Summary

Introduction. Since reproductive technologies are becoming increasingly popular among the couples with infertility problem, and having in mind that the success rate is still low, the clinicians tend to transfer more embryos in order to increase the probability of success. However, such a strategy increases the risk of multiple pregnancy, which brings about numerous risks to the health of both the mother and children. Therefore, an elective single-embryo transfer is set as imperative, which, on the other hand, would not be possible without selection and evaluation of the quality of embryos. Assessment of Embryo Quality. Embryos can be selected by various methods, from non-invasive to invasive methods. In non-invasive methods, the embryos are selected by their morphology or by the techniques based on the analysis of molecular components – analyses of the level of proteomes or metabolomes. A more detailed monitoring of the kinetics of the embryo development can be related to the introduction of time-lapse imaging and monitoring systems into laboratory practice. The invasive methods encompass the techniques such as preimplantation genetic diagnosis and preimplantation genetic screening. In preimplantation genetic diagnosis, the assisted reproduction technologies cycle is approached for the genetic reasons, whereas preimplantation genetic screening is used to enhance the success rate of the assisted reproduction cycles. Conclusion. In this paper we have shown that the application of elective single-embryo transfer requires the selection and assessment of the quality of embryos by the methods that have been developed in the last four decades, and still need further improvements.

Key words: Embryonic Development; Fertilization in Vitro; Selection, Genetic; Embryo Implantation; Genetic Testing; Preimplantation Diagnosis; Single Embryo Transfer; Pregnancy Rate; Risk Factors; Time-Lapse Imaging; Metabolomics; Proteomics

Introduction

The inability to obtain offspring represents a serious medical, psychological as well as sociological problem, both for an individual and for the partners, so that it is rightly considered as a “partners’ problem.”

The beginning of in vitro fertilization (IVF) dates back to the end of the 19th century, with the experiments on animal models [1]. As early as in 1965, Pro-
Abbreviations

ART – assisted reproduction technologies
ET – embryo transfer
hpi – hours post insemination
ICM – inner cell mass
ICSI – intracytoplasmic sperm injection
IVF – in vitro fertilization
PB – polar body
PGD – preimplantation genetic diagnosis
PGS – preimplantation genetic screening
TE – trophoectoderm
ZIFT – zygote intrafallopian transfer
ZP – zona pellucida

Professor Robert G. Edwards et al. attempted to fertilize oocytes in vitro, and after 13 years their efforts were crowned with success: on July 25, 1978 Louise Brown was born as the first test-tube baby [1]. Almost three decades later, when millions of children had been born this way, Professor Robert G. Edwards was awarded the Nobel Prize for physiology/medicine in 2010 for the development of IVF [2, 3]. Today, there are more than 2000 clinics involved worldwide, the biggest being the one in Tokyo, which treats more than 15,000 couples for infertility annually [2].

However, the success rates are still low; lower than the ones that would satisfy both the doctors and the patients, and a much bigger problem are high percentages of multiple pregnancies after the assisted reproduction. While physicians consider multiple pregnancies to be a problem, to the patients, due to their great desire to obtain offspring, they seem to be a great success. Because of that, a successful selection of embryos is a necessity in order to decrease the number of transferred embryos, and thus reduce the risk of multiple pregnancy and increase the success rate. Thus a solution would be a selective single-embryo transfer (eSET), the success of which is possible without the selection and assessment of the quality of the embryo.

Infertility – Basic Notions, Types and Causes

Infertility is defined as the state in which there is no conception after one year of regular sexual intercourse without contraception. The expression “infertility” denotes the incapability of carrying out the pregnancy and giving birth to a viable child, and we should distinguish it from the notion of sterility [4, 5]. Sterility may be primary and secondary. Primary sterility denotes the woman’s incapability of conception, whereas secondary sterility refers to the inability of the woman to get pregnant after one previous pregnancy.

Assisted Reproduction Technologies

According to the World Health Organization, assisted reproduction technologies (ART) include the following methods: embryo transfer (ET), intracytoplasmic sperm injection (ICSI), ovarian stimulation with exogenous gonadotropins, surgical laparoscopy, and surrogacy [6]. Besides, the ART methods include also gamet intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), and frozen embryo transfer (FET), but they are applied to a much lesser extent. In the literature, one can also find preimplantation genetic diagnosis (PGD), gamete and embryo donation, as well as gamete and embryo cryopreservation, mentioned as ART methods. It is thought that approximately 99% of ART cycles are performed by IVF-ET [7].

In IVF, as one of the most known ART method, egg(s) from the woman’s ovary are fertilized in laboratory conditions and after several days returned back, i.e. the embryo/s is/are implanted in the uterus of the same or another woman [4].

It is stated in the literature that over three million [4] or even five million [8] children were born worldwide thanks to IVF. In the recent years there has been an increase in the number of IVF newborns, but also an increase in the number of multiple pregnancies. The reason for this can be found in the practice of ovarian stimulation and pregnancy of women of older age, which represents a risk for a multiple pregnancy.

Assessment of Embryo Quality

Morphology is an appropriate marker of viability of the implantation potential of the embryo. Embryologists often describe a morphologically normal embryo as “nice”). However, morphology is not esthetics, and we cannot speak of an absolute correlation between the normal morphology and the positive outcome of the IVF treatment. In view of the fact that this assessment is subjective, it has been endeavored for years or even decades to find a way of transforming the embryo assessment into an objective and quantitative method [9, 10]. There are disputes over whether it is necessary to select embryos at all, bearing in mind the possibility of their freezing. However, if we take a look at the data it comes out that the rate of successful implantation is lower after the transfer of defrosted embryos, which would lead to the situation that the success rate could not be raised to a higher level. In addition, from the aspect of the patients who want to decrease the number of visits to the clinics and number of cycles in order to avoid additional costs and lessen emotional stress, embryo selection appears as a necessity.

Embryos can be selected by non-invasive and invasive methods. In non-invasive methods, the embryos are selected according to their morphology or by the techniques based on the analysis of molecular components – analyses at the level of proteomes or metabolomes [11]. Time-lapse imaging has recently been developed. Invasive methods encompass the techniques such as PGD and preimplantation genetic screening (PGS).

Morphology as a Tool for the Assessment of Embryo Quality

The quality of embryos is mainly assessed on the basis of their morphology, i.e. based on the key morphological characteristics that are in correlation with an enhanced implantation rate. Examination of the embryos is carried out under the microscope after taking them from the incubator. Because of the harmful effects
of taking out the embryo from the incubator, the procedure is limited to only several discrete observations, which limits the amount of collected information, and the embryo assessment is highly dependent on the timing of the information [12]. In order to achieve a more reliable assessment of the quality of the embryos, more frequent evaluation would be needed; however, this means also more frequent exposition of the embryo(s) to the changes in temperature, gas components and humidity [13]. When conventional incubators are used, there is a conflict between the need to obtain a detailed picture of the development of embryos and disturbance of the stable conditions of the culture [11]. There are two main approaches to embryo assessment, performed either sequentially in several stages of the embryo development or only once, immediately prior to the transfer [14].

**Timing of Observation of Fertilized Oocytes and Embryos**

It is accepted that standardization of the time of observation is critical for the possibility of comparison of the results among the laboratories. Assessments are uniformly expressed in hours post insemination (hpi). Thus, if the day of insemination is denoted by 0, then day 5 is the last possible day for embryo transfer.

**Assessment of the Quality of Oocytes**

In view of the fact that the gametes provide the future embryo with more than two haploid sets of chromosomes, it is clear that the quality of oocytes plays a crucial role in the determination of the development of the embryo and consequently to its viability [15]. A conclusion of the Istanbul Workshop was that optimal morphology of oocytes is a spherical structure surrounded by a uniform zona pellucida (ZP) with a uniform translucent cytoplasm with no inclusions, as well as with the corresponding size of the polar body (PB) [16]. Anomalies of oocytes can be classified as extracytoplasmic and intracytoplasmic dysmorphisms [17]. In the assessment of the quality of oocytes one can carry out scoring of cumulus-oocyte complex (COC), ZP, perivitelline space, PB, vacuolizations, etc. [16, 18].

**Assessment of Fertilization**

In the assessment of fertilization it is necessary to detect whether the fertilization took place in an appropriate way. It is necessary to analyze pronuclei and nuclear precursor bodies (NBPs) after 16-18 hpi [18]. Optimal appearance of a fertilized oocyte should be spherical with two PBs and two centrally localized pronuclei. There are different systems of scoring the nuclei, which use different criteria for the prediction of the further development and quality of the embryo [19-21].

**Embryos at the Stage of Cleavage**

According to the Istanbul consensus, the evaluation of embryos at the stage of cleavage is carried out 26±1 h after ICSI and 28±1 h after IVF [16]. The most common criteria that are used to evaluate the embryo quality at this stage are the number of cells and their morphology [16, 18], percentage of fragmentations [16, 22, 23], but also the cell size [16, 24], multinucleation [16, 25-27], and others. Numerous studies have shown retrospectively which embryos are best, i.e. the ones having the highest implantation potential. Some of the characteristics of such embryos are: 4 or 5 blastomers on day 2 and at least 7 blastomers on day 3 after the fertilization, absence of multinucleated blastomers, and less than 20% of fragmentations on day 2 and day 3 after the fertilization [18]. Furthermore, it has been shown that the frequency of mitotic divisions is associated with the embryo development potential since the cleavage rates, which can be faster and slower than the expected one, are associated with a poorer developmental potential of the embryo [12, 18, 20, 28].

**Embryo Evaluation on Day 4 (Morula Stage)**

The optimal embryo at the morula stage, i.e. 92±2 h after the insemination, should be compact or in the phase of compaction, and that it entered at the fourth cleavage [16, 29]. The compaction should include the whole embryo.

**Embryo Evaluation on Day 5 (blastocyst stage)**

Grading of the blastocyst, which is its morphological evaluation, includes the following stages according to the Istanbul Consensus: early, expanding, expanded, hatching or hatched, as well as the quality of inner cell mass (ICM) and trophoectoderm (TE) cells [16]. At this stage as well as at the stage of cleavage, the time of evaluation and morphology plays an important role in the selection of blastocysts for transfer. An optimal embryo at this stage of development, i.e. 116±2 h after insemination, should be a completely expanded blastocyst to the hatched blastocyst, with a pronounced ICM, which is easily discernable. It should have many cells which are in compaction and strongly connected, whereas TE consists of many cells that form a cohesive epithelium [16]. ICM is of great importance for the implantation potential and fetal development, as well as TE, which has been the subject of numerous studies [18, 30, 31]. It should be pointed out that many authors have proposed some other systems for grading of blastocysts as well [18, 32-34].

It has been shown that if the blastocyst collapses in the process of evaluation, it cannot be reliably evaluated [16]. Such blastocysts should be re-evaluated after 1-2 h, bearing in mind that a new expansion of a blastocyst can normally take place even in regular cycles. The time of blastocyst formation is of special importance, which has been confirmed by many studies [18].

**Transfer of the Blastocyst**

Despite numerous studies and generally accepted opinion that the blastocyst transfer increases the success rate, the published data still show that this hypothesis is not, at least partly, true [31]. The reason for this is that the blastocyst transfer increases the success rate only in the patients with good prognosis, i.e. the patients who have a large number of good-quality embryos on day 3, but only if they want to achieve pregnancy in the fastest way. In addition, the transfer of embryos at the stage of cleavage is
recommended to those women who want to have a cumulative pregnancy rate and possibility of giving birth to all their embryos.

Some authors are of the opinion that the embryos which are of good quality at the stage of cleavage should be transferred on day 3, otherwise they will not survive prolonged culturing to the stage of blastocysts. On the other hand, some researchers are of the opposite opinion, and they think that the embryos which did not reach the stage of blastocyst were not of good quality even at the stage of cleavage.

**OMICS Techniques – Noninvasive Techniques**

In view of the fact that the evaluation of morphological parameters does not provide information on the embryo physiology, the need for the development of techniques capable of doing that emerged, and therefore OMICS techniques, have been developed.

In order to apply these noninvasive methods, IVF centers have to fulfill several criteria such as the possibility of measuring the changes without damaging the embryo, the ability to perform fast measurement of the changes, and the possibility to carry out measurements in a strictly correct way [18].

**Metabolomics and Proteomics**

In contrast to genomics and transcriptomics, the analysis of proteins and other metabolites is not an invasive procedure. Besides, its advantage is also that the expendable medium is an excellent source of the material. However, its shortcoming is the low concentration of these components.

**Metabolomics**

Metabolomics is a new technique which enables measurement of the factors in the embryo culture medium, such as glucose, pyruvate and amino acids, as well as many others. In order to determine the metabolites associated with physiological and pathological states, different spectral (near infrared spectroscopy (NIR) and Raman spectroscopy) and other analytical approaches are applied. Studies of metabolites showed that the metabolic profile of the embryo that results in the pregnancy is different from that of the embryos that do not lead to pregnancy [18, 35]. However, a conclusion has been drawn that the morphology of the embryo is not fully associated with its physiology.

**Proteomics**

The proteomics profiling is most often performed by mass spectrometry, but also by other ionizing methods that enable precise, fast and cheap analysis of small-volume samples at the sensitivity level of picomols to femtomol. Numerous factors have been studied, such as human leukocyte antigen G (HLA-G), platelet activating factor (PAF), leptin, ubiquitin, etc. However, in spite of the significant development, the knowledge of proteomics of preimplantation embryos is still limited. The reason for this is the limited amount of the sample, low gene expression, and poor sensitivity of the proteomic platform [36]). Of course, one should understand that the development of the embryo depends on many factors and we cannot expect that one factor might be capable of predicting the embryo developmental or implantation potential.

Presently, the investigations are oriented towards non-invasive analyses of preimplantation genetic screening (PGS), resulting in non-invasive screening of the viability, including chromosomal constitution and the discovery of protein lipocalin-1 in the blastocyst secretome. It has been shown that this protein is associated with aneuploidy since its expression was elevated in the secretome samples of the aneuploid blastocysts [37].

Wang [38] mentioned that the process of embryo selection, which once was “competition in beauty”, i.e. a simple assessment of the appearance of the embryo will soon include metabolic, proteomic, and genomic markers as the evaluation markers.

**Time-Lapse Monitoring**

A more detailed monitoring of the kinetics of the embryo development can be related to the introduction of time-lapse imaging (TLI) and monitoring systems into laboratory practice. The continuous monitoring of the progress of the embryo development is possible by following the key indicators of this process (both positive and negative) such as formation of the pronuclei, early cleavage, cell cycle intervals, synchronization of cell division and initiation of blastulation, and multinucleation, etc., since all these contribute to the selection of the best embryos for transfer [11]. This enables the precise determination of the beginning, duration, and time lapse between the cell divisions [39]. Furthermore, it allows getting a detailed insight into the embry development and studying of the effects of exposure of the embryo to different factors, which can contribute to the improvement of optimal culture conditions, and thus enhance the success rate.

There are two most often used time-lapse systems – Primo Vision (Vitrolife) and Embryoscope (Fertilitech) based on bright field technology, whereas the third one, EEVA (Early Embryonic Viability Assessment, Auxogyn) uses dark field [8]. In all the systems the embryos are photographed in the intervals of 5-20 min, and the obtained photos can be joined into a short video. Time-lapse incubator supports the embryo development in the same way as the conventional incubator does [40].
of chromosomes, PCR (polymer chain reaction) – for the diagnosis of monogenic diseases, and CGH (comparative genomic hybridization) – for chromosomal rearrangements. It should be pointed out that the method of detection at the level of a single cell is the same as the method used for other tissues for prenatal diagnosis. Still, it is more difficult to work with one cell since certain difficulties are encountered (cell lysis, allele drop out or some other problems due to mosaicism) [41].

Preimplantation Genetic Screening is used in the infertile couples who at a low risk of transmitting hereditary diseases to the offspring (in contrast to the couples who pass through PGD), because of which it is often termed “low risk PGD” [42].

**Ethical and Legal Aspects of ART – Embryo Selection**

Besides bringing the benefits to the couples who cannot produce offspring in a normal way, assisted reproduction technologies raise numerous ethical, legal, cultural, and social questions. Great Britain was first to regulate the ART issues [2]. In Arabic countries, there are few acts regulating ART matter, and the majority of them follow the religious laws. In contrast to that, surrogates and donations are allowed in the United States, and there are no limitations of the number of transferred embryos. In Germany, it is permitted to create at most three embryos, and all of them have to be transferred. Therefore, selection of embryos is not permitted, which means that PGD and PGS are illegal. The same holds for Switzerland and Italy. In our country, neither the Orthodox nor Catholic Church accepts IVF fully, i.e. they consider it unethical, especially because of the creation of a number of embryos, so that there is a concern about the fate of the redundant embryos after the completed cycle. The act which regulates ART issues in the Republic of Serbia is the “The Law on Treatment of Infertility by Procedures of Biomedically-assisted Fertilization” [43]. It regulates the issues related to PGD, donation of the gametes and embryos, the number of transferred embryos, and the way of performing the PGD procedure. In view of the fact that our country is among ten states in the world with oldest population (the share of those over 65 is more than 16.5%) [44], it is obvious that it is necessary to stimulate the partners to become parents. But it is also necessary to help the partners with infertility problems to get acquainted with the methods of assisted reproduction, using different methods and forms of information and education. Because of the lack of information about the risks of multiple pregnancy, and because the state finances only two IVF attempts, the couples decide to transfer more embryos. Hence, it is necessary that our scientific and other system institutions, as well as governmental health-care institutions – the Ministry of Health, social politics, etc. should regularly gather information about modern achievements in the field of assisted reproduction, contribute to its advancement, and support research and more unpaid attempts.

**Conclusion**

In this work we have shown that the application of elective single embryo transfer requires the selection and assessment of the quality of embryos by the methods that have been developed in the last four decades, and still need further improvements.

It is hoped that these approaches will become a part of routine laboratory practice in the future, and, along with evaluation of embryo morphology parameters, will give a sound base for success in this pursuit. While we are waiting for the development of more precise non-invasive technologies of embryo quality assessment, good laboratory practice and close care in all steps of the in vitro fertilization cycle remain indispensable.

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