or cross-over errors during prophase I as described by Schmerler *et al.* 2011, or non-disjunction and premature separation as illustratrated by Vialard *et al.*, (2006) (Figure 1, p.170). Kuliev *et al.* 2011 also speculated that missing signals might be due to the hybridization failure that can lead to misinterpretation.

Fragouli *et al.*, 2009 reported overall aneuploidy of 65% in oocytes after CGH testing was performed. A further breakdown indicated that the aneuploidy rates for PB I (meiosis I) and for PB II (meiosis II) were 36 and 46%, respectively. These results support the findings in our study regarding the genetic status of PB I and PB II. However, in another study by Fragouli *et al.*, 2009b they reported only 3% aneuploidy when young oocyte donors underwent PB I CGH testing – these results contradict the findings in our study: we reported 58% of oocytes obtained from young egg donors were abnormal. Statistical analysis in the present study revealed that the correlation found between PB I, PB II and

blastomere chromosomal results is good (Kappa >0.7 for all three pair-wise comparisons). Therefore they could be used as a proxy for one another. However, we did find a negative association between embryo morphologic quality and corresponding genetic status of embryos on day 3. Baltaci *et al.*, 2006 reported that when embryo quality at the cleavage stage was correlated with chromosomal abnormalities, the morphological assessment might not reflect the chromosomal status of the embryo, because among the aneuploid embryos considerably high proportions were morphologically good quality, confirming our findings. We, therefore, suggest that an embryo's morphology can not be correlated with its ploidy prior to transfer. This supports data by Munné *et al.*, 2007 suggesting that the best way to select euploid embryos for transfer is to use a combination of both morphologic and genetic testing. We reported that 31/50 (62%) of abnormal Day 3 embryos developed into blastocysts. Our data indicated that there was no association between the expression of sHLA-G and embryo ploidy. In our study we reported a significant positive association between PB I and blastomere ploidy which furthermore confirms that sperm derived from young healthy donors likely contributes very little to any additional aneuploidy after fertilisation. Magli *et al.* 2004 concluded that the major application of PB biopsy is the detection of maternally derived chromosomal aneuploidies and translocations in oocytes, and could be applied as a viable alternative to blastomere biopsy. In a large study by Li *et al.* 2005, 42% embryos were screened for 16, 18, 21 and XY and underwent extended culture to the blastocyst stage and 20% progressed to the blastocyst stage. After re-testing of the blastocyst's chromosomal complement, 61% were confirmed abnormal. This study confirms the similar high percentage of blastocyst aneuploidy as reported in our study.

Interestingly, Hassold and Jacobs 1984 reported that at least 50% of first trimester spontaneous miscarriages were chromosomally abnormal. Veiga *et al.*, 1987 reported that 26% of oocyte and zygote were aneuploid by FISH. Munné *et al.* 1993 reported aneuploidy to be the most prominent chromosome anomaly found in normally developing monospermic embryos. Several other researchers (using FISH) have reported oocyte aneuploidy rates in excess of 50% in women older than 40 years of age (Sandalinas *et al.*, 2002, Kuliev *et al.*, 2003, Gutierrez-Mateo *et al.*, 2004, Fraguoli *et al.*, 2006 and Hassold *et al.* 2007). Gutierrez-Mateo *et al.* 2004 reported 48% aneuploidy after CGH was performed on PB I. Montag *et al.* 2009 concluded that the detection of the paternal influence on the genetic constitution of the embryo is not possible with PB diagnosis. However, PB

diagnosis can be applied in cases of monogenetic diseases, as well as cases of maternal structural and numerical chromosome aberrations.

In all the above cases – only a limited number of chromosomes were evaluated, (about one-quarter of the genome) – leaving three-quarters untested. This leads to the assumption that several “normal” embryos could have been aneuploid if the whole genome was screened. The conclusions drawn from these studies were based on incomplete genetic screening of embryos. The most recent study reported was a poster presentation at 2011 ESHRE, Magli *et al.* 2011 reported that 5 chromosomes (13, 16, 18, 21, and XY) were analyzed and 50% were aneuploid. Furthermore, they reported that PB I and PB II do not reveal identical chromosomal information - which supports the findings in our study (Table 4). Geraedts *et al.* 2010 suggested that the polar body (PB) approach be thoroughly investigated by a panel of researchers and reported annually at ESHRE PGD Consortium 2011. Array CGH screening of the first and second polar bodies added valuable information to the criteria needed in order to select an viable embryo for transfer as reported by Geraedts *et al.*, 2011 and Magli *et al.*, 2011. In spite of the fact that more than half of mature (M II) oocytes obtained from young donors were aneuploid; the majority were fertilizable and subsequently developed into blastocysts. We hypothesize that the ability to fertilize eggs and subsequent extended embryo culture to blastocysts in no way affirms the chromosomal integrity of those blastocysts. The outcome of our small study and the transfer of vitrified/warmed blastocysts that originated from normal oocytes/zygotes and Day 3 blastomeres as determined by sequential PGS of PB-I, PB-II and blastomere, respectively, resulted in encouraging implantation and pregnancy results and limiting high order multiples (Ta 5). We conclude that future studies regarding that sequential chromosomal screening of the whole genome to determine the chromosomal status of an embryo will shed more light on this controversial topic.

TABLE 1. Oocyte Grading used for CGH –study

Grade 1: Homogeneous cytoplasm, intact PB, normal oocyte shape, no cytoplasmic defect, no PV and zonae defect, normal oolemma.

Grade 2: Homogeneous cytoplasm, and at least 2 of following
:

- Fragmented PB
- Abnormal oocyte shape
- Cytoplasmic droplets
- Vacuoles
- Grainy spots
- Increased PV space
- Darken zonae or defect
- Double oolemma

Grade 3: No homogenous cytoplasm, and at least 3≥ above.

TABLE 2. Graduated embryo scoring (GES) of cleavage stage embryos.

Evaluation	Hours after insemination	Developmental milestone	Score
1	16–18	Nucleoli aligned along pronuclear axis	20
2	25–27	Cleavage regular and symmetrical Fragmentation[a]	30
		Absent	30
		<20%	25
		>20%	0
3	64–67	Cell number and grade[b] 7CI, 8CI, 8CII, 9CI	20
		7CII, 9CII, 10CI, 11CI, Compacting I	10
Total score			100

Fisch 2001. Graduated embryo score (GES).

[a] If the embryo was not cleaved at 25–27 hours, grading of fragmentation should occur at the 64–67 hour evaluation if the embryo reached the seven-cell stage and had <20% fragmentation.

[b] Grade I = symmetrical blastomeres and absent fragmentation. Grade II = slightly uneven blastomeres and <20% fragmentation. Grade III = uneven blastomeres and >20% fragmentation. Grade A embryos are seven or more cells with <20% fragmentation.

TABLE 3. Blastocyst grading:

Blastocyst grading according to Dokras *et al*., 1993 was performed as follows:

Grade 1 blastocysts were characterized by early cavitation resulting in the formation of an eccentric and then expanded cavity lined by a distinct inner cell mass region and trophectoderm layer.

Grade 2 blastocyst exhibited a transitional phase where single or multiple vacuoles were seen which over subsequent days developed into the typical blastocyst appearance of a grade 1 blastocysts.

Grade 3 blastocysts were defined as blastocysts with several degenerative foci in the inner cell mass with cells appearing dark and necrotic.

TABLE 4 Data for all 57 oocytes

Oocyte #	Oocyte Grade	1st PB	2nd PB	Blastomere	sHLA-G	Blast Grade
1	1	18L	N	18G	0.139	BII
2	1	N	N	N	0.119	BII
3	1	17L,22L	INC	INC	0.139	BII
4	1	INC	INC	INC	0.149	NO Blast
5	2	INC	N	N	0.178	BIII
6	2	4L,5L,17L,21G	22L,7G	22L	0.18	BI
7	1	INC	INC	INC	0.109	BI
8	1	1L,13L,16L	12G,15G,16G,20G	16L,1G,13G	0.146	BI
9	2	N	N	N	0.125	BIII
10	1	N	N	N	0.173	NO Blast
11	2	18L,21L,22L	21L,17G,22G	18L,21L	0.148	NO Blast
12	2	N	N	N	0.201	BII
13	2	22G	11L	N	0.149	BII
14	1	21L,22L,5G	4G,5G,13G,19G	3G,4G,5G,16G,22G	0.158	NO Blast
15	1	22L,5G	17L,4G,5G	5G,6G,22G	0.209	BII
16	1	20L,22L	12G,15G,22G	5L,22L	0.117	BII
17	2	N	N	N	0.138	BII
18	3	16L,20L,4G	4G,13G,17G	16L,9G	0.191	BI
19	1	N	N	N	0.135	BII
20	2	N	N	N	0.163	BII
21	1	N	N	N	0.136	EBIII
22	2	20L,22L,4G	22L,7G	20G	0.149	BII
23	3	1L,9L,15L	22L	1G,9G,15G,22G	0.145	BII
24	3	N	N	N	0.189	BI
25	3	16L,7G	15L,6G,13G	4G,7G,19G	0.141	BI
26	1	3L,16G,21G	5G	22L,3G,21G	0.127	EBIII
27	2	5L	5G	N	0.189	EBIII
28	1	7L	10G,14G	10L,19G	0.137	BII
29	1	N	N	N	0.161	BII
30	1	N	N	N	0.202	BII
31	2	21L,11G,12G,15G	9G	11L,12L,22G	0.155	NO Blast
32	2	N	N	N	0.131	NO Blast
33	3	N	12L	22L,5G,6G	0.122	BIII
34	3	N	N	N	0.167	BI
35	2	N	N	N	0.195	BIII
36	1	4G,10G,22L	N	24L,22G	0.153	NO Blast
37	2	19L,21L,1G	7L	1L,21G	0.197	NO Blast
38	1	N	N	N	0.149	BII
39	1	4L,16G,18G	3G	10L,22G	0.176	NO Blast
40	2	14G,15G	22L	11G,14G	0.164	BII
41	1	21L	14G,17G	13G	0.129	BII
42	1	7G,13G,17G,20G	4L	7L,17L,20L	0.205	BII
43	1	1L,21L	N	1G,4G,5G	0.201	NO Blast
44	2	N	N	N	0.284	NO Blast
45	1	1G,16G,17G	4L,5L	20G	0.107	EBIII

TABLE 4 continue

46	*1*	*6G,9G*	*N*	*9L,1G,8G*	*0.114*	*NO Blast*
47	*1*	*N*	*N*	*N*	*0.107*	*NO Blast*
48	*3*	*N*	*N*	*N*	*0.153*	*EBIII*
49	*2*	*18L,1G,17G,19G*	*6L*	*17L,19L*	*0.149*	*BII*
50	*3*	*N*	*N*	*N*	*0.176*	*BIII*
51	*3*	*1L,2L,21L*	*FRAG*	*12G,21G*	*0.226*	*NO Blast*
52	*3*	*5G,6G,17G,19G*	*5G,14G*	*5L,17L,19L*	*0.226*	*NO Blast*
53	*1*	*16L,5G,6G*	*7L*	*10G,21G*	*0.164*	*CIII*
54	*2*	*5L*	*7L,13L,17L*	*4G,5G*	*0.16*	*BIII*
55	*2*	*N*	*N*	*N*	*0.123*	*NO Blast*
56	*1*	*21L,22L,5G*	*4G,5G,19G*	*3G,5G*	*0.165*	*BII*
57	*1*	*1L,16L,20L*	*FRAG*	*16L,20L*	*0.191*	*BII*

L=Loss
G=Gain

N= NORMAL set of chromosomes

FRAG = Polar body was fragmented and DNA amplification was not possible

PB1 = Polar body 1

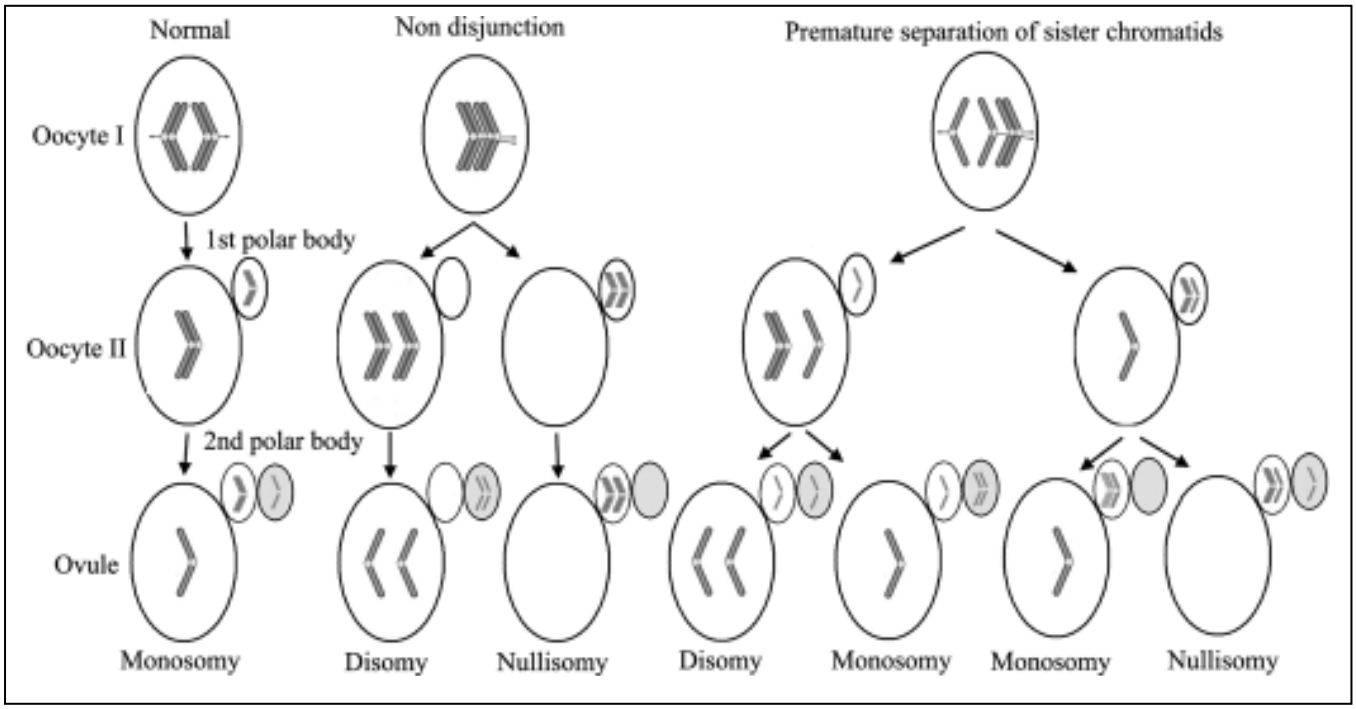
PB2 = Polar body 2

INC = DNA amplification was possible but imaging and analysis was not possible

TABLE 5 CGH –study pregnancy outcome

# Embryo recipients	5
Age years (ave)	37±1.5
# Euploid blastocyst vitrified	11
# Euploid blastocyst warmed	11
# Euploid blastocyst that survived warming (%)	10/11 (97.9)
# Euploid blastocysts transferred (total)	10
# Euploid blastocysts transferred (ave)	2.0
Implantation/euploid blastocyst transferred (%)	5/10 (50)
Live babies/euploid blastocyst transferred (%)	5/10 (50)
Birht rate per warmed euploid blastocyst (%)	5/11 (45.5)
Full term pregnancies (%)	4/5 (80)
Singletons	3
Twins (two babies)	1
Triplets	0
Miscarriages	0

Figure 1: Diagram of non-disjunction and premature separation as illustratrated by Vialard *et al.*, (2006)



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CHAPTER 6

Submitted to Human Ruproduction

CHAPTER 6

THE MORPHOLOGICAL, BIOCHEMICAL AND GENETIC FEATURES OF EMBRYOS THAT HAVE BEEN SHOWN TO BE PREDICTIVE OF IMPLANTATION AND PREGNANCY OUTCOME IN ART

ABSTRACT

Introduction

Identifying a developmentally competent embryo to transfer that has the highest probability to develop into a live baby has been an issue of debate and continues research.

Aim

The aim of this chapter is to discuss the morphological, biochemical and genetic features of an embryo that has been shown to be predictive of implantation and pregnancy outcome in ART using most current evidence.

Methods

A literature search was performed looking at the correlation between pronuclear morphology, early cleavage, cleavage stage embryos, blastocyst development, the presence of sHLA-G, CGH, PGS, embryo development and implantation/pregnancy rates in ART.

Results

Based on the available literature, a combination of observations could assist the scientist with embryo selection. The pronuclear stage morphology, the early embryo division, cleavage embryo stage and quality of the day 3 embryos provides limited guidance. However, choosing a blastocyst with a positive sHLA-G result on Day 5 is the optimal combination to make the final selection before embryo transfer or freezing

DISCUSSION

This non-invasive approach should improve pregnancy outcome and reduce multiple pregnancy rates. As far as the use of the more invasive technology such as aCGH is concerned, more research on pregnancy outcome is needed.

INTRODUCTION

Since the inception of assisted reproductive technology (ART) two decades ago, it has been constantly evolving as far as ovulation induction, embryo culture conditions, culture medium and embryo transfer techniques are concerned. However, identifying a developmentally competent embryo to transfer that has the highest probability to develop into a live baby have been an issue of debate and continuous research. Currently embryo selection is predominantly based on morphological appearance. Several developmental characteristics have been suggested as selection criteria in order to identify a viable embryo. In several earlier reported studies it was evident that morphological evaluations furnish clues that enhanced the ability in choosing the best embryos for transfer (Cummins *et al.*, 1986; Puissant *et al.*, 1987; Staessen *et al.*, 1992; Visser *et al.*, 1993; Roseboom 1995; Ziebe *et al.*, 1997; Shoukir *et al.*, 1997; Van Blerkom 1997). However, these methods were severely limited to provide reliable evidence for predicting normal embryo implantation and live birth outcome. Furthermore, it has been suggested that morphological evaluation does not fully reflect the developmental potential of a preimplantation embryo (Munne & Cohen, 1998). Scoring for Day 3 embryos were first reported by Desai *et al.* 2000, whereas all prior scoring systems were based on the second day of culture (Day 2). In 2001, Fisch *et al.*, introduced a graduated embryo scoring (GES) system in which embryos were individually cultured, allowing for sequential microscopic assessment of developmental stages starting on Day 1 through Day 3 of embryo culture. More recent, extended embryo culture to the blastocyst stage has been promoted by studies of Gardner *et al.*, 1999a; Gardner *et al.*, 2000a and Gardner *et al.*, 2000b. Jurisicova *et al.* 1996a/b reported that soluble Human Leukocyte Antigen-G (sHLA-G) expression was present during preimplantation human embryo development and was detected in culture; medium surrounding grouped embryos (Fuzzi *et al.*, 2002). This biochemical marker could be used as a non-invasive tool to identify embryos with high implantation potential. The first successful clinical application of PGD was reported by Handyside 1990. A few years later, Kallioniemi *et al.*, 1992 developed comparative genome hybridization (CGH) to screen the whole genome's DNA in tumors. This technique was adopted to study the DNA of single cells (like blastomeres) by Wells *et al.*, 1999 and Voullaire *et al.*, 2000. There have been reports on the successful use of array-CGH by Hu *et al.*, 2004 and Wells *et al.*, 2004 and more recent the use of SNP-array to detect any chromosome imbalances and genome wide linkage analysis by Handyside *et al.*, 2010.

The aim of this chapter is to discuss the best available evidence of morphological, biochemical and genetic features of an embryo that have been shown to be predictive of implantation and pregnancy outcome in ART. These features will be discussed under the headings non-invasive and invasive methods.

NON-INVASIVE METHODS

Pronuclear Morphology

The presence of two pronuclei has been the standard for assessing “normal” fertilization and has been associated with normal embryo development (Tesarik *et al.*, 1989) and early stages of RNA-synthesis (Tesarik *et al.*, 1986). Additional evaluation of pronuclei such as positioning within the ooplasm, their size, nucleoli distribution, orientation with respect to the polar bodies and the presence of a cytoplasmic halo have been suggested to be of value.

Several studies reported on the positive correlation between pronuclear morphology and embryo morphology/development, namely Salumets *et al*., 2001, Van Blerkom *et al.*, 2002; Ebner *et al.*, 2003; Scott 2003; Balaban *et al.*, 2004; Shen *et al.*, 2006; Sjöblom *et al.*, 2006; Depa-Martynow *et al.*, 2007; Alvarez *et al.*, 2008, Liao *et al*., 2006, Nicoli *et al*., 2007 and Zamora *et al.*, 2011. However, some studies by Wittemer *et al.*, 2000; Payne *et al.*, 2005, James *et al*., 2006 and Nicoli *et al.*, 1010 reported no correlation between pronuclear morphology and embryo morphology/development.

The most important point of interest is without doubt the correlation of pronuclear morphology and pregnancy rates. The following studies reported a positive correlation between pronulear morphology and implantation/pregnancy outcome in ART: Lundqvist *et al.*, 2001; De Placido *et al.*, 2002; Lukaszak *et al.*, 2003; Kattera & Chen, 2004; Shen *et al.*, 2006; Gianaroli *et al.*, 2007; Li *et al.*, 2007; Scott *et al.*, 2007; Alvarez *et al.*, 2008; Liu *et al.*, 2008 and a prospective study by Montag & Van der Ven 2001. However, there were some studies that reported no correlation between pronulear morphology and implantation/pregnancy outcome in ART: Salumets *et al.*, 2001; Payne *et al.*, 2005; Chen & Kattera, 2006; James *et al.*, 2006; Liao *et al.*, 2006; Nicoli *et al.*, 2007; Brezinova *et al.*, 2009, Arroyo *et al.*, 2007; and Arroyo *et al.*, 2010.

A few studies have been addressing the positive correlation between pronulear morphology and embryo ploidy: Sadowy *et al.*, 1998; Chen *et al.*, 2003; Coskun *et al.*, 2003; Gamiz *et al.*, 2003; Gianaroli *et al.*, 2003; Balaban *et al.*, 2004; Borges *et al.*, 2005 and Gianaroli *et al.*, 2007. However, there was a study by Arroyo *et al.*, 2010 which reported no association between pronuclear morphology and genetic status of an embryo.

Early zygote cleavage evaluation

Shoukir *et al.*, 1997 defined "early cleavage" as the timing at which the first mitotic division post fertilization takes place - this phenomenon has been extensively studied to be used as additional criteria for embryo selection prior to transfer and as a predictor of embryo development potential and improved implantation/pregnancy rates. As early as 1984, Edwards *et al.*, postulated that early cleavage resulted into a higher degree of developmental competence compared to those embryos that did not undergo early cleavage.

Several studies have also been published on the topic of "early cleaving/fast dividing" embryos and its subsequent effect on embryo morphology and implantation/pregnancy outcome in vitro. The effect of early cleaving zygotes on subsequent embryo development has been reported. Fenwick *et al.*, 2002 associated early cleavage with increased developmental potential, Van Montfoort *et al.*, 2004, associated early cleavage with improved blastocyst development and Fu *et al.*, 2009 associated early cleavage with improved embryo quality. Retrospective studies that reported a positive correlation between these "fast cleavers" and implantation and pregnancy outcome were published by Shoukir *et al.*, 1997, Ziebe *et al.*, 1997, Sakkas *et al.*, 1998, Bos-Mikich *et al.*, 2001, Petersen *et al.*, 2001, Tsai *et al.*, 2002, Salumets *et al.*, 2003, Van Montfoort *et al.*, 2004, Windt *et al.*, 2004, Ciray *et al.*, 2005, Giorgetti *et al.*, 2007, Rehman *et al.*, 2007 and Fu *et al.*, 2009. Prospective studies confirming the above is also of importance (Isiklar *et al.*, 2002; Brezinová *et al.*, 2003).

On the other hand prospective studies by Brezinová *et al.*, 2004 and Chen and Kattera 2006 reported that the transfer of early cleaved embryos did not benefit implantation/pregnancy rates. Furthermore, in 2008, Sundström & Saldeen confirmed the above findings. As with pronuclear stage embryo morphology there is no consensus in the international literature on potential advantages/disadvantages of early embryo cleavage.

Developmental stage: Blastocyst vs cleavage stage transfer

Since the inception of IVF, the majority of IVF clinics transferred embryos at the cleavage stage. However, extended culture of embryos to the blastocyst stage have since been reported by Gardner and Lane 1997; Menezo *et al.*, 1998 Gardner *et al.*, 2000a/b and Pool 2002. The main purpose of the trend towards blastocyst transfer is an attempt to reduce the number of embryos being transferred, resulting in a reduction of multiple implantations after IVF/ICSI-ET therapy.

Extended embryo culture has its benefits and shortfalls:

- a reduction in the number of available embryos, however, it has been speculated that extended culture distinguish between embryos with a poor or a high developmental potential (Tesarik 1994; Van Blerkom 1994).
- Improved implantation rates with a reduced number of embryos transferred resulted in a reduction of multiple pregnancies.
- Blastocyst stage transfers might eliminate some chromosomally abnormal embryos for transfer (Alfarawati *et al.*, 2011a/b) since Magli *et al.*, 2000 reported that many Day 3 embryos with a ”normal” morphology on Day 3 are actually chromosomally abnormal due to mosiacism. That said, extended embryo culture does provide information about an embryo’s viablity in vitro, however it should not be directly associated with pregnancy outcome. In Chapter 4 of this thesis it was reported that 58% of aneuploid day 3 embryos did reach the blastocyst stage of development, therefore refuting the belief that blastocyst stage transfers might eliminate some chromosomally abnormal embryos for transfer.

Blastocyst grading systems have been developed by Cohen *et al.*, 1985, Dokras *et al.*, 1993, Gardner and Schoolcraft 1999b, Richter *et al.*, 2001 and Kovacic *et al.*, 2004. These morphological grading systems take into account expansion of the blastocoel cavity, characteristics of the inner cell mass (ICM) and the trophectoderm (TE). In 2000, Balaban *et al.*, reported that the transfer of good quality blastocysts were associated with improved implantation rates compared to poor quality blastocysts. Racowsky *et al.*, 2000 suggested that extended culture of at least two, but preferably three more high quality eight-cell embryos on day 3, should develop into sufficient blastocysts for transfer and/or cryopreservation. Papanikolaou *et al.*, 2005 similarly suggested that four good quality Day 3 embryos should undergo extended culture and be transferred at the blastocyst stage,

providing patients with a better chance of achieving a live birth compared to cleavage stage embryo transfer.

Several studies have reported improved implantation/pregnancy rates when transferring blastocyst embryos compared to cleavages stage embryos: Blake *et al* 1004; Schwarzler *et al.*, 2004; Levitas *et al*, 2004; Pantos *et al.*, 2004a/b; Papanikolaou *et al.,* 2005; Blake *et al.,* 2005; Blake *et al.,* 2007 and Mangalraj *et al.*, 2009. Papanikolaou *et al.*, 2008 performed a meta-analysis to compare studies where equal number of embryos in both the cleavage-stage and the blastocyst-stage were transferred and compared clinical pregnancy rate, multiple pregnancy rate and live birth rate outcomes. They found that clinical pregnancy rates were significantly higher in patients who received a blastocyst-stage embryo for transfer compared to patients that received a cleavage-stage embryo for transfer. Live birth rates were also significantly higher in the blastocyst-stage compared to cleavage-stage embryo category.

Some studies reported no difference in ART outcome when blastocyst and cleavage stage transfers were compared (Bungum *et al.*, 2003; Hreinsson *et al.*, 2004; Utsunomiya *et al.*, 2004. Controversially, Levron *et al.,* 2002) reported a significant decrease in implantation rates associated with blastocyst transfer concluding blastocyst transfer had a negative effect on clinical pregnancy rates. Finally a few studies by Criniti *et al.*, 2005; Henman *et al.*, 2005 and Zech *et al.*, 2007, suggested that transferring of a single blastocyst could effectively reduce multiple pregnancies without compromising the live birth rates as reported by American Society for Reproductive Medicine 62nd Annual Meeting, 2006.

BIOCHEMICAL EVALUATION

Soluble HLA-G

The implantation mechanism is a very poorly understood aspect of reproduction. It is a highly complex dialog (cross-talk) between the embryo and the endometrium (Simón *et al.*, 1995; Hill 2001; Viganò *et al.*, 2003; Shimomura *et al.*, 2006. Implantation requires the successful suppression of the maternal immune system. The human body will identify and discriminate against foreign tissue by major histocompatability complex (MHC), human leukocyte antigen (HLA) originated from a group of genes located on the short arm of chromosome six (6). The MHC evokes T-cell intervention to non-self antigens expressed

by an individual of the same species. The human fetus is considered a non-self tissue to the maternal uterus due to paternal MHC antigens. However, during pregnancy the immune system develops fetal tolerance (Saito *et al.*, 2007). HLA-G produced by the extra-villous cytotrophoblast (the only fetal contact with maternal uterine cells), confers immuno-tolerance through interaction with maternal uterine membrane lymphocytes. This scenario would suggest that HLA-G protects the fetus from maternal immune response attack. HLA-G is expressed by the placenta throughout gestation and is also present in amniotic fluid. Soluble HLA-G (a spliced iso-form of membrane bound HLA-G) is in suspension and can be detected in culture medium. sHLA-G, a non-classic type I human leukocyte antigen, was first identified in the media surrounding groups of embryos and blastocysts in culture by Jurisicova *et al.*, 1996a/b. Menicucci *et al* 1999, first identified, sHLA-G (a non-classic type I human leukocyte antigen), in the culture media surrounding a group of Day 3 embryos. Fuzzi *et al.*, 2002 has shown that the presence of sHLA-G in the culture media harboring groups of three day old cleaved embryos, correlated both with their cleavage rate and with their overall subsequent implantation potential. Hviid *et al.*, 2004 postulated that the presence of sHLA-G protected the conceptus from destruction by the maternal immune response.

However, there has been some criticism of using the optical density (OD) value as our sHLA-G unit value; no previous reported studies performing sHLA-G analysis have reached consensus on the appropriate way to report these results (Fournel *et al.*, 2000; Rebmann *et al.*, 2004; Criscuoli *et al.*, 2005; Noci *et al.*, 2005; Yie *et al.*, 2005; Desai *et al.*, 2006; Rebmann *et al.*, 2007; Sargent *et al.*, 2007; Vercammen *et al.*, 2008).

The detection of soluble HLA-G in culture medium surrounding embryos has been reported by Jurisicova *et al.*, 1996a; Menicucci *et al.*, 1999 and Fuzzi *et al.*, 2002. Several studies regarding sHLA-G and its effect on pregnancy outcome have been reported since (Sher *et al.*, 2004; Sher *et al.*, 2005a; Sher *et al.*, 2005b; Criscuoli *et al.*, 2005; Noci *et al.*, 2005; Yie *et al.*, 2005; Desai *et al.*, 2006; Fisch *et al.*, 2007 Rebmann *et al.*, 2007; Kotze *et al.*, 2010). The prospective randomized trial by Kotze *et al.,* 2010 on more than 200 patients showed a significant benefit when selecting embryos on the basis of sHLA-G. In Chapter 3 the multicentre study also confirmed the value of sHLA-G to predict pregnancy outcome. It was also emphasized in Chapter 3 that this biochemical tool can also be used to reduce multiple pregnancies. In review studies by Sargent *et al.*, 2007 and Vercammen *et al.*, 2008 they thoroughly evaluated and discussed previous studies regarding sHLA-G

and ART outcome. In a meta-analysis by Vercammen *et al*. they reported that sHLA-G is moderately helpful in predicting pregnancy, however if the embryos are of good quality sHLA-G has much better diagnostic performance. Furthermore, Rebmann *et al.*, 2004 addressed specific issues dealing with sHLA-G ELISA protocols.

It is important that the threshold values should be standardised and one should consider this as a valuable non-invasive embryo marker to assist with embryo selection and the theoretical potential to improve pregnancy outcome and simultaneously reduce multiple pregnancies. A combination of sHLA-G expression and extended embryo culture to the blastocyst stage might provide future tools by which to select single embryos for transfer.

INVASIVE METHODS

Embryo Pre-implantation Genetic Screening (PGS)

Pre-implantation genetic screening of embryos is applied to patients at risk such as: advanced maternal age (AMA), recurrent pregnancy loss, repeat IVF failure or severe male factor infertility (Baruch *et al.*, 2008).

In the past the most common technique used for PGS was fluorescence in situ-hybridization (FISH) – a fast and relatively easy procedure to perform. However, recently the validity of FISH PGS was scrutinized by Staessen *et al.*, 2008 since this procedure did not improve implantation and delivery rates in women <36. In contrast, Hardarson *et al.*, 2008 reported that PGS caused a decrease in women with advanced maternal age (AMA). Possibly due to cleavage stage embyos affected by mosaicism that could have affected the accuracy of PGS (Munne *et al.*, 1994; Bielanska *et al.*, 2002; Li *et al.*, 2005). Finally another possible explanation why FISH PGS does not improve pregnancy/implantation rates is the fact that the complete genome (22 pairs of outosomal and the two sex chromosomes) is not screened – leaving the potential to transfer aneuploid embryos, (untested) which could potentially influence embryo development and implantation. Kallioniemi *et al.*, 1992 developed comparative genome hybridization (CGH) to screen the whole genome's DNA in tumors. This technique was modified to study the DNA of single cells (like blastomeres) by Voullaire *et al.*, 1999 and Wells *et al.*, 1999. The CGH technique is superior to FISH since it screens the whole genome for anomalies such as chromosome imbalances due to aberrant segregation and structural imbalances

(gains/losses) larger than 10-20 Mb as reported by Griffin *et al.*, 1998 and Malmgren *et al.*, 2002. A disadvantage of CGH is that it is very time/labor intensive – it takes up to four days to obtain results after hybridization and amplification of the DNA and comparing the test DNA to that of a normal template of chromosomes. First (PB-I) and second polar body (PB-II) screening (evaluating the oocyte and zygote's genome), allowing fresh embryos transfers, have been reported by Wells *et al.*, 2002; Sher *et al.*, 2007; Obradors *et al.*, 2008; Obradors *et al.*, 2009 and Fragouli *et al.*, 2009. Day 3 blastomere was evaluated to detect the influence of paternal contribution as reported by Wilton *et al.*, 2001; Voullaire *et al.*, 2002; Sher *et al.*, 2007; Sher *et al.*, 2009 and Kotze *et al.*, 2012.

Kotze *et al.*, 2012 reported no association between the chromosomal status of PB-I, PB-II, Day 3 blastomeres and subsequent blastocyst formation. However, they reported respectable live birth outcome when sequential screening was applied and normal embryos developed into blastocysts and were transferred.

Lately, the removal of several trophectoderm cells at the blastocyst stage has been reported by Fragouli *et al.*, 2009 and Schoolcraft *et al.*, 2010. Blastocyst biopsy required embryo cryopreservation, allowing time to complete CGH analysis. Euploid embryos can be thawed and transferred in a future FET cycle. However, potential detrimental effects of cryopreservation post PGD has been reported by Joris *et al.*, 1999, Edgar *et al.*, 2000 and Zheng *et al.*, 2005, resulting in a 30-40% reduction in implantation potential.

Recently the introduction of vitrification of blastocysts by Mukaida *et al.*, 2003 and Zhang *et al.*, 2009 has significantly improved the survival of warmed embryos. Sher *et al.*, 2007 and Kotze *et al.*, 2012 reported 50% live birth rates after the transfer of PGS vitrified/warmed blastocysts.

Lately, a faster technology has been developed, allowing a more rapid and more detailed analysis of PB-I, PB-II, Day 3 blastomeres and trophectoderm cells, namely array-CGH (Hu *et al.*, 2004; Wells *et al.*, 2004; Le Caignec *et al.*, 2006; Treff *et al.*, 2010; Treff *et al.*, 2011). Different approaches (by modifying earlier procedures) to screen the whole genome compliment within 16 hours and to potentially avoid embryo cryopreservation of PGS embryos have been suggested by Gutierrez-Mateo *et al.*, 2004 and Landwehr *et al.*, 2008.

Screening the whole genome has indicated that anomalies could be present in any of an embryo's chromosomes. In the study by Kotze *et al.*, 2012 and Traversa *et al.*, 2011, it has been shown that aneuploid embryos have the potential to develop to a blastocyst. These findings contradict the belief that aneuploidy of the larger chromosomes (1-12) are less likely to develop into a blastocyst. FISH indentifies a single point on a chromosome while CGH-probes cover the entire length of a chromosome, therefore structural anomalies can be detected such as partial duplications and deletions. Translocation errors can be detected by the ability of CGH to determine a loss/gain of parts of chromosomes. Traversa *et al.*, 2010 has shown that FISH suffers from high false positive abnormality rates. Improved PCR technologies (array-CGH) have been reported by Traversa *et al.*, 2010 to improve translocation screening. Furthermore, CGH can detect translocation in cases where the fragments are large enough – balanced translocation can thus be determined as well as the ploidy status of all other chromosomes. FISH analyzes each cell individually for the specific probe(s) that are applied.

Traversa *et al.*, 2010 demonstrated the reliability and feasibility of CGH to detect aneuploidy in blastocysts. Kotze *et al.,* 2012 used sequential CGH screening of PB-I, PB-II and Day 3 blastomeres to identify aneuploid embryos, subsequent blastocysts were vitrified. Blastocysts that were classified as euploid were warmed and transferred, resulting in excellent implantation and pregnancy outcomes.

Most recently a more rapid method to perform cyto-genetic screening has been introduced, namely array CGH. Currently there are two approaches that are being applied: single nucleotide polymorphism array (SNP–array) and CGH-array.

- SNP-array: Common polymorphic DNA sequences found throughout the genome is used to detect any chromosome imbalances and genome wide linkage analysis (Handyside *et al.*, 2010; Vanneste *et al.*, 2009; Treff *et al.*, 2010; Treff *et al.*, 2011).
- CGH-array: test and reference DNA is hybridized to DNA probes fixed to a slide. Several reports on the successful use of array-CGH: Hu *et al.,* 2004; Wells *et al.*, 2004 and Le Caignec *et al.*, 2006. Furthermore, some clinical applications have been reported by Hellani *et al.*, 2008, Fishel *et al.*, 2010 and Rius *et al.*, 2010.

The latest studies to improve the efficiency/accuracy of PGS are the use of BAC microarrays in order to detect aneuploidy in all chromosomes as reported by Fragouli *et al.*, 2011 and Guiterrez-Mateo *et al.*, 2011. Furthermore, this technique could also be used

to determine specific segmental errors during chromosomal rearrangement as reported by Alfarawati *et al.*, 2011a and Fiorentino *et al.*, 2011.

Based on the previous experience with FISH-PGS it is too early to comment on the true value/benefit of aCGH. More data regarding pregnancy outcome is needed before a final conclusion can be rendered, however, this technology (despite being invasive and costly) looks very promising in assisting clinicians and scientists with this embryo selection method for selective patients.

CONCLUSION

To conclude, based on the available literature it is logical to use all available tools for embryo selection. A combination of observations for embryo selection, starting with the pronuclear stage morphology, early zygote cleaving, cleavage-stage embryo morphology/quality on Day-3, but ultimately choosing a blastocyst on Day 5 with sHLA-G values available, will assist the scientist in making the final decision before selecting an embryo for transfer or cryopreservation. This non-invasive approach should improve pregnancy outcome and reduce multiple pregnancy rates. As far as the use of the more invasive approach technology such as aCGH is concerned, more research on pregnancy outcome is needed.

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Chapter 7

Chapter 7

CONCLUSIONS AND FUTURE PERSPECTIVE

ABSTRACT

A combination of observations for embryo selection, starting with oocyte grading, pronuclear stage morphology, early zygote cleaving and cleavage-stage embryo morphology/quality on Day-3, however, ultimately using extended embryo culture and choosing a blastocyst on Day 5 with positive sHLA-G values available, will assist the scientist in making the final decision before selecting an embryo for transfer or cryopreservation. The use of aCGH (for chromosomal analysis) is invasive and is still considered experimental.

DISCUSSION

Since the inception of IVF there has been great progress in the ART arena. However, there are some areas that still lack understanding and are in need of continuous research. The reduction of multiple gestations might be resolved if the mechanism of implantation, the identification of embryos to transfer with the highest probability to implantation are systematically investigated. In this thesis two approaches were ultimately investigated: non-invasive and invasive.

A literature review was thus performed to research the impact of the oocyte/zygote and the embryo on implantation/pregnancy rates. The morphologic characteristics of the oocyte;- cumulus –oocyte-complex (COC), zona pellucida, perivitelline space, cytoplasm, meiotic spindle and the polar body and its appearance was studied. The morphologic characteristics of the zygote;- pronuclei orientation, the embryo and its effect on embryo development, ploidy and blastocyst formation and its effect on implantation and pregnancy outcome was also reviewed. The pronuclear morphology, early cleavage and a biochemical marker that is expressed by developing embryos (soluble Human Leucocyte-Antigen-G) and its role in predicting pregnancy outcome was discussed as non-invasive markers. “Omics” is another non-invasive approach in order to identify the most competent embryos for transfer. “Omics” covers genomics, transcriptomics, proteomics, and metabolomics but provides limited information and more research and is required in this

field. The value of blastocyst transfer and the improved ongoing pregnancy rate compared to cleavage stage embryos was also highlighted based on a recent meta-analysis. Genetics testing using FISH, CGH and array-CGH and the future of genomics was discussed as invasive markers.

In the future the ultimate goal is to have a non-invasive set of criteria and parameters to apply to developing embryos in vitro by which to identify the most competent embryo/s for transfer that each will lead to pregnancy and a single live baby. In the first prospective randomized study in the international literature comparing the traditional way of embryo evaluation versus sHLA-G expression plus a cumulative GES-score on day 3 as selection criteria was reported. It was concluded that by combining a positive sHLA-G expression with the highest GES-score on Day 3 resulted into significantly improved pregnancy outcome. Furthermore a multicentre study evaluating the impact of sHLAG on pregnancy outcome was undertaken. This multi centre study indicates the benefit in the use of sHLA-G but the threshold values must be standardised. Clinics should consider this assay as a valuable non-invasive embryo marker to assist in improving pregnancy outcome with the theoretical potential to reduce multiple pregnancies. A combination of sHLA-G expression and extended embryo culture to the blastocyst stage might provide future tools by which to select single embryos for transfer and reduce the risk of multiple pregnancies, without compromising the pregnancy rates.

Another important fact is the ploidy status of the embryo. This invasive approach includes embryo biopsy for either polar bodies, day 3 blastomeres or day5/6 trophectoderm. FISH has been used extensively, using a single day 3 blastomere, to identify single gene anomalies and aneuploidy in a limited number of chromosomes. FISH was also successfully used for gender selection. The introduction of WGA/CGH was an improvement to FISH since all 23 pairs of chromosomes were analyzed. CGH however, only revealed gains/losses in chromosomes and the process was very time consuming. Since then, arrayCGH was introduced, it analyzes the whole genome in fine detail (small base-pairs). A real-time “same day” assay was recently introduced. Quantitative real time polymerase chain reaction is a rapid analysis of the whole genome and results are available within four (4) hours.

A study on the ploidy status of oocytes and embryos were conducted in chapter 5. In spite of the fact that more than half of mature (M II) oocytes obtained from young donors were

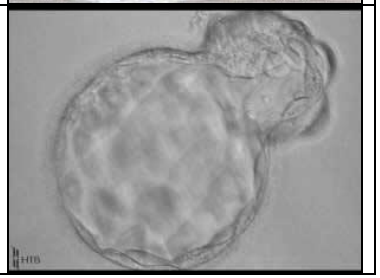
aneuploid; the majority were fertilizable and subsequently developed into blastocysts. We hypothesize that the ability to fertilize eggs and subsequent extended embryo culture to blastocysts in no way affirms the chromosomal integrity of those blastocysts. (Figure 1 – Pictures of Deceptive Blastocyst Development) The outcome of our small study and the transfer of vitrified/warmed blastocysts that originated from normal oocytes/zygotes and Day 3 blastomeres as determined by sequential PGS of PB-I, PB-II and blastomere, respectively, resulted in encouraging implantation and pregnancy results and limiting high order multiples. Based on the previous experience with FISH-PGS it is too early to comment on the true value/benefit of aCGH. More data regarding pregnancy outcome is needed before a final conclusion can be rendered, however, this technology (despite being invasive and costly) looks very promising in assisting clinicians and scientists with this embryo selection method for selective patients.

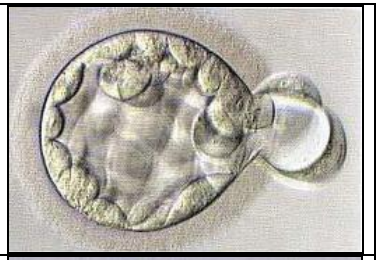
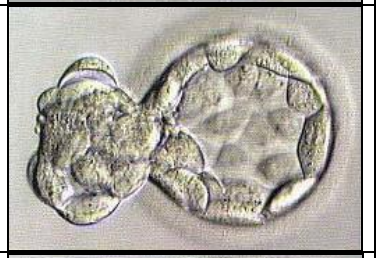
CONCLUSION

To conclude, based on the available literature it is logical to use all available tools for embryo selection. A combination of observations for embryo selection, starting with the pronuclear stage morphology, early zygote cleaving, cleavage-stage embryo morphology/quality on Day-3, but ultimately choosing a blastocyst on Day 5 with sHLA-G values available, will assist the scientist in making the final decision before selecting an embryo for transfer or cryopreservation. This non-invasive approach should improve pregnancy outcome and reduce multiple pregnancy rates. As far as the use of the more invasive approach technology such as aCGH is concerned, more research on pregnancy outcome is needed.

We conclude that despite all the above mentioned parameters to select an embryo for transfer that will develop into a live baby, extensive research and international corroboration is needed in order to improve and standardize such criteria.

Figure 1: Pictures of Deceptive Blastocyst Development -
(Four) 4 CGH abnormal blastocyst – (Five) 5 CGH normal blastocyst

Oocyte #	Oocyte grade	1st PB	2nd PB	Blastomere	sHLA-G	Blast	Picture
8	1	1L,13L,16L	12G,15G,16G,20G	16L,1G,13G	0.146	BII	
10	1	N	N	N	0,173	NO BLAST	
15	1	22L,5G	17L,4G,5G	5G,6G,22G	0.209	BII	
18	3	16L,20L,4G	4G,13G,17G	16L,9G	0.191	BI	

19	1	N	N	N	0.135	BII	
21	1	N	N	N	0.136	EBIII	
30	1	N	N	N	0.202	BII	
49	2	18L,1G,17G,19G	6L	17L,19L	0.149	BII	
55	2	N	N	N	0.123	NO BLAST	