British Journal of Medicine & Medical Research
4(15): 2910-2930, 2014

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A Review of Non-invasive and Invasive Embryo Characteristics to Indentify the Most Competent Embryo for Transfer

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Authors' contributions

This work was carried out between all authors. Author DK designed the study, wrote the protocol and wrote the first draft of the manuscript, managed the analyses of the study and performed the literature searches. The statistical analysis was performed by Dr. Lombard the biostatistician for the University of Stellenbosch. The others authors TFK promoter and GS co-promoter, SO evaluated the article and made suggestions. All authors read and approved the final manuscript.

Review Article

Received 5th December 2013
Accepted 1st February 2014
Published 10th March 2014

ABSTRACT

Identifying "competent embryos" (those with the greatest potential to develop into normal concept) for transfer to the uterus has been a matter of the highest priority and the subject of both hot debate and ongoing research, since the very inception of human in vitro fertilization (IVF). A thorough literature search was performed to evaluate the correlation between pronuclear morphology, early embryo cleavage speed, cleavage stage embryos, embryo/blastocyst development, "omics", sHLA-G expression, PGS, and implantation/pregnancy-generating potential in ART. Based on available literature, an array/combination of laboratory 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. We conclude that use

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of (invasive) PGS in specific patient populations is appropriate; however, more data are needed to determine its true value for overall impact in ART. Non-invasive selection of blastocysts on day 5 with optimal sHLA-G expression provides a very high degree of confidence to yield a viable pregnancy and potentially reduce multiple gestations.

Keywords: Morphology; omics; soluble human leu kocyte antigen-G (sHLA-G); blastocyst; cytogenetics.

1. INTRODUCTION

Since the inception of Assisted Reproductive Technology (ART) more than three decades ago, ovulation induction techniques, embryo culture conditions, and culture media and embryo transfer methods have been constantly evolving. However, identifying the embryos with optimized implantation competence for transfer, those that have the highest probability of developing into a live baby, has been an issue of debate and continuous research. Currently embryo selection is predominantly based on morphological appearance. Several developmental characteristics have been proposed as helpful criteria by which to best identify viable embryos. Earlier studies reported that morphological evaluations will furnish clues that enhance the ability to identify the best embryos for transfer [1]. However, all these methods have significant limitations when it comes to predicting the likelihood of successful implantation and live births. Furthermore, morphological evaluation does not fully reflect the developmental potential of a pre-implantation embryo as described by Munne and Cohen [2].

The presence of two pronuclei (2PN) has been the standard for assessing “normal” fertilization and has been associated with normal embryo development [3] and early stages of RNA-synthesis [4]. It has been postulated that fast cleaving embryos resulted in a higher degree of developmental competence than slower ones. Shoukir et al. [1] 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. Embryo scoring was initially performed on a single microscopic assessment done on day 2 (post-fertilization) embryos. In 2000 Desai et al. [5] introduced the first day 3 embryo score and in 2001, Fisch et al. [6] introduced a graduated embryo scoring (GES) system where embryos were individually cultured so as to allow for sequential microscopic assessment and scored during their development stages from day 1 through day 3 of culture. Each day’s embryo total cumulative score was out of 100 points. Those with a GES-score of greater than 70 were shown to have the greatest implantation and pregnancy potential. Jurisicova et al. [7,8] reported that pre-implanted human embryos express soluble Human Leukocyte Antigen-G (sHLA-G). Fuzzi et al. [9] thereupon demonstrated the presence of sHLA-G in the media surrounding grouped embryos and suggested that this could be used as a non-invasive biochemical marker by which to identify embryos with the highest implantation potential. In 2010, Kotze et al. [10] published the first prospective randomized study to conclude that higher ongoing pregnancies were achieved when embryos with a GES-score of >70 with optimal sHLA-G expression was transferred. (Level II evidence) Recently, these findings have been confirmed by a retrospective multi-center data set of 2040 ICSI patients [11].

Pre-implantation Genetic Diagnosis (PGD) was introduced in 1990 by Handyside et al. [12]. Genomics using Fluorescent In situ Hybridization (FISH) has been applied to identify

aneuploidy and known single-gene disorders in order to prevent their transmission. Extended embryo culture to the blastocyst stage has been promoted by Gardner et al. [13,14]. The issue of transferring cleaved versus blastocyst stage embryos has been controversial at best. However, a meta-analysis by Papanikolaou et al. [15] compared clinical pregnancy rate, multiple pregnancy and live birth rate outcomes where cleaved versus blastocyst stage embryos were transferred. They reported both clinical pregnancy and live birth rates to be significantly higher in the latter group. (Level I evidence)

The goal of this article was to review a wide array of available evidence in order to determine if morphological, biochemical and/or genetic features of an embryo are predictive of its implantation and pregnancy potential in ART. Each criterion will be addressed under the headings a) non-invasive and b) invasive methods.

2. NON-INVASIVE METHODS

2.1 Pronuclear Morphology

The detection of two pronuclei has been used as the first objective evidence of early stage of RNA-synthesis [4] and that fertilization might subsequently lead to orderly embryo development [3]. Additional evaluation of pronuclei such as: a) their positioning within the ooplasm b) size c) nucleoli distribution d) orientation with respect to the polar bodies and, e) the presence of a cytoplasmic halo has also been attributed with prognostic significance. Several studies reported on the positive correlation between pronuclear morphology and embryo morphology/development [16-27]. Contradicting however, other studies have reported no correlation between pronuclear morphology and embryo morphology/ development [28,29,30,31,32].

The associations between pronuclear morphology and pregnancy rates were controversial at best: the following studies reported a positive correlation between pronuclear morphology and implantation/pregnancy outcome in ART, [24,28-36] and was confirmed with a prospective study by Montag & Van der Ven [38]. Contradicting however, other studies have reported no correlation between pronuclear morphology and implantation/pregnancy outcome in ART: [16,25,26,37-42].

A few studies have reported on the positive correlation between pronuclear morphology and embryo ploidy, [20,33,43-48]. However, there was a contradicting report by Arroyo et al. [42] which found no association between pronuclear morphology and genetic status of an embryo.

2.2 Early Zygote Cleavage Evaluation

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. Moreover, the effect of early cleaving zygotes on subsequent embryo development has been reported. Fenwick et al. [49] associated early cleavage with increased developmental potential, Van Montfoort et al. [50], associated early cleavage with improved blastocyst development and Fu et al. [51] associated early cleavage with improved embryo quality. Furthermore, retrospective studies reported a positive correlation between these “fast cleavers” and implantation and pregnancy outcome [1,50-61]. It is important to

note that prospective studies by Isiklar et al. [62] and Brezinová et al. [63], confirmed the findings from the above mentioned retrospective studies. (Level III-2 evidence)

On the other hand, contradicting prospective studies by Brezinová et al. [64] and Chen and Kattera [38] reported that the transfer of early cleaved embryos did not benefit implantation/pregnancy rates. In 2008, Sundström and Saldeen [65] confirmed that the transfer of early cleaved embryos did not benefit ART outcomes. As with pronuclear stage embryo morphology there is no consensus in the international literature on potential advantages/disadvantages of early embryo cleavage.

2.3 Developmental Stage: Blastocyst versus Cleavage Stage Transfers

Until recently, the vast majority of programs favored the transferring of cleaved embryos. However, the development of extended culture systems that allow embryos to grow to blastocysts [Gardner et al. [14,66] and Pool [67] promises to change this. Since blastocysts tend to have a higher implantation/pregnancy-outcome potential compared to earlier, cleaved embryos, more and more IVF practitioners are transferring blastocysts preferentially, hoping that this paradigm will permit the transfer of fewer embryos, without negatively impacting success rates and at the same time reducing the risk of IVF multiple pregnancies.

2.3.1 Extended embryo culture has advantages

Extended culture yields blastocysts (a more developmentally advanced embryo) that theoretically have an improved implantation potential. Fewer embryos are needed for transferred and could potentially reduce multiple pregnancies. It has been reported that embryos that fail to reach the blastocyst stage of development are most often aneuploid and are thus incapable of developing into a normal conceptus [68].Magli et al. [69] reported that many day 3 embryos with a “normal” morphology are actually chromosomally abnormal. Alfarawati et al. [70,71] speculated that extended culture of embryos to the blastocyst stage could be used to eliminate many chromosomally abnormal, “incompetent” embryos prior to transfer. In contrast, Kotze et al. [11], reported that 67% of aneuploid oocytes did progress and develop into blastocysts. Therefore, reaching this stage of development by no means provides assurance that embryos are euploid and/or “competent”.

Blastocyst grading systems were developed by Cohen et al. [72], Dokras et al. [73], Gardner and Schoolcraft [74], Richter et al. [75] and Kovacic et al. [76]. These above mentioned grading systems accounts for blastocoel’s expansion, characteristics of the inner cell mass (ICM), and the trophectoderm (TE), respectively.

In 2000, Balaban et al. [77] reported that the transfer of morphologically good quality blastocysts were associated with improved implantation rates as compared to when morphologically “poor quality blastocysts” were transferred. Racowsky et al. [78] suggested that keeping at least two, but preferably three good quality eight-cell (day 3) in extended culture, would likely yield sufficient blastocysts for transfer and/or cryopreservation. Papanikolaou et al. [15] suggested that ideally at least four good quality day 3 embryos should remain in extended culture in order to be more assured that there will be “good morphologic blastocysts” available for transfer and that in so doing the chance of achieving a live birth would be significantly improved.

Several studies have reported improved implantation/pregnancy rates when transferring blastocysts as compared to cleaved embryos: Blake et al. [79] and Mangalraj et al. [80]. A

meta-analysis was performed by Papanikolaou et al. [15] to compare clinical pregnancy rate, multiple pregnancy and live birth rate outcomes where cleaved versus blastocyst stage embryos were transferred and found both clinical pregnancy and live birth rates to be significantly higher in the latter group. (Level I evidence)

Controversially, some studies have shown no difference in ART outcome when comparing blastocyst with cleaved embryo transfers [81,82]. (Level II evidence) In a surprisingly contradiction, Levron et al. [83] reported a decrease in implantation and pregnancy rate associated with the transfer of blastocysts as compared to cleaved embryos and suggested that blastocyst transfers had a negative effect on IVF outcome. (Level II evidence)

Criniti et al. [84] and Zech et al. [85], suggested that transferring a single blastocyst at a time would virtually eliminate the risk of multiple pregnancies. Furthermore, they reported that cryopreservation of supernumerary blastocysts and transferring them subsequently in hormone-prepared frozen/thawed cycles would not compromise the overall live birth rate. Similar suggestions were reported at the American Society for Reproductive Medicine's, 62nd Annual Meeting [86].

2.3.2 Genomics, transcriptomics, proteomics and metabolomics and its role in ART

Lately, advances in genomics, transcriptomics, proteomics, and metabolomics suggest a potential role for these novel techniques in aiding embryo selection.

Proteomics - (non-invasive) - describes the changes in all proteins expressed and translated from a single genome. Currently little is known about the genome and/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 bio-informatics to identify, quantify and characterize a proteome. Continuous research can lead to profiling and understanding the proteome of individual human oocytes and embryos, as well as the proteins produced by the embryo into the surrounding medium (the secretome) by Katz-Jaffe and McReynolds [87]. 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 as reported by Nyalwidhe et al. [88]. 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.

Transcriptomics - (non-invasive) - studies that evaluate the cumulus mass (surrounding oocytes), for the presence of specific messenger ribonucleic acid (mRNA). Katz-Jaffe et al. [89] reported that when these embryos were transferred, live births resulted compared with those that failed to deliver. Assou et al. [90,91] in a review study reported on the cumulus cells (CCs) transcriptomic profiling that predict oocyte and embryo competence. Using reverse transcription polymerase chain reaction (RT-PCR) or deoxyribonucleic acid (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. In 2004, Brison et al. [92] found that changes in the levels of some amino acids are associated with implantation rates. Sturmey et al. [93] 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 could provide 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. [94] 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. In 2010, Seli et al. 2011, [95] introduced a metabolomic Viability Score and claimed that this score alone or in combination with morphologic grading (rather than only using morphology grading) potentially improved pregnancy prediction for single embryos transfer on day 5. Gardner et al. 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 [96]. 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. In 2011, McReynolds et al. discovered the first protein, lipocalin-1, in the secretome of human blastocysts, that is associated with chromosome aneuploidy [97]. If confirmed, this finding could potentially eliminate the use of invasive PGS-techniques currently available.

2.3.3 Biochemical evaluation non invasive

Soluble HLA-G

- While still poorly understood, the embryo implantation mechanism represents a highly complex dialog (cross-talk) between the embryo and the endometrium [98,99]. For Implantation to succeed, the local uterine maternal immune system must undergo profound adaptations. The human body can identify and discriminate against foreign tissue by way of the major histocompatability complex (MHC), human leukocyte antigen (HLA) located on the short arm of chromosome six [6]. When confronted with non-self antigens expressed by the contribution made by a different member of the same species MHC evokes T-cell intervention (allo-immune response), The embryo is comprised cells made up (in part) by the contribution of paternal MHC antigens (in sperm) Once reaching the uterine environment it is immediately targeted as being non-self-tissue. Ordinarily one would anticipate that this would evoke an MHC-T-cell response that would reject the embryo. However, during pregnancy the immune system develops fetal tolerance [100]. HLA-G produced by the extra-villous cyto-trophoblast (the only fetal contact made with maternal endometrial cells), confers immuno-tolerance through interaction with maternal uterine membrane lymphocytes. This scenario would suggest that HLA-G helps protect the developing conceptus from maternal immune response attack. HLA-G is expressed by the trophoblast and then by the placenta throughout

gestation. It is also present in amniotic fluid. Soluble HLA-G (a spliced iso-form of membrane bound HLA-G) is released into the medium surrounding the early embryo in culture where it can be detected and measured 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. [7,8] and Menicucci et al. [101], 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. [9] demonstrated 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 [9]. In 2004, Sher et al. were able to detect sHLA-G in the media surrounding day 2 embryos [102]. Hviid et al. [103] postulated that the presence of sHLA-G protected the conceptus from destruction by the maternal immune response.

As of yet, no consensus has been reached with regard to the best manner by which to express sHLA-G in the culture media. In fact some criticism has been leveled regarding expressing sHLA-G in terms of optical density (OD) [104-108].

The detection of soluble HLA-G in culture medium surrounding embryos has been reported [8,9,101]. Several studies regarding sHLA-G and its effect on pregnancy outcome have been reported since [10,105-107,109,110,111]. A prospective randomized trial by Kotze et al. [10] conducted on more than 200 patients, showed a significant benefit in selecting embryos on the basis of their early sHLA-G expression while in culture. (Level II Evidence) In a multicentre study Kotze et al. [109] confirmed the value of using sHLA-G in the media surrounding early embryos in culture to predict embryo implantation and pregnancy-generating potential following ET. (Level III-2) Sargent et al. [107] and Vercammen et al. [108] performed a retrospective analysis of prior studies that assessed sHLA-G and ART outcome. In a meta-analysis Vercammen et al. [108] reported that embryo selection for transfer based upon advance assessment of sHLA-G expression can indeed augment the ability to predict IVF outcome, especially if the embryos so tested had a high morphologic grade. (Level I) Finally, Rebmann et al. [110] addressed specific issues associated with the use of sHLA-G, ELISA protocols.

The expression of SHLA-G by early embryo is a potentially valuable, non-invasive method by which to in selecting the most “competent embryos for transfer with the objective of limiting the number of embryos transferred while improving implantation and pregnancy rates and reducing multiple pregnancies. This potential could be augmented through selective transferring a single blastocyst derived from early embryos that expressed optimal sHLA-G adequately.

2.3.4 Morphokinetics

Cinematography using different devises: Hoffman Modulation optics Cohen et al. [111], digital imaging system Wright et al. [112], PolScope real time, Shen et al. [21], time lapse video cinematography by Payne et al. [113] and more recent, cinematography (time-lapse imaging) by Lemmen et al. [114], and scanning electron microscopy (SEM) [115] have been reported.

Meseguer et al. [116] and Rubio et al. [117] reported that the continuous monitor of embryo development, applying a noninvasive method, increase the precision and sensitivity of information obtained. An associations has been established between specific morphokinetic

events (during the early cleavage stages of the embryo), and embryo viability and implantation potential. They concluded that predictive morphokinetic markers could improve embryo selection of a single embryo for transfer. (The above two authors hold a financial interest in the "Embryoscope™" deeming this a potential bias group of researchers).

3. INVASIVE METHODS

3.1 Embryo Pre-implantation Genetic Screening (PGS)

Genomics - (invasive) - was used as early as 1990 by Handyside et al. [12] when pre-implantation diagnosis (PGD) was introduced. Fluorescence in situ hybridization (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. [118]. A few years later Kallioniemi et al. [119] 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. [120] and Wells et al. [121]. 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 [122-126].

More recently Treff et al. [127,128] introduced real-time qPCR.

The first successful clinical application of preimplantation genetic diagnosis (PGD) was reported by Handyside [12]. Pre-implantation genetic screening (PGS) of embryos has been selectively used in patients with advanced maternal age (AMA), recurrent pregnancy loss, repeat IVF failure, polycystic ovarian syndrome (PCOS) or in cases of severe male factor infertility [129]. In the past, the most common PGS technique used was fluorescence in situ-hybridization (FISH). FISH is a relatively rapid and easy procedure to perform. However, the validity of FISH-PGS as a method by which to select the "best" embryos for transfer has recently come under criticism. Staessen et al. [130] found that FISH-based embryo selection did not improve IVF embryo implantation and baby rates in women less than 36 years of age. Hardarson et al. [131] reported that PGS-based embryo selection was in fact associated with a decrease in IVF plantation/birth rates in women with advanced maternal age (AMA). In 2005, Li et al. [132] suggested that this finding could be attributable to the cleavage stage of such embryos and the influence of mitotic aneuploidy (mosaicism) which is more prevalent in embryos derived through the fertilization of the eggs from older women.). Another reason why the transfer of FISH-selected embryos does not appear to improve IVF pregnancy/implantation rates is that FISH cannot evaluate all 23 chromosome pairs. At best it can evaluate 12 pairs reliably. However, "FISH-euploid embryos" might still be aneuploid. The opposite scenario has been reported by Northrop et al. [133]. They used a single nucleotide polymorphism (SNP) microarray-based 24 chromosome aneuploidy screening technology to re-evaluate morphologically normal blastocysts that were diagnosed as "FISH-aneuploid embryos" at the cleavage stage, which could result in the erroneous disposal of reproductively competent blastocysts. Bases on the above findings it is strongly recommended that retesting be performed of morphologically normal blastocysts that develop despite an "FISH-aneuploid status".

Kallioniemi et al. [119] developed comparative genome hybridization (CGH) to screen the whole genome's DNA in certain tumors. This technique was subsequently modified to study the DNA in single cells (e.g., blastomeres) by Voullaire et al. [120] and Wells et al. [121].

CGH screens the whole genome and is thus superior to FISH when it comes to embryo selection. The procedure detects anomalies such as chromosome imbalances due to aberrant segregation and structural imbalances (gains/losses) larger than 10-20 Mb as reported by and Malmgren et al. [134]. One disadvantage of CGH is that it is very time/labor intensive – requiring up to four days to obtain results following hybridization and amplification of the DNA and comparing the test DNA to that of a normal template of chromosomes.

In 2007, Sher et al. [68] became the first to report pregnancies through the transfer of blastocysts derived from fresh metaphase-CGH (mCGH) tested, karyotypically normal human oocytes. The same researches went on to report a high pregnancy rate following the transfer of blastocysts derived from day 3, CGH-normal embryos [135,136]. Handyside et al. (2010) [36] reported on the use of single nucleotide polymorphism (SNP)-array to detect chromosome imbalances and genome-wide linkage analysis. (Level IV evidence)

CGH karyotyping of the oocyte's first polar body (PB-I) allows evaluation of the oocyte's genome, the main determinant of an embryo's karyotype and allows for the selection of the most "competent" (euploid) embryos for transfer [11,68,137,138]. However, in order to consider/account for the paternal (sperm) chromosomal contribution to the embryo's genome, Wilton et al. [139], Sher et al. [68], Sher et al. [135] and Kotze et al. [11] started to perform CGH on the embryo, rather than the oocyte.

Sher et al. [135], reported a strong correlation, between the chromosomal status of PB-I (oocyte), PB-II (zygote), day 3 blastomeres and subsequent blastocyst formation. Kotze et al. [11] also reported such correlation with a Kappa of >0.7. Both groups were able to show a high live birth outcome when sequential CGH screening was applied and euploid embryos that developed into blastocysts were transferred.

More recently, Fragouli et al. [138] and Schoolcraft et al. [140] reported on the value of performing CGH on the collective DNA derived through the removal of several blastocyst trophectoderm (TE) cells. However, the time taken to perform CGH required that biopsied blastocysts be cryopreserved and held until the results of CGH testing are available. The process of cryopreserving, storing and then delaying transferring warmed CGH-normal (euploid) embryos in a future frozen embryo transfer (FET) cycle is referred to as "staggered IVF".

Potentially detrimental effects of cryopreserving-PGS/D-tested embryos have been suggested by Zheng et al. [141]. Researchers reported a 30-40% reduction in their implantation potential. However, the recent the introduction of blastocyst verification (ultra-rapid freezing) by Mukaida et al. [142] and Zhang et al. [143] has significantly improved the gamete/embryo cryopreservation process and with it, the survival of warmed embryos. Sher et al. [68] and Kotze et al. [11] reported more than a 50% live birth rates after the transfer of PGS vitrified/warmed blastocysts.

It was recently suggested that a more rapid form of CGH testing known as array CGH (aCGH) [124,126,144,145], by permitting rapid analysis of PB-I, PB-II, Day 3 blastomeres and trophectoderm cells, subsequently avoiding/limiting the need for both embryo cryopreservation and "staggered IVF [146].

Screening the whole genome has shown that anomalies could be present in any of an embryo's chromosomes. Kotze et al. [11] and Traversa et al. [147] showed that some

aneuploid embryos do have the potential to develop to a blastocyst. These findings contradict the belief that embryos exhibiting aneuploidy of the larger chromosomes [1-12] are incapable of developing into blastocysts. While FISH indentifies a single point on a chromosome, CGH-probes cover the entire length of a chromosome, allowing for the detection of structural anomalies such as partial duplications and deletions. Translocation errors can likewise be detected through the ability of CGH to determine a loss or gain of parts of chromosomes. Interestingly, Traversa et al. [148] has shown that FISH suffers from high false positive rates. Furthermore, Traversa et al. [148] reported superior PCR technologies to improve translocation screening. CGH can also 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/targets each cell individually for the specific probe(s) that are applied.

In 2010 Traversa et al. [148] also demonstrated the ability of aCGH to reliably detect aneuploidy in blastocysts. Sher et al. [135] and Kotze et al. [11] used mCGH screening 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.

Currently the two full karyotyping techniques that are still under investigation are:

1. SNP-array: Common polymorphic DNA sequences found throughout the genome is used to detect any chromosome imbalances and genome wide linkage analysis [136,149-151].
2. CGH-array: test and reference DNA is hybridized to DNA probes fixed to a slide. Several reports on the successful use of array-CGH [124,144]. Furthermore, some clinical applications have been reported by Hellani et al. [152], Fishel et al. [153] and Rius et al. [154].

There are emerging developments that hold promise with regard to improving the efficiency/accuracy of PGS and involve the use of BAC microarrays [155,156]. This approach could also be used to determine specific segmental errors during chromosomal rearrangement as reported by Alfarawati et al. [71] and Fiorentino et al. [157]. In an interesting study in 2013 by Mertzanidou et al. [158] (analyzing the whole genome) evidence was presented that around 70% of good-quality embryos carry chromosomal abnormalities, including structural aberrations. In this study they analyzed the majority of the blastomeres from top-quality embryos that originated from a cohort of embryos showing normal developmental rates and high implantation potential.

Several studies have claimed the benefits in recent advances made in the area of the PGS, more specifically, array comparative genomic hybridization technique (aCGH) that is considered an advance in preimplantation genetic testing. A study by Scriven et al. [159], using polar body aCGH indicates that the test accuracy compares favorably with the fluorescence in situ hybridization technique although a substantial number of euploid zygotes are still likely to be excluded incorrectly. A sound argument against selection in principle has recently been published by Mastenbroek et al. [160] and Wikland et al. [161], based on accumulating evidence that potentially all embryos can now be cryopreserved and transferred in subsequent frozen replacement cycles without impairing pregnancy rates. We suggest that vitrification and serial transfer without testing are likely to give patients the best chance for a successful pregnancy, and avoid the use of an expensive technology. Treff et al. [127] and Scott et al. [162], provided evidence of accuracy, safety, clinical predictive

value, and clinical efficacy indicate that trophectoderm biopsy and quantitative real-time polymerase chain reaction (qPCR)-based comprehensive chromosome screening (CCS) and suggested this technique as useful to improve IVF success. Forman et al. [163] reported that transferring of a single blastocyst that underwent trophectoderm biopsy followed by rapid PCR-based comprehensive chromosome screening (CCS) increases ongoing pregnancy rates (OPR) and reduces the miscarriage rate. They furthermore suggested that effective single embryo transfer (SET) will laminate multi-zygotic multiple gestations without compromising clinical outcomes. While extremely encouraging, it is too early to comment or make a judgment on the true value/benefit of CGH. More data (Level I or II evidence) is needed before a final conclusion can be reached. This having been said, this technology (despite being invasive and costly) offers great promise for the future.

4. CONCLUSIONS AND STATE OF THE ART

4.1 Non-Invasive Approach

Non-invasive approach suggests using all available tools for embryo selection. However, a combination of assessment of the pronuclear stage morphology, early zygote cleaving and cleavage-stage embryo morphology/quality on day-3 appears to be inferior. Currently, ultimately choosing a day 5 blastocyst with optimal sHLA-G expression of its predecessor cleaved embryo (for transfer or cryopreservation). The above mentioned criteria should be very beneficial in assisting the technician in making the final decision to select the most competent embryo/s for transfer. This, approach should improve pregnancy outcome and reduce the incidence of multiple pregnancy.

4.2 Invasive Approach

Invasive approach current evidence of accuracy, safety, clinical predictive value, and clinical efficacy indicate that trophectoderm biopsy and quantitative real-time polymerase chain reaction (qPCR)-based comprehensive chromosome screening (CCS), suggested this technique as useful in a specific patient population to identify competent euploid embryos for single embryo transfer without compromising IVF success.

CONSENT

Since the treatments were standard operating procedures to all patients undergoing IVF treatment legally they had to sign the appropriate consent.

ETHICAL APPROVAL

RESEARCH PROJECT : "TO DETERMINE THE DEVELOPMENT POTENTIAL OF A HUMAN EMBRYO *IN VITRO* "
PROJECT NUMBER : N06/07/119

At a meeting of the Committee for Human Research that was held on 2 August 2006 the above project was approved and therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki."

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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