Noninvasive Assessment of Embryo Quality in Human *in vitro* Fertilization: Metabolomic Profiling of Embryo Culture Media with Raman Spectroscopy

by

Alaleh Asghari Roodsari

M.D., Tehran University of Medical Sciences, 2006

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA
(VANCOUVER)

September 2012

Abstract

Introduction:

Light microscopy has remained the primary tool for the assessment of embryo quality and the selection of embryos for transfer in clinical IVF practice. Recent studies have suggested that metabolomic profiling of embryo culture media can distinguish human embryos with better implantation potential. We therefore undertook the following study to further assess the usefulness of metabolomic profiling "spent" embryo culture medium using Raman spectroscopy.

Methods:

Patients undergoing IVF+/-ICSI treatment from the UBC Centre for Reproductive Health were recruited for study. Demographic and clinical information was collected. As part of routine clinical procedures, embryos were individually cultured in G1 media from Day 1-3 and in G2 media from Day 4-6. G1 and G2 culture medium (vitrolife, Englewood, CO) introduced specifically for cleavage embryo and blastocyst culture respectively. Embryo-free G1 and G2 droplets were placed alongside the embryo-containing ones as controls. For the study, fresh droplets of spent and control culture media were individually collected on Day 3 and 6 and prepared for assessment by Raman spectroscopy. The assessment score under light microscopy of the corresponding embryo and its fate were recorded for comparison and correlation. To validate the detection limits of Raman spectroscopy a wide range of glucose and glycine concentrations between 0 and 500 mM in distilled water were analyzed.

Results:

A total of 300 embryos/spent media droplets from 54 patients aged 27-43 years (mean age ± SD: 36.33 ± 3.26) were evaluated. Of 111 embryos transferred, 19 implanted and led to a pregnancy: 7 (12.96%) single and 6 (11.1%) twin pregnancies. Irrespective of pregnancy, there were no systematic differences between the Raman spectra generated from spent media of Day 3 and Day 6 embryos, or between spent media and control media.

Conclusions:

In contrast to published reports, our study does not show that metabolomic profiling of spent embryo culture media by Raman spectroscopy can differentiate embryos with better implantation potential or add value to light microscopic assessment as in clinical practice.

Preface

Chapter 3 is based on work conducted in both the IVF laboratory at the Children's & Women's Health Centre of British Columbia and the Department of Chemistry at the University of British Columbia by Dr. Anthony P. Cheung, Dr. Ed Grant, Zhiwen Chen and Alaleh Asghari Roodsari. I was responsible for collecting the "spent" embryo culture media and transferring them to the Department of Chemistry. Both Zhiwen Chen and I have applied Raman spectroscopy to samples. Most of the Raman analysis was done by Zhiwen Chen. I was responsible for making different glucose and glycine concentrations and for measuring their signals by Raman spectroscopy.

The Human Ethics with the Certificate Number H09-00420 was obtained from UBC Research Ethics Board for this study.

Table of Contents

List of Tables

List of Figures

Acknowledgements

- I offer my enduring gratitude to my teacher, Dr. Anthony P. Cheung, who makes the complex understandable, and who has dedicated his life to the education of physicians, and served as a role model for healers.
- I owe particular thanks to Dr. Ed Grant, for giving me the opportunity to fulfill my dreams.
- I thank Zhiwen Chen and Najmeh Tavassoli, in the University of British Columbia's Department of Chemistry, whose enthusiasm, curiosity and pursuit of excellence have provided a constant source of stimulation and of lifelong friendship.
- In addition, I express my sincere gratitude to Dr. Daniel Chen from Tianjin University, China.
- A world of thanks to all the IVF lab staff members at the Children's & Women's Health Centre of British Columbia, whose great help and patience have provided better sources for the completion of this project.
- This study was funded, in part, by a research grant from Women's Health Research Institute (WHRI), BC awarded to Anthony P. Cheung.
- In particular, I would like to acknowledge Dr. Mehdi Najafi, a great colleague and friend who provided me intellectual and scientific support within my study period and beyond.
- Special thanks are owed to my mom and my late father who instilled an early love of learning, and who were and are always there.

Dedication

With love and gratitude to my supportive parents, to my loving sisters and to my beautiful niece

1 Background

1.1 General Concept

Infertility is defined as the inability to become pregnant after 1 year of unprotected intercourse. It is a multifactorial disorder that is estimated to affect 8-15% of reproductive-age couples with significant medical and economic ramifications (1,2,3). In the United States, the prevalence of infertility has remained stable over the last several decades; in spite of this fact, the number of women undergoing infertility treatment has been increasing (4).

Of all the available treatments for infertile couples, assisted reproductive technologies (ART) offer the highest success rates to infertile couples (1,3). ART refers to *in vitro* fertilization (IVF), a process by which eggs are fertilized by sperm outside the body, and intracytoplasmic sperm injection (ICSI) in which a single sperm is injected directly into a mature egg microscopically. It has been estimated that over 3 million children worldwide have been conceived through assisted reproductive technology (ART) since the birth of Louise Brown in 1978 (5,6). Currently, approximately 1% of all children born in the United States are from ART-related conception (7). Intra-cytoplasmic sperm injection (ICSI) represents 52% of all IVF treatment in the UK in 2009. The remainder is conventional IVF. Despite wide application and increased improvements during the last decades, only 25.2% of non-donor egg, fresh IVF cycles resulted in live births in the UK in 2009, andtwo out of three IVF cycles fail to result in clinical pregnancy, causing significant physical, emotional and financial burdens for infertile couples (8,9).

One of the most pressing concerns in human IVF is how to increase implantation potential—in other words, how to improve the likelihood of a live birth for an infertile couple. The preferred choice in many clinics has been to transfer an increased number of embryos, and in this way to increase the infertile couple's chances of achieving pregnancy. This has led to an unacceptable major disadvantage of IVF treatment, resulting in a recognized level of high-order multiple pregnancies (8,10,11,12). In the United States, a 77% increase in the number of twin deliveries was observed and the number of higher order multiple births increased by 45% between 1980 and 2001 (13). Multiple pregnancies, in turn, are responsible for a high proportion of public health consequences such as preterm (<37 weeks) birth(one in eight), very low birth weight (<2000g) infants(1 in 4), cerebral palsy (8-fold increases in twins and 47-fold increases in

triplets), infant deaths in the first year of life (6-fold increases in twins and 17-fold increases in triplets and higher order gestations), and pregnancy-induced hypertension and postpartum hemorrhage (1,7,14,15,16). To overcome this problem, many countries legally restrict the number of transferrable embryos, in some instances limiting this number to one within a certain age group (7,17,18). Many centers have decreased the number of embryos transferred to two, known as double embryo transfer (DET) and even one which is called elective single embryo transfer (eSET). The practice of DET has markedly reduced the rates of triplets and higher-order multiple pregnancies, however twin pregnancy rates have remained high at around 36% after DET in women under the age of 35. The mean pregnancy rate is around 35% after eSET in all age group (CARTR 2010). Although eSET will reduces the twin pregnancy rate, it also reduces the pregnancy rate. The most significant contemporary goal of infertility research is to decrease the prevalence of multiple gestations while maintaining or improving overall pregnancy rates. One approach is to identify reliable methods that can select the embryo with maximum development for replacement in the uterus, thus reducing the number of embryos needed without compromising the prognosis for successful birth.

1.2 Morphological Assessment of Embryo

A key step in ART is the assessment of oocyte embryo quality, which helps determine the embryo(s) with the highest likelihood of implantation and the greatest pregnancy potential. Many methods have been proposed to assess the quality of embryos in IVF programs. Traditionally, the embryos selected for transfer are chosen according to morphological criteria and rate of development in culture on microscopic assessment, which serves as a relatively simple and reliable method for this purpose (1,7,19). Numerous systems have been used to grade embryos. A simple grading system assesses the overall appearance of the cleavage stage embryo, cell symmetry, number, size, position of the nucleoli and fragmentation between day 1 and 3. For the embryos at the blastocyst stage—a structure formed in the early embryogenesis of mammals after the formation of the morula, which consists of the inner cell mass (ICM) and the trophoectoderm (TE),grading takes into account the appearance of these two cell thypes, as well as the degree of blastocyst cavity expansion 5 to6 day after fertilization (20,21,22).

In 2005, the Society for Assisted Reproductive Technology (SART) charged one of its subcommittees with developing the standardized embryo grading system. A three point grading

system (Good, Fair and Poor) was developed for staging embryos using morphological features (22) summarized in Table 1.

Table 1: SART grading system

Growth Phase	Overall grade	Stage
Cleavage	Good, Fair, Poor	Cell #: 1 through >8 Fragmentation: 0%, < 10%, 11-25%, >25% Symmetry: Perfect, Moderately Asymmetric, severely Asymmetric
Morula	Good, Fair, Poor	Compaction: Complete, Incomplete Fragmentation: 0%, < 10%, 11-25%, >25%
Blastocyst	Good, Fair, Poor	Expansion: Early, Expanding, Expanded, Hatched Inner Cell Mass: Good, Fair, Poor Trophoectoderm: Good, Fair, Poor

Adapted from article 22

The visual assessment of oocyte/embryo quality with light microscopy is by far the least technically challenging and the most practical of all the methods for scoring the oocytes and embryos. While morphological evaluation is noninvasive and has been an integral part of ART practice, its accuracy is far from ideal and there are problems with relying solely on static morphology assessment of embryo quality. For example, a good scoring system on day 3 may not accurately predict an embryo's ability to implant. Two 8-cell embryos that look identical do not necessarily have the same implantation potential, as evidenced by the low implantation rates of transferred embryos - in other words, good embryo appearance cannot guarantee pregnancy success (23). In 2010, a mean number of 2.4 embryos from fresh oocytes were transferred in ART cycles leading to a 32.77% live birth rate. (24). Only one out of three IVF cycles resulted in pregnancy and less than two out of ten embryos implanted according to Society for Assisted Reproductive Technology (SART) statistics for 2005. Furthermore, cleavage embryos with fragmentation can develop to the blastocyst stage *in vitro* (25,26). There are numerous factors contributing to oocyte and embryo quality which are not necessarily reflected by morphology. Additionally, twenty-nine percent of embryos with good morphological appearance present chromosomal abnormalities (ref). Furthermore, embryo assessment under light microscopy also requires specialized training and can be difficult to standardize (1,7,8,27).

Taken together, these facts suggest that embryos with proper morphological appearance alone are not sufficient to predict a pregnancy and more reliable indices of embryo health are required. Therefore, many investigators have been pursuing adjunctive technologies for the assessment of the reproductive potential of a given embryo.

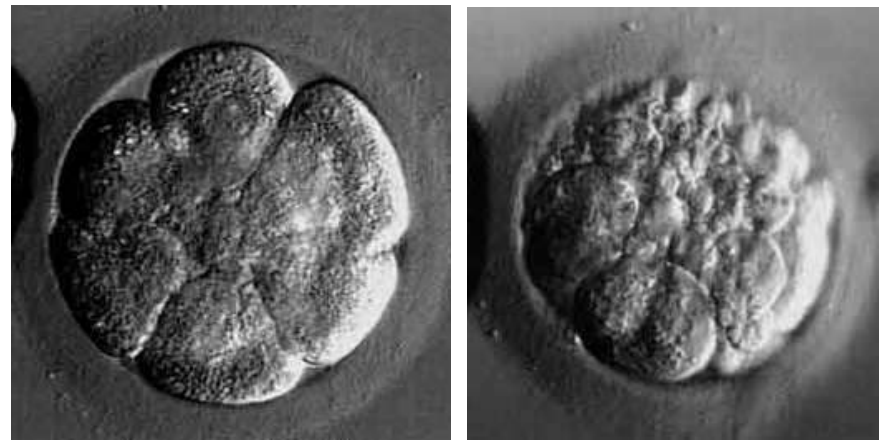


Figure 1: Morphological appearance of embryo on Day 3 and 6

Upper Left, "Perfect" looking Day 3 Embryo. The cells are very even, regular, and similarly sized. Upper Right, Poor quality Day 3 embryo, severely fragmented and has unevenly sized cells. Lower Right, Well expanded blastocyst, inner cell mass at 6 o'clock surrounded by trophectoderm cells

Adapted from Ref. 28

1.3 Embryo Metabolism

The changes in the metabolism of the embryo start in the pronuclear stage, in which a relatively quiescent oocyte is under the genetic control of maternally derived genes. The oocyte evolves to a metabolically and biosynthetically active group of cells that grow predominantly under their own genetic control by the blastocyst stage (29,30). Fertilization is a complex process of cell-cell interaction which starts with the recognition and binding of spermatozoa to oocytes. After sperm-oocyte fusion, the sperm derived calcium wave initiates oocyte activation. This cell, the zygote, starts to divide by mitosis into a number of smaller cells. This process of division, known as cleavage, starts some 21 h later; subsequently, cleavage divisions occur every 12-15h, while there is gradual activation of embryonic genome. At about the third cleavage division, the

embryo undergoes the process of compaction to form the morula. At the 32-cell stage, a second morphological change occurs which gives rise to the blastocyst which consists of the inner cell mass (ICM) and the throphoectoderm (29). The best known metabolic change in an embryo is the switch in ATP synthesis from the carboxylic acids pyruvate and lactate as primary energy sources to a glucose-based metabolism around the compaction time. At the blastocyst stage, glucose has become a key metabolite which is metabolized by both the TCA cycle and by aerobic glycolysis (3,8,19,29). The complex, stage-specific metabolic and nutritional requirements of mammalian embryos require precise regulation of many cell functions such as cellular homeostasis and gene expression. Table 2 shows the physiologic changes of the mammalian embryo from the zygote to the blastocyst stage (29).

Similar results with regard to the uptake and utilization of carbohydrates during the embryonic development of other animals such as mouse, hamster, sheep and cow show a limited capacity for glucose utilization until compaction. Instead, pyruvate, lactate and amino acids are the preferred energy substrates of the cleavage stage embryo (31,32,33,34,35). Significantly, the nutrients available within the human female reproductive tract mirror the changing nutrient preference of the developing embryo. Therefore, at the time when the embryo is in the female reproductive tract, a decline in pyruvate and lactate gradients from the oviduct to the uterus is observed, while the opposite is true for glucose. These changing levels of carbohydrates reflect the switch from a carboxylic-acid-based metabolism to a glucose-based metabolism in the embryo (36,37).

Table 2: Physiology of the mammalian embryo from the zygote to the blastocyst stage

Precompaction stage	**Postcompaction stage**
Low biosynthetic activity	High biosynthetic activity
Low QO_2 (Metabolic Quotient)	High QO_2 (Metabolic Quotient)
Pyruvate-based metabolism	Glucose-based metabolism
Maternal genome	Embryonic genome
Single cell	Transporting epithelium
Totipotent	Differentiation into inner cell mass and trophectoderm

Adapted from article 29

1.4 Development of Embryo Culture Media

Years of studying these metabolic processes have equipped scientists to develop embryo culture media which successfully support the growth and development of human embryos. Historically, human embryos were cultured in simple salt solutions such as Earle's, T6 and HTF medium, which contained balanced salt solutions with added carbohydrates glucose, pyruvate and lactate and which were supplemented with the patient's serum. But they lacked many important components which are essential for embryo physiology and health. Alternatively, complex tissue culture media were designed which supplemented with carbohydrates, amino acids, vitamins, nucleotides and metal ions. Although these media were supportive for human embryo development through the first three cleavage divisions, they were not able to support acceptable levels of blastocyst development (29,30). Over the last decade, a significant change has occurred: commercially produced sequential culture systems specifically for use in clinical IVF are widely used. These media mirror oviduct and uterus components to prevent intracellular stress to the embryo, thereby maintaining embryo viability; additionally, they are able to support high rates of blastocyst development in cultures of embryos from many species (30,38). Among the sequential serum-free culture systems are the G1 and G2 (vitrolife, Englewood, CO) systems. The composition of these culture systems mirrors the oviduct and uterus components except for some nutrients such as glycine which is 0.1 mM in both G1 and G2 media (39).

Table 3: Comparison of nutrients in mammalian oviduct and uterus

Component	Oviduct	Uterus
Glucose concentration	0.5 mM	3.15 mM
Pyruvate concentration	0.32 mM	0.10 mM
Lactate concentration	10.5 mM	5.2 mM
Oxygen concentration	8%	1.5%
Carbon dioxide concentration	12%	10%
pH	7.5	7.1
Glycine concentration	2.77	19.33
Alanine concentration	0.5	1.24
Serine concentration	0.32	0.80

Adapted from article 29

1.5 Measurement of Single or Specific Molecules in the Culture Media for Oocyte/Embryo Quality Assessment

Metabolic pathways in cells require the uptake of certain substances from the surrounding environment and secretion many products into the extracellular space. This holds true for oocyte complexes in culture as well. Hence, collection and measurement of the culture media may provide information reflecting cellular activities and overall developmental potential during the culture period. More recently, a number of noninvasive tests for embryo viability have been under investigation. These techniques are capable of measuring both nutrient consumption and metabolite release in real time and can measure the movement of ions and molecules between the cell and the surrounding media.

1.5.1 Pyruvate Metabolism

As stated previously, the embryo's capacity for glucose utilization is limited to the period after compaction or at the blastocyst stage. In the early stage, the carboxylic acids pyruvate and lactate, together with amino acids, are the dominant energy substrates of embryos. Therefore, at cleavage stage, ATP generation is completely reliant on mitochondrial-based metabolism via tricarboxylic acid (Krebs) cycle (3,8,19,39). The same results were obtained in animal studies, which showed that pyruvate consumption declines after 2-3 days, to be replaced largely by glucose (40,41). Consequently, pyruvate uptake has been investigated as a feasible marker of human embryo viability and developmental potential. Table 4 shows pyruvate metabolism as a predictor of embryo development and viability in human studies.

Table 4: Pyruvate metabolism as a predictor of embryo development in human studies

Year	Author	Media type	Pyruvate in media (mM)	Technology Used	Embryo stage examined	Consumption rate (pmol/embryo/h)	
						Development to Blastocyst stage/implanted	Arrested/Unfertilized
1989	Hardy et al. (42)	T6	0.47	Ultramicrofluorescence	Day 2.5-4.5	28-40	30
1990	Gott et al. (43)	T6	0.47	Ultramicrofluorescence	Day 2.5-4.5	36-52.5	30
1993	Conaghan et al. (44)	Earl's	0.47	Ultramicrofluorescence	Day 1-3	22.43-22.9	26.85-27.1
1994	Turner et al. (39)	HTF	0.47	Ultramicrofluorescence	Over 20-24h	21.5	23.5
2000	Devreker et al. (45)	EBSS	0.47	Ultramicrofluorescence	Day 1-2	13.3-15.6	14-15.5
2001	Gardner et al. (46)	G2.2	0.1	Ultramicrofluorescence	Day 4-6	33.4	25.8

As shown in table 4, the investigation into pyruvate metabolism in all human embryo studies has displayed a wide range of uptake values. Hence, whether pyruvate uptake is predictive of embryo development potential and viability appears to be inconclusive.

1.5.2 Glucose Metabolism

Upon compaction—the moment of transition from morula to blastocyst stage—uptaking and utilization of glucose by the embryo increases significantly, and glucose becomes a key metabolite for lipid, amino acid and nucleic acid synthesis and blastocyst hatching (2,8,47). Reports from bovine and mouse studies have shown that blastocysts with higher glucose uptake develop more successfully and result in more term pregnancies compared with those with lower glucose uptake (48,49). However, the work of Lane and Gardner (50) had a considerable effect on blastocyst development and selection. They have found that increased glucose uptake together with decreased lactate production was predictable for the healthiest mouse blastocysts before transfer, thereby directed to a significant increase in implantation rate. In contrast to animal

studies, several human studies failed to demonstrate a relationship between glucose uptake and positive blastocyst development (42,43,51). But in these studies, the media lacked pyruvate, lactate, amino acids and vitamins, which could potentially put significant stress on the embryos; therefore, the conclusion drawn was problematic. In a follow-up study, Gardner et al. (52) utilized sequential culture media and reported that, on Day 4, human embryos that formed blastocysts had higher glucose consumption, which could be consistently correlated with viability. Most recently, Gardner et al. 2011 presented a plausible relationship between increased glucose uptake from Day 3 onwards in individually cultured and transferred human embryos, and the embryos' subsequent viability and sex post-transfer. They also reported that this relationship appeared to be independent of morphological grade. Furthermore, on Day 4 of development, female human embryos consumed significantly more glucose than their male counterparts. They indicated that metabolic activity could serve as a valuable independent marker of developmental potential (53).

1.5.3 Amino Acids Uptake

Amino acids maintain the well-being of the embryo and serve a variety of functions. They are essential to protein and nucleotide synthesis, and they act as sources of energy, osmolytes, antioxidants, PH regulators, chelators and precursors of signaling molecules such as nitric oxide (19,54). The mixtures of amino acids during cleavage and blastocyst stage are added to human embryo culture media to improve blastocyst formation rates (54). Reverse-phase high performance liquid chromatography (HPLC) and proton nuclear magnetic resonance have been used to investigate the consumption and secretion of specific amino acids by human embryos at different stages of preimplantation development and their relation to overall embryo metabolism. For the first time, Houghton et al. (54) determined changes in the secretion and uptake of amino acids in human culture media. Their data indicated that preimplantation embryos which arrested before blastocyst stage had higher amino acid turnover rates than those which developed into blastocysts, consistent with the "quiet embryo" hypothesis - preimplantation embryo survival is best served by a relatively low level of metabolism (55). An interesting observation in the Houghton et al. study was that leucine—an essential amino acid—stimulates protein synthesis in a variety of cell types, this amino acid is depleted throughout development by those embryos which formed blastocysts. In a subsequent study from the same group, Brison et al. (56) showed

that a decrease in glycine and leucine and an increase in aspargine in the culture medium are significantly associated with the ability of an embryo to implant, and with clinical pregnancy and live birth. They also found that this association is largely independent of maternal age, ovarian reserve, embryo cell number and morphological grade. Using proton nuclear magnetic resonance (^{1}H NMR), Seli et al. (57) found a correlation between higher glutamate level in culture media and clinical pregnancy and live birth. The different technology used, as well as the volume and type of culture medium used, may explain the difference in identified amino acid biomarkers. Picton et al. (58) went a little further by profiling the level of amino acids with the ploidy status of the embryo. Based on their results, on Day 2-3, turnover of asparagine, glycine and valine was significantly higher among normal embryos. The significant differences were observed in tyrosine and lysine turnover among abnormal embryos. As development progressed, on Day 3-4, turnover of serine, leucine and lysine was significantly higher among abnormal embryos.

Although the findings regarding measurements of pyruvate, lactate, glucose and amino acids in human culture media are insightful and have presented some metabolic differences between embryos with different reproductive potential, the application of these technologies to a clinical setting has been limited. This is due to the fact that they are expensive, require significant expertise and time commitment and do not allow the information to be used clinically in the limited window of time acceptable for embryo transfer. In addition, an important technical drawback with many existing methodologies is the limited number of metabolites that can be determined by a single analytical platform.

1.6 Metabolomics

The preimplantation embryo needs proper metabolic turnover to remain viable and develop into a successful pregnancy. The metabolism of the embryo changes the concentrations of metabolites in the culture medium that could serve as biomarkers to signify the physiology and functional phenotype of embryos at a cellular level. As a result, studying nutrients and metabolites within the culture environment could be a predictor of embryo viability. The entire collection of metabolic pathway products secreted into the culture medium—which consists of the complete inventory of small-molecule (< 1 kDa), non-proteinaceous compounds, including amino acids, nucleotides, lipids, adenosine triphosphate (ATP), hormones, signaling molecules and secondary metabolites—is called the metabolome(3,19). These ultimate products of

metabolism have diverse physical and chemical properties with a wide concentration range. Metabolomics is the systemic study of unique chemical footprints that particular cellular processes leave behind. It provides a comprehensive analysis of all measurable metabolites under a given set of conditions (59). While genomics and proteomics can provide significant information on more than 25,000 genes in the human genome and 1 million proteins on the anticipated function, metabolomics provides a snapshot of all the most recent biological functions, reflecting up-to-the-minute events (3,14,60). Metabolomics provides an opportunity to investigate the relationship between an organism's genotype and its resulting phenotype, which also indicates a relationship between an organism's physiology and environmental conditions (8). This assessment can be targeted at the intracellular or extra-cellular metabolites. Intracellular or "intrametabolome" assessment is invasive and may affect cell viability. Assessment targeted at extra-cellular metabolites in the "spent" culture media left behind by the cell, or "exometabolome," is a noninvasive process and is the focus of this research proposal (61).

Studying the complex metabolic/metabolomic profiles of biological systems is performed by analytical technologies: spectroscopic/spectrometrics and chromatographic techniques. "Biospectroscopy" is the term used for the analysis of biological fluids with spectroscopic technologies. The combination of biospectroscopy with metabolic profiling is known as biospectriscopy-based metabolomics (BSM). The various techniques that have been applied in BSM studies include non-optical and optical spectroscopies (3,8,62). Common non-optical techniques applied in BSM studies include nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and separation methods such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-mass spectrometry (CE-MS). Fourier transform infrared (FT-IR), near infrared (NIR) and Raman spectroscopies, with similar physical mechanisms involved in each technique, are all optical spectroscopies that provide complementary profiles of the various components within biological fluids (8).

Despite the wide application of NMR and chromatographic MS technologies in research, their physical size, complexity of operation and cost limit the use of non-optical spectroscopy in clinical applications. The interaction of a chemical species with electromagnetic radiation is measured in optical spectroscopy. The main optical spectroscopies employed to detect vibrations in molecules are based on the processes of infrared absorption and Raman

scattering. They are widely used to quantitatively or semi-quantitatively determine the amount of substances in a whole range of physical states, as solids, liquids or vapors, in hot or cold states, in bulk, as microscopic particles, or as surface layers. Simple instrumentation, fast and highly reproducible measurements, easy maintenance and operation by minimally trained users are the advantages of optical spectroscopies in clinical research settings (8,14,63).

1.7 Raman Spectroscopy

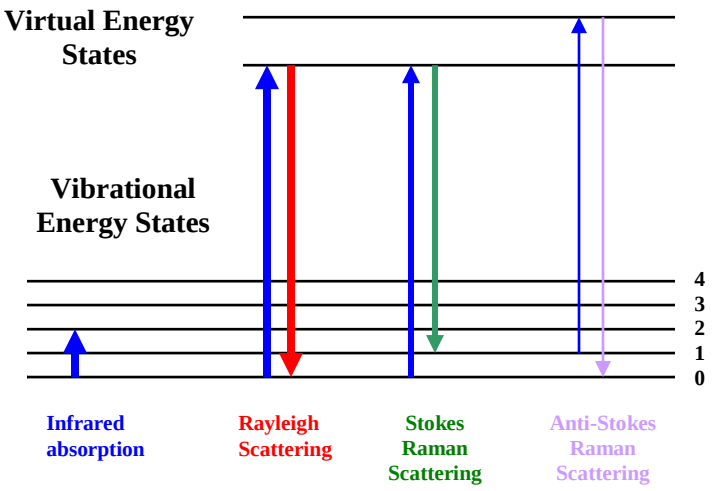
Light that is scattered from a molecule is primarily elastically scattered with the similar energy between the incident and the scattered photons. However, a small probability exists that a photon is scattered inelastically, resulting in either a net gain or loss of energy of the scattered photon. The phenomenon of inelastic scattering of light was first postulated by Smekal in 1923 and first observed in practice in 1928 by the Indian scientists Sir C.V. Raman and K.S. Krishnan (64). This phenomenon allows scientists to measure fundamental molecular vibrational transitions at a defined excitation wavelength.

Raman Spectroscopy is a spectroscopic technique which finds application in the field of metabolomics, and which is used to study vibrational, rotational and other low-frequency modes in a system. Vibrational spectroscopy has long been used for constituent analysis in the chemical industry—for example, in relation to agricultural products. It is dependent on inelastic scattering of monochromatic light, typically generated from a laser in the visible, near infrared, or near ultraviolet range (8).

In a Raman experiment, when monochromatic light interacts with the electron cloud and the bonds of a molecule, the light can be scattered or absorbed. Most of the scattered light will be at the same frequency as the incident light. This is called Rayleigh scattering or elastic scattering. For a fraction of the time, a molecule will scatter a photon inelastically. The energy of the molecule returns to a different vibrational state. This process of exchanging energy between scattering molecules and the initial light is the Raman effect (65,66).

Figure 2: Energy level diagram of vibrational spectroscopic transitions involved in Raman signals

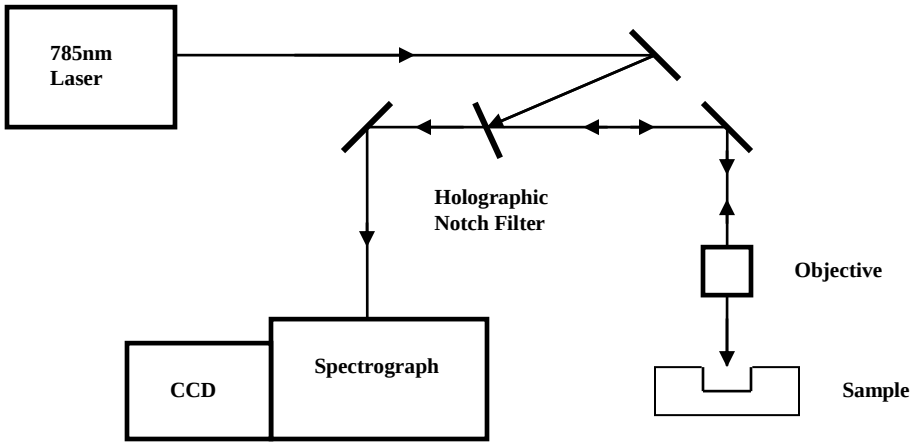
The line thickness is proportional to the signal strength from the different transitions.



Typically, a laser is utilized to illuminate a sample for promotion of a vibrational excitation or relaxation along a molecular vibrational mode. Light from the illuminated spot is collected with a lens and sent through a monochromator. By filtering out the elastic Rayleight scattering, the rest of the collected light is dispersed onto a detector (8,66).

Raman spectroscopy has similar levels of analytical sensitivity to other optical spectroscopy tools, with several added advantages: 1) less expensive to acquire and operate, 2) analysis requires little or no sample preparation, 3) little chemical bias, 4) smaller instrument size, 5) gives a low background response to water, and 6) rapid, simultaneous analysis of multiple biomarkers (3,14).

Figure 3: Schematic of a common sampling geometry for Raman spectroscopy

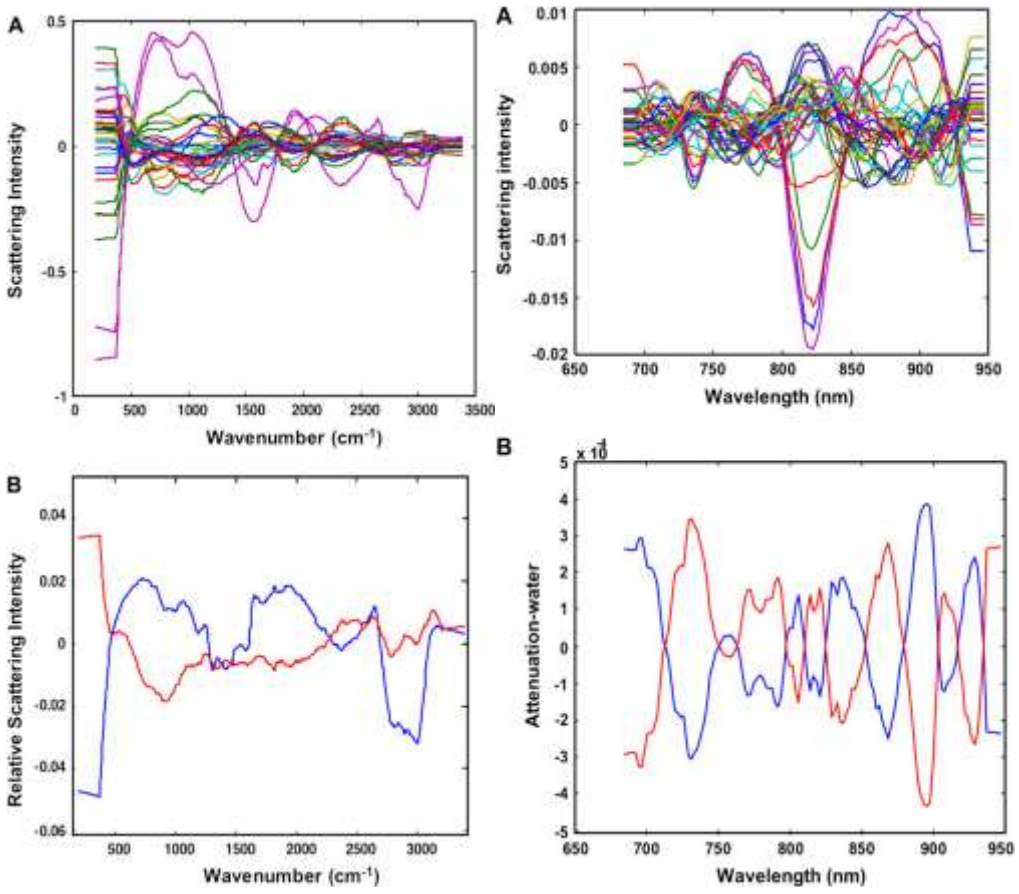


1.8 Human Embryo Culture Media and Metabolomic Profiling

The advent of metabolomics in the field of assisted reproduction is very recent. So far, there are only a few of studies evaluating spent media samples using optical spectroscopy for metabolomic profiling. The first report of optical spectroscopy to develop a model for embryo viability prediction was published in 2007. In this study, Seli et al. (1) analyzed Day 3 spent culture media of individually cultured embryos with known outcomes using both Raman and NIR spectroscopies. The authors compared the spectra of embryos that resulted in a pregnancy and live birth with those that failed to implant. They derived a score for the spectrum reflecting each embryo viability score using a genetic algorithm based on spectral regions of pregnancy outcome prediction. This study confirmed that, notwithstanding different culture media and samples from different IVF centers, the mean viability score (also called viability index) of embryos that implanted and resulted in a live birth was significantly higher than the score of those that failed to result in implantation and pregnancy. The authors concluded that Raman and NIR spectroscopies achieved a sensitivity of 76.5 and 83.3 % and a specificity of 86 and 75%, respectively. In addition, these tests were rapid (<1 min per sample) and needed a very small sample volume (<15 μl).

Figure 4: Signals from Raman and NIR Spectroscopy by Seli et al.

(A) Signals obtained by Raman Spectroscopy (left) and NIR spectroscopy (right). (B) Mean values for culture media of embryos that implanted and led to delivery (in red) and those that did not implant (in blue). Represented with permission; Ref. 1



The model developed in the initial study was then tested in a second study conducted by the same group (7). In a blinded design, they tested Day 3 and Day 5 spent culture media collected from a different center using Raman spectroscopy. In this study, embryos were cultured in a different volume (50 µl instead of 30 µl) and type (SAGE versus Vitrolife). The authors applied the previously developed algorithm to the Raman spectra of embryo culture media and successfully predicted the outcome of pregnancy for embryos transferred on Day 3 and 5. The viability indices of Day 3 and Day 5 samples associated with implantation were significantly higher than those that were not. Moreover, the viability indices on Day 3 were significantly

higher than those on Day 5 and the overall accuracy of biospectroscopic metabolomic profiling was 80%.

Subsequently, additional studies were performed from IVF centers where single embryo transfer (SET) was routinely practiced (67,68,69,70). In these studies, NIR spectroscopy and bioinformatics were applied to analyze culture media. Then independent regression algorithms for NIR spectra of embryos that underwent SET on Day 2 (69,70), Day 3 (70) and Day 5 (68) were obtained. The findings of these more recent studies, in agreement with the findings of the initial studies, showed that mean viability scores of embryos that resulted in a pregnancy with fetal heart activity are higher than those that did not (67,68,69,70). Their findings also showed that metabolomic profiling of embryo culture media was independent of morphology (68,69,70) and that identification of embryos with higher implantation potential may be improved by employing a combination of morphology and metabolomic profiling (69).

The data summarized above is encouraging, and also demonstrates rapid, non-invasive and reliable potentiality of these technologies as an adjunct for embryo assessment that can be applied on site. In addition, one of the advantages of a spectroscopic approach to assessing spent culture media is that the whole sample can be monitored simultaneously. However, to date, all published studies were derived from data using frozen spent culture media. Therefore, metabolomic profiling on fresh samples at the IVF site needs to be undertaken. Additionally, so far, metabolomic assessment has been practiced on different types of culture media, using different volumes and different days of preimplantation development. We assume that all findings, yet, are not independent of culture media or volume use. Thus, the accuracy affected by these factors will need to be further investigated. Finally, because of the small patient sample size, these studies were unable to assess the effects of other predictors such as female age, duration of infertility, and other treatment parameters that correlate with embryo quality (e.g., number of oocytes fertilized and number of embryos for transfer or cryopreservation) that might have confounded their findings.

2 Objectives

The objectives of this study were to assess embryo quality using metabolomic profiling of embryo culture media with Raman spectroscopy as a non-invasive approach to predicting embryo viability, and consequently, implantation and pregnancy. In addition, to compare metabolomic profiling with the existing morphological assessment of embryo quality and other predictors of pregnancy and to further characterize the micromolecules profiled by Raman spectroscopy in a sample subset as a preliminary approach to probing physiological functions. Due to insignificant results of first part, comparison of metabolomics profiling with the existing morphological assessment and characterization of micromolecules by Raman spectroscopy are not possible.

A secondary objective of this study was to determine the limits of detection of the Raman spectroscopy for glucose and glycine, corresponding to the main nutrient for Day 3 and Day 5-6 embryos, respectively.

The overall purpose of this study was to examine if Raman could improve our ability to predict positive outcomes and therefore, potentially modify the number of embryos replaced to minimize multiple pregnancies.

3 Materials and Methods

3.1 Patient selection

All patients participating in the study were recruited from the UBC Centre for Reproductive Health (UBCCRH). The approval of the institutional review board was obtained before the initiation of the study. UBCCRH performs about 300 IVF cycles annually. From January 2011 to October 2011 women undergoing IVF+/-ICSI treatment were approached and consented to be part of this study. Demographic and clinical information – including age, duration of infertility, prior IVF cycles attempted and ovarian reserve screening (e.g., day 3 FSH ± antral follicle count) - were obtained from each patient. Pregnancy outcomes were recorded for each patient. Serum pregnancy test (was done about 14 days after embryo transfer. Clinical pregnancy is defined as the presence of an intrauterine gestational sac on a 6-7 week ultrasound examination, irrespective of a fetal heart beat.

3.2 Treatment

At UBCCRH, stimulation protocols used were as follows:

- Micro-Dose Lupron Flare Protocol

This protocol utilized the initial pituitary stimulation with a small dose of the GnRH agonist, Lupron, and subsequent pituitary suppression with continuous use. Due to the theoretical concern of excessive LH release at standard lupron doses, a micro-dose lupron flare protocol was developed. This protocol as recommended for patients who met the following criteria in their pre-cycle evaluation:

1. Day 3 FSH $\geq$ 10 IU/L and/or
2. Day 3 Estradiol $\geq$ 250 pmol/L and/or
3. Antral follicle count (AFC) $\leq$ 8 and/or
4. Anti-Müllerian hormone (AMH) $\leq$ 0.4 ng/ml (US units)

Lupron is a GnRH agonist (leuprolid acetate) which is administrated by subcutaneous, self-administered injection. The micro-dose lupron was started on the third day after the last OCP and was continued daily up to and including the day of human chorionic gonadotropin (hCG) administration. Gonadotropin stimulation commenced on the third day of Lupron administration and the doses were adjusted according to estradiol levels, and follicle size and

number on serial blood tests and ultrasound assessments, respectively. hCG was administered when the three leading follicles were at least 17-18 mm and oocyte retrieval was planned 36 hours later.

- Long Luteal Agonist Protocol

This has been the most commonly used protocol for IVF, representing over 50% of cycles performed in Canada. It describes the appropriate instructions for producing pituitary down regulation and subsequent suppression of gonadotropin ovarian stimulation. This protocol was selected for patients with average ovarian reserve who were anticipated to be good responders.

1. Day 3 FSH ≤ 10 IU/L and day 3 Estradiol ≤ 200 pmol/L and
2. AFC 9-20 and /or
3. AMH 0.4-3 ng/ml (US units)
4. No previous excessive response (≥ 16 eggs) or ovarian hyperstimulation syndrome (OHSS) in a previous cycle

For the GnRH analogue, Synarel (intranasal), Lupron (subcutaneous) or Superfact (subcutaneous) is used.

- Estrogen Priming Antagonist Protocol

This protocol describes the appropriate instructions for using ovulation monitoring in the preceding natural cycle with estradiol patch administration starting in the mid-luteal phase to promote synchronous follicular development in potential poor responders. Gonadotropin stimulation is administered and monitored as per flow sheet (number), and GnRh antagonist is initiated to avoid premature ovulation. This protocol was recommended for patients who met two or more of the following criteria in their pre-cycle evaluation.

1. Day 3 FSH ≥ 10 IU/L and/or
2. Day 3 Estradiol ≥ 200 pmol/L and/or
3. AFC ≤ 8 and/or
4. AMH ≤ 0.4 ng/ml (US units)
5. Poor response in a long agonist protocol

With the help of staff from hospital, we acquired a set of preliminary samples for Raman spectroscopic analysis. These samples were acquired over a period of one year and kept at -10 degrees in the hospital with oil removed. We subjected these samples to Raman spectroscopic analysis all together during the methods development phase of this project. Without oil protection, these samples dried out. We reconstituted them for Raman analysis by adding 30 µl of water to dissolve metabolites. Raman spectra showed that these samples were contaminated by residual oil. This oil has a very strong Raman signal, which overlaps with signals from the culture media in some regions, making it impossible to subtract for classification according to embryo viability. In terms of evaporation, contamination and ease of sampling, have we found it best to transport fresh samples frozen under oil using dry ice. We planned the following steps for samples collection and measurement.

3.3 Sample Collection

The following steps summarize the clinical and laboratory procedures and required data collection in routine practice. Spent culture media otherwise discarded were collected for Raman spectroscopy and marked with an asterisk:

1. Controlled ovarian stimulation: gonadotropins were administered and ovarian response was monitored with serial ultrasound and serum estradiol assessment and recorded according to protocols.
2. Oocyte retrieval was performed with vaginal ultrasound-guided needle aspiration under local anesthetics and conscious sedation and the oocytes were inseminated or injected (ICSI) with sperm on the same day (day 0). Afterwards eggs were cultured in 0.8 mL G1 medium overlaid with oil.
3. On the next day (day 1), each oocyte was examined for evidence of fertilization by the appearance of two pronuclei.
4. Each of these fertilized oocytes was then mechanically placed into individual fresh droplets for culture to the cleavage stage (day 1-3).
5. Embryo-free control drops were also incubated alongside the embryo-containing drops.
6. Specifically, each embryo was cultured individually from days 1-3 in "≈30 µl" of G1 media (VitroLife, Englewood, CO).

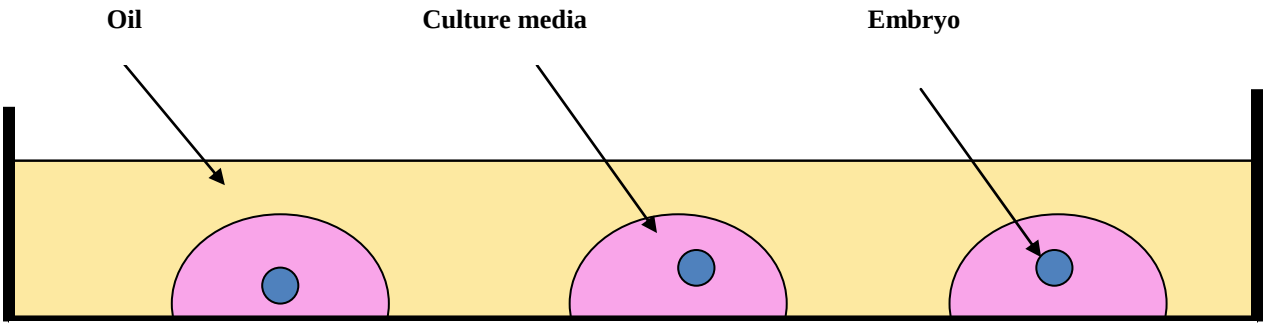
7. Treatment parameters such as number of follicles, number of oocytes obtained and fertilized, and embryo morphological assessment was recorded as in clinical practice, together with the relevant demographic and clinical information outlined earlier.
8. After the embryos have been removed on day 3 according to (9) below, the spent media drop (Day 3 media)* and a control media drop were immediately placed on dry ice box and transported to t the Department of Chemistry at UBC for Raman spectroscopic analysis on the same day.
9. On day 3, the destiny of the embryos was recorded:
    a. transfer to the patient's uterine cavity
    b. cryopreservation on day 3
    c. transfer to fresh G2 media drop (VitroLife, Englewood, CO) for culture to day 6 to the blastocyst stage before
        i. transfer to the patient's uterine cavity
        ii. cryopreservation
        iii. discarded due to poor development
10. In any of the three scenarios in (9c) above, the day 6 culture media plate was placed on dry ice box and then kept the plate temporarily frozen in dry ice for transported to UBC's Department of Chemistry for Raman spectroscopic analysis that same day.
11. Implantation and clinical pregnancy as documented by fetal sacs and fetal activities on ultrasound assessment were recorded and used as the gold standard for embryo quality assessed by Raman spectroscopy and light microscopy assessment.

To assess a "dose-response" relationship, the spent media drops were classified according to the percentages of embryo(s) implanted as observed on ultrasound and outlined earlier. The outcome of frozen-thawed embryos was similarly tallied and correlated with the metabolomic profiling of the spent media drops.

As mentioned before, oil has features in Raman signals. To obtain fresh samples, we firstly removed oil with a glass micropipette, and then further assessed the samples under direct light to make sure that they were not contaminated by oil before aspirating the sample with a new pipette tip for Raman spectroscopic analysis. Without the additional step of first removing the oil with the separate glass pipette, the tip of the sample pipette used to would be

contaminated with oil. The oil was then replaced to cover the remaining droplets to prevent evaporation, and the process was repeated. See Section 3.4.2.

Figure 5: Schematic picture of the Petri dish containing the embryo inside the culture media covered with oil



3.4 Development of Methods for Raman Spectroscopy

3.4.1 Instruments

The present measurements combined an Olympus BX51microscope with a reflex Raman probe that collected the Raman spectrum at the focus position of the microscope objective. The position of the microscope stage could be controlled under stepper-motor control with an accuracy of 1 μm by means of a custom LabVIEW user interface. This program also controlled Raman signal acquisition and offered on-line wavelet transform of acquired spectra. A 785nm single- mode diode laser had been applied in this system. A six-around-one fiber bundle carried the backscattered signal to a 0.3m spectrograph equipped with an advanced thermoelectrically cooled CCD detector.

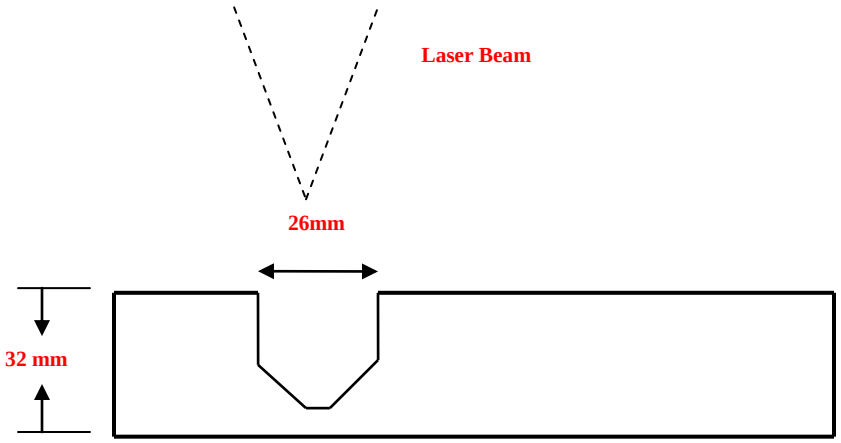
3.4.2 Experimental Methods for Raman Spectroscopy

- Conventional Raman spectroscopy

An aluminum well-plate cuvette with a diameter of 2.6mm and height of 3.2mm for holding a volume of 18 μl was designed and developed at the Department of Chemistry, University of British Columbia (UBC). For obtaining Raman spectra, each sample was exposed to a 785nm diode laser with a power at the source of 350 mW for 5 minutes. In this way, a high

signal-to-noise ratio would be obtained. Routinely, each patient's Petri dish had 6 to 10 sample drops under oil. In addition, each dish contained 1 to 3 control drops consisting of culture media without fertilized embryo. To obtain 18 µl of clean sample, we first removed the oil by a glass micropipette. After transferring each sample to the cuvette with a new clean plastic micropipette for Raman analysis, the oil was returned to the Petri dish. Oil covered the remaining samples to prevent evaporation and contamination. Samples with a volume less than 18 µl without Raman analysis were discarded. Fresh samples on Day 3 and Day 6 were available several times per week to trace the change in metabolites mirroring each embryo development.

Figure 6: Aluminum cuvette used to hold 18 µl of culture medium



- Sample transportation

Transportation the samples under oil in the Petri dish from BC Children's and Women's Hospital to UBC for Raman analysis results in the combination of all the drops. For preliminary Raman spectroscopic analysis, spent culture samples without oil were kept at -10 degrees over a period of one year by Dr. Cheung and his IVF lab staff. Due to the absence of oil protection, these samples dry out, so for Raman analysis we added 30 µl of water to dissolve metabolites. Obvious variances existing in the Raman signal (the region 1400-1600cm^{-1}) indicating that the

samples were contaminated by residual oil. Spectra from these samples clearly demonstrated that oil had a very strong Raman signal which overlapped with signals from culture media and made it difficult to subtract for classification according to embryo viability. As a result, we re-designed…as described for analysis. To minimize evaporation and oil contamination, every time fresh samples were available, we transported fresh samples under oil in a …temporarily frozen in dry ice for immediate transport to UBC and then thawed within half an hour for prompt analysis by Raman spectroscopy.

- Chemometrics in the analysis of Raman spectrum

After data collection, various pre-processing steps were undertaken to improve data quality. Background and noise always adversely affect Raman spectra analysis. We needed to generate a model analysis to identify spectral features associated with biomarkers. Discrete Wavelet Transform (DWT) is a powerful preprocessing method that has been applied to remove background and noise. DWT is performed by means of scaling and translating a mother wavelet.

$$\varphi a,b = \frac{1}{|\sqrt{a}|} * \varphi\left(\frac{t-b}{a}\right) \quad a \neq 0$$

Here, a is the scaling variable related to frequency and b is the position variable related to time, yielding a discrete wavelet transform (DWT) by (a,b)=$\sum f(t) * \varphi_{a,b}$ By choosing a and b, we can remove the background and noise from spectra.

Raw spectra of unused G-1 and G-2 media looked almost the same. Yet, after DWT, we could zoom in on the differences that made these samples easily distinguishable to the naked eye.

Raman analysis produces a large amount of data from each spectrum. Therefore, we had to use large-scale multivariate data analysis techniques (chemometrics) to characterize the samples and applied classification results to predict the implantation outcomes in terms of a quantitative model. Among multivariate techniques, Principal Component Analysis (PCA) offers the simplest picture of the distribution of a dataset based on its variance. Principal component analysis (PCA) is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables. The number of principal components is less than or equal to the number of original variables. Principal components are guaranteed to be independent only if the data set is jointly

normally distributed. PCA is sensitive to the relative scaling of the original variables. Each Raman spectrum contains data concerning 1340 wavelengths, but the information in these 1340 dimensions is highly correlated. PCA can reduce the dimension of the dataset by orthogonalizing the components of the data set, ordering the resulting orthogonal components (principal components) by their variations and eliminating those components with the least contribution of the variation to the data set.

One drawback of the PCA technique is that it captures only the characteristics of the *X*-vector or predictive variables. No importance is given to how each predictive variable may be related to the dependent or the target variable. Partial Least Square (PLS) allows us to achieve the balance between the predictive and target variables and provides an alternate approach to the PCA technique. This is a statistical method that finds a linear regression model by projecting the predicted variables and the observable variables to a new space. The PLS methods are known as bilinear factor models which try to find the fundamental relations between two matrices (*X* and *Y*). The PLS regression is today most widely used in chemometrics and related areas. Finally, by applying the root-mean-square deviation (RMSD) or root-mean-square error (RMSE), we measure the accuracy of the PLS model. RMSE is frequently used to measure the differences between values predicted by a model or an estimator and the values actually observed. In analytical biochemistry, RMSE less than 1 is scientifically acceptable.

- LabVIEW user interface program

The computer software used in conventional Raman systems gives only the real-time raw Raman spectra of samples. It generally does not offer any spectrum pre-processing functions or the capacity for developing chemometric model systems. In such conventional spectra, the dominance of background and noise can suppress and obscure the molecular vibrational peaks of interest. This makes it impossible to establish the feasibility of experimental methods or identify peaks for biomarkers in real time. With the great help of a Chemistry lab student and her supervisor, a LabVIEW platform was built which not only controls all the Raman hardware, but also integrated a sophisticated signal processing system, including a capacity for online DWT.

All spectra were recorded by Alaleh Asghari Roodsari and Zhiwen Chen. Some data was sent to Daniel Chen at Tianjin University, China for supplemental Raman processing.

3.5 Raman Spectroscopic of Glucose and Glycine Solutions

To validate our method, we had to design the experiments which could show that Raman has sensitivity to detect small changes in culture media. The capacity of glucose metabolism significantly reflects the embryo's developmental potential and viability; in addition, documented data have demonstrated that amino acids enhance embryo development to the blastocyst stage and subsequent viability. We demonstrated the detection of limitations of glucose and glycine by Raman spectra of samples with a wide range of glucose and glycine concentrations in distilled water. In analytical chemistry, the limit of detection (LOD), is the lowest quantity of a substance that can be distinguished from the absence of that substance (a *blank value*). The accuracy of the model used to predict concentration from the raw analytical signal can affect the detection limit as the mean of the blank and standard deviation of the blank affects it.

To conduct this experiment, molar solutions of both glucose and glycine were prepared. A mole is the molecular weight (MW) expressed in grams (sometimes referred to as the 'gram molecular weight' (gMW) of a chemical). For making the stock solution of glucose and glycine, we needed to have the molecular weight of these two chemicals. The molecular weight of glucose and glycine are 180.16 g/mol and 75.07 g/mol respectively. The stock solution for both chemicals was 500 mM in 100 ml distilled water. The first thing we needed to do was to make all units of measures similar. 0.1 litre x 0.5 mol/litre = 0.05 mol glucose/glycine, then we needed to weigh this amount of glucose/glycine in grams. So by multiplying this number by molecular weight, the amount of glucose and glycine in grams was obtained.

0.05 mol glucose x 180.16 g/mol = 9.008 g glucose

0.05 mol glycine x 75.07 g/mol = 3.753 g glycine

We weighed out these amounts of glucose and glycine and placed them separately in 100 ml volumetric flasks. Then we added a small volume of distilled deionized water to dissolve the chemicals and filled the flask to the 100 ml line. Thirty standard solutions of glucose and glycine were prepared from stock solution (250,100, 20,15,10,9,8,7,6.5,6,5.5,5,4.5,4.25, 4,3.5,3.25,3,2.5,2.25,2,1.5,1.25,1,0.5,0.25,0.1,0.05,0.025 and 0 mM). All solutions were freshly prepared and conditioned for calibration 1 h before the measurements, to allow the anomeric equilibrium to be established. Doubly distilled and deionized water was prepared in the

Department of Chemistry and its purity was tested. Higher concentrations of both nutrients ere needed to test linearity. Three high-quality Raman spectra of glucose and glycine of each solution were measured using the same preocedures as in the study samples. These data were generated to show how well Raman could differentiate variant levels at low concentrations.

3.6 Statistical Analysis

Student's t-test was used to compare mean values of the patient demographics. Alpha error of less than 0.05 is considered significant for all comparisons. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) package version 20.0.

4 Results

4.1 Demographic and Treatment Results

From January through October 2011, a total of 54 patients signed the consent form and participated in this study. Women in this study were aged 27-43 years (mean age ± SD: 36.33 ± 3.26). The following causes of infertility were found in our patients: 19 (35.18%) Male factor, 10 (18.51%) female tubal factor, 3 (5.5%) endometriosis, 5 (11.1%) ovulatory dysfunction including polycystic ovary syndrome and 22 (40.74%) unexplained. For some patients, more than one factor contributes to infertility. Twenty-seven (50%) patients used the long luteal agonist protocol; 13 (24.07%) used the estrogen priming antagonist protocol and 14 (25.92%) used the micro-dose lupron protocol.

A total of 300 day 3 and 40 day 6 spent embryo culture media samples from 54 patients were prospectively evaluated using Raman spectroscopy. Embryo-free control drops as a matched control group were evaluated concurrently. Thirty-one patients underwent IVF, 19 ICSI, and 4 patients both IVF and ICSI. All samples were analyzed successfully and included in data analyses; none of the patients were excluded before and after analyses. No samples were less than 18 µl.

Figure 7: Pregnancy outcome by different stimulation protocols

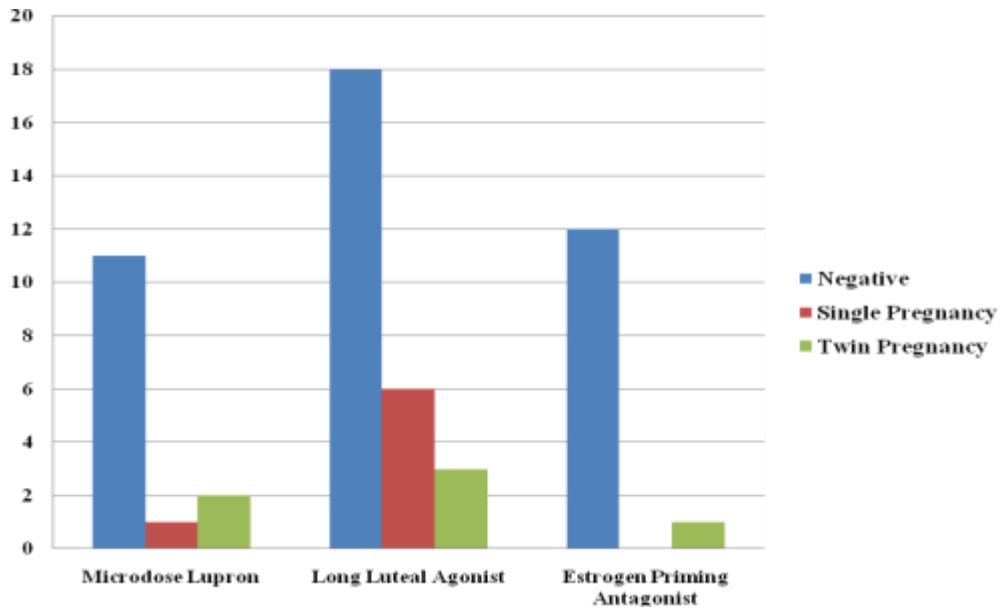
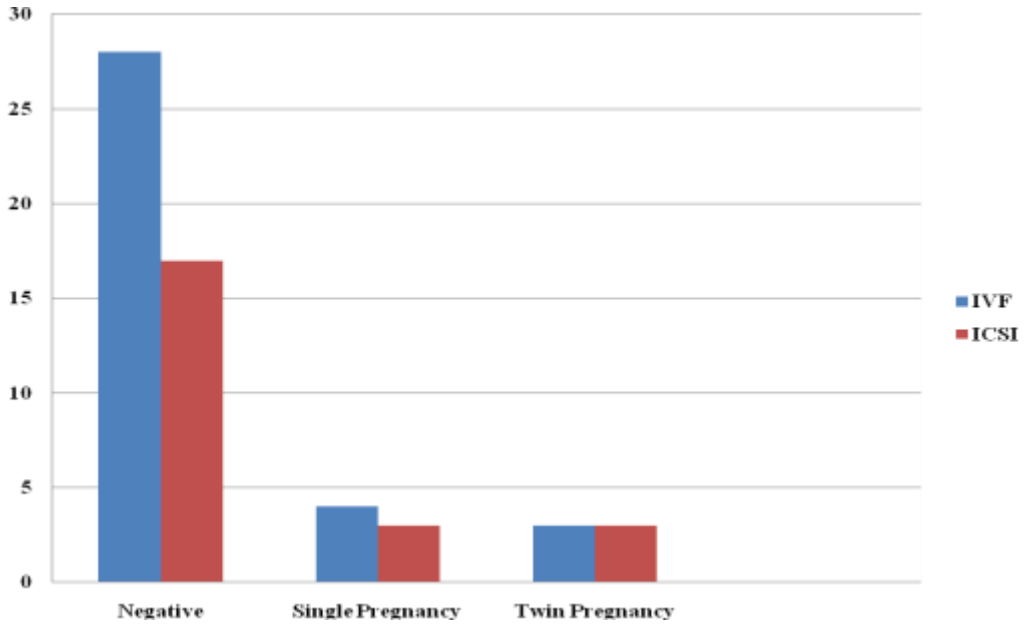


Table 5: Staging and Grading of transferred embryos on Day 3 with successful pregnancy outcome

# of Patients with positive pregnancy outcome	# of transferred embryo	Stage	Grade	Single/Twin pregnancy
1	1	8	1	Single Pregnancy
2	1	8	2	Single Pregnancy
3	3	6	3	Single Pregnancy
		4	1	
		6	3	
4	2	8	1	Twin Pregnancy
		8	3	
5	2	8	1	Single Pregnancy
		8	1	
6	3	8	2	Twin Pregnancy
		8	2	
		8	3	
7	2	8	2	Single Pregnancy
		8	3	
8	2	8	2	Single Pregnancy
		8	1	
9	2	6	4	Twin Pregnancy
		8	3	
10	2	8	1	Twin Pregnancy
		8	2	
11	3	8	4	Twin Pregnancy
		8	4	
		8	4	
12	2	7	2	Single Pregnancy
		8	2	
13	3	8	2	Twin Pregnancy
		8	2	
		8	2	

Of the 111 embryos transferred on day 3, 19 implanted and led to x pregnancies. Four single pregnancies and 3 twin pregnancies resulted from IVF, whereas 3 single pregnancies and 3 twin pregnancies resulted from ICSI. Twenty-four embryos on day 3 and 41 embryos at blastocyst stage on day 6 were cryopreserved. The remaining embryos did not have sufficient quality based on light microscopy scoring on Day 6 for cryopreservation. Seven (12.96%) single pregnancy, 6 (11.1%) twin pregnancy, 3 biochemical pregnancy and 1 ectopic pregnancy were observed.

Figure 8: Pregnancy outcome by means of fertilization



Based on morphological assessment, of these 300 embryos, 50 (16.66%) were grade 1/excellent quality, 63 (21%) were grade 2/good quality, 126 (42%) were grade 3/medium quality and 61 (20.3%) were grade 4 or more/poor quality. Among single pregnancies, two patients of age 40 and 27 had 1 embryo transferred (of grade 1 and grade 2, respectively); four patients of age 34, 38, 33, and 37 had 2 embryos transferred and all with single; one patient of age 35 had 3 embryos transferred, (one grade 1 and two grade 3). Among twin pregnancies, three patients had 2 embryos transferred (two embryos at grade 1, one embryo at grade 2, two embryos

at grade 3 and one embryo at grade 4); three patients had 3 embryos transferred (five embryos at grade 2, one embryo at grade 3 and three embryos at grade 4).

4.2 Analysis of Day 3 Samples

Evaluation of the day 3 samples demonstrated that the spectroscopic signals were similar for spent culture media droplets and control media droplets. There were also no differences in spectroscopic signals between spent media droplets from embryos with proven reproductive potential and those that failed to implant. There were no differences in Raman spectra associated with embryos fertilized by intracytoplasmic sperm injection and those following standard in vitro fertilization. In all graphs, each line represents the signal corresponding to each individual sample.

Figure 9: The Raman spectra of control samples on day 3

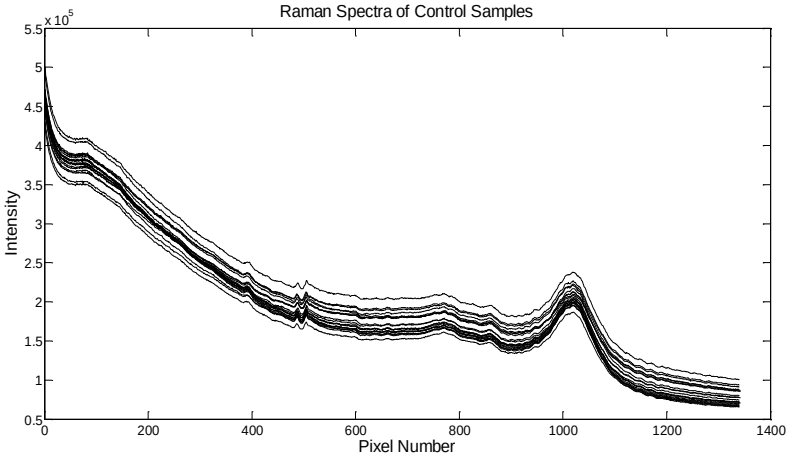


Figure 10: The Raman spectra of spent culture media on day 3

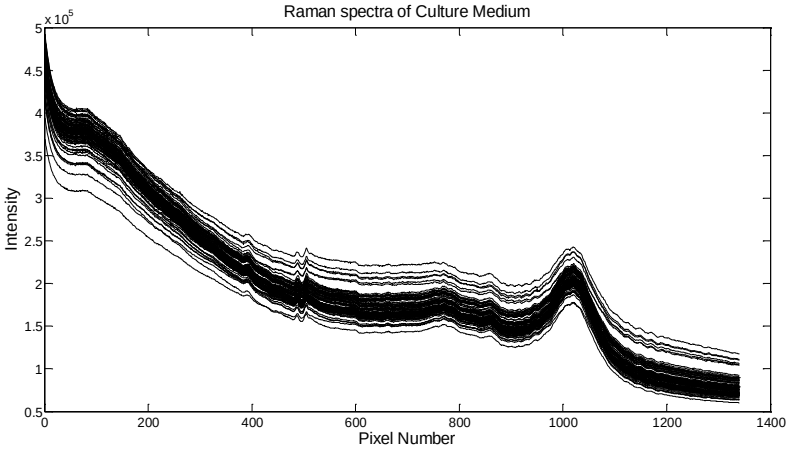


Figure 11: The Discrete Wavelength Transform (DWT) preprocessed Raman spectra of control samples on day 3

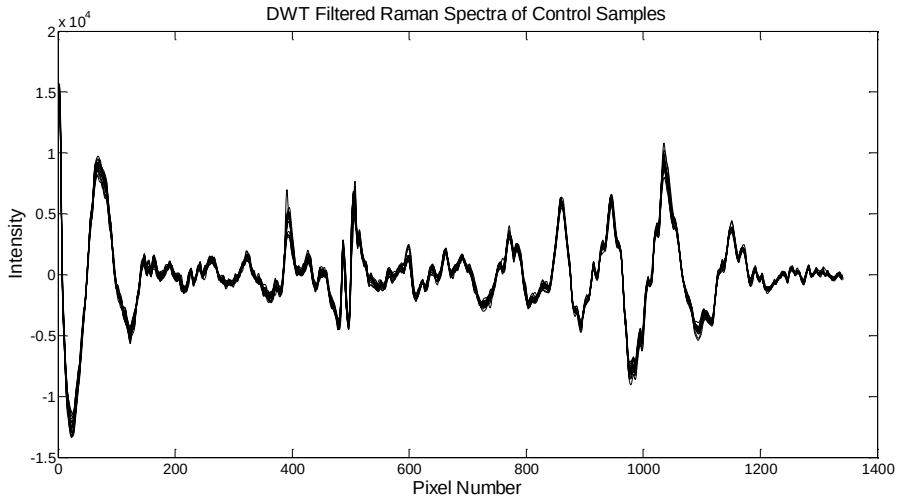


Figure 12: The Discrete Wavelength Transform (DWT) preprocessed Raman spectra of spent culture media on day 3

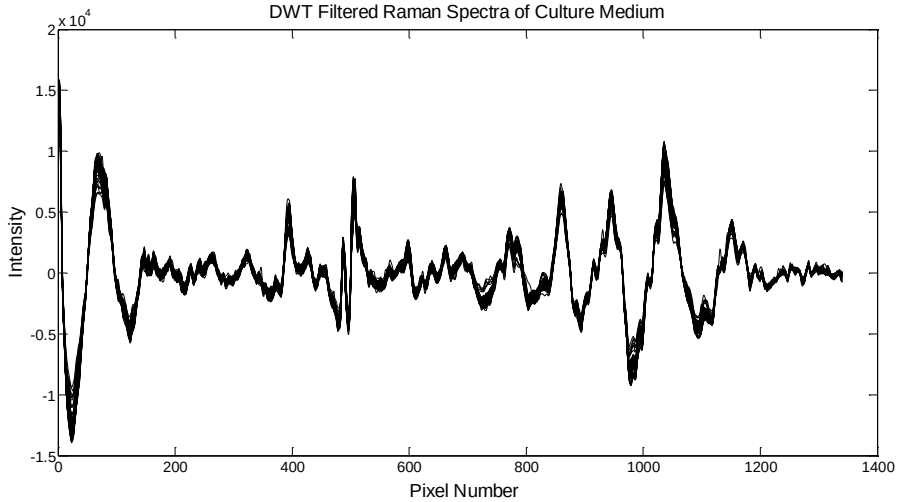
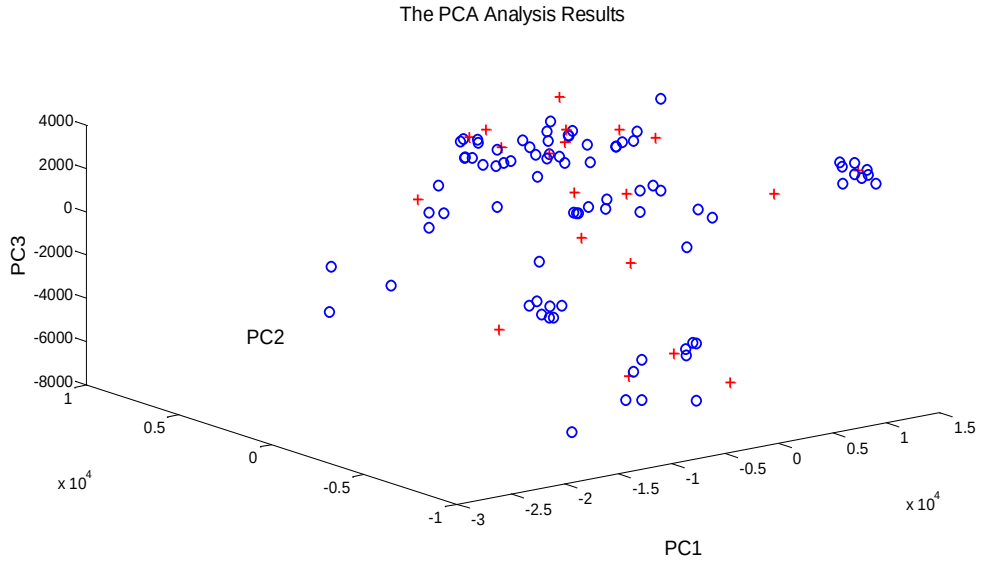


Figure 13: The PCA analysis results of all samples on Day 3

Including control samples and spent culture media. Blue circles represent spent culture media and red crosses represent control samples



4.3 Analysis of Day 6 Samples

As with Raman spectroscopic analysis on Day 3 samples, no differences were observed between the signals obtained from spent culture media and control media on day 6. Raman spectroscopic analysis of Day 6 samples also confirmed that there were no differences between embryos with proven reproductive potential and those that failed to implant.

Figure 14: The Raman spectra of control samples on day 6

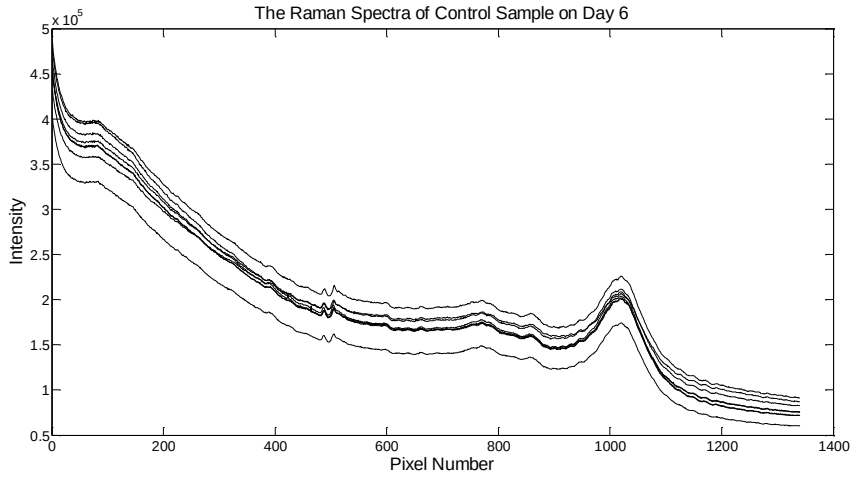


Figure 15: The Raman spectra of spent culture media on day 6

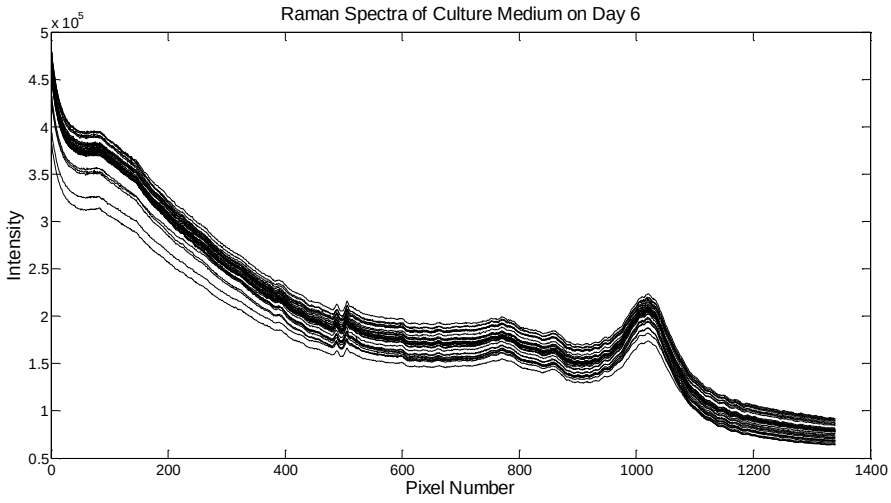


Figure 16: The Discrete Wavelength Transform (DWT) preprocessed Raman spectra of control samples on day 6

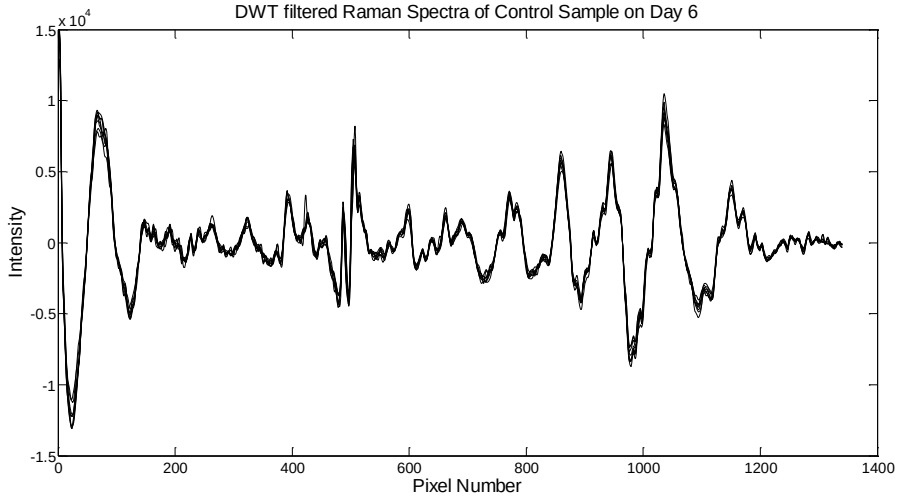


Figure 17: The Discrete Wavelength Transform (DWT) preprocessed Raman spectra of spent culture media on day 6

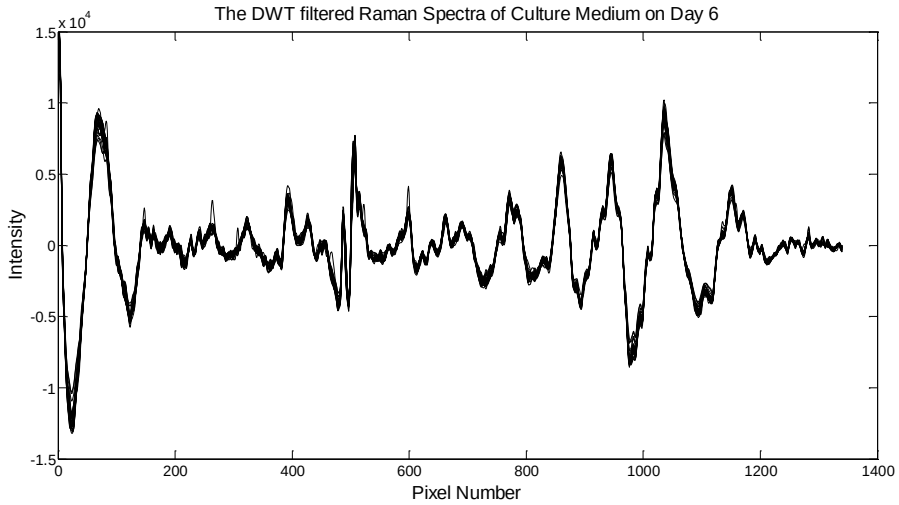
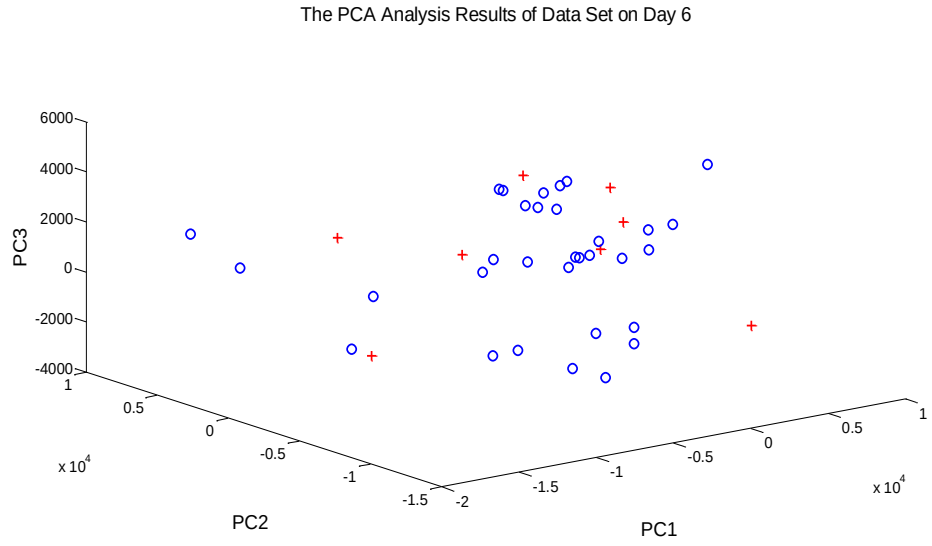


Figure 18: The PCA analysis results of all samples on Day 6

Including control samples and spent culture media. Blue circles represent spent culture media and red crosses represent control samples



Since our study results using Raman spectroscopy failed show significant signal differences between embryos with high implantation potential and those that failed to implant, and differences between the spent culture media containing an embryo and the control media, ,

we undertook detailed experiments to determine the limits of sensitivity of Raman spectroscopic analysis with respect to our biological samples. Given Raman advantages, we hypothesized that it should be a sensitive tool to detect small molecules that should produce Raman signals. To mirror the requirements of Day 5 and Day 3 embryos, we chose glucose and glycine, respectively to ascertain their corresponding detection limits using Raman spectroscopy.

4.4 Analysis of Glucose and Glycine Solutions

4.4.1 Glucose

Our detailed measurementsshowed that prominent signals could be detected in higher glucose concentrations (~ 250, 100 mM). However, in lower concentrations close to the physiologic concentrations of glucose in culture media, some of the Raman signals overlapped with pure distilled water and, in some instances, the signals were no longer detected.

Figure 19: Comparison of higher concentrations of glucose with pure distilled water to show linearity

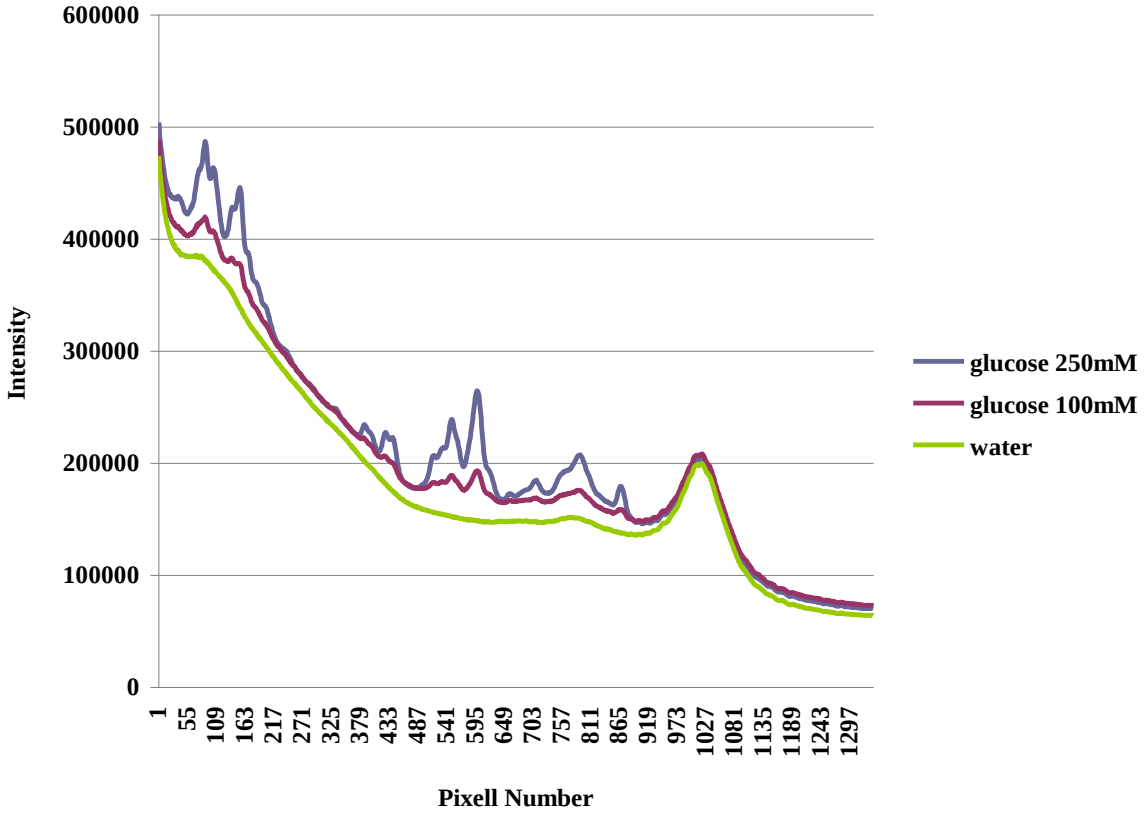
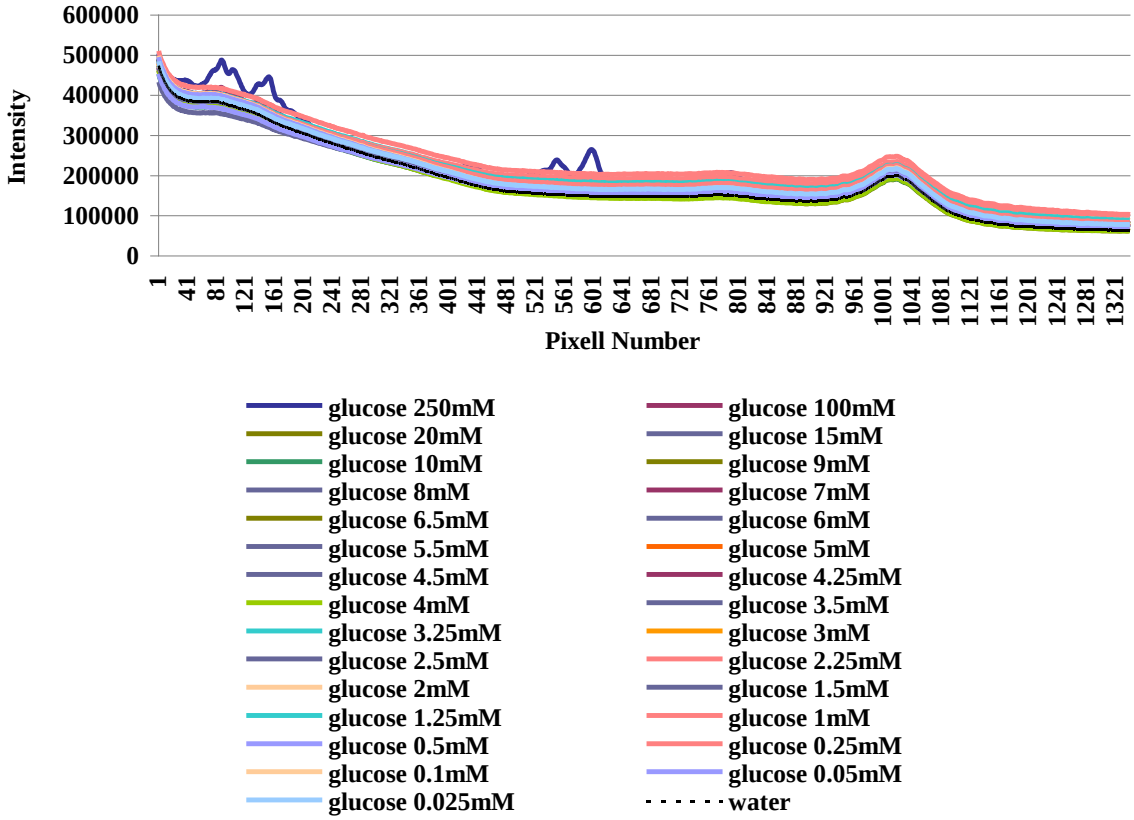
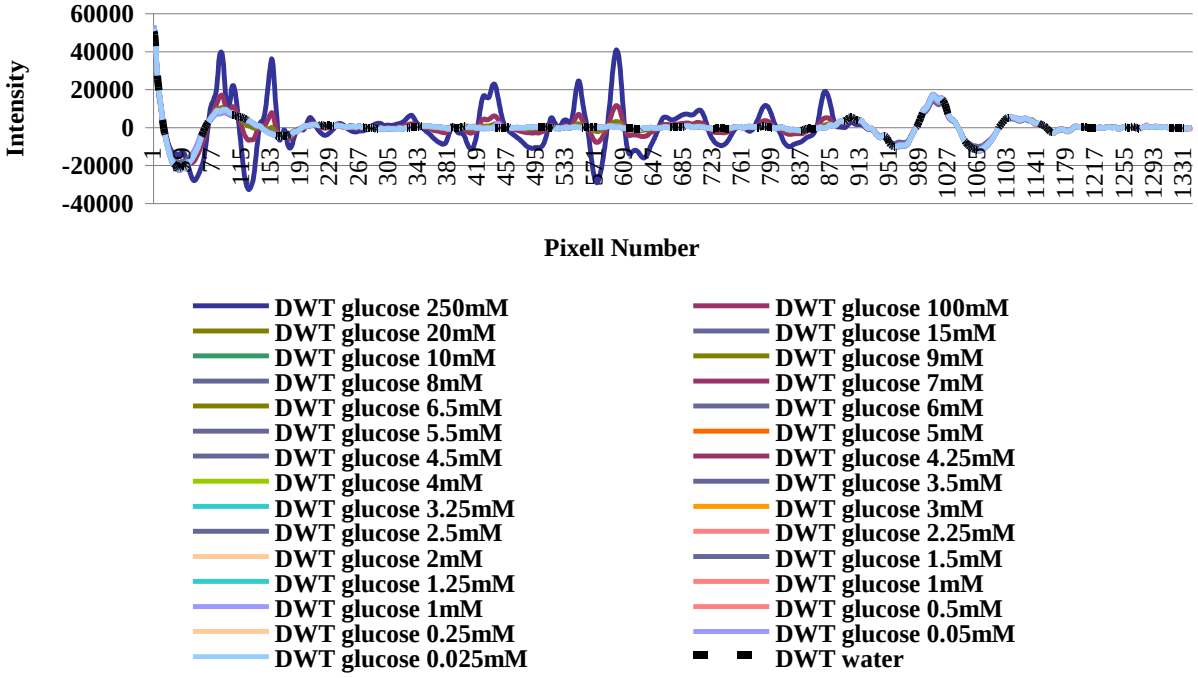


Figure 20: Comparison of different glucose concentrations with pure distilled water



A discrete wavelet transform (DWT) was applied to remove background and noise from each glucose signal. With the application of chemometrics techniques, and applying DWT, no significant differences were again observed between the lower glucose concentrations and pure distilled water.

Figure 21: Comparison of DWT preprocessed Raman spectra of different glucose concentrations with pure distilled water



The PLS model of glucose was obtained using 2 spectra of each concentration as a calibration and the remaining one as validation. Generating PLS calibration models of lower glucose concentrations and obtaining RMSE of those samples showed that there was a difference between glucose solutions in the lower concentrations and distilled water. Qualitative judgment offered by PLS and the calculated RMSE suggested the 0.5 mM of glucose concentration as a limitation of detection of glucose in our experiments.

Figure 22: The PLS analysis results of 5-20 mM glucose concentrations

Red circles represent calibration and blue asterisks represent validation. X axis shows real concentration and Y axis shows predicted concentration.

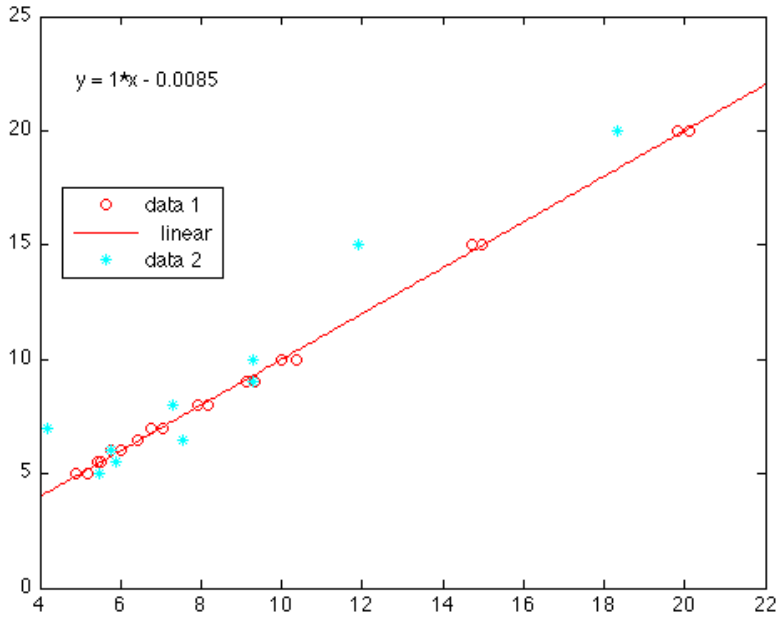


Figure 23: The PLS analysis results of 0-10 mM glucose concentrations

Red circles represent calibration and blue asterisks representsvalidation. X axis shows real concentration and Y axis shows predicted concentration.

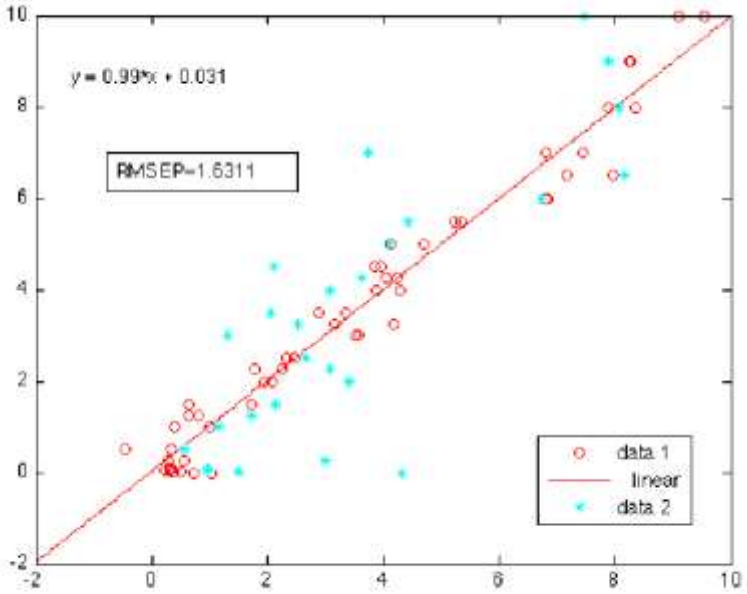


Figure 24: The PLS analysis results of 0-1 mM glucose concentrations

Red circles represent calibration and blue asterisks represent validation. X axis shows real concentration and Y axis shows predicted concentration.

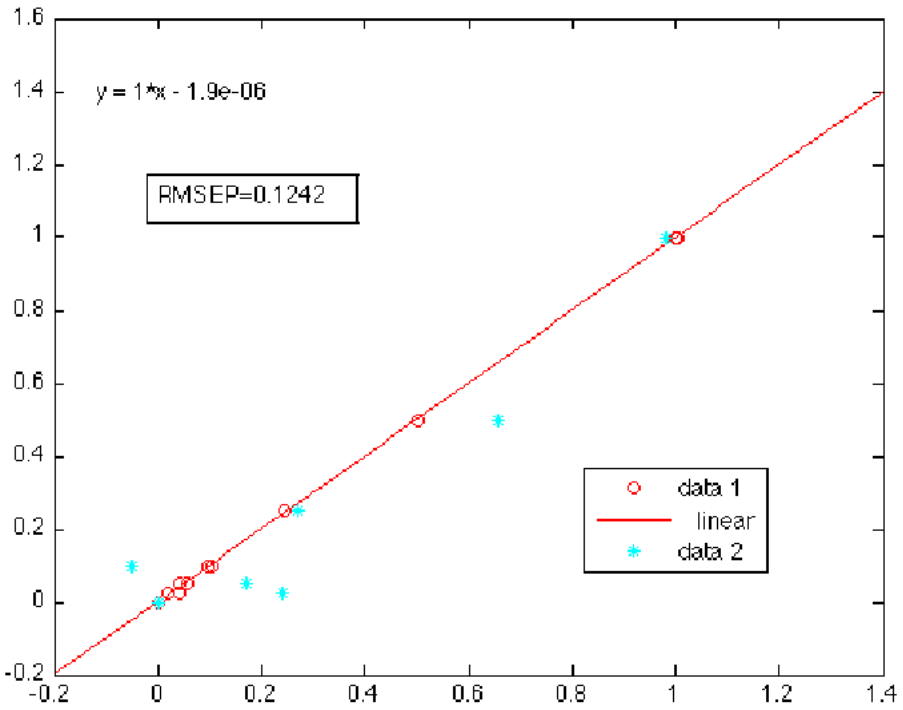


Table 6: Calculated errors to measure the accuracy of our PLS model in lower glucose concentrations

Glucose (mM) in Distilled Water	Root-Mean-Square Error (RMSE)
1	0.5606
0.5	**0.9597**
0.25	1.3540
0.1	2.0534
0.05	2.0468
0.025	1.88
0	5.8866

4.4.2 Glycine

Raman spectra of solutions of glycine in distilled water are presented in Fig 25. In general, spectral features for the different concentration and pure distilled water were the same. Following the application of DWT preprocessing, glycine spectra became sharper and more distinguishable. Despite being more distinct overall, these glycine Raman spectra still overlapped to some extent and future models must consider this spectral overlap into account. Based on DWT preprocessed Raman spectra of different glycine concentrations, it was not able to define the limitation of detection for glycine in our experiments.

Figure 25: Comparison of different glycine concentrations with pure distilled water

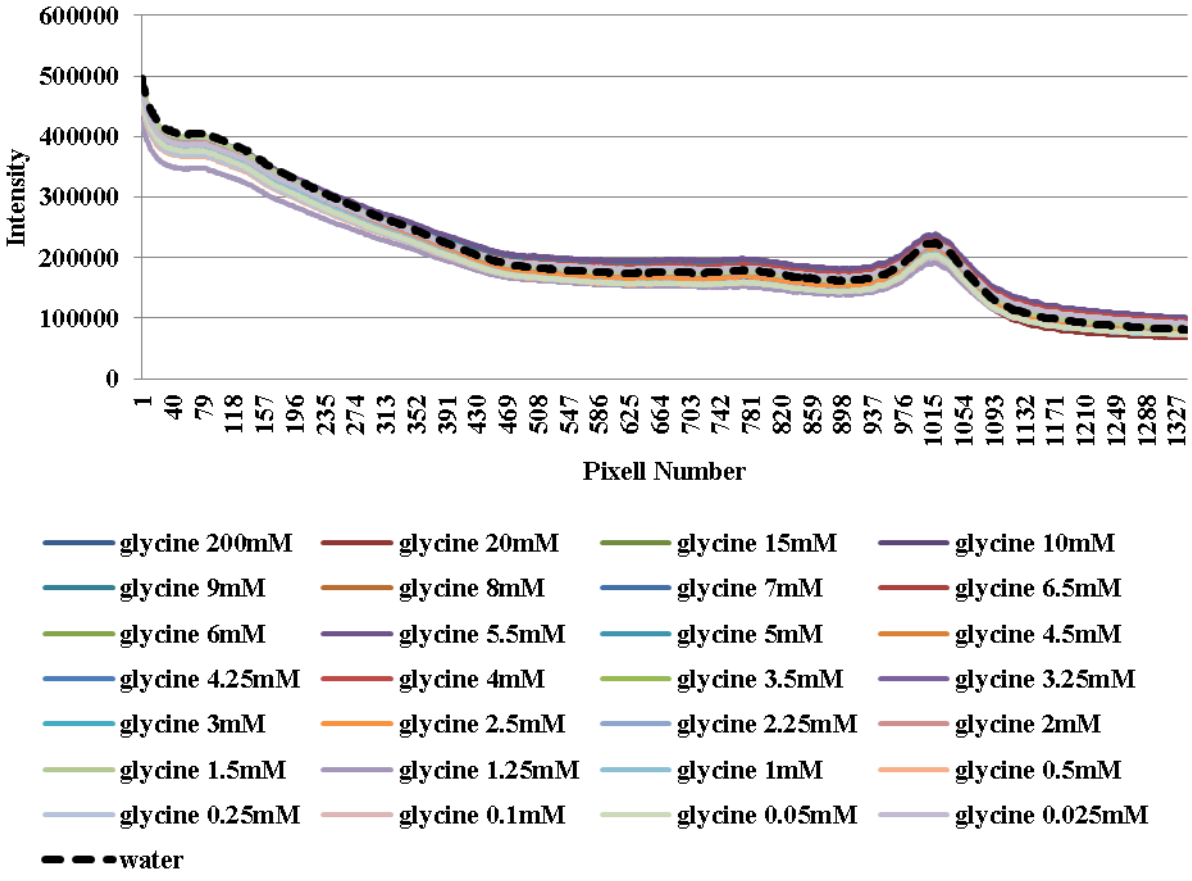
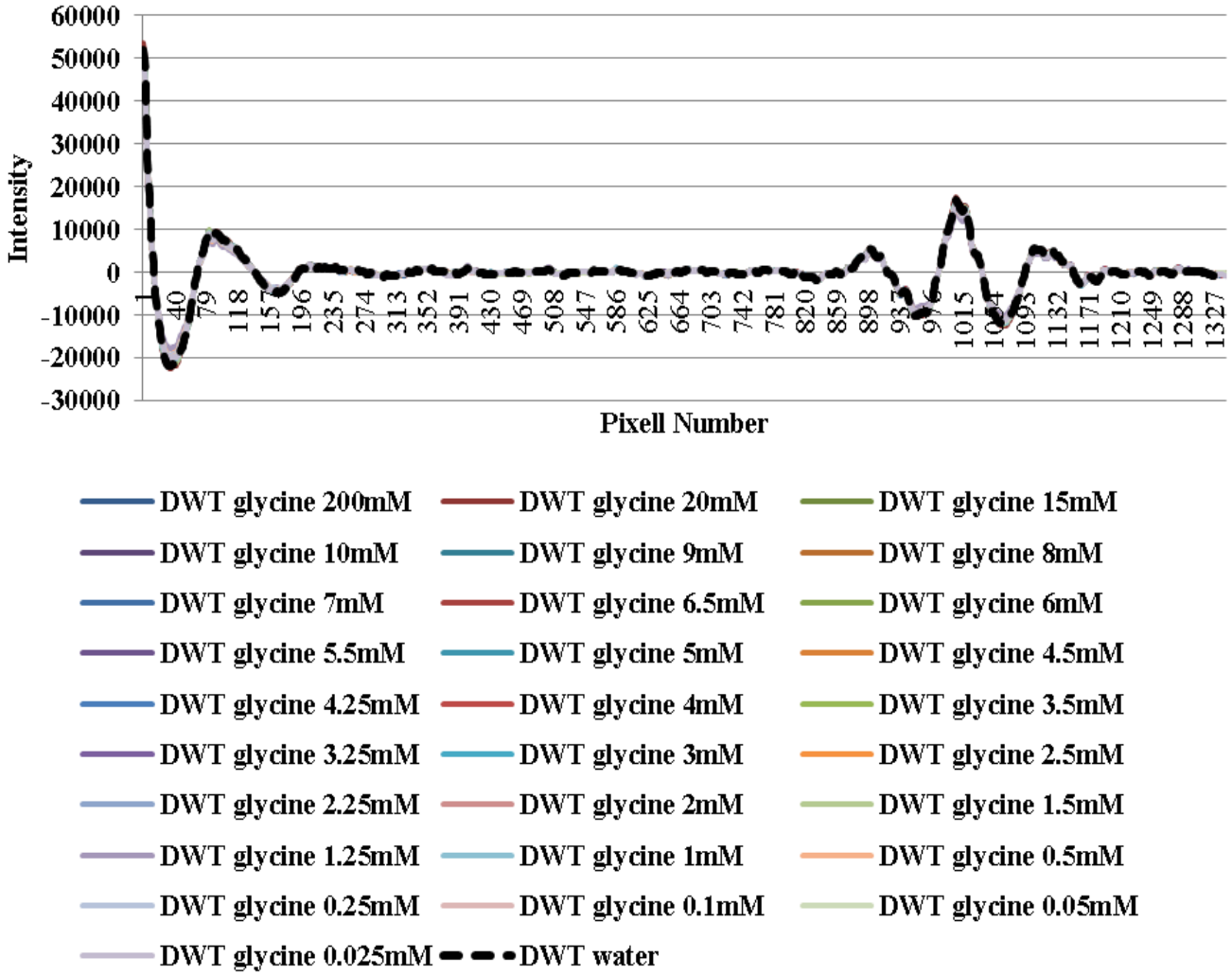


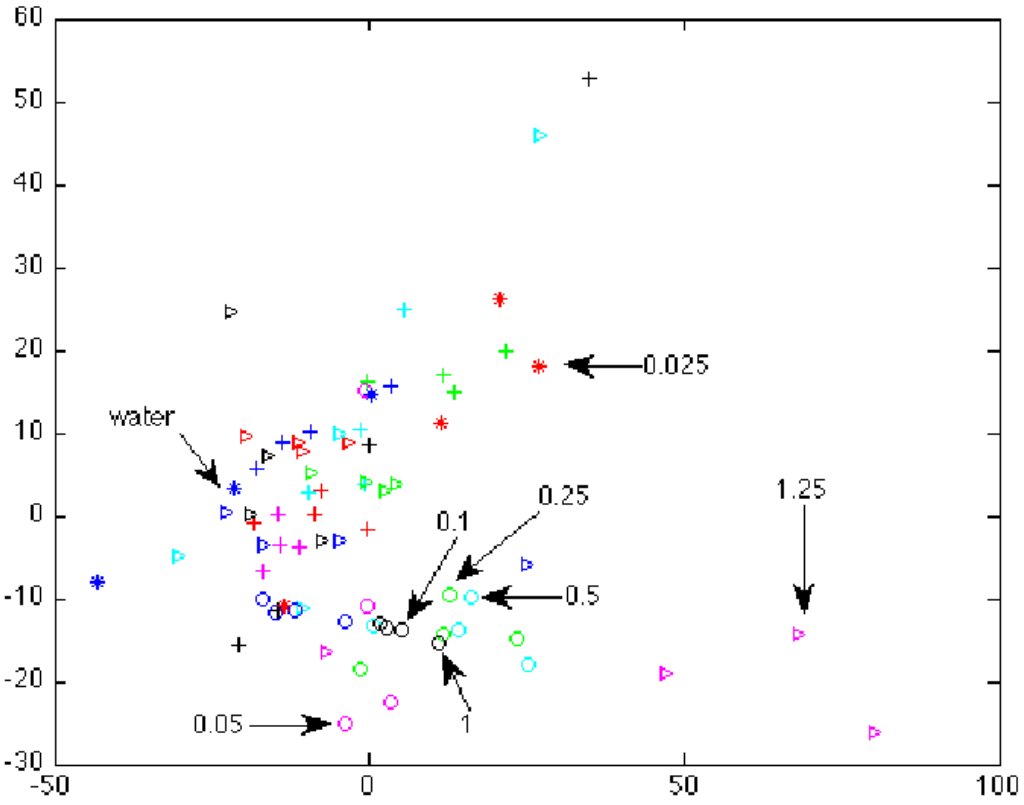
Figure 26: Comparison of DWT preprocessed Raman spectra of different glycine concentrations with pure distilled water



In addition, the PCA analysis of all glycine samples did not show any significant differences between Raman signals of distilled water and different glycine concentrations.

Figure 27: The PCA analysis results of 0-200 mM glycine concentrations

Including all concentrations and distilled water



5 Conclusions

The aim of this study was to assess embryo quality using metabolomic profiling of human embryo culture media with Raman spectroscopy in clinical IVF as a non-invasive approach to predicting embryo viability, and consequently, implantation potential and pregnancy. In addition, we compared metabolomic profiling with the existing morphological assessment of embryo quality and predictors of pregnancy. The result of our study demonstrates that metabolomic profiling from "spent" embryo culture media is not able to predict embryo development and implantation potential for day 3 and day 6 embryos because ofbecause of overlap in spectra in culture media from embryos on day 3 and day 6 as well as control media. We also observe that Raman does not have the sensitivity to differentiate changes in glucose and glycine at low concentrations.

In any IVF laboratory, the primary objective is to achieve a successful viable singleton pregnancy for infertile couples by selecting the best embryo with the most implantation potential for transfer. Even though this objective may seem straightforward in theory, it is much more complicated to achieve in reality. Clinics are constantly looking for new methods and techniques to increase conception rates. Thus far, morphological assessment by light microscopy is the only method for predicting embryo developmental and implantation potential. Although this assessment is one measure to better identify the best embryo(s) for transfer, its accuracy is still insufficient to reduce the number of embryos transferred to a point where twins or high-order multiple gestations can be prevented (4). Metabolomics is the systematic study of metabolites as small molecular biomarkers representing the functional phenotype in a biological system. However, contrary to published data, \our findings indicate that differences, if any, in spent culture media, from embryos with different developmental and implantation potential, are not detectable by targeted Raman spectroscopic analysis. The quality of our spectra was demonstrated by the highly reproducible features owing to the substances contained in the IVF medium. In addition, the variance of the Raman measurement of our control samples was comparable to the biological variance of cultured samples, and both were larger than the variance introduced by deterministic differences between embryos with differences either in scores or implantation outcomes. Recently, the usefulness concept of NIR and Raman spectroscopy for predicting embryo developmental and implantation quality has been presented by two published

articles. Hardarson et al. (72) and Vergouw et al. (73) showed that selecting embryo by metabolomics profiling of culture medium with NIR spectroscopy on Day 2 and 5 (72) and Day 3 (73) as an addition to morphology cannot improve the ongoing pregnancy and live birth rates compared with embryo selection by morphology alone. These two prospective double-blind RCT studies demonstrated that NIR spectroscopy of spent embryo culture media in its current form cannot improve the likelihood of a viable pregnancy after single embryo transfer. The results of these current studies support our results which conclude that metabolomics profiling technology requires further refinement before it can be used as a potential tool to assess of embryo viability.

To date, all published data refer to studies that used embryo culture media samples that were frozen upon collection and transported to a central laboratory for analysis (1,7,8,14,27). In this study, we used fresh culture media samples for measurement and the time between sample collection and Raman recording was on the same day; thus, the potential effect of freezing-thawing was removed. As Varga et al. (74) indicated the use of frozen-thawed and lyophilized culture medium, which is employed for the purposes of preservation, significantly decreases embryo developmental rates related to a fresh control group. They showed that both procedures affect its properties and the ability of the culture medium to support embryo development due to chemical reactions and enzymatic and non-enzymatic degradation processes that reduce the quality of the culture medium (74,75). As already noted some properties may precipitate after a freeze-thaw cycle and not regain their colligative properties. Upon thawing of the media, the precipitation may go unnoticed because of the low molar concentrations of some components in IVF culture media. Referring to this fact, that is the concern and it might therefore similarly affect the properties of culture media even after removing of embryo and change the obtained Raman signals. Therefore, such an approach fails to take into account that freezing and thawing may alter the components of in the small culture medium droplets and as a result, may introduce differences in Raman spectroscopic analysis. Thus, even if metabolomic profiling were proven to be a powerful test for embryo selection, assessment without freezing immediately in the vicinity of the clinical IVF laboratory would need to be investigated. As Vergouw et al. (73) discussed in their current work, the lack of reproducibility between their results and previous published data in terms of usefulness of spectroscopy application in the field of IVF might be due to different conditions used in previous retrospective trials. All investigators used snap frozen culture media

and all analysis were done in one laboratory. Thus, the viability score test might not be possibly as robust when performed at IVF site.

Another issue regarding the application of this technology is the fact that different types of culture media and volume have been used thus far. Different commercial culture media, all formulated according to the metabolic needs of the human embryos (28), may be used in ART laboratories. For commercial reasons, their formulas are regrettably protected and not available to the public. In theory, a valid and widely applicable methodology for spent culture media evaluation should be able to detect the changes associated with embryonic reproductive potential and not be impacted by the substantial differences in the content of commercial culture media, including batch-to-batch variability within one formulation. Although similar sensitivity and specificity scores of Raman spectra across two different types and volumes of culture media have been reported (1), the accuracy of Raman measurement could be affected by the small changes in different culture media composition, as demonstrated in our glucose experiments. Furthermore, the algorithm used to detect changes need to be standardized and validated. Indeed, published articles (1,7) have shown many random variances in Raman signals; thus, the application of an algorithm derived from directly from Raman spectra of the samples under study without further validation would not be complete and could be misleading.

One of the challenges in metabolomic profiling is that products of complex metabolomic pathways range greatly in embryo culture media. Various types of molecules are produced or secreted at different stages during embryo development. These include pyruvate, glucose, amino acids, oxygen, and leptin, to name a few (to review see (3,19)). Over the past decades, many targeted metabolic parameters for developing embryos have been studied using a variety of noninvasive assays (42-46,50,52-56). Although the results of these studies suggest metabolic differences between embryos with different reproductive potential, the application of these methods to a clinical setting is limited for a variety of reasons. These assays are technically difficult and require technical staff and time commitment, making them less suitable for clinical application. Moreover, in some situations, the obtained data are not consistent (42,43,45,46) and therefore may not provide a reliable biomarker for prediction of embryo viability. Thus, for a better understanding of the overall health of embryos, scientific efforts move toward measurement of various secreted metabolites in tandem with spectral approaches. At present, some published data obtained by NIR and Raman spectroscopy have been proposed for clinical

application. However, as mentioned above, unlike these published data, the findings of our study using Raman spectroscopy are not encouraging. This is not only because the result shows no significant differences between embryos with different developmental potential, but also because of the undetectable differences between spent culture media containing the embryos and the control culture media. Because of the reproducible features of our spectra, we the spectra generated from our IVF and control samples were considerably superior in quality to those published in this area. The spectra of our study showed that, while the degree of changes between different samples was small, the determined variation associated with embryo activity appeared even smaller. The scatter in control samples seemed to encompass the variation in patient samples. As a result, we further examined the accuracy and credibility of our analysis by doing controlled Raman measurement of standard samples with concentrations that were related to biochemical determinations of embryo metabolism. In other words, the basic principles of the method required testing under controlled conditions.

Due to the significant role of glucose during embryo development, specifically around the time of compaction—switching from a pyruvate to a glucose-based metabolism—and the higher amount of this metabolite in human embryo culture media for embryo consumption, we speculated that glucose might have a significant effect on Raman spectra. Similarly, because of the beneficial effects of amino acids on embryo development in culture to help regulate the activities of specific energy generation pathways of the embryo, we also tested the effect of glycine—one of the required amino acids for embryo maturation—on Raman spectra under controlled conditions. Thus, a wide range of concentrations of glucose and glycine between 0 and 250 mM in distilled water were prepared and analyzed to determine the corresponding limits of detection of Raman spectroscopy. For the development of our model, 3 high quality spectra acquired from each sample of glucose and glycine were used for data analysis. While the glucose spectra grossly appeared to have similar profiles in lower concentrations, careful visual inspections identified subtle but discernible spectral shape differences. In our experiment, the lowest concentration of glucose in which Raman spectra could be obtained was ~ 0.5 mM. Zhang et al. (76) has shown that the lowest concentration of glucose that can be detected by Raman spectroscopy is ~ 0.1 mM (5 times lower than our result). However, their result has been obtained using a drop coating deposition Raman (DCDR) method in which a microvolume of solution is deposited on a suitable substrate, followed by solvent evaporation and nondestructive

Raman detection. More recently, Yang's et al. (77) have shown that the liquid filled photonic crystal fiber (PCF) achieves signal intensity at a concentration of 50 mM glucose, while the conventional bulk Raman spectroscopy reaches the detection limit only at a concentration of 1 M. Their measurements have been obtained on a 50nL sampling volume under a 2mW laser power with 30 s integration time. Considering the level of signal intensity in conventional Raman detection and our data, we conclude that pushing the detection limit to such low glucose concentration in human IVF culture media within reasonable exposure time is extremely difficult. One alternative way is that to increase the laser power which consequently can increase the Raman intensity, however, application of high laser power and long exposure time is not suitable for the *in vitro* and *in vivo* diagnostic purpose.

Unexpectedly, no detectable signals could be recorded from the different concentrations of glycine solutions. We do not have a solid, reasonable explanation on why glycine was not detected in our experiment. The only explanation for this result is that glycine is quite a unique amino acid because of its simple side chain (78). Glycine is the simplest and smallest amino acid, thus, may represent the extreme detection limit achievable with Raman spectroscopy. The absence of observable differences in spectra among different glucose and glycine concentrations highlights the weak sensitivity of the proposed approach to detect very small structural variations in the culture media. Importantly, since the amount of glucose and glycine in human embryo culture media decreases during embryo development to levels below the detection limits demonstrated in this work, Raman may not be suitable for identifying and detecting changes in biomolecules between embryos with different developmental potential. Based on the result obtained here, this method (Raman spectroscopy) may not be an ideal tool for the prediction of the developmental and implantation potential of embryos. However, the results reported in this study demonstrate that the milestones along the path from laboratory research to clinical diagnostic application are still in the early stages. We propose that for getting more reliable results, we need, firstly, to improve the method using for measurement and analysis. Secondly, increasing the quality of samples is essential for meaningful conclusion. Last but not least, we should standardize and validate algorithms used to detect spectroscopic signals as applied to clinical culture medium samples.

Noninvasive approaches, such as optical spectroscopy, aim at the identification of culture media metabolites indicative of the state of the embryo. However, even in the best of situations,

where embryo quality is determined based on culture environment quality, investigators can only conclude the quality of embryos that result in a pregnancy. Without any doubt, other factors independent of the embryo such as female age, uterine factors or transfers technique will affect the final result. Thus, these factors may further reduce the tolerance range of the model and make it less accurate. In other words, the development of an accurate aid for the selection of high quality and viable embryos is multifactorial.

The application of metabolomic analysis to the field of oocyte and embryo assessment in some clinical trials is leading to the discovery of biomarkers associated with oocyte and embryo viability. Despite the fact that the available publications on metabolomic profiling by Raman and/or NIR spectroscopy have been quite encouraging over the last decade, its application has its own challenges. The installation and implementation of this technology into an IVF lab which does not specialize in the technology in question requires training of laboratory personnel. In addition, collecting the medium samples, loading the machine and interpreting the results could add to the workload of a busy IVF clinic. Thus, more randomized prospective trials assessing implantation and pregnancy rates using these technologies are still needed to test the true value and accuracy of this technology, if further refinements of the technology can be made. The aim of the present discussion is not to challenge any of the progress made in terms of the selection of embryos to increase pregnancy rates; however, it is only fair to mention that finding a fast, inexpensive, easy-to-use and non-invasive means for embryo selection is still needed. Further studies will be necessary to determine the value and limitations of the use of metabolomics in ART. This technology is mostly in the experimental phase and it does not seem to have the practical potential to be introduced as a standard selection procedure in the typical IVF laboratory.

Bibliography

1. Seli E, Sakkas D, Scott R, Kwok SC, Rosendahl SM, Burns DH. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. Fertil Steril. 2007;88(5):1350-1357
2. Mosher WD, Pratt WF. Fecundity and infertility in the United States: incidence and trends. Fertil Steril. 1991;56(2):192-193
3. Seli E, Robert C, Sirard MA. OMICS in assisted reproduction: possibilities and pitfalls. Mol Hum Reprod. 2010;16(8):513-530
4. Bromer JG, Seli E. Assessment of embryo viability in assisted reproductive technology: shortcomings of current approaches and the emerging role of metabolomics. Curr Opin Obstet Gynecol. 2008;20(3):234-241
5. Adamson GD, de Mouzon J, Lancaster P, Nygren KG, Sullivan E, Zegers-Hochschild F. World collaborative report on in vitro fertilization, 2000. Fertil Steril. 2006;85(6):1586-1622
6. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet. 1978;2(8085):366
7. Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study. Fertil Steril. 2008;90(1):77-83
8. Botros L, Sakkas D, Seli E. Metabolomics and its application for non-invasive embryo assessment in IVF. Mol Hum Reprod. 2008;14(12):679-690
9. http://www.hfea.gov.uk/ivf-figures-2006.html#1279
10. Dawson KJ, Conaghan J, Ostera GR, Winston RM, Hardy K. Delaying transfer to the third day post-insemination, to select non-arrested embryos, increases development to the fetal heart stage. Hum Reprod. 1995;10(1):177-182
11. Puissant F, Van Rysselberge M, Barlow P, Deweze J, Leroy F. Embryo scoring as a prognostic tool in IVF treatment. Hum Reprod. 1987;2(8):705-708
12. Tan SL, Royston P, Campbell S, Jacobs HS, Betts J, Mason B, et al. Cumulative conception and livebirth rates after in-vitro fertilisation. Lancet. 1992;339(8806):1390-1394

13. Martin JA, Hamilton BE, Ventura SJ, Menacker F, Park MM. Births: final data for 2000. Natl Vital Stat Rep. 2002;50(5):1-101
14. Nagy ZP, Sakkas D, Behr B. Non-invasive assessment of embryo viability by metabolomic profiling of culture media(metabolomics). Reprod Biomed Online. 2008;17(4):502-507
15. Luke B, Martin JA. The rise in multiple births in the United States: who, what, when, where, and why. Clin Obstet Gynecol. 2004;47(1):118-133
16. Keith LG, Oleszczuk JJ, Keith DM. Multiple gestation: reflections on epidemiology, causes, and consequences. Int J Fertil Womens Med. 2000;45(3):206-214
17. Thurin A, Hausken J, Hillensjö T, Jablonowska B, Pinborg A, Strandell A, Bergh C. Elective single-embryo transfer versus double-embryo transfer in in vitro fertilization. N Engl J Med. 2004;351(23):2392-2402
18. JOINT SOGC-CFAS. Guidelines for the number of embryos to transfer following in vitro fertilization. J Obstet Gynaecol Can. 2010;32(4):363-377
19. Nel-Themaat L, Nagy ZP. A review of the promises and pitfalls of oocyte and embryo metabolomics. Placenta. 2011;32 (Suppl 3):S257-263
20. Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring human embryo growth rates in in vitro fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. J In Vitro Fert Embryo Transf. 1986;3(5):284-295
21. Gardner DK, Schoolcraft WB. In-vitro culture of human blastocysts. In: Jansen R, Mortimor D, editors. Towards reproductive certainty:fertility and genetics beyond. Carnforth:Parthenon Publishing;1999.p.378-388
22. Racowsky C, Vernon M, Mayer J, Ball GD, Behr B, Pomeroy KO, et al. Standardization of grading embryo morphology. J Assist Reprod Genet. 2010;27(8):437-439
23. Milki AA, Hinckley MD, Fisch JD, Dasig D, Behr B. Comparison of blastocyst transfer with day 3 embryo transfer in similar patient populations. Fertil Steril. 2000;73(1):126-129
24. SART, https://www.sartcorsonline.com/rptCSR_PublicMultYear.aspx?ClinicPKID=0, 2010

25. Bromer JG, Seli E. Assessment of embryo viability in assisted reproductive technology: shortcomings of current approaches and the emerging role of metabolomics. Curr Opin Obstet Gynecol. 2008;20(3):234-241
26. Hardy K, Handyside AH, Winston RM. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development. 1989;107(3):597-604
27. Nagy ZP, Jones-Colon S, Roos P, Botros L, Greco E, Dasig J, et al. Metabolomic assessment of oocyte viability. Reprod Biomed Online. 2009;18(2):219-225
28. http://www.advancedfertility.com/embryoquality.htm
29. Lane M, Gardner DK. Embryo culture medium: which is the best?. Best Pract Res Clin Obstet Gynaecol. 2007;21(1):83-100
30. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology. 1998;49(1):83-102
31. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oöcyte and zygote. Proc Natl Acad Sci U S A. 1967;58(2):560-567
32. Kim JH, Funahashi H, Niwa K, Okuda K. Glucose requirement at different developmental stages of in vitro fertilized bovine embryos cultured in semi-defined medium. Theriogenology. 1993;39(4):875-886
33. Schini SA, Bavister BD. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol Reprod. 1988;39(5):1183-1192
34. Thompson JG, Simpson AC, Pugh PA, Tervit HR. Requirement for glucose during in vitro culture of sheep preimplantation embryos. Mol Reprod Dev. 1992;31(4):253-257
35. Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR, King WA. Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. J Reprod Fertil. 1991;93(1):125-132
36. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. Fertil Steril. 1996;65(2):349-353
37. Leese HJ, Tay JI, Reischl J, Downing SJ. Formation of Fallopian tubal fluid: role of a neglected epithelium. Reproduction. 2001;121(3):339-346

38. Lane M, Gardner DK, Hasler MJ, Hasler JF. Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. Theriogenology. 2003;60:407-419
39. Turner K, Martin KL, Woodward BJ, Lenton EA, Leese HJ. Comparison of pyruvate uptake by embryos derived from conception and non-conception natural cycles. Hum Reprod. 1994;9(12):2362-2366
40. Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. J Reprod Fertil. 1984;72(1):9-13
41. Gardner DK, Leese HJ. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. Hum Reprod. 1986;1(1):25-27
42. Hardy K, Hooper MA, Handyside AH, Rutherford AJ, Winston RM, Leese HJ. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. Hum Reprod. 1989;4(2):188-191
43. Gott AL, Hardy K, Winston RM, Leese HJ. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. Hum Reprod. 1990;5(1):104-108
44. Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. J Assist Reprod Genet. 1993;10(1):21-30
45. Devreker F, Hardy K, Van den Bergh M, Winston J, Biramane J, Englert Y. Noninvasive assessment of glucose and pyruvate uptake by human embryos after intracytoplasmic sperm injection and during the formation of pronuclei. Fertil Steril. 2000;73(5):947-954
46. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertil Steril. 2001;76(6):1175-1180
47. Deverker F. Uptake and release of metabolites in human preimplantation embryos. In: Cohen J, Elder K (eds). Human preimplantation embryo selection. London, UK: Taylor and rancis, 2007;325-336
48. Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. J Reprod Fertil. 1980;58(1):161-164

49. Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. J Exp Zool. 1987;242(1):103-105
50. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum Reprod. 1996;11(9):1975-1978
51. Jones GM, Trounson AO, Vella PJ, Thouas GA, Lolatgis N, Wood C. Glucose metabolism of human morula and blastocyst-stage embryos and its relationship to viability after transfer. Reprod Biomed Online. 2001;3(2):124-132
52. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertil Steril. 2001;76(6):1175-1180
53. Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. Hum Reprod. 2011;26(8):1981-1986
54. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, et al. Non-invassive amino acid turnover predicts human embryo developmental capacity. Hum Reprod. 2002;17(4):999-1005
55. Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. Metabolism of the viable mammalian embryo:quietness revisited. Mol Hum reprod. 2008;12(4):667-672
56. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum Reprod. 2004;19(10):2319-2324
57. Seli E, Botros L, Sakkas D, Burns DH. Non-invassive metabolomics profiling of culture media using proton NMR correlates with reproductive potential of embryos in women undergoing in vitro fertilization. Fertil Steril. 2008;90:2183-2189
58. Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, et al. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. Mol Hum Reprod. 2010;16(8);557-569
59. Posillico JT and the Metabolomics Study Group for Reproductive Technologies. Selection of viable embryos and gametes by rapid, non-invasive metabolomic profiling of oxidative stress biomarkers. In: Cohen J, Elder K (eds). Human Preimplantation Embryo Selection. London,UK:Taylor and Franci, 2007,325-336

60. Raamsdonk LM, Teusink B, Broadhurst D, Zhang N, Hayes A, Walsh MC, et al. A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol. 2001;19(1):45-50
61. Oldiges M, Lütz S, Pflug S, Schroer K, Stein N, Wiendahl C. Metabolomics: current state and evolving methodologies and tools. Appl Microbiol Biotechnol. 2007;76(3):495-511
62. Singh R, Sinclair KD. Metabolomics: approaches to assessing oocyte and embryo quality. Theriogenology. 2007;68 (Suppl 1):S56-62
63. Seli E, Vergouw CG, Morita H, Botros L, Roos P, Lambalk CB, et al. Noninvasive metabolomic profiling as an adjunct to morphology for noninvasive embryo assessment in women undergoing single embryo transfer. Fertil Steril. 2010;94(2):535-542
64. Raman CV, Krishnan KS. Nature. 1928; *121*: 501-502
65. http://en.wikipedia.org/wiki/Raman_Spectroscopy
66. http://www.nrc-cnrc.gc.ca/eng/projects/ibd/raman-spectroscopy.html
67. Kato O, Terramotos S, Morita H, Botros L, Roos P, Burns DH. Metabolomic assessment of day 2 embryos based on pregnancy outcome after single embryo transfer (SET). American Society for Reproductive Medicine, 2007
68. Hardarson T, Tucker M, Seli E, Botros L, Roos P, Sakkas D. Non-invasive metabolomic profiling of day 5 embryo culture media adds to the discriminatory power of blastocyst culture for single embryo transfer (SET). American Society for Reproductive Medicine, San Francisco, CA, 2008
69. Seli E, Vergouw CG, Kato O, Botros L, Roos P, Morita H, et al. A viability index determined by non-invasive metabolomic profiling of embryo culture media correlates with ART outcome. European Society for Human Reproduction and Embryology, Barcelona, Spain, 2008
70. Vergouw CG, Botros LL, Roos P, Lens JW, Schats R, Hompes PGA, et al. Metabolomic profiling by near-infrared spectroscopy as a tool to assess embryo viability: a novel, non-invasive method for embryo selection. Hum Reprod. 2008;23:1499-1504
71. Seli E, Bruce C, Botros L, Henson M, Roos P, Judge K, et al. Receiver operating characteristic (ROC) analysis of day 5 morphology grading and metabolomic Viability Score on predicting implantation outcome. J Assist Reprod Genet. 2011;28(2):137-144

72. Hardarson T, Ahlström A, Rogberg L, Botros L, Hillensjö T, Westlander G, et al. Non-invasive metabolomics of Day 2 and 5 embryo culture medium: a prospective randomized trial. Hum Reprod. 2012;27(1):89-96
73. Vergouw CG, Kieslinger DC, Kostelijk EH, Botros LL, Schats R, Hompes PG, et al. Day 3 embryo selection by metabolomics profiling of culture medium with near-infrared spectroscopy as an adjunct to morphology: a randomized controlled trial. Hum Reprod. 2012;27(8):2304-2311
74. Varga S, Diez C, Fernández L, Alvarez J, Katchicualula A, Hidalgo CO, et al. Culture system and long-term storage of culture media in the in vitro production of bovine embryos. Acta Vet Hung. 2011;59(1):129-139
75. Liao YH, Brown MB, Quader A, Martin GP. Protective mechanism of stabilizing excipients against dehydration in the freeze-drying of proteins. Pharm Res. 2002;19(12):1854-1861
76. Zhang D, Xie Y, Mrozek MF, Ortiz C, Davisson VJ, Ben-Amotz D. Raman detection of proteomic analytes. Anal Chem. 2003;75(21):5703-5709
77. Yang X, Zhang AY, Wheeler DA, Bond TC, Gu C, Li Y. Direct molecule-specific glucose detection by Raman spectroscopy based on photonic crystal fiber. Anal Bioanal Chem. 2012;402:687-691
78. Stewart S , Fredericks PM. Surface-enhanced Raman spectroscopy of amino acids adsorbed on an electrochemically prepared silver surface. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 1999, Volume 55, Issue 7, pp. 1641 - 1660