Chapter 6

Non-Invasive Assessment of the Embryo Viability via the Analysis of the Culture Media

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Abstract

Infertility in recent years is a growing public health issue throughout the developed world. Assisted reproductive techniques, especially *in vitro* fertilization, have the potential to partially overcome the low natural reproductive ratio. Nowadays, single embryo transfer gains grounds in clinical practice, urging the development of more reliable methods for selecting the best embryo. In the traditional clinical practice, embryos are selected for transfer based on morphological evaluation. *In vitro* culturing of embryos also provides a very important material for further non-invasive evaluation by means of examining a biomarker in the spent culture medium (SEC). Current measure methods concentrate on the metabolomic activity of the developing embryos none compounds. These studies are mainly utilizing the tools of modern analytics and proteomics. In a paper published by Montskó et al. in 2015, the alpha-1 chain of the human haptoglobin molecule was described as a quantitative biomarker of embryo viability. In a series of retrospective, blind experiments achieved more than 50% success rate. This chapter summarizes the currently available metabolomic and proteomic approaches as the non-invasive molecular assessment of embryo viability.

Keywords: *in vitro* fertilization, embryo viability, non-invasive analysis, proteomics, mass spectrometry, haptoglobin alpha-1 chain

1. Introduction

Nowadays, infertility is a major public health issue affecting couples in the developed world. With the widespread use of assisted reproductive techniques (ARTs), especially *in vitro* fertilization (IVF), there are more and more pregnancies conceived. Currently, approximately 3–4% of

all deliveries are IVF pregnancy and this number continues increasing. Availability of ART is a very relevant topic. The cultural and legal conditions, insurance/public funding systems and structure of data collection can influence not only the amount of treatment cycles per inhabitant but also success rates. The Assisted Reproductive Technology National Summary Report of the USA showed a total of 142.000 IVF cycles in 2007 [1], while the most current results in 2014 was 208.604 cycles [2]. The type of ART cycle applied (non-donor or donor egg cycle) is highly varied based on the woman's age. The women younger than 35 years of age often used their own eggs (non-donor) in the majority of cases and just about 4% used donor eggs. However, 38% of women aged 43–44 and 73% of women older than 44 needed to use donor eggs [2]. Similar to the USA, the numbers of ART cycles in Europe show a growing tendency. In 2007, the reported number [3] of treatment cycles was 493.134, while the latest available report describes 640.144 cycles in 2012 [4]. Among the 452.578 fresh cycles reported in 2012, the fraction of IVF and intracytoplasmic sperm injection (ICSI) was 139.978 (31%) and 312.600 (69%), respectively [4]. Despite evolving microsurgical technologies—such as ICSI and some new embryo culturing materials—the rate of successful delivery is far below expectations. In the European IVF monitoring report published in 2016 [4], this rate was 27.8-33.8% depending on the technique of the cycles.

Successful implantation is a complex and bilateral process that requires the selection of a viable embryo and the effective interaction with a receptive endometrium. It is highly unlikely, however, that for the low delivery rate following IVF only maternal reasons would be responsible. In Europe, the total proportion of single embryo transfers (SETs) was 30%. Double embryo transfers occurred in 55% of the cycles, triple embryo transfers were reported in 13% and four or more embryos were transferred in 1% of the cycles. The highest proportions of SETs in 2012 were found in Sweden (76.3%), Finland (75.0%), Norway (60.8%), Belgium (51.1%), Iceland (49.4%), the Czech Republic (47.4%), Austria (46.5%) and Denmark (46.4%) [4]. Nowadays, SET gains grounds in clinical practice. The adoption of an elective SET policy is spreading, urging the development of a reliable method for selecting the most viable embryo, that is, the embryo with the best implantation potential. In the traditional clinical practice of ART, embryos are selected for transfer based on non-invasive morphological evaluation. Several new morphological parameters such as the cleavage rate, blastomere shape and symmetry, and the presence of an adequate trophectoderm layer (TL) or an inner cell mass (ICM) are considered as indicators of implantation potency.

2. Embryo morphology

The most obvious approach for the viability assessment of *in vitro* fertilized embryos is the visual inspection using microscopy. The main reason is the use of any invasive technique such as genetic screening following on-cell embryo biopsy may raise a series of ethical questions. One must not forget that any impact, which affects the embryo during the first days of development, might have undesired late consequences. The choice of morphological parameter depends partly on the time spent after fertilization.

On the first day of development, the morphology of the two pronuclei (the interphase zygotic nuclei) can be graded at 1-cell stage zygotes. Zygote has two pronuclei as the female from

the oocyte and the male one from the spermium. Until the end of the interphase of the first embryonic cell cycle, the two pronuclei remain separated. Though on the first day of *in vitro* embryonic development, nucleoli screening is reported to be predictive of pregnancy rate, there are still some disagreements about the usefulness of this morphological marker [5].

The time point of the breakdown of the pronuclear membranes or the time of the first cleavage following fertilization is considered as an indicator of reproductive potential of embryos. Fancsovits et al. reported the relationship of the time point of the pronuclear breakdown with clinical pregnancy and implantation rates. The earliest pronuclear breakdown was at 18 hours after fertilization and the latest time was 31 hours post-insemination. Transferring embryos with the early pronuclear breakdown resulted in a significantly higher clinical pregnancy rate (48.3 vs. 27.3%) and the implantation rates (26.5 vs. 15.1%) [6].

On the second and other later days, the blastomere size, cleavage rate, and pattern of the developing embryo may be evaluated. The best quality embryos supposed to have developed to the four to five blastomere stage on day 2 and have seven or more blastomeres on the third day [5]. Along the number of blastomers, the symmetry of the cleavage is also considered as an indicator of embryo quality. The embryos with symmetric cleavage patterns have a tendency for significantly higher implantation than asymmetric blastomeric shape. Thus, the acceptable cleavage pattern can also be a predictor of implantation outcome [7].

Another important morphological parameter is the grade of fragmentation at the early embryonic development. Cytoplasmic fragments can be found in any human embryo irrespective whether they were fertilized *in vitro* or *in vivo*. The amounts of fragments vary highly, ranging from a few small fragments to a notably high extent of fragmentation involving even blastomere number loss in early cleavage stage embryos. The degree of fragmentation is widely used as an indicator of embryo quality and a predictor of implantation potential. Extensive fragmentation is commonly associated with reduced blastocyst formation and implantation potential. If the degree of fragmentation is below 15%, it seems no effect on blastocyst formation, but more that 15% fragmentation will quickly declines blastocyst formation [8].

The morphological scoring of embryos on 5 and 6 days is also possible by the populations of inner cells (inner cell mass precursors) and outer cells (trophoblast precursors) segregating at about 16-cell stage [9]. An appropriate quality blastocyst has a blastocoel, a trophectoderm layer (TE) and an inner cell mass (ICM). Therefore, the examination of the cell number or the area covered by these cells might be an important factor correlating with embryo viability [5].

It can be seen even on these highlighted examples that there are several options to study the morphology of *in vitro* fertilized embryos and to use these observations to predict implantation potential. It is advised not to select a single parameter, the combination of more than one serves as a better option. The full history of embryo development combining grading of zygotes, cleavage stages and if possible, blastocysts is required to maximize the reliability [5]. Morphological evaluation is an inexpensive method which can be easily implemented in the clinical environment. The biggest drawback of morphological evaluation is that it is a highly subjective method. Therefore, there was a need to form a consensus on these parameters, namely which morphological markers need to be used, what is the weighing of these parameters in the final score, and

a scale on which all individual parameters are graded. An international consensus was created in 2011 by the Alpha Scientists in Reproductive Medicine and the European Society of Human Reproduction and Embryology (ESHRE) Special Interest Group of Embryology, based on several morphology markers in different stages of development. The result of this agreement is known as the Istanbul Consensus scoring system. It was expected that standardization of laboratory practice related to embryo morphology assessment will result in more effective comparisons of treatment outcomes worldwide. The document set by the Alpha Scientists group intended to refer as a global standardized consensus for the accurate description of embryo development [10]. The scoring system is composed of several morphological aspects and also considers time spent after fertilization. Nowadays, the guideline sets here serve as the accepted methodology of viability assessment of *in vitro* fertilized embryos.

3. Analysis of the embryo culture medium

Because of ethical reasons, a huge effort is made to find ways of non-invasive viability assessment. The most obvious approach is to study the metabolomic activity of the embryo through the analysis of secreted compounds or by studying the alterations made by the embryo within the culture medium. Due to the importance of the surrounding environment of the embryo and the goal of single embryo transfer concept, and the maintenance of acceptable pregnancy rates, selecting the most optimal culture medium is a crucial point.

First human embryos were cultured in simple salt solutions or in more complex media originally designed for tissue culturing. These early media consisted of physiological salt solutions with added glucose, pyruvate and lactate, and was also supplemented with the patient's serum. Later, it was also revealed that the addition of amino acids to the culture medium increases reproductive potential. Research papers described in both animal and human models that the introduction of amino acids has a positive effect on embryo development and increases viability [11].

Using the experiences published in the literature, several clinics started to develop 'in-house' embryo culturing media, but this way the standardization of culturing circumstances is not an easy task [11]. Therefore, shortly, commercially produced media specifically designed for use in clinical IVF applications was developed satisfying the growing needs. These media are aseptically produced in a specialized factory under standardized conditions, regulations and quality control, and therefore an attractive alternative of 'in-house' embryo culturing media. Nowadays, two types of media exist: sequential culture systems and monoculture systems. Monoculture systems use a single medium composition to support zygote development to the blastocyst stage. The limitation of monoculture systems is that they do not adapt to the altering biochemical needs of the embryo during its development. A medium composition suitable for early cleavage state embryos might not be optimal for the blastocyst stage embryos. Therefore, the majority of IVF clinics use sequential culture systems. It has been determined that conditions that support blastocyst development might inhibit the development of early cleavage stage embryos. If the practice of the clinic covers blastocyst transfer, the sequential medium is the best choice [11].

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A very important additive of any type of embryo culturing medium is human serum albumin, which is the most abundant soluble protein constituent of blood described with several physiological roles. In culture medium, albumin serves as pH buffer, an osmotic regulator, membrane stabilizator, a surfactant and a scavenger of metals or toxic substances. Earlier, albumin supplementation was done using human or maternal serum but it has now shifted towards the use of purified albumin products, mainly because of the risk of transferring infectious diseases. With the use of purified albumin products this risk can be eliminated. However, the batch-to-batch stability of different lots of albumin products is sometimes questionable. The use of recombinant albumin might solve all the issues discussed above, but their use is not as widespread as the use of purified albumin products [12].

When dealing with purified albumin products, one must consider that these products are not a 100% pure. In Dyrlund et al.'s recent study [13], 110 proteins other than albumin were identified in commercially available unconditioned culture media supplemented with purified human serum albumin products. Probably it is not an issue in clinical practice since these products have been proven themselves for decades. However, if we use the culture medium as a material for research purpose, it is a very important question.

The measurement of the spent culture medium (SEC) may be served as an exceptional non-invasive alternative in the search of markers of embryo viability. In SEC, the interesting compounds can be divided into two major groups. One consists of compounds present in the unconditioned medium and these compounds may be quantitatively altered by the developing embryo (e.g. nutrients or peptide/protein compounds) [14]. The other group contains embryo-related molecules (e.g. proteins and metabolic end products) secreted by the embryo into the surrounding medium. In order to analyse the secretome of the developing embryo, especially the proteome, it has to be cleared which identified protein originates from the embryo and which was present (or altered in concentration) in the unconditioned medium.

4. Metabolomic studies

The current goal of IVF is to reduce the number of transferred embryos in a single cycle, preferably to only one. Therefore, there is an increasing need for new markers of viability. Numerous factors have been identified as suitable markers of implantation potential, started by the measurement of glucose uptake rate or the determination of pyruvate concentration in the culture medium. Papers reporting such applications in mouse and human models [15, 16] described that blastocysts implanted and developed properly after transferring to the uterus had a significantly higher rate of glucose consumption *in vitro* than those that failed to implant. During the *in vitro* development of human embryos, pyruvate and glucose uptakes were found to be significantly higher by embryos forming normal blastocysts than embryos failing to develop properly. In the first group, an average 22.1 pmol per embryo per hour glucose uptake was recorded, while in the latter group this was only 10.2 pmol per embryo per hour. Comparison of glucose uptakes with morphological embryo grading revealed that the highest glucose uptake was seen in blastocysts of highest grade. Among blastocysts of the same grade from the

same patient, there was a notable spread of glucose uptake, indicating that glucose consumption during *in vitro* development may report additional information on embryo viability. It is also described that the measurement of glucose in the medium is more important than that of pyruvate since pyruvate uptakes were similar irrespective of blastocyst grade.

Another option is the examination of amino acid turnover during the early embryonic development by analysing quantitative changes in the amino acid profile of the medium. Amino acids have numerous biological functions during the early period of embryo development. Houghton et al. [17] quantitatively analysed amino acid turnover using high-performance liquid chromatography of individual human embryos. Quantitatively different patterns of amino acid utilization were found between embryos that went on to form a blastocyst and those that failed to develop to blastocyst stage. In the group of normally developing embryos, an increased consumption of leucine from the culture medium was determined. It was also found that the profiles of alanine, arginine, glutamine, methionine, and asparagine predicted developmental potential significantly. Brison et al. [18] revealed alterations in the amino acid concentration of the medium of human zygotes cultured to the 2-cell stage. The turnover of three amino acids, that is, asparagine, glycine, and leucine, was found to be significantly associated with clinical pregnancy and live birth.

Not only selected metabolomic compounds can be examined, but also the analysis of the total metabolome is possible. This area of metabolomic experiments examines the overall metabolic content of the surrounding medium, rather than measuring known nutrients or metabolites. Using analytical techniques such as Raman or near-infrared (NIR) spectroscopy, it is possible to obtain the whole spectral profile of the culture medium surrounding the embryo. It has to be highlighted that it is not possible to identify specific components, it is only possible to detect specific changes to the obtained spectrum. The potential advantage of this approach is an overall analysis for the culture environment [19]. The concept is that after performing spectroscopy at multiple wavelengths in the medium samples of embryos with different implantation outcome, spectral alterations are searched for. These differences are calculated into viability scores or indexes using mathematical algorithms. The observed alterations in the spectra are due to differences in the amount of chemical groups which is a consequence of the metabolic activity of the embryo. The methodology cannot identify the compounds responsible for the spectral differences but indirectly reports information on the metabolomic activity of the developing embryo. For example, if spectral signatures from the near-infrared show differences through the 750–950-nm spectral region, it reports a change in the relative amounts of –OH, –CH and –NH groups [20]. Both Raman and NIR spectroscopic analyses of spent culture media of embryos with known implantation potential demonstrated significantly higher viability indices for embryos representing transfers resulting in clinical pregnancy. When embryos with similar morphology were examined using infrared spectroscopy, viability scores varied remarkably indicating that the analysis of the total metabolome also reports additional information on embryo viability [19]. When calculated viability scores were compared with live birth rates, it was found that embryos having viability scores <0.45 resulted in 19.4% live birth rate, while embryos having viability scores >0.578 resulted in 46.9% live birth rate. This is a very important observation because it clearly indicates that non-invasive metabolomic analysis of the medium of *in vitro* fertilized embryos has its place in the

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process of viability assessment. Probably a new and additional method cannot replace the existing methodology. However, it can add some new information by identifying markers of low implantation potential unnoticed by the morphological evaluation.

5. Proteomic studies

It is hypothesized that secretory compounds found in the culture medium might provide a characteristic molecular fingerprint. This pattern informs us about embryo growth, developmental competences, and implantation potential. With the emerging of sensitive and specific new analytical techniques, it is possible to carry out a comprehensive analyses of the surrounding environment of pre-implantation embryos [21]. These molecular profiles are supposed to utilize with high accuracy the differentiation of viable and non-viable embryos [22]. The identification of new biomarkers of the embryonic secretome can result in significant improvements in the efficiency of IVF cycles, increasing pregnancy rate per transfer and decreasing the costs of the procedure. There is also a more subjective aspect of more reliable viability assessment: the reduction of the patient's emotional stress [23]. Biological functions are often regulated or carried out by proteins, therefore to understand how a cell or in this case a small population of cells function can be crucial. The analysis of the proteome reports us how the embryo responds to external and also internal conditions. The analysis of the embryonic protein production into the surrounding medium provides a new, molecular perspective of the biochemical pathways activated during the early embryonic development [21].

The proteomic analysis of the embryonic secretome covers the use of the latest analytical tools, very often mass spectrometry (MS) or liquid chromatography-coupled mass spectrometry (LC-MS). MS is probably the most promising technique to study the embryonic secretome. The standard proteomic approach involves separation of intact proteins using 2D gel electrophoresis followed by immediate MS analysis or more likely by digestion and the analysis of the resulting peptide profile. The LC-MS analysis of tryptic digests of control and conditioned embryo culture media, characterization of embryo-related peptides and proteins is now also possible. More recent advances like involving nano-ultra-high pressure chromatography (nano-UPLC) and label-free quantification with mass spectrometry allows the use of minimal amounts of sample and the efficient identification of numerous peptides and proteins in a single analytical run [24]. Matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) and surface-enhanced laser-desorption ionization-time-of-flight mass spectrometry (SELDI-TOF) are also used to detect different proteins in embryo culture media. SELDI-TOF is a highly sensitive and more importantly a high-throughput method for proteomic analysis, especially for proteins having low molecular weight [21].

Candidates of markers of viability secreted by the embryo cover a broad range of molecules. Sher et al. [25] used the soluble human leukocyte antigen G (sHLA-G) as a predictor of implantation and pregnancy rate. sHLA-G was quantified using an immunoassay and two groups were made according to the quantitative results. Embryos producing sHLA-G above the geometric mean were considered as sHLA-G+ while the ones producing the antigen below the geometric mean were considered as sHLA-G−. In the previous group, significantly higher pregnancy and

implantation rates were observed. In the sHLA-G+ group, the pregnancy and implantation rates were 75 and 44%, compared to 23 and 14% of the sHLA-G− group, respectively.

The role of apolipoprotein A1 was also described in Ref. [26] after identification by gel electrophoresis followed by MALDI-TOF MS. Quantification was also performed by ELISA and by quantitative reverse transcriptase polymerase chain reaction of mRNA of apolipoprotein A1. It was found that the level of apolipoprotein A1 correlates with blastocyst grade, but it does not correlate with implantation and pregnancy rates. Contradictory to those findings, Nyalwidhe et al. [22] used MS, Western-blot, and ELISA to identify 14 differentially regulated peptides that were then used to generate genetic algorithms being able to identify embryo transfer cycles resulting in pregnancy and cycles with failed implantation. These genetic algorithms were able to recognize with 71–84% accuracy embryo transfer cycles, which resulted in pregnancy. Several of the 14 peptides were identified as fragments of apolipoprotein A-1, showing reduced expression in media samples representing transfer cycles resulting in viable pregnancies. McReynolds et al. reported an interesting approach based on proteomic analysis [27]. Potential biomarker candidates were selected using an Linear Trap Quadropole-Fourier Transform (LTQ-FT) ultra hybrid mass spectrometer operated in tandem mass spectrometric (MS/MS) mode. Using this proteomic platform, we identified lipocalin-1 to be associated with chromosome aneuploidy. The concentration of lipocalin-1 was determined using a commercially available lipocalin-1 ELISA kit. A clear discrimination of euploid and aneuploid embryos may be determined based on change of lipocalin-1 concentration in micro-drops of culture media. The lipocalin-1 concentration from aneuploid blastocysts showed more significant increase than euploid blastocysts. Pooled micro-drops of euploid embryos contained 3–4 ng/ml of lipocalin-1, while aneuploid embryos contained this compound in a concentration of 6–7 ng/ml. When analysing individual micro-drops of euploid and aneuploid embryos in the spent culture media samples, the results were 4–5 vs. 5–6 ng/ml of lipocalin-1, respectively.

These examples clearly indicate that the non-invasive proteomic analysis of spent culture medium samples has a great potential to determine embryo developmental potency. Thus, this method can be integrated to the existing viability assessing concepts.

6. Viability assessment using quantitative determination of the haptoglobin alpha-1 chain

By LC-MS analysis of spent culture medium samples incubated for 3 days, four different polypeptides were detected and the mass spectra revealed that the monoisotopic masses of the four molecules were 4787.4, 4464.6, 4622.4, and 9186.5 Da, respectively. These numbers showed quantitative difference between the viable (successful pregnancy) and the non-viable (no pregnancy) embryo groups [28]. As the result of various proteomic and statistical considerations, the number of biomarker candidates was reduced to a 9186.5 Da polypeptide. The respective mass spectrum is depicted in **Figure 1**.

Only this compound differed significantly in quantity between the viable and non-viable embryo groups ($p = 0.005$). Proteomic identification was carried out after digestion of the respective chromatographic fraction. By database search using MS data and manual investigation of

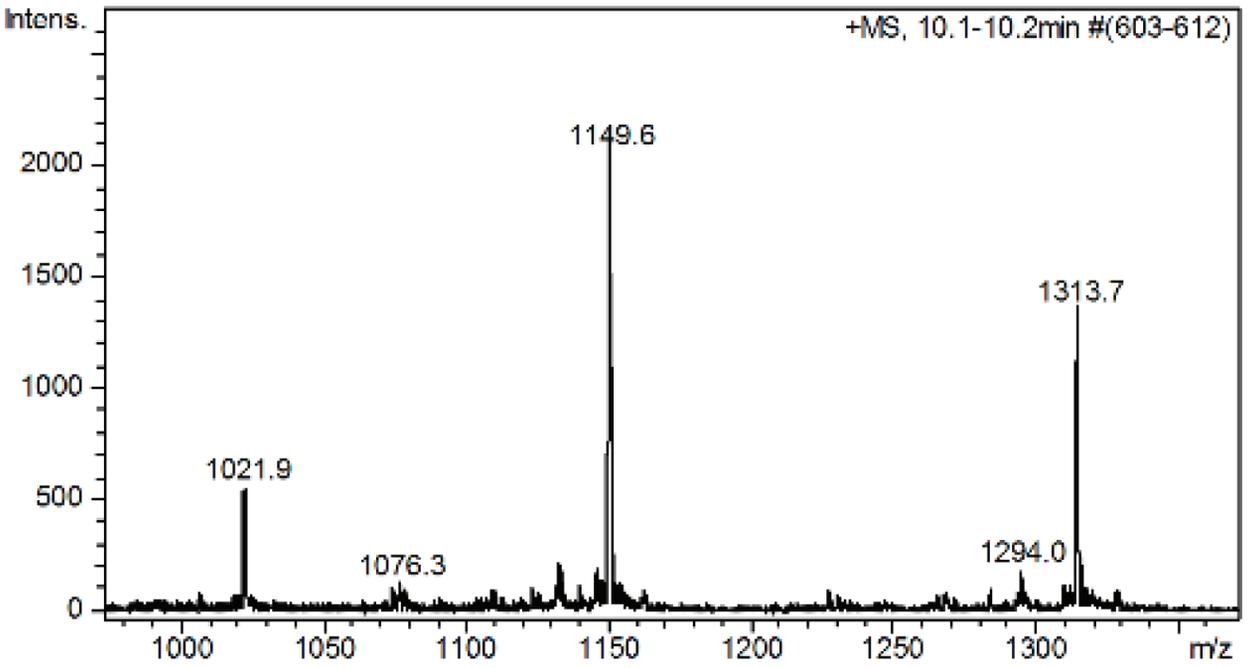

Figure 1. Mass spectrum of the haptoglobin alpha-1 fragment. The horizontal axis represents the measured mass to charge ratio values, displayed as m/z. Absolute peak intensity is shown on the vertical axis. The most intensive peak at m/z 1149.6 corresponds to the [M+8H]8+ ion of the molecule. The peaks at m/z 1021.9 and m/z 1313.7 represent the [M + 9H]9+ and [M + 7H]7+ molecular ions, respectively.

sequence annotations of entries, the protein was identified as the alpha-1 chain of human haptoglobin. The alpha-1 form of this subunit has a monoisotopic mass of 9186.4 Da. All enzymatic fragments identified by tandem mass spectrometry correspond to this region of the haptoglobin precursor protein.

In a set of blind and retrospective experiments including 161 haptoglobin alpha-1 chain measurements, 62 samples were found to be biochemically non-viable and 99 samples were biochemically viable. The biochemically non-viable 62 embryos did not result in any successful baby delivery, while in the biochemically viable group showed 55% pregnancy rate (**Figure 2**). This result revealed a significant difference between viable and non-viable embryo groups ($p < 0.001$) on the basis of the amount of the alpha-1 chain. Moreover, we have found a significant correlation ($p < 0.001$) between the amount of the peptide fragment and the pregnancy outcome.

The probable source of human haptoglobin in the unconditioned medium is the protein contamination of various purified albumin products. The sources of the haptoglobin alpha-1 chain in the culture medium are due to the reduction of the disulphide bonds connecting the chains of the matured haptoglobin molecule. The explanation for the increased amount of alpha-1 chain in the samples of non-viable embryos might be the fact that abnormally developing or damaged embryos often show the characteristics of apoptosis in a larger extent than normal embryos. Apoptosis later might be followed by secondary necrosis accompanied by increased membrane permeability. We hypothesize that these processes might result in the release of enzymes or other chemical factors from the cells of abnormally developing embryos altering the chemical environment in the medium.

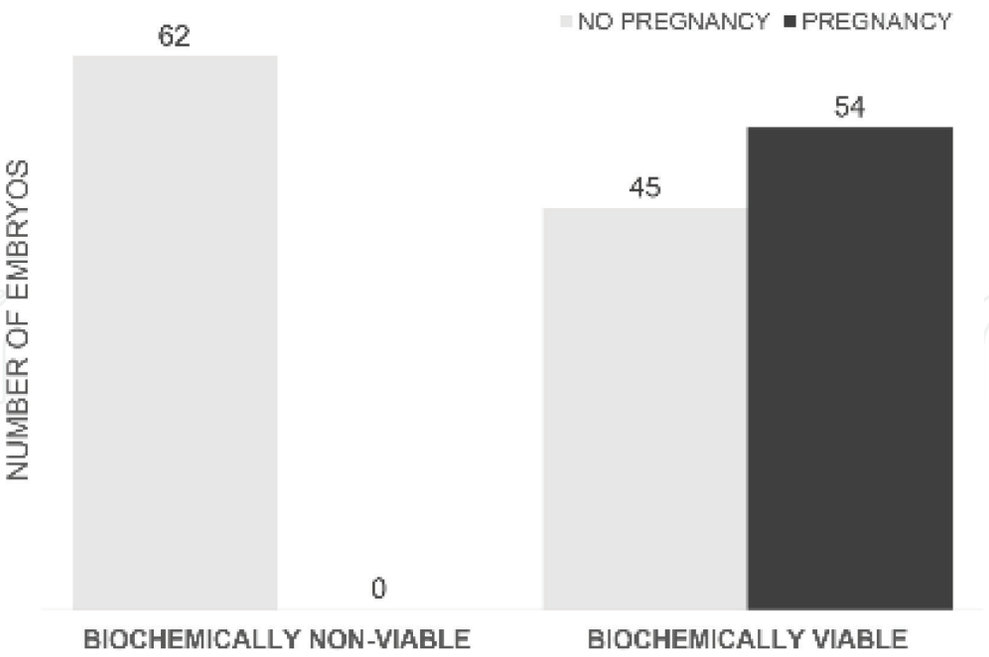

Figure 2. Results of the blinded analysis of embryo culture medium after 3 days of incubation (n = 161). In the group assessed as biochemically non-viable, no pregnancy was found. Embryos assessed as biochemically viable, showed an 55% pregnancy rate.

7. Apoptosis during early embryonic development

Programmed cell death (PCD)—also called apoptosis—is a well-known biological phenomenon. It is characterized by cell membrane blebbing, chromatin condensation and DNA fragmentation, involving several membrane receptors and the activation of signal transduction pathways. Classic signs of apoptosis are cell shrinkage, nuclear condensation, and the formation of vesicles called 'apoptotic bodies'. The most significant biochemical event associated with apoptosis is DNA fragmentation producing a specific gel electrophoresis picture called the DNA ladder. Apoptosis *in vivo* occurs in every multicellular organism and is an essential biological process [29].

Normal apoptosis in early embryos is crucial for proper development. In blastocysts, for example, both the inner cell mass and the trophectoderm layer undergo apoptosis [29]. Apoptosis during the normal development of the pre-implantation embryos has several functions. It is hypothesized that the cell number in the inner mass of the blastocyst follows an equilibrium and apoptosis helps to maintain cellular homeostasis. The other possible reason for PCD during early development is the elimination of cells with an abnormally altered genetic constitution or cells having other abnormalities or inadequate developmental potential. For example, within the inner cell mass, the appearance of aneuploid cells is well known. The markers of apoptosis are also considered as additional features for oocyte and embryo quality assessment. Arrested embryos tend to have a high grade of apoptosis [30].

Apoptotic cells should be normally phagocytosed, however, if it is not possible they may undergo secondary necrosis, which differs from apoptosis by an increase in membrane permeability and excretion of cytosolic structures. These events are observed in a variety of

different cell types [31]. The apoptotic program provides two alternative ways of cell elimination. Early surface signals can allow scavenger phagocytes to recognize apoptotic cells and remove them with a 'silent' elimination process. Secondary necrosis occurs in the absence of scavenger cells leading to a final autolytic disintegration. These cells exhibit specific apoptotic signs and also necrotic features, for example, the degradation of the cytoplasmic membrane. Secondary necrosis might also occur *in vivo* accompanying several pathological cases when functioning scavenger cells are not available [32]. *In vitro* apoptosis tends to proceed in a similar way involving the activation of hydrolytic enzymes and a damage of the cytoplasmic membrane, resulting in cell disruption. This process occurs if the removal of the apoptotic cells or apoptotic bodies fails. The events described in the process of primary necrosis are operating during secondary necrosis, too. The mechanism of cell death involves proteolysis due to the activity of proteinases causing an additional release of cytosolic compounds [33]. Studies on animal models indicate that *in vitro* culturing increases PCD and that the composition of the medium can affect the incidence of the process. The reason is that the culture medium lacks some crucial maternal 'survival' factors [31]. We hypothesize that during the *in vitro* culturing an increased PCD is observed, resulting in secondary necrosis because of the absence of scavenging cells in the artificial *in vitro* environment. The described phenomenon of haptoglobin cleavage might be a result of factors released from the embryonic cells due to secondary necrosis and increased membrane permeability.

8. Concluding remarks

Our detailed study showed that the alpha-1 chain of the human haptoglobin molecule may be used as a biomarker to distinguish the *in vitro* culture embryo implantation ability, which yet has not been proven earlier by others to be an indicator of embryo viability. The embryos diagnosed as biochemically non-viable did not lead to pregnancy at all. However, the embryos that were classified as biochemically viable showed a 55% pregnancy rate, while the control only showed the 30% pregnancy rate without the measurement of the haptoglobin alpha-1 fragment. The authors think that non-invasive metabolomic and proteomic approaches might have a place in the process of routine IVF but cannot substitute the process of morphological assessment. An ideal practice of IVF might contain a step ruling out the morphologically worst embryos followed by a laboratory measurement of the haptoglobin alpha-1 chain of media of the remaining ones. The main disadvantage of this technique is the application of mass spectrometry in the routine process of IVF, which requires an expensive laboratory background and is usually not available in the reproductive units. The developing field of lab-on-a-chip concept in combination with already existing point-of-care medical instruments can be a possible end point [34].

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References

[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. Fertility and Sterility. 2007;**88**:1350-1357. DOI :10.1016/j.fertnstert.2007.07.1390

[2] 2014 Assisted Reproductive Technology National Summary Report.[Internet] 2014. Available from: https://www.cdc.gov/art/pdf/2014-report/art-2014-national-summary-report.pdf

[3] de Mouzon J, Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, Kupka M, Nygren KG, Andersen AN. Assisted reproductive technology in Europe, 2007: Results generated from European registers by ESHRE. Human Reproduction. 2012;**4**:954-966. DOI :10.1093/humrep/des023

[4] European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE), Calhaz-Jorge C, de Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, Motrenko T, Scaravelli G, Wyns C, Goossens V. Assisted reproductive technology in Europe, 2012: Results generated from European registers by ESHRE. Human Reproduction. 2016;**31**:1638-1652. DOI: 10.1093/humrep/dew151

[5] Ajduk A, Zernicka-Goetz M. Quality control of embryo development. Molecular Aspects of Medicine. 2013;**34**:903-918. DOI: 10.1016/j.mam.2013.03.001

[6] Fancsovits P, Toth L, Takacs ZF, Murber A, Papp Z, Urbancsek J. Early pronuclear breakdown is a good indicator of embryo quality and viability. Fertility and Sterility. 2005;**84**:881-887. DOI:10.1016/j.fertnstert.2005.03.068

[7] Sela R, Samuelov L, Almog B, Schwartz T, Cohen T, Amit A, Azem F, Ben-Yosef D. An embryo cleavage pattern based on the relative blastomere size as a function of cell number for predicting implantation outcome. Fertility and Sterility. 2012;**98**:650-656.e4. DOI: 10.1016/j.fertnstert.2012.05.041

[8] Hardy K, Stark J, Winston RML. Maintenance of the inner cell mass in human blastocysts from fragmented embryos. Biology of Reproduction. 2003;**68**:1165-1169. DOI: 10.1095/biolreprod.102.010090

[9] Boiso I, Veiga A, Edwards RG. Fundamentals of human embryonic growth in vitro and the selection of high-quality embryos for transfer. Reproductive BioMedicine Online. 2002;**5**:328-350

[10] Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. Istanbul consensus workshop on embryo assessment. Proceedings of an Expert Meeting. Reproductive BioMedicine Online. 2011;**22**:632-646. DOI: 10.1016/j.rbmo.2011.02.001

[11] Lane M, Gardner DK. Embryo culture medium: Which is the best? Best Practice & Research Clinical Obstetrics & Gynaecology. 2007;**21**:83-100. DOI:10.1016/j.bpobgyn.2006.09.009

[12] Blake D, Svalander P, Jin M, Silversand C, Hamberger L. Protein supplementation of human IVF culture media. Journal of Assisted Reproduction and Genetics. 2002;**19**:137-143

[13] Dyrlund TF, Kirkegaard K, Poulsen ET, Sanggaard KW, Hindkjær JJ, Kjems J, Enghild JJ, Ingerslev HJ. Unconditioned commercial embryo culture media contain a large variety of non-declared proteins: A comprehensive proteomics analysis. Human Reproduction. 2014;**29**:2421-30. DOI: 10.1093/humrep/deu220

[14] Rødgaard T, Heegaard PM, Callesen H. Non-invasive assessment of in-vitro embryo quality to improve transfer success. Reproductive BioMedicine Online. 2015;**31**:585-592. DOI: 10.1016/j.rbmo.2015.08.003

[15] Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. Fertility and Sterility. 2013;**99**:1062-1072. DOI: 10.1016/j.fertnstert.2012.12.004

[16] Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertility and Sterility. 2001;**76**:1175-1180

[17] Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. Human Reproduction. 2002;**17**:999-1005.

[18] Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by noninvasive measurement of amino acid turnover. Human Reproduction 2004;**19**:2319-2324. DOI:10.1093/humrep/deh409

[19] Vergouw CG, Botros LL, Judge K, Henson M, Roos P, Kostelijk EH, Schats R, Twisk JW, Hompes PG, Sakkas D, Lambalk CB. Non-invasive viability assessment of day-4 frozen-thawed human embryos using near infrared spectroscopy. Reproductive BioMedicine Online. 2011;**23**:769-776. DOI: 10.1016/j.rbmo.2011.08.015

[20] 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. Fertility and Sterility. 2007;**88**:1350-1357. DOI: 10.1016/j.fertnstert.2007.07.1390

[21] Katz-Jaffe MG, Schoolcraft WB, Gardner DK. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. Fertility and Sterility. 2006;**86**:678-685. DOI:10.1016/j.fertnstert.2006.05.022

[22] Nyalwidhe J, Burch T, Bocca S, Cazares L, Green-Mitchell S, Cooke M, Birdsall P, Basu G, Semmes OJ, Oehninger S. Molecular Human Reproduction. 2013;**19**:250-263. DOI: 10.1093/molehr/gas063

[23] Poli M, Ori A, Child T, Jaroudi S, Spath K, Beck M, Wells D. Characterization and quantification of proteins secreted by single human embryos prior to implantation. EMBO Molecular Medicine. 2015;**7**:1465-1479. DOI: 10.15252/emmm.201505344

[24] Cortezzi SS, Garcia JS, Ferreira CR, Braga DP, Figueira RC, Iaconelli A Jr, Souza GH, Borges E Jr, Eberlin MN. Secretome of the preimplantation human embryo by bottom-up label-free proteomics. Analytical and Bioanalytical Chemistry. 2011;**401**:1331-1339. DOI: 10.1007/s00216-011-5202-1

[25] Sher G, Keskintepe L, Fisch JD, Acacio BA, Ahlering P, Batzofin J, Ginsburg M. Soluble human leukocyte antigen G expression in phase I culture media at 46 hours after fertilization predicts pregnancy and implantation from day 3 embryo transfer. Fertility and Sterility. 2005;**83**:1410-1413. DOI: 10.1016/j.fertnstert.2004.11.061

[26] Mains LM, Christenson L, Yang B, Sparks AE, Mathur S, Van Voorhis BJ. Identification of apolipoprotein A1 in the human embryonic secretome. Fertility and Sterility. 2011;**96**:422-427.e2. DOI: 10.1016/j.fertnstert.2011.05.049

[27] McReynolds S, Vanderlinden L, Stevens J, Hansen K, Schoolcraft WB, Katz-Jaffe MG. Lipocalin-1: A potential marker for noninvasive aneuploidy screening. Fertility and Sterility. 2011;**95**:2631-2633. DOI: 10.1016/j.fertnstert.2011.01.141

[28] Montskó G, Zrínyi Z, Janáky T, Szabó Z, Várnagy Á, Kovács GL, Bódis J. Noninvasive embryo viability assessment by quantitation of human haptoglobin alpha-1 fragment in the in vitro fertilization culture medium: an additional tool to increase success rate. Fertility and Sterility. 2015;**103**:687-693. DOI: 10.1016/j.fertnstert.2014.11.031

[29] Brill A, Torchinsky A, Carp H, Toder V. The role of apoptosis in normal and abnormal embryonic development. Journal of Assisted Reproduction and Genetics. 1999;**16**:512-519

[30] Haouzi D, Hamamah S. Pertinence of apoptosis markers for the improvement of in vitro fertilization (IVF). Current Medical Chemistry. 2009;**16**:1905-1916

[31] Hardy K. Apoptosis in the human embryo. Reviews of Reproduction. 1999;**4**:125-134

[32] Silva MT. Secondary necrosis: the natural outcome of the complete apoptotic program. FEBS Letters. 2010;**584**:4491-4499. DOI: 10.1016/j.febslet.2010.10.046

[33] Silva MT, do Vale A, dos Santos NM. Secondary necrosis in multicellular animals: An outcome of apoptosis with pathogenic implications. Apoptosis. 2008;**13**:463-482. DOI: 10.1007/s10495-008-0187-8

[34] Le Gac S, Nordhoff V. Microfluidics for mammalian embryo culture and selection: Where do we stand now? Molecular Human Reproduction. 2017;**23**:213-226. DOI:10.1093/molehr/gaw061

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