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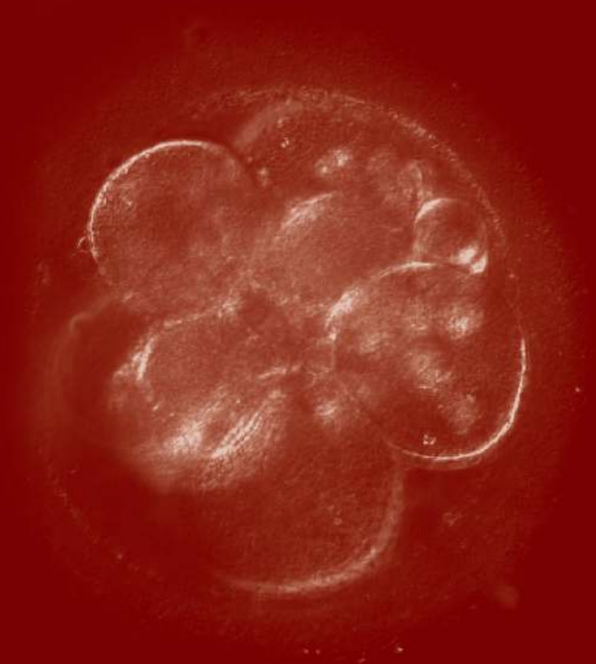

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PhD thesis, University of Amsterdam, the Netherlands

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Improving embryo quality in assisted reproduction

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Faculteit der Geneeskunde

We are at the end of the beginning - not the beginning of the end
Sir Robert Edwards
Time Magazine, August 7 1978

To Eleni
Για την Ελένη

To all couples in need of IVF/ICSI

Contents

CHAPTER 1

General introduction and outline of the thesis

The preimplantation stage of human development is a fascinating biological process where within a very short period of time the two gametes fuse to become a new diploid zygote that can give rise to all cells of the future body. The molecular events that occur during human preimplantation embryo development are poorly understood, but experimental studies in animal model systems (mainly mice) have provided considerable insights into these processes.

The entry of the sperm into the oocyte triggers a cascade of events before the formation of the zygote. These events include amongst others the completion of the second meiotic division of the oocyte, the extraction of the second polar body and chromatin remodeling of the paternal nucleus, thereby permitting the two nuclei to fuse and form the zygote [1]. The zygote is a totipotent cell capable of differentiating into every specialized cell type of the human body to be, including the extra embryonic tissues (placenta). Subsequently, the zygote undergoes a series of mitotic divisions (early cleavages) producing increasing numbers of progressively smaller cells, known as blastomeres, without changing the overall size of the embryo (Figure 1). In humans, the first cleavage occurs at about 24 hours after fertilization and the next cleavages take place at intervals of approximately 24 hours [2, 3]. The cell-cycle length varies not only among embryos but also among blastomeres of the same embryo; thus, the total blastomere number of an embryo can vary from the expected even numbers given in Figure 1. An intriguing characteristic of the early preimplantation embryo during the first four divisions is its adaptability. Exemplary of this is that early embryos can easily withstand removal, addition or rearrangement of cells [4, 5].

The advent of assisted reproductive technologies greatly boasted research on human preimplantation embryos. The first successful *in vitro* fertilization (IVF) of a human oocyte was performed in 1944 by John Rock and Miriam Menkin [6] but it took until 1978 until the first live birth after IVF was reported by Patrick Steptoe and Robert Edwards [7]. Since then, more than five million children have been conceived using assisted reproductive technologies (ART) [8]. In these thirty-five years, some of the major developments in assisted reproduction include improved ovarian stimulation, intracytoplasmic sperm injection (ICSI) to obtain fertilization using only a single spermatozoon [9], blastocyst culture [10, 11], embryo biopsy to diagnose genetic defects [12], egg and embryo cryopreservation [13, 14], and egg and sperm donation [15]. Even though assisted reproductive techniques are well-established treatments for infertility, and despite the above mentioned advancements, the major quest still is to increase success rates. Less than 20% of embryos selected for transfer implant and the two largest data collections report a delivery rate per started cycle of only 20% and 22% for IVF and ICSI respectively [16, 17].

During an IVF/ICSI treatment, oocytes and preimplantation embryos are cultured in the laboratory for a period of two to six days. An inappropriate culture environment could lead to changes in the (epi) genome, transcription, and metabolism of the embryos potentially impairing embryo quality and hence the chance of pregnancy [18-28]. Aspects of *in vitro* embryo culture that could affect embryo quality include culture media, oxygen

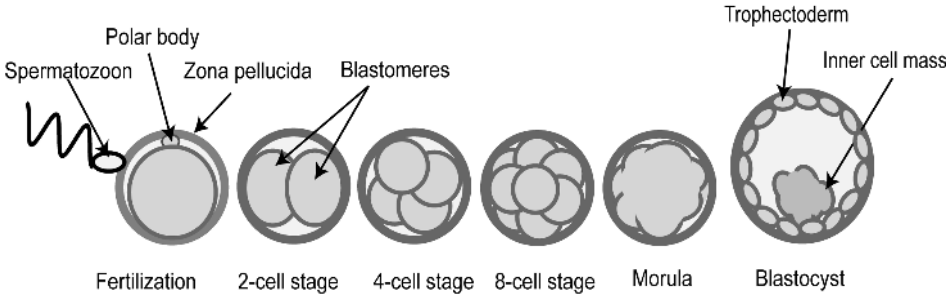

Figure 1: The stages of human preimplantation embryo development.

concentration, mineral oil overlay, temperature, embryo density, air quality, osmolality and pH [23, 27, 29-31].

The first culture media used for human IVF were those that were initially developed for somatic cell and tissue culture and then adapted for embryo culture, at first in laboratory animals (e.g. Ham's F10, Earle's balanced salt solution, Tyrode's T6 and WM1) [32]. Specific culture media for human embryos were further developed halfway the nineteen eighties with the addition of serum albumin as a source for amino acids [33] and the introduction of HTF medium [34] that was based on the then-known chemical composition of human tubal fluid [35]. Over the past decades considerable effort has been made to optimize culture media by reducing glucose concentrations [36], adding amino acids [37-40] and supplementing media with growth factors [41-43]. At the time the research in this thesis was initiated little was known on the response of developing human preimplantation embryos to their environment, but it was well accepted that optimizing culture media could increase embryo quality and hence success rates of the IVF/ICSI treatment.

Background

Molecular control of preimplantation embryo development

During the early cleavages, the human embryo is transcriptionally silent [44, 45] and cell divisions are under control of maternally inherited mRNA and proteins. During this period, maternally synthesized products that were essential for oocyte development are no longer necessary and products that are necessary for proper embryo development should start to be expressed. The time from fertilization to embryonic genome activation varies among different species; in humans, transcription of the embryonic genome does not start until the third day of development at the four-to-eight cell stage [44, 45], whereas in the mouse it already commences at day one at the two-cell stage [46].

The eight-to-sixteen cell human embryo subsequently undergoes compaction, where the intercellular adhesion is increased and the cells adopt a more flattened morphology. After

the fifth cleavage, a small cavity (the blastocoel) starts to form among the blastomeres. The cavity continues to expand and the embryo is then called a blastocyst. At this point, the first cell fate decision is made; external cells (now defined as trophectoderm, TE) adopt an epithelial morphology and surround the whole embryo whereas internal cells (now defined as inner cell mass, ICM) aggregate as a single mass that is attached to the TE [47]. TE is essential for implantation by directly interacting with the mother's uterus, and gives rise to the extra-embryonic tissues including the placenta. The ICM is the pluripotent progenitor of the embryo proper. A study in day 5 human blastocysts has shown that, unlike in mice blastocysts, human TE cells are not yet committed and they can still differentiate into ICM cells when repositioned in the center of an embryo [5].

The mechanisms that govern this decision involve cell polarity, cell position, transcriptional regulation and epigenetic modifications. At the level of transcription, TE cells have more *Cdx2* transcripts than cells that form the ICM. The expression of *Cdx2* down-regulates the expression of *Oct4* and *Nanog* which further promotes the specification of the TE [48]. In contrast, expression of *Oct4* and *Sox2* transcription factors promote the expression of *Nanog* that ensures the pluripotency of the ICM. Cell polarity affects the spatial distribution of *Cdx2* transcripts within a given cell and in turn, *Cdx2* expression reinforces cell polarity [49-51]. In regards to epigenetic regulation, during gametogenesis genome-wide demethylation occurs followed by remethylation before fertilization [52]. Early embryo development is characterized by a second genome-wide demethylation event [52]. At the blastocyst stage, *de novo* methylation is observed specifically in the ICM, but not in the TE, indicating an important role of DNA methylation in lineage specification [53]. DNA methylation of the TE occurs after the segregation of ICM and TE in the mouse preimplantation embryo [54]. Further, post-translational modifications of histone proteins restrict or facilitate access of transcription factors to the above mentioned genes (*Cdx2*, *Nanog*, and *Oct4*) involved in differentiation, controlling their expression in particular cell types [55-57].

Chromosomal aneuploidies in human preimplantation embryos

Studies into the chromosomal content of human preimplantation embryos have revealed a high rate of errors in the number of chromosomes (chromosome aneuploidy). Only 22% of human preimplantation embryos on day 3 have a normal number of chromosomes (diploid), 5% contain the same aneuploidy in all cells and 73% contain cells with different aneuploidies (mosaicism). From the mosaic embryos, 80% are diploid-aneuploid mosaics containing both diploid and aneuploid cells [58]. Chromosomal aneuploidy has a negative impact on embryo quality causing embryos to arrest in their development, to fail implantation, to spontaneously abort or to result in a child with a genetic defect [59-63].

Chromosomal aneuploidies can be inherited from an aneuploid oocyte or - more rarely - from an aneuploid spermatozoon or can arise *de novo* after fertilization. When every cell of an individual embryo carries the same aneuploidy, then the error most probably was caused during meiosis and aneuploidies that are present in some, but not all, cells most

likely have arisen during the mitotic divisions of the developing embryo. The exact causes of these chromosomal errors are not yet fully understood.

The in vitro environment of IVF embryos

It is known from IVF treatments that embryo quality varies among embryos and that the (morphological) quality of the embryo correlates with pregnancy chances after IVF [64, 65]. It is therefore important to define the basic mechanisms controlling human embryo quality, especially in relation to culture conditions. This will enable the design of better culture environments for embryos, thereby minimizing the negative influences of *in vitro* embryo culture, and improving healthy development during pregnancy and healthy post-gestational life. Although multiple embryo culture media are being used in daily IVF practice it is unknown if, and if so to what extent, these media vary in their effect on the embryos cultured in it, on the quality of the embryos, and on the outcomes and success rates of IVF/ICSI treatment.

Aim and outline of the thesis

The goal of this thesis was to improve embryo quality in assisted reproductive technologies by gaining more insight into human preimplantation embryo development and by improving *in vitro* culture conditions. To do so, we investigated an intriguing feature of the human preimplantation embryo, i.e. its mosaic nature, and the occurrence of mitotic aneuploidies. To improve *in vitro* culture conditions, we aimed to elucidate the effect of culture media on human preimplantation embryo development, and on the success rates of IVF/ICSI treatment.

In **Chapter 2** we assessed which preimplantation stages and divisions of human preimplantation development are most susceptible to aneuploidies.

Next, we reviewed the mechanisms causing these abnormalities, and the fate of aneuploid cells within human preimplantation embryos (**Chapter 3**).

To gain more insight into the molecular events that regulate early development of human preimplantation embryos, we developed a protocol that allows accurate and reproducible gene-expression analysis in single cells (**Chapter 4**).

In **Chapter 5**, we used this protocol to determine the influence of various biological and environmental factors, such as the medium used for culturing embryos and the maternal age, on the gene-expression profiles of human embryos.

In order to assess the clinical effect of culture media on IVF/ICSI success rates we first conducted a systematic review to assess their impact on embryo quality and IVF outcomes (**Chapter 6**).

Subsequently, we directly compared two commonly used culture media by means of a multi-center randomized controlled trial (**Chapter 7**).

In **Chapter 8** we discussed the relevance of the work performed in this thesis and presented suggestions for future research.

The results of this work were summarized in **Chapter 9**.

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CHAPTER 2

Temporal and developmental-stage variation in the occurrence of mitotic errors in tripronuclear human preimplantation embryos

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Biology of Reproduction 2013; 89: 42, 1-7

Abstract

Mitotic errors during early development of human preimplantation embryos are common, rendering a large proportion of embryos chromosomally mosaic. It is also known that the percentage of diploid cells in human diploid-aneuploid mosaic embryos is higher at the blastocyst than at the cleavage stage. In this study, we examined whether there is temporal and/or developmental-stage variation in the occurrence of mitotic errors in human preimplantation embryos from the first day of development onwards using mitotically stable digynic tripronuclear human embryos as a model system. All the cells of the 114 digynic tripronuclear human preimplantation embryos included were analyzed by fluorescence *in situ* hybridization for chromosomes 1, 13, 16, 17, 18, 21, X and Y. Embryos were grouped according to day of development (1 - 6) and developmental stage (2-cell to blastocyst stage). The possibility of a mitotic error was highest in the first and second mitotic divisions. The percentage of cells with mitotic errors increased during preimplantation development and was highest at the 9 - 16 cell stage (76%, P=0.027). Thereafter, the percentage of cells with mitotic errors decreased to 64% at the morula and 56% at the blastocyst stage. The pattern found correlates with the activation of the embryonic genome at the 8 - 16 cell stage. A better insight in the timing of occurrence of mitotic errors in human preimplantation embryos could help in understanding and prevention of these errors and is relevant in the context of PGS.

Introduction

Chromosomal mosaicism, the phenomenon that cells in an embryo differ in chromosomal constitution, is common in human preimplantation embryos. A recent systematic review on the prevalence of mosaicism in spare human embryos showed that 73% of all human preimplantation embryos are mosaic [1]. The observed high incidence of mosaicism suggests that human preimplantation embryos are prone to errors during the first mitotic divisions after fertilization. Moreover, it was shown that the percentage of diploid cells in human diploid-aneuploid mosaic embryos was higher at the blastocyst stage (74%) than at the cleavage stage (62%) [1-3]. This suggests that there is a temporal and/or developmental-stage variation in the occurrence of mitotic errors in human preimplantation embryos.

Studies on the occurrence of mitotic errors in human preimplantation embryos are hampered by the fact that only spare embryos, that is, embryos of lower quality that will not be transferred or cryopreserved, can be examined. Another problem is that the examination of such embryos can only start on the day of embryo transfer (usually day 3 after injection or insemination) or later. Therefore, little is known on the occurrence of mitotic errors during the first cleavages of human preimplantation development. The first cleavages can be critical for further embryo development because the embryonic genome is not active yet and the embryo is depended on prestored maternal mRNA and proteins [4].

Here we use human tripronuclear (3PN) embryos to study the occurrence of mitotic errors during the first cleavages of human development. Tripronuclear embryos are embryos that show three pronuclei the first day after insemination and occur with an incidence of 2% - 9% in *in vitro* fertilization (IVF) cycles [5, 6] and 3% - 6% in intracytoplasmic sperm injection (ICSI) cycles [5-7]. In clinical practice, these embryos are never transferred into the uterus as these embryos are considered to be triploid.

Tripronuclear embryos can be divided in dispermic 3PN embryos and digynic 3PN embryos [8, 9]. Dispermic 3PN embryos are most often found in IVF, originating from oocytes that correctly excreted two polar bodies after fertilization but were fertilized by two spermatozoa. Dispermic embryos do not show bipolar spindles, presumably because there is an additional set of centrioles from the second spermatozoon, and as a consequence, they become mosaic from first cleavage onwards [10, 11]. Thus, dispermic 3PN embryos are not a good model for studying the temporal/developmental incidence of mosaicism.

Digynic 3PN embryos are most often found in ICSI, originating from oocytes that were fertilized by a single spermatozoon but failed to extrude the second polar body following fertilization. Digynic 3PN embryos contain only a single bipolar spindle, undergo regular chromosome segregation [12], and have a low rate of mosaicism [11, 12]. In fact, the removal of one pronucleus restores the embryo to normal diploid state [11]. Furthermore, the existence of stable triploid cell lines [13] and the capability of triploid embryos to

develop into blastocysts [14, 15] and even to live births [16] let us assume that these cells are not necessarily mitotically unstable.

In this study, we aimed at determining the difference in susceptibility to mitotic errors at different time points or developmental stages, during human preimplantation development. A better understanding of the developmental and temporal occurrence of mitotic errors will aid in evaluation of the consequences of mosaicism on preimplantation genetic screening (PGS), help determine the optimal developmental stage to perform PGS and, ultimately, help in attempting to prevent these errors in order to increase overall embryo quality and success rates in assisted reproductive technologies (ART).

Materials and Methods

Samples

The study was approved by the Dutch Central Committee on Research involving Human Subjects (CCMO). Only ICSI-derived (digynic) 3PN embryos were used for this study. Couples undergoing ICSI treatment at the Academic Medical Center (Amsterdam, the Netherlands) and University Medical Center Groningen (Groningen, the Netherlands) were asked to participate in this research, and written informed consent was obtained in all cases. A total of 114 fresh 3PN embryos were donated for research.

Donated embryos were randomly fixated on glass slides at six different time points, that is, Day 1 - 6 after fertilization. Embryos were transferred into 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween 20) in 0.01 N HCl to remove the zona pellucida, to dissociate the blastomeres, and to separate the individual nuclei. Fixation was performed by adding methanol:acetic acid (3:1) followed by dehydration in an increasing ethanol series [17, 18].

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) was performed in two rounds for the analysis of chromosomes 1, 13, 16, 17, 18, 21, X and Y as previously described [19]. In the first round, all the available nuclei were analyzed for chromosomes 1, 16 and 17 using chromosome enumeration probes (Abbott Molecular); for the second round, the same nuclei were analyzed for the other five chromosomes with MyltiVysion PGT probes (Abbott Molecular). In case of abundant cytoplasm, the slides were pretreated with 0.0025% pepsin to optimize hybridization results. The following scoring criteria were used for the assessment of chromosome copy numbers: 1) two signals represented two chromosomes if at least one signal fitted in between these two signals, 2) if there was contact between two signals, the signal was judged as one, and 3) in case there was doubt about a signal, then the copy number of that signal was judged as unknown [20].

Assessment of aneuploidy

The chromosomal makeup of each blastomere of an embryo was used to reconstruct the ploidy status of that embryo at the zygote stage (Figure 1). To assess whether an embryo has acquired mitotic errors, we compared its chromosomal content to the presumed chromosomal content of the zygote. When the two did not differ, we assumed no mitotic errors had occurred. When the two chromosomal contents differed, blastomeres with altered content were assumed to have acquired mitotic errors. The probability that an individual blastomere acquires mitotic errors at a given cell division was calculated using the following formula: $P_d = ((\Phi_{d+1} - \Phi_d) / (1- \Phi_d)) / N$, where Φ_d is the observed incidence of chromosome aneuploidy among all embryos at the initiating cell stage and N is the number of cells at the initiating cell stage [21].

Statistical Analyses

All embryos were grouped according to time point (Day 1 to Day 6), and in a second, separate analysis, the same embryos were grouped according to developmental stage (2-cell, 4-cell, 8-cell, 16-cell, morula or blastocyst stage). Embryos with 3 - 4 cells were grouped at the 4-cell stage, embryos with 5 - 8 cells at the 8-cell stage and embryos with 9 - 16 cells at the 16-cell stage. Because part of the analyzed embryos were fixated at Day 1 (2-cell stage), the grouping of the embryos to the different ploidy statuses was based on a minimum of two blastomeres. For the analysis of the data, we assumed that all blastomeres,

Blastomeres within embryo	FISH round 1			FISH round 2				
	#1	#16	#17	#13	#18	#21	#X	#Y
1	1	3	3	3	3	4	4	0
2	3	3	3	4	3	4	3	0
3	3	3	3	3	3	4	4	0
4	3	3	3	5	5	5	4	0
Presumed zygote makeup	3	3	3	3	3	4	4	0

Figure 1: An example of assessing the presumed zygote makeup from the individual blastomeres of a 4-cell embryo. For every chromosome (for example chromosome 1) the number of copies that appeared most often (for example 3) was presumed to be the number of chromosome copies present at the zygote stage.

Table 1: Characteristics of the women that donated the 3PN embryos.

Characteristic	All cycles (n=99)	Cycles of embryos analyzed on Day 1 (n=7)	Cycles of embryos analyzed on Day 2 (n=8)	Cycles of embryos analyzed on Day 3 (n=23)	Cycles of embryos analyzed on Day 4 (n=28)	Cycles of embryos analyzed on Day 5 (n=18)	Cycles of embryos analyzed on Day 6 (n=15)
Age (yr)	35.5 ± 4.2	36 ± 3.6	35 ± 4.2	35 ± 3.9	35 ± 4.0	35 ± 4.8	38 ± 4.0
Gravidity	0.7 ± 1.0	1.0 ± 1.2	1.0 ± 1.1	1.0 ± 1.2	1.0 ± 1.1	0.0 ± 0.6	1.0 ± 0.8
Parity	0.4 ± 0.8	0.7 ± 1.1	1.0 ± 1.1	0.0 ± 1.1	0.0 ± 0.7	0.0 ± 0.4	1.0 ± 0.7
Duration of infertility (yr)	3.3 ± 1.9	2.4 ± 1.3	3.0 ± 0.6	4.0 ± 2.4	4.0 ± 2.0	4.0 ± 1.9	2.0 ± 1.4
Cause of infertility, no. (%)							
Tubal	2 (2.0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (11.1)	0 (0)
Male factors	79 (79.8)	6 (85.7)	6 (75)	19 (82.6)	21 (75)	14 (77.8)	13 (86.7)
Endometriosis	4 (4.0)	0 (0)	0 (0)	1 (4.3)	3 (10.7)	0 (0)	0 (0)
Ovarian factors	3 (3.0)	0 (0)	0 (0)	1 (4.3)	1 (3.6)	1 (5.6)	0 (0)
Unexplained	11 (11.1)	1 (14.3)	2 (25)	2 (8.7)	3 (10.7)	1 (5.6)	2 (13.3)

whether diploid or aneuploid, divided at a similar rate during early embryo development [21]. To determine whether there was a temporal and developmental window during which mitotic errors occur more frequently, we plotted the percentages of blastomeres with mitotic errors against the developmental stage (number of blastomeres present in an embryo) or day of development. To investigate whether the occurrence of mitotic errors followed a certain pattern over time, we used regression analysis. The data were fitted to three different regression models (following linear, quadratic, and cubic patterns), and the *P* value for each model was calculated to select the pattern that described our data most accurately.

Results

Clinical parameters like maternal age, gravidity, parity, duration and causes of infertility of the women that donated the 3PN embryos are provided in Table 1. The average maternal age of all women was 35 yr of age. The embryos that were analyzed on Day 6 of development were provided from women of an average of 38 yr of age, but this difference was not significant.

Chromosomal makeup of 3PN embryos

Seven hundred and fifty-seven blastomeres from 114 tripronuclear embryos were analyzed. The precise findings of all the embryos are shown in Table 2. In total, we found that at the time of analysis two embryos (2%) were uniformly diploid, six (5%) were uniformly aneuploid, and 106 (93%) were mosaic. Upon reconstruction of the chromosomal makeup of the zygote, 21 (18%) were diploid, 25 (22%) were triploid, six (5%) haploid and 62 (54%) were aneuploid (Table 2).

Table 2: The chromosomal makeup of 3PN human preimplantation embryos (n=114) at the assumed zygote stage and at the time of analysis.

Chromosomal makeup	Criteria	Analysis per developmental stage							Assumed zygote stage
		Number of embryos (%)							
		Total (n=114)	2-cell stage (n=7)	4-cell stage (n=36)	8-cell stage (n=35)	16-cell stage (n=14)	Morula (n=12)	Blastocyst (n=10)	Number of zygotes (%)
Diploid	All blastomeres contain two chromosomes for each chromosome pair tested	2 (2)	1 (14)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	21 (18)
Mosaic		106 (93)	4 (57)	32 (89)	34 (97)	14 (100)	12 (100)	10 (100)	0 (0)
Diploid-aneuploid mosaic	A mosaic embryo with one or more diploid blastomeres but without triploid blastomeres	20 (18)	2 (29)	0 (0)	7 (20)	2 (14)	4 (33)	2 (20)	0 (0)
Diploid-triploid mosaic	A mosaic embryo with both diploid and triploid blastomeres	3 (3)	0 (0)	3 (8)	2 (6)	0 (0)	0 (0)	1 (10)	0 (0)
Triploid-aneuploid mosaic	A mosaic embryo with one or more triploid blastomeres but no diploid blastomeres	26 (23)	1 (14)	7 (19)	4 (11)	3 (21)	5 (42)	6 (60)	0 (0)
Aneuploid mosaic	A mosaic embryo without the presence of diploid or triploid blastomeres	57 (50)	1 (14)	22 (61)	21 (60)	9 (64)	3 (25)	1 (10)	0 (0)
Aneuploid		6 (5)	2 (29)	4 (11)	0 (0)	0 (0)	0 (0)	0 (0)	93 (82)
Haploid	All blastomeres contain one chromosome for each chromosome pair tested	2 (2)	0 (0)	2 (6)	0 (0)	0 (0)	0 (0)	0 (0)	6 (5)
Triploid	All blastomeres contain three chromosomes for each chromosome pair tested	3 (3)	2 (29)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	25 (22)
Polyploid	All blastomeres contain more than three chromosomes for each chromosome pair tested	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Aneuploid	All blastomeres contain the same abnormality for one chromosome pair tested	1 (1)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	62 (54)
Complex abnormal	All blastomeres contain the same abnormalities for multiple chromosome pairs tested	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Occurrence of mitotic errors

A high percentage of blastomeres with mitotic errors was observed at all days and at all stages of human preimplantation development. The percentage of blastomeres with mitotic errors increased during the first divisions, showing the highest percentage (67%) at Day 5 (Figure 2). The percentage of blastomeres with mitotic errors decreased to 61% at Day 6. When the embryos were analyzed according to developmental stage, the same pattern was observed, with an increase in the percentage of blastomeres with mitotic errors up to the 9 - 16 cell stage (76%) and then a decrease to 64% at the morula stage and 56% at the blastocyst stage (Figure 3).

Regression analysis showed that the data could follow all three regression models tested, but the data followed the quadratic model best (linear model: P=0.018 and P=0.041 according to day of development and developmental stage, respectively; quadratic model: P=0.010 and P=0.001 according to day of development and developmental stage, respectively; and cubic model: P=0.027 and P=0.002 according to day of development and developmental stage, respectively) (Figure 4).

Using the approach described from Bean *et al*. [21], we calculated the probability that a specific cell acquired mitotic errors at a given cell division. The results of this analysis showed that the probability of a specific cell to acquire mitotic errors decreased with each division and leveled out at the morula and blastocyst stage (Figure 5). At the morula stage, the possibility of a given cell to malsegragate has a negative value as the incidence of the observed occurrence of aneuploidy at the morula stage is lower than the incidence of the observed occurrence of aneuploidy in the developmental stage before that (16-cell stage).

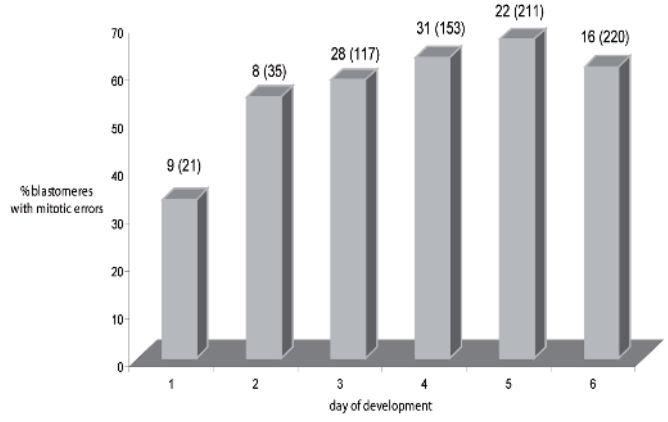

Figure 2: Percentage of blastomeres with mitotic errors in human preimplantation 3PN embryos at different days of development. The number of embryos (and blastomeres) analyzed per group is indicated above each bar.

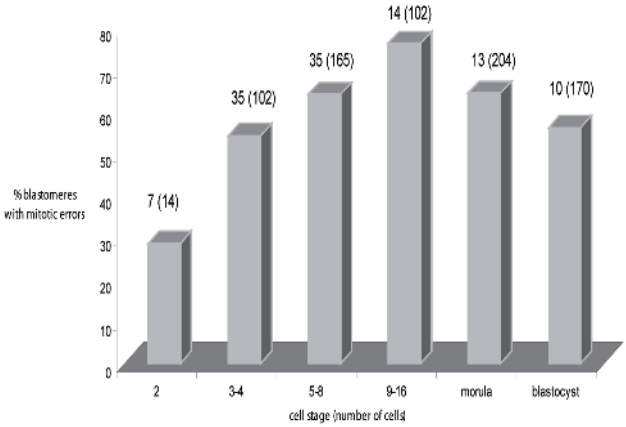

Figure 3: Percentage of blastomeres with mitotic errors in human preimplantation 3PN embryos at different developmental stages. The number of embryos (and blastomeres) analyzed per group is indicated above each bar.

Discussion

Our results showed that the possibility of any given cell to malsegregate is highest in the first two cleavages after fertilization. These results are in accordance with previous studies on mouse and human embryos that showed that nondisjunction events predominantly pertain to the earliest cleavage divisions [21, 22]. The percentage of cells with mitotic errors increased during early development, peaking at the 9 - 16 cell stage and decreasing thereafter. This pattern resembles normally fertilized (2PN) human preimplantation embryos where the percentage of diploid cells in blastocysts was found to be higher than in cleavage embryos [1].

Regression model	Formula	*P* value	
		Per day	Per stage
Linear	$y = \alpha + \beta x$	0.018	0.041
Quadratic	$y = \alpha + \beta x^2 + \gamma x$	0.010	0.001
Cubic	$y = \alpha + \beta x^3 + \gamma x^2 + \delta x$	0.027	0.002
$\alpha, \beta, \gamma, \delta$ constants and $\beta \neq 0$			

Figure 4: The obtained *P* values per regression model used.

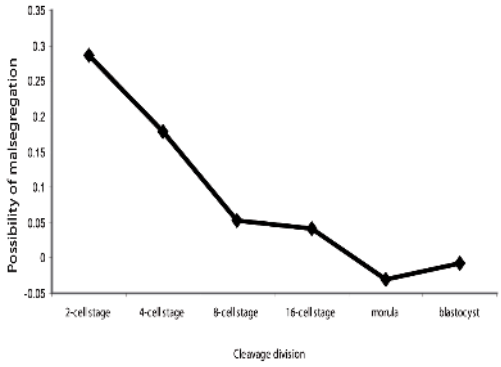

Figure 5: Possibility of a given cell to malsegregate at different developmental stages.

Limitations of the study

In this study, we used the most commonly used technique, FISH to define the chromosomal status of 3PN embryos. We were therefore unable to detect embryos that were aneuploid for the chromosomes that we did not test. Moreover, FISH does not allow the detection of partial or segmental aneuploidy since the FISH probes hybridize to a specific locus or the centromere and provide information only about that segment of the chromosomes [23]. Another technical limitation of FISH is that scoring errors can potentially occur due to loss or damage of nuclear material, overlapping of signals, split signals, diffused signals, hybridization failure, and probe inefficiency. FISH analysis has 92% - 99% accuracy per probe, so when using a multiprobe panel on one cell, the risk of misdiagnosis is indeed significant [24-26]. Nonetheless, FISH can be considered a reliable technique for aneuploidy determination for research purposes [24]. Because the focus of this study is not to report the exact rate of aneuploidy at the different developmental stages, which could be affected by scoring errors, but rather to study the pattern of its occurrence, and FISH scoring errors are not likely to occur more in one developmental stage compared to the other, we believe that the use of FISH did not affect our results.

Chromosomal makeup of 3PN embryos

In our study the percentage of 3PN embryos at the cleavage stage that were fully triploid was 3%. This percentage is in contrast to a study that reported a percentage of 55.7% of fully triploid 3PN embryos derived after ICSI [12]. This difference might be due to the fact that the other study analyzed only three chromosomes (18, X and Y) while we analyzed eight. The effect of the number of analyzed chromosomes on the percentage of mosaicism reported was also described in a recent review [1]. Also, we reported a slightly higher rate of triploid embryos at the cleavage stage (38% Days 2 - 4) in comparison to a study that found 32% of the ICSI-derived tripronuclear embryos to be triploid [14]. In contrast to our study, the study by Grau *et al*. analyzed five chromosomes in one hybridization round and only one blastomere per embryo.

When we reconstructed the chromosomal makeup of the zygotes using the chromosomal composition of the analyzed blastomeres, we found that 25 (22%) of the embryos were presumably triploid at the zygote stage. Our findings on the percentage of 3PN zygotes that are fully triploid are in contrast to those of other studies that showed a percentage of 7% - 88% of the 3PN zygotes to be triploid [5, 27]. This difference might be explained by the different criteria of assessing triploidy of zygotes. We used the analysis of individual blastomeres to reconstruct the presumed chromosomal makeup of the zygote while the other studies analyzed 3PN zygotes that were arrested before the first cleavage division. In addition, we strictly followed the rule that the zygotes had to show three copies of all analyzed chromosomes, while one study assessed zygotes as triploid if the majority of chromosomes were present in three copies [5].

Occurrence of mitotic errors

We found high rates of mitotic errors in the first days after fertilization that are reduced after the 9 - 16 cell stage. The first mitotic divisions are almost completely dependent on stored mRNA and proteins in the oocyte because there is no transcription from the embryonic genome in this early stage of development [4, 28, 29]. The quality of human oocytes, their proteins, and gene transcripts could diminish over time because of factors such as the accumulation of radiation or toxic agents, oxidative stress [30], compromised mitochondria [31], or telomere shortening [32]. This compromised environment could then result in absence or reduced stringency of cell-cycle checkpoint mechanisms during the earliest stages of human preimplantation development, thereby facilitating chromosome segregation errors, especially in women of advanced maternal age [29, 33, 34]. Once the embryonic genome is activated, cell-cycle checkpoints could become functional again, resulting in relatively less aneuploid cells afterwards. It could therefore be hypothesized that the incidence of mosaicism will be highest in human preimplantation embryos around the 8-cell stage or Day 3 after fertilization, just before the embryonic genome is activated [35].

Apart from reinstating the mitotic machinery, the activation of the embryonic genome potentially initiates other phenomena that could explain the lower rate of aneuploidies at later stages of development. First, embryonic genome activation could induce the correction of aneuploidies via mechanisms such as anaphase lagging, corrective non-disjunction or chromosome fragmentation [36]. Second, it could induce preferential proliferation of diploid cells and induce arrest or apoptosis of aneuploid cells [37]. Indeed, data indicate that apoptosis does not become activated in preimplantation embryos until the morula stage [38]. Third, it could induce preferential allocation of diploid cells to the inner cell mass or embryo proper [35]. Confined placental mosaicism, in which placental tissue was trisomic whereas the fetus was diploid, has been reported as a result of the loss of trisomic chromosomes in the embryonic tissue [39]. However, further research on the fate of the abnormal blastomeres within an embryo is necessary to confirm this hypothesis [40-42].

In conclusion, our data indicate that there is clear temporal and developmental-stage variation in the occurrence of mitotic errors in human preimplantation embryos. As mitotic errors are most likely to occur during the first three cleavages, more research should be conducted into which elements (either biological or technical) control their occurrence during this stage of development. Possibly, improved ovarian stimulation or *in vitro* culture systems will limit the rate of mitotic errors and thereby increase overall embryo quality and, with that, treatment success in ART. The high prevalence of mosaicism at all stages of preimplantation development causes a serious diagnostic dilemma to PGS during which the selection of embryos for transfer into the uterus is based on the chromosomal content of a single blastomere that is removed from each embryo. Embryo biopsy for PGS has often been performed at the third day of embryo development, and our data indicate that this is in fact the time when the percentage of blastomeres with mitotic errors is highest.

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CHAPTER 3

Molecular origin of embryonic mitotic aneuploidies

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Abstract

Mitotic errors are common in human preimplantation embryos. The occurrence of mitotic errors is highest during the first three cleavages after fertilization and as a result about three quarters of human preimplantation embryos show aneuploidies and are chromosomally mosaic at day three of development. The origin of these preimplantation mitotic aneuploidies and the molecular mechanisms involved are being discussed in this review.

At later developmental stages the mitotic aneuploidy rate is lower. Mechanisms such as cell arrest, apoptosis, active correction of the aneuploidies and preferential allocation of the aneuploid cells to the extra-embryonic tissues could underlie this lower rate.

Understanding the mechanisms that cause mitotic aneuploidies in human preimplantation embryos and the way human preimplantation embryos deal with these aneuploidies might lead to ways to limit the occurrence of aneuploidies, in order to ultimately increase the quality of embryos and with that the likelihood of a successful pregnancy in IVF/ICSI.

1. Introduction

The introduction of human IVF into clinical practice made it possible to study human embryos in the earliest stages after conception and it was rapidly discovered that aneuploidies, i.e. numerical chromosome abnormalities, exist in human preimplantation embryos [1-3]. In 1993, chromosomal mosaicism, the phenomenon that not all cells in an embryo have the same chromosomal content was described in human preimplantation embryos for the first time [1] and since then many studies have been published on this topic. Aneuploidies have been found in embryos from women of different age, in arrested and developing embryos, in fresh and in frozen-thawed embryos, and in fragmented and good morphology embryos [4-11].

Mosaic embryos can consist of both diploid and aneuploid cells (diploid-aneuploid mosaics) or of cells with multiple aneuploidies involving more than one chromosome (aneuploid mosaics). The application of high resolution DNA techniques such as array comparative genomic hybridization (CGH) revealed that structural abnormalities, apart from numerical abnormalities, also occur in cleavage stage human embryos, leading to partial mosaicism of certain chromosomal segments [12-14].

Mitotic aneuploidies have been suggested to affect the developmental potential of human preimplantation embryos, possibly leading to developmental arrest or embryo loss at later stages of development [5, 9, 15, 16]. Mitotic aneuploidies may contribute to implantation failure or when compatible with implantation, may result in fetal or confined placental mosaicism [5]. It might also cause serious fetal complications like intrauterine growth delay, congenital malformations, mental retardation, and uniparental disomy [17].

The goal of this review is to describe the frequency of mitotic errors in human preimplantation embryos and to provide insight into the cause and the fate of these mitotic errors.

2. Frequency of mitotic aneuploidies

The frequency of mitotic aneuploidies in human preimplantation embryos can be deducted from the frequency of mosaic embryos. However, there is considerable heterogeneity in the reported frequency of mosaic embryos in the literature, with frequencies varying from 15% to more than 90% [5, 18, 19]. To resolve this heterogeneity, a systematic review and meta-analysis of studies on the chromosomal constitution of human preimplantation embryos has recently been performed [20]. This review shows that 73% of all human preimplantation embryos after IVF are mosaic, 22% are diploid and 5% contain other abnormalities [20]. Of the mosaic embryos, diploid-aneuploid mosaic embryos are most common (59% of all embryos) followed by aneuploid mosaic embryos (15% of all embryos).

Several factors contribute to heterogeneity in the reported frequency of mosaic embryos in the literature; the definition of mosaicism that is used, the number of chromosomes that

have been analyzed, the type of embryos that have been studied, the developmental stage at which the embryos have been analyzed and the method of analysis that was used. One of the main reasons is the definition of mosaicism that is used in a study. While some studies classify embryos as mosaic as soon as a single cell has a different chromosomal content, others classify an embryo as diploid even when a percentage up to 50% of aneuploid cells is present [4, 21, 22].

The number of chromosomes that is analyzed also plays an important role in the varying rates of mosaicism reported [20]. Since the aneuploidies found in human preimplantation embryos are not limited to specific chromosomes, more mosaic embryos will be detected when more chromosomes are tested. Most of the studies target the sex chromosomes and the smaller autosomes as these chromosomes are more often found in aneuploidies in prenatal samples and miscarriages but they might not necessarily be the most frequent in preimplantation embryos [7]. Several studies try to increase the number of probes used in FISH, with 12 probes being the maximum number of probes used in three consecutive hybridization rounds [23]. Mitotic aneuploidies involve all chromosomes, with some chromosomes being implicated more frequently; sex chromosomes (24.1%) and chromosomes 8 (12.1%), 2 (8.6%), 16 (8.6%), 7 (5.2%), 13 (5.2%), 18 (5.2%), 20 (5.2%) and 21 (5.2%) [24, 25]. However, these studies investigate only a few chromosomes. Some information concerning the frequency of mitotic errors for all 24 chromosomes is available but no extensive study comparing the frequency of mitotic errors among all chromosomes in a substantial number of embryos has been performed yet [12-14].

Almost all studies on the frequency of mitotic aneuploidies use spare embryos of compromised quality rather than embryos of good morphology that are transferred or cryopreserved. In one study, a cohort of embryos without selection, transfer or cryopreservation was studied, displaying a rate of diploid-aneuploid mosaic embryos not different from the rate reported for spare embryos (53% and 59% respectively) [20, 22].

Mitotic aneuploidies have been observed at all developmental stages of human preimplantation development, with the first three mitotic divisions appearing more error-prone [15, 26].

Comparisons of aneuploidy rates between cleavage stage embryos and blastocysts are conflicting with some studies reporting similar rates [6], some studies reporting a lower rate in blastocysts compared to cleavage stage embryos [5, 11, 27-29] and some studies reporting a higher rate in blastocysts compared to cleavage stage embryos [30, 31]. Meta-analysis shows that mitotic aneuploidies increase from 63% at the cleavage stage to 95% at blastocyst stage [20]. Even though the percentage of mosaic embryos increases at the blastocyst stage, the proportion of aneuploid cells within the embryos decreases so that blastocysts contain relatively more diploid cells (74%) compared to cleavage stage embryos (62%) [5, 10, 18, 20, 30, 32, 33]. The percentage of cells with mitotic aneuploidies decreases further in the fetus and after birth. Mosaicism, if thoroughly analyzed, would

most probably be present in all individuals in the general population but in an insignificant, low level that remains undetected and has no apparent phenotypic effect.

Mosaicism has not only been found in human embryos but also in embryos from other species, such as cattle (22% - 42%) and nonhuman primates (22% - 32.1%) [34-37]. While meiotic aneuploidies are present in mouse embryos albeit at a very low rate, data on mitotic aneuploidies in mice are lacking [38]. In order to study the occurrence and fate of mitotic aneuploidies in mice, mitotic errors are usually induced by, for example, exposure to acrylamide, a carcinogen present in tobacco that induces chromosomal damage at first cleavage and abnormal preimplantation development, or use of spermatozoa that had been exposed to γ-rays [39, 40]. In addition, several mice models have been developed such as the *Sycp3*-null mice or the *BALB/cWt* mice that have an increased rate of nondisjunction events [41, 42].

2.1. Technology

The methodology used to analyze human preimplantation embryos also contributes to the variation in the reported frequency of mitotic aneuploidies [20]. Some of the first studies reporting on mitotic aneuploidies in human embryos used karyotype analysis [43-45]. Although karyotyping allows for analysis of all chromosomes, it requires dividing, metaphase-stage cells. Only 24% - 36% of the embryos analyzed by karyotyping produced metaphases of sufficient quality for accurate chromosome quantification and in less than 25% of the embryos all cells could be analyzed [43, 45]. This technique is also biased towards developing cells and embryos as arrested cells cannot be analyzed since these cells do not produce metaphases. Other disadvantages of the karyotyping technique are the difficulty to obtain optimal chromosomal banding, making individual chromosomes hard to identify, and the possible loss of chromosomes during fixation of the nuclei [43, 45]. Karyotyping is no longer used for the analysis of chromosomal aneuploidies in human preimplantation embryos.

The technique that has been used most often for the analysis of chromosomal aneuploidies in human preimplantation embryos is fluorescence *in situ* hybridization (FISH) [1, 7, 10, 16, 18, 22, 23, 46-48]. FISH can be applied to single cells and allows analysis of chromosome number both in metaphase and interphase nuclei. However, the technique is limited by the number of probes that can be used simultaneously as only a limited number of spectrally distinct fluorochromes are available for labeling the probes. To overcome this limitation, multiple sequential FISH rounds can be used. However, during sequential hybridization the efficiency of hybridization decreases with each round so it has been suggested to use no more than three rounds [48]. Also, FISH has an estimated accuracy of 92% - 99% per probe so there is a substantial risk of misdiagnosis when multiple probes are used [16]. It has been calculated by a mathematical model that the predictive value of FISH for an abnormal test result is 90% when two chromosomes are tested but could be as low as 41% when five chromosomes are investigated [49]. A limitation of FISH is the necessity for

fixation and spreading of the nucleus of a single cell on a microscope slide which could lead to chromosome loss or scoring errors that arise from split signals, overlapping signals, damage to the nucleus, loss of micronuclei, hybridization failure and probe inefficiency [16]. In order to improve accuracy, a sequential FISH protocol using additional probes that bind to different loci of the same chromosomes can be used. Subsequent hybridization rounds can also be performed for any non-conclusive result and for confirmation of specific aneuploidies like monosomies [50, 51]. The error rate of FISH for aneuploidy screening has been described to lie between 3.7% - 17.3% [50-52]. Moreover, FISH does not allow the detection of partial or segmental aneuploidy since the FISH probes hybridize to a specific locus or the centromere and provide information only about that segment of the chromosome [53].

More recently, the examination of the copy number of all 24 chromosomes has become possible with CGH, CGH-microarrays (aCGH) and single-nucleotide polymorphism-based (SNP) microarrays following whole genome amplification (WGA) [12-14, 54-57]. With CGH, chromosome copy number analysis is achieved by co-hybridization of differentially labeled DNA samples (a reference, most often diploid sample and a test DNA sample of unknown chromosomal status) to metaphase spreads derived from cells of a chromosomally diploid individual and comparison of the relative levels of hybridization. aCGH is based on the same principle as CGH but uses genomic clones from selected regions of the genome that are spotted on a slide, instead of metaphase cells. These techniques are applicable to cells at any phase of the cell cycle and avoid the use of fixation and spreading. They also have increased resolution and their analysis can be automated. Another advantage is the possibility to detect segmental aneuploidy [53]. The major challenge in performing genome-wide, single-cell analysis is the possible introduction of bias as a result of failure to amplify one of the parental alleles at random or excess amplification of specific regions [54, 57-59]. CGH has proven to work at least equally as well as FISH, having further the ability to test all 24 chromosomes [53, 60, 61]. The recent advances in the use of array technology for determining the chromosomal status of a single blastomere will lead to important new insights and will more accurately determine the actual preimplantation aneuploidy and mosaicism rates. However, as no test with 100% accuracy is yet available, one should be careful regarding the clinical use of these tests as it could result in discarding viable embryos [61].

3. Origin of mitotic aneuploidies

3.1. Mechanisms leading to mitotic aneuploidy

Anaphase lagging in mitosis describes a delayed movement of one chromatid during anaphase, where the chromatid fails to connect to the spindle apparatus and is not incorporated in either nucleus of the daughter cells. The lagging chromosome is lost, resulting in a monosomy in one of the two daughter cells. Nondisjunction is the failure of the sister chromatids to separate properly during mitosis, resulting in a cell with a loss

and a cell with a gain of a chromosome. These two mechanisms are the most common mechanisms leading to mitotic aneuploidies in human embryos [6, 7, 18, 26, 30]. Other mechanisms that could cause aneuploidies are chromosome demolition, premature cell division, errors in cytokinesis, cell fusion and chromosome breakage (Figure 1). A study analyzing 299 blastocysts using FISH for chromosomes X, Y and 18 reports that two-thirds of all complex mosaic blastocysts are abnormal due to anaphase lagging of both an autosome and a sex chromosome [30]. Cells from the remaining blastocysts contain both nondisjunction and anaphase lagging events with nondisjunction occurring mainly in the sex chromosomes and anaphase lagging in the autosomes [30]. Another study analyzing 47 day 5 embryos using three rounds of FISH reports that chromosome loss is the main mechanism leading to aneuploidy [18]. Even though the authors conclude that the observed chromosome loss is probably the result of anaphase lagging, the equally high observed frequency of chromosome gains in this study could also suggest a high rate of mitotic nondisjunction.

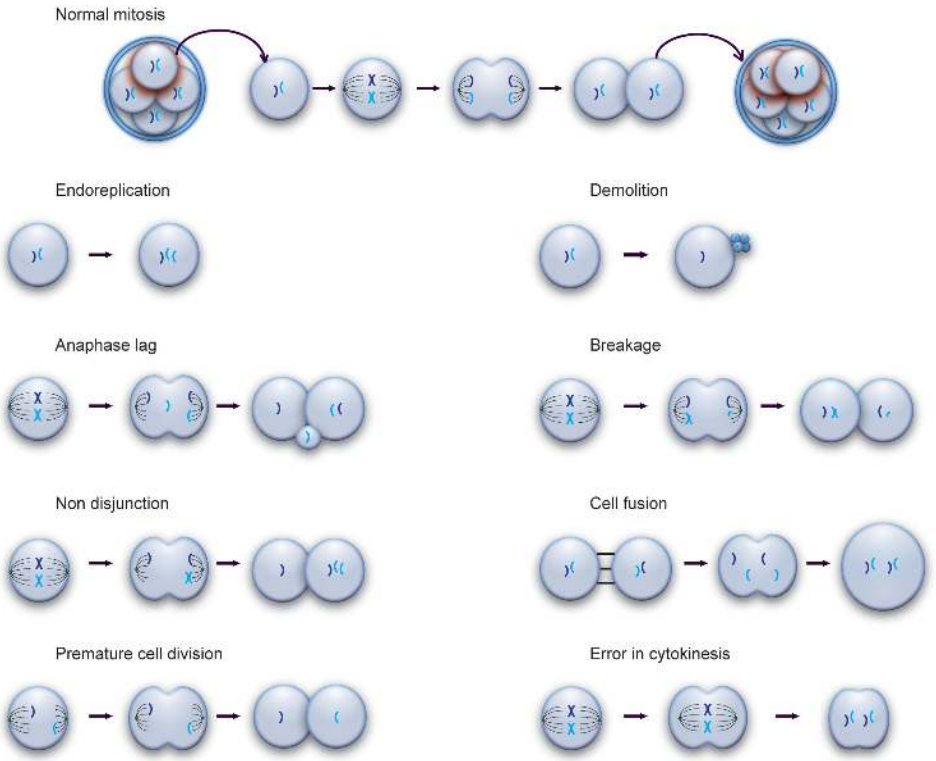

Figure 1: Mechanisms leading to mitotic aneuploidy. Schematic representation of the mechanisms that could lead to aneuploidy.

The observed monosomies should be interpreted with caution when FISH is used since monosomies could be FISH artifacts either due to overlapping signals or due to hybridization failure. Also, it might be that nondisjunction, resulting in both losses and gains, occurs in a higher rate but the cells with complementary chromosomal content are eliminated or are not analyzable. More recently, a combination of FISH, CGH and aCGH shows no excess of chromosome losses compared to chromosome gains [60].

Chromosome demolition, which involves the destruction and fragmentation of one chromosome, has been proposed as a mechanism for chromosomal rescue of trisomic zygotes [62]. However, if chromosome demolition occurs in a normal diploid cell it will lead to aneuploidy.

Premature cell division without prior duplication could result in haploid cells and polyploid cells may be caused by endoreplication of the chromosomes two or more times. Endoreplication has been observed in human zygotes and in trophoblasts of first trimester human placenta [63, 64]. In human embryos, tetraploidy caused by endoreplication in a single two-cell embryo has been observed [65].

Errors in cytokinesis are also likely to contribute to mitotic aneuploidy [10, 66-69]. Failed or asymmetric cytokinesis results in the formation of binucleated cells, tetraploidy or spindle pole abnormalities and chromosomal chaos [70]. Analysis of the cells from cleavage stage human embryos at the 2 - 4 cell stage reveals multinucleated cells in 17% of cases, the incidence of which peaked during the third cleavage division (65%) [68]. The proportion of binucleate blastomeres within normally fertilized embryos increases from 5% in 2 - 4 cell embryos to 10% in 9 - 16 cell embryos, whereas in polyspermic or parthenogenetic embryos the proportion is higher during early cleavage stages but decreases at the nine- to 16-cell stage (25% and 6%, respectively), due to arrest of these embryos [68]. Comparison of the size of the multinucleated blastomeres to that of normal mononuclear blastomeres suggests that multinucleated blastomeres arise from failure in cytokinesis. This may result from deficiencies in maternal molecules involved in polarization, cell division and compaction or from defects at the cell surface affecting cell-cell interactions. Cell-cell interaction in cytokinesis has been shown to be important in normal mitosis by the observation of reversible uncoupling of gap junctions between blastomeres in mice [71]. The formation of binucleated blastomeres could also arise from haploid mononucleated blastomeres where endoreplication occurred followed by karyokinesis but not cytokinesis [72].

Cell fusion of blastomeres is another possible mechanism contributing to mitotic aneuploidies. Cell fusion has been demonstrated in frozen-thawed human embryos resulting in polyploid or diploid-polyploid mosaic embryos [73]. It is suggested that the occurrence of blastomere fusion could be associated with changes of the cell membrane, for instance fluidity that might happen normally during embryo development or might occur after freeze/thaw. Also, changes in pH, temperature or osmotic pressure might

cause blastomere fusion. However, so far cell fusion has been linked to treatment effects and there is no evidence that it will occur spontaneously in embryos. Even if it occurs spontaneously, cell fusion is a less frequently described mechanism compared to the other proposed mechanisms.

Another mechanism leading to aneuploidy is chromosome breakage causing partial chromosome loss and gain [6, 13, 14]. The breakage affects almost all chromosomes [6, 74], with some chromosomes having defined fragile sites implicated more often [75]. Further, structural aberrations are reported in embryos from women with repeated implantation failure or advanced maternal age; gain/loss of entire chromosome arms is most common but aberrations affecting only part of an arm are also present [53, 76-78]. Partial aneuploidy is likely to result in an unstable karyotype due to the formation of acentric and dicentric chromosomes [78]. Despite the importance of the data reported, these studies are limited by the resolution of the CGH which is 40Mb and so chromosome breakage resulting in smaller deletions or duplications would not have been detected. SNP array and array CGH technologies have a higher resolution [57, 79-81]. Using such techniques, it has been shown that seventy percent of top quality embryos from young and fertile patients (couples entering the IVF-program for sex selection) are mosaic for segmental deletions, duplications and amplifications [12]. Based on the aneuploidy patterns observed it is hypothesized that these aneuploidies occur via interstitial or terminal DNA double-stranded breaks and fusions in the zygote or during the first two cleavages [74]. These high levels of partial aneuploidy have yet to be confirmed by other investigations.

3.2. Molecular mechanisms involved in aneuploidy

Preimplantation mitotic aneuploidy could be the result of several interrelated events. The mechanisms contributing to mitotic aneuploidy in human embryos will be discussed in the following sections.

3.2.1. Maternal factors leading to aneuploidy

The activation of the human genome occurs at the 4- to 8- cell stage and many of the proteins regulating correct chromosome segregation during the first divisions are provided by the oocyte [82]. A defective maternal mRNA and protein pool could lead to failure of the mechanisms that guide and control cell division. Free radicals that are accumulating in the oocytes throughout the years until fertilization, exposure to external factors like radiation or chemicals, or poor vascularization of the antral follicle during oocyte maturation might harm the oocyte protein pool. Examples of mechanisms that are affected by maternal proteins involve microtubule kinetics, cell-cycle checkpoints, DNA repair proteins, chromosome cohesion, telomere shortening and mitochondrial function [83-90]. Although the overall mosaicism rate in human embryos does not increase with maternal age [26, 91], data suggest that mitotic nondisjunction does [76, 92, 93].

In mice oocytes, the mRNA and proteins are stored in specific compartments in the cytoplasm [94]. The transmission of mRNA and proteins to daughter blastomeres during the first cleavages could be asymmetrical, resulting in differences of the maternal pool among the blastomeres. Evidence for such asymmetrical divisions in human embryos is lacking.

Cell divisions could also result in depletion of critical nutrients, such as nucleoside precursors, required for chromosomal integrity leading to chromosome fragmentation and aneuploidy [95]. For example, folic acid deficiency increases chromosomal instability and aneuploidy in human lymphocytes [96, 97]; however such studies have not been performed in human embryos.

3.2.1.1. Cell-cycle checkpoints. Cell-cycle checkpoints ensure correct cell division by checking whether the processes at every cell cycle stage have been correctly completed before progression to the next stage. When an error occurs the checkpoint sends a signal to halt cell division until repairs are completed, and if repair is not possible then the checkpoint directs the cell towards apoptosis. Cell-cycle checkpoints function at the G1, G2 and metaphase (spindle assembly checkpoint – SAC) stages of the cell cycle [98].

The G1 checkpoint ensures that all conditions required for DNA synthesis are present (cell size, environment, quantity of energy, presence of nucleotides and nutrients) and the G2 checkpoint ensures proper duplication of the DNA during the S phase of the cell cycle and progression to mitosis. It has been recently reported that RB, the key protein of the G1 cell-cycle checkpoint, and WEE1, the key protein of G2 cell-cycle checkpoint, are lacking from normal appearing 8-cell stage human embryos [99]. At the same time, many genes involved in cell division are over-expressed, suggesting that early cleavage stage human embryos depend more on cell divisions rather than functional checkpoints [100].

Further, the SAC protein is essential for normal mitotic progression as it senses failure of kinetochore attachment to microtubules and halts the cell cycle until all chromosomes are attached. Overexpression of SAC components (BUB3, BUBR1 and MAD2) in mice embryos inhibits metaphase-anaphase transition. Depletion of SAC accelerates metaphase-anaphase transition during the first cleavage leading to formation of micronuclei, chromosome misalignment and aneuploidy [101].

Relaxation or absence of cell-cycle checkpoints during early human preimplantation development may therefore cause aneuploidy by allowing a blastomere with chromosome errors to enter and proceed through mitosis [29, 69]. Also, the presence of extra chromosomes in a blastomere might result in incorrect spindle formation and erroneous chromosome-microtubule attachments leading to genome instability during subsequent divisions.

3.2.1.2. Cohesins. Cohesins are a group of proteins regulating sister chromatid cohesion and ensuring proper chromosome segregation. The cohesins hold the two sister chromatids together to prevent them from premature separation from S-phase till anaphase. Upon entry into anaphase, the connection between the sister chromatids is destroyed to allow their separation. Malfunction of these proteins results in premature chromosome separation while delay in their removal may result in nondisjunction.

The role of cohesins in maintaining diploidy during meiosis is well described [102-104] but only indirect data is available on the effect of cohesins in mitosis during human preimplantation embryo development. Depletion of BUB 1 in mice fibroblasts causes these cells to fail to align their chromosomes or sustain SAC function resulting in an aberrant mitosis [105]. Further, the centromeres separate prematurely; however, this might be due to SAC disfunction rather than a direct role of BUB1 in protecting chromosome cohesion. BUB1 expression has been found to be low after fertilization in human embryos up to the 4-cell stage followed by an increase, with the greatest gene expression detected in hatched blastocysts [88]. In combination, these results suggest that the absence of BUB1 and other proteins important for protein cohesion in the cleavage stage human embryo may be in part responsible for mitotic aneuploidy caused by premature chromosome segregation.

3.2.1.3. Other maternal factors contributing to aneuploidy. FILIA is a protein provided by the oocyte that was found to be necessary for successful early embryogenesis in mice. Depletion of FILIA from the mouse oocyte impaired preimplantation embryo development resulting in a high incidence of mitotic aneuploidies from abnormal spindle formation, chromosome misalignment and SAC inactivation [90]. FILIA regulates the proper allocation of key spindle assembly regulators (AURKA, PLK1 and γ-tubulin) to the microtubule-organizing center. Further, FILIA is required for the placement of MAD2 to kinetochores to enable SAC function [90]. Since this gene is highly conserved in humans, absence of the FILIA protein from the maternal RNA/protein pool might impair proper embryo development but this hypothesis needs to be properly studied.

Another protein present in the oocyte that is required for correct embryo development is the a-thalassemia/mental retardation X-linked protein (ATRX). ATRX has been found in mouse oocytes to bind to pericentric heterochromatin domains at the metaphase II stage where it mediates chromosome alignment at the meiotic spindle. Absence of ATRX from oocytes and 1-cell embryos exhibits chromosome fragments and centromeric DNA-containing micronuclei [83].

STELLA, ZAR1, MATER, PADI6 and FLOPED, maternal proteins which are required for proper early embryo development, have been identified in mouse embryos and most of these proteins are also present in human embryos [85]. None of these proteins has been studied in relation to mitotic aneuploidy in humans but they are, together with FILIA, part of a subcortical complex in mouse embryos [106]. Since FILIA has a role in maintaining diploidy

in mouse preimplantation embryos, these proteins could also have a direct or indirect effect on euploidy.

In mouse oocytes, telomere dysfunction leads to disruption of functional meiotic spindles and misalignment of chromosomes during meiotic division [86]. It has been demonstrated that telomere DNA length is also associated with aneuploidy in human preimplantation embryos [87]. Aneuploid human blastomeres display significantly reduced telomere DNA quantity relative to diploid blastomeres from sibling embryos, but the difference in telomere length between aneuploid and diploid blastomeres is lost at the blastocyst stage [87].

Mitochondrial activity in developing human embryos could also correlate with chromosome abnormalities [89, 107]. The mitochondrial activity of chaotic human mosaic embryos was found to be significantly less than that of diploid- or aneuploid-mosaic embryos [89]. Also, a significant difference in the mitochondrial activity was observed when embryos that contained only diploid blastomeres were compared to embryos that contained only aneuploid blastomeres [107].

Absence of DNA repair proteins can also cause mitotic aneuploidy. DNA repair proteins are responsible for detecting and repairing different DNA lesions and errors during replication. DNA repair genes are expressed at high rates in human oocytes and blastocysts, but at low rates after fertilization up to the 4-cell stage [84, 88].

3.2.2. Paternal factors leading to aneuploidy

The centrosome, the organizing center of the mitotic spindle, is paternally inherited [108]. The centrosome consists of two centrioles from which the spindle microtubules are generated. Too few or too many centrioles may result in abnormal spindle formation and chromosome malsegregation. Indeed, a higher frequency of mosaicism, originated from an abnormal spindle organization, has been observed in dispermic human embryos compared to monospermic or digynic embryos [109]. Severe sperm defects may also result in a higher percentage of mitotic abnormalities and chaotic mosaic embryos. Embryos deriving from patients with nonobstructive azoospermia (NOA) undergoing testicular sperm extraction (TESE) have an increased rate of mosaic embryos compared to embryos generated with ejaculated sperm (53% and 26.5% respectively) [110]. The incidence of chaotic mosaicism is also significantly higher in embryos from NOA patients compared to embryos from all other sperm categories confirming the hypothesis that testicular spermatozoa might have difficulties in organizing the first mitotic spindle [111].

3.2.3. Effect of in vitro procedures on the occurrence of mitotic aneuploidy

The high percentage of mitotic errors found in preimplantation embryos could be different from the *in vivo* situation and it might be induced by components of IVF/ICSI procedures.

Indeed, different mosaicism rates have been observed among embryos obtained from different IVF centers at different chronologies and subjected to different culture protocols [112]. Temperature fluctuation, oxygen concentration, culture medium and hormonal stimulation regimes could affect spindle assembly and chromosome segregation.

Data from several studies confirm that ovarian stimulation affects embryo quality and chromosomal constitution, including postzygotic errors [26, 113]. An increase in the proportion of chromosomally abnormal embryos has been described with a conventional high dose exogenous gonadotropin regimen and GnRH-agonists co-treatment compared to a mild stimulation regimen using GnRH-antagonists co-treatment [113]. The increase in chromosomal abnormalities observed is due to an increased incidence of chromosomal mosaicism [113]. Furthermore, a higher daily FSH dose has been linked to an increase in mitotic division errors of chromosome 21 in mosaic embryos [26]. Chromosomal abnormalities are not solely the result of ovarian hyperstimulation as they are also observed in embryos from unstimulated cycles in young women (average age of 31 years-old) [114].

Increased oxygen concentration has been shown to increase nondisjunction events in the early divisions of nondisjunction-prone mice embryos suggesting also that subtle changes in the IVF setting can significantly influence chromosome segregation [115].

Despite the possible influences of IVF procedures in the frequency of aneuploidies, the high incidence of mosaicism found in early human abortions [116, 117] suggests that a high incidence of mosaicism is present in *in vivo* conceptuses as well. Strikingly, the mosaicism rate in chorionic villus samples (CVS) in the late first trimester from IVF/ICSI pregnancies and spontaneous pregnancies shows no difference in the frequency of mosaicism [24].

4. Fate of mitotic aneuploidies

In contrast with the high frequency of mitotic aneuploidies in early preimplantation embryos, a lower percentage of blastomeres with mitotic aneuploidies is observed in later stages of human preimplantation development and subsequently in established pregnancies and live births, suggesting a natural selection against aneuploid blastomeres or embryos [20].

The chromosomes involved in mitotic aneuploidies in human preimplantation embryos are different from those found later in embryonic development indicating that not all types of errors have the same fate [118, 119]. It might be that errors concerning specific chromosomes are detrimental for further embryo development causing embryos with such errors to be absent at later stages. Some preliminary data suggested that aneuploidy of chromosomes 1 - 12 is very common in cleavage stage human embryos but in the majority of the cases these abnormalities do not persist to the blastocyst stage [118]. Aneuploidy of chromosome 13, 18, or 21 is able to persist throughout development and is even compatible with life.

It is unclear whether there is a difference in this respect between aneuploidies of meiotic origin and aneuploidies of mitotic origin, although the difference in the frequency of chromosome 21 errors originating from mitosis between early embryos and clinically recognized trisomy 21 pregnancies suggests that the majority of embryos or blastomeres with mitotic nondisjunction involving chromosome 21 are not viable [15].

Selection against or correction of aneuploidies is already present in the preimplantation stage, which is demonstrated by the relatively decreasing number of aneuploid blastomeres from the cleavage to the blastocyst and implantation stage [20]. Three underlying mechanisms have been suggested: (i) cell arrest or apoptosis of aneuploid blastomeres and or embryos, (ii) active correction via anaphase lag, nondisjunction or chromosome demolition and (iii) preferential allocation of diploid/aneuploid blastomeres to embryonic or extra-embryonic tissues (Figure 2). These hypotheses for the fate of mitotic aneuploidies will be discussed in the following paragraphs.

4.1. Cell arrest

Within aneuploid embryos, arrest and apoptosis has been observed more in embryos containing blastomeres containing the same aneuploidy when compared to embryos containing both aneuploid and diploid blastomeres and these mosaic embryos reach

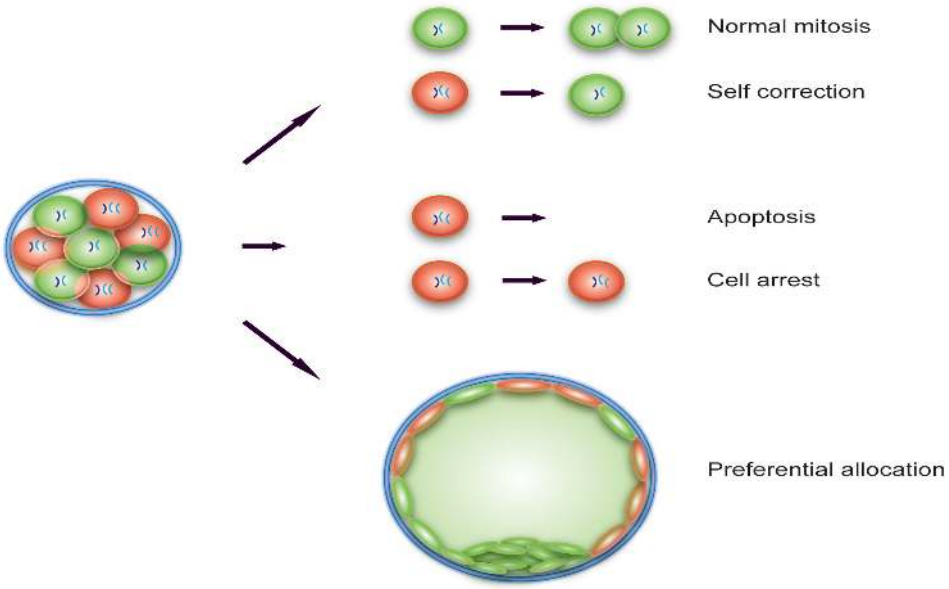

Figure 2: Fate of mitotic aneuploidy. Schematic representation of the proposed mechanisms regarding the fate of aneuploid (red) and diploid (green) cells within human preimplantation embryos.

blastocyst stage more easily than aneuploid embryos [29]. Cell arrest occurs more likely at the time of compaction and cavitation [11, 120-123].

This coincides with the appearance of the first embryonic transcription proteins [82]. The initiation of cell arrest around the time of embryonic genome activation presumably works as one of the mechanisms to prevent further development of chromosomally abnormal blastomeres.

4. 2. Apoptosis

Apoptosis, the process of programmed cell death, might also be responsible for the selection against aneuploid blastomeres. Apoptotic nuclei, fragmented and TUNEL labeled, are significantly increased at morula stage and TUNEL labeled nuclei are not seen until the morula stage, suggesting lack of apoptosis in early cleavage embryos [124]. The onset of the appearance of apoptotic markers seems to increase closely after activation of the embryonic genome [124].

The lack of apoptosis in early cleavage stage embryos could be either due to the ability of the embryos to suppress apoptosis or from the absence of (components of) the apoptotic pathway. In order to elucidate this, several studies have examined the expression of proteins of the apoptotic pathways in human oocytes and embryos. mRNA and proteins for the *BAX* and *BCL* genes that are involved in the regulation of apoptosis are expressed from fertilization to the blastocyst stage [125, 126]. Other apoptotic markers, like PDCD5, BAD, caspases and Harakiri are down-regulated or present in relatively small amounts at cleavage stage and increase in blastocysts during human preimplantation development [125-127]. A study in mice embryos suggested that all components of the apoptotic machinery are present even in the early 2 - 4 cell embryo and when these embryos are chemically induced for apoptosis some caspase activity and DNA fragmentation is indeed observed [128]. Induction of apoptosis after chemical treatment in the early developmental stages has also been observed in bovine embryos [129]. These few studies confirm that early cleavage embryos possess the complete apoptotic machinery throughout early cleavage stage but this machinery is not active.

Apoptosis is controlled by extracellular or intracellular signals that can both induce or suppress apoptosis. Unlike most types of mammalian cells, data suggest that blastomeres do not require external signals to avoid apoptosis [130-132]. Mice embryos, from the 1-cell to 16-cell stage can survive and divide in the absence of exogenous signal even when cultured as isolated single cells [130]. Further, in cleavage stage human embryos there is limited cell-cell communication via gap junctions, that are necessary to propagate apoptotic signals between cells and gap junctions are expressed extensively only after compaction [131, 132]. It is still not clear though whether activation of apoptosis in later developmental preimplantation stages is a result of the establishment of cell-cell communication, the activation of cell-cycle checkpoints that identify defective cells or other factors.

Upon activation of apoptosis, some aneuploid blastomeres among blastocysts could undergo apoptosis leaving the blastocyst with a higher proportion of diploid blastomeres [118]. Weaker mitotic checkpoints could also initiate apoptosis of aneuploid blastomeres as shown by deletion of genes coding for checkpoint proteins like BUB and MAD, in mice [118, 133].

According to one hypothesis, early cell death would be naturally prevented during the early cleavage stages to maintain adequate cell numbers that are needed in order for the embryo to proceed through development [129]. After the onset of apoptosis, and if apoptosis would result in excessive cell death then implantation failure or embryonic loss might occur. However, there are no experimental data to prove or disprove this hypothesis.

4.3. Self-correction

Uniparental disomy (UPD) where both copies of a certain chromosome in an individual originated from the same parent has been suggested as proof for the occurrence of trisomic zygote rescue by loss or removal of one of the three chromosomes or monosomic zygote rescue by replication of the missing chromosome [25, 26, 134, 135].

The exact mechanism for such self-correction is not known but the same mechanisms that cause mitotic errors, i.e. anaphase lagging and nondisjunction might also be able to correct them [33, 62]. Whether the correction of the abnormal blastomeres and embryos is an active mechanism or an accidental event needs further investigation.

4.4. Preferential allocation

Human preimplantation embryos might deal with aneuploid blastomeres by preferential allocation of these blastomeres to the trophectoderm where chromosome aneuploidy might be better tolerated, and displacing diploid blastomeres to the inner cell mass or even the embryo proper [136]. The finding of confined placental mosaicism, i.e. the presence of aneuploid cells only in the placenta and not in the fetus, is the primary experimental data to support such a preferential allocation [137]. Confined placental mosaicism can be diagnosed by finding aneuploid cells by chorionic villus sampling without confirmation in a follow-up amniocentesis, and has been reported in 1% – 2% of all chorionic villus samples [138]. The degree of aneuploid cells in confined placental mosaicism seems to correlate with pregnancy outcome like intra-uterine growth retardation [139, 140]. On the other hand, in cases of an abnormal embryo (trisomy 13 or 18), the presence of diploid cells in the placenta could facilitate complete embryonic and fetal development [141].

Studies in human blastocysts showed a similar degree of mosaicism in the inner cell mass compared to the trophectoderm or the overall blastocyst and could thus not confirm the theory of preferential allocation [27, 28, 31, 95, 121]. Preferential allocation of diploid cells to the embryo proper has not been studied yet [27, 121].

In mice, injection of only a small percentage of diploid donor ES cells (20% diploid cells combined with 80% cells with chromosomal abnormalities) in tetraploid blastocysts resulted in fully diploid normal adults [142]. Since tetraploid cells are excluded from the embryo proper, offspring resulting from these injected blastocysts must have originated from the injected ES cells, possibly indicating preferential allocation of diploid cells to the embryo proper.

Nonetheless, it remains to be seen whether this preferential allocation is indeed an active process or that for example in cases of confined placental mosaicism it was simply a matter of chance that diploid cells could form the embryo.

4.5. Threshold for the percentage of aneuploid blastomeres

Similar to the specific chromosome involved in the aneuploidy, the percentage of diploid blastomeres in a preimplantation embryo could be related to the ability of that embryo to develop into a child. The ratio of diploid blastomeres and aneuploid blastomeres may need to be above a certain threshold for development into a normal fetus [27]. The minimum ratio or number of diploid blastomeres needed for proper development can only be speculated on, and could be dependent on other factors such as culture environment and uterine receptivity. But, limited indirect evidence supports this hypothesis. Human frozen-thawed embryos that have lost nearly half of their blastomeres due to the cryopreservation procedure are still able to result in live births, implying that not all blastomeres of human preimplantation embryos are necessary for proper development into a child [21, 143]. The latter can also be concluded from the experiment mentioned above, where the injection of a small percentage of diploid donor ES cells in tetraploid mice blastocysts resulted in fully diploid normal adults [142]. This experiment could not demonstrate a correlation between chance of offspring and the percentage of diploid cells due to its design.

5. Conclusions

This review shows that mitotic errors are common in human preimplantation embryos and that these errors could be caused by several interrelated mechanisms. During early human development important mechanisms that are required for the regulation of genomic integrity, like cell-cycle checkpoints, cell arrest or apoptosis are relaxed or absent leading to an increased rate of aneuploidies. Upon embryonic genomic activation these mechanisms are re-established and aneuploid blastomeres might be removed either by cell arrest, apoptosis, active self-correction or allocation to the trophectoderm. Whereas the high prevalence of mitotic errors might be an intrinsic phenomenon of human development, aspects of the IVF/ICSI treatment such as the ovarian stimulation, the use of suboptimal sperm, or culture conditions might also contribute to the occurrence of mitotic aneuploidies. A better insight in the cause and fate of aneuploidy in human preimplantation embryos could help in limiting these preimplantation aneuploidies, with the ultimate goal of increasing overall embryo quality and with that treatment success in ART.

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CHAPTER 4

Evaluation of RNA amplification protocols for human oocyte transcriptome analysis

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Submitted

Abstract

Single cell gene-expression profiling is commonly used in several research fields. However, despite the advances of the last years, the strategies employed have not been adequately validated for reproducibility and sensitivity, with only four studies validating their local strategies in actual single cells. First, we investigated five commercially available protocols for their amplification efficiency. Two of the protocols resulted in sufficient yields and high success rates and were further validated for their performance in obtaining reliable, reproducible and sensitive expression profiles from individual human oocytes. Evaluation of these two protocols demonstrated that they both displayed low technical variation and produced highly reproducible profiles ($r \geq 0.95$). One of these two protocols identified significantly more transcripts but had also a higher number of false discoveries. These two protocols could also be used to generate ample amounts of mRNA for (q)PCR and sequencing techniques.

Introduction

Global gene-expression profiling using microarrays or next-generation sequencing has become an invaluable tool for the understanding of gene function and regulation. Until recently, large numbers of cells were required to obtain enough RNA for global gene-expression profiling. Although highly informative, this profile is often of limited value since it represents an average profile of all cells from the tissue under investigation while the actual expression in any single cell most likely varies [1-6]. Furthermore, in some conditions, such as in studies investigating gametes or preimplantation embryos, only one or a few cells can be analyzed.

To overcome the inability to analyze single cells, many studies have pooled samples from the same experimental procedure to obtain sufficient RNA for microarray analysis [7-13]. Pooling is also often used to reduce the number of arrays or subject-to-subject variability [14, 15]. Although pooling can be useful in some studies, it can introduce bias since the gene-expression profile derived from a pooled sample is not equivalent to the gene-expression profile of the individual samples [15].

Recent developments now allow transcriptome analysis of single cells. Obtaining reproducible gene-expression profiles using microarray technology in combination with limited starting material, such as single cells, requires amplification of the hybridization signal, mRNA amplification, or both. The methods aiming at intensifying fluorescence hybridization signal have reduced the required RNA input a few folds but not to the level of a single cell, i.e. picogram amounts [16]. Also, there are many practical issues, like the difficulty in isolating and labeling low abundant transcripts [17].

For mRNA amplification nowadays several commercial kits are available. mRNA amplification protocols fall in two categories: those based on exponential and those based on linear amplification [18-21]. The exponential amplification PCR protocols introduce PCR priming sites at both ends of each reversed transcribed cDNA molecule, followed by global amplification via a number of PCR cycles. Most linear amplification protocols employ the Eberwine method where double-stranded cDNA, generated by reverse transcription of mRNA and RNA polymerase utilizing a poly(T) oligonucleotide primer carrying a T7 promoter, undergoes *in vitro* transcription by T7 RNA polymerase. Alternatively, the Ribo-SPIA protocol uses a combination of chimeric DNA/RNA primers (3' end and random primers), DNA polymerase and RNase-H to linearly and isothermally amplify total RNA [22].

PCR protocols are relatively simple and can easily amplify a RNA sample about a million-fold in only a few hours. Limitations include the use of DNA polymerase having low efficiency in the amplification of GC rich sequences and low fidelity leading to errors and their subsequent exponential amplification (reviewed in [23, 24]). Also, the PCR reaction can lead to saturation if excess RNA input is used favoring the amplification of high-abundant transcripts and distorting the original RNA distribution [23]. Linear amplification

does not disturb the original RNA distribution as the RNA polymerase is almost not affected by template sequences or the concentration of templates and the reaction has a higher fidelity as errors do not accumulate exponentially [25]. A single round produces up to 1000-fold amplification, which is not sufficient for most microarray-based protocols when subnanogram amounts are used. Therefore, additional amplification rounds are required that are time consuming and can lead to RNA degradation [23, 26-28]. Optimized protocols for both PCR-based and linear amplification protocols have been described to overcome the previously mentioned disadvantages [29, 30].

However, irrespective of the protocol, there are common limitations associated with RNA amplification (reviewed in [24]). For example, since most of these methods make use of the poly(A) tail of the mRNA, partially degraded RNA or non-coding RNA cannot be readily detected. Methods that use random primers together with oligo(dT) primers may overcome this problem, but then the primers should not be targeting the rRNA and tRNA, or these RNA types should be depleted prior to amplification [31].

By analyzing single cells, the effect of biological difference(s) between cells can be measured. Technical replication is not possible as these samples have to be analyzed as a whole in one amplification step. Therefore, it is essential to find a protocol that can robustly and accurately amplify picograms input RNA from a single cell into micrograms necessary for microarray analysis. Also, the technique should exhibit high amplification efficiency, high signal enrichment, and produce reproducible gene-expression patterns. Since the RNA content of individual cells can vary per tissue, it is important to validate the protocols directly on the cell type to be used in each study [32].

There are several successful strategies to reduce the required RNA input and several studies have been published using few pooled or even single cells [7, 8, 11, 18, 32-38]. However, the lower sensitivity limits have not adequately been validated with respect to accuracy and reliability of gene-expression analysis. Many studies reported on generated expression profiles from picograms of RNA. Some of these studies reported only on a small number of genes [2, 3, 39-42], while at the whole transcriptome level, studies have utilized populations of few cells [7-13, 32, 43], titrated dilutions of stock total RNA to picogram amounts, or single cells [1, 18, 35, 38, 44-48]. Few studies though have validated the performance of whole transcriptome profiling from picogram amounts of input RNA [8-12, 18, 32-34, 36, 37, 49, 50] and only four, to our knowledge, used actual single cells [38, 45-47].

In this study, we determined the technical and biological variability of samples, using commercially available single-cell based global gene-expression protocols to establish an optimal and robust workflow when working with human single oocytes.

Methods

Biological materials

Oocytes in the germinal vesicle stage (GVs) were used for the technical validation. Couples undergoing ICSI treatment at the Center for Reproductive Medicine of the Academic Medical Center of the University of Amsterdam (Amsterdam, the Netherlands) were asked to donate their immature oocytes for research and written informed consent was obtained in all cases. Since spare immature human oocytes were used, no ethical approval was deemed necessary by the Central Ethics Committee on Research involving human subjects. All couples did sign informed consent to donate their spare immature oocytes for research.

Individual oocytes were transferred shortly to a drop containing 0.05% HCl/Tween 20 to remove the zona pellucida, subsequently they were washed using oocyte culture medium and transferred to PCR tubes. The oocytes were snap-frozen in liquid nitrogen and stored at -80°C till analysis.

RNA isolation and amplification

RNA isolation was performed using the Arcturus PicoPure isolation kit (Life Technologies) following the instructions of the manufacturer. RNA extraction was performed with 50 µl extraction buffer and the recommended DNase treatment was performed. The RNA was then amplified using the following kits: ExpressArt pico (Amsbio), Ovation PicoSL WTA System V2 (NuGEN), WT-Ovation One-Direct (NuGEN), Ambion Amino-Allyl MessageAmp II (Life Technologies) and Arcturus RiboAmp HS Plus (Life Technologies). All experiments were performed according to the manufacturer's instructions. The ExpressArt pico, Amino-Allyl MessageAmp II and Arcturus RiboAmp HS Plus kits are based on a linear amplification protocol and produce RNA as the final product. The Ovation PicoSL WTA System V2 and WT-Ovation One-Direct kits are SPIA-based methods and result in cDNA as end product.

RNA yield and quality

The concentration of RNA or cDNA was measured on the NanoDrop ND-1000 (Thermo Scientific). The quality of the amplified product was investigated with the BioAnalyzer (Agilent Technologies) using the RNA Nano 6000 kit (Agilent Technologies) as recommended by the manufacturer.

Experimental design for protocol validation

Two of the protocols for mRNA amplification were further validated using microarray technology. For the validation, RNA was isolated from 25 human oocytes. From twelve oocytes, RNA was individually isolated. To eliminate biological variation, thirteen human oocytes were pooled together right after the addition of extraction buffer of the isolation

protocol. The pooled material was homogenized and split into twelve identical samples for further RNA isolation. Six individual and six pooled samples were subsequently amplified using the NuGEN WT-Ovation One-Direct (called NuGEN Single; NS 1 - 6 and NuGEN Pooled-and-Split; NPS 1 - 6) and the rest were amplified using the Arcturus RiboAmp HS Plus (called RiboAmp Single; RS 1 - 6 and RiboAmp Pooled-and-Split; RPS 1 - 6). All samples amplified with one protocol were processed in the same round.

RNA labeling and microarrays

5 µg of amplified RNA obtained with the Arcturus RiboAmp HS Plus protocol (Arcturus RAHSP) was dried in a speedvac, dissolved in 5 µl 50 mM carbonate buffer (pH 8.5) and placed at 37°C for 10 min. 10 µl of CyDye (1 µg/µl) (Cy3 for test samples and Cy5 for reference samples) diluted in DMSO was added to each reaction. The samples were subsequently incubated in the dark at room temperature for 60 min. The reactions were quenched by addition of 5 µl of 4 M hydroxylamine for 15 min. at room temperature in the dark. The RNA was purified according to the MicroElute RNA Cleanup protocol (Omega Bio-Tek). The labeled RNA was finally eluted in 15 µl RNase/DNase free water.

2 µg of amplified cDNA obtained with the NuGEN WT-Ovation One-Direct protocol (NuGEN WTOOD) was labeled and purified using the SureTag DNA labeling kit (Agilent Technologies) according to the sample labeling and clean-up protocols for 4x microarrays described in the Agilent Oligonucleotide Array-based CGH for Genomic DNA Analysis manual. In both cases, pooled RNA/cDNA from the amplified samples was used as reference. The yield and CyDye incorporation of labeled RNA/cDNA were measured with the NanoDrop ND-1000 (Thermo Scientific).

Microarrays were custom designed and obtained from Agilent technologies using the 4x180k format. The 180k microarray (design ID: 028004) contains ~42,000 probes in quadruplet and 11,200 control probes.

Microarray hybridization, scanning and data processing

For the Arcturus RAHSP samples, each hybridization mixture contained 825 ng of "test RNA" and 825 ng "reference RNA". For the NuGEN WTOOD samples, each hybridization mixture contained 3 µg of "test cDNA" and 3 µg "reference cDNA". The hybridization mixture was prepared according to the Two-Color Microarray-Based Gene Expression Analysis protocol. Labeling and array hybridization of the NuGEN WTOOD samples was performed according to the NuGEN recommendations [51]. The RNA/cDNA was hybridized for 17 hours at 65°C and 10 rpm. Afterwards, the slides were washed and scanned in an ozone-free room with the Agilent scanner GA2505C (Agilent Technologies). Slides were scanned at 3 µm resolution. Microarray data were extracted with Feature Extraction software version 10.7.1 (Agilent Technologies). To avoid technical variation introduced by these procedures, labeling and hybridization were performed for all samples at the same day.

All data analysis was performed on non-normalized log2-transformed data using R (version 2.14.1) (http://www.R-project.org). The variance between the samples was investigated with a principle component analysis (PCA). All correlations were calculated using Spearman correlation coefficients. The number of present/absent transcripts was generated by setting a cut-off on the intersection between distribution of the negative control probes and the distribution of the transcript probes. The list of differentially expressed genes (DEGs) was generated based on the Expectation Maximization Algorithm were the real signal ratios (test/reference) were compared to the expected normal distribution using a cut-off Benjamini-Hochberg, false discovery rate (FDR)-corrected *P* value of 0.05. The microarray data presented in this study are published in the Gene Expression Omnibus database under accession number GSE48141.

Results

Quantitative analysis of amplification products

In this study, we evaluated five commercially available amplification kits that after a literature search were identified to be frequently used for amplification of small RNA samples (Table 1). The analysis was performed using single human oocyte samples, a non-template control sample to account for background amplification, and a control sample of human total RNA. All procedures were performed by a single investigator at one laboratory facility.

Yields sufficient to perform microarray analysis were obtained from all protocols tested, apart from amplification with the Message Amp II (Table 2, Figure 1). Moreover, all protocols generated a certain yield of non-specific amplification products when amplification was performed without RNA (non-template control, NTC) (Table 2). Amplification reactions that yielded over 3 µg of aRNA or 1.5 µg of cDNA were considered successful and success rates varied between 21% and 100% (Table 2). Based on the best yields and success rates, only the Arcturus RAHSP and the NuGEN WTOOD protocols were further characterized (Figure 2).

Table 1: Overview of commercially available RNA amplification kits.

Protocol	Manufacturer	Principle*	Minimum input total RNA
RiboAmp HS Plus	Arcturus	RT	100 pg
Message Amp II	Ambion	RT	35 ng
ExpressArt Pico	Amsbio	RT	100 pg
Ovation PicoSL WTA V2	NuGEN	SPIA	500 pg
WT-Ovation One-Direct	NuGEN	SPIA	10 pg

* RT: Reverse Transcription, SPIA: Single-Primer Isothermal Amplification

Table 2: Amplification yields.

Protocol	Times tested	Sample	Total samples	Success* rate (%)	Maximum yield (µg)
		NTC	2	0	2.8
RiboAmp HS Plus	3	Positive control	2	100	8
		Sample	16	94	73
		NTC	2	0	0.2
Message Amp II	2	Positive control	2	0	0.4
		Sample	3	0	1.5
		NTC	11	9	7.4
ExpressArt Pico	11	Positive control	9	33	34
		Sample	19	21	18
		NTC	3	0	0.2
Ovation PicoSL WTA V2	3	Positive control	3	100	3.8
		Sample	6	50	3.6
		NTC	1	100	4.3
WT-Ovation One-Direct	1	Positive control	1	100	19
		Sample	12	100	23

NTC= non-template control, * amplification is considered a success if the yield is above 3 µg for aRNA and above 1.5 µg for cDNA

Protocol validation

The experimental design to determine technical variation used single as well as artificial identical samples (Figure 3). Given the limited amount of RNA in a single cell, assessment of RNA quantity and quality after RNA isolation could not be performed and we had to rely on the quality of the amplified products (aRNA or cDNA) (Figure 4A). The size of the products ranged from 50 to 3,000 nucleotides for both protocols and all samples gave reproducible patterns. Although both protocols generated sufficient quantity of amplified product for further microarray processing, the yields of aRNA obtained from Arcturus RAHSP were higher than the yields of cDNA obtained by NuGEN WTOOD (Figure 4B). The Arcturus RAHSP yielded a median of 60 µg and the NuGEN WTOOD a median of 19 µg. Except for the non-pooled samples in the NuGEN WTOOD protocol, all samples cluster together per sub experiment (Figure 4B). Both protocols produced similar yields of about 3 - 4 µg of non-specific amplification products from the NTC samples (Table 2).

After amplification, aRNA or cDNA from the four best (based on amplification yield and bioanalyzer profile) single and pooled-and-split samples was labeled and used in microarray analysis to assess the impact of each protocol on gene-expression profiles. As expected, the PCA analysis showed that the major variance observed (PCA1; accounting for 38% of the total variability) distinguishes the two amplification protocols (Figure 5A). The NuGEN WTOOD protocol resulted in more variability among the technical replicates compared to the Arcturus RAHSP protocol, as shown by the closer clustering of the pooled-and-split

samples (Figure 5A). In both protocols the pooled-and-split samples were very similar with correlation coefficients above 0.95 and the correlation coefficients for the single samples were above 0.80 (Figure 5B). The correlations between samples of different amplification protocols were weak (r=0.66). The NuGEN WTOOD protocol resulted in significantly more genes called present than Arcturus RAHSP (Figure 5C). The number of genes detected as differentially expressed, therefore in our experiment the false positive genes, were in the NuGEN WTOOD protocol on average higher than in the Arcturus RAHSP protocol, but this difference was not statistically significant (Figure 5D).

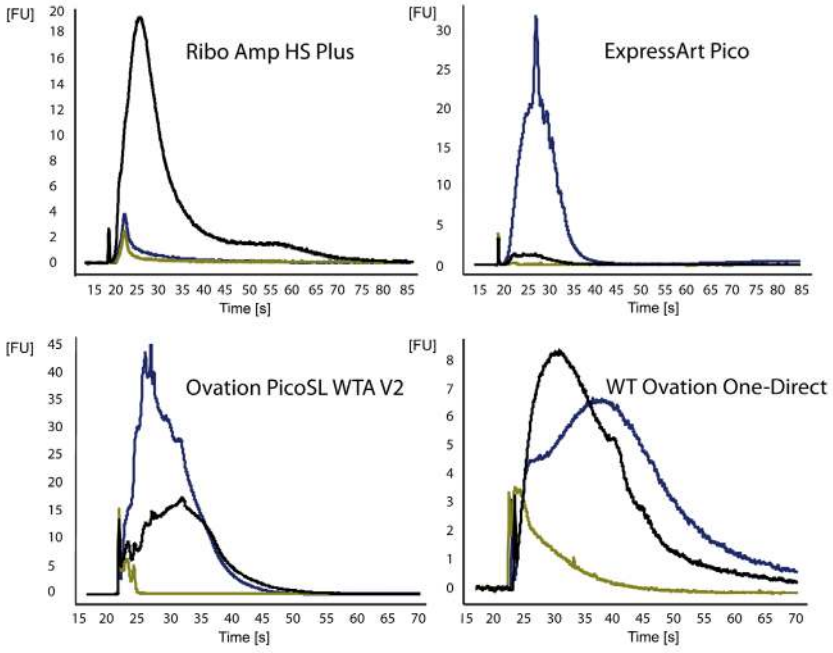

Figure 1: Amplification quality. Examples of the Bioanalyzer electrophoretic profiles of amplified products (single oocyte: black line; positive control: blue line; NTC: green line) from four amplification protocols.

Discussion

Over the last years, tools and methods have been developed to facilitate the generation of expression profiles from as little as one cell or even cellular fractions. Improvements have been made in all steps of the process from sample collection, to RNA isolation, RNA amplification, data acquisition and analysis. In this study, we evaluated the performance of five commercially available protocols for RNA amplification using single human oocytes. Three of them resulted in poor yields and success rates, whereas two provided good sensitivity and reproducibility. The failure of the Message Amp II protocol to amplify RNA

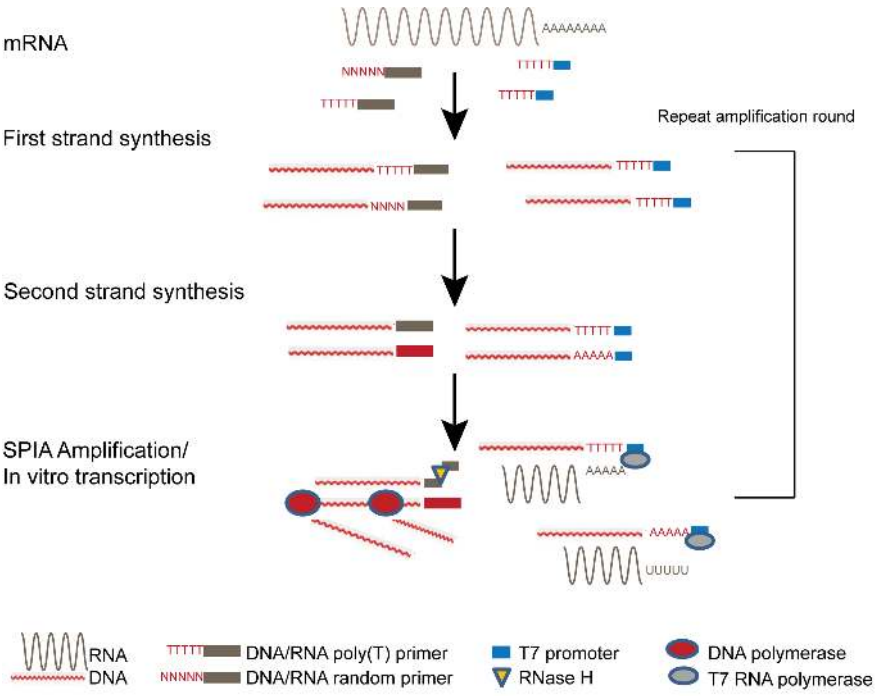

Figure 2: Amplification protocols. Amplification process of NuGEN WT Ovation One-Direct (left) and Arcturus RiboAmp HS PLUS (right).

from human single oocytes might be caused by the low amount of total RNA present in a single human oocyte (~55 pg [7]), which is less than the recommended minimum input (35 ng). For the other two protocols, no obvious explanation for their failure is available. The protocols with the best performances were the Arcturus RAHSP and the NuGEN WTOOD. These two protocols were further used to validate the procedure of generating expression profiles as a whole.

Even though the gene-expression profiles within each protocol were very reproducible (correlation coefficient ≥ 0.95), the gene-expression profiles obtained by the two protocols were quite variable (correlation coefficient r=0.66). The NuGEN WTOOD protocol identified more transcripts, which is most likely due to the utilization of random primers that recognize mRNA without poly(A) tails next to mRNA with poly(A) tails not only from the 3' end but also within the mRNA. Both protocols introduced errors into the microarray data. However, the NuGEN WTOOD protocol resulted in more false positive differentially expressed genes in the pooled-and-split samples.

Of the four studies that validated their protocols on actual single cells, one validated an amplification method for generating robust expression profiles by microarray technology using three single HeLA and three primary brain tumor cells [45]. Intra-sample

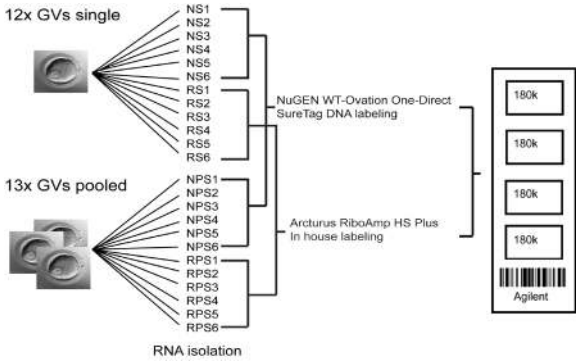

Figure 3: Experimental design for validating the two protocols. Pooled-and-split oocytes (NPS and RPS) were used to determine the technical variation introduced by the two protocols and single oocytes (NS and RS) were used to determine the biological variation among single human oocytes.

reproducibility was much lower than the protocols analyzed in the current study (r=0.76 and r=0.74 respectively versus r≥0.95 in the current study). Another study validated a local mRNA sequencing protocol on single oocytes and single cells of a mouse 4-cell embryo [46] and demonstrated very similar oocyte transcriptome profiles (r=0.99). Also, another study developed and validated a single cell PCR-based amplification protocol in combination with microarray-based analysis using single cells from the olfactory system [38]. Comparison of the transcriptional profiles of two individual cells revealed a correlation coefficient of 0.66. Even though all these studies proved that their methods were able to produce reliable and reproducible expression profiles from single cells of the same type, they were not able to validate the technical reproducibility since the observed variation could be attributed to either technical errors or biological differences between the cells. The fourth study validated a PCR-based amplification protocol by microarray experiments on single and pooled cells from the murine mammary carcinoma cell line TUBO [47]. The correlation coefficient was above 0.87. A similar design like that of our study was used, where pooled cell lysates were split to obtain identical single cell equivalents. Using such a design, one can validate a protocol using actual single cells while correcting for biological variation which allows conclusions for the technical variation. An exact comparison between these studies and our study is not possible, since neither the experimental design, nor the cell types are the same. More importantly, in our study we compared multiple protocols that are commercially available, whereas all other studies validated their own locally developed protocols.

As we have clearly demonstrated, different mRNA amplification methods can generate different transcriptome profiles. In order to obtain more valid comparisons, researchers should generate their datasets using a standardized protocol. The decision as to which protocol to use should be based on the number of detected transcripts and an accepted false positive rate. Other factors include the quality and quantity of input RNA, sample size, cost and time per reaction, experience with the techniques and experimental goal.

This study used human oocytes to validate the two protocols. The unique nature of oocytes should therefore be considered. During the first three days of preimplantation embryo

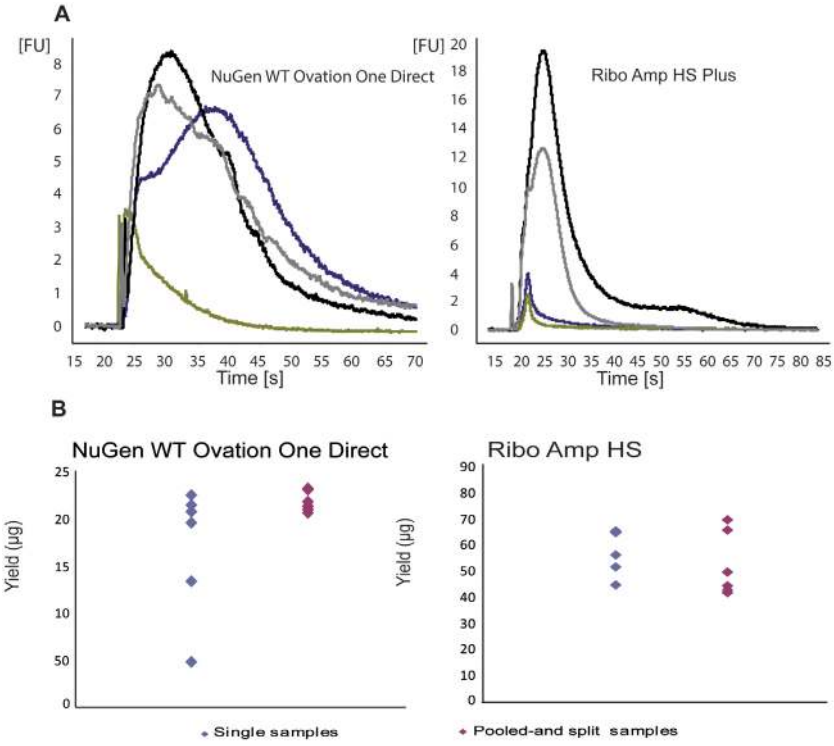

Figure 4: Quality and quantity assessment of amplification products. A: Examples of the Bioanalyzer electrophoretic profiles of cDNA obtained using NuGEN WT-Ovation One direct and aRNA obtained using Arcturus RiboAmp HS Plus (single oocyte: black line; pooled-and-split oocyte: grey line; positive control: blue line; NTC: green line). B: cDNA and aRNA amplification yields obtained with the two protocols.

development, transcription is absence and protein synthesis is sustained by the translation of mRNA stored during oogenesis [52]. As such, oocytes contain a higher amount of mRNA compared to single cells from other tissues [7]. It might be that the two protocols described here will not work as optimal when single cells of another tissue that contain lower amounts of mRNA are analyzed.

Nowadays, another technique (RNA-sequencing) is available to obtain expression profiles from as little as 10 ng input cDNA [46, 53, 54]. As such, the success rates reported in Table 2 have to be recalculated when sequencing is used. In our hands, both Message Amp II and ExpressArt Pico kits repeatedly failed to amplify RNA in various experiments and as such the success rates were low (also see Figure 1). Non-reliable techniques are not useful, either for microarray or sequencing so our conclusion that these two kits cannot be used for downstream applications remains. Ovation PicoSL WTA V2 could be used for downstream applications but WT-Ovation-One-Direct, both provided by NuGen, utilizes the same experimental principle and it is more sensitive compared to Ovation PicoSL WTA V2.

In summary, we compared several commercially available protocols for amplification of mRNA from single human oocytes. The two protocols that gave sufficient yields and high success rates were further validated to assess reproducibility and sensitivity of the obtained expression profile using a design that can discriminate between biological and technical variation. It was shown that both protocols introduced limited technical variation and produced highly reproducible transcriptome profiles among the technical replicates. One of them was able to identify more transcripts, but gave a higher false discovery rate. These protocols are correctly validated and can be used for single cell gene-expression profiling.

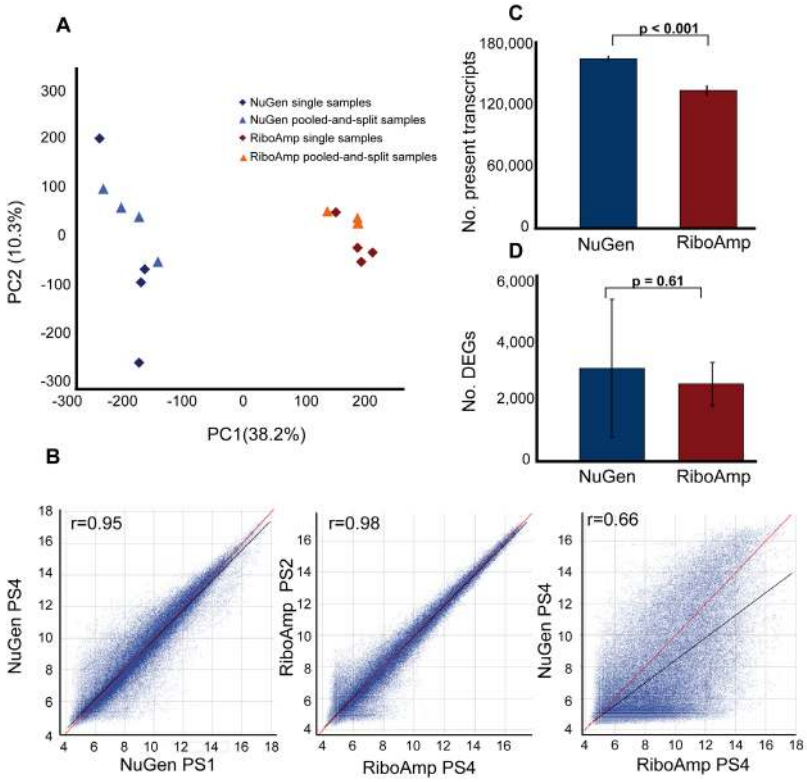

Figure 5: Validation of the protocols' performance on gene-expression profiling. A: Principal component analysis of the unnormalized log2 ratios (test/reference). B: Spearman correlations showing the similarity of the unnormalized microarray data from single and pooled-and-split samples obtained by the two protocols. C: Mean number of present transcripts per protocol. D: Number of differentially expressed genes per protocol.

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CHAPTER 5

Factors affecting the gene expression of *in vitro* cultured human preimplantation embryos

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Abstract

Preimplantation embryo development is a highly dynamic process and involves a series of critical events. Recent technological advances allow study of the expression profile of single preimplantation embryos. The effect of developmental stage on gene-expression profile of human preimplantation embryos has been demonstrated. Here we show the effect of maternal age and *in vitro* culture conditions next to developmental stage on the gene-expression profile of human preimplantation embryos. Eighty-nine day 4 human embryos were randomized to two IVF culture media and to two oxygen concentrations, with stratification for maternal age. Developmental stage and maternal age had a larger effect on the gene-expression profile than the *in vitro* culture conditions. Interactions between all factors were found, indicating that the effect of culture conditions might depend on developmental stage or maternal age. Our results indicate that one should take into account the confounding effect of all these variables when designing and analyzing gene-expression studies in single preimplantation embryos.

Introduction

Preimplantation embryo development is a highly dynamic process and involves a series of critical events. In humans, within about six days the embryo develops from a single-cell zygote to a multicellular blastocyst by initiation of mitosis, reorganization of chromatin, embryonic genome activation around day three, compaction and at the blastocyst stage differentiation into the inner cell mass (ICM) and trophectoderm (TE) [1]. Until recently, studying the underlying differences in the expression profile by these important biological milestones in preimplantation development has been hampered by the inability to analyze single cells or embryos. Recent technological advances in mRNA amplification in combination with microarray analysis now allow the simultaneous analysis of transcript levels of thousands of genes in a few or even single cells (Chapter 4) [2-5]. This makes it possible to explore the molecular and cellular mechanisms controlling preimplantation human embryo development.

Most available knowledge of the molecular basis of preimplantation embryo development comes from gene-expression studies on mouse and bovine embryos [6-12]. Few studies have investigated overall gene-expression profiles in individual or pooled human preimplantation embryos [13-21]. These studies documented the transcription dynamics as the embryo progresses from the cleavage stage to the morula and blastocyst stage [15-21], the time of embryonic genome activation [15, 17] and the molecular mechanisms that govern differentiation of the ICM and TE cell lineages [13, 14, 22].

Environmental factors, such as culture media and oxygen concentration in the incubator, influence embryo quality and pregnancy outcomes in an IVF program [23-25]. Several animal studies confirmed that embryos developing under different culture conditions have altered gene expression compared to their *in vivo* counterparts [26-35]. Affected molecular and cellular mechanisms, recognized from animal studies, involved metabolism, mitochondrial activity, oxidative stress, cellular integrity, cellular development and proliferation, cell-cell signalling and communication, apoptosis, imprinting, and protein synthesis [25, 27, 32, 36-47]. Studies on the effect of *in vitro* culture environment on human embryonic transcriptome are lacking.

In this study, we used a microarray technique, recently validated by our laboratory on single human oocytes (Chapter 4) to dissect the effect of different biological and environmental factors on gene-expression profiles of single human embryos. Apart from developmental stage and *in vitro* culture conditions, we also investigated whether maternal age affects the embryonic transcriptome, as this is an important determiner for reproductive success in humans and for embryo quality and clinical outcomes in IVF treatments [48-51]. Understanding the gene-expression profile differences in response to different biological and environmental factors will aid in selecting candidate genes as markers for embryo quality, improve our knowledge on regulation of embryonic development and improve success of embryo culture. Furthermore, understanding possible confounding effects

of inherent biological variation among human embryos will aid in designing better experiments to assess gene-expression profiles of human embryos.

Materials and Methods

Embryos

Left over, good quality, day 4 cryopreserved human preimplantation embryos, which were donated to research by couples who had finished their IVF/ICSI treatment and fulfilled their childbearing wish, were used for this study. The study protocol was approved by the Dutch Central Committee on Research involving Human Subjects (CCMO).

Embryo culture

All embryos were cultured in HTF (Lonza, Verviers, Belgium) supplemented with 10% Albuman (Sanquin, Amsterdam, the Netherlands) under 5% CO_2 and 20% O_2 as part of normal patient treatment until day 4 when they were cryopreserved. Forty women (23 undergoing IVF and 17 undergoing ICSI) were randomized to have their embryos cultured for two additional days after thawing (till embryonic day 6) in one of the following groups: (i) HTF medium and 20% O_2, (ii) HTF medium and 5% O_2, (iii) G2version5 medium and 20% O_2 and (iv) G2version5 medium and 5% O_2. During experimental culture, the two culture media were prepared according to the manufacturers' instructions. HTF medium was supplemented with 10% GPO and was equilibrated in 5% CO_2, while G5 medium (Vitrolife, Göteborg, Sweden) was supplemented with Human Serum Albumin (HSA) (5 mg/ml) and was equilibrated in 6% CO_2.

Randomization of the women to the four experimental groups was performed in the morning before thawing using sealed envelopes and stratification was performed for maternal age (≤ 35 and >35 years). The embryos were morphologically assessed according to the Gardner criteria, by two (SR, SM) embryologists that were blinded for the culture conditions used, right after thawing, at the end of the first day of culture and at the end of the second day of culture. Parameters like cell number, percentage of fragmentation and presence of dead cells were noted [52]. At the end of the culture, individual embryos (n=89) were transferred in PCR-tubes, flash frozen in liquid nitrogen and stored at -80^{0}C until RNA extraction. Only embryos that had developed to the morula and blastocyst stage (n=41) were further analyzed.

Sample preparation and microarray hybridization

For sample preparation we used a protocol that was recently developed and validated for analysis of picogram amounts of RNA (Chapter 4). Briefly, RNA isolation was performed using the Arcturus PicoPure isolation kit (Applied biosystems) following the instructions of the manufacturer. RNA extraction was performed with 50 µl extraction buffer and the

recommended DNase treatment was performed. The RNA was then amplified using the Arcturus RiboAmp HS Plus (Applied Biosystems). The concentration of the aRNA was measured on the NanoDrop ND-1000 (Thermo Scientific) and the quality of the amplified product was investigated with the BioAnalyzer (Agilent Technologies) using the RNA Nano 6000 kit (Agilent Technologies). 5 µg of amplified RNA was dried in a speedvac and then dissolved in 5 µl 50 mM carbonate buffer (pH 8.5) and placed at 37^{0}C for 10 min. 10 µl of CyDye (Cy3 for test samples and Cy5 for reference samples) diluted in DMSO was added to each reaction. Then, the samples were incubated in the dark at room temperature for 60 min. The reactions were quenched by 5 µl of 4 M hydroxylamine for 15 min at room temperature in the dark. The labeled RNA was purified according to the MicroElute RNA Cleanup protocol (Omega Bio-Tek). The RNA was finally eluted in 15 µl Rnase/DNase free water. Pooled aRNA from the amplified samples was used as a reference in all experiments. The yield and CyDye incorporation of labeled aRNA were measured with the NanoDrop ND-1000 (Thermo Scientific). 825 ng of Cy3 labeled sample aRNA and 825 ng Cy5 labeled reference aRNA were hybridized to 180k custom designed Agilent arrays (Agilent technologies). The 180k microarray (design ID:028004) contained ~42,000 probes in quadruplet and 11,200 control probes (Chapter 4). The hybridization mixture was prepared according to the Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent technologies). The aRNA was allowed to hybridize for 17 hours at 65^{0}C and 10 rpm. Afterwards, the slides were washed and scanned in an ozone-free room with the Agilent scanner GA2505C (Agilent Technologies). Slides were scanned at 3 µm resolution. Microarray data were extracted with Feature Extraction software 10.7.1 (Agilent Technologies).

Microarray data analysis

The data were analyzed in R-2.14.1 (http://cran.r-project.org/). All arrays were subjected to a set of quality control checks, such as visual inspection of the scans, checking for spatial effects through pseudo-color plots, and inspection of pre- and post-normalized data with box plots, density plots, ratio-intensity plots and principal component analysis. Expression values were calculated by using the robust multi-array average (RMA) algorithm [53]. When multiple embryos per woman were correlated based on gene-expression profiles only one of those samples was selected for inclusion in the analysis to avoid effects of pseudoreplication. For each experimental condition, mean expression values of all samples per gene were calculated. The conditions were compared using Spearman correlation coefficients.

Differentially expressed genes (DEGs) caused by the biological factors "Developmental stage", "Maternal age" and the experimental factors "Culture medium" and "Oxygen concentration" were identified using ANOVA, taking into account all interactions between any two factors. Preliminary analyses and biological considerations suggested that the biological factors are not necessarily relevant for every gene. The following strategy was chosen. The effects of the main factors "Culture medium" and Oxygen concentration" (the culture conditions) were tested for every gene, and the biological factors and interactions

were only included in the model once selected by the Akaike Information Criterion, in a gene-by-gene analysis [54, 55]. To control the number of false positive DEGs, a false discovery rate correction (FDR) was performed across all genes and all statistical tests [56].

Genes were subjected to biological interpretation in the context of an experimental factor or interaction term, when they were associated to a FDR corrected *P* value ≤ 0.05. Normalized expression values of these genes (Z-scores) were calculated from the model coefficients and their profile were explored across the experimental factor levels using self-organizing maps (SOMs) or hierarchical clustering. KEGG-pathway and gene ontology analyses were performed using the DAVID analysis wizard [57, 58]. The microarray data presented in this study are published at the Gene Expression Omnibus database under accession number GSE49374.

RESULTS

Two of the forty-one embryo samples did not pass our quality control criteria for microarray hybridization and were therefore excluded from further analysis. Two women had two embryos each that were highly correlated on gene-expression profile; one of these two embryos from every woman was therefore also excluded. The baseline characteristics of the women for the remaining thirty-seven embryos in the four groups are shown in Table 1.

The correlation coefficient between gene expression in embryos of different developmental stage and maternal age was smaller (ranged from 0.983 to 0.992) compared to embryos cultured in different culture conditions (0.996) (Figure 1). Principal component analysis (PCA) using all genes present on the arrays showed a clear clustering of samples according to developmental stage but there was no clear clustering of samples according to maternal age, culture media or oxygen concentration (Figure 2, A-D).

Table 1: Baseline characteristics in the four groups.

	HTF - 20% O_2	HTF - 5% O_2	G5 - 20% O_2	G5 - 5% O_2
Number of embryos	10	9	11	7
Number of women	7	7	9	6
Mean maternal age*	33.3 ± 5.0	35.2 ± 2.8	35.7 ± 3.1	33.4 ± 4.4
Treatment				
IVF	5	7	7	3
ICSI	5	2	4	4
Embryo development				
Developing	6	9	8	6
Arrested	4	0	3	1
Embryo score				
Morula	9	5	7	2
Early blastocyst	0	1	2	3
Blastocyst	1	3	2	2

*Data presented as mean years ± SD

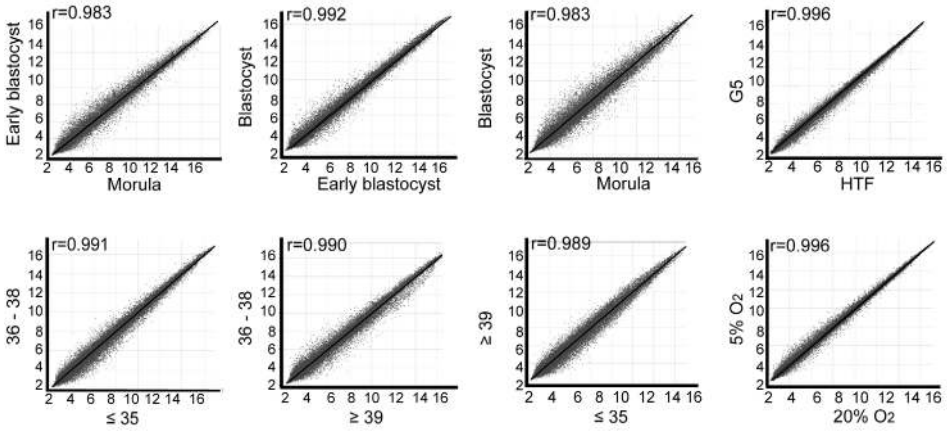

Figure 1: Pairwise comparisons of the transcriptome of human embryos according to the different factors.

Effect of developmental stage on gene expression

Out of the thirty-seven embryos included in the analysis, twenty-three developed to the morula stage, six became early blastocysts and eight were fully developed blastocysts. Statistical analysis taking into account the confounding effects of the other factors, revealed 1,532 genes that were significantly differentially expressed among the developmental stages (Table 2). A PCA was performed to visualize the differential expression among the developmental stages (Figure 2E). The PCA revealed that the difference between morulas and blastocysts is larger than the difference between blastocysts and early blastocysts (Figure 2E).

Using self-organizing-maps (SOM) clustering, patterns of expression differences among the different developmental stages were examined (Figure 3A). Clusters 1, 2 and 3 include genes that are up-regulated as embryos progress from the morula to the blastocyst stage. These genes are involved in phosphorus/phosphate metabolic processes, oxidation reduction, regulation of cell proliferation, phosphorylation, generation of metabolites and energy, response to organic transport and ion transport (Figure 3B). Clusters 5, 6 and 7 include genes whose expression decreases as embryos progress from the morula to the blastocyst stage. The top biological functions involved included regulation of transcription, cell adhesion, regulation of cell proliferation, cell death, cell motion and transmembrane transport (Figure 3C).

Subsequently, we analyzed the expression levels of a set of pluripotency genes previously investigated in human oocytes and embryos [17]. The results showed an increase in expression of KN motif and ankyrin repeat domains 3 (*ANK3*) gene from the morula to the blastocyst stage (Figure 4). In contrast, podoplanin (*PDPN*), zinc finger protein 42 homolog (*ZFP42*) and teratocarcinoma-derived growth factor 1 (*TDGF1*) gene expression declined

Table 2: Number of differentially expressed genes (DEGs).

Factors	Number of DEGs
Developmental stage	1,532
Maternal age	368
Oxygen concentration	183
Culture medium	174
Oxygen concentration * Culture medium	518
Developmental stage * Maternal age	462
Oxygen concentration * Maternal age	220
Culture medium * Developmental stage	274
Oxygen concentration * Developmental stage	181
Culture medium * Maternal age	142

*Indicates interaction among factors

from the morula to the blastocyst stage (Figure 4). The expression of grancalcin (*GCA*), spermatogenesis and oogenesis specific basic helix-loop-helix 2 (*SOHLH2*), zinc finger protein 462 (*ZFP462*), nanog homeobox (*NANOG*), POU class 5 homeobox 1 (*POU5F1*) and SRY sex determining region Y-box 21 (*SOX21*) genes was not altered during the transition from the morula to the blastocyst stage (Figure 4).

Effect of maternal age on gene expression

Maternal age was grouped in three categories: ≤35 (twenty-two embryos; mean 31.7 years), 36-38 (eight embryos; mean 37.1 years) and ≥39 (seven embryos; mean 39.7 years). Statistical analysis taking into account the confounding effects of the other factors, revealed 368 genes that were significantly differentially expressed among the three age categories (Table 2). PCA analysis showed that the difference between the young and old age group is relatively large (albeit one outlier among the old age group) and that the difference between the middle and the young and old age groups was relatively small (Figure 2F).

Examined patterns of gene differences among the different maternal ages are depicted in Figure 5A. Clusters 1, 2 and 3 include genes whose expression decreased with advanced maternal age. The top ten biological functions of these genes are shown in Figure 5B and include phosphate/phosphorus metabolism and cell cycle. Clusters 5, 7 and 8 contain genes with higher expression in embryos of advanced-age women as compared to embryos of young women. These genes are involved in five biological functions, mostly related to cell communication (Figure 5C).

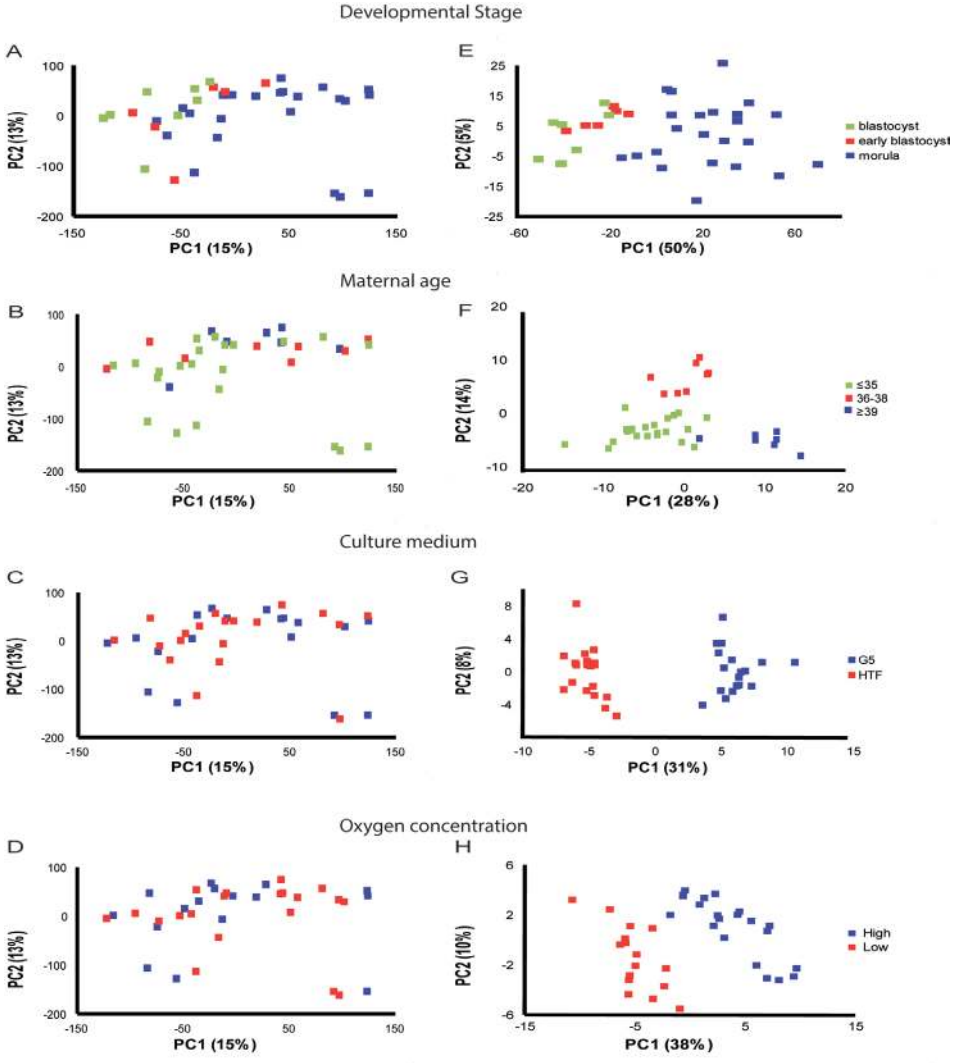

Figure 2: Principal component analysis (PCA). In A-D all genes on the array were used for PCA while in E-H only the genes affected per factor were used. (A) PCA of all genes among the three developmental stages. (B) PCA of all genes among the three maternal ages. (C) PCA of all genes between the two culture media. (D) PCA of all genes between the two oxygen concentrations. (E) PCA of the DEGs showing the clustering of the samples among the three developmental stages. (F) PCA of the DEGs showing the clustering of the samples among the three maternal ages. (G) PCA of the DEGs between the two culture media. (H) PCA of the DEGs between the two oxygen concentrations.

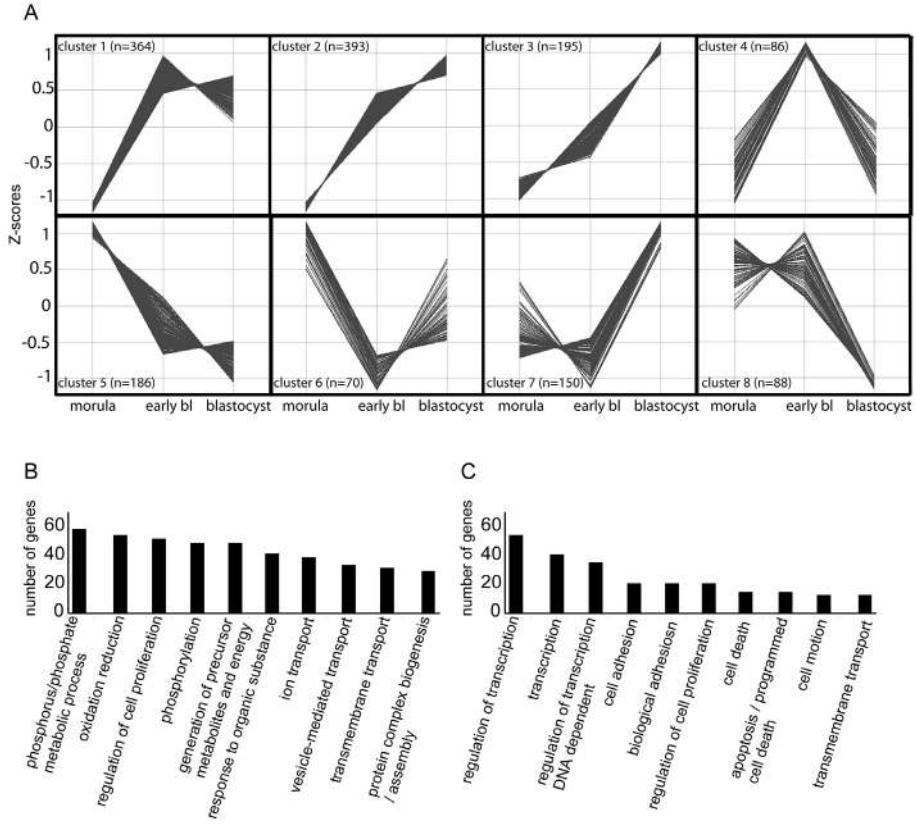

Figure 3: Analysis of embryonic transcriptome per developmental stage, corrected for the confounding effect of the other factors. (A) Patterns of gene expression as found by SOM clustering. The number of genes included in each cluster is stated in parenthesis. (B) Top ten biological functions of genes with increasing expression during development (clusters 1-3). (C) Top ten biological functions of genes with decreasing expression during development (clusters 5-7).

Effect of culture conditions on gene expression

When we analyzed the data according to the culture medium used (19 HTF and 18 G5), taking into account the confounding effects of the other factors, 174 genes were significantly differentially expressed. One hundred and four were up-regulated during culture with HTF and seventy were up-regulated during culture with G5. Biological functions that were up-regulated in embryos cultured in HTF involved cell death and apoptosis while biological functions that were up-regulated in embryos cultured in G5 involved regulation of activities and cell cycle (Figure 6A & B).

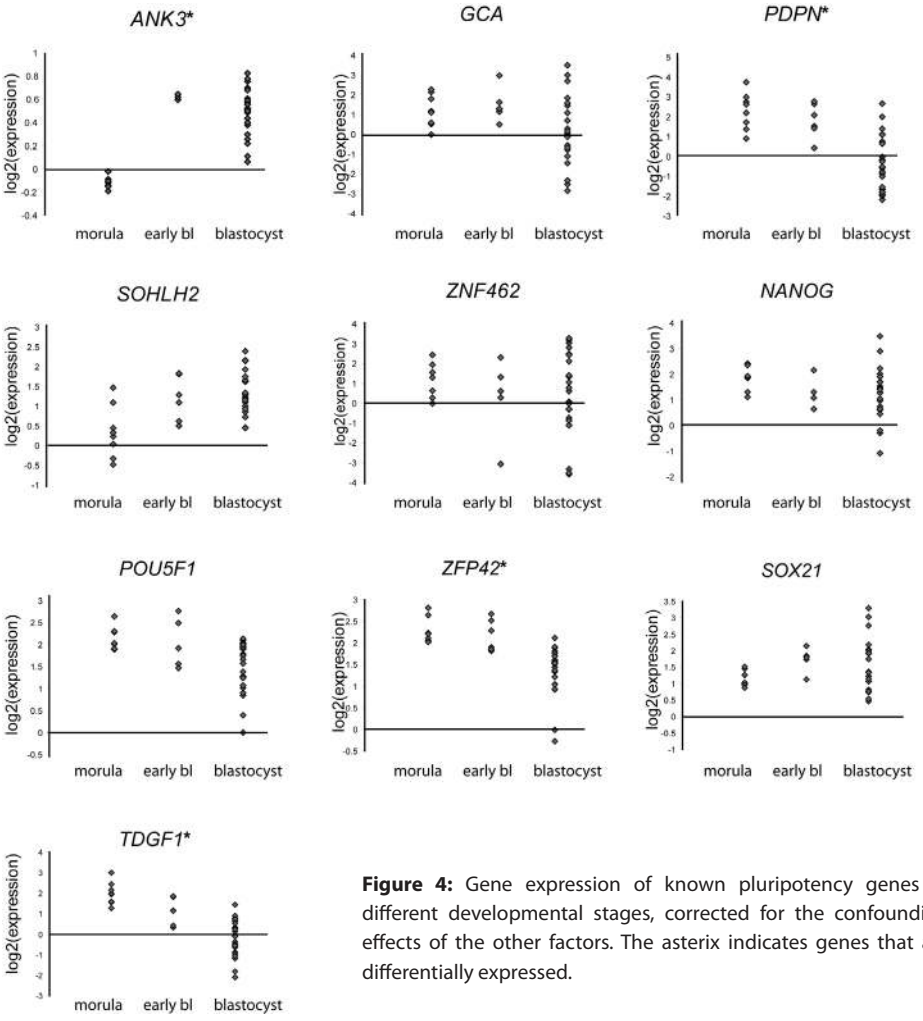

Figure 4: Gene expression of known pluripotency genes at different developmental stages, corrected for the confounding effects of the other factors. The asterix indicates genes that are differentially expressed.

The difference in oxygen concentration, when also taking into account the confounding effects of the other factors, caused significant differential expression for 183 genes. One hundred and seventeen genes were up-regulated in low oxygen concentration (n=16) and involved genes with biological functions in cell morphogenesis and muscle contraction while sixty-six genes were up-regulated in high oxygen concentration (n=21) and involved genes with biological functions in sensory perception of stimulus (Figure 6C & D).

A PCA was performed to visualize the differential expression caused by the difference in culture medium (Figure 2G) and oxygen concentration (Figure 2H). It was observed that the effect of culture medium on embryo gene expression was clearer than the effect of oxygen concentration.

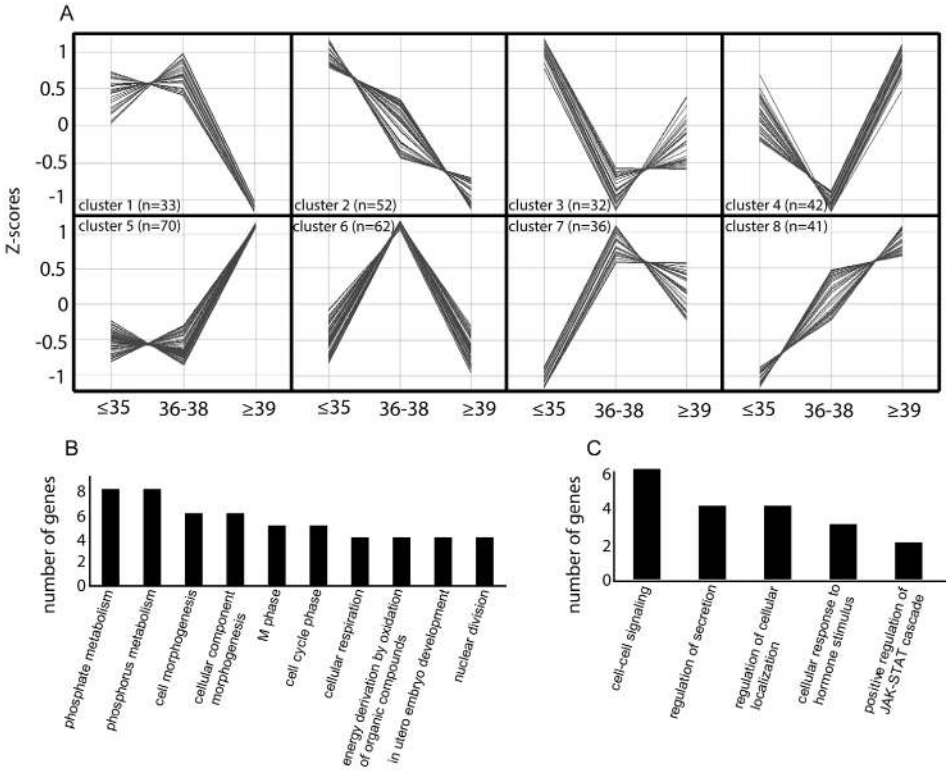

Figure 5: Analysis of embryonic transcriptome for embryos of different maternal age, corrected for the confounding effects of the other factors. (A) Patterns of gene expression as found by SOM clustering. The number of genes included in each cluster is stated in parenthesis. (B) Top ten biological functions of genes with decreased expression in embryos from older women (clusters 1 - 3). (C) All biological functions of genes with increased expression in embryos from older women (clusters 5, 7 and 8).

Interactions among the factors

To assess whether culture media have a different effect on the gene expression of embryos of different maternal age, we clustered the 142 genes that were found differentially expressed for the interaction of culture media and maternal age (Table 2). The majority of these genes (65 genes) followed a pattern where their expression was up-regulated in HTF compared to G5 in embryos of women 38 years old and below while this expression pattern was reversed in embryos of women above 39 years of age. These genes were involved in functions such as regulation of kinase and transferase activity, regulation of phosphorylation, intracellular signaling and cell morphogenesis.

The effect of culture medium was also different among the developmental stages (Table 2). One hundred and eighty genes (out of two hyndred and seventy-four) were down-regulated when the embryos were cultured in HTF compared with G5 in the blastocyst

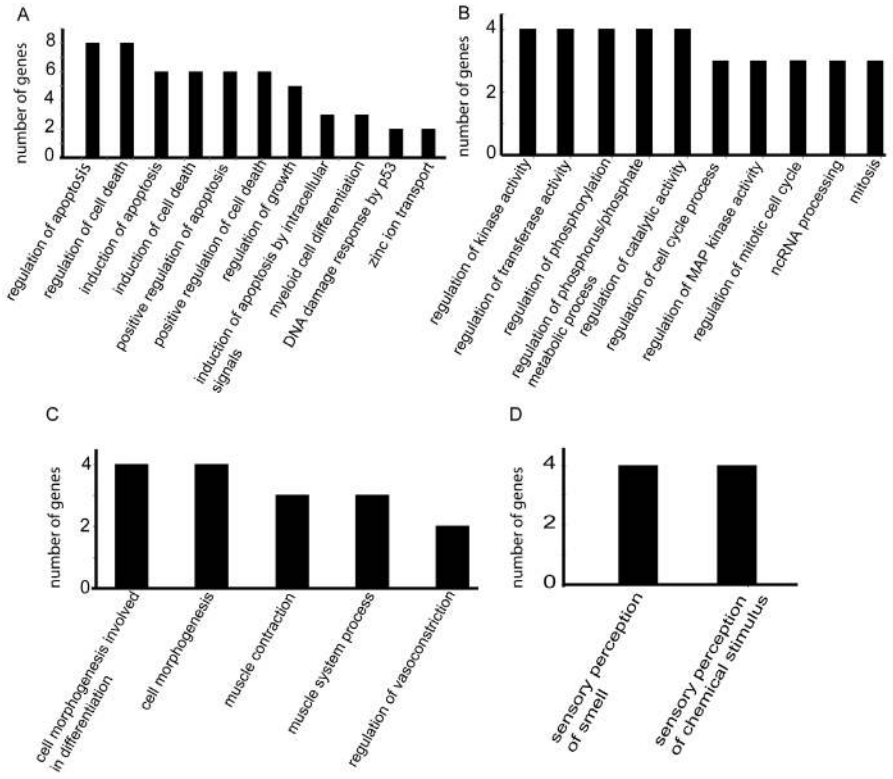

Figure 6: Biological functions of genes differentially expressed in different culture conditions, corrected for the confounding effects of the other factors. (A) All biological functions of the genes that are up regulated in embryos cultured in HTF. (B) Top ten biological functions of genes that are up regulated in embryos cultured in G5. (C) The biological functions of the genes that are up regulated in embryos cultured in low oxygen concentration. (D) The biological functions of the genes that are up regulated in embryos cultured in high oxygen concentration.

and morula stage embryos but the same genes were up regulated when the embryos were cultured in HTF in the early blastocyst stage. Biological functions of these genes included, among others, cell signaling, secretion, regulation of growth and regulation of exocytosis.

An interaction was also found between the oxygen concentration and maternal age (Table 2). The majority of the genes that were differentially expressed were part of two clusters. In one of the clusters (108 genes), the genes were down-regulated when the embryos were cultured in low oxygen concentration compared to high when the maternal age was below 38 years and the opposite pattern was observed when the maternal age was above 39 years. The second cluster (73 genes) involved genes whose expression was up-regulated in low oxygen concentration compared to high in embryos of women below 38 years and down-regulated in embryos of women above 39 years old. Biological functions of these

genes included cell-cell signaling, calcium ion transport, regulation of protein kinase cascade and cell activation.

One hundred and eighty-one genes were found to be affected by the interaction of oxygen concentrations and developmental stage (Table 2). However, the number of genes in the individual clusters was too small for any relevant pattern to be identified. The interaction between developmental stage and maternal age involved 462 genes (Table 2). It was shown that the same genes in blastocysts or morulas have a different expression pattern in women of younger compared to women of older age. These genes were involved in cell cycle, cell adhesion and regulation of cell size.

Lastly, the effect of culture medium was different when embryos were cultured in the two oxygen concentrations, with 518 genes found differentially expressed (Table 2, Figure 7A). The two main clusters recognized involved genes that are involved in regulation of transcription, cell cycle, ncRNA and rRNA metabolism, ribosome biogenesis, methylation, regulation of metabolites and energy, oxidative phosphorylation, nucleotide biosynthesis and ATP synthesis (Figure 7B).

Discussion

By analyzing the gene-expression profile of good quality human embryos, we have identified factors, both biological and environmental, that cause significant gene-expression differences during human preimplantation development. The number of DEGs was higher for developmental stage and maternal age (1,532 and 368 respectively) compared to the number of DEGs for culture medium and oxygen concentration (174 and 183 respectively). Also, the correlation coefficient of gene-expression levels among embryos of different developmental stage and maternal age was smaller compared to embryos cultured in different culture conditions. These data suggest that developmental stage and maternal age have a larger effect on the gene-expression profile of human preimplantation embryos than culture conditions (culture medium and oxygen concentration) used.

As expected, a difference in expression level in a considerable number of genes (1,532) was found as embryos progress through development and the cells undergo differentiation.

This study also demonstrated differences in the embryonic transcriptome relevant to the maternal age. We found more than 300 genes that were significantly up- or down-regulated as maternal age increased. One of the most profound differences in the genes that were down-regulated with advanced maternal age was found in genes associated with cell-cycle function. Genes up-regulated with advanced maternal age were mostly associated with cell signaling. The same functions were influenced, in the same direction, by advanced maternal age in human oocytes [59, 60]. Despite the believe that maternal age affects mainly oocytes, our study has shown that maternal age influences gene-expression profiles in human embryos in the same way as human oocytes. Having identified the

pathways and functions that are altered by advanced maternal age in human embryos allows further research to study the specific mechanisms and genes involved.

Although previous studies on the gene-expression profile of embryos produced *in vitro* have been conducted for mouse, bovine and pig, this is the first study to generate gene-expression profiles of single human embryos under different culture conditions [26, 28, 32, 33]. Even though the effects of the culture conditions were small (174 DEGs for culture medium and 183 DEGs for oxygen concentration) it was clear that HTF caused up-regulation of expression of genes related to cell death and apoptosis while culture in G5 caused up-regulation of genes involved in regulation of phosphorylation and mitosis, possibly implying that human embryos develop better in G5 compared to HTF. Two studies presented as abstracts in conferences compared HTF to G2 (an older version of G5 that is no longer available on the market) and reported better embryo quality in G2 compared to

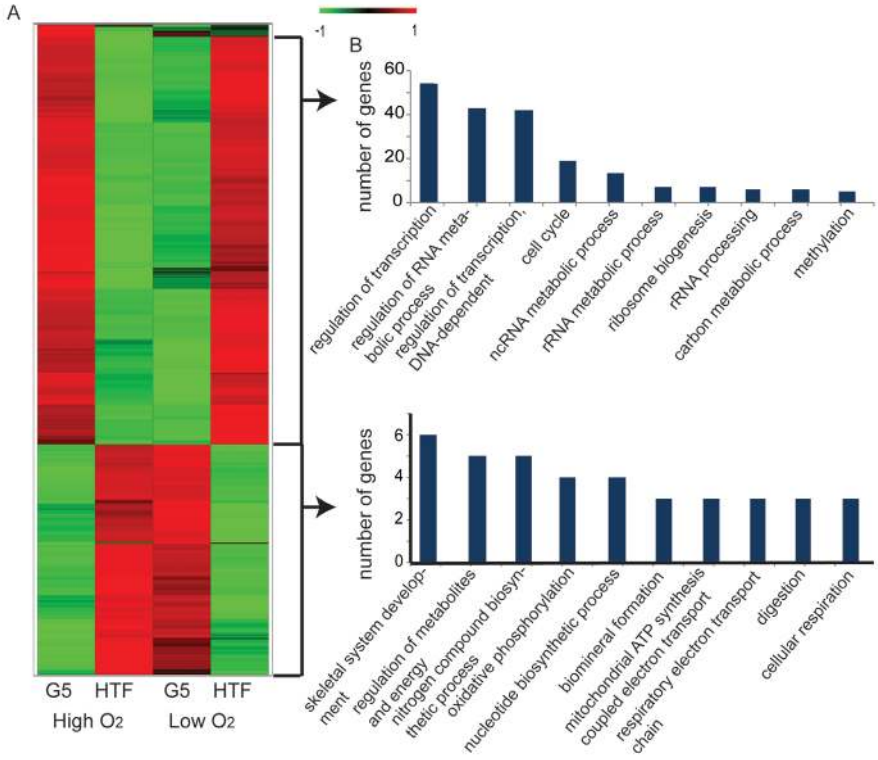

Figure 7: An example of the interactions found in this study: the interaction between culture media and oxygen concentration, corrected for the confounding effect of the other factors. (A) The heat map of the effect of culture media according to oxygen concentration shows that for the same genes the direction of differences in gene expression between G5 and HTF is different when embryos are cultured in high compared to low oxygen concentration. (B) The top ten biological functions of the differentially expressed genes clustered according to the two observed patterns of gene expression.

HTF [61, 62]. These studies point to the same direction as the data presented in this article. The effect of oxygen concentration was less pronounced. We found that culturing embryos in low oxygen led to up-regulation of genes involved in cell morphogenesis, which should be very relevant for embryo development and blastocyst formation. Indeed, clinical data show that reduced oxygen concentrations lead to better quality embryos and improved success rates [23]. Genes involved in functions like oxidative phosphorylation and stress processes were not found differentially expressed between the two oxygen concentrations.

We also found a relevant interaction effect between maternal age and culture medium, maternal age and oxygen concentration, developmental stage and culture medium, developmental stage and maternal age and culture medium and oxygen concentration. These interactions suggest that embryos of different maternal age or developmental stage could respond differently to various culture conditions. The mechanism through which this occurs, and what culture conditions would be better for specific developmental stages or maternal age groups needs further investigation.

Finally, in our study we identified pathways and molecular functions that are affected by the factors under study and their interactions. Even though microarray studies produce a lot of data, many of which not easily interpreted, determination of the affected molecular functions provides the first step in understanding the effect of maternal age or *in vitro* culture on human embryo quality. These data can be combined with clinical results and lead to further research aiming in optimization of embryo culture systems. For example, IVF success rates are decreasing with advanced maternal age and culture of human embryos in low oxygen concentrations lead to better quality embryos and higher live birth rates [23, 49-51]. It is still unknown which culture medium (HTF or G5) leads to better quality embryos and higher live birth rates [24].

A strong point of this study is the experimental designed applied. Embryos were randomized for culture conditions and stratified for maternal age in order to have equal distribution of confounding factors among the experimental groups. We further investigated every factor using a multi-variate model to isolate its effect from confounding effects of the other factors, while also looking at their interactions. Many of the current studies on gene-expression analysis of human preimplantation embryos at different developmental stages do not report correcting for confounding effects of maternal age. Sometimes, even the information on maternal age or what culture media were used is not described in the materials and methods. Our results showed that such correction is definitely needed. Especially when working with human preimplantation embryos since the limited availability of embryos does not always allow matching for maternal age or for developmental stage.

It should be mentioned that the effects observed in our study are the result of culture of human embryos for two days, from day 4 to day 6 of development. It remains to be

elucidated if earlier stages of development (day 1 - 4) as well as prolonged culture has similar effects on gene expression.

In conclusion, we showed that developmental stage and maternal age have a larger effect on the gene-expression profile of human preimplantation embryos than the culture medium or oxygen concentration used during *in vitro* culture. The data described here are a valuable addition to the few studies investigating gene-expression differences during human preimplantation development. For the first time data on gene-expression differences in regards to maternal age and culture conditions were described for human embryos. Our results indicate that one should take into account the confounding effect of biological variables, such as developmental stage and maternal age when designing and analyzing experiments studying gene expression in single human preimplantation embryos under various experimental conditions.

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CHAPTER 6

Embryo culture media and IVF/ICSI success rates: a systematic review

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Abstract

BACKGROUND: The media that are used to culture human preimplantation embryos are considered to be an important factor for the success rates of IVF/ICSI. Here, we present a systematic review of randomized controlled trials (RCTs) on the effect of culture media on IVF/ICSI success rates.

METHODS: RCTs published between January 1985 and July 2012 were eligible for inclusion. The primary outcome was live birth. Secondary outcomes were health of babies born, ongoing pregnancies, clinical pregnancies, miscarriages, multiple pregnancies, implantation rate, cryopreservation rate, embryo quality and fertilization rate. For those media that were evaluated in more than one comparison, an unconventional meta-analysis was performed by pooling the data of the media they were compared to.

RESULTS: Twenty-two RCTs were included that evaluated thirty-one different comparisons. Conventional meta-analysis was not possible for any of the outcomes as nearly all trials compared different culture media. Only four trials reported on live birth, and one of them reported a significant difference. Nine trials reported on ongoing and/or clinical pregnancy rates, of which four showed a significant difference. Pooling the data did not reveal a superior culture medium.

CONCLUSION: It is yet unknown what culture medium leads to the best success rates in IVF/ICSI. Given the potential importance of culture media for treatment outcome, rigorously designed RCTs are needed for currently available, as well as newly introduced culture media.

Introduction

Subfertility is of major clinical, social and economical concern. The most frequently used interventions are *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Despite their frequent use, the two largest data collections report a delivery rate per started cycle of only 18.4% and 25.2% respectively [1, 2].

For a successful pregnancy to occur, good quality preimplantation embryos are essential. Several studies have suggested that culture media have an impact on the quality of embryos generated in IVF/ICSI cycles thereby influencing implantation and pregnancy rates [3-5]. Currently, many culture media are commercially available, each with a different composition. The first culture media in IVF/ICSI were balanced salt solutions to which glucose and phosphate were added. Subsequently, more complex culture media formulations with the addition of non-essential amino acids, chelators (EDTA), vitamins and antibiotics were introduced [6, 7]. More recently, sequential culture media were designed to take into account the changing metabolic needs of the embryo from the cleavage to the blastocyst stage [8].

Despite all these changes in culture media, it is still unclear whether the composition of the media affects embryo quality and IVF/ICSI success rates and which culture medium leads to the best IVF/ICSI success rates.

Here, we present a systematic review of randomized controlled trials (RCTs) describing the effect of embryo culture media on IVF/ICSI success rates.

Methods

Computerized searches were conducted using MEDLINE, EMBASE, the Cochrane Central Register of Controlled Trials (CENTRAL), the National Research Register, the Medical Research Council's Clinical Trials Register, and the NHS Centre for Reviews and Dissemination databases using the following medical subject headings and text words: (Keywords CONTAINS "IVF" or "*in vitro* fertilization" or "in-vitro fertilisation" or "ICSI" or "intracytoplasmic sperm injection" or "Embryo" or "ET" or "Embryo Transfer" or "in-vitro fertilization" or Title CONTAINS "IVF" or "*in vitro* fertilization" or "in-vitro fertilisation" or "ICSI" or "intracytoplasmic sperm injection" or "Embryo" or "ET" or "Embryo Transfer" or "in-vitro fertilization") AND (Keywords CONTAINS "embryo culture" or "embryo culture media" or "Culture-Media" or "culture" or "culture incubator" or "cumulus coculture" or "blastocyst culture technique" or "blastocyst media" or "media" or "G1" or "G1.2" or "G2" or "G2.2 sequential" or "Medicult" or "Medicult Sequential Medium " or "Vitrolife" or "sequential culture" or "sequential media" or "fetal bovine serum" or "fetal cord serum" or "P1" or "P1 culture medium" or "human tubal fluid" or Title CONTAINS "embryo culture" or "embryo culture media" or "Culture-Media" or "culture" or "culture incubator" or "cumulus coculture" or "blastocyst culture technique" or "blastocyst media" or "media" or "G1" or "G1.2" or "G2" or "G2.2 sequential" or "Medicult" or

"Medicult Sequential Medium " or "Vitrolife" or "sequential culture" or "sequential media" or "fetal bovine serum" or "fetal cord serum" or "P1" or "P1 culture medium" or "human tubal fluid"). Since the first papers comparing culture media for human IVF/ICSI were published in 1985, our search strategy starts from 1985. More detailed information on search strategies is available in Appendix 1 (http://humupd.oxfordjournals.org/content/19/3/210/suppl/DC1). A Cochrane review on this subject will be developed and it will be updated regularly [9].

The citation lists of relevant review articles and included studies were also searched. Conference abstracts from the annual meetings of the European and American societies on human reproduction (ESHRE and ASRM) from 1985 to 2012 were manually searched. Authors of included studies were contacted for any additional information about their study when necessary. RCTs that compared commercially available media for the *in vitro* culture of human preimplantation embryos were included without any limitation to language.

All identified articles were independently assessed by two investigators (E.M. and M.A.F.M.Y.) and disagreements were discussed further with a third investigator (S.R.). Quality assessment on the included studies was based on the recommendations of the Cochrane Handbook of Systematic reviews and was performed by one of the authors and checked by the other [10]. The overall study quality was assessed as good (+) if the study fulfilled the key requirements of a randomized control trial (allocation concealment, blinding and randomization), moderate (+/-) if the study fulfilled some of the requirements and poor (-) if the study fulfilled none of the above requirements or if the study design regarding these parameters was unclear. In case no data were available for any of the studied outcomes, the primary author of the study was contacted and if there was no response the paper was excluded.

The primary outcome of this review was live birth rate presented per woman randomized. Other outcomes were: health of the babies born (defined by the presence of congenital anomalies), birthweight, ongoing pregnancy rate (number of viable gestations with positive fetal heart beat per randomized woman), clinical pregnancy rate (number of clinical pregnancies demonstrated by the presence of a gestational sac on ultrasound scan per randomized woman), miscarriage rate (number of failed pregnancies up to 22 weeks of gestation per randomized woman) and multiple pregnancy rate (number of multiple pregnancies per randomized woman). Embryo outcomes were: fertilization rate (number of oocytes fertilized per oocytes retrieved), number of top quality embryos at Day 3 per number of embryos, cryopreservation rate (number of embryos cryopreserved per randomized woman), and implantation rate (number of fetal sacs detected by ultrasound per number of transferred embryos).

Our analysis was on an intention-to-treat basis, hence studies not reporting on exact numbers of women per group were excluded from the analysis. To investigate whether any potential effect of the culture media on IVF/ICSI success rates depended on the day

of embryo transfer, we subdivided the data according to the time of embryo transfer (Day 2 - 3 or Day 4 - 6).

We anticipated that conventional meta-analysis might not be possible. To be able to recognize the potential superiority of one medium over several others, an unconventional hypothesis-generating meta-analysis on pregnancy rate per woman was done for media that were involved in multiple comparisons. For this meta-analysis, we used the random effect analysis model and the Mantel-Haenszel statistical method. As a criterion for heterogeneity among the studies, the I^2 was calculated for every comparison. The control group consisted of the pooled data of the combined comparison media. Data on clinical pregnancies, which were reported in the majority of the studies, were pooled and if these were not available, then the data from ongoing pregnancies were used. No subgroup analysis was performed and data from early and late embryo transfers were pooled together.

Results

Characteristics of included studies

Five hundred and sixty-six potentially relevant abstracts were identified by our search strategy. After reading the abstracts, 461 abstracts were excluded because they were comparing early versus late embryo transfer, because they were comparing media supplementation or because they were studying different species. Of the remaining 105 abstracts that appeared to meet the inclusion criteria, the full papers were retrieved. Another 65 papers were excluded after in depth assessment of presented data. Reasons for exclusion were: the trial was not relevant to the topic under study (25), the trial reported incomplete data and no response was received after contacting the corresponding author (18), the trial was not an RCT (14), the trial was a republication of a previous trial (7) and the trial appeared to be a review (1) (Figure 1 and Supplementary data, Table 1). Further, 18 studies were excluded because they were published as abstracts in conference books more than five years ago and were never published in peer reviewed journals (Supplementary data, Table 2). Thus, 22 trials were included in our review [11-32].

The basic characteristics (number of women, oocytes and embryos, mean female age, mean number of embryos transferred, whether it was a multicenter study, whether donor oocytes were included, time of embryo transfer and whether frozen embryos were included) are shown in Table 1. Fifteen studies randomized women or cycles, six studies randomized oocytes and one study randomized embryos. All studies were performed at single private or university-based clinics apart from three studies for which the location in which they were performed was unclear. IVF was performed in three studies, ICSI was performed in five and the other studies used both IVF and ICSI. Three studies reported on donor and non-donor oocytes, one only on donor oocytes and the rest of the studies used non-donor oocytes. Twelve studies performed early embryo transfer (Day 2 - 3), four studies performed late embryo transfer (Day 4 - 6) and six studies performed transfers on Day 3 or

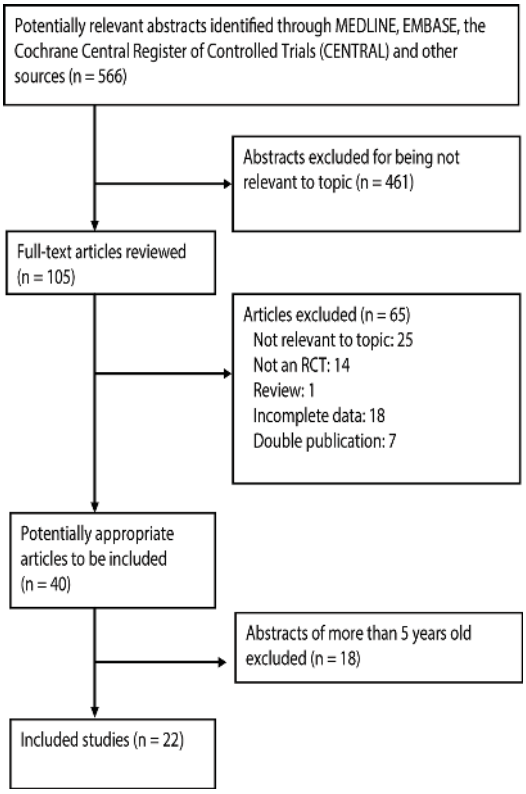

Figure 1: Flow chart of search for RCTs on culture media for human preimplantation embryos.

Day 5. Three studies transferred fresh as well as frozen-thawed embryos and all other studies transferred fresh embryos only.

The quality of the included studies (whether it was a full paper or not, allocation concealment, blinding, method of randomization, power calculation and whether it was an intention-to-treat analysis) is shown in Table 2. There were twenty-one studies published as full papers and one was published as abstract. Seven studies reported concealment of allocation, four studies reported no concealment while in the rest of the studies it was unknown whether concealment was performed. Blinding was performed in six studies, five studies reported no blinding and for the rest of the studies it was unclear. Two studies randomized women using a computer program [13, 15], two used sealed envelopes [14, 25], one randomized by drawing lots [21] and the compared culture media were used alternately in nine studies, while for the remaining studies the exact method of randomization was not reported. One study randomized patients according to patient number for a part of the study and according to oocytes in a second part of the study [20].

The reported comparisons and outcomes per study are shown in Table 3. In our analysis, pregnancy outcomes from trials that randomized oocytes or embryos were excluded because of the difference in the unit of analysis. From trials that randomized women (or cycles), both outcomes that are analyzed per woman (or cycle) and outcomes that are analyzed per oocyte or embryo were included. Many studies did not report all data necessary for analysis; in this case, the principal investigators of the studies were contacted for additional information. Of the 22 contacted authors, 11 responded to our questions. When we received no answer from the authors, we included the studies only for the outcomes for which data were available.

Table 1: Baseline characteristics of included studies.

Included studies	Women /Oocytes	Age (mean)	ET (mean)	Multicenter	IVF/ICSI	Donor oocytes	Time of embryo transfer	Fresh/frozen embryos
Studies randomizing women/cycles								
Quinn *et al.* (1985)	113	-	-	-	IVF	No	Early	Fresh
Parinaud *et al.* (1998)	416	-	2.8	-	ICSI	No	Early	Fresh
Mauri *et al.* (2001)	182	34.4	2.8	No	ICSI	No	Early/late	Fresh
Utsunomiya *et al.* (2002)	114	34.1	3	No	IVF/ICSI	No	Late	Fresh
Zollner *et al.* (2004)	176	33	3	No	IVF/ICSI	No	Early/late	Fresh
Ben-Yosef *et al.* (2004)	349	35.3	3	No	IVF/ICSI	No	Early	Fresh
Summers-Chasse *et al.* (2004)	280	-	-	-	IVF/ICSI	No	Early	Fresh/frozen
Balaban *et al.* (2005)	473	32.1	2.8	No	IVF/ICSI	No	Early/late	Fresh/frozen
Hoogendijk *et al.* (2007)	39	-	-	No	ICSI	No	Early/late	Fresh
Sepulveda *et al.* (2009)	79	40.5	-	No	IVF/ICSI	Yes	Late	Fresh
Campo *et al.* (2010)	172	34.6	1.6	No	IVF/ICSI	No	Early	Fresh
Dumoulin *et al.* (2010)	826	33.2	-	No	IVF/ICSI	No	Early	Fresh
Paternot *et al.* (2010)	178	29.9	-	No	IVF/ICSI	No	Early/late	Fresh
Khoury *et al.* (2012)	163	34.7	1.7	No	ICSI	Yes	Late	Fresh
Nelissen *et al.* (2012)	1,732	33	-	No	IVF/ICSI	No	Early	Fresh/frozen
Studies randomizing oocytes/embryos								
Staessen *et al.* (1998)	6,330	32.4	2.1	No	IVF	No	Early	Fresh
Parinaud *et al.* (1999)	493	-	-	No	IVF/ICSI	No	Early	Fresh
Artini *et al.* (2004)	1,014	35.4	-	No	IVF/ICSI	No	Early	Fresh
Findikli *et al.* (2004)	1,434	-	-	No	IVF/ICSI	No	Late	Fresh
Reed *et al.* (2009)	60	37.4	2.3	No	IVF/ICSI	Yes	Early/late	Fresh
Hambiliki *et al.* (2010)	1,206	33.9	-	No	IVF	No	Early	Fresh
Di Falco Cossiello *et al.* (2011)	2,289	33.6	2.2	No	ICSI	Yes	Early	Fresh

ET, embryos transferred; -, unknown

Table 2: Quality assessment of the studies included.

	Quinn 1985	Parinaud 1998	Mauri 2001	Utsunomiya 2002	Zollner 2004	Ben-Josef 2004	Summers-Chase 2004	Balaban 2005	Hoogendijk 2007	Sepulveda 2009	Campo 2010	Dumoulin 2010	Paternot 2010	Staessen 1998	Khoury 2012	Nelissen 2012	Parinaud 1999	Artini 2004	Findikli 2004	Reed 2009	Hambiliki 2010	Di Falco Cossiello 2011
	Studies randomizing women/cycles																Studies randomizing oocytes/embryos					
Full paper																						
Allocation concealment																						
Blinding																						
Randomization																						
Power calculation																						
ITT																						
Overall study quality	-	+/-	+	-	+/-	+/-	+/-	+/-	+/-	-	+	+	+	+/-	+	+	-	+/-	-	-	+/-	+

= yes, = no, = unknown, + = good, +/- = moderate, - = poor

ITT = intention to treat protocol

Characteristics of the culture media used

There were 20 culture media from 11 commercial companies represented in the review. These companies were: Irvine Scientific (HTF, P1, MultiBlast); Vitrolife (G2, G3, G5 series); MediCult (Universal IVF, BlastAssist, ISM, EmbryoAssist); Cook (Sydney IVF cleavage/ blastocyst media); Biopharma Sage (Quinn's Advantage); Scandinivian IVF (IVF); InVitroCare (HTF); IVF Online (Global), Ellios Bio-Media (EllioStep2, BM1, SMART2), Api-System (Menezo B2) and Gynemedia (GM501). G series media were used in eight studies, Sydney IVF in six, HTF, Global and P1 in four studies each, Quinn's Advantage and IVF media in three studies each, MultiBlast, BM1, ISM, Universal IVF, GM501 and EllioStep2 media in two studies each and the other media were used in one study each. The comparisons among the various culture media are shown in Figure 2. All studies involved individual comparisons between two media apart from P1 versus HTF and Sydney IVF versus G3 that were compared in two studies each.

Pregnancy outcomes

An overview of all available pregnancy data is provided in Figure 3. For each outcome, results are sorted based on the effect size.

Four studies reported on live birth rate; two after Day 2 - 3 embryo transfer and two in both subgroups. No evidence of a statistical difference was observed between the compared media apart from one study where significantly more live births were observed in embryos cultured in G3 compared to embryos cultured in Sydney IVF [risk difference (RD) 0.06, 95% confidence interval (CI): (0.02, 0.11)] [22]. Meta-analysis was not possible since all studies compared different culture media.

Table 3: Included studies and reported outcomes per study, including their suitability for review.

Included studies	Compared media	Pregnancy outcomes						Embryo outcomes			
Studies randomizing women		LBR	HN	OPR	CPR	MR	MPR	FR	EQ	CR	IR
Quinn *et al.* (1985)	HTF vs. T6[a]				×						
Parinaud *et al.* (1998)	EllioStep2[a] vs. BM1[a] vs. IVF				×				x[1]		×
Mauri *et al.* (2001)	P1 vs. IVF					×		×	x[1]		×
Utsunomiya *et al.* (2002)	HTF/MultiBlast vs. G2[a] vs. HTF/Sydney IVF			×	×	×	×	×			×
Zollner *et al.* (2004)	G2[a] vs. BlastAssist	×			×	×	×	×	x[1]		×
Ben-Josef *et al.* (2004)	P1 vs. Sydney IVF	x[1]			x[1]		x[1]	x[1]	×		×
Summers-Chase *et al.* (2004)	HTF vs. P1 vs. Quinn's				x[1]		x[1]				×
Balaban *et al.* (2005)	G3[a] vs. G2[a]				×		×	×	×		×
Hoogendijk *et al.* (2007)	Sydney IVF vs. Quinn's			x[1]				x[1]	×		
Sepulveda *et al.* (2009)	Global vs. MultiBlast			×	×	×			x[1]		×
Campo *et al.* (2010)	ISM1 vs. GM501	×		×	×	×		×		×	×
Dumoulin *et al.* (2010)	Sydney IVF vs. G3[a]	x[2]	x[2]		x[2]	x[2]	x[2]	x[1]	x[1]		×
Paternot *et al.* (2010)	Sydney IVF vs. GM501	×		×	×	×	×	×	×	x[1]	×
Khoury *et al.* (2012)	Global vs. Quinn's				x[1]			×		×	×
Nelissen *et al.* (2012)	Sydney IVF vs. G3[a]	×	×		×	×	×				
Studies randomizing oocytes/embryos											
Staessen *et al.* (1998)	MB2[a] vs. Universal IVF vs. BM1[a]				x[3]	x[3]	x[3]	×			×
Parinaud *et al.* (1999)	EllioStep2[a] vs. SMART2[a]							×	×		
Artini *et al.* (2004)	HTF vs. P1				x[1,3]			×	×		×
Findikli *et al.* (2004)	ISM vs. G2[a]							×	x[1]		
Reed *et al.* (2009)	Global vs. G5			x[1,3]	x[1,3]			x[4]	x[1]		x[1]
Hambiliki *et al.* (2010)	EmbryoAssist vs G5	x[1]			x[1]			x[4]	×	×	×
Di Falco Cossiello *et al.* (2011)	HTF vs. Universal IVF vs. Global vs. IVF								x		

LBR, live birth rate, HN, health of newborns, OPR, ongoing pregnancy rate, CPR, clinical pregnancy rate, MR, miscarriage rate, MPR, multiple pregnancy rate, IR, implantation rate, CR, cryopreservation rate, EQ, embryo quality, FR, fertilization rate. Outcomes were excluded from the analysis, reasons being: [1]the exact number of women/oocytes/embryos were not known, [2]reported also by Nelissen *et al.* 2012, [3]oocyte/embryo randomization, [4]fertilization was performed in other media

[a] indicates media that are no longer commercially available

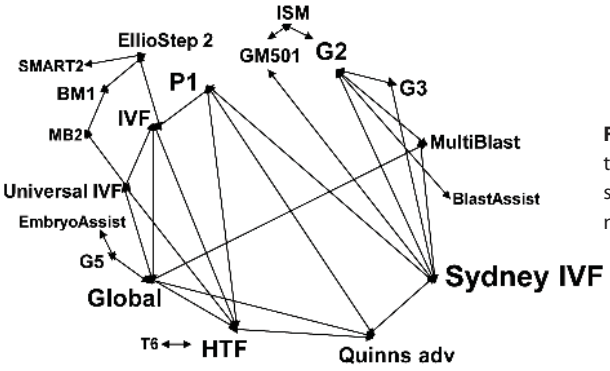

Figure 2: Schematic representation of the comparisons made in the included studies. The font size represents the number of studies on each medium.

One of the included studies reported on the birthweight of babies born [22]. This study showed that embryos cultured in Sydney IVF resulted in singletons with lower birthweight compared with embryos cultured in G3.

Four studies reported on ongoing pregnancy rate: one after Day 2 - 3 embryo transfer, two after Day 4 - 6 embryo transfer and one in both subgroups. The gestation weeks up to when an ongoing pregnancy is defined were unclear in most of the studies. One study comparing three media with each other found a significant difference in ongoing pregnancies after late embryo transfer [31]. Culture in both MultiBlast and Sydney IVF resulted in more ongoing pregnancies compared with culture in G2 medium [RD 0.26, 95% CI: (0.08, 0.44) and RD 0.26, 95% CI: (0.10, 0.42) respectively].

Nine studies reported on clinical pregnancy rate: four of them after Day 2 - 3 embryo transfer, two studies after Day 4 - 6 embryo transfer and three in both subgroups. Six comparisons were found to be significantly different. For early embryo transfer, HTF resulted in more clinical pregnancies than T6 [RD 0.19, 95% CI: (0.04, 0.33)] and G3 resulted in more clinical pregnancies than Sydney IVF [RD 0.06, 95% CI: (0.01, 0.10)]. For late embryo transfer, MultiBlast and Sydney IVF resulted in more clinical pregnancies compared to G2 [RD 0.26, 95% CI: (0.08, 0.44) and RD 0.26, 95% CI: (0.10, 0.42), respectively]. G3 resulted in more clinical pregnancies than G2 both after early and late embryo transfer [RD 0.12, 95% CI: (0.03, 0.22) and RD 0.14, 95% CI: (0.00, 0.27), respectively].

Seven studies reported on miscarriage rate: three after Day 2 - 3 embryo transfer, two after Day 4 - 6 embryo transfer and two after both time points. Three comparisons were found to be statistically significant. BlastAssist, MultiBlast and Sydney IVF all three resulted in more miscarriages compared with G2 [RD 0.10, 95% CI: (0.01, 0.19), RD 0.14, 95% CI: (0.02, 0.26) and RD 0.10, 95% CI: (0.00, 0.20), respectively].

Five studies reported on multiple pregnancy rate: one for Day 2 - 3 embryo transfer, one for Day 4 - 6 embryo transfer and three both for Day 2 - 3 and Day 4 - 6 embryo transfer. G3

medium resulted in a higher multiple pregnancy rate compared with G2 after early embryo transfer but the difference was not significant after late embryo transfer [RD 0.11, 95% CI: (0.03, 0.18) and RD 0.09, 95% CI: (-0.01, 0.19), respectively].

We performed an unconventional hypothesis-generating meta-analysis on pregnancy rates per woman for five media that were involved in multiple comparisons. Each of these media was compared with the pooled data of the combined comparisons (Figure 4). Only one culture medium (G2) resulted in significantly less pregnancies compared to the group of other media [RD -0.12, 95% CI: (-0.22, -0.03)] but this result should be interpreted with caution as the heterogeneity among the studies was high (I^2= 57%). For the other four culture media, no significant differences were found.

Embryo outcomes

An overview of all available embryo data is provided in Figure 5. For each outcome, results are sorted based on the effect size.

Ten studies reported on fertilization rate (the definition of fertilization was heterogeneous among the studies) while studies that transferred the embryos to the compared media after assessment of fertilization were excluded for this outcome measure. Two comparisons showed a significant difference. GM501 resulted in a higher fertilization rate compared with Sydney IVF [RD 0.11, 95% CI: (0.07, 0.16)] and G2 resulted in a higher fertilization rate compared with BlastAssist [RD 0.08, 95% CI: (0.04, 0.11)].

Given that the assessment and reporting (mean, average or percentage) of embryo quality was very heterogeneous, only eight studies that reported on the number of good quality embryos could be included in our analysis. Eight comparisons showed a significant difference. Global resulted in more good quality embryos compared with Universal IVF, HTF and IVF media [RD 0.29, 95% CI: (0.21, 0.37), RD 0.19, 95% CI: (0.12, 0.26) and RD 0.15, 95% CI: (0.08, 0.23), respectively]. Quinn's advantage performed better than Sydney IVF [RD 0.18, 95% CI: (0.02, 0.34)], IVF and HTF better than Universal IVF [RD 0.14, 95% CI: (0.09, 0.19) and RD 0.10, 95% CI: (0.06, 0.15)], G3 better than G2 [RD 0.12, 95% CI: (0.09, 0.15)] and Sydney IVF better than GM501 [RD 0.10, 95% CI: (0.04, 0.16)].

Three studies reported on cryopreservation rate: two after Day 2 - 3 embryo transfer and one after Day 4 - 6 embryo transfer. None of the comparisons resulted in a statistically significant difference.

Fifteen studies reported on implantation rate: ten after 2 - 3 days of culture, three studies reported on implantation rate after Day 4 - 6 embryo transfer and two studies reported on implantation rate after both Day 2 - 3 and 4 - 6 embryo transfer. Six comparisons resulted in statistically significant differences after early embryo transfer and four comparisons gave significant differences in the implantation rate after late embryo transfer. After early

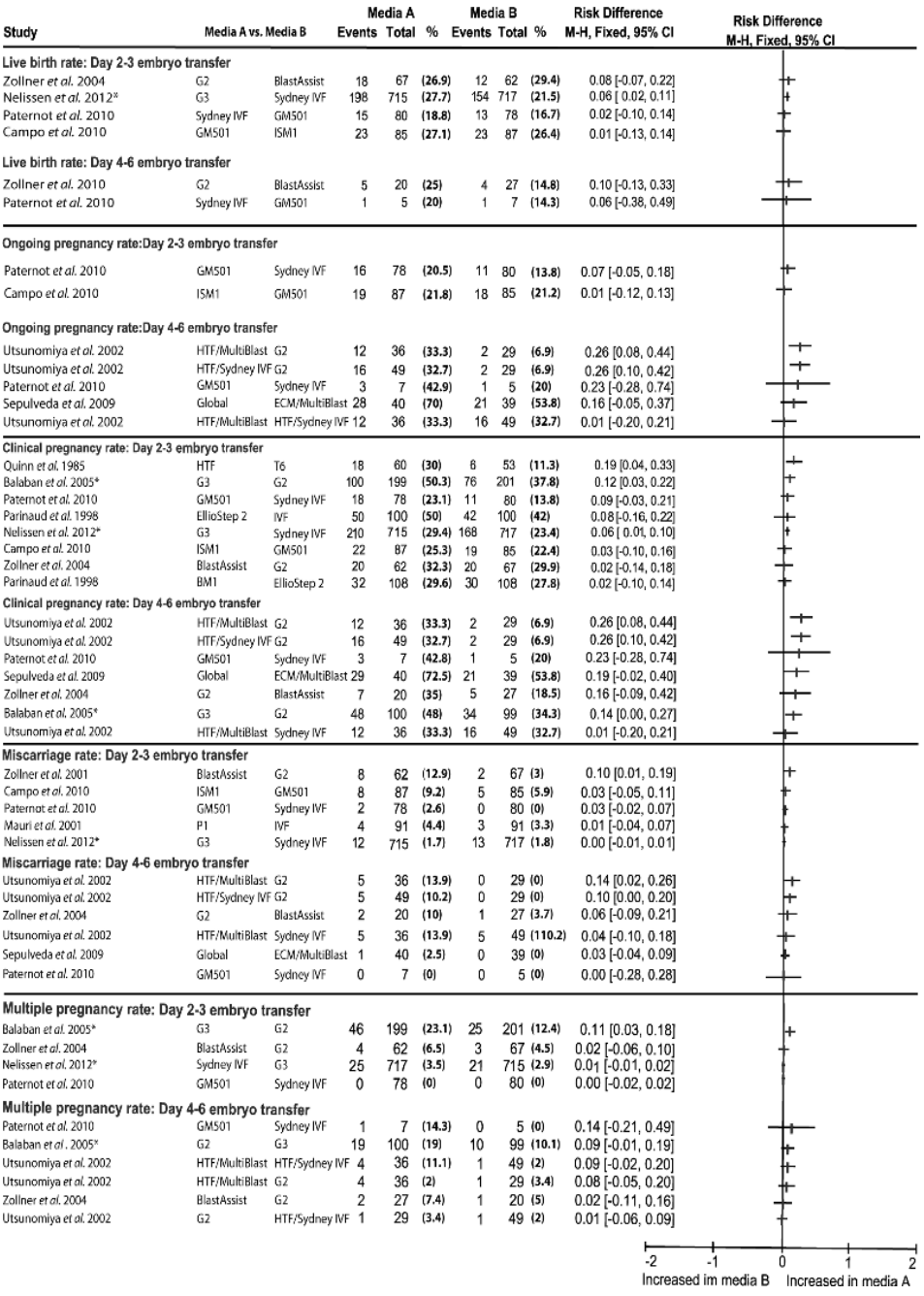

Figure 3: Overview without meta-analysis of the pregnancy outcomes of all included studies where women or cycles (*) were randomized. Women or cycles are used as unit of analysis in this overview. For each outcome, studies are sorted based on effect size.

embryo transfer, BM1 resulted in a higher implantation rate than MB2 [RD 0.34, 95% CI: (0.05, 0.63)], Quinn's advantage and P1 resulted in a higher rate than HTF [RD 0.12, 95% CI: (0.08, 0.16) and RD 0.07, 95% CI: (0.02, 0.11), respectively), P1 resulted in a higher rate than Sydney IVF [RD 0.04, 95% CI: (0.01, 0.07)] and G3 resulted in a higher rate compared with G2 and Sydney IVF [RD 0.11, 95% CI: (0.07, 0.16) and RD 0.10, 95% CI: (0.03, 0.17), respectively]. After late embryo transfer, Global resulted in a higher implantation rate compared with MultiBlast [RD 0.21, 95% CI: (0.05, 0.36)] and G3, Sydney IVF and MultiBlast resulted in higher rate compared to G2 [RD 0.16, 95% CI: (0.02, 0.30), RD 0.09, 95% CI: (0.02, 0.16) and RD 0.08, 95% CI: (0.02, 0.14), respectively].

Discussion

In this systematic review, we assessed the effect of commercially available culture media on IVF/ICSI success rates to indentify the medium with the best clinical outcomes. We were unable to identify such a medium, due to the paucity of data available in the literature. The 22 included studies involved different comparisons or reported data in such a way that proper meta-analysis was not possible for neither primary nor secondary outcome measures. Only four studies reported on live births and one found a significant difference. Although meta-analysis was not possible, in the majority of the studies a difference in pregnancy rate of more than 5% (RD ≥ 0.05) between the culture media was observed, indicating the clinical relevance of culture media for IVF/ICSI success rates. We were able to perform an unconventional hypothesis-generating meta-analysis by combining data from studies that shared the same culture medium in one of the treatment arms. With this analysis we addressed the question of whether these media consistently show good or bad results. Out of the five media that were part of this meta-analysis, G2 resulted in less pregnancies compared with the group of other media but this result is based on studies with high heterogeneity (I^2=57%) so it should be cautiously interpreted.

The overall quality of the included studies was low. Only four studies reported on live birth rate per woman and four on ongoing pregnancy rate while it is commonly accepted that

Medium A			Medium B			Risk Difference			
medium	Events	Total	# of media compared with	Events	Total	RD	CI	I^2 (%)	Risk Difference M-H, Random, 95% CI
G2	141	445	4	201	473	-0.12	[-0.22, -0.03]	57	
Sydney IVF	212	900	4	245	865	0.01	[-0.12, 0.14]	80	
MultiBlast	45	111	3	47	118	0.03	[-0.23, 0.30]	82	
EllioStep2	80	208	2	74	208	0.02	[-0.07, 0.12]	11	
GM501	40	170	2	34	172	0.04	[-0.06,0.14]	18	

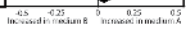

Figure 4: Meta-analysis of clinical/ongoing pregnancy rate. Medium B refers to all other media with which medium A has been compared.

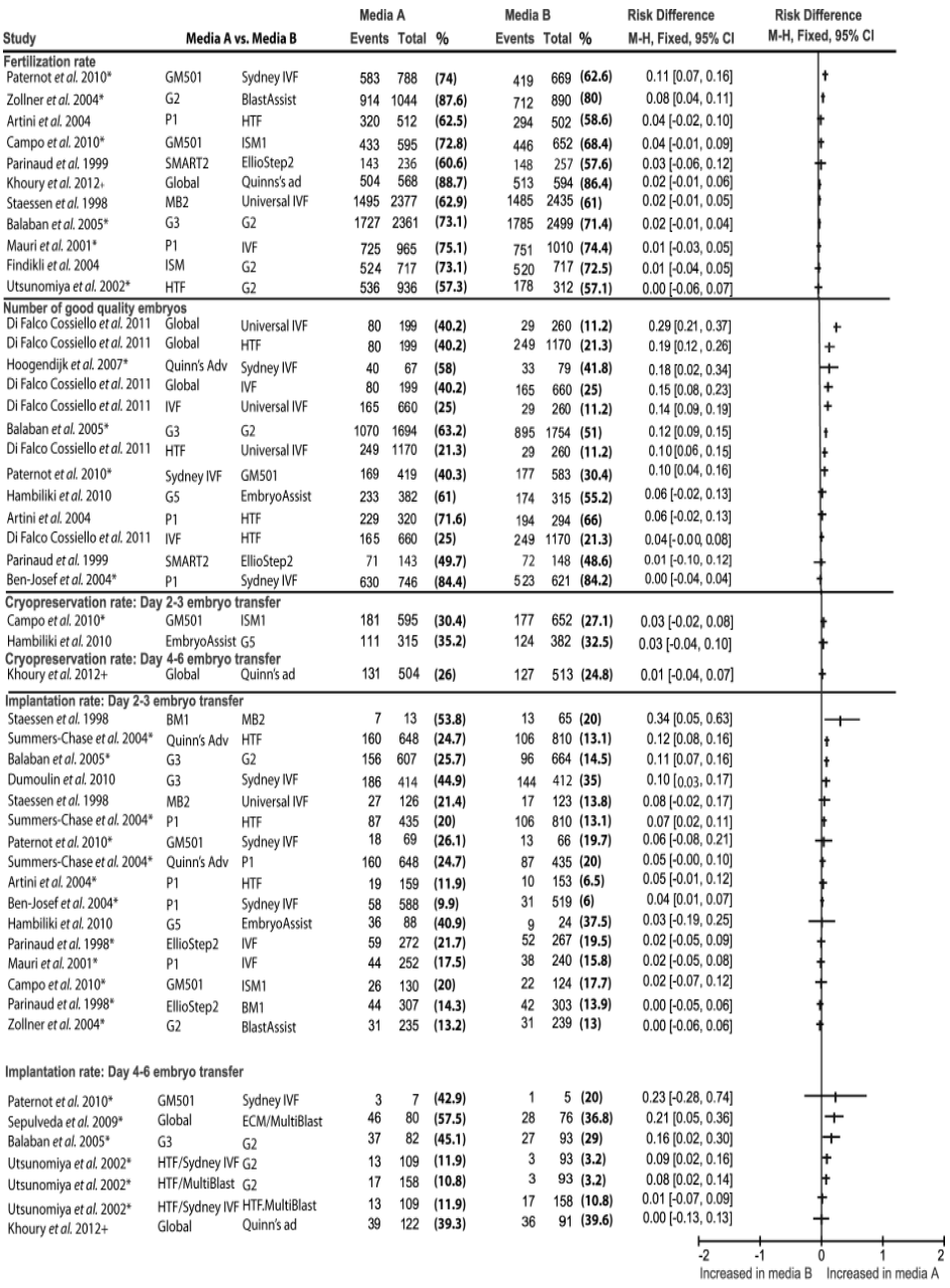

Figure 5: Overview without meta-analysis of the embryological outcomes of all included studies. Oocytes or embryos are used as unit of analysis in this overview. The asterisk indicates studies that have randomized women instead of oocytes or embryos. + indicates a study that randomised both oocytes and women. For each outcome, studies are sorted based on effect size.

at least ongoing pregnancy and preferably live birth should be the primary outcome of clinical studies to assess IVF/ICSI success rates [33-36]. The majority of the included studies had methodological limitations such as a weak randomization protocol, randomization of oocytes and embryos rather than women, small sample size and absence of a power calculation. In addition, not all studies commented on all outcomes and many studies reported part of their outcomes as percentages or means without providing exact numbers thereby preventing extraction of relevant data. This was also the case for 22 trials that had to be excluded since no relevant data could be extracted at all. Exact definitions of pregnancy (clinical and ongoing), miscarriages, and fertilization were not provided which could result in discrepancies among the studies. Finally, the way that embryo quality was reported was very heterogeneous with some studies providing number of good quality embryos as percentages, means or averages without providing exact numbers. In addition, the way embryo quality was scored varied greatly among the different studies. This heterogeneity led to exclusion of much potentially interesting data.

In our analysis we excluded pregnancy data from studies that randomized oocytes and embryos. This decision was based on several reasons. First, by using this data from trials randomizing embryos and oocytes, a unit of analysis error is introduced. Secondly, in case of oocyte and embryo randomization, oocytes and embryos from one patient are randomized between the compared media but only the best morphological embryos will be selected for transfer. This design introduces bias since the morphology of the embryos that are transferred might depend on the medium used. Lastly, the analysis of the clinical outcomes is based on the number of women and it is impossible to calculate the live birth and pregnancy rates per woman from trials that analyzed outcomes per oocytes or embryos. Embryo data from trials that randomized patients were included in the analysis. The potential confounding effect of including multiple oocytes/embryos from one woman/cycle was neglected as these concerned only secondary outcomes of our review and since only very limited data would otherwise be available for review.

To clarify whether culture media do have an effect on IVF/ICSI success rates and to determine the magnitude of such an effect, more good quality trials need to be conducted. The data presented in this review can be used as a guideline for researchers planning to conduct such RCTs on different culture media in the future. Changing the scope from embryological to clinical outcomes, performing proper randomization methods to ensure blinding and allocation concealment, and clear data reporting by providing exact numbers together with percentages or means will lead to better quality studies being available to be used for meta-analysis.

It should be noted that some of the media described in the included studies, such as EllioStep, BM1, SMART2, Menezo B2, T6 or the G2 and G3 series of Vitrolife, are no longer used for human IVF. Of importance is the fact that even today new culture media are introduced into clinical care without properly designed RCTs. Obviously there are commercial aspects to this as large scale RCTs are both time and money consuming. Moreover, if an RCT

Supplementary data, Table 1: Characteristics of excluded studies.

Study	Reason for exclusion	Study	Reason for exclusion
Adaniya *et al.* (2000)	Comparison with in house media	Kyono *et al.* (2002)	Comparison of day of embryo transfer
Alan *et al.* (2003)	Retrospective study	Lambert *et al.* (2005)	Unknown compared media
Aoki *et al.* (2005)	Retrospective study	Lilli *et al.* (2003)	Retrospective study
Auwera *et al.* (2002)	Comparison of day of embryo transfer	Macklon *et al.* (2002)	Comparison with in house media
Balaban *et al.* (2004)	Double publication, the same as Balaban *et al.* (2005)	Mahadevan *et al.* (1996)	Comparison with in house media
Barak *et al.* (1998)	Incomplete data	Meintjes *et al.* (2009)	Study on medium supplementation
Barrett *et al.* (1997)	Incomplete data	Michaeli *et al.* (2011)	Retrospective study
Ben-Josef *et al.* (2001)	Study on medium supplementation	Nelissen *et al.* (2010)	Double publication, same as Dumoulin *et al.* (2010)
Biggers *et al.* (2002)	Incomplete data	Nelissen *et al.* (2011)	Double publication, same as Nelissen *et al.* (2012)
Binda *et al.* (2010)	Double publication, same as Campo *et al.* (2010)	Parinaud *et al.* (1987)	Incomplete data
Chatzilioannou *et al.* (2010)	Incomplete data	Perri *et al.* (2001)	Incomplete data
Cummins *et al.* (1986)	Incomplete data	Prados *et al.* (2002)	Retrospective study
Bungum *et al.* (2002)	Study on medium supplementation	Psalti *et al.* (1989)	Study on medium supplementation
Bungum *et al.* (2003)	Incomplete data	Quinn *et al.* (1995)	Incomplete data
Catriona *et al.* (1986)	Study on medium supplementation	Raja *et al.* (2002)	Comparison of day of embryo transfer
Chesmore *et al.* (2005)	Double publication, the same as Lambert *et al.* (2005)	Romano *et al.* (2004)	Incomplete data
Choi *et al.* (2004)	Study with cryopreserved embryos, not an RCT	Sakkas *et al.* (1994)	Study on co culture system
Cooke *et al.* (2002)	The second medium is a modification of the first	Sifer *et al.* (2009)	Incomplete data
Coskun *et al.* (2000)	Comparison of day of embryo transfer	Staessen *et al.* (1994)	Comparison with in house media
De Silva *et al.* (1998)	Review	Steinkampf *et al.* (2001)	Retrospective study
Devaux *et al.* (2001)	Retrospective study	Stoddart *et al.* (2002)	Study on medium supplementation
Eaton *et al.* (2012)	Retrospective study	Sun *et al.* (2001)	Incomplete data
Gardner *et al.* (1997)	Double publication, the same as Gardner *et al.* (1998)	Torres *et al.* (2012)	Incomplete data
Gardner *et al.* (1998)	Comparison of day of embryo transfer	Urman *et al.* (2004)	Double publication, the same as Balaban *et al.* (2005)
Hashimoto *et al.* (2008)	Study on medium supplementation	Van den Bergh *et al.* (2004)	In house media
Huisman *et al.* (1992)	Study on medium supplementation	Vasteenburgge *et al.* (2007)	Retrospective study
Ignoul-Vanvuchelen *et al.* (1996)	Comparison with in house media	Verheuen *et al.* (2003)	Incomplete data
Jindal *et al.* (2005)	Retrospective study	Warnes *et al.* (1997)	Study on medium supplementation
Karamalegos *et al.* (1999)	Comparison with in house media	Wirleitner *et al.* (2010)	Retrospective study
Kemeter *et al.* (2012)	Comparison of different media and oxygen levels	Xella *et al.* (2009)	Retrospective study
Khan *et al.* (2004)	Incomplete data	Yoon *et al.* (2011)	Comparison with in house media

indicates equal or even lower success rates than already available media, years of research and development are wasted. Nevertheless, it is our firm belief that such an essential component of IVF should be treated with the highest level of scrutiny and that companies should report what studies have been performed and which endpoints were analyzed upon introduction of new media.

Only one of the studies reported data on neonatal outcomes. Recent studies have indicated that the type of culture media used to culture human preimplantation embryos can affect birthweight of newborns [16]. In addition, animal data suggest that the type of culture media used can affect gene expression and the imprinting status of bovine and mouse embryos [37-39]. This suggests that the *in vitro* culture of human embryos might have prolonged effects on the health of offspring, similar to the effect of *in utero* undernutrition on disease susceptibility in adulthood or the effect of *in vitro* maturation media on human oocytes and embryos [40-42]. We therefore suggest that next to the live birth rate, also the health of offspring should be included in future RCTs investigating culture media.

Retrospective studies have been excluded from our analysis as their design is inferior to that of prospectively randomized studies [43-47]. These retrospective studies, like the randomized studies that are included in our analysis, report on different comparisons and

Supplementary data, Table 2: Excluded abstracts.

Study	Presented at
Stevens *et al.* (2000)	Fertility and Sterility; 74 S106
Van Langendonckt *et al.* (2001)	Fertil Steril; 76 S1023-31
Cano *et al.* (2001)	Abstracts for the 17th annual meeting of the ESHRE; 161
Hazlett *et al.* (2002)	Fertil Steril; 78 S143
Bisioli *et al.* (2003)	Fertil Steril; 80 S57
Mortimer *et al.* (2003)	Fertility and Sterility; 80 S168
Westin *et al.* (2003)	Abstracts for the 19th annual meeting of the ESHRE; xviii151
Rubino *et al.* (2004)	Abstracts for the 20th annual meeting of the ESHRE; i142
Von During *et al.* (2004)	Abstracts for the 20th annual meeting of the ESHRE; i148
Moodie *et al.* (2004)	Fertil Steril; 82 S328-S29
Atchajaroensatit *et al.* (2005)	Abstracts of the 21st Annual Meeting of the ESHRE; i158
Arenas *et al.* (2007)	Fertil Steril; 88 S324
Mayer *et al.* (2001)	Fertil Steril; 76 S229
Mayer *et al.* (2003)	Fertil Steril; 80 S298
Baum *et al.* (2004)	Abstracts for the 20th annual meeting of the ESHRE
Fechtali *et al.* (2004)	Abstracts for the 20th annual meeting of the ESHRE; i147-i48
Sieren *et al.* (2006)	Fertil Steril; 86 S233-S34
Yamamoto *et al.* (2006)	Fertil Steril; 86 S228

different outcomes. Of interest are two studies that investigated the effect of culture media on the neonatal birthweight [44, 45]. In contrast to the study of Nelissen *et al*. [22] that reported a significant difference in birthweight between Sydney IVF and G3, these studies found no significant differences in birthweight for embryos cultured in G3, Global and G5 media or HTF and Quinn's advantage, respectively.

It should also be mentioned that other factors during embryo culture could influence IVF/ICSI success rates. Such factors include the number of embryos per drop and culture dish and embryo produced factors [48]. None of the included studies reported explicitly on these factors.

This systematic review shows an effect of culture media for human preimplantation embryos on embryo quality and success rates during IVF/ICSI treatment cycles. The existing data, especially on ongoing pregnancies and live births, are insufficient to allow the selection of the best culture medium for IVF/ICSI and thus more rigorously designed RCTs are necessary for both currently used culture media as well as new to be introduced culture media.

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CHAPTER 7

A multi-center double-blind randomized trial on the use of G5 or HTF medium for human preimplantation embryo culture in IVF/ICSI

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Abstract

Study question: Should G5 or HTF be used for culturing embryos in IVF/ICSI?

Summary answer: A higher, but non-significantly different ongoing pregnancy rate was obtained in the G5 group compared to the HTF group. Other outcomes such as clinical pregnancy, number of utilizable embryos, number of embryos implanted after a fresh transfer and the number of cryopreserved embryos available for transfer at a later date at the end of the study all significantly favored G5.

What is known already: A wide variety of culture media for human preimplantation embryos in IVF/ICSI treatments currently exists. It is unknown which medium is best in terms of clinical outcomes.

Study design, size, duration: Between September 2010 and May 2012, 836 couples (419 in the HTF group and 417 in the G5 group) were included in this multi-center, double-blind randomized controlled trial. Allocation was performed centrally by an online computer program. Each couple was treated until a live birth was obtained within a maximum timeframe of one year of IVF/ICSI treatment. The allocated medium was used in all treatment cycles, including transfers with frozen/thawed embryos.

Participants/materials, setting, methods: Couples, who started IVF/ICSI treatment at one of the six participating centers and their affiliated clinics for a first cycle, or first cycle after a successful pregnancy.

Main results and the role of chance: A higher, but non-significantly different ongoing pregnancy rate was obtained in the G5 group compared to the HTF group [45% (189/417) vs. 39% (163/419); RR: 1.2; 95% CI: (0.99-1.38); *P*=0.07]. Clinical pregnancy [48% (199/417) vs. 40% (168/419); RR: 1.2; 95% CI: (1.02-1.39); *P*=0.03] favored G5. Number of utilizable embryos, number of embryos implanted after a fresh transfer and the number of cryopreserved embryos per woman left for transfer at a later date at the end of the study were all significantly in favor of G5.

Limitations, reasons for caution: This study was powered to detect a 10% difference in live births while a smaller difference could still be clinically relevant.

Study funding/competing interest(s): This study was funded by the NutsOhra foundation.

Trial registration number: NTR1979

Introduction

Subfertility is of major clinical and social concern. The most frequently used interventions to treat subfertility are *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Despite their common use, IVF and ICSI are far from optimal treatments: the two largest data collections report a delivery rate per started cycle of only 20% and 22% respectively [1, 2].

A well-known factor that contributes to IVF/ICSI success is the medium that is used for oocyte and embryo culture. Several studies have shown the importance of the choice of embryo culture medium in this respect as it seems to impact embryo quality, pregnancy outcomes, and even the birthweight of newborns [3-10].

Despite the importance of culture media, a recent systematic review showed that randomized studies that compare clinical outcomes of different culture media are very limited in number and of low methodological quality [7]. Evidence-based selection of the best culture media from the wide range of available brands is currently not possible.

The aim of this multi-center, double-blind, randomized, controlled trial was to compare the clinical outcomes of two widely used embryo culture media, HTF medium and G5 medium.

Materials and methods

Study Design

We conducted a multi-center, double-blind, randomized, controlled trial in six hospital-based IVF centers [Academic Medical Center in Amsterdam (AMC), Catharina Hospital in Eindhoven, St. Elisabeth Hospital in Tilburg, Maastricht University Medical Center in Maastricht (MUMC), University Medical Center Groningen in Groningen (UMCG) and Radboud University Medical Center Nijmegen in Nijmegen (UMCN)) and five of their affiliated clinics (the Onze Lieve Vrouwe Gasthuis in Amsterdam, the Gemini Hospital in Den Helder, the Scheper Hospital in Emmen and the Maxima Medical Center in Veldhoven) in the Netherlands. The study protocol was approved by the institutional review boards of all participating centers and by the Central Committee on Research Involving Human Subjects (CCMO) in the Netherlands.

Allocation between two embryo culture media, G5 series (Vitrolife, Göteborg, Sweden) and HTF (Lonza, Verviers, Belgium), was performed centrally, one day before oocyte retrieval of the first cycle, by an online computer program with a 1:1 allocation using random block sizes of two and four couples. Stratification was performed for maternal age (<38 years of age and ≥ 38 years of age) and fertilization technique (IVF or ICSI) for each individual center. The allocated medium was used for all treatment cycles, including transfers with

cryopreserved embryos, which the couple received during one year of treatment in the specific center.

Decisions to include a couple and written informed consent of the couples were obtained via their gynecologists that were unaware of the allocation sequence. Participating couples, attending gynecologists and outcome assessors were fully blinded as to the allocated treatment. The allocation sequence was revealed to the primary investigators only at the end of the study. The allocation of the couples to one of the two groups was performed by the embryologists based on the outcome of the online allocation. Blinding of the embryologists was not possible since they performed the procedures in the laboratory.

Study population and sample size calculation

All couples that were scheduled for an IVF/ICSI treatment at one of the participating centers or their affiliated clinics for their first, or first after a successful pregnancy, cycle were eligible to participate in this study. Couples undergoing preimplantation genetic diagnosis (PGD), couples for whom IVF was used to prevent the transmission of HIV and couples undergoing a modified natural cycle [11] were excluded.

Based on a cumulative live birth rate of 40% after one year of IVF/ICSI treatment (2 - 3 cycles) in the participating centers in the years preceding this study, we calculated that a sample size of at least 784 couples would be needed to detect an increase of 10% (from 40% to 50%) in live birth rate after one year of treatment with a power of 80% at a significance level of 0.05.

Study procedures

All procedures that the included women underwent, such as ovarian hyperstimulation, follicular aspiration and oocyte fertilization were the routine IVF/ICSI procedures that these women would receive when not participating in the study in the particular center where they received treatment.

The G5 series are sequential media that include G-IVF medium for fertilization, G1 medium for culturing embryos from day one to day three and G2 medium for culturing embryos from day three to day six. HTF is a medium for continuous uninterrupted culture of oocytes and embryos from fertilization up to day three of culture. To keep the same methodology as when using G5 media, the embryos were moved to a new culture dish containing HTF on day three for further culture. HTF was supplemented with 10% pasteurized plasma solution (Central Laboratory of Blood Transfusion, Amsterdam, the Netherlands).

In case of IVF, the oocytes were incubated in dishes containing G-IVF (Vitrolife, Göteborg, Sweden) or HTF, according to the culture medium allocated to every woman, with 10.000 - 100.000 progressively motile spermatozoa per ml for fertilization. The next morning, the

cumulus cells were removed and all fertilized and unfertilized oocytes were transferred to a clean dish containing the allocated medium, G1 or HTF. On day three of culture the embryos were transferred to a new dish containing G2 or HTF.

In case of ICSI, the oocytes were denudated using cumulase (Origio, Malov, Denmark), injected with a single immobilized spermatozoon and directly thereafter cultured in dishes containing G1 or HTF according to treatment allocation. On day three of culture the embryos were transferred to a new dish containing G2 or HTF. Transfer of the embryos was performed in G5 or HTF accordingly. Transfer of one, two or three embryos was performed according to the local policies of each participating center.

Embryo culture in each of the culture media was performed following the instructions of the manufacturers, i.e. at 37^0C and 6% CO_2 for G5 and 37^0C and 5% CO_2 for HTF. Three centers cultured oocytes and embryos in 20% O_2 (University Medical Center Groningen, Catharina Hospital and University Medical Center Nijmegen) while the other three cultured oocytes and embryos in 5% O_2 (Academic Medical Center, St. Elisabeth Hospital and Maastricht University Medical Center). Embryo morphology was assessed daily and the number of cells as well as the percentage of fragmentation were scored based on a structured scoring sheet available in all centers [12]. All embryologists in all centers participated in a national external online embryo scoring quality control scheme (www.embryoonline.eu). Embryos of sufficient quality were transferred to the uterine cavity of the women on day 2 or 3 after culture. Cryopreservation of supernumerary good quality embryos was performed one or two days after embryo transfer.

In case of multiple cycles per woman, the allocated medium was used for all treatment cycles that the woman received during a one year time frame after allocation. In case of a transfer with cryopreserved embryos, the allocated medium was used for culture and transfer of these embryos as well.

Outcome measures

The primary outcome of the study was live birth rate. Live birth was defined as a pregnancy that resulted in the birth of at least one baby born alive, independent of gestational age. Since at the time of writing of this thesis data on live births were not yet complete, we focus here on the results of the secondary outcome measures, with ongoing pregnancy as the main outcome. Data analysis of live births is expected to be complete in the beginning of 2014.

Other secondary outcomes included clinical pregnancy, biochemical pregnancy, multiple pregnancies, miscarriages, implantation, embryo quality and fertilization. Ongoing pregnancy was defined as a viable intrauterine pregnancy after 12 weeks of gestation. Clinical pregnancy was determined by the presence of a gestational sac confirmed by transvaginal ultrasound examination at 6 - 8 weeks of gestation. Biochemical pregnancy

was defined as a serum β human chorionic gonadotropin level of at least 50 IU per liter 2 weeks after embryo transfer. Multiple pregnancies were defined by the presence of two or more fetal sacs at 12 weeks of gestation. Miscarriages were defined by fetal loss till 12 weeks of pregnancy. Implantation was determined by the number of fetal sacs as identified by transvaginal ultrasound examination at 6 - 8 weeks of gestation. The end of study participation for a particular couple was the achievement of a live birth, the passing of one year after allocation or withdrawal of consent by the couple. Only pregnancies achieved via IVF/ICSI at the participating center were included in the data analysis.

Statistical analysis

The rates of clinical and laboratory outcomes were calculated in each group with 95% confidence intervals. We used 2-sided chi-square statistics to test for significance of categorical variables and a one-way ANOVA for continuous variables. Data were analyzed according to an intention-to-treat principle and involved analysis of all women who were randomized according to their original group allocation.

One interim analysis of efficacy was planned to be performed one year after the initiation of the study by an independent data and safety monitoring committee. Primary outcome of this interim analysis was ongoing pregnancy for first, second, and third cycles, including transfers with cryopreserved embryos, that had been performed at that time. The data and safety monitoring committee had to use these proxy outcomes as otherwise an interim analysis would not have been possible before the completion of the inclusion of the study. Live birth data were only available at the end of the study, due to the design and inclusion rate of the study. For the same reason no formal stopping rules or adjusted statistics were applied. A blinded overview, which was available only to the data and safety monitoring committee, was used for the interim analysis.

Results

Between September 2010 and May 2012, a total of 836 couples were randomly allocated to undergo IVF/ICSI using either G5 (n=417) or HTF (n=419) medium for embryo culture (Figure 1). There were 412 couples in the G5 group and 395 couples in the HTF group that underwent the allocated intervention in all their cycles. Five couples in the G5 and twenty-four couples in the HTF group did not receive the allocated treatment in one of the performed cycles because of human error in identifying the couple as participating in the study. Only one couple (in the G5 group) withdrew informed consent prior to finishing the year of treatment, after two cycles. This woman was not pregnant and was analyzed as such. Nineteen couples were allocated in either of the two culture media without fulfilling the inclusion criteria; the primary reason being that they had underwent one or more previous unsuccessful IVF/ICSI treatments. Since our analysis is based on an intention-to-treat protocol, these couples were included in the analysis.

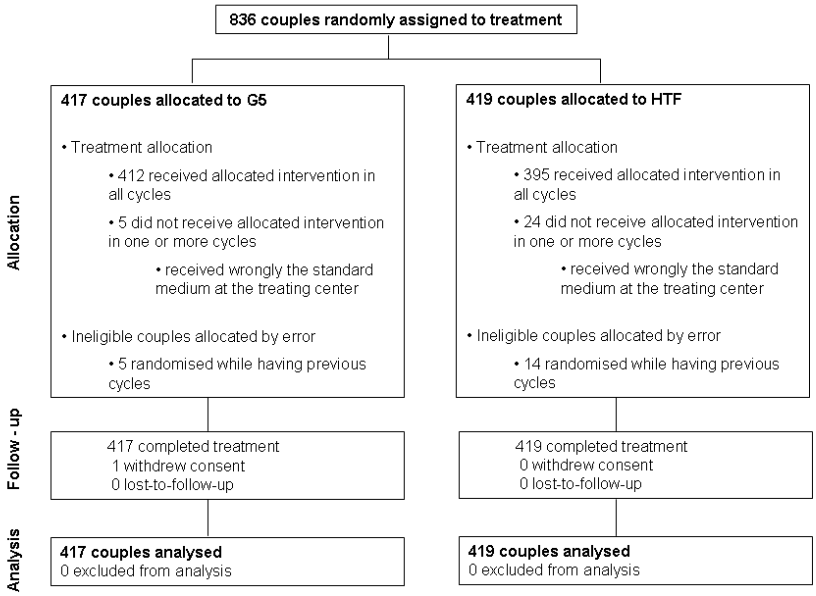

Figure 1: Flow chart of allocation, follow-up and analysis of the included couples.

The couples remained enrolled in the study for one year after allocation and in the case of pregnancy they were followed until delivery. No couples were lost-to-follow-up. The baseline characteristics of the couples are presented in Table 1. Four couples (three in the G5 group and one in the HTF group) received oocyte donation and twenty-two couples (eight in the G5 group and fourteen in the HTF group) had treatment with sperm from a donor. In these cases, the age of the donor was used for the calculation of the mean maternal and paternal age.

The number of women with an ongoing pregnancy in the G5 group was higher than the number of women achieving an ongoing pregnancy in the HTF group, but the difference was not statistically significant [45% (189 of 417) vs. 39% (163 of 419); RR: 1.2; 95% CI: (0.99-1.37); *P*=0.07]. Compared to women in the HTF group, significantly more women in the G5 group had a clinical pregnancy [48% (199 of 417) vs. 40% (168 of 419); RR: 1.2; 95% CI: (1.02-1.39); *P*=0.03]. The number of women with biochemical pregnancy was higher, but not significantly, in the G5 group compared to women in the HTF group [57% (236 of 417) vs. 50% (210 of 419); RR: 1.1; 95% CI: (0.99-1.28); *P*=0.06]. The number of miscarriages did not differ significantly between the groups (Table 2).

Clinical characteristics according to treatment cycle are shown in Table 3. A total of 1,496 cycles [714 (mean of 1.7) for the G5 and 782 (mean of 1.9) for the HTF group] were performed (*P*=0.57).

Table 1: Characteristics of the couples*.

Characteristic	G5 (n=417)	HTF (n=419)
Maternal age (years)	33.9 ± 4.3	33.8 ± 4.4
Paternal age (years)	37.4 ± 6.5	37.2 ± 6.3
Primary indication for IVF/ICSI		
Tubal	47 (11)	37 (9)
Anovulation	25 (6)	37 (9)
Endometriosis	22 (5)	25 (6)
Cervical	12 (3)	11 (3)
Male subfertility	211 (51)	225 (54)
Unexplained	100 (24)	84 (20)
Duration of subfertility (years)	3.1 ± 1.9	3.1 ± 2.3
Gravidity	0.62 ± 1.1	0.59 ± 1.0
Parity	0.30 ± 0.58	0.30 ± 0.58
Treatment Center		
AMC	72 (17)	73 (17)
Catharina zh.	88 (21)	87 (21)
Elisabeth zh.	69 (17)	68 (16)
MUMC	91 (22)	94 (22)
UMCG	52 (12)	50 (12)
UMCN	45 (11)	47 (11)

* Data presented as means ± SD or numbers (%)

Although not significant, the cumulative percentage of ongoing pregnancies was higher in the G5 group compared to the HTF group at all time points in treatment window of one year (Figure 2A). Women in both groups underwent a similar number of transfers, when using fresh embryos (714 vs. 783; *P*=0.57). The number of transfers using cryopreserved embryos was higher in the G5 group compared to the HTF group but the difference was not statistically significant (276 vs. 204; *P*=0.08) (Table 3 and Figure 2B). At the end of the study period, significantly more cryopreserved embryos per woman were remaining in the freezer in the G5 group compared to the number of embryos in the HTF group (1.3 vs. 0.8; *P*<0.001) (Figure 2C).

The embryo characteristics of all treatments are shown in Table 4. The number of fertilized oocytes in the HTF group was significantly higher compared to the G5 group [69% (4,346 of 6,279) vs. 63% (3,667 of 5,822); P<0.001] (Figure 3). The number of embryos that were transferred or cryopreserved, i.e. the number of utilizable embryos, and the number of embryos that implanted after a fresh transfer were significantly higher in the G5 group compared to the HTF group [1,974 vs. 1,778; P<0.001 and 20% (196 of 967) vs. 15% (170 of 1,116); P<0.001, respectively].

Discussion

In this randomized controlled trial we evaluated the effect of two commonly used culture media on embryo characteristics and pregnancy outcomes. A higher but not statistical significantly different ongoing pregnancy rate was obtained in the G5 group compared to the HTF group. Clinical pregnancy and embryo outcomes were significantly in favor or G5.

Table 2: Clinical outcomes in women per culture medium used.

Outcome	G5 (n=417)	HTF (n=419)	Risk Ratio (95% CI*)	*P* Value
Women with ≥1 ongoing pregnancy	189 (45)	163 (39)	1.2 [0.99-1.37]	0.07
Total no. of ongoing pregnancies†	191	164		
Women with ≥1 clinical pregnancy	199 (48)	168 (40)	1.2 [1.02-1.39]	0.03
Total no. of clinical pregnancies	206	170		
Women with ≥1 biochemical pregnancy	236 (57)	210 (50)	1.1 [0.99-1.28]	0.06
Total no. of biochemical pregnancies	256	221		
Women with ≥1 miscarriage§	61 (15)	51 (12)	1.2 [0.85-1.70]	0.31
Total no. of miscarriages	65	57		

Data presented as numbers (%)

* CI denotes confidence intervals

† 18 women had a multiple pregnancy in the G5 group and 21 women had a multiple pregnancy in the HTF group

§ miscarriages calculated up to 12 weeks

We now found a 6% difference in ongoing pregnancies. Despite the absence of a statistical difference [borderline significance (RR 1.2 (0.99-1.37))] this difference could be of clinical relevance, especially if we take into account that the study was powered to detect a 10% difference in live birth rates. Designing the study to be able to find a smaller difference between the media was not considered feasible at the time of study design due to the higher number of couples needed. This argues strongly in favor of broadening cooperation in large multi-center trials, as this is necessary to be able to establish smaller differences, which are still clinically relevant.

Based on the effect size for ongoing pregnancy rate and the fact that other secondary outcomes such as clinical pregnancy rate, implantation rate, and embryo utilization rate, all favor G5 we feel it is justified to conclude that G5 provides better treatment success than HTF. Complementary, the number of transfers using cryopreserved embryos was higher in the G5 group compared to the HTF group (276 vs. 204; P=0.08), and women in the G5 group had more cryopreserved embryos available in the freezer at the end of the study for transfer at a later date compared to the women in the HTF group (1.3 vs. 0.8; P<0.001), which further adds to the beneficial effect of G5. We will collect data from cycles transferring these cryopreserved embryos that are still in the freezer, and conduct a secondary analysis to compare the cumulative live birth rates over time.

To explain the differences between the outcomes of the two media we looked at the composition of the media. Even though a complete list with their components and concentrations used is not available because of patent issues, one of the differences is that the G5 medium has certain amino acids that the HTF medium lacks. Studies in human

Table 3: Clinical characteristics according to treatment cycle.

Characteristic	G5 (n=417)	HTF (n=419)	*P* value
No. of fresh cycles started*			
Cycle 1	417 (100)	419 (100)	1.00
Cycle 2	223 (53)	260 (62)	0.22
Cycle 3	72 (17)	101 (24)	0.52
Cycle 4	2 (<1)	3 (<1)	0.17
Fertilization procedure			
IVF	297 (42)	335 (43)	0.13
ICSI	417 (58)	448 (57)	0.63
No. of fresh embryo transfers	664 (93)	732 (94)	0.76
No. of fresh cycles with ongoing pregnancy			
Cycle 1	98 (24)	80 (19)	0.13
Cycle 2	54 (24)	48 (19)	0.14
Cycle 3	11 (15)	12 (12)	0.65
Cycle 4	0 (0)	0 (0)	n.a.
No. of transfers with cryopreserved embryos			
As part of cycle 1	206	148	0.09
As part of cycle 2	55	49	0.19
As part of cycle 3	15	7	0.14
As part of cycle 4	0	0	n.a.
No. of transfers with cryopreserved embryos leading to an ongoing pregnancy§			
As part of cycle 1	21 (10)	20 (14)	0.4
As part of cycle 2	6 (11)	5 (10)	1.00
As part of cycle 3	1 (7)	0 (0)	1.00
As part of cycle 4	0	0	n.a

Data presented as numbers (%), percentages calculated per number of fresh cycles started

* Percentages calculated per number of fresh cycles 1

†One woman in each group had two embryo transfers using fresh and vitrified oocytes

§ percentage calculated per number of embryo transfers with cryopreserved embryos

n.a. denotes not applicable

and animal embryos have shown that the addition of amino acids in the culture medium can indeed be beneficial for embryo development *in vitro* [13-18]. However, no studies in humans looked specifically at the effect of amino acids on pregnancy rates or live births.

As far as we know, there are no other randomized controlled trials comparing the efficacy of these two culture media for human IVF/ICSI treatment [7]. The reason why G5 and HTF culture media were used for this study is because HTF was the culture medium initially used by the majority of the Dutch IVF centers and that many centers considered switching to G5. Two previous studies compared the efficacy of HTF medium and G2 medium; G2 is an earlier version of G5 and is no longer on the market [19, 20]. The exact differences in components and their concentrations between these two culture media are not known. One study was a quasi-randomized trial with 294 couples which showed that culturing embryos using G2 media resulted in a significantly better embryo quality and reproductive performance (implantation and birth rate) compared to culturing embryos using HTF [19]. The other study in 558 frozen-thawed zygotes showed that G2 medium supported better the development of frozen-thawed human embryos *in vitro* and provided better embryo quality and higher pregnancy rates than HTF medium [20]. Both studies are indicative of a better efficacy in embryo development and clinical outcomes of G2 over HTF but they fall short in study design and number of included couples.

Although we conclude that G5 is superior to HTF, there are still many other media available. A trial comparing all available culture media simultaneously is simply not

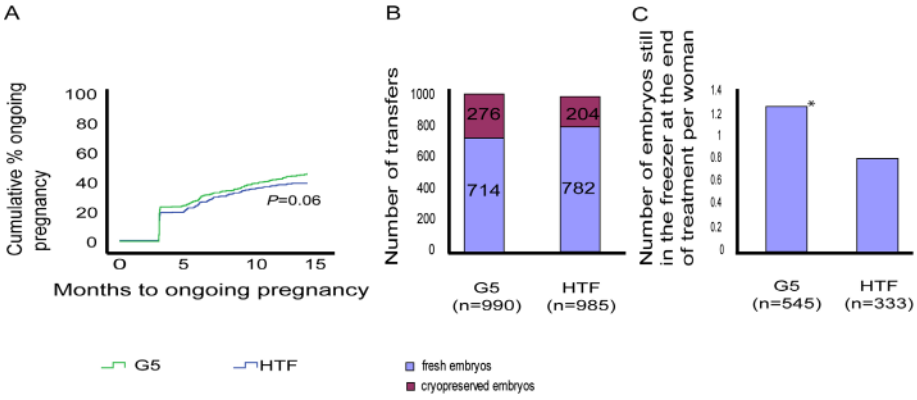

Figure 2: Clinical outcomes per medium used. (A) Cumulative ongoing pregnancy rate was higher throughout the study period for the G5 group compared to the HTF group. (B) Number of transfers performed using either fresh or cryopreserved embryos per group. The overall number of transfers is the same, but women that were allocated in the G5 group had more transfers with cryopreserved embryos compared to women that were allocated in the HTF group (P=0.08). (C) The number of cryopreserved embryos that are still available in the freezer is higher in the G5 group compared to the HTF group (P<0.001). That could lead to more transfers in the future and more ongoing pregnancies for women in the G5 group compared to women in the HTF group. * Indicates a significant difference.

possible. Nevertheless, such an essential component of IVF should be treated with the highest level of scrutiny, and there is a need for more properly randomized trials on media for human embryo culture. In addition, the full composition of the culture media should become available from the companies that produce them. The formulations should have a scientific rational and the introduction of new media into clinical practice should be based on properly conducted RCTs, as also agreed in the Dutch technical specification on

Table 4: Embryo characteristics per culture medium used.

Characteristic	G5	HTF	*P* Value
Cumulus-oocyte complexes – no. (mean no. per cycle ± SD)	6,477 (9.1 ± 5.3)	7,090 (9.1 ± 5.4)	0.95
Oocytes inseminated/injected – no. (mean no. per cycle ± SD)	5,822 (8.2 ± 5.0)	6,279 (8.0 ± 5.0)	0.61
Fertilized oocytes (embryos)* – no. (mean no. per cycle ± SD)	3,667 (5.1 ± 3.9)	4,346 (5.6 ± 4.0)	0.04
Fertilization rate - % per oocytes inseminated/injected	63	69	<0.001
Utilizable embryos† – no. (mean no. per cycle ± SD)	1,974 (2.8 ± 2.4)	1,778 (2.3 ± 1.8)	<0.001
Embryos transferred from a fresh cycle – no. (mean no. per transfer ± SD)	967 (1.5 ± 0.6)	1,116 (1.5 ± 0.6)	0.19
Implanted embryos from a fresh cycle – no. (% of embryos transferred)	196 (20)	170 (15)	<0.001
Embryos cryopreserved – no. (mean no. per cycle ± SD)	1,007 (1.4 ± 2.4)	662 (0.9 ± 1.8)	<0.001
Cryopreserved embryos transferred – no. (mean no. per transfers with cryopreserved embryos ± SD)	324 (1.2 ± 0.6)	225 (1.1 ± 0.6)	0.22
Implanted cryopreserved embryos – no. (% of cryopreserved embryos transferred)	36 (11)	25 (11)	0.89

* 2PN zygotes and 1PN/0PN zygotes that cleaved in 48 hours

† Number of embryos transferred or cryopreserved

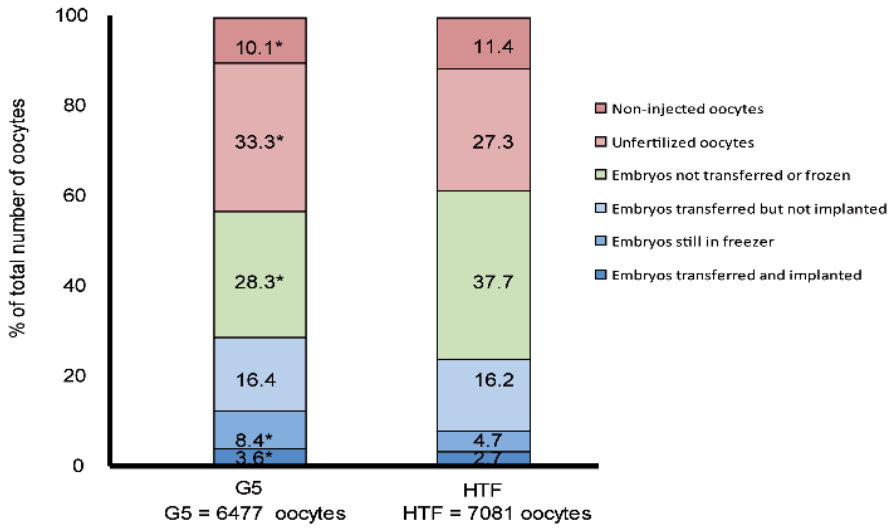

Figure 3: Graphical representation of the embryo characteristics per medium used. * Indicates a significant difference.

devices for assisted reproductive technologies [21]. Companies should also report what studies have been performed to test these media and which endpoints were analyzed. The responsibility for proper introduction of culture media with new formulations lies with the manufacturers that should disclose the composition of the culture media and the rational behind it but also with the reproductive medicine professionals that should be more critical and compare success rates of new culture media to the older ones. As illustrated here by six participating centers, the randomized use of two culture media can be implemented easily in daily routine.

Since there is evidence that culture media can affect not only pregnancy rates but also neonatal health [5, 8], future studies should also include follow-up data on the children born. We are currently gathering these data form the children born in the current trial and will publish them at a later time.

In conclusion, we found a higher but not statistically significant difference in the number of women achieving an ongoing pregnancy after a year in the G5 group as compared to the HTF group. Given the fact that this difference reached borderline significance, the power calculation was based on finding a difference of 10%, the culture in G5 resulted in significantly better embryos and higher implantation and clinical pregnancy rates than HTF, we recommend the use of G5 for culture of human embryos during IVF/ICSI treatments over HTF.

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Conflict of interest

None

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CHAPTER 8

General discussion and implications for further research

In the thirty-five years since the birth of the first IVF child, about five million children have been conceived worldwide using assisted reproductive technologies [1]. Despite the broad use of these technologies and all the advancements in the field, the two largest data collections available [i.e. of the European Society for Human Reproduction and Embryology (ESHRE) and of the American Society for Reproductive Medicine (ASRM)] report a delivery rate of only 20% and 22% per started cycle of IVF and ICSI respectively [2, 3]. Impaired embryo quality could explain these low success rates after IVF/ICSI treatment. Given the high percentage of human preimplantation embryos that arrest in culture (>50%) or fail to implant upon transfer (>80%, as shown in **Chapter 7**), it is evident that the human preimplantation embryo, although it has the possibility to adapt to a certain degree, is vulnerable [4].

Early preimplantation embryo development is a highly dynamic process where many important events take place in a short period of time. In humans, after fertilization, the embryo undergoes a series of mitotic divisions (early cleavages) where cells are progressively increasing in number and decreasing in size while the overall embryo size remains the same. These early cleavages rely almost solely on mRNA and proteins provided by the oocyte. Around day three of development, the embryonic genome gets activated. In later developmental stages (day four to six) the embryo compacts to form a morula, and subsequently the first differentiation occurs where two lineages, the trophectoderm and the inner cell mass, are formed in the blastocyst (day six). The molecular mechanisms that drive these events in humans are not fully understood.

The main aim of this thesis was to study the mechanisms that could lead to improved quality of human preimplantation embryos and to investigate factors - with an emphasis on culture media - which can affect the quality of the embryo. A better insight into these factors could help embryologists optimize the *in vitro* culture conditions. This hopefully will lead to improved quality of embryos and therefore higher success rates of IVF/ICSI treatment.

A basic limitation when studying human embryo quality is the availability of normally fertilized (2PN) human embryos of good morphology. These embryos only become available for research when they are no longer needed in clinical practice, as couples finished their childbearing wish. In the Netherlands, generating human embryos specifically for research is not allowed; something which is possible in other countries such as the United Kingdom. Due to the obvious ethical and practical limitations with working with normally fertilized (2PN) human preimplantation embryos, a model is needed to be able to study human embryo quality from the first day of development onwards. Possible models to consider include animal embryos, trophectoderm and embryonic stem cell lines (ESC) and abnormally fertilized embryos (3PN and 1PN embryos). Supernumerary human embryos after an IVF/ICSI treatment can also be used, which could be left-over lower quality embryos that otherwise will be discarded or cryopreserved good quality embryos that are donated for research.

Animal models are an attractive alternative as they overcome many of the logical, ethical and financial challenges of working with human embryos. Common animal models include mouse, bovine, porcine and nonhuman primate embryos. Nevertheless, there are important differences between animal and human embryos. The timing of embryo development in animals for instance is different compared to human embryos (e.g. the mouse embryo undergoes fast mitotic divisions and reaches the expanded blastocyst stage already on day four [5]). The timing of embryonic genome activation (EGA) also varies among different species (e.g. the mouse EGA happens at day two [6, 7]). Moreover, animal embryos appear less sensitive to mitotic errors than human preimplantation embryos (e.g. the incidence of chromosomal mosaicism in nonhuman primate preimplantation embryos is 31.2%) [8, 9]. Nonhuman primate embryos are the closest to human embryos with similar timing of embryo development and EGA [10, 11].

The use of trophectoderm and embryonic stem cell lines is another alternative but these cells are not representing the entire embryo. They respectively represent just the trophectoderm and the ICM of the blastocyst. Working with supernumerary human embryos or abnormally fertilized embryos after IVF could be a better option. Supernumerary embryos are often of lower quality since the best available embryos were already transferred or cryopreserved. Furthermore, supernumerary embryos are often only available from day four onwards, limiting research on the critical period of the first three days. Abnormally fertilized 3PN embryos are believed to contain a triploid chromosomal content and as such they are never transferred to a woman. Therefore, these embryos can be investigated from the first day onwards. For digynic 3PN embryos, the fact that they are triploid does not make them chromosomally more unstable than normal 2PN embryos as they contain a single bipolar spindle and undergo regular chromosome segregation [12, 13]. Cryopreserved embryos are of good quality and can be available at different developmental stages, from the zygote stage to the blastocyst stage, depending on the cryopreservation policy of the IVF center.

The selection of which model to use at a given time depends on the scientific question to be answered and the design of the experiment.

Culture media and embryo quality

An essential factor in daily IVF practice is the culture medium used to culture embryos *in vitro*. Surprisingly, even after 35 years of clinically culturing human embryos in the lab we know very little about the efficacy of culture media and about the optimal composition to achieve the best results not only in numbers of live births but also on the health of children born. Over these years effort has been made to optimize the components of culture media by reducing glucose concentrations [14], adding amino acids [15-18] and supplementing with growth factors [19-21]. Unfortunately, a simple list with essential components of media for human embryo culture is not available since no broad consensus exists. Moreover, a literature search showed that most of the studies on the components of culture media for

human preimplantation embryo culture have been performed in the eighties and nineties and only a couple of such studies have been performed in recent years [22-26].

No broad consensus exist either on whether the choice of culture medium actually matters for obtaining good results. In recent years, there has been a renewed interest in culture media after several studies showed an effect of culture media on human intrauterine growth, neonatal birthweights and epigenetic alterations in the placenta [27-30]. Some studies on both animals and human showed that *in vitro* culture and culture media in specific can affect embryo quality and pregnancy outcomes [31-38].

The data presented in this thesis show an effect of culture media at the transcriptome level as well as at the level of embryo development and clinical outcomes.

However, other studies showed no effect of culture media either on embryo quality or success rates [39-43]. On neonatal outcomes, in contrast to the one quasi-randomized study that showed an effect, three retrospective studies reported no difference for the compared media [44-46]. All these studies compared different culture media so the different outcomes could be attributed to a general lack of effect of culture media. Alternatively, some culture media might be equally effective and therefore when compared to each other they give similar results. The systematic review described in this thesis (**Chapter 6**) demonstrated that there is very limited published data on the effect of culture media on IVF/ICSI success rates. Furthermore, the plethora of culture media used currently in IVF laboratories did not allow proper meta-analysis of all these data. This is an important conclusion in itself, and as such is highly relevant for the reproductive community at large. Such an essential component of IVF should be treated with the highest level of scrutiny, before it is introduced in clinical care.

The points raised in the discussion section of our systematic review (**Chapter 6**) can be used as guidelines for future research and can lead to better designed randomized trials that are urgently needed to develop evidence based laboratory practice [40]. It can be argued that, given the high number of commercially available culture media, even with addition of good designed trials a meta-analysis will not be possible. New analytical methods, such as network meta-analysis that allows for indirect treatment comparisons could potentially overcome this problem [47]. Also, the best trial that would answer this question adequately would be one comparing all available culture media simultaneously. Of course, such a trial is methodologically and practically not feasible given the high number of culture media and the fact that such trial needs to be repeated again upon introduction of any new culture media.

What is feasible though, and should actually go without saying, is conducting properly designed RCTs when new culture media are introduced into clinical care evaluating their (cost-) effectiveness and safety. Surprisingly, this is not a common practice most likely because large-scale RCTs are both time and money consuming. When it comes to

commercial companies the exact components of culture media and the concentrations used are not always revealed, prevailing further research. Moreover, if an RCT indicates equal or even lower success rates than already available media, years of research and development are wasted; thus, from a commercial perspective, an RCT carries a serious risk of harm for a company. In our view, the responsibility for proper introduction of culture media with new formulations lies with companies that should disclose the composition of each medium and should report what studies have been performed and which endpoints were analyzed to confirm effectiveness and safety. But it is not solely a responsibility of companies; embryologists should also aim to properly evaluate any new culture medium before its introduction to the clinic. In order to detect small differences in ongoing pregnancies or live births when comparing two culture media, large numbers of women are necessary. We have demonstrated that with proper collaboration of the reproductive medicine community, in this case in the Netherlands, a large, well-designed multi-center RCT can be performed. With further collaboration at an international level, and utilization of the two reproductive societies in Europe and in America (ESHRE and ASRM) inclusion of high numbers of women should not be an issue.

Molecular analysis of human preimplantation embryos

Until recently, molecular analysis of preimplantation embryos was hampered by the inability to analyze small quantities of material. Recent technological advances in mRNA amplification in combination with microarray or sequencing analysis now allow the simultaneous analysis of transcript levels of thousands of genes in a few or even in single cells [48-52]. Given that single cell analysis is an exciting new technology that yields a lot of data and opens up a totally new and undiscovered area of research on preimplantation embryos, this technology has found immediate application.

The implementation of single cell technology in the field of human reproduction has come without proper validation of the reproducibility and accuracy of the protocols used on actual single oocytes and embryos. Prior to the study presented in this thesis where we developed and validated protocols for microarray analysis of single oocytes and embryos (**Chapter 4**), only one study had validated their protocol to perform sequencing analysis of single mouse blastomeres [50].

Most data on gene expression in preimplantation embryos came from studies on mouse and bovine embryos [53-59]. In humans, few studies investigated overall gene-expression profiles in individual or pooled human preimplantation embryos [60-68]. These studies documented the transcription dynamics as the embryo progresses from the cleavage stage to the morula and blastocyst stage [62-65], the time of embryonic genome activation [62, 64, 68], the molecular mechanisms that govern differentiation of the ICM and TE cell lineages [60, 61, 67] and the heterogeneity in gene expression of embryos that could reflect their developmental competence [66].

The influence of environmental factors, such as culture media and oxygen concentration in the incubator, on embryo quality and pregnancy outcomes in an IVF program is well demonstrated in the literature and, for culture media, becomes more clear in this thesis [40, 69-70]. Several studies tried to find an explanation for these clinical observations based on altered gene expression of *in vitro* cultured animal embryos compared to their *in vivo* counterparts or when cultured in different culture media [33-35, 37, 38, 71-80]. The recognized affected molecular and cellular mechanisms involve metabolism, mitochondrial activity, oxidative stress, cellular integrity, cellular development and proliferation, cell-cell signaling and communication, apoptosis, imprinting, and protein synthesis [34, 37, 69, 76-79, 81-88]. Studies on the effect of *in vitro* culture environment on the transcriptome of human embryos were lacking. The study in this thesis (**Chapter 5**) presents the first data on the effect of culture media on gene expression of human preimplantation embryos. It was clear that HTF caused up-regulation of expression of genes related to cell death and apoptosis while culture in G5 caused up-regulation of genes involved in regulation of phosphorylation and mitosis, possibly implying that human embryos develop better in G5 compared to HTF between day four and six of development. Indeed, this assumption was substantiated by the RCT presented in this thesis (**Chapter 7**) that showed that more embryos in the G5 arm were utilized (transferred or cryopreserved) compared to the embryos in the HTF arm. Also, more embryos in the G5 group implanted compared to the HTF group.

Another factor that can influence embryo quality is maternal age. An effect of maternal age on oocyte quality is well established, where oocytes from older women have a high rate of meiotic aneuploidies [89, 90]. Maternal age has also an effect on genomic stability of human preimplantation embryos, with nondisjunction events increasing with maternal age [91-93]. At the level of the transcriptome, few studies have reported differences in gene expression between oocytes from younger and older women. For the first time, in this thesis, an effect of maternal age on gene-expression level in human embryos was shown (**Chapter 5**). In fact, the number of genes of which transcription was altered with varying maternal ages was higher than the number of genes affected by the culture environment in our experiment. The differences found in embryos of varying maternal ages involved genes associated with cell cycle and cell signaling, similar to the genes in the oocyte found to be affected by maternal age [89, 90]. In addition, genes that were affected by maternal age in embryos included many genes involved in phosphate metabolic processes.

These data, taken together with clinical data that show that pregnancy rates are decreased in women of advanced age [94], could suggest a model of how the effect of advanced maternal age works. During the first days of embryo development, the embryo is dependent on maternally inherited products from the oocyte until activation of the embryonic genome. The pool of maternally inherited products in oocytes of older women would be suboptimal, lacking mRNAs and proteins that are important for correct progression of the cell cycle. That would mean that the embryos derived from such oocytes have diminished cell cycle function and relaxed cell-cycle checkpoints. This could allow nondisjunction

events to occur and the incidence of aneuploidy (both meiotic and mitotic) to increase. Even after activation of the embryonic genome at day three, embryos from older women would have less or a different expression of genes regulating the cell cycle resulting in suboptimal development and for example a lowered potential to deal with aneuploidies in comparison to embryos from younger women. The direct link between decreased gene expression and incidence of aneuploidy, or vice versa, has yet to be confirmed.

It would be worthwhile to conduct an additional analysis on the occurrence of mitotic errors in relation to maternal age. The data reported in **Chapter 2** could potentially be used for such an analysis. Furthermore, genes involved in phosphate metabolism appear to be less active in embryos of older women as compared to embryos of younger women. Phosphate metabolism is essential for phosphorylation of many proteins, enzymes and transcriptional factors thereby turning them on and off. Embryos of older women could be diminished in this ability, disturbing further the presence of essential active proteins for embryo development. Genes associated with mitochondrial energy production and oxidative phosphorylation were also found to be less expressed in embryos of older women compared to younger women. Since embryo development requires a high amount of energy, diminished mitochondrial function could affect the rate of cell division and the correct formation of the morula and the blastocyst.

Another interesting finding is the interaction between maternal age, culture medium, oxygen concentration and developmental stage (**Chapter 5**). These interactions suggest that embryos of different maternal age or developmental stage respond differently to various culture conditions. Theoretically, this could be translated into personalized IVF/ICSI treatments where some embryos and/or the embryos of some women require different culture conditions.

In this thesis, several factors (maternal age, culture media and oxygen concentration) have been investigated for their effect on embryo quality and emphasis was put on culture media. Other factors that could influence embryo quality are culture associated factors like CO_2 levels, culture medium volume, number of embryos per drop of culture medium, pH, stability of the incubator, temperature, and embryo produced factors [95-100], but also physiological factors such as ovarian reserve and maternal body mass index (BMI) and exogenous factors such as hormonal stimulation [101-105].

All molecular work presented in this thesis, has been performed using microarray technology, which has been the method of choice for many years for gene-expression analysis. Meanwhile, RNA sequencing techniques (RNA-seq), which allow DNA fragments to be repeatedly sequenced in a very short time, have been developed and validated for whole transcriptome analysis of single cells albeit not of single human embryos or oocytes [50, 106-108]. Since microarray probes are designed on the basis of prior genomic data, microarrays potentially miss novel expressed regions while these should be detectable using RNA-seq [109]. The main advantage of microarrays over sequencing is that they

are better validated and mature analysis strategies and experimental designs have been developed [109, 110]. Additionally, microarrays currently still have relative low costs compared with sequencing [109].

How to assess embryo quality

Embryo quality can be assessed from two different perspectives. The first one is the ability of an embryo to develop to a blastocyst, to implant and to lead to a live birth, i.e. the embryo's developmental potential. The second perspective is the embryo's genomic and epigenetic integrity that is necessary to lead to a delivery of a healthy offspring with lifelong health. A proper outcome to assess embryo quality from both perspectives is thus the delivery of a healthy baby. However, in the laboratory, other tests must be used to evaluate embryo quality and assume its developmental potential. Morphological evaluation by embryologists is currently the preferred method of choice [111]. It is however hampered by the lack of parameters with perfect sensitivity and specificity, and has inter- and intra-observer variability that is inherent to any imaging technique. Embryos with the expected cell number according to their time in development, and low percentage of fragmentation are generally considered of good quality [111]. However, this evaluation is considered suboptimal as less than 20% of embryos selected for transfer implant while some embryos considered of less good quality and therefore less suitable for transfer on day 2 - 3 are able to develop to blastocysts and implant following transfer to the uterus [112]. Time-lapse imaging is an emerging tool that allows the identification of additional morphological parameters indicative of good embryo quality and may allow more accurate prediction of embryonic developmental and implantation potential. Predictive parameters include duration of the first cytokinesis, the time between the first and second cytokinesis and the time between second and third cytokinesis [113-116]. Time-lapse imaging is a non-invasive, computer-automated test and as such it could be able to overcome the above mentioned disadvantages of morphological evaluation by the embryologists. Validation of the efficacy of time-lapse imaging with clinical trials is currently underway, and is needed before it can routinely be applied into clinical practice [113]. Given the high expense of both the equipment and the disposables necessary for time-lapse imaging, a cost-effectiveness analysis would be necessary as well.

Other methods have emerged producing a comprehensive analysis of the embryo's genome, transcriptome, proteosome, epigenome and metabolome [117]. These procedures can be applied at different stages during preimplantation embryo development.

Preimplantation genetic screening (PGS) has been proposed as an alternative method to assess embryo quality at the genomic level by screening for embryos that are aneuploid. However, the clinical trials performing PGS using FISH showed that PGS not only did not increase but instead significantly reduced IVF success rates [118, 119]. New technologies that enable in depth assessment of the embryonic genome (comparative genomic hybridization arrays or single nucleotide polymorphism arrays) have recently

been developed [120-122] but whether performing PGS using these techniques results in better outcomes remains to be tested. No matter which technique is used to assess the ploidy status of an embryo, the high percentages of mitotic errors occurring during the first three cleavages (**Chapter 2**) resulting in mosaicism, especially diploid-aneuploid mosaicism, hinders the efficacy of PGS on day 3 [123]. Biopsy of an aneuploid blastomere from a diploid-aneuploid embryo will lead to the discarding of a potentially viable embryo containing normal blastomeres. Alternatively, biopsy of a diploid blastomere will lead to transfer or cryopreservation of an embryo that has one less diploid blastomere, thereby potentially hampering its developmental potential. Performing the biopsy on day 5 might reduce these risks since more blastomeres can be biopsied. Further, it is possible to remove only blastomeres from the trophectoderm leaving the inner cell mass intact. However, whether PGS on day 5 is better than no biopsy should be evaluated.

Transcriptomic analysis reveals the cell's phenotype and could yield information on how the embryo's transcriptome reflects its quality and provide candidate genes as markers of embryo quality. Assessment of proteomics and metabolomics is the next step to find biomarkers of human embryo developmental potential through investigating proteins and metabolites that are secreted by the embryo in the culture medium [124-128]. However, these methods are all still at the very early stage of development. The first randomized controlled trials on selection of embryos by metabolomic profiling as an adjunct to morphology showed no significant difference in success rates when selection of embryos by metabolomic profiling and morphology was compared to embryo selection by morphology alone [129, 130].

Some first attempts have been made to assess epigenetic regulation in the preimplantation embryo, also in regards to the effect of *in vitro* culture [131-135]. There are still important limitations with this technique that prohibits its current application as a method to assess embryo quality. Firstly, the required input does not allow for global DNA methylation and histone modification studies in single cells. Secondly, the studies have thus far investigated epigenetic regulation in relation to ART only in animal embryos and little is known about epigenetics in the human preimplantation embryo.

Very recently, the presence of both genomic and mitochondrial DNA (mtDNA) in used culture media of human embryos was demonstrated in 99% of the 800 cases examined [136]. Moreover, a positive correlation between DNA release and embryo fragmentation on days 2 and 3 of development was found [136]. An increase of mtDNA release into the culture media was also found in correlation to advance maternal age. Such results may provide a new non-invasive model to assess embryo quality.

Despite all these technological advancements, which easily excite the human brain that constantly strives for innovation, a critical note has to be made on the usefulness of any form of embryo assessment and subsequent embryo selection. Assessment of embryo quality and selection of the best embryo for transfer was deemed highly relevant when

embryo transfer policies were implemented to decrease the rate of multiple pregnancies and cryopreservation protocols of supernumerary embryos were far from optimal. In this situation the best embryo clearly had to be transferred first. Recent developments in embryo cryopreservation however, challenged the current role of embryo selection in IVF [137]. An RCT in a selected population, that is, women with a high response to ovarian hyperstimulation showed that vitrification of all embryos followed by a transfer in a subsequent non-hyperstimulated cycle resulted in significantly improved ongoing pregnancy rates compared with a fresh transfer in a cycle with ovarian hyperstimulation [138]. In a scenario where all available embryos can be cryopreserved and transferred in subsequent cycles without impairment of embryo quality, no selection method will ever lead to improved live birth rates, as, by definition, the live birth rate per stimulated IVF cycle can never be improved when all embryos are serially transferred. The only parameter that could possibly be improved by embryo selection would be time to pregnancy, if embryos with the highest implantation potential are transferred first [137]. Despite these potential limitations for the use of assays of embryo quality in a clinical setting, investigating embryo quality at the (epi) genetic, transcriptomic, proteomic and metabolomic levels could lead to interesting observations and provide ideas on how to enhance embryo quality in general.

Another assessment of embryo quality should be performed at the level of the health of the offspring born after IVF/ICSI treatment. There are currently data suggesting that culture medium can affect intrauterine growth as early as in the second trimester as well as neonatal birthweight [27-29]. Data on the effect of prenatal undernutrition on the health of the offspring born are also striking. These data showed that men and women who were conceived during the Dutch famine during the Second World War have an increased risk for coronary heart disease, diabetes, an atherosclerotic lipid profile, obesity and breast cancer compared to people that were born before or conceived after the famine [139-143]. This risk was greater in those who were exposed to Dutch famine during early gestation compared to those exposed during mid and late gestation. Moreover, a trans generational effect was found in offspring of prenatally undernourished fathers that were heavier and more obese than offspring of fathers that had not been undernourished prenatally [144]. Even though these data do not assess the effect of undernutrition directly on preimplantation embryos the fact that greater effects were found during the first trimester might be indicative that undernutrition of the early embryo by culturing it in a suboptimal culture medium could lead to similar findings. This needs to be investigated.

How to enhance embryo quality

This thesis clearly shows that the choice of culture medium can improve embryo quality. Even though this is an important finding and research on culture media should continue, there is a limit on how much culture media can improve embryo quality. For example, if the embryo derives from an old oocyte that has impaired mRNA and protein pools, has acquired meiotic errors and lacks control mechanisms, the embryo quality most probably will not fully improve just by embedding it in the best culture medium.

Investigation of the molecular machinery of good and bad quality oocytes and embryos could identify the mRNA and proteins that are necessary for successful embryo development. Theoretically this could open up ways for manipulation of the oocyte and/or embryo, where important mRNA or proteins for cell-cycle checkpoints, DNA repair and chromosome cohesion that help maintain genomic integrity could be injected into the cytoplasm of oocytes and embryos to guide embryos through the first mitotic divisions. Of course, such forms of oocyte or embryo optimization should be treated with the greatest level of scrutiny and would involve a significant amount of preclinical work to assess effectiveness and safety. Even so, being able this way to 'repair' old oocytes would overcome the greatest threat to reproductive potential, i.e. advanced maternal age.

The same could hold for mitochondria. Mitochondrial activity is important for the developing embryo since it provides the embryo with the necessary energy [145]. Following fertilization, the embryo depends on the function of existing mitochondria that were provided by the oocyte. The number of mitochondrial DNA (mtDNA) copies in metaphase II oocytes varies from 2×10^4 to 9×10^5 [146, 147]. This variability in mtDNA copy number is thought to reflect oocyte quality, with oocytes containing higher copy numbers displaying higher fertilization rates [148, 149]. A reduction in mitochondrial copy number affects further the embryonic developmental potential [150]. Moreover, uncleaved embryos in women who were older than 40 years of age showed a significantly lower mtDNA copy number compared to uncleaved embryos of women of a younger age [151]. As the cells within an embryo divide, the total amount of mitochondria within each cell decreases due to the absence of mitochondrial synthesis until genomic activation. mtDNA replication is strictly down-regulated from the fertilized oocyte through the preimplantation embryo in those mammalian species so far investigated (mouse and porcine embryos) [152]. At the blastocyst stage, the onset of mtDNA replication is specific to the trophectodermal cells. The inner cell mass cells restrict mtDNA replication until they receive the key signals to commit to specific cell types [152]. Mitochondrial mutations and subsequent reduced energy production are increased in women of advanced age [153]. Therefore, another possibility for oocyte and/or embryo optimization will be the transfer of mitochondria from healthy young oocyte donors to support the development of embryos from older women. Such transfers have already been performed in human oocytes and led to the birth of normal healthy children [154-156]. A critical point of mitochondrial transfer is the occurrence of mitochondrial heteroplasmy in the offspring where mitochondria from young donors will co-exist with the suboptimal mitochondria of older donors. Another option that avoids the use of donor oocytes is the synthetic generation of mitochondrial DNA and its subsequent microinjection into the ooplasm. Commercial gene-synthesis services are already available from numerous companies worldwide.

The concept of oocyte or embryo optimization is still in its infancy and evidence of clinical usefulness is lacking. The idea of oocyte/embryo optimization requires manipulation of the embryo and injection of molecules (genes, mRNA or proteins) that are not intrinsically present. As such it raises important ethical issues. The technology should only be used to

avoid transmission of genetic diseases and improve embryo quality to yield higher success rates in cases of subfertility.

Implications for clinical practice and further research

The results described in this thesis have significant clinical relevance. We clearly demonstrate lack of evidence-based practice when it comes to selecting the medium for *in vitro* culture of human preimplantation embryos. After 35 years of IVF there is no consensus whether culture media do have an effect on IVF/ICSI success rates and what the magnitude of such an effect is. We believe that there is enough evidence to suggest an effect of culture media on pregnancy rates and that such an important part of daily IVF practice should be treated with the highest scrutiny. Further, the introduction of new culture media in the IVF laboratory should be accompanied by properly designed studies evaluating both readily available commercial media as well as individual components. When G5 culture media was compared to HTF culture medium in a prospective randomized trial, G5 culture media resulted in better embryo development, increased clinical pregnancy rate and increased ongoing pregnancy rate (although borderline non-significant). The primary outcome for this RCT was the number of women with an ongoing pregnancy after a year of treatment. Data on the number of women who achieved a live birth where still not complete at the time of the writing of this thesis. It remains therefore to be seen what the effect of the two compared culture media on live births will be. Based on the currently available data we suggested using G5 instead of HTF.

Moreover, given the described effects of culture media on neonatal birthweight [28], it is highly recommended that every study investigating the effect of treatment interventions during IVF/ICSI treatment also provides data on the follow-up of the children born. Reproductive technologies should aim not for more children alone but for more and healthier children. For the RCT presented in this thesis, follow-up data of the children born are currently being gathered and will be presented separately in the near future. The effect of culture media even later in life should be also examined.

The results of the RCT are especially relevant for the Dutch IVF centers that have been changing their culture systems from HTF to G5 over the last years. Very recently, some of these centers have moved to Quinn's advantage (SAGE) as the culture medium of choice. In the meantime, the next multi-center RCT comparing G5 to Quinn's advantage is being set up. The Dutch reproductive community is well organized via the professional organizations of embryologists and gynecologists [society for clinical embryology (KLEM) and the Dutch association for obstetrics and gynecology (NVOG), respectively], and the research consortium for studies in women's health and reproduction (ObsGyn consortium). These forums provide a unique environment to conduct good designed, multi-center RCTs with high number of participants in a cost- and time- effective way.

The data presented in this thesis also suggest that the decision as to which culture medium to use could depend on the maternal age and developmental stage of the embryos. Such suggestion could have important implications for clinical practice leading to personalized treatment but needs further investigation before it could be implemented. A sub analysis of the RCT data presented in this thesis (**Chapter 7**) based on maternal age can be the first step to investigate this relationship. If the direction of effect between the two culture media is different in young versus old women then a new randomized trial should be performed. The results of an RCT in a given population (e.g. women below 35 years of age with a medical indication) are often extrapolated to other populations (e.g. older women or women with unexplained infertility). Such conclusions are not substantiated and might even harm women undergoing IVF/ICSI treatments.

From a research perspective, this thesis brings forward new interesting research questions. In regards to culture media, the clinical trial provided a unique randomized source of materials, in this case supernumerary frozen embryos and placentas, that enables further research on the effect of culture media on the gene-expression profile of these embryos and placentas. Many placentas from the pregnancies derived from the RCT have been collected and the effect of G5 and HTF culture media on expression, methylation, and maternal/paternal expression of specific imprinted genes will be investigated. This research is relevant as the placenta is important for proper development of the fetus in the uterus.

Supernumerary embryos that were cultured during the RCT in G5 or HTF in the IVF center of Maastricht University Medical Center have also been collected to investigate the effect of culture media on embryonic gene expression. This study resembles the study described in this thesis (**Chapter 5**) but utilizes embryos of supposedly lower quality, but with the advantage that they have been cultured in the experimental conditions from day one onwards. Moreover, supernumerary embryos cultured in two different oxygen concentrations (20% and 5%) were collected in the IVF center of University Medical Center Groningen and they will be analyzed in a similar design. Comparing these datasets will give more insight into the regulation of embryonic transcription and could establish an overall embryonic behavior, irrespective of embryo quality or duration of culture. It could also be that differences in the results are found, indicating that good quality embryos are better equipped to culture in suboptimal conditions compared to embryos of lower quality. All of these studies are underway.

The epigenetic regulation in preimplantation embryos cultured in different media should be studied especially since this thesis suggests an environmental role on early embryo quality. For this purpose, supernumerary human preimplantation embryos that reach the blastocyst stage in different culture media can be collected to allow investigation of the effect of culture on epigenetic regulation. This effect can be studied in blastomeres of arrested or normally developed embryos and in TE and ICM samples. Such study is currently challenged by the above mentioned technical limitations. Also, analysis of single

or few blastomeres might not be representative of the entire embryo, especially since human embryos have a high rate of mosaicism as described in this thesis.

As mitotic errors were found most likely to occur during the first three cleavages, more research is required on early cleavage embryos to assess the mechanisms causing these errors. Also, the role of suboptimal environmental conditions or increased maternal age in those early developmental stages needs further investigation. For example, do culture conditions (embryo culture medium or oxygen concentration) also affect the first three cleavage divisions? If the embryos of earlier developmental stages (day 1 - 4) are more vulnerable to their environment one could argue that use of suboptimal culture conditions could aggregate the problem. Another possibility could be that embryos of women of advanced age that have already a disadvantage from compromised maternal mRNA and protein pool acquire additional disadvantage from growing in suboptimal culture conditions during these first crucial days of development before the embryonic genome gets activated. To investigate the mechanisms leading to mitotic errors during the first cleavages, supernumerary human embryos cryopreserved in the zygote stage or 3PN embryos could be analyzed for their chromosomal content using array CGH. Embryos could also be created for this kind of research, using donated gametes. However, this is currently in the Netherlands not allowed by law. If all blastomeres of a given embryo are analyzed the causative events of the aneuploidies found can be assessed (e.g. monosomies and trisomies of the same chromosome within one embryo are indicative of nondisjunction events). Similar experiments have been performed for later developmental stages mainly using FISH [157-159]. If this approach is repeated, using more advanced and accurate analysis techniques, with embryos of women of different ages and in different culture conditions, the specific mechanisms and relationships can be further investigated. Such experiments are hampered by the limited availability of human embryos. Moreover, transcription analysis of blastomeres from early cleavage embryos will shed more light into which molecular mechanisms cause these events and whether they are similar to the mechanisms found in the later developmental stages. Finally, a simultaneous analysis of the genome and transcriptome of a given cell will provide insight into the correlation of ploidy status and transcription. This is however not yet technically possible in single cells.

The data on gene expression of human preimplantation embryos presented in this thesis are a valuable addition to the few available studies investigating gene-expression changes at different developmental stages [62-65]. For the first time, data on gene-expression changes in regards to maternal age and culture conditions were described for human embryos. Given the limited availability of human preimplantation embryos, scientists working on reproductive biology have to work with a limited, heterogeneous embryo population that does not always allow matching for maternal age, fertilization method, developmental stage and culture conditions. This heterogeneity is difficult to avoid, but our results showed that strong interactions exist among these factors, stressing the importance of correction for confounders in such analyses. With this in mind, the findings of previous studies evaluating gene expression of human preimplantation embryos should

be re-assessed. Studies on single embryos are in this way different from studies analyzing expression changes in an experimental setting in, for example, cell lines, where all other factors can be accounted and controlled for.

Our data on gene-expression changes in regards to maternal age and culture conditions are suggestive of new and interesting questions for further research. For example, what are the specific transcripts and proteins that are necessary for proper embryo development? Should we provide oocytes/embryos from older women with these proteins to improve IVF/ICSI success rates? Would that really result in improved success rates? Should the intervention target the oocyte, the embryo or both? In regards to culture media, is there a correlation between the components of the media and gene expression? If this correlation exists, could we drive gene expression just by altering the composition of the culture medium? What culture conditions are better for women of older ages? Further, since culture media affect apoptosis, what is the effect of culture media in number of cells in the two cell lineages at the blastocyst level? Would changes in cell number affect the proper formation of these lineages? And how do culture media affect cell fate gene expression and cell lineage allocation?

To answer these questions, the specific genes affected per maternal age and culture conditions should be further investigated. The transcriptome data that are published on human embryos of different developmental stages could be re-analyzed in regards to maternal age and/or culture conditions to identify genes that are commonly found affected irrespective of study population (women of different ethnic backgrounds and subfertility indication), materials (supernumerary embryos, 3PN embryos, zygotes, good quality embryos) and techniques applied (microarrays and microarray platforms and sequencing). When a shorter list of candidate genes is identified, further in depth experiments can be performed on interesting genes and pathways. Such experiments could involve knockout or over expression studies using animal models or perhaps even human embryo models (as long as it does not involve transfer to the uterus) with outcomes such as embryo quality, embryo development, and for the experiments with animal models implantation and generation of healthy offspring. Alternatively, mRNA for the candidate genes could be microinjected into the cytoplasm of oocytes or early zygotes from women of advanced age to check whether the resulting embryo will develop similarly to embryos of younger women (for example in regards to the number of mitotic errors) especially during the first days of development. Moreover, human embryos can be cultured using culture media other than HTF and G5, different pH values of the culture media and different temperatures of the incubator. At the end of the culture, PCR analysis of the candidate genes could help in deciding the general conditions that are best for embryo culture.

Eventually, by combining the best culture conditions and the right interventions to 'correct' or 'avoid' the effects of maternal age on oocyte and embryo competence, better success rates during IVF/ICSI could be obtained.

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CHAPTER 9

Summary
Samenvatting
Περίληψη

Summary

Preimplantation embryo development is biologically a very interesting process since many events happen in a short period of time. It is known through IVF that embryo quality varies among embryos and that embryo quality affects clinical outcomes including the success rates of an IVF treatment. It is therefore important to study embryo quality (**Chapter 1**).

One of the intriguing findings in recent years is the frequent occurrence of mitotic errors in human preimplantation embryos [1]. It was not known though which preimplantation stages and divisions are most prone to such errors since the first three days of embryo development normally can not be analyzed. To be able to answer this question, we used mitotically stable digynic tripronuclear human embryos as a model system that gave us the possibility to analyze human preimplantation embryos from the first day of development onwards (**Chapter 2**). All cells of 114 digynic tripronuclear human preimplantation embryos were analyzed by fluorescence *in situ* hybridization for chromosomes 1, 13, 16, 17, 18, 21, X and Y. Embryos were grouped according to day of development (Day 1 - 6) and developmental stage (2-cell to blastocyst stage). We found that the possibility of a mitotic error was highest during the first and second mitotic division. The percentage of cells with mitotic errors therefore increased during early preimplantation development and was highest at the 8 - 16 cell stage (76%). Thereafter the percentage of cells with mitotic errors decreased to 64% at the morula and to 56% at the blastocyst stage. This pattern correlates with the activation of the embryonic genome at the 8 - 16 cell stage. A better insight in the timing of occurrence of mitotic errors in human preimplantation embryos could help in understanding and preventing these errors and is relevant in the context of preimplantation genetic screening.

To understand the mechanisms causing these mitotic errors and the fate of abnormal cells within an embryo, a review was performed (**Chapter 3**). The review showed that during the first mitotic divisions in the human important mechanisms that are required for the regulation of genomic integrity, such as cell-cycle checkpoints, cell arrest and apoptosis, were relaxed or absent thereby potentially increasing the occurrence of mitotic aneuploidies. Three main hypotheses about the fate of aneuploid cells within an embryo (cell arrest and apoptosis, active correction of the aneuploidies and preferential allocation of the aneuploid cells to the extra-embryonic tissues) were discussed and data favouring or opposing those hypotheses were presented. Finally, this review discussed various aspects of IVF/ICSI treatment that have been associated with increased occurrence of mitotic aneuploidies, including ovarian stimulation, the use of suboptimal gametes and culture conditions. Understanding the mechanisms that cause mitotic aneuploidies in human preimplantation embryos and the way human preimplantation embryos deal with these aneuploidies might lead to ways to limit their occurrence, in order to ultimately increase the quality of embryos and with that the likelihood of a successful pregnancy in IVF/ICSI.

The review on the mechanisms causing mitotic errors in human preimplantation embryos provided significant insight into the events that regulate early development but it also showed that there are many more unanswered questions. Further analysis on the molecular events that govern human preimplantation embryo development appeared necessary. To be able to do so, we developed and validated a protocol that allowed us to perform gene-expression analysis from very small quantities of materials. We demonstrated that our method was very robust and reliable in determining the transcriptome of single human oocytes (**Chapter 4**).

We then proceeded to use this method to determine genome-wide gene expression of human preimplantation embryos (**Chapter 5**). Since embryos have different backgrounds (embryo quality, developmental stage and biological variation), intrinsic variation in gene expression is to be expected. It is therefore important to take this variation into account when designing a study on the transcriptome of single embryos, and before any conclusions in regards to gene expression can be drawn. We controlled for this variation using a design where human preimplantation embryos where randomized and stratified according to their biological background (maternal age and developmental stage) and environmental variables (culture medium and oxygen concentration). Eighty-nine day 4 human preimplantation embryos were randomized to two culture media (G5 medium or HTF medium) and to two oxygen concentrations (5% or 20%), with stratification for maternal age. Next to these variables, developmental stage after culture was also taken into account in gene-expression analysis. After thawing, the embryos were cultured for two days under the randomized conditions. Only embryos developing to morula or blastocyst stage (41/89, 46%) were assessed for genome-wide gene expression using the developed and validated protocol described in **Chapter 4**. Based on the number of differentially expressed genes (DEGs), developmental stage (1,532 DEGs) and maternal age (368 DEGs) had a larger effect on the gene-expression profile of human preimplantation embryos than the oxygen concentration (183 DEGs) or the culture medium (174 DEGs). Interactions between all factors were found, indicating that culture conditions might have a different effect depending on the developmental stage or the maternal age of the embryos. Pathways affected by the studied factors included metabolism, cell cycle processes and oxidative phosphorylation. Embryos though, were cultured for only two days and this might have limited the effect on gene expression by the investigated culture conditions. Earlier stages of development (day 1 until day 4) were not analyzed and might respond differently to the experimental conditions.

The above described effects of culture conditions are very relevant when considering embryo quality. The effect of oxygen concentration on embryo quality and pregnancy rates was already shown, with low oxygen concentration (5%) accounting for better results [2]. For culture media, the effect on embryo quality and pregnancy rates was less clear. Thus, we performed a systematic review to investigate which culture medium was the best, leading to best embryos and IVF outcomes (**Chapter 6**). For this review all randomized controlled trials (RCTs) published between January 1985 and July 2012 were eligible for inclusion.

Primary outcome was live birth. Secondary outcomes were health of babies born, ongoing pregnancies, clinical pregnancies, miscarriages, multiple pregnancies, implantation rate, cryopreservation rate, embryo quality and fertilization rate. For those media that were evaluated in more than one comparison, an unconventional meta-analysis was performed by pooling the data of the media they were compared to. Twenty-two RCTs were included that evaluated thirty-one different comparisons. Conventional meta-analysis was not possible for any of the outcomes as nearly all trials compared different culture media. Only four trials reported on live birth, one of them reported a significant difference. Nine trials reported on ongoing and/or clinical pregnancy rates, of which four showed a significant difference. Pooling the data did not reveal a superior culture medium. It appeared to be unknown what culture medium would lead to the best success rates in IVF/ICSI. Given the potential importance of culture media for treatment outcome, it was concluded there was a need for rigorously designed randomized controlled trials on currently available, as well as newly introduced culture media.

At the time this review was performed, many IVF centers in the Netherlands changed their culture medium from HTF to G5 based on apparently good results with G5 media in some centers. In order to properly investigate whether this change was justifiable, six IVF centers in the Netherlands cooperated and performed a multi-center, randomized, controlled trial to determine which of the two culture media would result in better quality embryos and improved success rates (**Chapter 7**). Between September 2010 and May 2012, a total of 836 couples (417 in the G5 group and 419 in the HTF group) were included in this trial. All couples who underwent an IVF/ICSI treatment at one of the participating centers and their affiliated clinics for their first, or first after a successful pregnancy, cycle were eligible to participate in this study. Randomization was performed using an online computer program. Participating couples, attending gynecologists and investigators were blinded as to the allocated treatment. At the time of writing of this thesis, the data on live birth rate were not yet available, but more women had an ongoing pregnancy in the G5 group compared to the HTF group but the difference was not significant [45% (189/417) vs. 39% (163/419); rate ratio (RR): 1.2; 95% confidence interval (CI): 0.99 to 1.38; P=0.07]. Other outcomes such as clinical pregnancy, number of utilizable embryos, number of embryos implanted after a fresh transfer and the number of cryopreserved embryos available for transfer at a later date at the end of the study all significantly favored G5. Based on these aggregate findings, we recommended the use of G5 for culture of human embryos during IVF/ICSI treatments over HTF.

The work described in this thesis provided new insights into the biology of human preimplantation embryo development, as well as into ways of optimizing human embryo culture, which directly resulted in better evidence based laboratory practice. The connection and synergetic effect between a fundamental understanding of human preimplantation embryo development, new ideas for clinical practice, and evaluation of such ideas using clinical evaluation research tools is evident. Clinical practice is improved via RCTs, but in

order to get ideas for improving clinical practice and also to be able to explain the results obtained, biological research is necessary. The difference in pregnancy outcomes in the clinical trial we conducted was explained by the observed (morphological) difference in embryo quality between HTF and G5, which in turn was clearly reflected by the gene-expression patterns we found in our culture experiments (**Chapter 8**).

References

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Samenvatting

De ontwikkeling van preïmplantatie embryo´s is zeer interessant, omdat er in korte tijd veel biologische processen plaatsvinden. Door het gebruik van IVF is bekend geworden dat de kwaliteit tussen embryo's varieert en dat de kwaliteit van het embryo invloed heeft op de klinische uitkomsten, zoals de kans van slagen na een IVF-behandeling. Om deze reden is het van belang de kwaliteit van het embryo te bestuderen (**Hoofdstuk 1**).

Een van de meest intrigerende bevindingen van de afgelopen jaren is het veelvuldig voorkomen van mitotische fouten, leidend tot numerieke chromosoomafwijkingen, in humane preïmplantatie embryo's [1]. Drie kwart van de embryo´s in een IVF laboratorium heeft een afwijkend aantal chromosomen in één of meerdere cellen [1]. Het was echter onbekend welk preïmplantatie stadium en welke deling het meest gevoelig voor dergelijke fouten is, omdat de eerste drie dagen van de embryonale ontwikkeling normaal gesproken niet geanalyseerd kunnen worden. Door gebruik te maken van mitotisch stabiele humane embryo's met drie voorkernen (3PN) als modelsysteem hebben we deze vraag kunnen beantwoorden. Dit model gaf de mogelijkheid om humane preïmplantatie embryo's te analyseren vanaf de eerste dag van de ontwikkeling (**Hoofdstuk 2**). Alle cellen van 114 3PN humane preïmplantatie embryo's werden geanalyseerd met behulp van fluorescentie *in situ* hybridisatie voor de chromosomen 1 , 13 , 16 , 17 , 18 , 21, X en Y. De embryo's werden gegroepeerd op basis van de dag van ontwikkeling (dag 1 - 6) en het ontwikkelingsstadium (2-cellig tot blastocyste stadium). De kans op een mitotische fout bleek het grootst tijdens de eerste en tweede mitotische deling na bevruchting. Het percentage cellen met mitotische fouten nam daardoor toe tijdens de vroege preïmplantatie ontwikkeling en was het hoogst in het 8 - 16 cellig stadium (76%). Vervolgens nam het percentage cellen met mitotische fouten af tot 64% in het morula stadium en tot 56% in het blastocyste stadium. Dit patroon komt overeen met de activatie van het embryonale genoom tijdens het 8 - 16-cellig stadium. Een beter inzicht in het moment waarop mitotische fouten ontstaan in humane preïmplantatie embryo's zou kunnen helpen bij het begrijpen en voorkomen van deze fouten en is relevant in het kader van preïmplantatie genetische screening.

Om de mechanismen die tot deze mitotische fouten leiden en het lot van deze afwijkende cellen in een embryo te begrijpen, werd een literatuuronderzoek verricht (**Hoofdstuk 3**). Uit dit onderzoek bleek dat gedurende de eerste mitotische delingen belangrijke mechanismen die nodig zijn voor de regulering van de genomische integriteit, zoals cel-cyclus controle mechanismen, verminderd aanwezig waren, waardoor mitotische fouten kunnen optreden. De drie voornaamste hypothesen over het lot van cellen met numerieke chromosoomafwijkingen in een embryo (blokkade in celdeling en apoptose, actieve correctie van de numerieke chromosoomafwijkingen en preferentiële toewijzing van de cellen met numerieke chromosoomafwijkingen naar de extra-embryonale weefsels) werden besproken en data die deze hypothesen bevestigden of tegenspraken werden gepresenteerd. Ten slotte werden in dit literatuuroverzicht verschillende aspecten van de IVF/ICSI behandeling die geassocieerd worden met een toename van het aantal

numerieke chromosoomafwijkingen in preïmplantatie embryo´s (ovariële stimulatie, gebruik van suboptimale gameten of kweekomstandigheden) bediscussieerd. Inzicht in de mechanismen die leiden tot mitotische fouten in humane preïmplantatie embryo's en de manier waarop humane preïmplantatie embryo's met deze afwijkingen omgaan, leidt tot mogelijkheden die het optreden hiervan beperken, met als uiteindelijk doel de kwaliteit van embryo's te verbeteren en daarmee de kans op een succesvolle zwangerschap na IVF/ICSI te verhogen.

Het literatuuroverzicht over de mechanismen die leiden tot mitotische afwijkingen in humane preïmplantatie embryo's gaf inzicht in de processen die de vroege ontwikkeling reguleren, maar toonde ook aan dat er veel onbeantwoorde vragen zijn. Nader onderzoek naar de moleculaire gebeurtenissen tijdens de humane preïmplantatie embryo ontwikkeling bleek nodig. Om dit te faciliteren, hebben we een protocol ontwikkeld en gevalideerd dat het uitvoeren van genexpressie analyse van zeer kleine hoeveelheden materiaal mogelijk maakt. We toonden aan dat onze methode zeer robuust en betrouwbaar is voor het bepalen van het transcriptoom van individuele humane eicellen (**Hoofdstuk 4**).

Vervolgens hebben we deze methode gebruikt om het transcriptoom van humane preïmplantatie embryo's in verschillende condities te onderzoeken (**Hoofdstuk 5**). Gezien de intrinsieke verschillen tussen embryo´s (verschil in embryokwaliteit, ontwikkelingsstadium en biologische variatie) is variatie in genexpressie te verwachten. Het is belangrijk om met deze variatie rekening te houden bij het ontwerpen van een studie naar het transcriptoom van individuele embryo's. Wij hebben dit gedaan door een gerandomiseerde opzet te gebruiken, waarbij rekening gehouden werd met de biologische achtergrond van de embryo´s (maternale leeftijd en het ontwikkelingsstadium) en met omgevingsvariabelen (kweekmedium en zuurstofconcentratie). Negenentachtig dag 4 ingevroren humane preïmplantatie embryo's werden gerandomiseerd tussen twee kweekmedia (G5 medium of HTF medium) en twee zuurstofconcentraties (5% of 20%), met stratificatie voor de maternale leeftijd. Naast deze variabelen werd in de genexpressie analyse ook rekening gehouden met het ontwikkelingsstadium na de kweek. De embryo's werden na ontdooien namelijk gedurende twee dagen gekweekt onder de gerandomiseerde condities. Alleen de embryo's die zich ontwikkelden tot het morula stadium of blastocyste stadium (41/89, 46%) werden beoordeeld op genoombrede gen-expressie middels het opgezette en gevalideerde protocol beschreven in **Hoofdstuk 4**. Op basis van het aantal differentieel tot expressie gebrachte genen (DEGs) bleken het ontwikkelingsstadium (1,532 DEGs) en de maternale leeftijd (368 DEGs) een groter effect op het genexpressieprofiel van de humane preimplantatie embryo's te hebben dan de zuurstofconcentratie (183 DEGs) of het kweekmedium (174 DEGs). Tussen alle factoren werd een interactie gevonden. Dit zou kunnen betekenen dat, afhankelijk van het ontwikkelingsstadium of de maternale leeftijd van het embryo, de kweekomstandigheden een ander effect zouden kunnen hebben. Genen die werden beïnvloed door de onderzochte factoren waren betrokken bij metabolisme, cel cyclus processen en oxidatieve fosforylering. De embryo's werden slechts twee dagen gekweekt en dit zou mogelijk een beperkende factor kunnen zijn op

het effect van onderzochte kweekomstandigheden op de genexpressie. Eerdere stadia van ontwikkeling (dag 1 tot dag 4) werden niet in deze studie geanalyseerd en zouden anders kunnen reageren op de experimentele omstandigheden.

De hierboven beschreven effecten van de kweekomstandigheden zijn erg relevant voor de embryokwaliteit. Het effect van de zuurstofconcentratie op de embryokwaliteit en daarmee het percentage zwangerschappen na IVF werd reeds eerder aangetoond, waarbij een lage zuurstofconcentratie (5%) leidde tot betere resultaten [2]. Voor kweekmedia was het effect op embryo kwaliteit en zwangerschap minder duidelijk. Om te onderzoeken welk kweekmedium het beste is, in de zin van betere embryo kwaliteit en IVF uitkomsten, hebben we een systematische analyse van de literatuur uitgevoerd (**Hoofdstuk 6**). Voor deze analyse kwamen alle gerandomiseerde gecontroleerde studies (RCTs) gepubliceerd tussen januari 1985 en juli 2012 in aanmerking. De primaire uitkomstmaat was een levendgeborene. De secundaire uitkomstmaten waren de gezondheid van deze levendgeborenen, doorgaande zwangerschappen, klinische zwangerschappen, miskramen, meerlingzwangerschappen, implantatiegraad, cryopreservatiegraad, kwaliteit van het embryo en fertilisatiegraad. Indien er sprake was van evaluatie van een medium in meerdere vergelijkingen dan werd een onconventionele meta-analyse uitgevoerd door het samenvoegen van de data van het betreffende medium en het samenvoegen van alle data van de andere media waartegen het werd uitgezet. Tweeëntwintig RCT's werden geïncludeerd waarbij eenendertig verschillende vergelijkingen waren geëvalueerd. Het uitvoeren van een conventionele meta–analyse was voor geen van de uitkomstmaten mogelijk, omdat bijna alle studies verschillende kweekmedia vergeleken. Slechts vier studies rapporteerden over levendgeborenen, waarvan één studie een significant verschil vond. Negen studies rapporteerden over doorgaande en/of klinische zwangerschappen, waarvan er vier studies een significant verschil toonden. Het samenvoegen van de data leidde niet tot het onthullen van een superieur kweekmedium. Het bleef onbekend welk kweekmedium tot de beste succes kansen zou leiden na IVF. Gezien het potentiële belang van kweekmedia voor de behandelingsuitkomsten werd geconcludeerd dat er behoefte was aan rigoureus opgezette gerandomiseerde studies, zowel voor kweekmedia die reeds beschikbaar zijn als voor kweekmedia die nieuw geïntroduceerd worden.

Op het moment dat dit literatuuroverzicht werd uitgevoerd veranderden veel Nederlandse IVF-centra hun kweekmedium van HTF naar G5, gebaseerd op goede resultaten met G5 in sommige centra. Om netjes en zorgvuldig te onderzoeken of deze verandering gerechtvaardigd was, werd er een gerandomiseerde multicenter studie uitgevoerd om te bepalen welk van de twee kweekmedia zou resulteren in betere kwaliteit embryo's en betere succes kansen na IVF (**Hoofdstuk 7**). Aan deze studie deden zes Nederlandse IVF-centra mee. Tussen september 2010 en mei 2012 werden in totaal 836 koppels (417 in de G5-groep en 419 in de HTF-groep) geïncludeerd in deze studie. Alle koppels die voor hun eerste behandelingscyclus, of eerste behandelingscyclus na een succesvolle zwangerschap, een IVF/ICSI behandeling ondergingen in één van de deelnemende centra of in de bijbehorende IVF-transportklinieken, kwamen in aanmerking voor

deelname aan deze studie. Randomisatie werd uitgevoerd met behulp van een online computerprogramma. Deelnemende koppels, gynaecologen en onderzoekers waren geblindeerd voor de toegewezen behandeling. Op het moment van schrijven van dit proefschrift waren de gegevens van de levengeborenen nog niet beschikbaar. Wel hadden meer vrouwen een doorgaande zwangerschap in de G5 groep dan in de HTF groep, al was dit verschil niet significant [45% (189/417) versus 39% (163/419); rate ratio (RR): 1,2; 95% betrouwbaarheidsinterval (CI): 0,99-1,38; P=0.07]. Andere uitkomsten zoals klinische zwangerschap, het aantal bruikbare embryo's, het aantal geïmplanteerde embryo's na een verse terugplaatsing en het aantal ingevroren embryo's voor terugplaatsing waren allemaal significant in het voordeel van G5. Op basis van deze geaggregeerde bevindingen raadden wij het aan om het G5 medium te gebruiken voor de kweek van humane embryo's tijdens IVF behandelingen.

Het beschreven werk in dit proefschrift verschaft nieuwe inzichten in de biologie van de humane preïmplantatie embryo-ontwikkeling alsmede in de optimalisering van de humane embryo kweek, hetgeen direct leidt tot betere ´evidence based´ praktijk in het laboratorium. Het verbindende en synergetische effect tussen een fundamenteel begrip van de humane preïmplantatie embryo ontwikkeling, nieuwe ideeën voor de klinische praktijk en evaluatie van dergelijke ideeën met behulp van klinisch evaluatieonderzoek is evident. De klinische praktijk wordt verbeterd met behulp van RCTs, maar zowel voor het creëren van nieuwe ideeën om de klinische praktijk te verbeteren als voor het kunnen verklaren van de resultaten, is basaal biologisch onderzoek noodzakelijk. Een goed voorbeeld hiervan in dit proefschrift is dat het verschil in zwangerschapsuitkomsten in de klinische studie kon worden verklaard door het verschil in embryokwaliteit (morfologie) tussen HTF en G5, wat overeen kwam met de genexpressie patronen zoals die in het kweek-experiment gevonden werden (**Hoofdstuk 8**).

Referenties

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Περίληψη

Η προ-εμφυτευτική ανάπτυξη του εμβρύου είναι μια πολύ ενδιαφέρουσα, από βιολογικής άποψης, περίοδος, καθώς πραγματοποιούνται πολλές διαδικασίες απαραίτητες για την άρτια ανάπτυξη του εμβρύου και μάλιστα σε σύντομο χρονικό διάστημα. Με την ανάπτυξη της υποβοηθούμενης αναπαραγωγής, έγινε γνωστό ότι η ποιότητα των εμβρύων διαφέρει από έμβρυο σε έμβρυο και μπορεί να επηρεάσει τα κλινικά αποτελέσματα, όπως τα ποσοστά επιτυχίας μετά από μια προσπάθεια εξωσωματικής γονιμοποίησης (IVF) ή ενδοωαριακής έγχυσης σπερματοζωαρίων (ICSI) (**Κεφάλαιο 1**).

Ένα από τα πιο συναρπαστικά ευρήματα τα τελευταία χρόνια είναι η συχνή εμφάνιση μιτωτικών σφαλμάτων (που επηρεάζουν τον φυσιολογικό αριθμό χρωμοσωμάτων) στα ανθρώπινα προ-εμφυτευτικά έμβρυα [1]. Αυτό που παρέμενε άγνωστο είναι ποια προεμφυτευτικά στάδια και ποιες κυτταρικές διαιρέσεις είναι πιο επιρρεπείς σε τέτοια σφάλματα επειδή κατά κανόνα οι πρώτες τρεις μέρες της προ-εμφυτευτικής ανάπτυξης του εμβρύου δε μπορούν να μελετηθούν. Για να μπορέσουμε να απαντήσουμε σε αυτό το ερώτημα, χρησιμοποιήσαμε μιτωτικά σταθερά, τριπύρηνα ανθρώπινα έμβρυα ως πειραματικό μοντέλο, κάτι που μας έδωσε την δυνατότητα να αναλύσουμε ανθρώπινα προ-εμφυτευτικά έμβρυα από την πρώτη κιόλας μέρα της προ-εμφυτευτικής τους ανάπτυξης (**Κεφάλαιο 2**). Όλα τα κύτταρα από 114 τριπύρηνα ανθρώπινα έμβρυα αναλύθηκαν με φθορίζουσα *in situ* υβριδοποίηση (fluorescence *in situ* hybridization, FISH) για τα χρωμοσώματα 1, 13, 16, 17, 18, 21, X και Y. Τα έμβρυα ομαδοποιήθηκαν ανάλογα με την ημέρα (μέρα 1 - 6) και το στάδιο ανάπτυξής τους (στάδιο 2 κυττάρων μέχρι το στάδιο της βλαστοκύστης). Τα αποτελέσματα μας έδειξαν ότι η πιθανότητα να συμβεί ένα μιτωτικό σφάλμα ήταν πιο υψηλή στην πρώτη και δεύτερη κυτταρική διαίρεση. Κατά συνέπεια, το ποσοστό των κυττάρων με μιτωτικά σφάλματα ήταν αυξημένο στα πρώτα στάδια της προ-εμφυτευτικής ανάπτυξης, φτάνοντας το υψηλότερο ποσοστό (76%) στο στάδιο των 8 - 16 κυττάρων. Στη συνέχεια, το ποσοστό των κυττάρων με μιτωτικά σφάλματα μειώθηκε στο 64% στο στάδιο του μοριδίου και στο 56% στο στάδιο της βλαστοκύστης. Αυτό το μοτίβο σχετίζετε με την ενεργοποίηση του εμβρυϊκού γονιδιώματος στο στάδιο των 8 - 16 κυττάρων. Μια καλύτερη εικόνα στη χρονική περίοδο εμφάνισης μιτωτικών σφαλμάτων στα ανθρώπινα προ-εμφυτευτικά έμβρυα θα μπορούσε να βοηθήσει στην κατανόηση και πρόβλεψη τέτοιων σφαλμάτων και είναι σημαντική στο πλαίσιο του προ-εμφυτευτικού γεννητικού ελέγχου.

Για να κατανοήσουμε τους μηχανισμούς που προκαλούν αυτά τα σφάλματα και την τύχη των ανώμαλων κυττάρων σε ένα έμβρυο, πραγματοποιήσαμε μια ανασκόπηση της βιβλιογραφίας (**Κεφάλαιο 3**). Η ανασκόπηση αυτή έδειξε ότι κατά τις πρώτες κυτταρικές διαιρέσεις στον άνθρωπο, σημαντικοί μηχανισμοί που είναι απαραίτητοι για τον έλεγχο της γονιδιωματικής ακεραιότητας, όπως ο έλεγχος του κυτταρικού κύκλου και η κυτταρική σύλληψη και απόπτωση, ήταν πιο χαλαροί ή απουσίαζαν αυξάνοντας έτσι ενδεχομένως την εμφάνιση μιτωτικών ανευπλοειδιών. Επιπλέον, παρουσιάστηκαν τρεις βασικές υποθέσεις για την τύχη των ανευπλοειδικών κυττάρων εντός των εμβρύων (κυτταρική σύλληψη

και απόπτωση, ενεργή διόρθωση των ανευπλοειδιών και η υπό πρόθεση κατανομή των ανευπλοειδικών κυττάρων στους εξωεμβρυϊκούς ιστούς) και δεδομένα που ενισχύουν ή απορρίπτουν αυτές τις υποθέσεις. Τέλος, στην ανασκόπηση αυτή συζητήθηκαν διάφορες διαδικασίες της εξωσωματικής γονιμοποίησης που έχουν συσχετισθεί με αυξημένη εμφάνιση μιτωτικών ανευπλοειδιών, όπως η διέγερση των ωοθηκών, η χρήση γαμετών μη βέλτιστης ποιότητας και οι συνθήκες καλλιέργειας των εμβρύων. Η κατανόηση των μηχανισμών που προκαλούν μιτωτικές ανευπλοειδίες στα ανθρώπινα προ-εμφυτευικά έμβρυα και ο τρόπος με τον οποίον τα έμβρυα αντιμετωπίζουν αυτές τις ανευπλοειδίες ίσως οδηγήσει σε τρόπους που περιορίζουν την εμφάνιση τους, προκειμένου τελικά να αυξηθεί η ποιότητα των εμβρύων και έτσι η πιθανότητα για μια επιτυχημένη εγκυμοσύνη μετά απο εξωσωματική γονιμοποίηση.

Η ανασκόπηση της βιβλιογραφίας για τους μηχανισμούς που προκαλούν μιτωτικά σφάλματα στα ανθρώπινα προ-εμφυτευτικά έμβρυα παρείχε σημαντικές πληροφορίες για τις διαδικασίες που ελέχγουν την πρώιμη εμβρυϊκή ανάπτυξη αλλά έδειξε επίσης οτι υπάρχουν ακόμα πολύ περισσότερα αναπάντητα ερωτήματα. Η περαιτέρω ανάλυση των μοριακών διαδικασιών που διέπουν την ανάπτυξη των προ-εμφυτευτικών εμβρύων κρίθηκε αναγκαία. Για να μπορέσουμε να μελετήσουμε αυτές τις διαδικασίες, αναπτύξαμε και επικυρώσαμε μια μέθοδο που μας επέτρεψε να αναλύσουμε την έκφραση των γονιδίων από πολύ μικρές ποσότητες γονιδιωματικού υλικού. Δείξαμε οτι η μέθοδος μας ήταν πολύ αξιόπιστη για τον καθορισμό της γονιδιακής έκφρασης από μεμονωμένα ανθρώπινα ωάρια (**Κεφάλαιο 4**).

Στη συνέχεια χρησιμοποιήσαμε αυτή τη μέθοδο για να καθορίσουμε την γονιδιακή έκφραση ολόκληρου του γονιδιώματος στα ανθρώπινα προ-εμφυτευτικά έμβρυα (**Κεφάλαιο 5**). Δεδομένου οτι τα έμβρυα έχουν διαφορετικό υπόβαθρο (ποιότητα του εμβρύου, αναπτυξιακό στάδιο και βιολογικό υπόβαθρο), η ύπαρξη εγγενής διακύμανσης στην έκφραση γονιδίων είναι αναμενόμενη. Επομένως, κατά τον σχεδιασμό μιας μελέτης για την γονιδιωματική έκφρασης σε μεμονωμένα έμβρυα, και πριν εξαχθούν οποιαδήποτε συμπεράσματα για την έκφραση των γονιδίων, είναι σημαντικό να ληφθεί υπόψιν αυτή η διακύμανση. Αυτή η διακύμανση ελέγθηκε με τον σχεδιασμό μιας μελέτης όπου τα ανθρώπινα προ-εμφυτευτικά έμβρυα κατανεμήθηκαν τυχαία ανάμεσα στις πειραματικές ομάδες και ομαδοποιήθηκαν με βάση το βιολογικό τους υπόβαθρο (ηλικία της μητέρας και αναπτυξιακό στάδιο) και περιβαλλοντικές μεταβλητές (μέσο καλλιέργειας και συγκέντρωση οξυγόνου στον επωαστικό θάλαμο). Ογδόντα εννιά ανθρώπινα προ-εμφυτευτικά έμβρυα που είχαν καταψυχθεί την τέταρτη μέρα καλλιέργειας κατανεμήθηκαν τυχαία για να καλλιεργηθούν σε δυο μέσα καλλιέργειας (G5 και HTF) και σε δύο συγκεντρώσεις οξυγόνου (5% ή 20%), αφού ομαδοποιήθηκαν με βάση την ηλικία της μητέρας. Παράλληλα με αυτές τις μεταβλητές, για την ανάλυση της γονιδιωματικής έκφρασης λάβαμε υπόψιν επίσης το αναπτυξιακό στάδιο των εμβρύων στο τέλος της καλλιέργειας. Μετά την απόψυξη, τα έμβρυα καλλιεργήθηκαν για δύο μέρες στις παραπάνω συνθήκες. Μόνο τα έμβρυα που αναπτύχθηκαν στο στάδιο του μοριδίου και της βλαστοκύστης (41/89, 46%) αναλύθηκαν για την έφκραση του συνολικού γονιδιώματος χρησιμοποιώντας την μέθοδο που

αναπτύξαμε και επικυρώσαμε στο **Κεφάλαιο 4**. Με βάση τον αριθμό των γονιδίων που εκφράζονται σε διαφορετικό επίπεδο (DEGs), το αναπτυξιακό στάδιο (1.532 DEGs) και η ηλικία της μητέρας (368 DEGs) είχαν μεγαλύτερη επίδραση στην έκφραση των γονιδίων των ανθρώπινων προ-εμφυτευτικών εμβρύων από την συγκέντρωση οξυγόνου (183 DEGs) ή το μέσο καλλιέργειας (174 DEGs). Βρέθηκαν επίσης αλληλεπιδράσεις μεταξύ των μεταβλητών, υποδεικνύοντας ότι οι συνθήκες καλλιέργειας ίσως έχουν διαφορετική επίδραση ανάλογα με το αναπτυξιακό στάδιο του εμβρύου ή την ηλικία της μητέρας. Οι διαδικασίες που επηρεάζονται από τους παράγοντες που μελετήθηκαν συμπεριλάμβαναν τον μεταβολισμό, τον κυτταρικό κύκλο και την οξειδωτική φωσφορυλίωση. Τα έμβρυα όμως καλλιεργήθηκαν μόνο για δύο μέρες και αυτό μπορεί να επηρρέασε την επίδραση των υπό ανάλυση συνθηκών καλλιέργειας. Τα προηγούμενα αναπτυξιακά στάδια (πρώτη μέρα καλλιέργειας μέχρι τέταρτη μέρα καλλιέργειας) δεν αναλύθηκαν και ίσως ενδεχομένως να αντιδράσουν διαφορετικά στις συνθήκες καλλιέργειας.

Η παραπάνω επίδραση των συνθηκών καλλιέργειας είναι πολύ σηματική για την ποιότητα των εμβρύων. Η επίδραση της συγκέντρωσης οξυγόνου στην ποιότητα των εμβρύων και στα ποσοστά εγκυμοσύνης ήταν ήδη γνωστή, με την χαμηλότερη συγκέντρωση οξυγόνου (5%) να δίνει καλύτερα αποτελέσματα [2]. Για τα μέσα καλλιέργειας, η επίδραση στην ποιότητα των εμβρύων και στα ποσοστά εγκυμοσύνης ήταν λιγότερο σαφή. Έτσι, πραγματοποιήσαμε μια συστηματική ανασκόπηση της βιβλιογραφίας για να ερευνήσουμε ποιό μέσο καλλιέργειας ήταν το καλύτερο, δίνοντας τα καλύτερα έμβρυα και τα καλύτερα αποτελέσματα μετά απο την εξωσωματική γονιμοποίηση (**Κεφάλαιο 6**). Γι'αυτήν την ανασκόπηση επιλέχθηκαν όλες οι κλινικές δοκιμές (RCTs) που δημοσιεύθηκαν από τον Ιανουάριου 1985 μέχρι και τον Ιούλιο 2012. Το κύριο αποτέλεσμα ήταν ο αριθμός των γεννήσεων. Δευτερεύοντα αποτελέσματα ήταν η υγεία των μωρών που γεννήθηκαν, ο αριθμός των κυήσεων 12 εβδομάδων, ο αριθμός των κλινικών κυήσεων, οι αποβολές, οι πολλαπλές κυήσεις, το ποσοστό εμφύτευσης, το ποσοστό κρυοσυντήρησης, η ποιότητα των εμβρύων και το ποσοστό γονιμοποίησης. Για εκείνα τα μέσα καλλιέργειας που αξιολογήθηκαν σε παραπάνω από μια κλινική δοκιμή, πραγματοποιήθηκε μια αντισυμβατική ανάλυση όπου τα δεδομένα από τα διάφορα μέσα καλλιέργειας ομαδοποιήθηκαν. Η ανασκόπηση συμπεριέλαβε είκοσι δύο κλινικές δοκιμές που αξιολόγησαν τριάντα μία συνγκρίσεις μεταξύ μέσων καλλιέργειας. Συμβατική ανάλυση των δεδομένων δεν ήταν δυνατή για κανένα από τα αποτελέσματα καθώς σχεδόν όλες οι κλινικές μελέτες σύγκριναν διαφορετικά μέσα καλλιέργειας. Μόνο τέσσερις κλινικές δοκιμές αναφέρθηκαν στον αριθμό γεννήσεων και μόνο μία απο αυτές ανέφερε σημαντική διαφορά στα αποτελέσματα. Εννιά δοκιμές ανέφεραν τον αριθμό κύησης 12 εβδομάδων ή τον αριθμό κλινικών κυήσεων, από τις οποίες τέσσερις έδειξαν σημαντική διαφορά. Η ομαδοποίηση των δεδομένων δεν έδειξε κάποιο καλύτερο μέσο καλλιέργειας. Ποιό μέσο καλλιέργειας δίνει τα καλύτερα ποσοστά επιτυχίας κατά την εξωσωματική γονιμοποίηση δεν έγινε γνωστό. Δεδομένης της πιθανής σημασίας του μέσου καλλιέργειας για το αποτέλεσμα της εξωσωματικής γονιμοποίησης, το συμπέρασμα αυτής της ανασκόπησης ήταν οτι υπάρχει ανάγκη για σωστά σχεδιασμένες κλινικές δοκιμές για τα μέσα καλλιέργειας που είναι διαθέσιμα σήμερα αλλά και για νεοεισαχθέντα μέσα καλλιέργειας.

Κατά την περίοδο που πραγματοποιήθηκε αυτή η ανασκόπηση, πολλά κέντρα εξωσωματικής στην Ολλανδία άλλαζαν το μέσο καλλιέργειας τους από HTF σε G5 βασιζόμενοι στα εμφανή καλά αποτελέσματα με το G5 ως μέσο καλλιέργειας σε μερικά απο τα κέντρα. Για να ερευνήσουμε σωστά αν αυτή η αλλαγή ήταν δικαιολογημένη, έξι κέντρα εξωσωματκής στην Ολλανδία συνεργάστηκαν για την πραγματοποίηση μιας πολυκεντρικής τυχαιοποιημένης κλινικής δοκιμής για να καθοριστεί ποιό από τα δύο μέσα καλλιέργειας θα έδινε καλύτερης ποιότητας έμβρυα και βελτιωμένα ποσοστά επιτυχίας (**Κεφάιο 7**). Από τον Σεπτέμβριο 2010 μέχρι και τον Μάϊο 2012, 836 ζευγάρια (417 στην ομάδα με G5 ως μέσο καλλίεργειας και 419 στην ομάδα με HTF ως μέσο καλλίεργειας) συμμετείχαν σε αυτήν την δοκιμή. Όλα τα ζευγάρια που υποβλήθηκαν σε IVF/ICSI σε ένα από τα συμμετέχοντα κέντρα και στις κλινικές τους για πρώτη φορά, ή πρώτη μετά απο μια επιτυχή κύηση, μπορούσαν να συμμετέχουν σε αυτήν την δοκιμή. Η τυχαία κατανομή των ζευγαριών στις δύο ομάδες έγινε με τη βοήθεια ενός online προγράμματος. Οι γυναίκες που συμμετείχαν, οι γυναικολόγοι τους και οι ερευνητές δεν γνώριζαν ποιό μέσο καλλιέργειας χρησιμοποίθηκε για κάθε ζευγάρι. Μέχρι και την στιγμή που γράφτηκε αυτή η διατριβή, τα δεδομένα για τον αριθμό των γεννήσεων δεν ήταν διαθέσιμα, αλλά περισσότερες γυναίκες είχαν μια κύηση 12 εβδομάδων όταν τα έμβρυα τους καλλιεργήθηκαν σε G5 σε σχέση με τις γυναίκες τα έμβρυα των οποίων καλλιεργήθηκαν σε HTF, αν και η διαφορά αυτή δεν ήταν στατιστικά σημαντική [45% (189/417) vs. 39% (163/419); αναλογία ποσοστού (RR): 1.2; 95% confidence interval (CI): 0.99 to 1.38; *P*=0.07]. Άλλα αποτελέσματα όπως ο αριθμός των κλινικών κυήσεων και ο αριθμός των κρυοσυντηρημένων εμβρύων που είναι διαθέσιμα στο τέλος της κλινικής δοκιμής για μεταφορά σε μεταγενέστερη ημερομηνία είναι σημαντικά μεγαλύτερος στην ομάδα με G5 ως μέσο καλλιέργειας. Με βάση αυτά τα συγκεντρωτικά αποτελέσματα, προτείνουμε τη χρήση του G5 αντί του HTF ως μέσο καλλιέργειας για τα ανθρώπινα έμβρυα κατά την εξωσωματική γονιμοποίηση.

Η δουλειά που περιγράφεται στην παρούσα διατριβή προσφέρει νέες γνώσεις για τη βιολογία της ανάπτυξης του ανθρώπινου προ-εμφυτευτικού εμβρύου, καθώς και τρόπους για τη βελτιστοποίηση της καλλιέργειας ανθρώπινων εμβρύων , η οποία είχε ως άμεσο αποτέλεσμα την καλύτερη εργαστηριακή πρακτική βάσει στοιχείων. Η σύνδεση και η συνεργική δράση μεταξύ μιας θεμελιώδης κατανόησης της ανάπτυξης του ανθρώπινου προ-εμφυτευτικού εμβρύου, νέων ιδεών για την κλινική πρακτική, καθώς και της αξιολόγησης αυτών των ιδεών με τη χρήση κλινικών ερευνητικών εργαλείων αξιολόγησης είναι προφανής. Η κλινική πρακτική βελτιώνεται μέσω των κλινικών δοκιμών, αλλά για να πάρουμε ιδέες για τη βελτίωση της κλινικής πρακτικής, αλλά και να είμαστε σε θέση να εξηγήσουμε τα αποτελέσματα, η βιολογική έρευνα είναι απαραίτητη. Η διαφορά στην έκβαση της εγκυμοσύνης στην κλινική δοκιμή που πραγματοποιήσαμε εξηγήθηκε από την παρατηρούμενη (μορφολογικά) διαφορά στην ποιότητα των εμβρύων μεταξύ HTF και G5, η οποία με τη σειρά της σαφώς αντανακλάται από τα πρότυπα γονιδιακής έκφρασης που βρέθηκαν στα πειράματα καλλιέργειας που διεξάγαμε (**Κεφάλαιο 8**).

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Since my university years back in Patras, human embryology and IVF was my passion. After searching for a PhD position in the field for a few months, I came across a position for an IVF technician at the AMC. I decided to apply to get the connection to the field and the practical skills. I was happy when I got a reply from Dr. (back then) Sjoerd Repping that I was invited for an interview. I was even happier when during that interview I was told by Sjoerd, Sebastiaan and Annemieke that they had, at the department, received an AMC bonus and they could therefore offer me a PhD position. An offer that I happily accepted and that brought me, today, four and a half years later to this thesis.

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"Hora est"

Eleni

Curriculum Vitae

About the author

Eleni Mantikou, the author of this thesis, was born on February 19th, 1985 on Rhodos, Greece. In December 2006, she obtained her Bachelor's degree from the University of Patras, Department of Biology. For her Bachelor's thesis she did an internship of six months at the Department of Human Genetics at Patras University.

After graduating, she decided to pursue her academic interests in the Netherlands with a Master's education in Biomedical Sciences at Leiden University. During this study she conducted three internships. The first internship, during eight months, was performed at the Department of Immunohematology and Blood transfusion (Prof. dr. F. Class) and at the Department of Obstetrics (Dr. S. Scherjon) at Leiden University Medical Center (LUMC). She then worked for three months at the Department of Cardiology, LUMC (Prof. dr. A. van der Laarse). Her thesis internship lasted seven months and was performed at the Department of Clinical Genetics at LUMC (Prof. dr. P. C. Giordano) and the Sanquin Blood Bank in Leiden. She received her Master's degree in May 2009.

From June 2009 until December 2013 she was working at the Center for Reproductive Medicine, Academic Medical Center Amsterdam where she finished her PhD thesis under supervision of Prof. dr. S. Repping and Dr. S. Mastenbroek.

Portfolio

PhD training

Courses
Computing in R, Graduate School University of Amsterdam 2012 (12 hours / 0.4 ECTS)
Advanced Topics in Clinical Epidemiology, Graduate School University of Amsterdam 2010 (32 hours / 1.1 ECTS)
Clinical Data Management, Graduate School University of Amsterdam 2010 (7.5 hours / 0.3 ECTS)
Systematic Reviews, Graduate School University of Amsterdam 2009 (8 hours / 0.3 ECTS)
Clinical Epidemiology, Graduate School University of Amsterdam 2009 (18 hours / 0.6 ECTS)
AMC World of Science, Graduate School University of Amsterdam 2009 (20 hours / 0.7 ECTS)
Developing a Cochrane Systematic Review, Dutch Cochrane Center 2009 (8 hours / 0.3 ECTS)

Seminars, workshops and master classes
Developmental Origins of Health and Disease, 22 November 2012 Amsterdam (8 hours / 0.3 ECTS)
Array technologies, ESHRE, 22 April 2010 Brussels (8 hours / 0.3 ECTS)

Presentations at scientific conferences
7 - 10 July 2013, 29th Annual Meeting of ESHRE, London, United Kingdom, selected poster presentation (1 hour / 0.07 ECTS)
10 January 2013, Research meeting of the Dutch society for clinical embryologists, Nijmegen, the Netherlands, invited oral presentation (1 hour / 0.07 ECTS)
3 - 6 July 2011, 27th annual meeting of ESHRE, Stockholm, Sweden, selected oral presentation (1 hour / 0.07 ECTS)
19 - 20 May 2011, 39th Gynaecongress, Zwolle, the Netherlands, selected oral presentation (1 hour / 0.07 ECTS)
13 July 2010, 15th International conference on Prenatal Diagnosis and Therapy, Amsterdam, the Netherlands, selected oral presentation (1 hour / 0.07 ECTS)
27 - 30 June 2010, 26th Annual Meeting of ESHRE, Rome, Italy, selected poster presentation (1 hour / 0.07 ECTS)

Other activities
One week visiting the laboratory of Prof. dr. M. Zernicka-Goetz, Cambridge, UK, 2011 (40 hours / 1.4 ECTS)

Teaching

Supervising (72 hours / 2.6 ECTS)

Previous non-PhD related activities

27 May 2009, The Menarini Diagnostics symposium for diabetes, Utrecht, the Netherlands, invited oral presentation
13 - 14 March 2009, Leiden International Medical Student Conference, Leiden, the Netherlands, selected oral presentation
6 June 2008, 19th Biomedical Sciences Symposium, Leiden, the Netherlands, selected oral presentation
12 September 2007, mini-symposium on Immunoregulation, Leiden, the Netherlands, invited oral presentation
12 - 15 December 2007, 7th annual panhellenic immunology congress, Thessaloniki Greece, selected poster presentation

Grants

Huygens Scholarship Programme (2007 - 2008)
Charitable society "Dodecanissiaki Melissa" (2005 - 2006)
The 'Andreas Diacomichalis' Foundation of Scholarships (2002 - 2006)

Awards

Cath Ollongren Fund (2006)
Lions Club, award for school achievements (2002)
Hellenic ministry of education, awards for school achievements (1997 - 2001)

Publication list

Mantikou E*, van Echten-Arends J, Sikkema-Raddatz B, van der Veen F, Repping S, Mastenbroek S. Temporal and developmental-stage variation in the occurence of mitotic errors in tripronuclear human preimplantation embryos. Biol Reprod 2013; 89 (2): 42, 1-7

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Bontekoe S, **Mantikou E**, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. Cochrane Database Syst Rev 2012; 7, DC008950

Mantikou E*, Wong KM, Repping S, Mastenbroek S. Molecular origin of mitotic aneuploidies in preimplantation embryos. Biochim Biophys Acta 2012, 1822 (12): 1921-1930

Mantikou E, Harteveld CL, Giordano PC. Newborn screening for Hemoglobinopathies using capillary electrophoresis technology: Testing the Capillarys Neonat Fast Hb device. Clin Biochem 2010, 43 (16-17): 1345-1350

Umar S, de Visser YP, Steendijk P, Schutte CI, Laghmani el H, Wagenaar GT, Bax WH, **Mantikou E**, Pijnappels DA, Atsma DE, Schalij MJ, van der Laarse A. Stem cell therapy attenuates right ventricular pressure overload, hypertrophy, and dysfunction by improving lung pathology in rats with pulmonary hypertension. Am J Physiol Heart Circ Physiol. 2009, 297 (5): H1606-1616

Mantikou E, Arkesteijn SG, Beckhoven van JM, Kerkhoffs JL, Harteveld CL, Giordano PC. A brief review on newborn screening methods for hemoglobinopathies and preliminary results selecting beta thalassemia carriers at birth by quantitative estimation of the HbA fraction. Clin Biochem 2009, 42 (18): 1780-1785

* = part of this thesis