RECENT ADVANCES IN *IN VITRO* FERTILIZATION: PROTEOMICS, SECRETOMICS, METABOLOMICS AND *IN VITRO* MATURATION

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SUMMARY

Since its first successful result in 1978, clinicians and researchers have been working on increasing the efficiency and safety of in vitro fertilization (IVF). As a result of advances in technology and understanding of human reproduction, IVF success rates have increased while high-order multiple pregnancy (triplets and more) rates have decreased. On the other, there is opportunity for further improvement as many couples still face 'unexplained infertility' and high rates of twin pregnancies. Latest technologic and scientific improvements in IVF are promising.
The aim of this review is to present the latest advances in the fields of proteomics, secretomics, metabolomics and oocyte culture, how they can potentially improve embryo selection and in vitro maturation (IVM) and subsequently their possible impact on the safety and efficacy of IVF.

Key words: *in vitro fertilization, in vitro maturation, metabolomics, proteomics, secretomics*

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IN VİTRO FERTİLİZASYON'DA GÜNCEL YAKLAŞIMLAR: PROTEOMİKLER, SEKRETOMİKLER, METABOLOMİKLER VE IN VİRO MATURASYON

ÖZET

1978'de insanlarda yapılmış olan ilk başarılı in vitro fertilizasyon (IVF) uygulamasından beri, IVF'nin etkinliğini ve güvenliğini geliştirme çabaları uzmanlar tarafından sürdürülmektedir. Teknolojideki gelişmeler ve insan üremesi hakkında artan bilgiler ışığında, IVF başarı oranları artarken, çoklu gebelik (üçüz, dördüz ve üstü) oranları düşmüştür. Çoğu çift 'açıklanamayan infertilite' yaşarken, IVF'e bağlı ikiz gebelik oranları halen yüksek olduğu için kat edilecek çok mesafe vardır. Ancak son dönemdeki teknolojik ve bilimsel gelişmeler, IVF'te ciddi iyileşme ihtimali vaat etmektedir.
Bu derlemenin amacı, proteomikler, sekretomikler, metabolomikler ve oosit kültürü ile ilgili son gelişmeleri irdelemek, embriyo seçimi ve in vitro maturasyon (IVM) üzerindeki klinik yansımalarına ışık tutmaktır. Ayrıca yakın gelecekte, bu gelişmelerin IVF'in etkinliğini ve güvenliğini nasıl etkileyebileceği de tartışılacaktır.

Anahtar kelimeler: *in vitro fertilizasyon, in vitro maturasyon, metabolomikler, proteomikler, sekretomikler*

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INTRODUCTION

As the rate of infertility increases all over the world, assisted reproductive technologies (ART) have started to play a more vital role to meet the needs of infertility patients. On the other hand, rate of live birth in women under 35 years old is currently around 40%[1]. Limited knowledge on the development and implantation of the embryo may be the reason for such low rates. However, recent technological and scientific advances have led to significant improvements in *in vitro* fertilization (IVF) treatment.

There have main two major developments in the field of IVF: (1) to increase the effectiveness and (2) to enhance safety. Briefly, most of the developments in recent years have helped clinicians and researchers to understand the mechanisms on the development of the embryo and pathophysiology of infertility. The development of pre-implantation genetic diagnosis, embryo screening for genetic disorders (which is beyond the scope of this review) and advances in the field of proteomics have deepened our knowledge about the environment and secretions of the embryo and the differences between a normal embryo growth and an abnormal embryo growth. The results of these studies, while providing selection of the 'best' embryo for transfer, can help increase the success rate of IVF. Increased accuracy in the selection of the embryo will result in transferring fewer embryos with a high potential for implantation. This will improve both the maternal and fetal health.

Due to the *in vitro* maturation treatment of pre-fertilized oocytes, ovarian stimulation may no longer be needed for patients who are at high-risk of hyperstimulation. Widespread usage of this technique will increase success rates in the future in other specific group of patients (i.e. patient with polycystic ovarian syndrome). Lastly, advances in oocyte cryopreservation play an important role in establishing a common practice of fertility.

The aim of this review is to present the latest advances in the fields of proteomics, secretomics, metabolomics and oocyte culture, how they can potentially improve embryo selection and *in vitro* maturation (IVM) and subsequently their possible impact on the safety and efficacy of IVF.

PROTEOMICS, SECRETOMICS AND METABOLOMICS

Even if pre-implantation genetic diagnosis tests provide information about the structure of the embryo, the benefit is limited because of the limited knowledge on the development and implantation potential of the embryo. Clinicians still apply a classification method that assesses embryo quality by morphological criteria to decide which embryo should be transferred. However, the morphology cannot predict the implantation rate by itself[2]. A reliable, effective and non-invasive method in assessing the development potential of the embryo can increase IVF success rates, while optimizing the single embryo transfer protocol and reducing the incidence of multiple pregnancy.

Researchers have been focusing on proteomics to better understand the function of embryo cells. Proteomics include the analysis of content of the embryo protein and its protein profile. Protein profiles of different embryos can potentially provide information about the outcome of the methods applied such as IVF or cryopreservation. Since proteomic analysis requires cell lysis, it is not possible to utilize it during pre-transfer embryo. Therefore, secreted proteins (secretomics) into the IVF culture media have been investigated. Basic premise is that secretory profile of any embryo can be correlated with reproductive success rate. For instance, profiles of successfully implanted embryos can be examined and biomarkers for the optimum embryo transfer may be found. Even genetic profiles of the embryos to be transferred can be examined in a non-invasive ways. As McReynolds et al. have revealed recently lipocalin-1 is a blastocyst's secretome and is in association with aneuploidies[3].

Lately, metabolites of the cell are examined (metabolomics) as well as the proteins in the culture media. Metabolites reflect physiological and metabolic status of the embryo. Some markers can be found to improve reproductive potential by comparing profiles of implanted embryos. Hence, more accurate methods can be established to decide which embryo will be transferred in IVF cycles. In addition, secretomics may also be halepful for aneuploidy embryos. Since aneuploidy embryos have shown various amino acid turnovers compared to genetically normal embryos, *in vitro* amino acid profiling can give an idea about the health of the embryo's genetic structure[4].

Analyze techniques in secretomics and proteomics

In earlier studies a two-dimensional gel electrophoresis [5], Western blot[6] or ELISA test[7] were employed to identify protein in the mouse embryos. However, these techniques required large amount of sample material, or had sensitivity limitations (electrophoresis), or limitation of the number of protein that can be defined at a single time (Western blot, ELISA test).

Recently, mass spectrometry brought a great impetus to embryo proteomics research. Several proteins have been identified by mass spectrometry. The most commonly used technique is surface-assisted laser desorption / ionization technique (SELDI) together with 'time-of-flight' (TOF). It helps to ionize bound proteins by laser. The resulting gas ions passing through a vacuum TOF tube reach the detector plate. Because ions have different dimensions, reaching speeds to the plate are also different. Hence, the ions are separated according to the mass-charge ratios and profiles are then examined. By this method Katz-Jaffe et al, compared the protein profiles in developing and degenerating embryos[8]. These authors have demonstrated the inadequacy of morphological evaluation for embryo selection revealing differences of similar protein in morphology embryos. Since some proteins are released in the media that surrounds the embryo, researchers examined the protein in embryo culture media to create an embryo secretome profile. Katz-Jaffe et al. who conducted a research with surface-assisted laser desorption / ionization technique together with TOF analysis described different secrotomic profiles in different stages in the development of the embryo and identified that blastocyst development is associated with ubiquitin which is a protein biomarker [9]. Ubiquitin is a part of ubiquitin-dependent proteasome system that leads protein to degradation. This system plays a role in proliferation and apoptosis. Reportedly, it increases body fluids in various disease states[10]. In addition, it plays a critical role in activities and destruction of key signal molecules during the implantation process[11].

Protein microarray technology has recently been used to compare secretomic profiles in culture medium of implanted blastocysts. Results have showed increased levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokine (C-X-C motif) ligand 13 (CXCL13) in successfully implanted embryos[12]. In this matter, unknown proteins are firstly analyzed and later described by mass spectrometer. In microarray technology protein-specific antibodies have to be used. Therefore, the microarray technology may be more useful to reveal individual markers than identifying protein profile.

Evaluation techniques of metabolomics

Waste culture media as a result of IVF can be examined by different methods. It is possible for metabolites to be examined. Consequently, information is available about the metabolic status of the embryo. Several spectroscopic techniques used to identify and compare the samples.

Near-infrared spectroscopy and Raman spectroscopic techniques are both vibrational spectroscopic techniques. They produce a metabolite profile according to vibrational characteristics of molecules in the sample. Both techniques have advantages and disadvantages in terms of density of the signal produced and defining specific sample components. Seli et al. have identified differences in metabolite profiles of implanted and non- implanted embryos using both techniques[13]. Then, the information obtained was used to create a viability index that was useful in measuring reproductive potential of other embryos. Although retrospective, both techniques evaluated the viability of embryos successfully.

Magnetic resonance spectroscopy is used for identification of metabolites by molecular behavior in the magnetic field. Consequently, differences of the embryos' metabolite profiles were described. In a study with magnetic resonance spectroscopy by Seli et al., implanting embryos revealed a higher glutamate concentrations in culture media than non-implanted embryos[14]. In addition, although not statistically significant, the implanting embryo media had lesser extent alanine, pyruvate and glucose. Researchers formed viability index that can predict the success of reproduction in light of these results (sensitivity of 88.2%).

Although magnetic resonance spectroscopy and Raman spectroscopy results have been similar so far, magnetic resonance spectroscopy is a more expensive and time-consuming method in terms of data collection and analysis in clinical practice[15]. In clinical practice, an ideal analytical technique for metabolomic profiling of embryo culture media has not been found as of yet. The combination of more than one test may be necessary

for successful prediction. In addition, in current practice, frozen culture media samples have been transported to another laboratory for analysis. When metabolomics profiling can be performed in the IVF centers, we will be able to see exactly how sensitive and successful results really are. In addition, different types of culture media are used in IVF centers, and transfers are done in different stages of pre-implantation. Metabolic profiling appears to have the ability to give independent results after normalization of these factors. On the other hand, how these factors affect the sensitivity of the profiling is still not clearly recognized. Finally, as aneuploidy plays a major role in the loss of embryos in IVF treatment, metabolomics profiling success will depend on its ability to determine aneuploidy embryos.

Clinical Practice

Secretomic analysis of culture media, which is not invasive for the embryo, has started to find its place in clinical practice. In 2002, Fuzzi et al. found a correlation between successful embryo implantation and soluble human leukocyte antigen G (HLA-G) in IVF culture media[16]. As a result, Sher et al. selected embryos according to the state of HLA-G[17]. HLA-G-positive embryos' implantation and pregnancy rates were significantly higher in comparison to the HLA-G negative embryos. Therefore, HLA-G can be a predictive marker for selection of embryos.

Metabolomics have not been still utilized prospectively in the selection of embryos for transferring. Recent research gives hope for potential clinical applications. Scott et al. were able to estimate the reproductive potential of day-3 and day-5 embryos prospectively with Raman spectroscopy on the basis of calculated viability index[18]. Successful pregnancy outcome or unsuccessful implantation was estimated with 80.5% diagnostic sensitivity. With this technique, culture media metabolites are evaluated rapidly. Therefore, it has potential use for selection of embryos before transferring.

In single embryo transfer, as well as the traditional morphological classification, metabolomics evaluation of embryo quality has been the subject of two recent studies[19,20]. These authors could not find a correlation between metabolomics viability index and morphological classifications of day-2 or day-3 embryos. Although their studies were retrospective, Seli et al. proved that they were able to predict embryo's reproductive potential while being blinded to pregnancy outcome[20]. All these data suggest there are potential benefits to metabolomics evaluation for embryo selection process. As the researchers pointed out, this method is more applicable to estimate higher reproductive potential between two embryos with the same morphological range particularly in single embryo transfer.

IN VITRO MATURATION (IVM)

Collection of oocytes, fertilization and the transferring of embryos into the uterine cavity are the basic stages of IVF process. In the conventional method, exogenous gonadotropins are administered to the patients and a large number of oocyte development is stimulated. Then oocytes are aspirated via transvaginal route. Antral follicles, which occur due to follicle-stimulating hormone (FSH), reach into Graafian stage before the oocyte pick-up process. The greatest benefit of this method is that it mimics the development of normal follicles in vivo (although in supraphysiologic mileu). On the other hand, the process of controlled ovarian stimulation has many disadvantages as well. The high cost of drugs and the risk of ovarian hyperstimulation syndrome (accounted for 10% of the risky patients)[21] are just a few examples of the disadvantages.

Since utilizing unstimulated antral follicles, there is no need for gonadotropin therapy in IVM, which is an alternative method. Antral follicles containing oocytes are arrested in prophase stage of meiosis I and cultured *in vitro* till metaphase stage of meiosis II for 24-48 hours. At the end of this period, maturing oocytes are fertilized either via standard insemination or intracytoplasmic sperm injection method[22,23].

The first baby that was born live by IVM was reported by Cha et al. in 1991[24]. As we understand the process of folliculogenesis better in the recent years, several progresses have been made in IVM techniques, like culture media and oocyte collection (with prior preparation with FSH or hCG). Today, researchers work on follicular growth in ovarian tissue IVM and subsequent development before IVF. On the other hand, most of the literature on IVM is so far consists of case reports or contains a small number of patients. Therefore, the data obtained is still inadequate. In the future, after more comprehensive studies on IVM, it

may offer more patient-friendly alternative than ovarian stimulation to infertile patients as well as the option of fertility preservation.

Culture Media

Oocytes' culture media plays a major role in their development during IVM. Different compositions of culture media for maturation of oocytes have been compared evaluated[25], but there is still no consensus on the optimum composition of the culture media. On the other hand, it is evident that the selected media effects both oocyte maturation rate and the energy consumption[26].

Studies showed that the energy source of oocyte is pyruvate during IVM[26]. Because of the role of gonadotropins in IVM, recombinant FSH, luteinizing hormone (LH), or hCG is added IVM to culture media. Although there is some research on this approach[27,28], advanced studies on the role of gonadotropins are still needed to better understand the process of oocytes IVM. Serum in the culture media is a source of albumin and steroid precursor as well as growth factors for cells. If there is no serum in the media, additional albumin support is important[22].

Since oocytes epigenetic modifications occur during the process of maturation, culture conditions and their potential effects of IVM are important[22,29]. More studies are needed to reveal the net effect of IVM on epigenetic modification. In the future, oocytes' epigenetic profiling may be necessary prior to fertilization and transfer.

Oocyte pick-up

Oocyte collection technique in IVM is very similar to IVF. However, the pre-treatment improvements have increased the success rate of collection. As a result of many studies, hCG administration in vivo has increased the rate of oocyte *in vitro* maturation before oocyte pick-up[30-32]. This approach has been utilized in patients with out without polycystic ovaries[33,34].

Prior to oocyte maturation in vivo, impact of FSH treatment on pregnancy rates reveals inconsistent findings. Many researchers have shown increased number of oocytes collected and the maturation potential of these oocytes with FSH treatment in patients without PCOS[35], and in patients with PCOS[36]. However, other studies of FSH treatment revealed no increase in maturation, fertilization or pregnancy rates in these two groups[37,38]. Fadini et al. recently prospectively administered different gonadotropins, prior to the oocyte pick-up process[32]. In this study, no gonadotropin was administered to the control group. One group received only FSH, and the other received only hCG. Another group received both of FSH and hCG. Oocyte maturation rates were higher in only hCG and hCG plus FSH groups. Clinically, the most valuable finding was the highest clinical pregnancy rate in the group given FSH and hCG (29.9%). Results of this study proved that gonadotropins can be useful for the multiple usages.

The timing of oocyte pick-up is also very important for the success of IVM. Son et al. compared the results of measurement of dominant follicle at the time of collection and IVM results in PCOS patients[39]. They showed that large follicles (>14 mm) had higher implantation and clinical pregnancy rates than small follicles (<14mm). These results have to be confirmed by additional prospective studies, yet it appears to be an important approach to improve IVM protocols.

Clinical practice

As *in vitro* oocyte maturation does not require extensive gonadotropin stimulation, it provides a great advantage to patients who are at risk of ovarian hyperstimulation syndrome. For this reason, many studies on clinical application of IVM have been carried out in PCOs patients who were at high risk of ovarian hyperstimulation syndrome. The results of such studies were as follows: fertilization rate of 73.3%[34], implantation rate of 21.6%[36], clinical pregnancy rate of 40.3%[39] and live birth rate of 15.9%[40] IVM was successfully implemented in patients with poor responsive ovarian stimulation[39]. If the growth of oocytes was not sufficient, or the number of oocytes that responded to exogenous gonadotropins was not enough, the cycle was canceled. However, Liu et al. aspirated such immature oocytes (≤14mm) and demonstrated their maturation *in vitro*[41]. As a result of this study: fertilization rate of 78.8%, implantation rate of 20% and a total of 8 cycles, 2 live births (1 ongoing pregnancy) have emerged. Therefore, IVM can be an interesting option for poor responders as well as hyper-responders.

In Vitro Follicular Maturation

Those at risk of hyperstimulation or show poor response

to gonadotropins present a number of exciting options for IVM, whose oocytes in the stage of antral follicles are required. On the other hand, primordial or pre-antral stage follicles have to be collected *in vitro* maturation. The main objective of this technique is to ensure the development of follicles up to the antral stage. For fertility preservation in patients undergoing chemotherapy or radiation therapy, achieving success in IVM can a groundbreaking development. Although a successful pregnancy has not reported yet, researchers continue to work on both humans and animals.

Cortical tissue biopsy in humans is usually performed during gynecologic surgery or cesarean section[42-44]. Before culturing, follicles are isolated either mechanically[45] or by melting enzymatic stroma around [42]. Despite a sufficient number of follicles isolated, follicles undergo atresia after a few days in culture because of being away from ovarian stroma support. Hence, researchers began to culture ovarian cortical strips carrying primordial follicles[43,44]. In a study using this method, over 66% of cortex follicles remained intact for four weeks in culture and most of them reached the primary and secondary stages of development[44]. More research is still needed to evaluate whether oocytes obtained by this method provide adequate maturation and fertilize successfully.

Telfer et al. matured *in vitro* primordial / primary follicles up to the antral stage by the two-digit cultural system[43]. Ovarian cortical tissue biopsies in serum-free media cultured for six days and pre-antral follicles were isolated for cultuvation again with activin A. Though antral formation was reached only in 30% of the oocytes, this technique can provide an alternative for the development of follicles *in vitro* before IVM.

Somatic cells (granulosa and theca cells surrounding the oocyte inside the follicle, etc.) need glucose, regardless of the technique used for the development in-vitro prior to IVM, while oocytes use pyruvate as an energy source. Hence, the ingredients added to the media should be carefully selected and set to create a right development environment.

Conclusion

Despite of the differences in the above-mentioned developments, the common goal is the same to increase the success rate while improving patient safety. As multiple pregnancy is an important concern of ART, single-embryo transfer is the only solution to reduce the incidence of twin or multiple pregnancy. As long as single embryo transfer does not provide comparable results to multiple embryo transfers, its usage will be limited to mandatory laws like in Turkey. If our ability to choose genetically healthy embryos with the highest reproductive potential with help of metabolomics and secretomics increases, single embryo transfer may be optimized. IVM technique reduces the risk of ovarian hyperstimulation syndrome and increases patient safety. In addition, IVM may be an interesting alternative for patients who are poor responders or patients who require fertility preservation. Although extensive research is needed before routine clinical practice, the above-mentioned recent developments are quite promising in improving ART outcomes.

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