Instituto Politécnico de Lisboa

Instituto Superior de Engenharia de Lisboa

Escola Superior de Tecnologia da Saúde de Lisboa

MECHANISMS AND EFFECTS OF CELL DENSITY OF EMBRYOS IN CULTURE IN *IN VITRO* FERTILIZATION PROCESSES IN EMBRYONARY DEVELOPMENT

Inês Barradas Ribeiro

Thesis to obtain the Master Degree in

Biomedical Engineering

Supervisors:

Dr. Miguel Gallardo Molina (Ginemed Lisboa)

Prof. Dra. Cecília Ribeiro da Cruz Calado (ISEL, IPL)

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Success does not come from physical capacity. It comes from an indomitable will.

Mahatma Gandhi

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This work was partially conducted at Ginemed Lisboa Clinic and at Engineering & Health Laboratory, Instituto Politécnico de Lisboa, that resulted from a collaboration protocol established between Universidade Católica Portuguesa and Instituto Politécnico de Lisboa.

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I will not forget this year, unusual for all of us, but with an achievement that makes me proud.

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Abstract

Background and Objectives: The objective of achieving higher pregnancy rates in the area of human reproduction has promoted the search for new insights that could lead to a better embryonic development. The culture of embryos in groups aims to promote embryotrophic interaction, mediated by the secretion of paracrine and autocrine factors that induce mutual embryonic development, being crucial to evaluate the presence of factors that lead this evolution to fully understand the process. The present study aimed to explore what crucial knowledge could be obtained for the medically assisted reproduction area through the use of mid-infrared spectroscopy in the analysis of culture media spent by murine embryos and to evaluate retrospectively ovodonation cycle data for evaluation of the benefit of group human embryo culture (CG) versus individual culture (IC). In this way, it would be possible to take a position regarding the theory of the benefit of group culture and to understand whether the FTIR spectroscopy tool would be useful in evaluating the spectral profile of media spent by embryos.

Methods: This thesis is divided in three distinct methodological chapters: I) the analysis and comparison of the FTIR spectral profile of different commercial human embryo culture media. In order to increase the accuracy of this tool, pre-processing methods were carried out as atmospheric and baseline correction, normalizations and derivatives that minimize distortions and resolve overlapping bands. II) the analysis of the spectral profile of spent culture media of grouped murine embryos: the molecular profile of 47 culture media spent by murine embryos up to the state of two cells was studied, with a density variation between 3 to 114 embryos per drop were evaluated before and after fertilization. III) the retrospective analysis to assess the impact of group culture on human embryonic development rates using data from Ginemed's IVF unit: An analysis was performed on each ovodonation cycle to compare the results of individually culture embryos and those culture in groups.

Results: In chapter I), it was observed through the pattern recognition method of Principal Component Analysis (PCA) and the pre-processing methods applied that FTIR spectroscopy is sensitive enough to recognize different molecular profiles between culture media, highlighting differences at aminoacids (aa) and lipids molecules. In chapter II), The analysis of culture media spent of murine embryos showed different spectral profiles between media analyzed individually, without being able to establish any link between variables (species, number of embryos, pre/post fertilization). In chapter III), the retrospective comparison of culture of human embryos in individual and groups, revealed similar developmental rates to the blastocyst stage between both strategies; i.e. it was not detected a positive effect of group culture.

Conclusions: The FTIR spectroscopy enables to acquire the molecular profile of human embryo culture media. Despite this, it was not possible to acquire by PCA of spectral data of spent media from the murine embryos patterns associated to species, number of embryos, etc. In the human embryo population and conditions evaluated it was not possible to observe advantages of culture the embryos in group in relation to culture embryos individually. It is proposed as future work, to evaluate other spectral processing techniques and increase the dimension of the human embryos evaluated.

Keywords: Embryos, Culture media, Group embryo culture, human embryonic pre-implantation, *in vitro* fertilization.

Resumo

Enquadramento e Objetivos: O objetivo de atingir melhores taxas de gravidez na área da reprodução humana tem promovido a procura de novas e melhoradas técnicas. A cultura de embriões em grupo visa promover a interação embriotrófica e avaliar a importância da secreção de fatores parácrinos e autócrinos que induzam um desenvolvimento embrionário mútuo mais promissor, sendo para isso crucial avaliar a presença de fatores que impulsionem este desenvolvimento. O presente trabalho teve como objetivo explorar que conhecimentos cruciais poderiam ser obtidos para a área de reprodução medicamente assistida através do uso da espectroscopia do infravermelho médio na análise de meios de cultura gastos por embriões de murinos e avaliar retrospectivamente dados de ciclos de ovodoação para avaliação do benefício da cultura de embriões humanos em grupo (CG) versus cultura individual (CI). Desta forma, seria possível assumir uma posição relativamente à teoria do benefício da cultura em grupo e perceber se a ferramenta FTIR seria útil na avaliação do perfil espectral dos meios gastos por embriões.

Métodos: A presente dissertação foi estruturada fundamentalmente em três capítulos metodológicos distintos: I) a análise e comparação do perfil FTIR espectral de diferentes meios de cultura de embriões humanos comerciais. Com o intuito de assegurar a precisão desta ferramenta, foram aplicados métodos de pré-processamento como correções atmosféricas e de linha de base, normalizações e derivativas que minimizam distorções e resolvem bandas sobrepostas. II) a análise do perfil espectral de meio de cultura gasto por embriões de murinos agrupados: o perfil molecular de 47 meios de cultura gastos por embriões murinos até ao estado de duas células foi avaliado, com uma variação de densidade entre 3 a 114 embriões por gota, antes e após fertilização. III) a análise retrospetiva com o intuito de avaliar o impacto da cultura em grupo nas taxas de desenvolvimento embrionário, através de dados obtidos na clínica Ginemed Lisboa: realizou-se uma análise a cada ciclo de ovodoação para comparar os resultados obtidos na cultura individual e em grupo de embriões humanos.

Resultados: No capítulo I) observou-se através do método padrão de reconhecimento – Análise de Componentes Principais – e com a aplicação dos métodos de pré-processamento que a ferramenta FTIR revelou sensibilidade suficiente para reconhecer que as amostras obtidas de um dos meios de cultura analisados apresentam um perfil molecular que se diferencia dos restantes, destacando diferenças em aminoácidos e moléculas lipídicas. No capítulo II, a análise do meio de cultura gasto por embriões murinos apresentou perfis espectrais diferentes entre meios de cultura analisados mas sem qualquer ligação entre grupos que permitisse estabelecer uma distinção entre as variáveis (espécie, número de embriões por gota, estado pré-fertilização *vs* pós-fertilização). No capítulo III) a comparação retrospetiva da cultura de embriões individual e em grupos revelou taxas de desenvolvimento embrionário semelhantes em ambas as estratégias aplicadas; o efeito benéfico da cultura em grupo não foi detetado nesta população específica através do protocolo utilizado.

Conclusões: A ferramenta FTIR demonstrou a sua eficiência na avaliação do perfil molecular de meios de cultura de embriões humanos. Ainda assim, com recurso a esta técnica não foi possível adquirir através da análise de PCA, dados espectrais dos meios gastos pelos embriões de murinos com padrões associados a espécies, densidade embrionária ou outra variável. Na população de embriões humanos e condições avaliadas não foi possível observar quaisquer vantagens na cultura de embriões em grupo relativamente à cultura de embriões individual. Propõe-se como futuro projeto a avaliação de outras técnicas de pré-processamento e o aumento da dimensão da população avaliada.

Palavras-chave: Embriões, Meios de cultura, Cultura de embriões em grupo, pré-implantação embrionária humana, fertilização *in vitro*.

Relevant publications in the field

E-POSTER

Inês Ribeiro; Filipa Pires; Cecília R. C. Calado; Miguel Gallardo Molina. FTIR spectroscopy analysis reveals differences between human embryo culture media composition by type of formulation and by manufacturer. EUROPEAN SOCIETY HUMAN REPRODUCTION AND EMBRYOLOGY virtual 37th Annual Meeting 26 Junho- 01 Julho 2021 Online

POSTER & ORAL PRESENTATION

Inês Barradas-Ribeiro; Joana Santos; Ana Braula-Reis; Miriam Castro; Micaela Fernandes; Pedro Ferreira; José-Luís Metello; Ana Paula Soares; Cecília R.C. Calado; Samuel Santos-Ribeiro; Miguel Gallardo. The introduction of a group embryo culture protocol in donor oocyte-recipient cycles did not increase the total useable blastocyst rates: a retrospective study. AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE 16-20 Outubro 2021 Scientific Congress & Expo, Baltimore, Maryland (USA)

POSTER & ORAL PRESENTATION

Inês Barradas-Ribeiro; Joana Santos; Ana Braula-Reis; Miriam Castro; Micaela Fernandes; Pedro Ferreira; José-Luís Metello; Ana Paula Soares; Cecília R.C. Calado; Samuel Santos-Ribeiro; Miguel Gallardo. The introduction of a group embryo culture protocol in donor oocyte-recipient cycles did not increase the total useable blastocyst rates: a retrospective study. SOCIEDADE PORTUGUESA DE MEDICINA DA REPRODUCAÇÃO 2021 XXXVII Jornadas de Estudos da Reprodução, 25 set 2021, EPIC SANA Marquês Hotel, Lisboa

SUBMITTED ARTICLE

Inês Barradas-Ribeiro; Joana Santos; Ana Braula-Reis; Miriam Castro; Micaela Fernandes; Pedro Ferreira; José-Luís Metello; Ana Paula Soares; Cecília R.C. Calado; Samuel Santos-Ribeiro; Miguel Gallardo. The introduction of a group embryo culture protocol in donor oocyte-recipient cycles did not increase the total useable blastocyst rates: a retrospective study. Reproductive Biomedicine Online

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List of Abbreviations

AA - Aminoacid
ADP - Adenosine di-phosphate
AHCA- Adaptive Hierarchical Clustering Analysis
ART - Assisted Reproduction Techniques
ATP - Adenosine triphosphate
COC- Cumulus oocyte complex
CPR - Clinical pregnancy rate
D5UBR - day 5 usable blastocyst rate
DHCA- Dual Head Cluster Analysis
DNA - Deoxyribonucleic acid
EDTA - Ethylenediaminetetraacetic acid
ESHRE - European Society for Human Reproduction and Embryology
FAP - Factor 1-0-alkyl-Sn-glycerol-3-phosphocholine
FIR – Far-infrared
FPA - Focal plane array
FTIR - Fourier Transform Infrared Spectroscopy
GC- Group culture
GEE - gene*ra*lized estimating equation
Gln - L-glutamine
GM-CSF - Granulocyte-macrophage colony-stimulating factor
GQUBR - good quality usable blastocyst rate
HBEGF - Heparin-binding epidermal growth factor
HCA- Hierarchical Clustering Analysis
IC – Individual culture
ICM - inner cellular mass
ICSI - Intracytoplasmic sperm injection
IUD – Intrauterine Devices
IVF - *In vitro* fertilization
LDH - Lactate dehydrogenase
LIF - Leukemia inhibitory factor
MIR – Medium-infrared
NAD+ - Nicotinamide adenine dinucleotide molecule
NADH - Nicotinamide adenine dinucleotide with hydrogen
NIR – Near-infrared
PAF - Platelet activation factor
PC - Principal components
PCA - Principal Component Analysis
PID - Pelvic Inflammatory Disease
RCT - Randomized controlled trial
RNA - Ribonucleic acid
ROS - reactive oxygen species
SG - Savitzky-Golay
TCA - Tricarboxylic acid
TE - Trophectoderm
TUBR - the total usable blastocyst rate
UAF - Use animals first
WHO - World Health Organization
β-hCG- beta human chorionic gonadotropin

Chapter 1: Introduction

1.1 Infertility and Assisted Reproduction Medicine

One of the most prized desires of humanity in adulthood is the ambition to have children and combine parenthood with a family's generation. In the last few decades, the number of couples who have reached for help at assisted reproduction techniques (ART) with difficulties to conceiving natural has increased exponentially (Inhorn and Patrizio, 2015), revealing significant social and psychological consequences (Rouchou, 2013). This inability to conceive translates into the concept of infertility, which is defined by the World Health Organization (WHO) as a disease of the reproductive system, diagnosed when the couple is unable to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (WHO, 2016). Infertility is primary when a woman cannot deliver a child due to the inability to conceive or maintain pregnancy and secondary when, after conceiving a live child, she becomes unable to conceive again (WHO, 2016).

The scientific progress for human assisted reproduction started in the middle of 1970 when *in vitro* fertilization (IVF) was still only an experimental project (Wang and Sauer, 2006). The first significant steps for the formation of an *in vitro* embryo occurred at this time. A patient called Lesley Brown, with nine years of primary infertility due to a bilateral obstruction of the fallopian tubes (Kamel, 2013), underwent a laparoscopy during an unstimulated ovulatory cycle to collect oocytes (Wang and Sauer, 2006). After the laparoscopic procedure, the collected oocyte was fertilized with her husband's sperm by a British physiologist, Robert Geoffrey Edwards. The final result of the first well succeeded human *in vitro* fertilization was revealed in an 8-cell embryo transferred to the uterine cavity of Lesley Brown. On 25 July 1978 (Figure 1.1), Louise Brown was born, the first baby originated from assisted reproduction techniques (Kamel, 2013).

1.1.1 Assisted Reproduction Technics Development

This event was a milestone in the history of human assisted reproduction. It triggered those that followed (Figure 1.1) to support the distinct conditions responsible for infertility diagnosed in couples. Thus, in 1983, a 25-year-old woman with premature ovarian failure achieved a successful pregnancy through a donated egg cell and inseminated with her husband's sperm (Wang and Sauer, 2006).

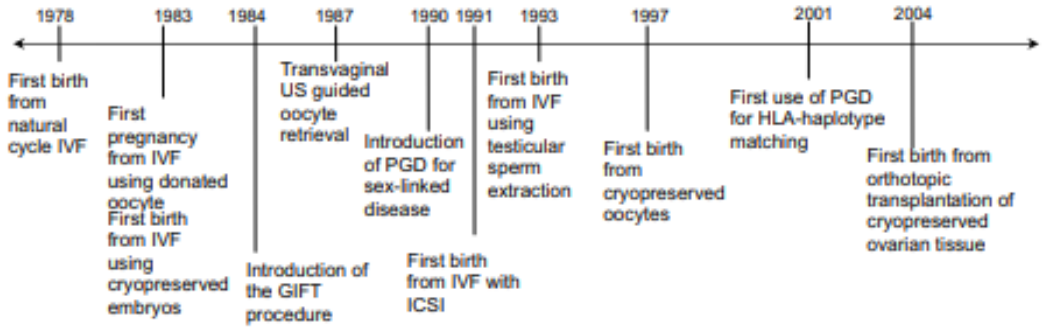


Figure 1.1. - Chronological advance of AR techniques (Wang and Sauer, 2006)

Currently, the evolutionary development of assisted reproduction techniques has allowed many infertile couples to achieve pregnancy that they could not achieve naturally (Farquhar *et al.*, 2015). It has also allowed homoparental couples or monoparental women to have a child using male donors (Montagut and Menezo, 2003).

According to the report on ART activity carried out by the National Council for Medically Assisted Procreation (CNPMA), in 2015, there was a record of 978 children born with ART techniques in Portugal (Table 1.1).

Table 1.1 - Total number of children born with ART (Adapted from CNPMA, 2017)

	Single Newborn	*Twins*	*Triplets*	*Total childbirth*
Intra-marital IVF/ICSI	459	109	3	686
IVF/ICSI with donated sperm	7	4	0	15
IVF/ICSI with donor oocytes	5	1	0	7
FET intra-marital donated sperm	80	22	0	124
FET with donor oocytes	1	0	0	1
FET with donated embryos	1	0	0	1
PGD	3	0	0	3
CAI	99	12	1	126
DAI	9	3	0	15
Total	664	151	4	978

According to the same report, it is still possible to see that implantation is not always successful despite the evolution of ART techniques. The non-pregnancy rate resulting from each cycle of assisted reproduction treatments remains high (60.8%), failing to satisfy all couples' desire for parenting (Chart 1.1).

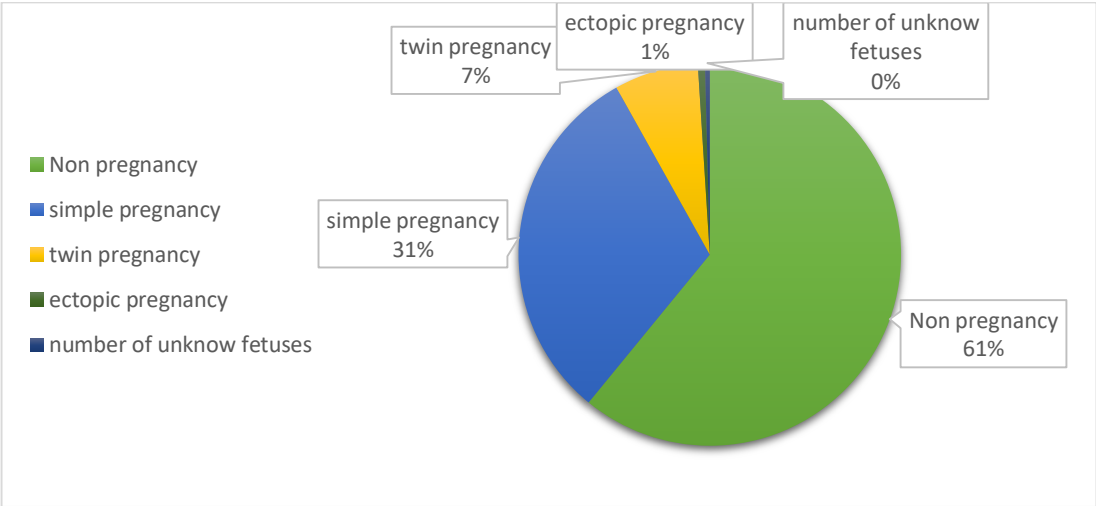


Chart 1.1 - Percentage of IVF/ICSI cycles that resulted in non-pregnancy and multiple pregnancies (Adapted from CNPMA,2017)

The rate of non-pregnancy presented refers to the rates per cycle. There is a distinction that must be valued concerning success rates in medically assisted reproduction: success rates for embryo transfer and cumulative success rates. Embryo transfer rates can be increased by improving the quality of transferred embryos, improving transfer techniques, and embryo-endometrial synchronization. The cumulative success rates (shown in Chart 1.1) can be enhanced by increasing the number of viable embryos obtained through ovarian puncture and stimulation. These embryos will be used for successive transfers until the desired pregnancy is obtained. Thus, the greater the number of embryos obtained, the greater the number of attempts to achieve pregnancy.

With the development of the use of assisted reproduction techniques, there is a need to improve practical aspects in the daily routine of the assisted reproduction laboratory, such as: improving the culture conditions, improving the selection of the embryo to transfer, and extension of the embryonic culture until the blastocyst state (Galliano *et al*.,

2015). These and other changes have allowed a significant increase in the results obtained, in the reliability of this area, and, consequently, in the number of people who use this service (Dyer *et al*., 2016)

1.2. Embryo Culture

1.2.1 An overview of the metabolism of the human preimplantation embryo

The pre-implantation embryo is characterized by noticeable changes in its genetic control, physiology, and morphology, comprising the phases from fertilization to the blastocyst formation implanted in the uterine cavity (Figure 1.2) (Niakan *et al*., 2012). The first step, fertilization, is defined as a sequence of coordinated molecular events that culminate in the fusion of male and female gametes. This fusion will form a cell called a zygote, the first human life form (Georgadaki *et al*., 2016).

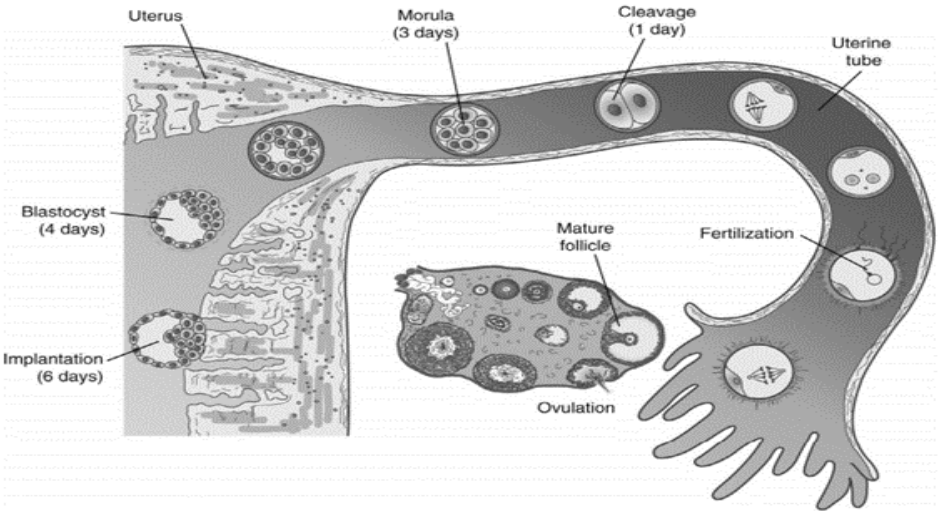


Figure 1.2 - Fertilization and embryonic development (Guyton e Hall, 2016)

After fertilization, the zygote begins the cleavage process. During cleavage, the fertilized egg's cytoplasm divides into numerous smaller nucleated cells, defined as blastomeres, through a series of consecutive mitotic divisions (Guyton and Hall, 2016). This process begins with the displacement of the zygote from the fallopian tubes, where fertilization occurs, following the path to its final destination: the uterus. In an initial phase, the zygote is divided in half, then in fourths, eighths, and so on, exponentially, until it forms a structure of about 16 to 32 cells, at which point the compaction phase begins (Figure 1.3) (Watson and Barcroft, 2001).

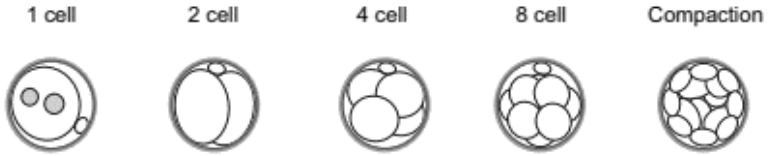


Figure 1.3 – Embryo development up to compaction stage (Devreker and Englert, 2000)

At the pre-compaction stage, the embryo's viability depends on its ability to generate energy through an appropriate metabolic pathway to support the essential processes for its development. Oxidative metabolism is the primary source of adenosine triphosphate (ATP), being pyruvate the preferred substrate (Leese *et al*., 1993; Martin, 2000) obtained as a sub-product of glycolysis but mainly directly from the external environment (Figure 1.4) (Martin, 2000). Pyruvate enters the embryo passively, using a carrier via facilitated diffusion, being the primary nutrient for the development of the embryo in the phase of cleavage (Gardner, 1998).

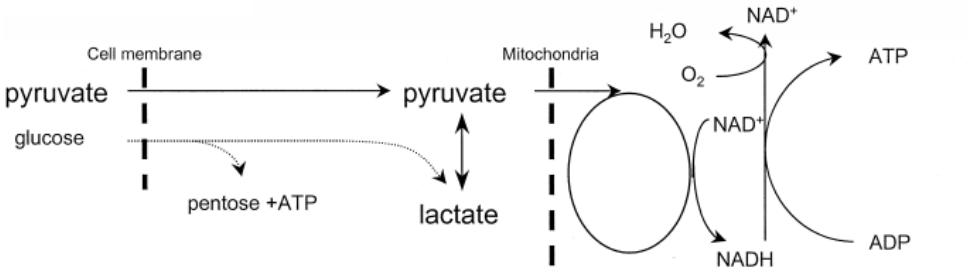


Figure 1.4 – Embryo cleavage metabolism (Thompson, 2000)

Pyruvate is metabolized through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. In this process, pyruvate is converted into lactate (about 34%), and the rest of the pyruvate is oxidized (Conaghan *et al*., 1993). In this way, pyruvate is the primary source of energy used by the embryo in its first mitotic division, thus being essential for development in the period immediately after fertilization. After the two cells stage, the embryo can use lactate as an energy source, suggesting that the differences found in the use of pyruvate or lactate in the different stages of development are due to differences in the regulation of lactate dehydrogenase (LDH). This enzyme catalyzes the oxidation of lactate by a nicotinamide adenine dinucleotide (NAD+) molecule, leading to pyruvate and nicotinamide adenine dinucleotide with hydrogen (NADH) (and vice versa), allowing the cell to obtain an additional pyruvate source (Lane and Gardner, 2000).

The low metabolic level in the embryonic pre-compaction phase is associated with the oocyte's quiescent state that remains energetically dormant inside the ovary for a long time (Gardner *et al*., 2011). During this period, despite the noticeable changes in deoxyribonucleic acid (DNA) replication and successive cell division, there is no increase in volume or mass (Turner *et al*., 1992). For this reason, during the cleavage period, the embryo has relatively reduced metabolic activity, and oxygen consumption remains at low levels (Lewis and Sturmey, 2018). This fact supports the theory that embryos in the cleavage phase are quieter than blastocysts, not exhibiting the need for a high ATP expenditure as observed in the post-compaction embryo (Baumann *et al*., 2007). At this stage, the embryo does not need to use the glycolytic pathway and therefore does not express the genes responsible for protein synthesis of the glycolytic pathway (Gardner and Wale, 2013)

The phenomenon called compaction takes place around the fourth day of embryonic development. After successive divisions of the zygote, events such as microvillous redistribution and cytoplasmic polarization promote the embryo's compaction, potentiating an interblastomeric contact that blurs the boundaries of the distinct individual cells until the formation of a uniform structure called a morula (Leese *et al*., 1993). Thus, the compaction comprises events that begin with the development of Ca2+ dependent cell adhesion, the establishment of interblastomeric cellular communication mediated by the junction, the onset of cell polarization induced by cell contact, the appearance of focal junctions that will divide the plasma membrane from outside the blastomeres. This whole process aims to prepare the cellular structure for the formation of the blastocyst (cavitation) (Leese *et al*., 1993).

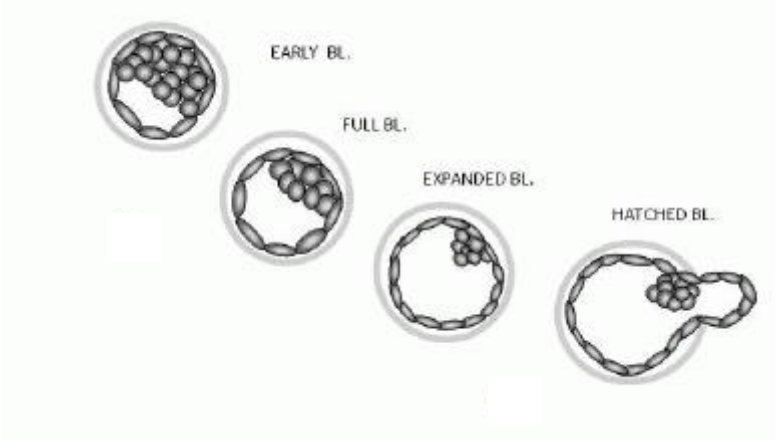


Figure 1.5 – Post-compaction phase (Sepúlveda *et al.*, 2011)

The cavitation consists of fluid accumulation by actions in the membrane NA^{+}/K-ATPase channels that allow the increase of salt concentration within the embryo, causing water to enter by osmosis and, consequently, causing a gradual increase in the size of the cavity (Watson *et al.*, 2004). Therefore, an increase in volume occurs to promote a cavity's appearance that will form the blastocelium. Thus, during the blastocyst formation, the energy requirement increases drastically due to the pumping of sodium ions by Na+, K+ (ATPase transport system located in the trophectoderm (TE)) that leads to the formation of the blastocelium cavity. On the other hand, the gradual increase in the volume of the blastocelium is accompanied by an increase in the number of cells, thus expanding the blastocyst and causing a decrease in the thickness of the pellucid zone. This process culminates with the pellucid zone's rupture and the embryo's hatching (Figure 1.5) (Hardarson *et al.*, 2012) at this more expensive phase of energy, the most energetic events are aimed at protein biosynthesis (a theory supported by declining protein concentration), increasing the number of cells in the division (Gardner *et al.*,2002).

Supporting the theory that embryos at an early stage depending on oxidative metabolism, to reverse what occurs at later stages, in a post-compaction phase, the embryo exhibits the main source of ATP from glucose via glycolysis (Figure 1.6). Some processes that occur during this period show notorious productivity with the adequate consumption of glucose (Martin, 2000). Thus, glucose is metabolized via pentose phosphate, allowing the formation of a viable substrate for the biosynthetic pathways indispensable during cell proliferation (DNA replication and RNA transcription) (Newsholme *et al.*,1985).

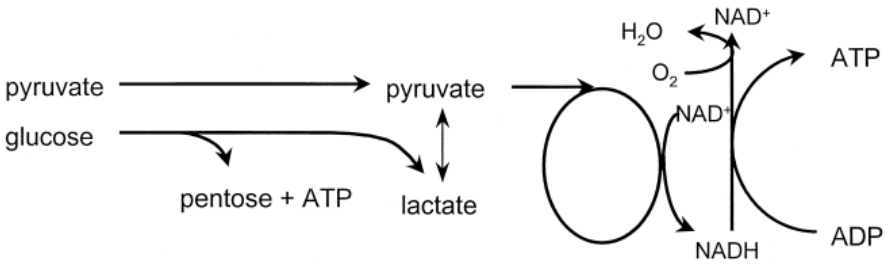


Figure 1.6 – Embryo Compaction metabolism (Thompson, 2000)

The early embryo has reduced biosynthetic activity, resulting in a significant value in the ATP/ADP ratio within the embryo, inhibiting glucose flow through the glycolytic pathway. Its transcriptional activity is activated with its continuous development, resulting in an increase in protein synthesis and a valuable need for ATP associated with the formation of blastocelium through ATPases, limiting the ATP/ADP ratio and, consequently, increasing glycolytic flow and glucose absorption (Gardner and Kaye, 1995). This accelerated metabolism allows the generation of energy necessary

to expand the blastocele and monitor mitosis and the subsequent synthesis of components essential to embryonic development, such as triacylglycerols and phospholipids (Reitzer *et al*., 1980).

The accumulation of fluid during cavitation benefited from this substrate's consumption since it thus obtains the necessary energy for the referred process (Brison and Leese,1994). Glucose metabolism presents intermediate precursors for the synthesis of macromolecules that suffer a valuable increase in the blastocyst stage (Brinster, 1969). The increased use of this substrate is regulated by glucose transporters, controlling uptake in embryo cells or catalyzing enzymes that control the glycolytic pathway (Gardner and Leese, 1988; Hogan *et al*., 1991).

When the blastocyst reaches a notorious degree of expansion, two populations of newly formed cells can be easily distinguished. The group of cells distributed by the peripheral contour takes the name of trophoectoderm, developing the blastocyst's spherical structure and contributing to the formation of the placenta, where contact occurs between the embryo and the progenitor at the site of implantation. On the other hand, the group of cells that form a compact mass is called the internal cellular mass, referring to the blastocyst's inner cells, originating the fetus's definitive structures. This set of cells are characterized as pluripotent stem cells capable of creating all cell lines of the fetus (Magli *et al*., 2013). At this stage, the blastocyst has the necessary conditions to implant in the woman's uterine cavity (Hardarson *et al*., 2012).

The alteration of the metabolic profile along the stages of embryonic development is due to the variation of energy expenditure according to embryonic needs and activation of glycolytic pathway protein transcription, which characterizes the regulatory mechanism of glycolysis. The pre-compaction embryo exhibits a suppression in the glycolytic pathway caused by reduced ATP expenditures that causes allosteric phosphofructokinase inhibition. As previously mentioned, the embryo exhibits an increased need to generate energy to keep its embryonic development viable in the blastocyst stage. This increase of activity supported by the rise of biosynthesis promotes a reduction in the ratio ATP: ADP and, consequently, causes a positive allosteric reaction in phosphofructokinase, facilitating a greater glucose flow glycolysis. At this stage, the embryo initiates the transcription of glycolytic pathway proteins and consequently promotes this activation. Both mechanisms enable the glycolytic pathway after the compaction phase (Leese, 2012).

1.3. Embryo Culture Medium

1.3.1. Brief history of culture media

The optimization of culture media that supports embryonic development has demonstrated an evident evolution that reflects the deepening of knowledge and progress in assisted reproduction. The culture media available in the current market transmit decades of animal and human research and studies that have allowed progress from simple solutions of salts to the more complex culture media with diversified components that support the evolution of the pre-implanted embryo (Pool, 2004; Chronopoulou and Harper, 2015).

The first steps in human embryos' thriving culture occurred when researchers Rock and Menkin collected the eggs through a laparotomy, fertilized and cultivated them in human serum, observing after 40 hours the first mitotic division (Rock and Menkin, 1944). In 1969, Edwards and his colleagues introduced human embryos' culture to the blastocyst stage through the Ham F10 medium (initially experimentally tested on animals) with human serum (Steptoe *et al*., 1971). Seven years later, the first baby conceived through assisted reproductive techniques is born, and the team exposes the positive results resulting from their research. They demonstrated that the embryos were cultivated in Earle's simple saline solution supplemented with pyruvate of the patient's serum (Steptoe and Edwards, 1978). All the media used up this time were based on undefined serum, which was being implemented and tested according to the study. There were still no defined concentrations or compositions to which specific embryonic procedures or phases could be associated.

At this time, the culture of embryos remained fundamentally until day 3 of embryonic development since the available culture media were not rich enough to support the embryo's evolution until the state of the blastocyst (Chronopoulou and Harper, 2015). In the intensive search for improvement of success rates, compositions of individual components in the *in vivo* environment, in the fallopian tubes, and the uterus were studied, evaluating a "back to Nature" perspective by accompanying the embryo along its path to the time of implantation. Through this evaluation, a change in the embryo's metabolic profile was demonstrated from the compaction phase, and, as a result of this data, the sequential media emerged. From this moment on, the production of defined compositions media was being produced on a large scale to support assisted medical reproduction techniques in a consistent and reproducible way (Chronopoulou and Harper, 2015).

1.3.2. Sequential media

Sequential culture media arise intending to mimic the *in vivo* conditions that support embryonic development. The perception of the post-compaction metabolic profile change promoted the appearance of culture media that valued the metabolic and nutritional needs according to the embryo's specific state. In this way, the fertilized eggs are placed from day 1 to day 3 in an adapted solution with a high pyruvate concentration and low in glucose. On the third day after fertilization, the pre-compacted embryos are transferred to a different culture media characterized by a high concentration of glucose that acts as the primary substrate in this phase (Reed *et al*., 2009; Balaban and Urman, 2005).

1.3.3. Continuous Media

Through the ambition of continuous development and scientific research, to achieve better results at the level of implantation, a new variant of non-sequential culture media appears. This new approach reveals an uninterrupted culture. Although it is still the subject of studies, it is based on providing the embryo all the nutrients necessary for its development. The embryo will select the components it will need throughout its development (Ciray *et al*., 2012).

This type of continuous culture has some benefits in the embryo's culture, notably the absence of embryo manipulation for the exchange of culture medium on day 3 of embryonic development and, consequently, the permanence of the incubator's culture media. Thus, allowing less disturbance of the culture conditions of pH, osmolarity, temperature, and air quality,essential for the embryo's viability. Despite the positive aspects presented, there are also concerns related to the degradation of the culture media resulting from ammonia production, depletion of substrates essential to embryonic development, and accumulation of organic components (Swain, 2019).

1.3.4. The transition of sequential to continuous

Three main factors challenged the use of constant media. However, currently, they can be considered to be overcome. First, an inhibitory effect of glucose, necessary for blastocyst development, in the cleavage stage embryos. Secondly, ethylenediaminetetraacetic acid (EDTA) inhibition of blastocyst formation and finally, the media's degradation, mainly by ammonium accumulation (Biggers and Summers, 2008).

The presence of glucose in all stages of embryonic development despite the alteration of the pre-implanted embryo's metabolic profile is pointed out as a factor against the use of continuous media by the researchers defend their point of view by arguing that it is essential to use the two-step culture protocol to support the embryo's physiological and metabolic changes and promote embryonic viability (Gardner and Lane, 1997). On the other hand, Biggers presents a different perspective (Biggers, 2004) and supports his theory based on the *in vivo* environment in which glucose is present even in the early stage of embryonic cleavage, albeit in reduced concentrations. Previous studies have demonstrated that the presence of glucose at the low concentrations needed for blastocyst development (1.0 - 2.0 mmol/L) does not inhibit the growth of the embryo in the pre-compaction period. For this reason, the author claims the need for a continuous means of presenting the components that meet embryonic needs that comprise all specific states and thus allowing the embryo to select the critical elements for its adaptation from the *in vitro* environment (Summers and Biggers, 2003; Biggers and Summers, 2008).

The inhibitory effect of EDTA on the development of blastocysts and internal cell mass (ICM) worries researchers who support the absence of EDTA in the second sequential medium to prevent embryonic unviability (Gardner and Lane, 2006). This factor has been avoided through the application of low concentrations in the single environment since studies have shown that EDTA at a concentration of 0.01 mmol/L allows overcoming the dilemma of blocking two cells and, additionally, it does not have a harmful post-implantation effect as mentioned above (Lane and Gardner, 2001). This recent advance in knowledge eliminates the need for a protocol with two different culture media and supports single media with a reduced concentration of this component (Biggers *et al*., 2006).

Finally, the chemical degradation of L-glutamine (Gln) in aqueous solution and the accumulation of ammonia and its adverse effects on embryonic development has been visibly exceeded by replacing glutamine in the continuous medium by stable dipeptides of glutamine such as glycyl glutamine or alanyl-glutamine that do not originate ammonia and thus its deleterious effects on embryo culture are avoided (Biggers, 2004).

1.3.5 Other aspects of the composition of culture media

1.3.5.1 Aminoacids

In the mid-1980s and 1990s, the need to improve culture systems provided a milestone in optimizing culture media: the addition of amino acids (Leese, 2015). Analysis of female reproductive system fluids revealed the presence of free amino acids. For this reason, the high concentrations of non-essential amino acids and the low concentrations of essential amino acids present at different stages of development have been demonstrated and associated as representative of the needs of the embryo (Miller and Schultz, 1987). Following previous research, incorporating amino acids into embryo culture media revealed an increased implantation rate in assisted medicine treatments (Gardner *et al*., 1994; Gardner and Lane, 1997). The benefits of including amino acids in the culture of embryos are referenced separately according to their classification: Non-essential aa are crucial during the embryo's cleavage period, while the essential aa play a significant role in a post-compaction phase (Van Winkle, 2001; Devreker *et al*., 2001). The importance of the presence of these components in the culture of embryos is reflected in their functions. Amino acids, as essential nutrients for embryonic development, play a crucial role in signaling, regulation, and differentiation of trophoblasts and, consequently, prove to be critical to the implantation of the blastocyst in the uterus (Martin and Sutherland, 2001; Kilberg *et al*., 2016)

1.3.5.2 Growth Factors

The female reproductive tract is exposed to fundamental growth factors that promote embryonic development, embryo implantation, and the fetus and placenta (Pampfer *et al*., 1991; Kaye and Harveyt, 1995). For this reason, the study of their incorporation into the *in vitro* culture of embryos has attracted interest. Some studies have been referencing factor 1-0-alkyl-Sn-glycerol-3-phosphocholine (Paf) as an essential factor that causes changes in the maternal reproductive tract and directly affects receptors expressed by the embryo as an autocrine factor, promotes metabolic processes and cell cycle progression and embryonic viability (O'Neill, 2005). Another currently evaluated factor is the granulocyte-macrophage colony-stimulating factor (GM-CSF), a multifunctional cytokine presents in the female tract during the preimplantation period (Robertson, 2011; Group, 2020). This factor has shown benefits in increasing the proportion of early cleavage embryos that develop up to the blastocyst stage and an increase in the number of viable ICM cells (Sjoblom *et al*., 1999; Sjoblom *et al*., 2002). Cytokines are characterized as biological regulators synthesized by epithelial cells and *in vivo* leukocytes that regulate embryonic development, cell stress, and apoptosis and promote implantation (Fawzy *et al*., 2019). Recently, an randomized controlled trial (RCT) reported the effectiveness of the integration of GM-CSF, heparin-binding epidermal growth factor (HBEGF), and leukemia inhibitory factor (IPL) into human embryo culture

media with improved clinical results after ICSI (Fawzy *et al*., 2019). The LIP factor promotes blastocysts' formation, and the HBEGF factor has been shown to encourage the development and outbreak of blastocysts and positively regulates integrins (Richter, 2008; Fawzy *et al*., 2019). Supplementation of culture media with growth factors has shown positive results in embryonic performance, although there is still not enough evidence of the long term benefits in the area for its consistent application in embryo culture (Group, 2020; Fawzy, 2019; Robertson and Thompson, 2014).

1.3.5.3 Vitamins

Vitamin supplementation in the culture medium is a subject that is still little addressed despite its high potential. Currently, its presence in commercial culture media is in undefined concentrations since ideal concentrations or long-term effects have not yet been determined (Sunde *et al*., 2016). One of the critical points for advancing the study of vitamins in assisted reproduction focuses on their antioxidant capacity (Zaken *et al*., 2001). During the metabolic processes of oocytes and embryos, reactive oxygen species (ROS) production, proven to be harmful to embryonic development, occurs (Guerin *et al*., 2001). When an imbalance occurs between ROS production and its removal through antioxidant systems, the embryo faces oxidative stress (Wang *et al*., 2002). This biological condition is responsible for severe damage that disturbs the phospholipids of the cell membrane, proteins, and nucleic acids that promote mitochondrial changes, embryonic cell block, and apoptosis (Kowaltowski and Vercesi, 1999; Wang *et al*., 2002). In the *in vivo* environment, both oocytes and embryos somehow overcome this oxidative stress condition through oxygen eliminators and antioxidant enzymes present in follicular and uterine fluids (Wang *et al*., 2002). This advantage is eliminated in the culture of *in vitro* embryos. For this reason, the need arises to incorporate into the media and culture components that provide antioxidant activity and protect the embryo from the risks associated with oxidative stress (Wang *et al*., 2002).

1.3.6. Culture Conditions of Preimplantational embryos in the IVF Laboratory

The process of *in vitro* fertilization undeniably induces additional stress on the embryo, to which it would not usually be exposed in the system *in vivo*. Minimizing this effect and enhancing an ideal culture environment is essential to obtain positive results (Swain, 2015). The mass production of culture media allowed an increase in their consistency and quality. However, additional factors can affect the success rates such as the optimum levels of pH, osmolarity, temperature, and air quality maintained by the incubators, directly influencing embryonic development (Higdon *et al*., 2008; Fujiwara *et al*., 2007; Morbeck, 2015).

1.3.6.1 Incubators

Ensuring the consistency of culture conditions is essential for achieving success rates. Besides the importance of culture media in embryonic viability, incubators are the key point that allows the maximum mimicking *in vivo* conditions of oxygen tension, pH, temperature stability, and humidity (Swain *et al*., 2016). This equipment should be controlled routinely through the evaluation of the mentioned parameters (Group, 2020). Also, unnecessary openings/closures leading to instability of the conditions promoted by the incubator shall be avoided. The application of continuous culture media has made it possible to achieve this objective since the culture medium does not need to be modified on Day 3, thus promoting stability in terms of the necessary parameters to avoid additional stress to the embryo (Swain, 2019).

1.3.6.2 Temperature

One essential factor for embryonic development controlled by incubators is temperature. Fluctuations can compromise the mitotic spindle's stability and produce harmful alterations at the embryonic metabolism level (Wang *et al*., 2001; Leese *et al*., 2008; Swain, 2015). The recommended values for embryo culture and handling are 37 ºC. This value is based on an estimate of the human central body temperature, although the temperature of the female reproductive tract is slightly lower: the temperature of the human follicle has a temperature of 2.3˚C and the Fallopian tube 1.5˚C below body temperature (Grinsted *et al*., 1985). Following these premises, the effect of slightly lower temperatures on embryo culture has been assessed in several studies, yet the alternative temperatures tested always resulted in lower embryo development rates (Hong *et al*., 2014; Ronny *et al*., 2019; Fawzy *et al*., 2018).

1.3.6.3 Humidity

The success of embryonic development is influenced, among other factors, by the evaporation of the culture medium supplemented with essential nutrients of the embryo evolution (Fawzy *et al*., 2017)., Incubators play a crucial role in providing the humidity necessary to prevent this factor from compromise embryo viability. Currently, dry incubators and humidified incubators are commercially available (Fawzy, 2017; Group, 2020). Dry incubators arise with the need to avoid the risk of contamination present in wet incubators, which lead to the development of harmful microorganisms. However, evaporation remains a problem in this equipment, and osmolarity changes represent a crucial factor that promotes the use of wet incubators instead (Fawzy *et al*., 2017, Group, 2020; Geraghty *et al*., 2014). To overcome this condition, the application of oil overlay was one of the promising approaches investigated to reach stability and avoid evaporation of the culture medium; however, even with this adaptation, wet incubators have achieved more positive results on development and embryo implantation (Fawzy *et al*.; 2017, Group, 2020). These incubators have shown the advantage of maintaining the correct osmolarity for successful embryonic evolution. With the ambition of promoting these incubators' commercialization, the disadvantage related to possible contaminations has been easily overcome with the application of recommended aseptic techniques (Fawzy, 2017, Group, 2020). According to this, it is currently advisable to use humidified incubators, although the optimal humidity level has not yet been determined (Group, 2020).

1.3.6.4 pH e CO_2

To promote embryonic viability and minimize the *in vitro* stress to which the embryo is exposed, maintaining a physiological pH is essential. The pH of the culture medium is defined by the concentration of sodium bicarbonate in the medium, and the concentration of carbon dioxide present in the incubator (Swain, 2015). The CO_2 dissolves in the solution and produces carboxylic acid, reaching equilibrium with sodium bicarbonate concentration in the culture medium (Swain, 2010). The embryo needs to generate a differential gradient between intracellular pH and extracellular pH to regulate transport mechanisms and compensate for the internal acidification of embryonic metabolism; for this reason, pH stability is essential (Lane *et al*., 1999; Group, 2020). Researchers have demonstrated through experimental studies that intracellular pH should be maintained between 7,2-7,3. The variation required for the execution of mechanisms essential to embryonic development revealed to be a value of 0.1 units and, consequently, the desired physiological extracellular pH presents if between 7,2 and 7,4 it is necessary to provide the culture medium with CO_2 concentrations between 5-6% (Bavister, 1995; Swain 2012; Group, 2020).

1.3.6.5 Low oxygen tension

Physiological oxygen concentrations in the female tract are 5%, contrasting with 21% atmospheric oxygen. Studies report adverse effects of the exposure of embryos to atmospheric oxygen, directly affecting transcriptome and proteome changes, comprehending the acid metabolism of carbohydrates and amino acids, and interfering with homeostasis (Lane and Gardner, 2005; Wale and Gardner, 2013; Guo *et al*., 2014). The variation of oxygen outside the referenced parameters has shown an increase of reactive oxygen species (ROS), characterized by altering most cell

molecules and inducing an embryonic developmental block (Bedaiwy *et al*., 2004). Some RCTs have attempted to demonstrate comparisons between 20% and 5% oxygen tension in improving embryonic development rates. However, no definitive results have yet been presented; the rates of live births seemed higher when the embryo is exposed to an oxygen tension of 5% (Bontekow *et al*., 2012; De Munck *et al*., 2019).

1.3.6.6 The culture dish: volume, oil overlay, evaporation

Besides using the right culture media and incubation conditions, the preparation of the culture dishes can influence the performance of the culture system. The volume of media used, the type, and the volume of oil overlay will determine media stability (Swain, 2019). The volume is supposed to play a role as well, a small volume is more unstable, but more significant volumes might dilute auto and paracrine interactions. The optimal volume for culture is still a subject of debate, and the number of embryos cultured per drop (Rijnders and Jansen, 1999; Swain, 2019). The key is to find an ideal balance that limits the concentration of toxic metabolic molecules, such as ammonia, and simultaneously benefits from the positive aspects of autocrine factors for embryonic development (Virant-Klun *et al*., 2006; Rijnders and Jansen, 1999). This balance requires an additional element to maintain the functionalities of the appropriate volume of culture medium, in this case, the use of an oil overlay to prevent evaporation of the culture medium and avoid it from becoming hyperosmotic (Gardner and Lane, 2007; Baltz, 2012; Reed, 2012). In the absence of the oil overlay, there would be an increase in osmolarity, detrimental to the survival of the developing embryo (Wale and Gardner, 2016; Swain, 2019)

1.4 Group culture of human embryos: The possible synergic role of group human embryo culture

1.4.1 Hypothesis

The development of optimized culture systems, although presenting significant improvements, has not yet reached the desired level of fully mimicking *in vivo* conditions and offering higher implantation rates (Swain, 2015; Rebollar-Lazaro and Matson, 2010). A hypothesis developed by the researchers is related to the need to include signaling factors that modulate cell growth and division *in vitro* culture of embryos (Wydooghe *et al*., 2017). In this sense, the valuation of elements derived from autocrine and paracrine pathways present in mammalian embryos has been evaluated (Rebollar-Lazaro and Matson, 2010). To maximize their potential, new strategies have been implemented, such as reducing the dilution of beneficial factors by reducing the volume of culture medium, the co-culture of single embryos with somatic cells, and finally, the use of embryo culture in groups (Ebner *et al*., 2010; Tao *et al*., 2013; Rebollar-Lazaro and Matson, 2010). Human embryos are often cultured individually, in microdroplets of culture medium, to select the best embryo for transfer or biopsy, based on monitoring its morphology (Rebollar-Lazaro and Matson, 2010). This methodology adapted to embryonic culture derives from the existence of data demonstrating the regulation of embryonic development motivated by growth factors and receptors derived from the embryo and the maternal uterus, and for this reason, group culture has been a topic of debate leading to the development of comparative studies between individual and group culture of embryos, intending to promote an improved culture system that meets the needs of successful embryonic development (Swain, 2015; Wydooghe *et al*., 2017).

1.4.2 Mechanisms

The hypothesis in question is based on the positive effect of the interaction between embryos that promotes the embryo's mutual development and the neighboring embryos through the synthesis of factors released among

themselves. In this case, to achieve this trophic stimulation, it is suggested to change the embryo's microenvironment through the secretion or elimination of various factors in the culture medium, resulting from the culture's cooperative communication in the group. The secretion of these factors occurs through different mechanisms (Wydooghe *et al*., 2017), among them:

(i) Active secretion - is based on the synthesis of secretory proteins in the ribosomes, which are subsequently referred to as secretory granules on the cell surface and released into the culture medium (Burgess & Kelly, 1987; Wydooghe *et al*., 2017).

(ii) Passive flow - Proteins formed in ribosomes can be passively released extracellularly (Wydooghe *et al*., 2011).

(iii) Protein-facilitated transport - Hydrophobic factors can be released through a molecule-transporting protein (Wydooghe *et al*., 2017).

(iv) Extracellular vesicles - Lipophilic factors, micro ribonucleic acid (RNA), and proteins can be secreted through extracellular vesicles and exosomes that assume essential functions and are triggered by biological processes such as cellular communication (Pavani *et al*., 2017).

1.4.3 What happens in animals

The effect of group culture was evaluated early in animal studies. Lane and Gardner determined the benefit of embryonic communication by demonstrating that the pre-implanted mouse embryo produces one or more factors that stimulate embryonic development and promote viability after the transfer by increasing the density of mouse embryos' culture (Paria and Dey, 1990; Lane and Gardner, 1992). The study of group culture in mono-ovulatory animals in sheep embryos' culture also revealed improvements in blastocyst hatching rates when cultivated in groups of four embryos in 20 µl microdroplets of culture medium (Gardner *et al*., 1994). Later studies have also demonstrated the positive effect of group culture on bovine embryos cultivated in groups of five embryos, with a blastocyst formation rate of 41% versus 28% obtained through individual embryo culture (Keefer *et al*., 1994). According to the data obtained, the subject revealed more interest in scientists and different investigations derived from these results (O'Doherty *et al*., 1997; Khurana and Niemann, 2000). The number of embryos per microdroplet was increased at the same time as the rates of blastocyst formation. It was later determined that, despite the aforementioned beneficial effect, there is a limit for embryonic density from which embryo development appears to be compromised (Nagao *et al*., 2008; Reed, 2011). This information promoted researchers to determine the optimal embryonic density for each species, maintaining the permissive that animal embryos' group culture promoted embryonic development. The data obtained in the animal field suggest a visible advantage in the culture of group embryos and the potential of embryotrophic factors beyond the species (Reed *et al*., 2011). This revelation allowed the insistence of a more in-depth investigation into human embryos' study, which could also provide the same benefits (Reed *et al*., 2011).

1.4.4 Human results

Although presenting a lower number of references compared to an animal study, the beneficial effect of group culture of human embryos exposes data that reveal the influence of embryonic density on the development of the embryo *in vitro* (Ali, 2004). In 1995, Moessner and Dodson presented a comparative study of fifty-five patients with embryos cultured individually and in groups. The result showed increased significant cleavage rates, although the morphology showed no improvement compared to individual embryo culture (Moessner and Dodson, 1995). A year after this demonstration, positive data emerged that revealed a noticeable increase in the group culture's pregnancy rate (Almagor *et al*., 1996). Although the results' reliability is reduced due to patients' variability, these were the first references in humans that indicated the possibility of the benefit of group culture (Reed *et al*., 2011). Later studies compared the culture of embryos up to day 3 of development and passed to an individual culture from this stage to

select the transferable embryo morphologically. This methodology aimed to assess the advantages of group culture during the cleavage process. (Rebollar-Lazaro and Matson, 2010). The results showed that the group culture did not influence the rate of pregnancy or implantation, although it showed a higher rate of formation of usable blastocysts (Rebolar-Lazaro and Matson, 2011). In this follow-up, the later projects added a study variable: a group culture with a physical barrier between embryos that share the same culture medium. This view would allow researchers to approach embryonic interactions and viability while maintaining the theory of factors released in the culture media. The results produced evidence that valued the interactions between embryos; the group culture of embryos without physical barrier showed rates of compaction and blastocyst formation superior to the group culture of embryos with a physical barrier (Ebner *et al*., 2011). This evidence demonstrated that the sharing of the culture medium is essential and the physical contact or proximity between embryos, possibly due to their interactions (Reed *et al*., 2011). The use of group culture is not yet standard practice in medically assisted reproduction laboratories since individual embryo culture can achieve viable pregnancies, albeit at rates that could be improved. One of the disadvantages of this methodology is the pejorative effect of low-quality embryos and their low influence on neighboring embryos (Smith *et al*., 2012). Further studies are needed to prove the group culture's efficiency and promote its reproduction (Smith *et al*., 2012; Tao *et al*., 2013).

1.5 Characterization of the embryo through the analysis of the culture media

One of the points in favor of this theme is the rise of the study of a transferable embryo selection methodology based on the culture medium's composition. This study boosts the in-depth knowledge of the culture media spent and introduces the theme of a systematic investigation of embryo metabolism. It is enhancing this way additional studies related to the composition of the culture media in evaluating embryos. Thus it is imperative to discover autocrine or paracrine factors that promote embryonic development, being essential in producing techniques that facilitate the described investigation.

1.5.1. Omiccs Science

The growing need for better and more effective techniques that embryonic aid development has been revolutionizing the analytical field to obtain promising and easily reproducible results. One of the emerging technologies in this context is presented as "omics" that encompass genomic, transcriptomic proteomic (Manzoni *et al*., 2018), and metabolomic tolls (Dettmer and Hammock, 2004). In this follow-up, through innovative techniques based on RNA, metabolites, or proteins analysis, it is possible to predict new complex interactions. Individually, genomics has contributed to the evolution of techniques that allow predictive diagnosis of embryonic viability: e.g., aneuploidy is a factor preventing embryonic development (Hassold *et al*., 1996; Hassold and Hunt, 2001) and identified as the most common cause of spontaneous abortion (Hassold and Chiu, 1985), this omic has been developing a technique that allows chromosome number analysis, through an invasive blastomere biopsy or trophectoderm procedure to thereby exclude aneuploid embryos. This advance in assisted reproduction has allowed the selection of viable embryos through standardized processes. It is duly approved by the European Society for Human Reproduction and Embryology (ESHRE) as approaches categorized by preimplantation genetic testing. At the proteomic level, the importance of analyzing secreted proteins for embryonic evaluation through methods such as MS and matrix hybridization is apparent. Scientists have studied the hypothesis that viable embryos have a unique proteome and that some of these proteins are secreted by the embryo to the medium that supports embryonic development. The analysis discovered that mammalian embryos secrete a PAF that acts autocrine as a trigger of embryonic viability, enhancing the embryo's survival (O'neill, 2005).

Metabolomic is also a promising methodology in studying metabolic regulation through the comprehensive analysis of metabolome, classified as including metabolic intermediates (amino acids, nucleotides, lipids), ATP hormones, secondary metabolites, and other signaling molecules. Metabolomics employs spectroscopic and chromatographic methods for the analysis of metabolomic profiles of complex biological systems. This technology's intended applications

have been based on the evaluation of the culture medium spent by the embryos. Initially, investigators analyzed means of individually cultured Day 3 embryos to obtain an average spectrum of successful pregnancies compared with the spectrum of embryos with failure in implantation and to generate an algorithm that gives rise to a viability score. They achieved positive results, which transmitted the concept of a selection system through metabolomic analysis (Seli *et al*., 2007). This study has developed interesting theories in the area that drives constant deepening to obtain better implantation rates. In the ascending embryonic selection method, the ultimate goal will be to determine a biomarker that can clearly and concisely predict the best transferable embryo. Simultaneously, and the basis of this project, the application of metabolomics to the optimization of the culture medium presents itself as a valuable tool in promoting a more extensive understanding of embryonic development. In this way, it is intended through metabolomics processes to evaluate the culture medium's ideal composition based on spectra that have value fertilization rates.

1.5.2 Cytokine analysis

The integration of cytokines in the embryonic culture media has been based on scientific studies that value this incorporation and ensure its practical support in embryonic development. These components synthesized by epithelial cells and leukocytes are characterized as biological regulators that regulate embryonic development, cellular stress, apoptosis, and promote implantation. Among the studies presented, we highlight those that focused on interleukine-1 that acts as an immune regulator, in the cytokine T helper cells as an aid to the mediation of reproductive events, the GM-CSF as regulators of embryonic development, support in embryo development and implantation, HB-EGF assists in implant failure through a genetic knockout and LIF also promotes embryonic development and implantation. For this reason, studies to deal with these components as biomarkers are essential for improving the performance of culture media through the integration of cytokines from the medium. In this sense, cytokine detection techniques are valuable ways to increase the rate of embryonic implantation (Simón *et al*., 1998; Lim *et al*., 2000; Hegde and Behr, 2012; Robertson *et al*., 2018).

1.5.3 Fourier Transform Infrared (FTIR) Spectroscopy

1.5.3.1 General concepts

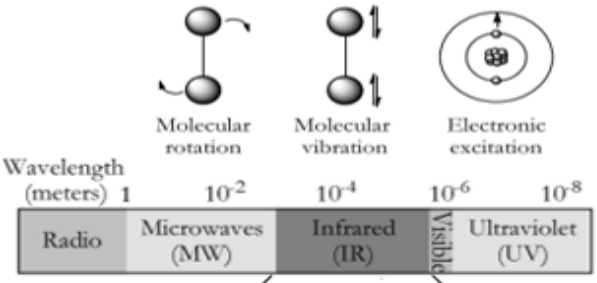
Following the need to characterize the environment that supports embryonic development, techniques with high potential are revealed, such as spectroscopies, highlighted by their accuracy and speed in analyzing samples and their components and by been non-invasive methods (Munoz *et al*., 2014). This analytical method is based on the production and interpretation of spectra obtained through the interaction between electromagnetic radiation and matter (Huang, 1971). To meet a wide range of analytical investigations, different spectroscopic methods arise depending on the species to be analyzed (atomic or molecular), the type of interaction (absorption, emission, or reflection), and the region of the spectrum that promotes different wavelengths of the emitted radiation (Pérez-Juste and Faza,2015) (Figure 1.7).

As shown in the figure 1.7, the electromagnetic spectrum comprises different regions with different characteristics to help identify substances by measuring the amount of radiation absorbed or emitted by the component under study. This tool promotes the characterization of the atomic structures that form the solutions and obtaining information on the types of bonds between atoms (Griffiths, 1972), the concentration of the component in the sample, and other increasing benefits (Agarwal and Atalla, 2000; Collingwood *et al*., 2005; Krafft and Sergo, 2006). This is determined by the observed frequencies characteristic of specific atomic species and methodically allows the analyses to be performed (Griffiths, 1972).

The spectrum presented translates into the set of electromagnetic waves, characterized by the oscillation of electric and magnetic fields, differentiated by frequency or wavelength. Electromagnetic waves are originated through the

movement of accelerated electric charges or during transitions (electronic, nuclear, or vibrational) between two quantized energy levels. In the same way that electric charges originate from radiation, electromagnetic radiation can also cause the movement of oscillation of the electric charges through the transfer of energy from electromagnetic waves to the component in which the radiation effects. In this case, molecules or chemical bonds with an electrical dipole suffer this effect from radiation (Keresztury, 2006).

The most perceptible type of electromagnetic wave for humans is visible light, composed of a range of 400 and 730 nm wavelengths. Although infrared radiation appears invisible to the human eye, it has proven to be valuable in research and analysis of unknown components and concentrations in a sample (Hsu, 1997).



	NIR	MIR	FIR
Wavelength (μm)	0.78 – 2.5	2.5 – 25	25 – 1000
Wavenumber (cm-1)	13500 – 4000	4000 – 200	200 – 10
Frequency (THz)	120 – 384	12 – 120	0.3 – 0.05
Energy (eV)	0.5 – 1.59	0.05 – 0.5	0.0012 – 0.05

Figure 1.7 – Electromagnetic Spectrum (Adapted from Verhoeven, 2017)

Infrared spectroscopy stands out as a technique that allows the analysis of samples in the three states of matter (gaseous, liquid, and solid). This technique is widely used in analytical processes due to its low cost of support and maintenance, ease of implementation, and efficiency in spectral acquisition (Ferrari *et al*., 2004; De Bruyne *et al*., 2018). By understanding that the bonding of two atoms in molecules presents several types of energy, infrared spectroscopy reveals itself as the technique that, through the radiation absorbed by the bonds, studies the atoms' vibrational energy. Infrared spectroscopy emerges as a promising analytical method in identifying numerous components and evaluating sample composition, applying to a large scientific scale. The infrared region in the electromagnetic spectrum is between the visible and the microwave regions (Fig 1.7), and the area referred to is further subdivided into near-infrared (NIR), medium infrared (MIR), and far-infrared (FIR). The wavelength associated with this region comprises values between 780 nm and 1 mm. For the characterization of a sample, the necessary procedure according to the methodology presented begins with the incidence of an infrared beam on the sample. Through specialized equipment, the frequencies passing through and the frequencies absorbed by the sample shall be determined. The absorption in the infrared region causes intermolecular vibrations, and the identification of functional groups is based on the detection of characteristic molecular vibrations of the components (Stuart, 2004; Wilson *et al*., 1980; López-Lorente, 2016).

1.5.3.2 Molecular Vibrations

Assuming a molecule is composed of atoms linked together with electromagnetic origin forces, it becomes understood that there are different possibilities of vibration. The various modes of normal vibrations of a polyatomic molecule, assuming N as the number of atoms, are represented by 3N-5 for linear. Three of these degrees are rotational, three translational, and the rest translate-in fundamental vibrations in a nonlinear molecule, thus giving rise to 3N-6 degrees of freedom (López-Lorente, 2016). The types of vibration differ fundamentally between the increase or decrease in the distance between atoms (elongation) and the change in the atoms' angle of connection (bending). Figure 1.8 shows the elongation variations (symmetrical or asymmetrical) and the bending movement (includes distinct movements such as scissoring, rocking, wagging, and twisting). (Griffiths, 1972; Berthomieu and Hienerwadel, 2009).

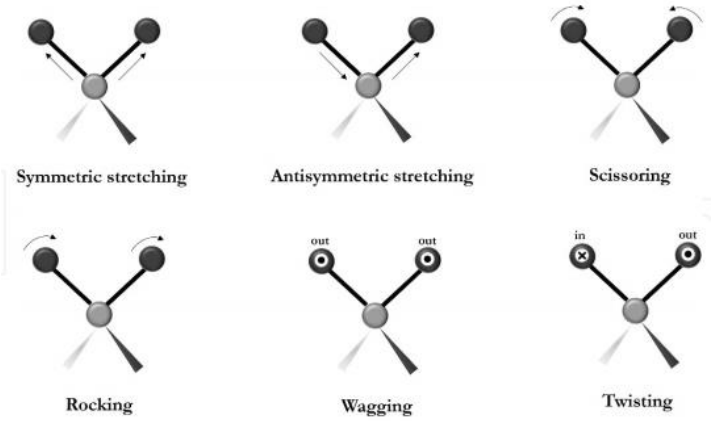


Figure 1.8 Variation modes of a molecule (Da Silva *et al.*, 2017)

The basis for the technique of characterizing atoms and molecules is that different functional groups absorb radiation at different wavelengths, translating into spectral peaks representing bonds that allow identifying a sample's components. In this sense, the IR spectrum represents molecules individually and uniquely as a fingerprint. This fact comes from the fact that the molecular vibrations suffer variance dependent on the atoms' mass, making up the molecules and organizing geometrically and binding forces. For this reason, the degrees of freedom are varied, being possible diversity of vibrational combinations valuable since the more complex the molecule presents, the more degrees of freedom will present (Smith, 2011).

1.5.3.3 MIR Spectral Analysis

The central region of red spectroscopy directly related to vibrational transitions is called MIR. This region is characterized by identifying molecular species through the analysis of specific vibratory absorptions. The MIR spectrum is divided into four distinct areas (represented in Table 1.2). This region is indicated to support the identification of biological samples since much of the infrared bands of interest occur in the wavelengths comprising the MIR region, thus promoting spectra with greater intensity and less complexity (Turker-Kaya and Huck, 2017).

Table 1.2 - The three regions from infrared region (near-IR, mid-IR and far-IR) (adapted from Atanasov,2018)

	MIR Regions			
	X-H stretching	Triple Bond stretching	Double-bond stretching	Fingerprint
Frequency (cm^{-1})	4000-2250	2250-2000	2000-1500	1500-400

1.5.3.4 IR Equipment

The equipment for spectrometric data analysis consists of a light source, optical system, and detector. The basic principle applied is the initiation of energy from a controlled source (usually carbon-silicon) directed to the sample, the phenomenon described above will be processed, and through a detector, the measurement of the intensity of the emerging beam takes place. How the incident beam is modulated will determine the optical principle of the spectrometer. Initially, the most widely used principle was dispersive. Different wavelengths of the radiation are applied separately from each other, using a prism inserted in a monochromator, which causes the light beam to diffract and produces the desired angular dispersion (Fuller and Smyrl, 1985).

With the development of techniques that promoted the improvement and improvement of existing equipment, the interferometric principle emerged that allows the temporal separation of radiation, originating at different times of arrival to the detector. An interferometer divides the light into two beams in which one of which reflects from a fixed mirror and the other beam from a movable mirror. The two beams are then recombined, resulting in an interferogram that translates into a sinusoidal variation of light intensity, whose frequency is a function of the wavelength (Fuller and Smyrl, 1985; El-Azazy, 2018).

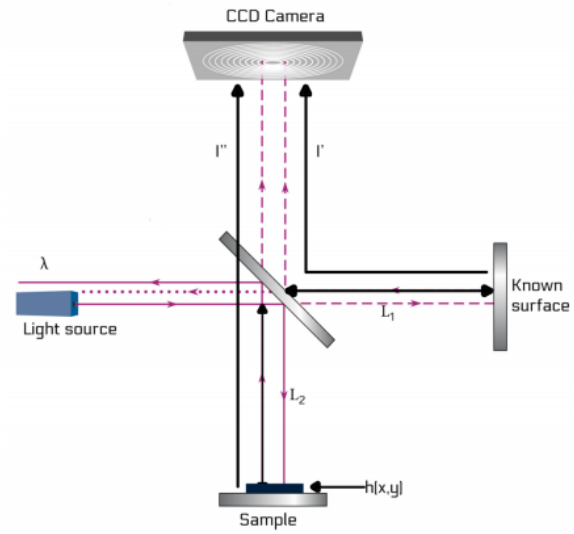


Figure 1.9 - Schematic diagram of IR dispersive spectrometer (López *et al.*, 2018)

1.5.3.5 Fourier - Transform Infrared (FTIR) Spectroscopy

In the Fourier transform spectroscopy, the beam emitted in the sample consists of several frequencies simultaneously. The device that compiles all the data can infer the amount of sample absorption at each wavelength. The main difference that distinguishes the two infrared spectrometers lies in the Michelson interferometer's presence introduced in the mid-1880 with notable advantages in optimizing the spectrometer traditionally used before its appearance. Interferometry is the application of a monochromatic radiation beam divided into two separate beams upon reaching an ideal beam splitter (Danzer, 1994). In this sense, 50% of the incident radiation will be reflected in one mirror, and the remaining 50% will be transmitted to the other mirror. The two beams are recombined, resulting in a single beam transmitted or reflected in the sample. This technique results in an interferogram that translates into a sinusoidal variation of the intensity of light, of which the frequency is the wavelength function (Movasaghi *et al.*, 2008).

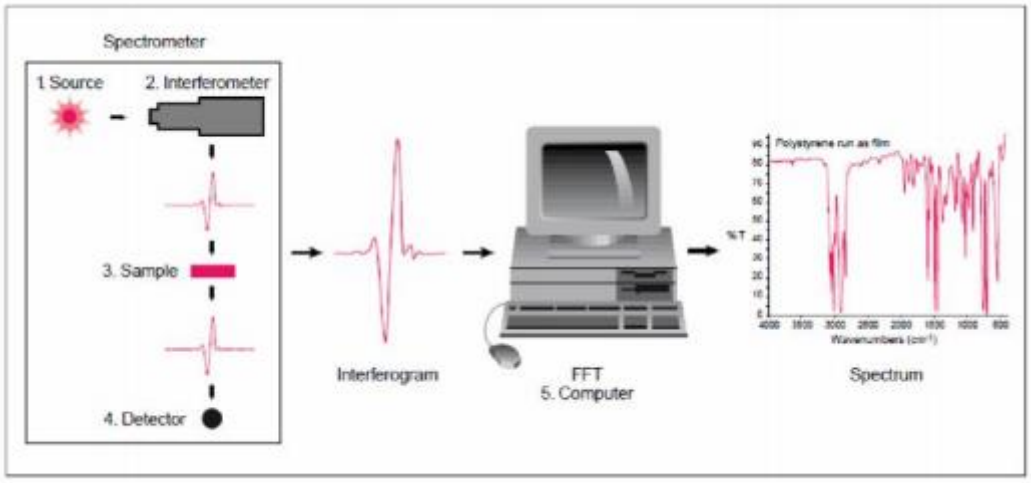


Figure 1.10 – Basic Components of a spectrometer (Gurumurthy and Ramesh, 2017)

This technology presents fundamental advantages to reducing the spectral acquisition time that passes from minutes to seconds, compared to dispersive spectroscopy. Time is saved once all wavelengths are evaluated simultaneously. Besides, this analysis allows a reduction of noise and the appearance of non-essential components for analysis. This analysis is more sensitive due to the optical system that enables the signal to be reached. Equipment maintenance features a user-friendly system, and no external calibration is required. The resolution of the analyzed spectrum is coherent for all frequencies, presenting greater accuracy in obtaining real results. However, FTIR spectroscopy requires concise protocols for the user to prepare samples that must be dehydrated as water absorption drastically alters the analysis results (El-Azazy, 2018).

1.5.3.6 General Applications

FTIR spectroscopy analysis has been applied in several areas, revolutionizing the spectroscopy era and boosting new investigations and interests by more recent authors. From areas such as agriculture, in the prediction of soil macronutrients and characterization of edible oils and fats (Vlachos et al., 2006), in environmental engineering to access the atmospheric, detection and identification of pollutant clouds in the atmosphere (Andreas et al., 1998). To advances in more scientific areas, such as biomedical, as in an identification of microorganisms in microbiology (Wenning and Scherer, 2013), application in drug resistance (Rak et al., 2014), characterization of leukocytes (Wang et al. .., 2018), deoxyribonucleic acid (DNA) methylation analysis (Banyay and Gräslund, 2002), studies on lung cancer cells in the pleural fluid (Wang et al., 1997).

1.5.3.7 Assisted Reproduction Techniques Applications

The use of the FTIR spectroscopy tool to support the investigation of assisted reproduction techniques has been gradually appearing in the scientific community. Sieme and colleagues studied the damage to the sperm cell membrane that compromises its function caused by cryopreservation. For this, they followed the characteristic peaks in the cell spectra and related them to the different phase changes of cryopreservation (Sieme *et al*., 2015). In 2013 Wiecheć *et al.* evaluated the FTIR spectroscopy technique's ability to determine the oocyte quality in bovine embryos, achieving promising results that boosted human embryo study (Wiecheć *et al*., 2013). In 2014, Giocchini *et al.* studied human oocyte quality using focal plane array (FPA) FTIR image spectroscopy after observing that the same technique had been applied to zebrafish oocytes to explore biological maturation processes (Giorgini *et al*., 2010; Carnevali *et al*., 2009). The

FPA FTIR spectroscopy image was used on individual oocytes to analyze the composition and molecular distribution of female gametes. Scientists were able to demonstrate a decline in oocyte quality in line with age, pointing to metabolic changes, in the plasma membrane, in the protein pattern, and the composition and structure of nucleic acids (Gioacchini *et al*., 2014). In another field, in 2017, Marchetti *et al.* tried to predict the blastocyst's potential in generating a biochemical pregnancy by analyzing the metabolic profile of the culture medium spent by the embryo, using the FTIR spectroscopy technique. They concluded that the spectral profiles of embryos that resulted in biochemical pregnancy are different from those that did not obtain through metabolomic differences in the regions of proteins, lipids, and carbohydrates (Marchetti *et al*., 2017).

1.5.4 Pre-processing methods and Multivariate Data Analysis

1.5.4.1 Spectra Pre-processing Methods

In the acquisition and analysis of spectral data, data pre-processing is fundamental for obtaining coherent data; undesirable components naturally arise that interfere with the intended signal analysis as noise and background. In this case, it is essential to remove the spectra's baseline to perform the spectra's useful quantitative analysis (Schulze *et al*., 2005) With this need, algorithms are developed to separate the nonfundamental signals according to the spectrum's characteristics, providing optimized requirements such as visual presentation, numerical processing, and the required accuracy of any baseline correction methodology (Jarvis e Goodacre, 2005)

1.5.4.2 Atmospheric Correction

In the spectral acquisition, there are several barriers to be overcome to obtain reliable data. The presence of notorious atmospheric absorptions can corrupt the data set by including substances not corresponding to the sample composition to be analyzed, as is the case of components such as CO_2, H_2. In this follow-up, the strategy applied translates into an atmospheric correction (Maiolino *et al*., 1996).

Contamination of the test samples is present in additional bands or spectrum distortions, severely disrupting multivariate analysis. Another frequently used method in atmospheric correction is subtraction to eliminate non-fundamental components of the sample, such as water vapor and carbon dioxide. One way to assist this correction is by removing spectral data from the water vapor. This step is achieved through the second spectrum derivative in the region 1750-1900 cm^{-1}, where water vapor lines are well defined (Lasch, 2013).

1.5.4.3 Baseline Correction

Data pre-processing involves fundamental steps that allow the correction of baseline distortions in the spectra. In this case, baseline correction is required due to environmental factors such as the light source, temperature, humidity, and vibration. This interference often occurs during continuous working periods that give rise to background variation due to non-core overlapping spectra with well-defined characteristics that influence the correct interpretation of analyzed data (Rowlands e Elliott, 2011).

For the quantitative and qualitative analysis of the baseline shift spectrum, it is essential to correct the spectrum base to promote the model forecast's improvement. This optimization can be achieved manually or automated (Peng *et al*., 2010). The diversity of correction techniques is presented in more straightforward methods that translate the spectra's shift from zero to a constant value, where only the subtraction of the spectrum in question will be necessary. In more complex methods, such as the use of linear or polynomial functions, in cases where the data meet away from the

baseline by an inclination, the difficulty is based on discovering the function suitable for adjustment, achieved through algorithms developed for this purpose (Peng *et al*., 2010).

1.5.4.4 Normalization

This optimization will result in the rescaling of data, presenting in a regularized way variables equivalent in terms of magnitude and minimizing the effects of fluctuations and variations of the data set analysed. A normalized spectra will be scaled and offset correctly (Lasch, 2012). There are mainly two types of normalization: a simpler ones that use the information directly from the spectra as peak normalization, where the intensity corresponding to the frequency of a certain band is used as a reference (usually Amide I band, at around 1650 cm^{-1}). And more complex ones, where are used more complex reference spectra, for example, the Multiplicative Scatter Correction (MSC) that need prior data modelling and estimation of the correction coefficeints to remove unwanted scatter effects (Gautam *et al*., 2015).

1.5.4.5 Derivatives

The application of derivatives in the pre-processing of the spectra aims to obtain an amplification that translates into the treatment of overlapping bands and selection of wavelengths that reveal more useful analytical information that would be hidden without using this method (Griffiths, 2010). The first derivative is related to peak resolution, while the second derivative is applied to reveal peaks in more complex spectra through the resolution of overlapping bands. The last one proves to be engaging in investigating biomarkers, highlighting differences between groups or clusters. These derivatives reduce the signal and amplify the noise but these issues can be easily minimized applying a Savitzky-Golay (SG) filter, (Savitzky and Golay, 1964; Vivó-Truyols and Schoenmakers, 2006).

1.5.4.6 Multivariate Data Analysis

Multivariate analysis translates into a study based on pattern recognition methods that allow determining the degree of interaction and finding the relationship between the samples through evaluating their spectra. This tool allows the simultaneous analysis of many variables and complex data sets (De Bruyne *et al*., 2018). The main multivariate analyzes used in this FTIR spectroscopy project are listed below.

1.5.4.6.1 Principal Component *Analysis (PCA)*

This mathematical method allows the reorganization of information from a data set, proving to be quite useful for occasions when one requires the analysis of a high number of variables, as is the case of spectroscopic data (Davies e Fearn, 2004) This methodology is widely used in pathological classifications on the evaluation of the origin and pathogenicity of Hodgkin's lymphoma (Brune *et al*, 2008), on the category of astrocytomas and malignant astrocytomas (Mckeown e Ramsay, 1996), studies based on K562 leukemia cells (Ong *et al*., 2012) and in the differentiation of hyperplastic colon polyps, among others.

In this case, the PCA presents new independent and orthogonal variables, called "Main Components," vectors in variance scale, representing most data variability. This scenario allows the samples evaluation in fewer variables than those presented, revealing only the essential information to be taken into account. The intention is based on the detection of patterns to facilitate future analyses. In a first perspective, the first revealed pcs present a maximum variation in the database. The following pcs represent the most significant variation according to the orthogonal direction to PC1 (Abdi e Williams, 2010).

1.5.4.6.2 Hierarchical Cluster Analysis (HCA)

The HCA is presented as an effective methodology in grouping different groups under investigation, complementing the previously mentioned techniques. It translates into a data analysis procedure that gives rise to a hierarchy in a data set classification. This classification organizes the groupings into levels ordered from an empirical criterion. In this case, two main approaches are revealed: Adaptive Hierarchical Clustering Analysis (AHCA) and Dual Head Cluster Analysis (DHCA). In the first instance, all data set characteristics are evaluated as belonging to a single cluster until they are referred to as smaller sets based on the variation of spectral data sets. This division follows the calculation of a distance matrix that analyses the similarity of the obtained spectra. The distance between two clusters is defined as the minimum of all distances between the two clusters. An interpretation is observed in the AHCA method (Gautam *et al*., 2015; Köhn and Hubert, 2014).

In the AHCA method, which is the focus of this investigation, an agglomerating interpretation is observed through a bottom-up approach where the user decides the number of initial clusters clustered according to the distance criterion is routed to a single cluster (Köhn and Hubert, 2014).

Chapter 2: Thesis Objectives and Work Structure

Culture procedures have been studied and optimized to increase the quality of embryos in *in vitro* fertilization processes and the consequent implantation rates. One factor that positively affects the quality of embryos is the culture of embryos in groups of 2 to 3 instead of individual culture. This thesis aims to understand the biological mechanisms of embryonic interaction to identify which factors can increase the development rate and, consequently, the cumulative success rate of PMA treatments.

This dissertation's objective initiates with a comparative FTIR spectroscopy analysis of several human culture media, available commercially. Here we intended to demonstrate that this tool is capable of detecting significant differences between molecular profiles of human culture media. Once we verified its capabilities, we aimed to employ it for analysing used media culture, in which embryos have grown, to gain insight into the factors linked to increased embryo development.

The next step was a FTIR spectroscopy analysis of the spent culture media by murine embryos, provided by the Champalimaud Foundation. This investigation aims to relate the culture's metabolic profile with the number of embryos cultivated, thus reinforcing the general objective of confirming the benefit of group culture.

Finally, a retrospective study with data from a private fertility clinic was carried out, comparing existing data of embryo group culture versus individual culture, with the objective of verifying if there are differences, in order to be able to characterize them in the future through the FTIR spectroscopy.

Chapter 3: FTIR spectroscopy as a tool to compare the spectral profile of human embryo culture media

3.1 Introduction

Human embryo culture for *in vitro* fertilization (IVF) is a system composed of several factors that support and enhance embryonic development. This system aims to create conditions that mimic the *in vivo* environment during the natural conception (Biggers, 2004; Smith, 2012; Chronopoulou and Harper, 2015). Culture media used in human-assisted reproduction laboratories can be produced "in-house" or obtained commercially. Commercial options are preferable as they follow stricter quality control and good manufacturing practices and standardized systems, i.e. the commercial media presents greater coherence in the culture media produced, specifically in incorporating the necessary nutrients in constant concentrations, leading to increased confidence in the performance of assisted reproduction procedures (Mauri *et al*., 2001; Lane and Gardner, 2007; Consensus, 2019).

There are sequential media and continuous media. The sequential media aims to mimic the different environments that the embryo is exposed to during its transit from the fallopian tubes to the uterus. Usually in this type of culture, media is changed at day 3 of the embryo development due to high metabolic changes that the embryo undergoes on that period. The continuous or single-step culture media, implies a unique media during the *in vitro* embryo development, minimising contamination process, but also minimizing alterations of the embryo environmental conditions, as temperature and CO_2.There are disadvantages and benefits of each approach. Continuous culture media have a strong point on reducing embryonic manipulation and the resultant destabilization of the culture conditions (pH, osmolarity, temperature, and air quality). Consequently, this avoids increased stress embryo that could lead to embryonic unviability. However, the "single step" media cause a concern related to the degradation of the continuous culture media and the consequent depletion of substrates essential to embryonic development and the accumulation of organic components that can compromise the embryo's viability (Reed *et al*., 2009; Cimadomo *et al*., 2018; Swain, 2019). Choosing the appropriate type of culture media and controlling the other laboratory factors that may impact its performance is one of the most critical tasks in the IVF laboratory. As reported, evidence shows that the culture media can affect the embryonic quality and the total number of viable embryos ultimately obtained. (Swain, 2011; Swain, 2015; Group, 2020). This fact points out as relevant because the cumulative success rates of an IVF cycle are very dependent on the number of viable embryos obtained (Swain, 2011; Swain, 2016; Group, 2020). Since there is still no consensus among clinical programs about which is the most appropriate media type, usually, assisted reproduction clinics may use both culture media strategies (Ciray *et al*., 2012; Morbeck *et al*., 2017; Gruber and Klein, 2011).

The need to characterize the environment that supports embryonic development to obtain better rates has resulted in the emerging and use of techniques, such as infrared spectroscopy (Munoz *et al*., 2014). This technique presents the advantages of offering a high sensitivity for the study of media constituents, being used in various fields of biomedical research (Bellisola and Sorio, 2012; Seli *et al*., 2007; Zandbaaf *et al*., 2020), and with the possibility of being applied to the area of assisted reproduction This method is based on the study of vibrational energy of molecular bonds due to radiation absorbed (Stuart, 2004; Wilson *et al*., 1980; López-Lorente, 2016). Table 3.1 represents some of the vibrational frequencies in the mid infra-red spectra associated to molecules presents in biological samples, as cancer cells (Bellisola and Sorio, 2012) and biological tissues (Movasaghi *et al*., 2008)

The present study aimed to compare the composition of seven distinct culture media (including sequential and single culture) using FTIR spectroscopy and, thus, to correlate possible differences and similarities between commercially available culture media.

Table 3.1. Examples of vibrational frequencies within the mid-infrared region (MIR) of the electromagnetic spectrum present in biological samples (adapted from Bellisola and Sorio, 2012; Coates 2006).

Wavenumber	Functional Group	Biochemical Component
3500-2500	*X-H stretching vibrations (where X is C, O or N)*	
3300	N-H	Amide A: peptide, protein
3100	N-H	Amide B: peptide, protein
2957	$C\text{-}CH_3$	Lipids
2920	$-(CH_2)_n-$	
2872	$C\text{-}CH_3$	
2851	$-(CH_2)_n-$	
2000-1500	*Fundamental stretching vibrations of double bounds*	
1740	$-CH_2\text{-}COOR$	Phospholipid esters
1725	C=O	Citric acid
1655	O=C-N-H	Amide I: peptide, protein
1645	H-O-H	Water
1545	O=C-N-H	Amide II: peptide, protein
1500-600	*The "fingerprint region": many overlapped vibrations*	
1450	$-(CH_3)_n-$ $-(CH_2)_n-$ $-(CH_3)_n-$	Lipid, protein
1395	$-(CH_2)_n-$ -O-C=O	Lipid, protein
1380	$C\text{-}CH_3$	Phospholipid, fatty acid, triglyceride
1400-1200	O=C-N-H, CH_3	Amide III peptide, protein, collagen
1245-1230	$RO\text{-}PO_2\text{-}OR$	DNA, RNA, phospolipid, phosphorylated protein
1170	R-COO-R’	Ester
1150	C-O, C-O-H	Carbohydrates
1105	C-O, C-C	Polysaccarides, Glycogen
1095, 1084 and 1070	C-O,C-O,H	DNA, RNA, phospolipid, phosphorylated protein
1078	$C\text{-}CH_3$	Glycogen
1060,1050 and 1015	C-O	DNA and RNA ribose
1050	C-O-P	Phosphate ester

1031	C-O-H	Glucose
965	C-O	DNA and RNA ribose
950	P-O	Phosphorylated protein
920	C-O-P	Phosphorylated protein
895-895	C=C	Alkene
750-700	N-H	Secondary amine
730-665	C=C	Alkene
710-685	CH_2-S-(C-S-stretch)	Thiol or thioether
690-500	C-Br and C-I	Halo compounds

3.2 Materials and Methods

Culture Media

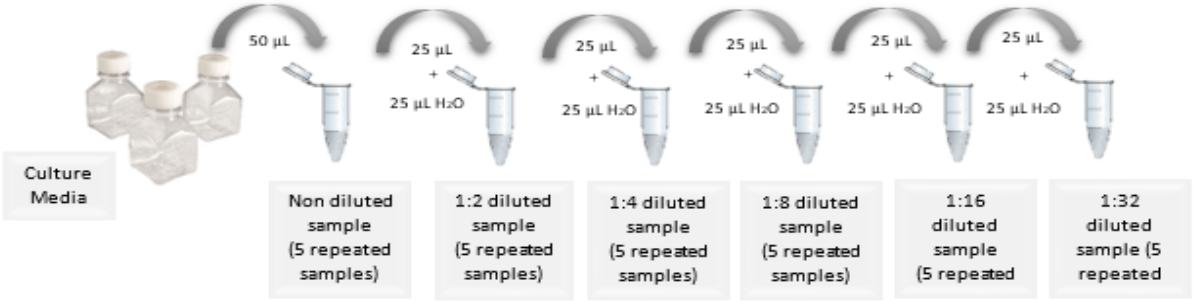
Seven commercially available human preimplantation embryo culture media were collected from five fertility centers and analyzed by FTIR-spectroscopy. Three one-step culture media "("SAGE 1-STEP", "G-TL Vitrolife" and "GERI") and four continuous culture media (two cleavage stage – "G1 PLUS with albumin" and "Sequential Cleav with phenol red" - and two blastocyst stage – "G2 PLUS with albumin" and "Sequential Blast with phenol red") were considered (Table 3.2).

Table 3.2 – Summary of collected culture media

Culture	Reference	Lot	Culture Media	Composition's details according to the manufacturer.
CONTINUOUS	67010010AS	20290097	SAGE 1-STEP	Magnesium sulphate, Potassium chloride, Potassium phosphate, Sodium chloride, Aminoacids, L-alanyl-L-glutamine, Calcium-L-lactacte, Glucose, Sodium pyruvate, Sodium bicarbonate, EDTA, Gentamicin, Phenol red.
	509043	508812	G-TL Vitrolife	Bicarbonate buffered media containing hyaluronan and human serum albumin.
	ONE-50	J000004267	GERI	Geri® Media is supplemented with human serum albumin and gentamicin. Contains glutamine as a dipeptide to reduce harmful ammonia levels
SEQUENTIAL	10132	508974	G1 PLUS w/ albumin	G-1 PLUS is a bicarbonate buffered media containing hyaluronan. G-1 PLUS also contains human serum albumin.
	509043	508812	G2 PLUS w/ albumin	G-2 PLUS is a bicarbonate buffered media containing hyaluronan. G-2 PLUS also contains human serum albumin.
	83060060	20330063	Sequential Blast with phenolred	Sodium chloride, Sodium dihydrogen Phosphate dehydrate, Magnesium Sulfate heptahydrate, Sodim Bicarbonate, D-(+)-glucose, Calcium Lactate, Sodium Pyruvate, Non-essential amino acids, L-alanyl-L-glutamine, Calcium pantothenate, Folic acid, Gentamicin sulfate, Tri-sodium citrate dehydrate, EDTA, Albumin, Potassium chloride, Taurine, Essential amino acids, Sodium hyaluronate.
	83040060	20320064	Sequential Cleav with phenolred	Sodium chloride, Sodium dihydrogen Phosphate dehydrate, Magnesium Sulfate heptahydrate, Sodim Bicarbonate, D-(+)-glucose, Calcium Lactate, Sodium Pyruvate, Non-essential amino acids, L-alanyl-L-glutamine, Calcium pantothenate, Folic acid, Gentamicin sulfate, Tri-sodium citrate dehydrate, EDTA, Albumin, Essential amino acids, Sodium hyaluronate, Potassium Sulphate; SSR™, Choline chloride, D-Biotin, Inositol, Niacinamid, Pyridoxine HCL, Riboflavin, Thiamine HC

FTIR Spectral Acquisition

The 7 culture media were diluted in water in a sequential mode as represented in Figure. 3.1, from ½ till 1/32.



Dilutions (5 repetitions each)	No dilution	Dilution ½	Dilution ¼	Dilution 1/8	Dilution 1/16	Dilution 1/32

Figure 3.1. Sequential dilution of the 7 culture media conducted in water, from ½ till 1/32.

Triplicates of 5 µL of each culture media without dilution and diluted from ½ till 1/32 were transferred to a 384-wells Si plate and then dehydrated for about 2.5 h, in a desiccator under vacuum. Spectral data was collected using a FTIR spectrometer (Vertex 70, Bruker) equipped with an HTS-XT (Bruker) accessory. Each spectrum represented 64 coadded scans, with a $2cm^{-1}$ resolution, and was collected in transmission mode, between 400 and 4000 cm^{-1} . The first well of each column of the 384-wells plate did not contain a sample and the corresponding spectra was acquired and used as background, according to the HTS-XT manufacturer.

Spectral Data Analysis

Spectra were pre-processed by atmospheric correction, baseline correction and normalization to amide I (Table 3.3). First and second derivative spectra were computed from raw spectra using a Savitzky-Golay filter based on a second order polynomial over a 15- point window. Atmospheric and baseline corrections were conducted with OPUS® software, version 6.5 (Bruker, Germany) and all remaining pre-processing work and principal component analysis (PCA) and Hierarchical cluster analysis (HCA) were conducted with Unscramble®.

Table 3.3 Description of Pre-Processing methods used in the evaluation of culture media spectras.

Pre-processing Methods	Description
Atmospheric Correction	H_2O and CO_2 compensation
Baseline Correction	Rubber-band method
Normalization	Normalization to Amide I
1^{st} and 2^{nd} derivatives	2^{nd} order polynomial, with a Savitzky-Golay (SG) filter and a 15-points window

Absorbance ratios were determined based on spectra pre-processed with atmospheric and baseline correction and normalization.

The PCA and HCA were applied after combination of different pre-processing methods, such as:

- Atmospheric correction;
- Atmospheric and baseline correction;
- Baseline and atmospheric correction and normalization to amide I;
- Atmospheric and baseline correction, normalization to amide I and 1^{st} Derivative

- Atmospheric correction and 2nd Derivative;
- Atmospheric, 2nd Derivative and Normalization to Amide I

PCA identifies correlations between a set of variables and transforms the original data set into a new set of correlated variables, called principal components (PC). These PC's are a linear combination of absorvances at different wavelengths. The distance between samples corresponds to a similarity distance.

The HCA is based on comparing and categorizing the samples according to their variability. In this way, the data is divided into the number of clusters determined by the user. Each sample is included in the group according to its similarity based on Spearman's coefficient correlation (Trygg *et al*., 2009; Bombalska *et al*., 2011; Lindon *et al*., 2017).

3.3 Results

Figure. 3.2 represents spectra obtained from samples after diverse dilution degrees, were the undiluted samples showed non saturated signal, and a good signal to noise ratio. Thus, the following analyses were performed only on undiluted samples. Figure 3.3 shows the 5 replicas of spectra analysis of each non-diluted media. The median spectra were considered in the following analysis.

Culture media's median spectra

Figure 3.4 shows the median spectra to each culture media. The most relevant peaks in these spectra appear at 1122 cm^{-1} reporting the presence of carbohydrates; 1545 cm^{-1} associated with amide II, related to the presence of peptides or proteins; 1400 cm^{-1} can be related to the presence of phospholipids, fatty acids or triglycerides. Finally, one of the peaks with greater absorbance reveals itself at 1656 cm^{-1} and 3345 cm^{-1} associated with stretching vibrations of Amide I and flexion vibrations of Amide A, respectively, both related to the presence of peptides or proteins.

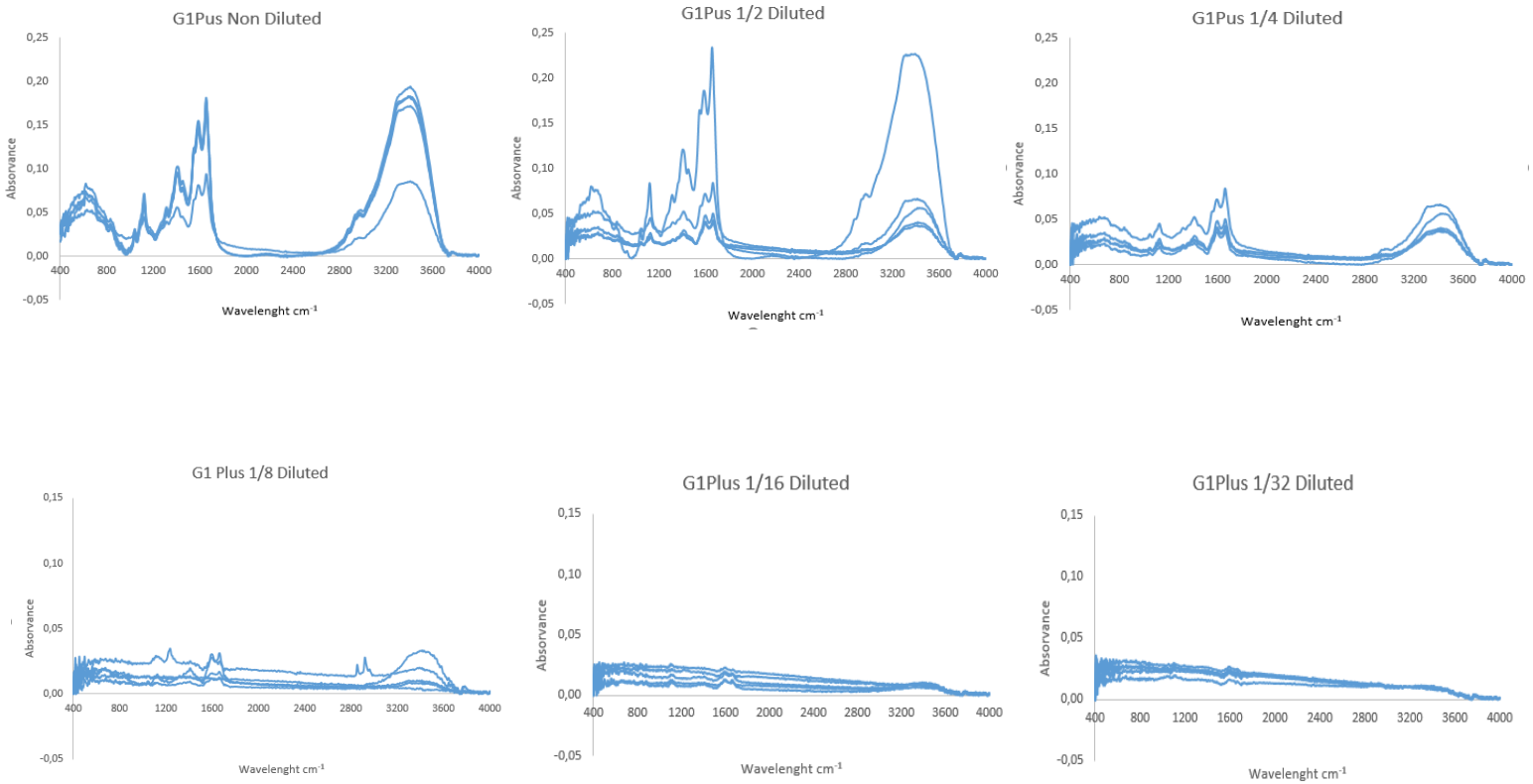


Figure 3.2. Spectra of all dilutions of five repetition samples, with atmospheric and baseline correction of G1Plus culture Media.

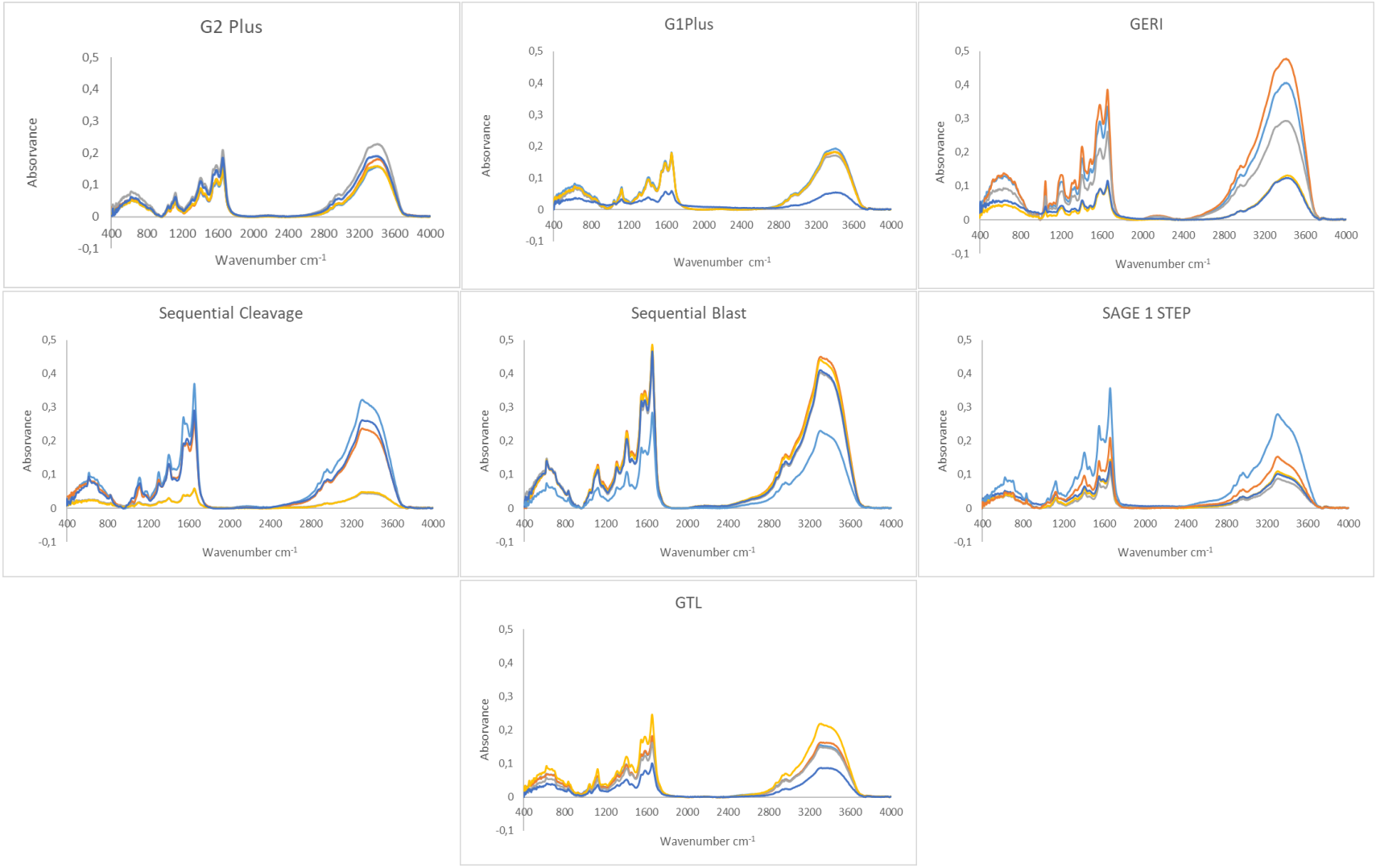


Figure 3.3. Quintuplicate spectra of non-diluted culture media samples, with atmospheric and baseline correction: (A) G2Plus Media; (B) G1Plus Media; (C) GERI; (D) SequentialCleavage Media; (E) SequentialBlast Media; (F) SAGE1STEP Media; (G) GTLVitrolife Media (each color represents a sample of the five repetitions of each culture media)

Detailed analysis of the media's average spectra in a specific range (from 400 to 1700 cm-1)

Considering the overall similarity between the median spectra of diverse media, and to highlight differences between spectra the region between 400 to 1700 cm^{-1} was analysed (Figure 3.4). The most interesting result was the GERI media that presented two peaks not presented in the other media at 1040 cm-1 and 1215 cm-1, corresponding to phosphate ester and Amide III of peptides, respectively.

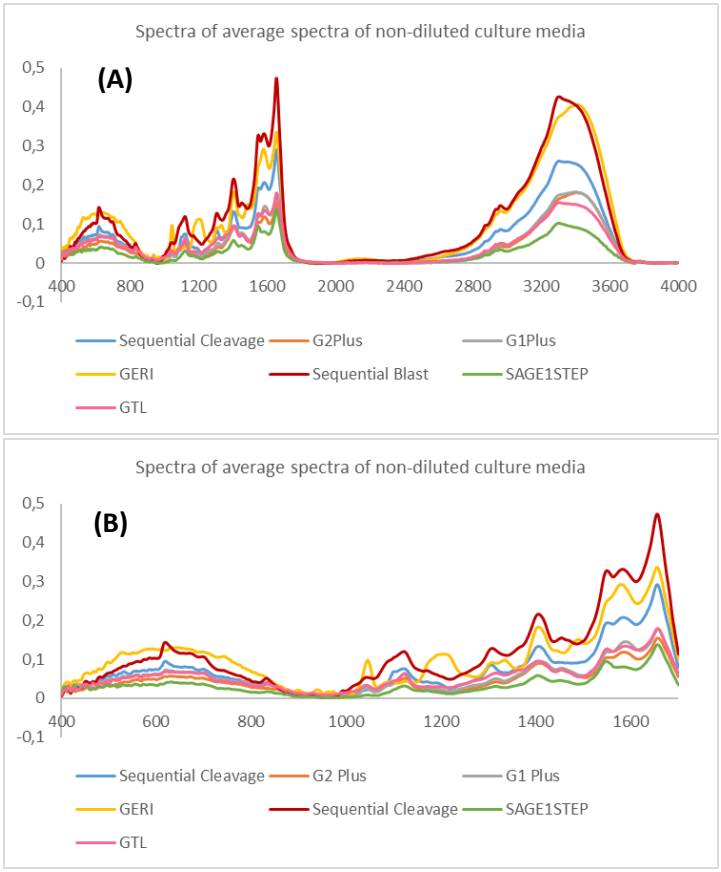


Figure 3.4 (A) Whole spectra of average spectra of non-diluted culture media (B) Extension of the lengths from 400 cm^{-1} to 1700 cm^{-1} obtained through Media's Average Spectra

PCA with different spectra pre-processing methods

The raw spectra were pre-processed with atmospheric correction to CO_2 and water. Baseline correction was applied to minimize dissimilarity between spectra due to baseline deviation. Normalization is used to rescale samples with the aim to gather all spectral data on the same scale. The first spectral derivative it is used to minimize the distortions, enhancing the resolution of peaks and the second derivative is used to rectify the overlapped bands. The application of these derivatives can lead to an increase noise, to minimize this effect and smoothing the spectral data, SG filters were applied (Griffiths, 2006).

The PCA enables to evaluate differences between samples, were similar samples are closed together in the score plot. The PCA also enables to discover which spectral regions discriminates the different clusters on the score plot.

When observing the PCA of the raw spectra (Figure 3.6), i.e. without pre-processing, there is no noticeable separation between samples of different culture media. Through the application of atmospheric correction, normalization to amide I and the 1st derivative (Figure 3.5A), the separation of the GERI culture media samples from other media is more evident, showing this pre-processing method as the best one applied. The application of the 2nd derivative does not show significant improvements in sample separation.

PCA based on sub-regions, 600 to 1800 cm^{-1}, 2800 to 3850 cm^{-1} and 600 to 1800 cm^{-1} with 2800 to 3850 cm^{-1} was also conducted. It was observed that the normalised spectral 1st derivative on these sub-regions also resulted on the GERI culture media separation from the other media.

Applying the PCA to the processed spectra (atmospheric and baseline correction peak normalization and 1st Derivative of whole spectra) also allowed us to view separately the PC2 (Y axis) loadings of this PCA, showed in Figure 3.5. In this Figure we can observe the PC2 loadings of PCA, based on the pre-processed spectra; this graphic highlights which spectral regions of the GERI media samples are different from the other culture media, as shown in graph A.

Thus, it is reported that the spectral regions that contributed the most to distinguishing GERI from the other culture media were 400-900 cm^{-1} and 1000-1800 cm^{-1}. From the literature, it is possible to verify that these peaks are characteristic of the presence of torsion of methoxy group; bonds in oligosaccharides such as mannose and galactose, phosphate vibration, carbohydrate residues and amide III vibration, present in glycogen, phosphate and oligosaccharides pointing out also relevant regions including amide I and II from proteins, phospholipids and carbohydrates (Belissola and Sorio, 2012; Movasaghi *et al.* 2008).

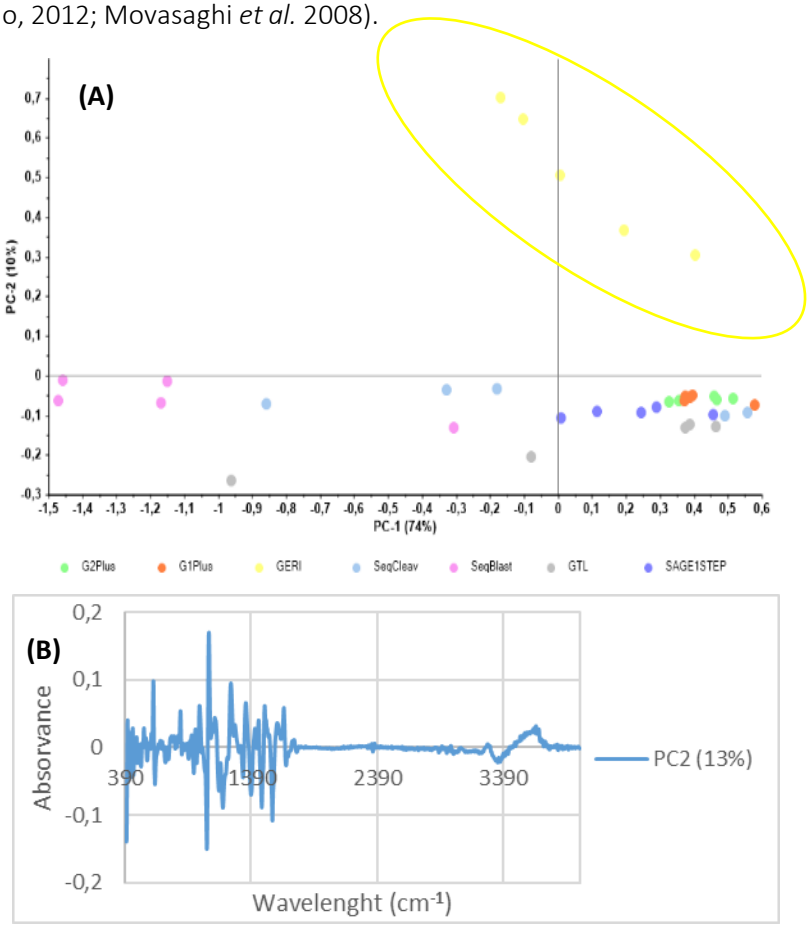
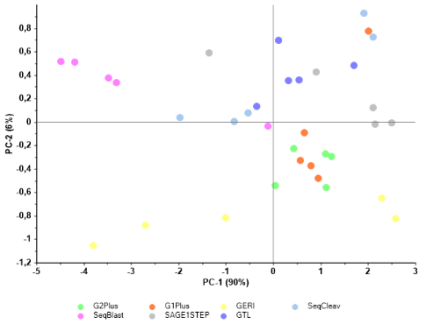
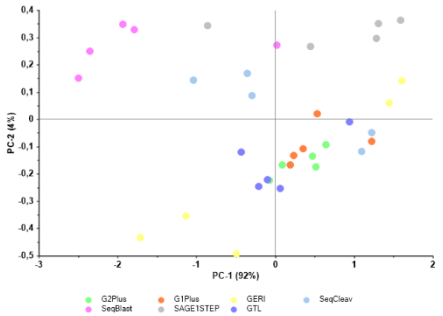
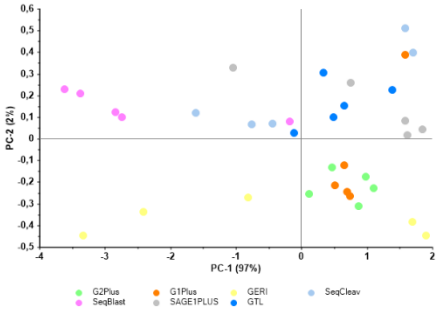
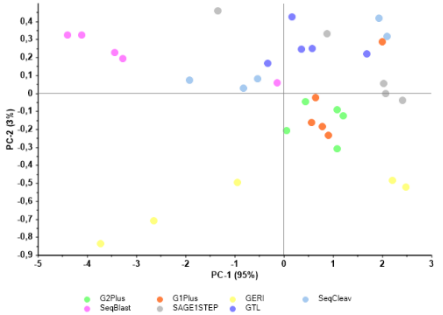
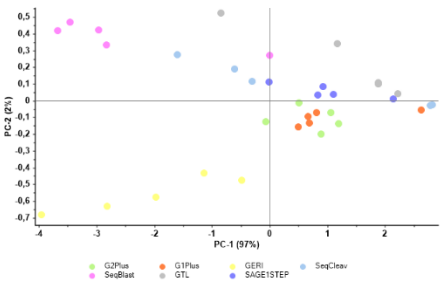
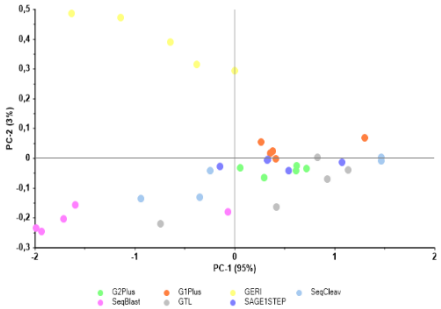
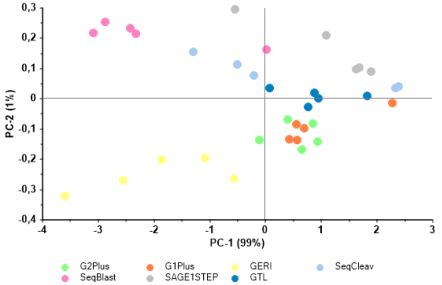
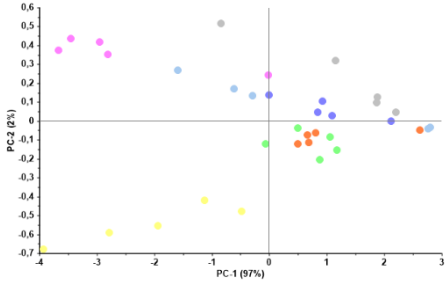
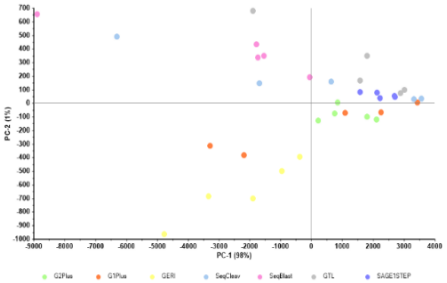
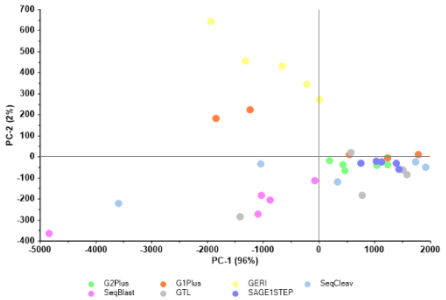
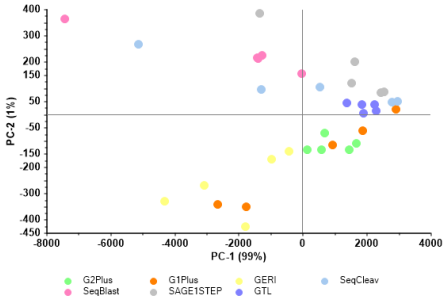
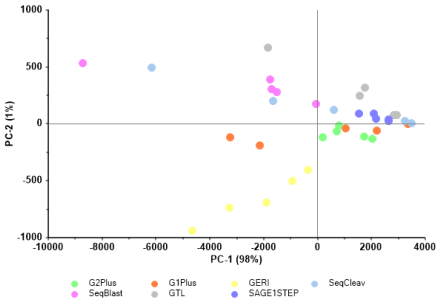
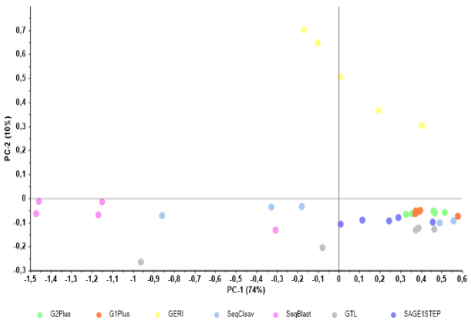
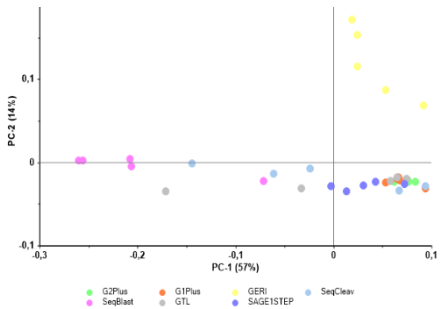
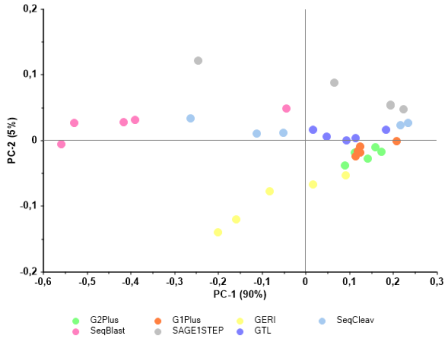
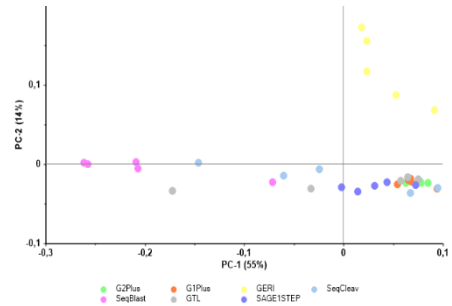


Figure 3.5 (A) PCA for spectra of all samples of culture media with atmospheric and baseline correction peak normalization and 1st Derivative.
(B) Loadings of PC2 of PCA with atmospheric and baseline correction, amide normalization, and 1st Derivative

	Whole Spectra	From 600 to 1800 cm^{-1}	From 2800 to 3850 cm^{-1}	From 600 to 1800 cm^{-1} and 2800 to 3850 cm^{-1}
No Pre-processing				
Atmospheric and Baseline Correction				
Atmospheric and Baseline Correction and Normalization				
Atmospheric Correction, Normalization and 1^{t} Derivative				

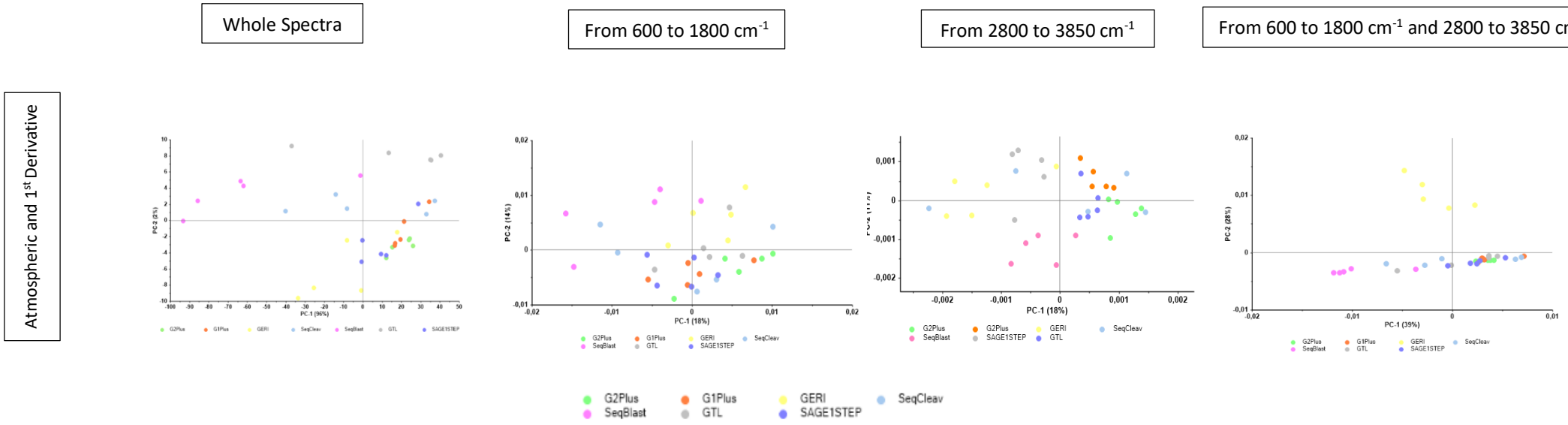


Figure 3.6 PCA with several pre-processing methods at differrent spectral ranges and sample grouping by culture media.

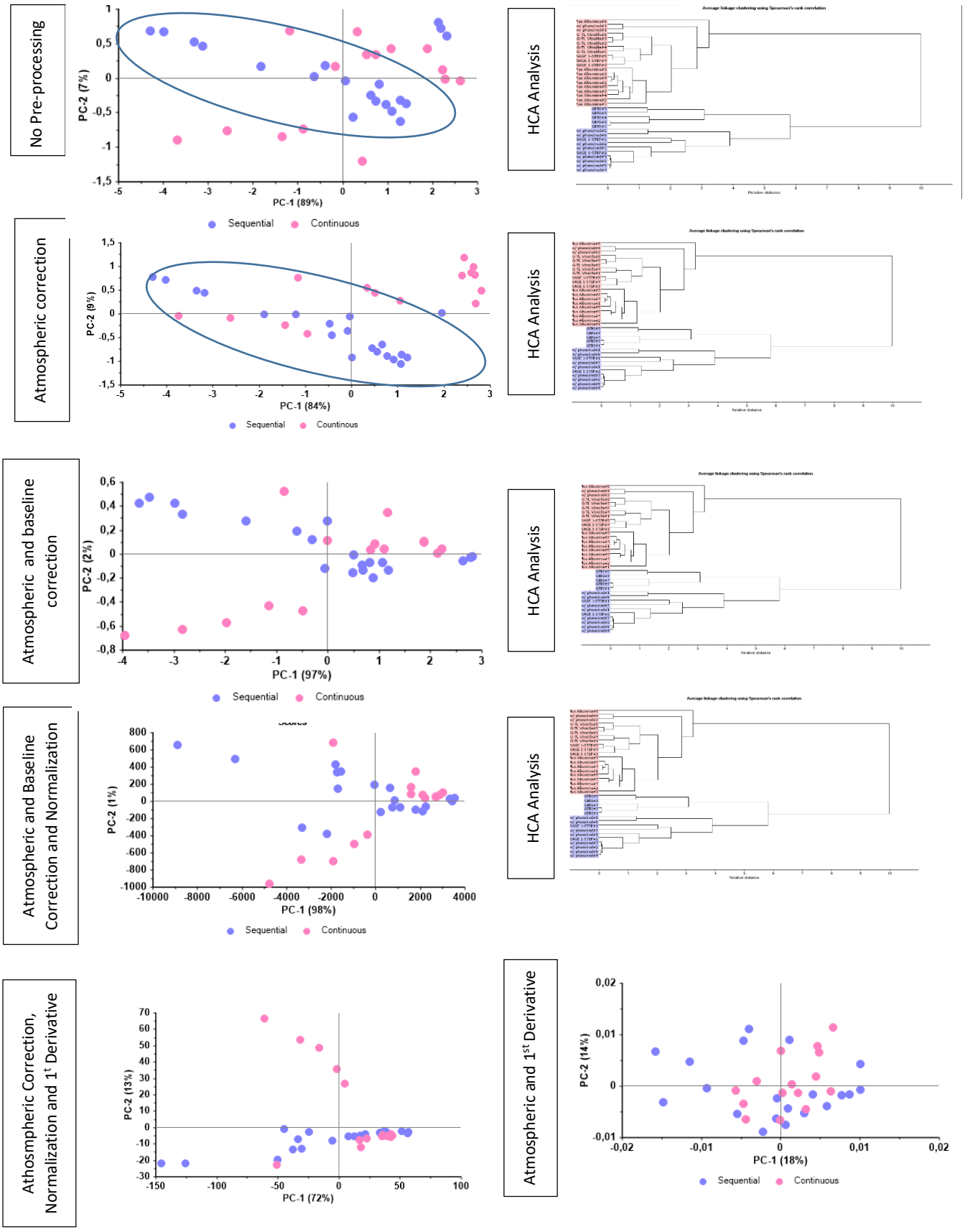


Figure 3.7 PCA with several pre-processing methods at different spectral ranges and sample grouping by type of cluture media with HCA analysis (Sequential – Blue dots vs. Continous – Pink dots

Comparison between sequential and continuous media through PCA

The same pre-processing methods were applied to assess differences between continuous and sequential culture media (Figure 3.7). In this regard, there is a clear relation between sequential media when no pre-processing methods are applied. This trend conveys a signature in the composition of these culture media, distinguishing them from continuous culture media. HCA analysis could not show two distinct clusters by type of culture (continuous *vs*. sequential) with neither pre-processing methods applied.

- Sequential culture media

Figure 3.8 shows the PCA's of sequential media spectra, with pre-processing of atmospheric and baseline correction and first Derivative. (A) shows a clear distinction between G1 and G2 Plus culture media compared with Sequential Cleavage and Sequential Blast culture media. The two samples of the Sequential Cleavage culture media (A) were excluded in order to obtain the loading of PC1, to visualize the spectra areas that most contributed to the separation of the remaining culture media (B).

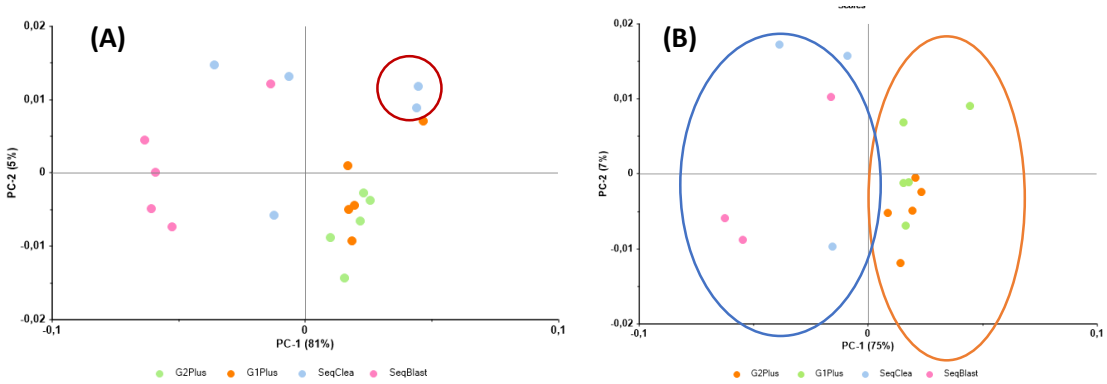


Figure 3.8 - PCA for sequential culture media spectra with atmospheric and baseline correction and 1st Derivative (A) evaluation of all samples from the four culture media (B) all samples but without Sequential Cleavage cultura media.

In Figure 3.9 it is shown PC1 loadings of PCA represented in Fig. 3.8(A), pointing the relevance of spectral regions between 600 cm^{-1} and 1700 cm^{-1} to discriminate the G plus media from the others culture media, pointing the contribution of phospholipid esters, lipids, proteins, fatty acids, and triglycerides (Belissola and Sorio, 2012; Movasaghi et al., 2008).

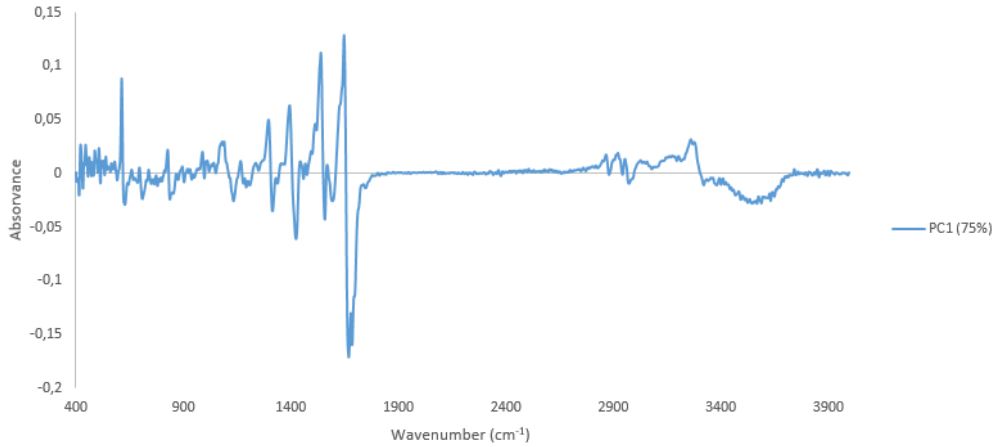


Figure 3.9 Loadings of PC1 of PCA of Figure 3.8(B) with atmospheric and baseline correction and 1st Derivative.

- Comparative analysis between G1 and G2 Plus culture media

In order to evaluate differences between culture media G1 and G2 (Figure 3.10), a PCA focusing only these samples were conducted (A) and the corresponding HCA (B) based on atmospheric and baseline correction. Although with a low data variance represented in the PC2 between G1 and G2 spectra obtained (3%), the loadings of PC2 are presented (C). Alternatively, HCA was carried out, confirming that G1 samples were different in composition in relation to G2.

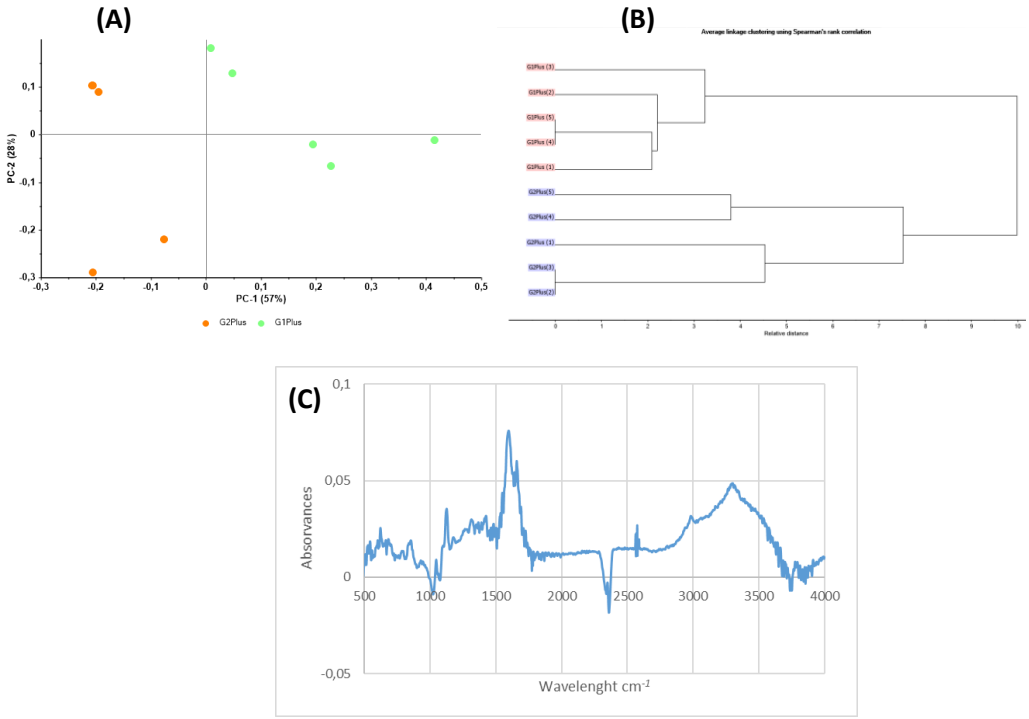


Figure 3.10 (A) PCA for spectra of samples of sequential culture media G1 and G2 Plus (only) with 2nd derivative and Amide I normalization. (B) HCA for spectra of continuous culture media samples with Atmospheric correction and 2nd Derivative. (C) Loadings of PC2 of PCA of Figure 3.1A(B) with 2nd derivative and Amide I normalization.

- Continuous culture media

The comparative analysis exclusively for the continuous media was presented in figure 3.11. This evaluation was also carried out with PCA support (A) and HCA (B), with the atmospheric correction and second derivative pre-processing methods. The results confirmed the first conclusions drawn at the beginning of the analysis: the GERI media stands out visibly from the rest in both analyses. The PCA also allowed to display the loadings of PC1(C) with a variance of 24%, showing peaks similar to those obtained previously, but with emphasis on the wavelengths 400 cm^{-1} and 1100 cm^{-1}, where a more significant variation is observed. These peaks suggest that the GERI media is distinguished from the others, namely in the regions of C-O stretching mode of C-OH groups of serine, threonine, & tyrosine residues of cellular proteins, polysaccharides, C-O stretching around peak 1164 cm^{-1} (Nandiyanto *et al.*, 2019).

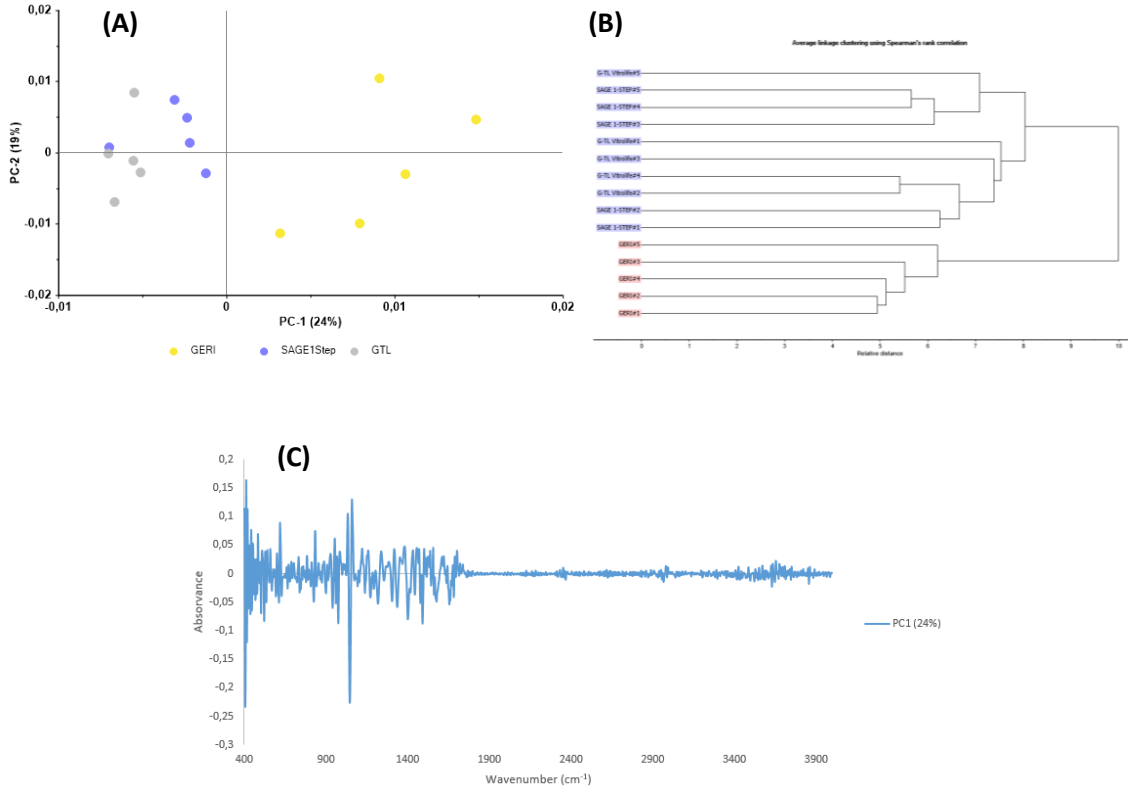


Figure 3.11 (A) PCA for spectra of samples of continuous culture media with atmospheric correction and 2nd Derivative. (B) HCA for spectra of continuous culture media samples with Atmospheric correction and 2nd Derivative. (C) Loadings of PC1 of PCA of Figure 3.12(A) with atmospheric and 2nd Derivative.

3.4 Discussion

The composition of seven distinct culture media, including sequential and single culture, was compared using FTIR spectroscopy, allowing to identify differences and similarities between the whole molecular composition of commercially available culture media used in *in vitro* fertilization laboratories. After different spectra pre-processing and multivariate analysis methods evaluation, it was observed a high variation in replicated analysis, most probably due to handling errors when conducting the FTIR spectroscopic analysis. Even so, it was found that samples of different culture media are grouped, revealing a distinction between composition of the two types of media of media, i.e. sequential *vs*. continuous. These findings suggest in the first phase that FTIR spectroscopy is a useful tool to acquire the whole molecular composition of culture media used in human-assisted reproduction.

It was observed that the GERI culture media presented spectral bands absent in the other media (Figure 5.4). This points out to the presence of specific compounds in this media, not present in the other formulations, such as phospholipids, esters and peptides. The continuous GERI culture media has been used in assisted reproduction clinics and also referenced in a study that comparatively evaluated its performance in the rates of excellent cleavage stage embryos, excellent or good blastocysts, biochemical pregnancy, and early pregnancy ultrasound, concluding that the embryologic outcomes obtained with this culture media were equivalent to the sequential medium of the same range (Hobson *et al*., 2016).

Similar to our investigation, Morbeck *et al.* (2014, 2017) compared two continuous media and five sequential media in 2014 and the composition of unique culture media in 2017. This work showed significant variations in the composition of the culture media analysed and proved that murine embryo culture composition had impact on the embryo development. These authors pointed that factors external to the composition of the media may also have influenced the results, such as oxygen tension. Also, this study applied animal embryo culture resulting in outcomes that cannot correspond to those available in human embryo culture. Also, in an attempt to determine an association between embryonic implantation and the metabolic profile of embryos, Scott *et al.* (2008) observed that Raman spectroscopy culture of media spent by human embryos offers excellent potential for rapid non-invasive assessment of the reproductive potential of embryos. These researchers observed changes in the spectra of the spent culture media according to the embryonic viability (Ali *et al*., 2013).

FTIR spectroscopic analysis do not allow the direct identification of the components in the solution. This technique only allows to indicate which functional group they belong. For the identification and direct quantification of the elements present, it would be recommended to complement this work with the use of liquid chromatography or gas chromatography-based methods (Morbeck *et al*., 2014; Morbeck *et al*., 2016)). Additionally, this work points to the necessity to a higher number of replicate samples.

3.5 Conclusion

Culture media have been developed based on the metabolic and physiological needs of human embryos. The commercially available media for use in ART laboratories, although suggesting obvious similarities, do not reveal their formulations for commercial reasons.

The present work, points the potential of FTIR spectroscopy in acquiring the whole molecular composition of these media, enabling to evaluate similar media, as sequential versus continuous, and between each type. The high resolution of the FTIR spectroscopy points to its potential application for spent culture media analysis to evaluate the effect of embryonic development, viability, euploidy or reproductive potential. The knowledge obtained in this work suggests a first step in the use of FTIR spectroscopy in the field of embryology, and that may have future applications.

Chapter 4: FTIR spectroscopic analysis of spent culture media of murine embryos

4.1 Introduction

In the previous chapter, it was concluded that FTIR spectroscopy could be an efficient tool in IVF studies. For that reason, the present study aimed to compare the spectral profile of culture media spent by individual and group-cultured murine embryos, in order to evaluate if FTIR spectral profiles revealed any differences between culture type in this animal model.

The growing need to support infertility patients to achieve the desire to conceive has triggered the development of new techniques and culture conditions in the area of assisted reproduction. Unfortunately, many of these innovations are applied without sufficient pre-clinical studies and evidence of their medium and long-term benefit (Dondorp and Wert, 2011; Harper *et al.,* 2012, Sharpe, 2018). This fact is derived from the moral and ethical sensitivity of the human embryo, resulting in less invasive studies in the human model for the advancement of assisted reproduction (Harper *et al.*, 2012). With this in mind, the animal model reaches an important level in the investigation of this area in validation and predictive studies of the efficacy of new techniques and culture conditions that can be introduced in day-to-day fertility clinics (Harper *et al.*, 2012). Currently, the regulation requires the "use animals first" (UAF) rule, were the use of human embryos are conducted previous to animal models only when animal models cannot be considered in advance (Jan *et al.*, 2018). Laboratory species, in particular mammals, provide irreplaceable *in vivo* systems and therefore contribute significantly to the advancement of biomedicine in humans (Harper *et al.*, 2012).

In this way, the conditions for the culture of animal embryos have been assessed from an early stage in order to promote their application to the human model and thereby improve embryo development *in vitro* (Garner and Lane, 2014). Numerous *in vitro* mammalian embryonic development studies have been presented and boosted the increasing evolution of assisted reproduction. In early 1890, Walter Heape reported the first successful embryonic transfer in rabbits and 1941 Kuhl presented himself as the first scientist to cultivate embryonic mice in a blood clot (Hammond, 1949; Biggers, 1991). Eight years later, Hammond recovered and

incubated up to the blastocyst stage mouse embryos, which later led to the attainment of live births of mice in 1958 (Biggers, 1991). From these achievements new techniques of cultivation and fertilization appeared and revolutionized the area of assisted reproduction in the animal and human model (Leese, 1998; Baldassarre and Karatzas, 2004; Ecker *et al.*, 2004; Galli, 2018).

Despite the progress shown, not all embryos develop to blastocyst suitable for implantation and, for this reason, the improvement of this parameter becomes important in increasing cumulative success rates (Medicine, 2012). In this context, evidence has shown *in vitro* fertilization process undeniably induces additional stress for the embryo, which would not normally be exposed in the *in vivo* system (Vaita *et al.*, 2010; Swain 2015). Minimizing this effect and promoting an ideal culture environment is essential to achieve positive results (Swain, 2015). One of the hypotheses developed in the field of improvement of culture systems is related to the need to include signaling factors that modulate cell growth and division in the *in vitro* culture of embryos (Wydooghe *et al.*, 2017). These factors are associated with autocrine and paracrine pathways inherent to embryonic development and which are promoted through group culture, which stimulates embryonic interactions through secretion or elimination of various factors in the culture media, beneficial for the mutual development between neighbouring embryos (Swain, 2015; Rebollar-Lazaro and Matson, 2010).

4.2 Materials and Methods

All animals and procedures with them were conducted at the Champalimaud Foundation Assisted Reproduction Service by Rodent Platform, with their own technicians. Animals used were used for purposes of 2-cell embryo cryopreservation, meaning that no animal was used specially for this project. Animals were from different strains of musculus with ages between 4 and 18 weeks old. In this project we only used data from IVF fertilization rates. The protocol used is summarized and illustrated in Figures 4.1 to 4.4.

4.2.1 Superovulation of females

1. Induce superovulation by injecting 0.1 mL of CARD HyperOva i.p. into a female mouse at 16.30pm.
2. Follow this up 48 hours later with a 7.5 IU (0.15 ml) i.p. injection of human chorionic gonadotropin (hCG).
3. 16 hours after hCG females are ready to be sacrificed.

4.2.2 IVF PROTOCOL

Dishes preparation (evening before day of IVF): all dishes are kept in an incubator at 37˚C with 5% CO_2.

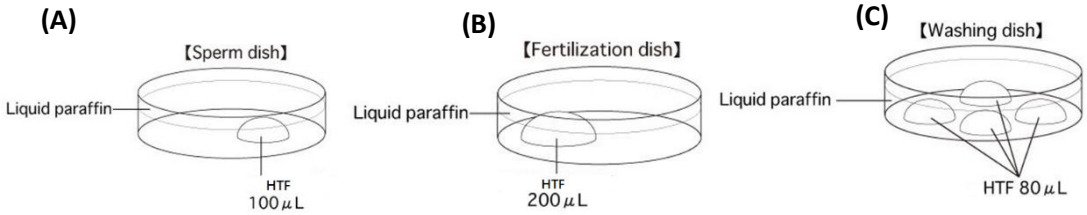


Figure 4.1 – A: Sperm dish (one drop (100µl) of HTF medium into a petri dish covered with liquid paraffin); B: Fertilization dish (1 drop (200ul) of HTF per female used in IVF, covered with paraffin); C: Washing dish (2 drops (80ul) of HTF per female used, covered with paraffin (we used 4 drops in each petri dish using 2 drops per female)).

4.2.3 COLLECTING OF SPERMATOZOA

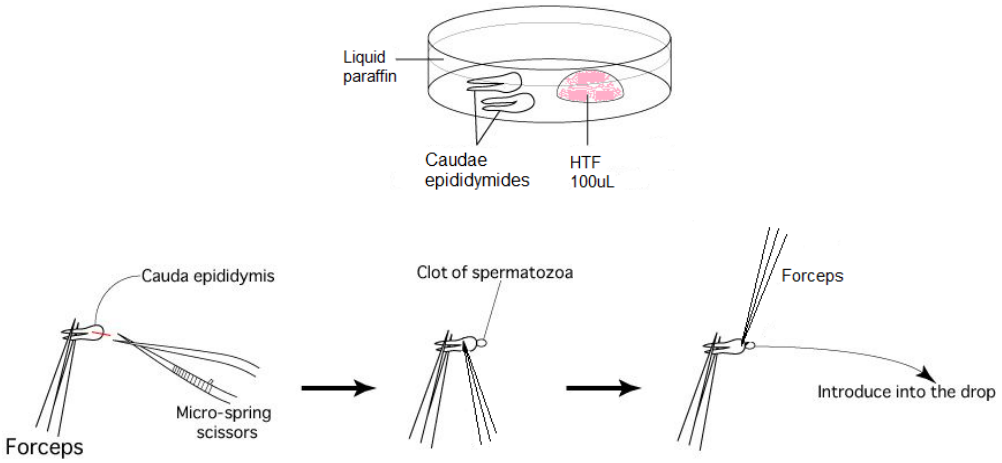


Figure 4.2 – Protocol used to collect spermatozoa

1- Sacrifice the mature male mice (10 weeks or older) and remove their cauda epididymides, avoiding as much fat, blood and tissue fluid as possible.
2- Place the removed cauda epididymides in the sperm dish.
3- Cut the duct of each cauda epididymis using a pair of micro-spring scissors, then use another forceps to gently press the surface of the cauda epididymis and release the sperm within.
4- Use the forceps to introduce the clots of spermatozoa released from the cauda epididymis into the drop of HTF.
5- Allow the sperm to capacitate by placing the suspension in an incubator (37°C, 5% CO_2 in air) for 60 minutes before insemination.

4.2.4 COLLECTION OF OOCYTES

1- Sacrifice a superovulating mature female mouse.
2- Dissect the mouse to expose the abdominal cavity.
3- Remove the oviducts (ampullae) only, avoiding as much fat, blood and tissue fluid as possible.
4- Immerse the removed oviducts in liquid paraffin contained within a fertilization dish.
5- Use forceps nº5 to hold the oviduct against the base of the fertilization dish, then use another one to open the ampulla of the oviduct and release the “cloud”, cumulus-oocyte-complexes (COCs) from within. Drag them into the drop of HTF.
6- Use one drop of HTF (200 µL) per female (2 oviducts).
7- Keep the fertilization dish including COCs in an incubator (37°C, 5% CO2 in air) for 30 minutes before insemination.

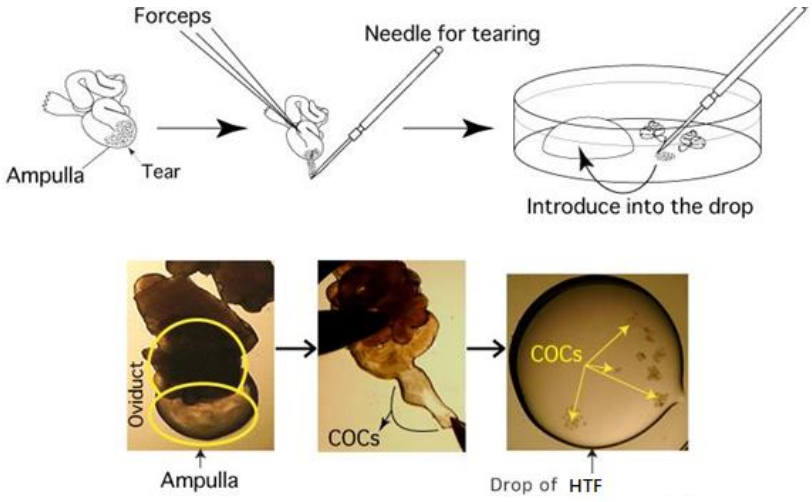
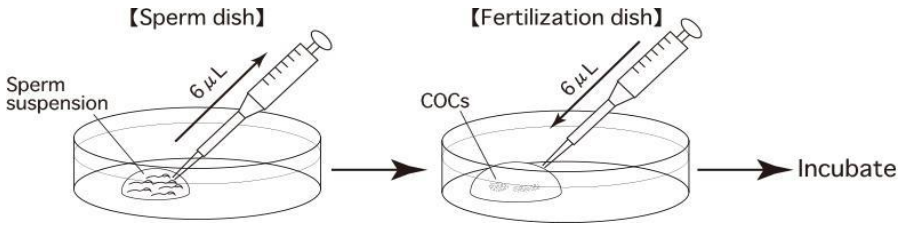


Figure 4.3 – Protocol used to collect oocytes.

4.2.5 INSEMINATION

1. After 1 hour of the sperm incubation, use the tip of a pipette to add appropriate amounts (usually about 6 µL) of the sperm suspension to the drop of HTF containing the COCs.
2. Place the fertilization dish in an incubator (37°C, 5% CO_2 in air).
3. 3 hours after insemination:
   a) wash the oocytes 2 times in fresh mHTF (80 µL) in a washing dish, avoiding the transfer of cumulus cells.
   b) Collect the drop from the fertilization dish, after removing the embryos from it, to a Eppendorf and freeze.
   c) After overnight culture of the oocytes:
   d) Transfer the obtained 2-cell stage embryos only to a drop of M2. These embryos can be vitrified, transferred to recipient females, or cultured to the blastocyst stage.
   e) Collect the drop from the washing dish, after removing the embryos, to an Eppendorf and freeze.



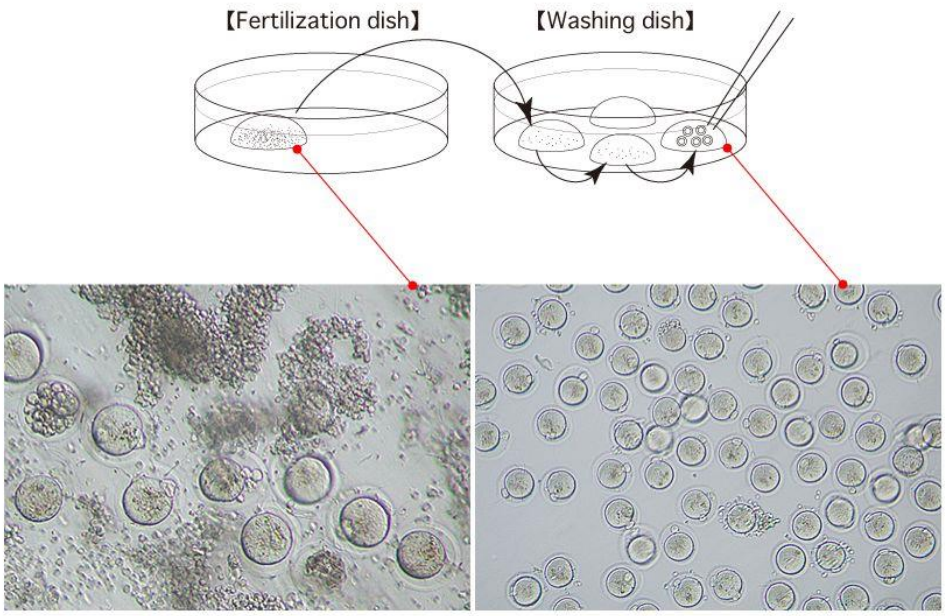


Figure 4.4 – Protocol used to insemination and collect the samples to analyse.

Study design

After freezing the samples of both groups (pre-fertilization obtained from the fertilization dish and post-fertilization obtained from the washing dish), they were transported in conditions that allowed maintaining the temperature favorable to freezing and stored in the engineering and health laboratory of the instituto superior de engenharia de lisboa.

FTIR Spectral Acquisition

All media were analyzed without performing any dilution. From the 94 samples summarized at Table 4.1, 25µl from each one were transferred to a 96-wells Si plate and then dehydrated for about 3 hours in a desiccator under vacuum. Spectral data was after collected using a FTIR spectrometer (Vertex 70, Bruker) equipped with an HTS-XT (Bruker) accessory. Each spectrum represented 64 coadded scans, with a $2cm^{-1}$ resolution, and was collected in transmission mode, between 400 and 4000 cm^{-1}. According to the HTS-XT manufacturer, the first well of the 96-wells plate did not contain a sample and the corresponding spectra was acquired and used as background.

Spectral Data Analysis

The software used to transfer the spectral data acquired from the spectrometer and atmospheric and baseline (Rubber-band method) correction was carried out by OPUS (Bruker). The normalizations to amide I, 1^{st} and 2^{nd} derivatives 2^{nd} order polynomial with Savitzky-Golay filter and multivariate analysis techniques as PCA (Principal Component Analysis) were done by The Unscramble®. The PCA was applied after a distinct combinations of pre-processing methods, such as atmospheric correction, atmospheric and baseline correction, atmospheric and baseline

correction and normalization to amide I, atmospheric and baseline correction and normalization to amide I and 1st derivative and finally atmospheric and baseline correction and 2nd derivative.

Table 4.1. Data collected from the culture media spent by murines to FTIR analysis.

Sample	Strain	Drop	#embryos	% Fertilization	Sample	Strain	Drop	#embryos	% Fertilization
1	D1-CRE X PWD	Fertilization	41		15,1	TH-IRES-Cre	overnight	30	40,00%
1,1	D1-CRE X PWD	Overnight	38	92,68%	16	D2GFPD	Fertilization	25	
2	D1-CRE X PWD	Fertilization	63		16,1	D2GFPD	overnight	25	100,00%
2,1	D1-CRE X PWD	Overnight	47	74,60%	17	D2GFPD	Fertilization	29	
3	D1-CRE X PWD	Fertilization	63		17,1	D2GFPD	overnight	28	96,55%
3,1	D1-CRE X PWD	Overnight	35	55,56%	18	D2GFPD	Fertilization	18	
4	Spretus	Fertilization	15		18,1	D2GFPD	overnight	17	94,44%
4,1	Spretus	Overnight	5	33,33%	19	D2GFPD	Fertilization	25	
5	Sert-cre	Fertilization	88		19,1	D2GFPD	overnight	25	100,00%
5,1	Sert-cre	Overnight	3	3,41%	20	D2GFPD	Fertilization	46	
6	Sert-cre	Fertilization	69		20,1	D2GFPD	overnight	46	100,00%
6,1	Sert-cre	Overnight	3	4,35%	21	D2GFPD	Fertilization	56	
7	Alpha6cre	Fertilization	88		21,1	D2GFPD	overnight	55	98,21%
7,1	Alpha6cre	Overnight	58	65,91%	22	D2GFPD	Fertilization	13	
8	Alpha6cre	Fertilization	102		22,1	D2GFPD	overnight	11	84,62%
8,1	Alpha6cre	Overnight	25	24,51%	23	Rag1KO. Rorc-iCre.NFiL3	Fertilization	72	
9	Alpha6cre	Fertilization	104		23,1	Rag1KO. Rorc-iCre.NFiL3	overnight	34	47,22%
9,1	Alpha6cre	Overnight	70	67,31%	24	Rag1KO. Rorc-iCre.NFiL3	Fertilization	68	
10	Alpha6cre	Fertilization	103		24,1	Rag1KO. Rorc-iCre.NFiL3	overnight	46	67,65%
10,1	Alpha6cre	Overnight	30	29,13%	25	Rag1KO. Rorc-iCre.NFiL3	Fertilization	67	
11	TH-IRES-Cre	Fertilization	114		25,1	Rag1KO. Rorc-iCre.NFiL3	overnight	35	52,24%
11,1	TH-IRES-Cre	Overnight	70	61,40%	26	CD2.R25RaraT403	Fertilization	58	
12	TH-IRES-Cre	Fertilization	87		26,1	CD2.R25RaraT403	overnight	18	31,03%
12,1	TH-IRES-Cre	Overnight	55	63,22%	27	CD2.R25RaraT403	Fertilization	78	
13	TH-IRES-Cre	Fertilization	106		27,1	CD2.R25RaraT403	overnight	8	10,26%
13,1	TH-IRES-Cre	Overnight	53	50,00%	28	CD2.R25RaraT403	Fertilization	43	
14	TH-IRES-Cre	Fertilization	113		28,1	CD2.R25RaraT403	overnight	35	81,40%
14,1	TH-IRES-Cre	Overnight	92	81,42%	29	CD2.R25RaraT403	Fertilization	78	
15	TH-IRES-Cre	Fertilization	75		29,1	CD2.R25RaraT403	overnight	36	46,15%

Sample	Strain	Drop	#embryos	% Fertilization	Sample	Strain	Drop	#embryos	% Fertilization
30	CD2.R25RaraT403	Fertilization	78		39	Ntsr1-Cre	Fertilization	104	
30,1	CD2.R25RaraT403	Overnight	55	70,51%	39,1	Ntsr1-Cre	Overnight	72	69,23%
31	Cerl2	Fertilization	63		40	Ntsr1-Cre	Fertilization	63	
31,1	Cerl2	Overnight	1	1,59%	40,1	Ntsr1-Cre	Overnight	42	66,67%
32	Cerl2	Fertilization	62		41	Ntsr1-Cre	Fertilization	68	
32,1	Cerl2	Overnight	0	0,00%	41,1	Ntsr1-Cre	Overnight	41	60,29%
33	CCL19	Fertilization	78		42	Ntsr1-Cre	Fertilization	88	
33,1	CCL19	Overnight	7	8,97%	42,1	Ntsr1-Cre	Overnight	53	60,23%
34	CCL19	Fertilization	103		43	Ntsr1-Cre	Fertilization	94	
34,1	CCL19	Overnight	13	12,62%	43,1	Ntsr1-Cre	Overnight	57	60,64%
35	Tbet-Cre	Fertilization	99		44	Ai32	Fertilization	61	
35,1	Tbet-Cre	Overnight	42	42,42%	44,1	Ai32	Overnight	28	45,90%
36	Tbet-Cre	Fertilization	65		45	Ai32	Fertilization	92	
36,1	Tbet-Cre	Overnight	24	36,92%	45,1	Ai32	Overnight	39	42,39%
37	Tbet-Cre	Fertilization	37		46	Ai32	Fertilization	100	
37,1	Tbet-Cre	Overnight	28	75,68%	46,1	Ai32	Overnight	39	39,00%
38	Ntsr1-Cre	Fertilization	91		47	Ai32	Fertilization	86	
38,1	Ntsr1-Cre	Overnight	44	48,35%	47,1	Ai32	Overnight	29	33,72%

4.3 Results and Discussion

The present analysis aimed to evaluate the usefulness of the FTIR spectroscopy technique in the comparative analysis of metabolic profiles of group embryos. This study was based on the detection of group metabolic changes, which promote mutual development through autocrine and paracrine factors. Since the samples received only include data up to the two-cell state, the study focused on a validation method. Since the samples received only include data up to the two-cell state, the study focused on a validation method to be applied subsequently to samples with the desired conditions. In this context, after the evaluation of the data obtained, three distinct variables were analyzed: the metabolic profile of the variable number of embryos per drop, the metabolic profile of the embryos before and after fertilization and the murine strains.

In order to minimize interferences that severely affect the FTIR spectrum, different processing techniques were applied as presented from Figure 4.5 to Figure 4.8. The spectra has shown some important components as the peak in the range between 3300 and 3400 translates into the absorption of amide A bonds that represent peptides or proteins and the peaks represented between 2800 cm^{-1} and 3000 cm^{-1} show the presence of lipids. After the observation of the figures presented, although the results were inconclusive, since there was no noticeable separation between the distinct groups after several methods of pre-processing and multivariate data analysis, the best combination presented reveals as the atmospheric and baseline correction and the normalization to amide I. The dispersion presented is greater, i.e. a distinct separation from the selected groups is not visible.

Atmospheric and Baseline Correction

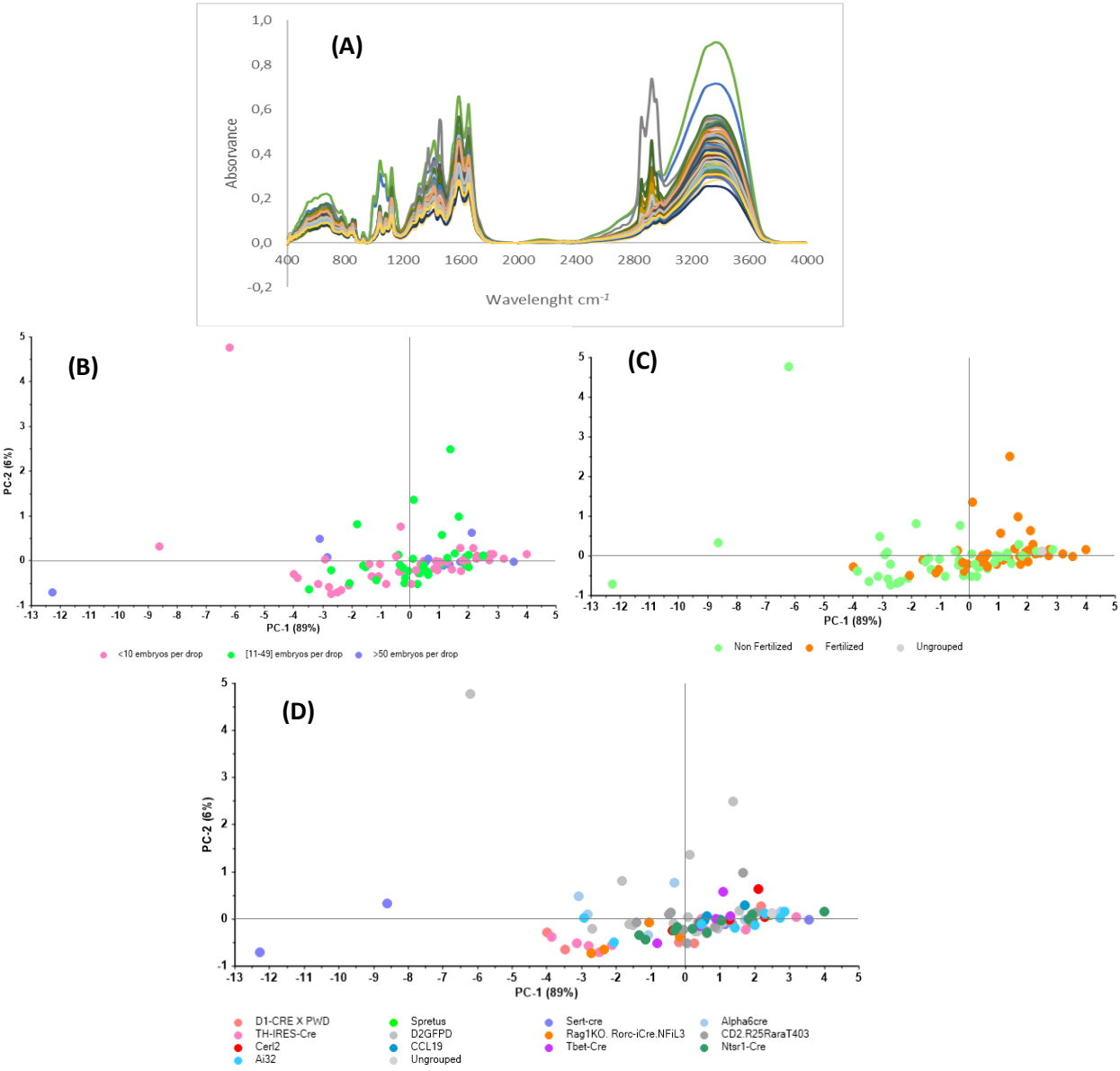


Figure 4.5 –Culture media spectra withour pre-processing (A): whole spectra; (B): PCA with sample grouping based on sample grouping based on embryo number per drop; (C): PCA with sample grouping based with sample grouping based on pre and post fertilization (D) PCA with sample grouping based on murine strain.

Atmospheric and baseline correction and Normalization to Amide I

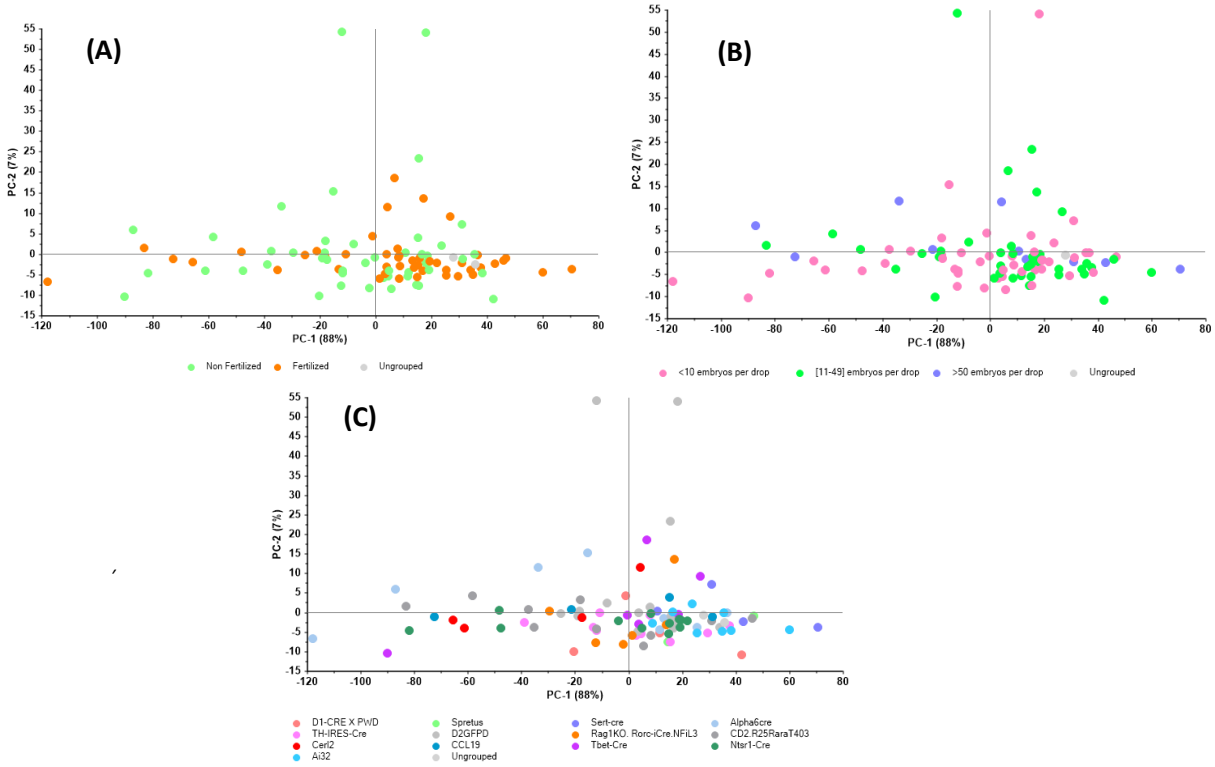


Figure 4.6 – Data with atmospheric and baseline correction and Normalization to amide I: (A) PCA with sample grouping based on pre and post fertilization; (B): PCA with sample grouping based on embryo number per drop; (C) PCA with sample grouping based on murine strain.

Atmospheric and baseline correction and Normalization to Amide I and 1st derivative

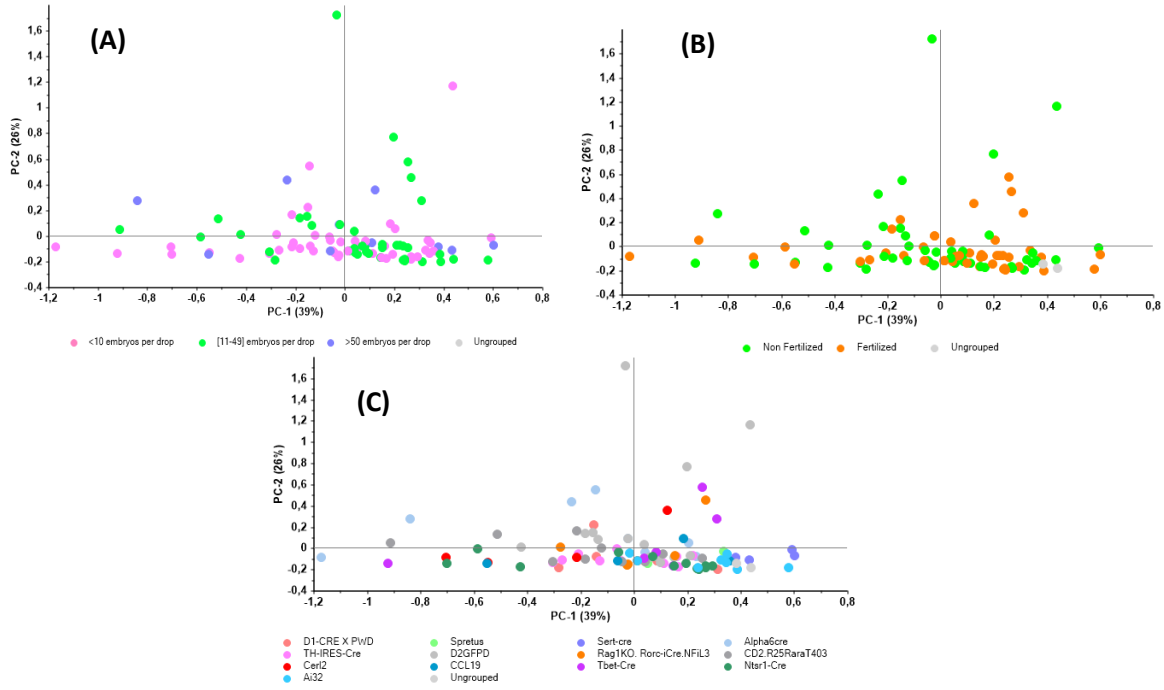


Figure 4.7 – Data with atmospheric and baseline correction, Normalization to amide I and 1st derivative: (A) PCA with sample grouping based on pre and post fertilization; (B): PCA with sample grouping based on embryo number per drop; (C) PCA with sample grouping based on murine strain.

Atmospheric and baseline correction and 2nd derivative

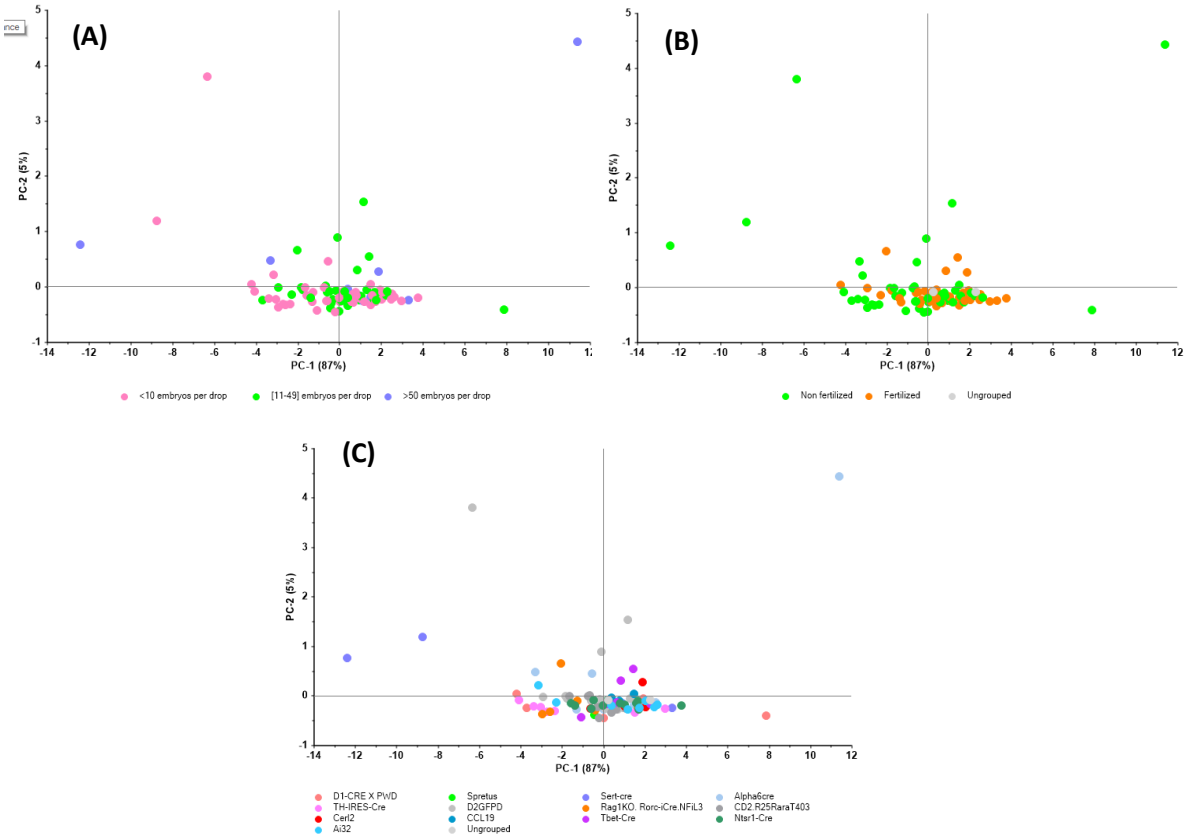


Figure 4.8 – Data with atmospheric correction and 2nd derivative: (A) PCA with sample grouping based on pre and post fertilization; (B): PCA with sample grouping based on embryo number per drop; (C) PCA with sample grouping based on murine strain.

The spectra shown in Figures 4.5-4.8 contain data on the functional groups detected through the absorption of infrared light in specific wavenumbers. Most of these regions can be identified according to the typical bonds observed. In this way, we can assume that these spectra present themselves as metabolic fingerprints of the analyzed media. All spectra revealed broad and complex outlines. There was a need to apply multivariate analysis techniques for a deeper and more complete analysis. For this reason, the graphics presented underwent the application of various pre-processing techniques for later evaluation of PCA.

After the application of different pre-processing, no conclusive results were obtained. In Figures 4.6, a more evident dispersion of data is observed, when the atmospheric and baseline correction are used together with the normalization to Amide I. Even so, there was no noticeable separation that revealed the metabolic distinction between the groups evaluated.

4.4 Conclusion

Through the results obtained, it was observed, that although the FTIR spectroscopy analysis presents itself as a useful tool in the distinction of different metabolic profiles, it did not reveal a noticeable distinction in the variables studied in the present work. One of the main limitations of this study was the excess number of variables applied, which promoted an infrequent dispersion. The number of embryos per drop of culture media varies from 0 to 113, without concise repetitions of embryos per drop. Additionally, the murine strain changes in varying proportions. In the future, a study should be designed to improve the conditions mentioned.

One of the limitations of the study was the absence of blastocyst development data. Since the media was only used until the mouse embryos reached two cells, it is not equivalent to clinical practice in human embryos. At the neurological research centre of the Champalimaud Foundation, it is not common practice for murine embryos to reach the blastocyst stage, which is why it was not applied.

FTIR spectroscopy seems to be a powerful tool to compare spent culture media by murine embryos, and hopefully in humans. It could allow us to identify profiles associated with positive development, elucidate the biological mechanisms implicated and study the role of group culture on developmental synergy.

Chapter 5: Group versus individual *in vitro* culture in the human preimplantational embryo

5.1 Introduction

In vitro embryo culture techniques and conditions is an area in continuous development. The optimization of culture media that support embryonic development has demonstrated an evident evolution that reflects the deepening of knowledge and progress in the area that helps maximize the chances of infertile couples to build a family (Chronopoulou and Harper, 2015). In several mammalian species, it has been reported that embryos grown in groups develop better than embryos grown alone, proving that cooperative communication could be happening by the embryo and its sibling. Initially, Lane and Gardner demonstrated that pre-implanted mouse embryo produces one or more factors that stimulate embryonic development and promote viability by increasing the density of the culture of mouse embryos (Paria and Day, 1990; Lane and Gardner, 1992); later, studies also demonstrated the positive effect of group culture on bovine embryos cultivated in groups of five embryos, with a blastocyst formation rate of 41% versus 28% obtained through individual embryo culture (Keefer et al, 1994). The study of group culture in mono-ovulatory animals in the culture of sheep embryos also revealed improvements in the rates of blastocyst hatching when cultivated in groups of four embryos in 20 µl microdroplets of culture medium (Gardner et al, 1994). This revelation allowed the insistence of a deeper investigation into the study of human embryos, which could also provide the same benefits. In 1995, Moessner and Dodson presented a comparative study of fifty-five patients with embryos cultured individually and in groups. The result showed increased significant cleavage rates although the morphology showed no improvement compared to individual embryo culture (Moessner and Dodson, 1995). A year after this demonstration, positive data emerged that revealed a noticeable increase in the pregnancy rate in the group culture (Almagor et al, 1996). Although the reliability of the results is reduced due to the variability of patients, these were the first references in humans that indicated the possibility of the benefit of group culture (Reed et al, 2011). The benefits of this modality are based on the secretion of autocrine and paracrine factors that promote a favorable microenvironment for embryonic development, with signaling pathways that reach receptors that decode the signaling agent's message, whether they are growth factors, proteins, lipids, neurotransmitters, saccharides or microRNAs (Wydooghe *et al*., 2017). It is estimated that 35% of all *in vitro* fertilization laboratories practice some form of group culture, in which a number of developing embryos share the same culture medium (Christianson *et al*., 2014). Despite the aformention studies, the evidence of the superiority of human embryo culture in group over individual culture is substantial limited. One of the possible factores is the variability in culture techniques, type of culture medium, number of embryos, volume and renewal of the medium. Currently, there is no consensus or standardization regarding the most adequate strategy regarding key group culture variables such these mentioned above and the role of poor quality embryos. There are some disadvantages associated with the embryo group culture, such as the accumulation of embryotoxic factors that can decrease embryonic viability or deplete substrates.

Therefore, it is imperative to have more in-depth studies than facts prove that the penalties have no value than group culture benefits.

5.2. Methods and Material

5.2.1 Study Design

All consecutive donor oocyte-recipient cycles meeting the inclusion criteria from December 2018 to May 2020 conducted in a single, private IVF unit were retrospectively analyzed for this study. Inclusion criteria were couples with <3 previous IVF cycles using autologous oocytes and with at least 1 useable blastocyst by day 6. Cycles in which the male recipient had an abnormal karyotype or in which surgically retrieved sperm was used were excluded from the analysis.

In the current study, we analyzed two different embryo culture systems: from December 2018 to April 2019, and from January 2020 to May 2020, standard single embryo culture was carried out. These cycles were compared to the GC strategy, which was implemented in our centre from May 2019 to December 2019. A total of 134 cycles were analyzed: in total, 497 zygotes were cultured until blastocyst stage. IC was used in 69 cycles, and GC in 65 cycles, with a total of 244 zygotes in IC and 253 in GC.

All patients signed informed consents for Assisted Reproduction Treatments which foresaw the participation in interventions aiming at the continual improvement of the quality of the processes performed in routine practice. The protocol of the study was approved by the Internal Review Board of Clínicas Ginemed.

5.2.2 Ovarian stimulation

Oocyte donors began stimulation with 225 IU daily of r-FSH. After day 8 of stimulation, ultrasound scans (US) were performed for eventual dose adjustment. After 6 days of ovarian stimulation, daily GnRH antagonist treatment was started. GnRH agonist triptoreline (2x0,1 mg) 35 hours prior to retrieval was used for ovulation trigger.

5.2.3 Endometrium Preparation

Endometrial preparation cycles for embryo transfer were carried out with 6 mg daily estradiol until endometrial thickness reached ≥7 mm. At that point, 800 mg daily of vaginal progesterone supplement was started. Blastocyst transfer was carried out on Day 5 of progesterone supplementation. Alternatively, some patients had embryo transfers in natural cycle, according to ultrasound and hormonal evaluations.

5.2.4 Oocyte retrieval

Ovarian puncture starts at 36 hours after the post-ovulation trigger. In this process, the COCs are separated from the follicular fluid and washed in GEMS Sperm Medium SPM-50 and later transferred to a GEMS Fertilization Medium. After two hours of recovery, the oocytes are stripped through hyaluronidase's chemical action (HYASE, Vitrolife). Then the mechanical movement is used when pipetting with a 135 µm capillary (CooperSurgical). When the number of mature oocytes is counted, the oocytes are either vitrified (when applicable) or injected 4 hours after the

puncture and subsequently placed in a unique GEMS GERI culture media until day 5 of development, where the transfer occurs.

5.2.5 *In vitro* fertilization

In vitro fertilization was carried out by ICSI. Fresh donor oocytes were denuded 2 hours after retrieval, by exposure to hyaluronidase (HYASE, Vitrolife) and mechanical pipetting with a 135 µm capillary (CooperSurgical), and microinjected 4 hours after retrieval. Frozen donor oocytes were warmed following the manufacturer's recommendation (VitKit-Thaw, IrvineScientific-Fujifilm) and microinjected 2 hours after warming. Following ICSI, the oocytes were transferred to continuous culture media (Geri, GeneaBiomedx).

5.2.6 Single and group embryo culture

After 17±1h of individual culture, zygotes showing two pronuclei were either maintained in the same 30 µL drop of media for individual culture (IC), or grouped in up to 3 2PN zygotes per droplet (GC) as shown in Figure 5.1. Irrespective of the culture strategy, embryo culture was carried on 30 µL drops of media, under 9-12 mL of paraffin oil (SAGE, Cooper Surgical), in 60 mm Petri dishes (Oosafe, Dibimed) containing 8 washing droplets and 8 culture droplets. Culture dishes were pre-equilibrated for >12 hours at 37ºC, 6% CO2, 5% O2 and 90% humidity, and embryo culture was carried out in the same conditions.

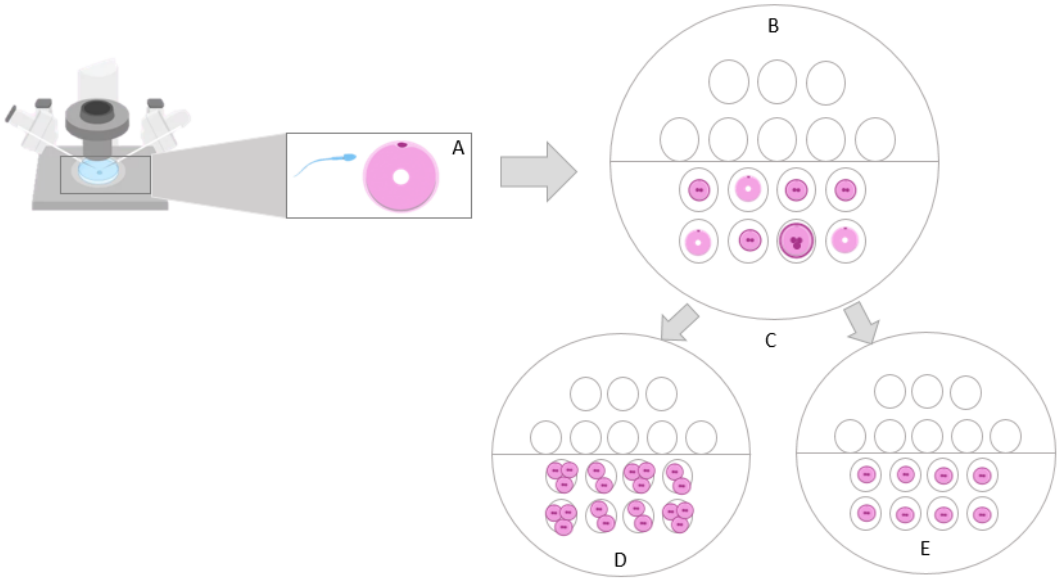


Figure 5.1 – Protocol used in evaluation of embryo development on group versus individual cuture. (A) 4 hours after retrieval, mature oocytes were microinjected. (B) The oocytes microinjected were transferred to continuous culture media. (C) After 17±1 hour of individual culture, 2PN zygotes were either maintained in the same 30 µL drop of media for IC (E), or grouped in up to 3 2PN zygotes per droplet, during the GC period (D).

5.2.7 Laboratory and clinical outcomes

Blastocyst status and quality was assessed 114-118 and 136-140 hours (day 5 & 6 of development, respectively) after ICSI, grading the expansion, inner cell mass (ICM) and trophoectoderm (TE). Fully formed blastocysts of fair or good ICM and TE were considered suitable for transfer or cryopreservation (≥3CC, according to ASEBIR Criteria, 2015).

Circulating beta human chorionic gonadotropin (β-hCG) levels were test 14 days after embryo transfer, with values ≥5 UI/mL considered positive. Clinical pregnancy rate (CPR) was confirmed by the determination of a gestational sac by ultrasound at 7 weeks.

5.2.8 Statistical considerations

Cycle data was collected in the clinical record management software, and retrospectively accessed and analysed, including baseline characteristics of donor and oocyte-recipients (age, BMI, smoking habits, reproductive history), and cycle characteristics (stimulation days, number of Metaphase-II oocytes collected, use of donor sperm, use of vitrified oocytes). The donor's and oocyte-recipient's baseline characteristics in IC and GC cycles were compared using Student's t-test for continuous variables and Fisher's exact test for categorical variables.

The primary laboratory outcome analyzed was the total usable blastocyst rate (TUBR), defined as the number of good quality blastocysts obtained on Day 5 and 6, which were suitable for either embryo transfer or cryopreservation, divided by the number of 2PN zygotes. The secondary outcomes assessed were the day 5 usable blastocyst rate (D5UBR) and the good quality usable blastocyst rate (GQUBR, ≥3BB blastocysts). These per zygote laboratory outcomes were compared between groups using generalized estimating equation (GEE) modelling to account for the correlation induced by the clustering of multiple zygotes in each donor/oocyte-recipient cycle. The clinical outcome of the first embryo transfer of each donor-oocyte recipient was also evaluated and compared using Fisher's exact test. The statistical analysis was performed using SPSS version 27 (IBM).

5.3 Results

Baseline characteristics of the population

A total of 134 cycles were analyzed; 69 with IC and 65 with GC of the zygotes obtained. As summarized in Table 5.1, there were no statistically significant differences in donor age at oocyte retrieval, donor BMI, donor smoking habits, number of nulliparous donors, number of first-time donors, ovarian stimulation days, number of M-II collected, oocyte-recipient age, BMI and smoking habits. The number of oocyte-recipients with secondary infertility was lower in the IC group *vs*. the GC group (30,7% *vs*. 47,8%). The number of cycles in which donor sperm was used was also similar in between groups, as well as the average number of M-II used in each cycle, the proportion of cycles using vitrified oocytes, and the mean survival rate for vitrified oocytes. The average number of zygotes obtained per cycle was of 3,7 (±1,4 and ±1,5, respectively) in both IC and GC groups.

Table 5.1. Baseline characteristics of oocyte donor and oocyte-recipient cycles.

	Individual Culture	Group Culture	p-value
# cycles	69	65	n/a
Donor's age at oocyte retrieval, mean (SD)	25,7 (3,2)	25,6 (3,6)	0,809
Donor's BMI, mean (SD)	21,6 (2,4)	21,7 (2,6)	0,824
Donors with smoking habits, # (%)	23 (35,4)	22 (31,9)	0,716
Nulliparous Donors, # (%)	42 (64,6)	38 (55,1)	0,293
First time donors, # (%)	40 (61,5)	49 (71,0)	0,276
Stimulation days in donation cycle, mean (SD)	12,4 (1,6)	12,5 (1,4)	0,614
MII collected in donation cycle, mean (SD)	14,8 (7,0)	15,7 (6,7)	0,498
Oocyte recipient age at oocyte fertilization, mean (SD)	43,0 (3,5)	42,8 (3,4)	0,731
Oocyte recipient BMI, mean (SD)	24,2 (3,5)	24,1 (3,5)	0,868
Oocyte recipients with smoking habits, # (%)	19 (29,2)	16 (23,2)	0,439
Oocyte recipients with secondary infertility, # (%)	20 (30,7)	33 (47,8)	0,053
Cycles using donor sperm, # (%)	11 (16,9)	6 (8,7)	0,197
MII oocytes per cycle, mean (SD)	5,1 (1,8)	5,5 (2,2)	0,867
Cycles using vitrified-rewarmed oocytes, # (%)	54 (83,1)	60 (86,9)	0,630
Vitrified-rewarmed MII oocytes survival rate (%)	92,8%	89,1%	n/a
2PN fertilized oocytes, mean (SD)	3,7 (1,4)	3,7 (1,5)	0,737

Embryology outcomes

In total, 497 zygotes were cultured to the blastocyst stage during the study period; 244 in IC and 253 in GC. The average embryo density in the GC period was of 2,6 (±0,5) embryos per droplet, and 50,8% developed into a useable blastocyst *vs*. 51% in the IC period (Table 1.10). The proportion of useable embryos reaching the blastocyst stage on Day 5 was of 77,2 in IC *vs*. 71,9 in GC, and the proportion of blastocysts of good quality was of 95% in both groups (Table 5.2).

Table 5.2. Embryology outcomes

	Individual Culture	Group Culture	p-value
Total usable blastocysts /Zygotes (%)	127/249 (51,0)	135/266 (50,8)	0,849
Day 5 usable blastocysts/Usable blastocysts (%)	98/127 (77,2)	97/135 (71,9)	0,337
Good quality usable blastocysts/Usable blastocysts (%)	114/127 (95,0)	126/135 (95,5)	0,984

Clinical outcomes

A follow-up of the 1st embryo transfer of each oocyte-recipient was carried out. During the study period, all IC oocyte-recipients underwent an embryo transfer, while 68/69 oocyte-recipients did of those cycles in which GC was used. Most of the embryo transfers were frozen embryo transfers in both groups (89,2% *vs*. 92,5% in IC *vs*. GC), and the mean number of blastocysts transferred did not differ between groups (1,17 *vs*. 1,22, in IC and GC, respectively). The rates of embryos transfers with positive β-hcg and clinical pregnancy confirmed by ultrasound is reported in Table 5.3.

Table 5.3. Clinical outcomes

	Individual Culture	Group Culture	p-value
# of cycles in which the 1st embryo transfer was carried out/Total cycles (%)	65/65 (100)	68/69 (98,5)	n/a
# frozen embryo transfers/Total 1st embryo transfers (%)	58/65 (89,2)	65/68 (92,5)	0,144
# of embryos transferred, mean (SD)	1,17 (0,38)	1,22 (0,45)	0,479
# patients with positive β-hcg/1st embryo transfers (%)	41/65 (63,1)	49/68 (72,1)	0,354
# clinical pregnancies/1st embryo transfers (%)	33/65 (50,8)	42/68 (61,8)	0,224

5.4 Discussion

The efficiency of two human embryo culture protocols were retrospectively compared in the oocyte donor-recipient model. Of the zygotes cultured with the GC strategy, 50.8% developed to blastocysts that were transferred or cryopreserved (total usable blastocyst rate; TUBR), *vs*. 51% in IC: the developmental and utilization rates of the zygotes was not improved when cultured under the studied group culture strategy. The total usable blastocyst rate (TUBR), used in the present study, is a very important laboratory key performance indicator (Hammond and Morbeck, 2019), and has been used to assess their performance of other laboratory parameters, such as low *vs*. ultra-low O_2 tension (Minasi *et al*., 2015) single *vs*. sequential media (Swain, 2019) or 36.5 *vs*. 37.0 ºC culture temperature (Ziebe *et al*., 2013).

Our group culture strategy was based on previous human model studies which reported increased development to the blastocyst stage using embryo grouping (Tao *et al*., 2013; Ebner, 2010; Rebollar-Lazaro, 2010; Trial *et al*., 2020). A prospective study showed that IC in 30 µL drops of sequential media – 1 embryo isolated in each micro-drop of culture media - performs worse than GC of 3 to 5 embryos (Ebner, 2010). The same study also reported that the benefit of GC is increased if there is no physical separation between the embryos, as in the case of micro-wells inside a single drop of culture media (Ebner, 2010). This agrees with the hypothesis that close contact (<150 µm apart) may be necessary to benefit from embryonic autocrine secretions (O'Neill., 2008). Tao *et al.* (2013) also found better embryo development grouping 2-5 embryos on day 3 (D3) in 15 µL drops of sequential media, but excluding poor quality D3 embryos from

the cohort to exclude a possible negative effect. In the GC protocol of this study, embryo development was not assessed on day 3, only on day 5, so it was not possible to exclude poor quality embryos from the groups, in order to avoid a possible deleterious effect of these embryos on the culture micro-environment. On the other hand, it allowed for continuous, uninterrupted culture from day 1 to day 5 of development, maintaining a steady culture environment, without disturbance of the proximal embryonic milieu.

Rebollar-Lazaro (Rebollar-Lazaro, 2010) found an increase in total useable blastocyst rates when culturing 2-5 embryos in 15 µL drops of sequential media up to day 3, followed by single culture to the blastocyst stage, since in mice, exposure to embryotrophins was more effective during the first two cell cycles (Brison and Schultz, 1997). In our setting, continuous media was used, so the microenvironment surrounding the embryos providing the putative embryotrophic effect of group culture was not disturbed. More recently, it was reported that grouping up to 5 oocytes at the time of ICSI resulted in an increase in blastocyst utilization rates (43.9% to 56.4%), with separation of non-fertilized and degenerated oocytes on Day 1, and without media renewal (Trial *et al*., 2020). However, the putative benefits of GC in the second study may be due to the simultaneous use of low O2 tension and benchtop incubators solely in the study group, whereas both of low O2 tension and benchtop incubators were employed in all cases in the present study.

The present study, on the contrary with others (Tao *et al*., 2013; Ebner, 2010; Rebollar-Lazaro, 2010; Trial *et al*., 2020; Glatthorn *et al*., 2021) did not achieve an improvement in embryo developmental rates by GC. It could be the case that other aspects of current, state of the art embryo culture media and laboratory conditions already supports embryo development to an extent that the effects of auto and paracrine activity are lessened and harder to detect in the developmental rates (Cohen *et al*., 2020). For instance, culture media composition, which is not fully disclosed by manufacturers (Sunde *et al*.; 2016), and external media supplementation has been shown to impact embryo development rates (Fawzy *et al*., 2019). Our study presents the limitation of its retrospective nature, however, laboratory staff, key laboratory practices and consumables remained constant throughout the study period. Also, the homogeneity of oocyte donors and recipients baseline characteristics in each group was assessed without the identification of potential confounders. The present study is the first to assess GC in good prognosis cycles with the use of donor oocytes exclusively, for that reason, the results obtained should not be extrapolated to poor prognosis patients.

Despite potential benefits, there are certain disadvantages in culturing embryos in groups with close physical contact. First, there is an inherent loss of traceability amongst embryos grouped in the same media droplet, which prevents clear association with previous developmental events, potentially impairing the accuracy of blastocyst grading and selection (Huang *et al*., 2021). Additionally, if there is a need of pipetting to regroup embryos, it can lead to increased observation and handling times outside the incubator (Cohen *et al*., 2020). Also, direct physical contact group culture is not compatible with any of the commercial time-lapse platforms currently available, and novel approaches such as non-invasive PGT-A and proteomic profiling from spent culture media (Fawzy *et al*., 2019; Vajta *et al*., 2021); Working *et al*, 2020; Huang *et al*., 2021).

5.5 Conclusion

The biological mechanisms that could explain the embryotrophic role of culturing human preimplantational embryos in groups with close physical contact are in the center of several studies (Reed *et al*., 2011), and GC strategies have widespread application in the IVF laboratory (Christianson *et al*., 2014). However, it could be argued that human embryo GC is lacking methodological standardization (Tao *et al*., 2013; Ebner, 2010; Rebollar-Lazaro, 2010; Trial *et al*., 2020; Glatthorn *et al*., 2021) and it is not clear how to maximize its putative benefits, which could result in increased utilization rates of the zygotes obtained in an IVF cycle, and consequently in the cumulative success rates of the treatment. More insight into the characterization and quantification of autocrine, as well as paracrine and endocrine embryotrophic factors, could allow to maximize group culture's benefits and overcome its pitfalls, by improving current culture strategies.

Additionally, it would be interesting to carry out an FTIR spectroscopy analysis of the media used for individual versus group embryo culture. After this step, if the study presents positive outcomes, a more detailed analysis of the spent culture media composition should be done. This last step aims to identify possible autocrine or paracrine factors that support the theory that embryos benefit from a group culture, explained by the release of embryotrophic factors that enhance mutual development, these factors could be identified using cytoquine or immunochemical analysis (Usami *et al*., 2010).

Chapter 6: Conclusions and Future Work

The main objective of the present work was to study the mechanisms and effects of density of embryos in culture in in vitro fertilization processes on embryo development through FTIR spectroscopy tool.

For this reason, the structure of this thesis was designed in order to first, validate the FTIR spectroscopy methodology as reliable in the evaluation of culture media, try to apply this methodology in animal study and then evaluate the culture media spent by human embryos to prove the benefit of the secretion of embryotrophic factors. This last goal was not achieved since an ethics committee approval to study the spent culture media by human embryos, the signing of an informed consent and the collection of data and samples from IVF cycles would be mandatory. Alternatively, the third phase was dedicated to a retrospective study to compare the results of individual and group culture of ovodonation cycles performed at the Ginemed clinic, to understand if grouped embryos presented any developmental advantage with the current protocol

Summarily, the potential of the FTIR spectroscopy was explored in the first analysis performed on different culture media, which allowed us to conclude that it is a sensitive technique with interest for human embryology. The results showed that the spectral profile of one of the analyzed culture media (GERI), pre-processed with atmospheric and baseline correction, amide I normalization and 1st derivative, differs from the others, pointing to the presence of different analytes in its composition. It was also possible to verify that the composition of the analyzed media is grouped by type of culture (sequential *vs*. continuous) and by manufacturer. These results are promising because it is known from the outset that the composition of the culture media varies in the type of culture and, since most manufacturers do not fully reveal the quantitative and qualitative composition, we assume that there is also variance between manufacturers, pointing to a spectral signature that allows you to differentiate them. In this way, it can be said that the technique used corresponded to expectations. The link made in this project between the comparative evaluation of culture media and the evaluation of embryonic density in different situations (animal and human embryos) was intended to highlight the existence of variables that can interfere with embryonic performance in different protocols.

The next phase was based on our previous results with FTIR spectroscopy, which would allow us to distinguish different metabolic profiles from culture media spent by murine embryos. This would be a transitional phase to apply the methodology to research with human embryos. After evaluating the results obtained and the variables under study, it was concluded that the expected results were not achieved, due to the limitations identified. At this stage, it was not possible to get any insight due to methodological defects. Although it was verified that the FTIR spectroscopy technique remained sensitive and capable of presenting different metabolic profiles for the analyzed culture media, it was not possible to establish any link between the studied groups: number of embryos per drop, strain, pre-fertilization *vs* post-fertilization. The major limitation that influenced the failure of the results obtained was the fact that the analyzed culture media were from fertilization stage. The established theory of secretion of embryotrophic factors is not applicable in this sense, since this interaction occurs during a later phase of embryo development, in late cleavage andblastocyst stage. In addition, the study variables disturbed the main objective of the project: different strains, number of embryos per drop too variable and with little

consistency. Ideally, in a next project, the main goal would be to obtain the culture media spent by murine blastocysts, from the same strain and with well defined and more concordant number of embryos per drop.

Finally, data from human embryos in group and individual culture were retrospectively analyzed. After evaluation of the obtained results, a benefit of group culture was not observed in this specific population since both culture strategies resulted in similar rates of embryo development, despite following a group culture protocol previously described in the literature as beneficial. These results show how important it is to better understand the group culture of human embryos and the potential of the FTIR spectroscopy methodology for this research: understanding which variables (volume, density, contact days) need to be optimized to obtain the desired paracrine synergy between embryos in close proximity, by comparing the spectral profile of spent culture media. The research into the identification of these growth promoting factors secreted during the group culture should be continued, for future application in culture media formulation which can result in better embryonic development and improve the rates of success of assisted reproduction treatments.

Chapter 7: References

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