The invasive and new non-invasive methods of mammalian oocyte and embryo quality assessment: a review

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ABSTRACT: The quality of oocytes-embryos can be determined by several techniques, including morphological, molecular, cellular and biochemical ones. The morphological methods of female gamete or embryo quality assessment often use the following *in vitro* manipulation procedures such as: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo production (IVP). However, these methods are highly subjective and the morphological classification of oocytes or embryos is not always compatible with their ability to grow and develop. Additionally, molecular biology methods are objective and present parametric results, which are more or less comparable to the real oocyte-embryo "health". Although these techniques enable us to determine markers of oocyte-embryo developmental potential, when applied they lead to destruction of the analysed cells. Therefore, the need still exists to search for new methods that will be highly objective (parametric) and, which is most important, non-invasive. In this review, the morphological and molecular methods of oocyte-embryo quality assessment are presented. Moreover, we described a new system based on microfluidic technology (Lab-on-Chip) which allows the creation of a new device for mammalian oocyte as well as embryo quality evaluation: by using their spectral characterisation following embryo transfer (ET) procedures in the cattle and the pig.

Keywords: oocyte; embryo; quality; microfluidics

Contents

1. Oocyte-embryo quality assessment-recent studies

Since the 19th century, embryologists have most frequently used light microscopic observation as the preferred method in oocyte or embryo quality assessment. However, this method is highly subjective and often leads to an incorrect classification of cells. Therefore, in the middle of 20th century, with the advance of genetic and molecular methods, a new era of female or male germ cell line quality evaluation using new objective parametric meth-

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ods began. The development of metabolomics made it possible to evaluate the *in vitro* culture agents which significantly influence maturation, fertilization of oocytes as well as the growth and the development of embryos in *in vitro* culture (IVC) systems. Several other molecular biology methods, such as confocal microscopic observation, QR-PCR, PCR-microarrays or two-dimensional electrophoresis of proteins enable us to determine the influence of oocyte-specific cytoplasmic factors, oocyte-surrounding somatic cells, supplements of *in vitro* culture media or hormones and small peptides from follicular fluid, on the achievement of MII stage by oocytes or of proper stages of embryo growth at early stages of preimplantation development (Bender et al. 2010). However, most of these methods are invasive and finally, when applied, lead to the destruction of the analysed cells. Therefore, the search for new, non-invasive methods of oocyte-embryo quality assessment, is now of high significance.

The development of new methods aimed at the determination of oocyte-embryo quality allows the focus of attention on new features of gamete-embryo biology, such as developmental potential (competence). Developmental competence determines the ability of oocytes to mature (nuclear and cytoplasmic) and undergo successful monospermic fertilization as well as the potential of embryos to grow and develop. The developmental competence or potential of oocytes and embryos can be assessed using several methods and techniques, including molecular, metabolomical or cellular ones. The specific features of oocytes-embryo "health" are evaluated by several predictors of their quality in the form of molecular and cellular markers. In this review, the most frequently used methods of oocyte-embryo quality assessment are presented. Moreover, we describe a novel non-invasive method used by our team, based on microfluidic technology. This Lab-on-Chip system enables us to determine the spectral parametric characterisation of the analysed single cell in real-time and real-life, with the possibility of recovering the investigated oocytes or embryos and subsequent transfer to the recipient, without destruction of the analysed cell.

2. Morphological classification of embryos-recent studies and perspectives

The assessment of embryo quality is one of the most important factors, which determine a successful embryo transfer. Its significance is related to the essential correlation between embryo quality, the stage of its development and the percentage of received pregnancies in embryo recipients. Thus, the higher class of embryo is related to the higher probability of pregnancy. Numerous statistical analyses have revealedthat the embryo quality class carries a superior significance for obtaining pregnancy in comparison to the stage of its development, the level of donor and recipient synchronization, individual features of recipients, the bull used to inseminate, the season, the person assessing the embryos, the breed (race) and kind of superovulation protocol as well as environmental factors (Callesen et al. 1995; Chebel et al. 2008). The assessment of embryo quality is carried out under a stereomicroscope on grounds of morphological criteria, usually after acquiring or culturing embryos directly before their transfer to recipient's uterus, after a short, conditioning culture of proper quality embryos and before their potential transfer as well as before freezing or after freezing. Because currently the majority of embryos are frozen in ethylene glycol and are destined for direct transfer without the evaluation of their condition after defrosting, the early definition of their quality takes on a special significance. The main aim of embryo quality assessment is to define its developmental potential, chances to obtain pregnancy and to define its sanitary condition. The most important factors, taken into consideration during the morphological assessment of embryos, include damage and fracture of the zona pellucida, shape, size, colour and the number of damaged blastomeres, as well as the content of granules and other cellular remnants in the subvitelline space. Damage to the zona pellucida is defined as morphological deviation from the standard shape. ZP fractures enable harmful substances to penetrate the embryo, which also may result in its lower viability. ZP surface should be smooth. In cases of reproductive system infection, pathogens may stick to its surface. In cases of massive infection, insemination does not take place. Based on the criteria proposed by Lindner and Wright (1983), embryos are categorised into five quality classes. The first class embryos (very good) are round and blastomeres have the same size, colour and consistency. The second class (good) embryos are slightly irregular, for example, as manifested by one swollen blastomere and an irregular shape of blastomeres. In the third class (sufficient quality)

embryos more extensive irregularities are present. In the fourth class (poor) embryos, on the other hand, loose blastomeres, degenerated cells, damaged cell membranes, cells of various sizes, and colour changes are noted. Degenerated embryos are included into the fifth class embryos. A similar system of assessment was adopted by other authors as well (Donaldson 1986; Abe et al. 2002). This division differs slightly from the criteria accepted by IETS, which divides embryos into four classes. Despite that, embryos have been assessed in a 2-grade scale (good and poor) (Schneider et al. 1980) and currently in 3-grade scale (excellent, good, regular (poor) (Aguilar et al. 2002; Peixoto et al. 2007). The criteria of morphological assessment of oocytes are similar. The criteria of oocyte assessment, described by de Loos et al. (1992), enables investigators to divide oocytes into four classes. One of the elements included in the morphological criteria of embryo assessment is embryo diameter. According to some investigators, the size of embryos enables us to define the number of blastomeres, the sex of the embryo and the development potential after its transfer to the recipient's uterus (Hoelker et al. 2006).

The shortcoming of the morphological assessment of embryos involves its extensive subjectivity. The assessment relativity depends, among other, on the variable experience of the examining person, not always identical criteria of evaluation and the quality of equipment (microscope zooming). In order to improve the quality of assessment, special training of examining people seems necessary. The quality of embryos may depend on a whole range of various factors which cannot be noted under the stereomicroscope. Extensive differences can be noted in the morphological assessment of embryo quality carried out by different investigators. More experienced ET teams evaluate embryos in a more strict manner than less experienced ones. It also seems that it is easier to assess the quality of less developed embryos (morulas) than more developed ones (blastocysts). Finally, it seems that the correct evaluation of embryos of a very good or insufficient quality is easier; however, it is more difficult to differentiate between good and sufficient embryos. However, even upon embryo evaluation by many different people the morphological assessment of embryos proved to be a useful way to evaluate their quality since a fundamental dependence was documented between the fertilization index and embryo quality class (Donaldson 1986). The subjective nature of the morphological assessment was proved by Farin et al. (1995) who compared the results of six different people, evaluating 15 embryos received *in vivo* and produced *in vitro*. The assessment of embryo quality made by them differed significantly. The relatively least pronounced differences were recorded in reference to embryos of the poorest and the best quality. Persons evaluating embryos agreed just in 68.5% of cases, carrying out the assessment of embryos in different stages of development and various degeneration levels. It was most difficult to differentiate between embryos of good and sufficient quality (Farin et al. 1995). On the other hand, no significant differences in the proportion of pregnant recipients were recorded between evaluating investigators. The percentage of fertilized heifers in the case of embryos recognised as very good or good was 66–72 and 62–69% respectively; in cases of sufficient embryos, however, the figure was 54–60%. It was demonstrated that the morphological assessment was unanimous. It seemed, however, that the lack of significant differences reflected the relatively small comparative material. In other studies, the embryo quality assessment carried out by means of stereoscopic microscope was compared to the analogical evaluation carried out by means of light and electron microscopes (Aguilar et al. 2002). The conclusion of this study were that morphological assessment is extremely subjective in nature. About 50% of embryos recognised as very good and good, were in fact, found to be of lower quality after their evaluation by means of light and electron microscopes. In morulas of good quality two typical types of cells were identified – the first one constituted original trophoblast cells located in peripheral layers of embryo mass, characterized by low optical density of the cytoplasm and containing numerous cell organelles, such as mitochondria and Golgi's apparatuses, as well as microvilli penetrating into perivitelline space. The other type of cells involved the proper embryo cells, located in the internal embryo mass and manifesting a cytoplasm which seemed more dense than that in the brighter looking cells. Good quality morulas had always well-developed mature nucleoli. Many embryos classified as sufficient contained both types of cells while in some sufficient and weak (poor) morulas they could not be identified under the microscope on the basis of their cytoplasm and ultra-structure. Morulas of

poorer quality had less intercellular connections, less developed microvilli on blastomeres and contained more lipid drops, vacuoles, immature mitochondria and nucleoli with low transcriptional activity (Abe et al. 2002). Thus, what is the optimum method of embryo assessment? Certainly, it should be completely non-invasive, useful in single embryo evaluation, providing immediate results and allowing the separation of living embryos from dead ones (Donnay and Lesse 1999). Here, it should be added that some of these conditions are fulfilled by morphological assessment of embryo quality. As we have seen, the reservations in this case concern, however, its average objectivity.

3. Selected methods of embryo quality assessment

Subjectivity of morphological assessment was one of the main reasons for seeking new, more accurate, and above all, objective methods of embryo quality assessment. Non-invasive lab tests to evaluate their quality belong to the more important new methods. In a great measure, they consist in the detection of enzymes, metabolites, proteins, gene products and other traits. The earliest described tests were the ones that allowed the evaluation of lactic acid dehydrogenase activity (Johnson et al. 1991). Its increased activity, detected by means of the bioluminescent test in the breeding medium, was informative regarding the degree of embryo cell damage, enabling investigators to differentiate between living and dead embryos. This test provided a non-invasive and immediate (about 5 min per embryo) assessment of embryo viability. This method, however, despite unquestionable advantages, neither did nor does receive recognition in the field. Some years ago a test was introduced to evaluate changes in the concentration of carbon dioxide produced by the embryo as a result of glucose consumption (Khurana and Niemann 2000). The assessment of carbon dioxide production was held in a closed system, in an environment rich in glucose, over the course of two hours. The application of this system made it possible to determine some essential facts. It appeared that oxidative metabolism was similar in embryos in the stages of the morula and blastocyst, that it was twofold higher in fresh embryos than in frozen and defrosted ones, and finally, that the low production of carbon dioxide correlated with the low viability of embryos. However, probably the considerable time required for performing the test meant that the test was not applied in field conditions.

The development of methods such as transmission electron microscopy, immunocytochemistry for confocal laser-scanning microscopy and fluorescent hybridization *in situ* made it possible to significantly develop the assessment of embryo and oocyte quality and development potential. Application of these techniques in combination enabled investigators to examine amino acids, metabolites as well as gene products, transcripts and proteins and other variables in parallel.

Recently, an interesting assessment of embryo quality was presented by Lopes et al. (2005, 2007). They introduced the nanorespirometer – a device for the evaluation of the oxygen usage by embryos. Previous investigations on oxygen consumption by embryos employed various methods. Techniques based on microspectrophotometry, ultramicrofluorescence, electrochemical methods, automatic scanning electrode, electrochemical scanning microscope (SCAM) (Shiku et al. 2001) or loop mediated isothermal amplification (LAMP) (Agung et al. 2005) belong to the more significant ones. Application of these techniques permitted, among other things, to establish differences in the oxygen use between embryos of very good and good quality. It was also found that embryos of male type used less oxygen than embryos of female type. Most of these methods had essential disadvantages: they were invasive, demanded technical resources, time to carry out measurements, application of UV light or radioactive reagents, and were dangerous for the life of the assessed embryos. In this aspect, the device introduced by Lopes et al. (2005) seemed especially promising. The nanorespirometer is a modern microsensoric technology that provides accurate and quick assessment of single embryos breathing in various stages of development. The whole procedure is simple and it involves an 8-minute measurement. The initial results showed that the breathing index of 7-day embryos blastocysts of very good quality was indeed higher than in embryos of low quality. Simultaneously, in studies on embryos produced *in vitro* it was established that the usage of oxygen increased with embryo development and it was generally higher than in embryos produced *in vivo*. Despite that, the usage of oxygen did not differ significantly between embryos from which pregnancy was achieved and the ones which failed to yield pregnancy.

4. "Ideal oocytes" versus methods of their quality assessment

For over twenty years many scientists have searched for the best method of oocytes/embryo quality assessment (Bukowska et al. 2008). In this period of time the definition of "the ideal oocyte" was modified several times, with the addition of some new predictors in quality evaluation. The first classification of oocyte quality was based on the optical microscopy evaluation of oocyte/embryo morphology. The morphological criteria that characterised "the ideal oocytes" included the cumulus-corona cell structure, morphology of oocyte cytoplasm with special attention given to its colour, inclusions and granularity, and extracytoplasmic structures, such as the *zona pellucida*, the first polar body and the perivitelline space (Kempisty et al. 2009, 2011a). Thus, a metaphase II (MII) oocyte was considered "normal or ideal" when under light microscopy it had a round, clear *zona pellucida*, a small perivitelline space containing a single, non-fragmented first polar body (I PB), and a pale, moderately granular cytoplasm, which contained no inclusions. However, with the development of molecular genetic techniques as well as metabolomics the classic morphological assessment of oocyte quality was substituted by several new methods (Kempisty et al. 2008). The several existing methods present definitions of "ideal oocyte" quality which stem from experimental data and include certain traits in common. (1) A morphological definition of "the good oocyte" involves the female gamete which is characterized by appropriate normal structures, such as first polar body, perivitelline space, cytoplasmic granularity and localization of granular area. (2) In the biochemical definition, a "the good oocyte" is characterized by an appropriate content of lipids, stored mainly as lipid droplets in the cytoplasm. (3) In molecular terms "the good oocyte" is defined as a cell, which at every step of maturation and development contains large amounts of nucleic acids (mRNA) and proteins, necessary for further embryo growth and development. (4) The biological "external" definition of "the good oocyte": a cell the growth of which is determined by ovarian follicular microenvironment and maternal signals (mediated by granulosa and cumulus cells). (5) The metabolomical definition of "the good oocyte": follicular fluid and culture media are characterised by normal level of several substrates such as glycolytic intermediates and amino acids, which support oocyte growth. (6) The cellular definition of "the good oocyte": this includes intrinsic markers (such as mitochondrial status and glucose-6-phosphate dehydrogenase 1 activity) and extrinsic markers (such as apoptosis of follicular cells and level of TGF-beta superfamily in follicular fluid or serum) (Antosik et al. 2010; Bukowska et al. 2011). Thus, in order to assess oocyte quality we can used several predictors, most of which are morphological, molecular, biochemical, cellular, and metabolomical. The molecular predictors of "the good oocyte" include assessment of EP45-serpin (serine protease inhibitors), follistatin (plays a functional role in the regulation of early embryogenesis), activin (treatment mimicks positive effects of follistatin on time to first cleavage and blastocyst development), TFIID (could be used as biomarkers of oocyte quality), lysosomal cysteine proteinases, cathepsins B, S, K, and Z (abundance of cathepsin mRNA in cumulus cells may be predictive of oocyte quality), hyaluronan synthase 2 (HAS2), inhibin betaA (INHBA), epidermal growth factor receptor (EGFR), gremlin 1 (GREM1), betacellulin (BTC), CD44, tumor necrosis factor-induced protein 6 (TNFAIP6), and prostaglandin-endoperoxide synthase 2 (PTGS2)-(candidates expressed in cumulus cells, which could provide valuable and indirect markers of oocyte competence), as well as local growth factors, such as IGFs and BMPs, IGFBPs, caspase-3 activity (interaction between extra-ovarian and intra-ovarian factors determines fate of the follicle and quality of the oocyte). There are also several biochemical predictors of oocyte quality which can be recognized to represent markers of an "ideal oocyte" (Jaskowski et al. 2010). Most of them include BCB staining test (glucose-6-phosphate dehydrogenase 1 activity) to assess oocyte developmental competence, mitochondrial status and apoptosis of follicular cells in the evaluation of gamete developmental potential and induction of degeneration processes in the cell as well as several biochemical substances, the proper concentration or activity of which may be used as a marker, such as gonadotropins (intrafollicular concentrations of FSH and LH), growth hormone (GH), prolactin (PRL), estrogens, progesterone (P) and androgens, corticoids, level of TGF-beta in follicular fluid or serum, other growth factors and interleukins (insulin-like growth factors (IGF), Reactive Oxygen Species (ROS) and antioxidant factors, lipids droplets in cytoplasm, sugars (hyaluronan), and prostanoids (Wongsrikeao et al.

2006; Uhm et al. 2010; Catala et al. 2011; Larson et al. 2011; Kempisty et al. 2012). The metabolomic predictors of oocyte quality mostly include amino acid (asparagine, glycine and leucine) or fatty acid concentration, non-invasive foot-printing of glycolytic activity; differences in –CH, –NH and –OH concentrations exhibit discrete differences between the culture, and the ratio of –CH to R–OH content in the media, which reflects oxidative stress.

5. Microfluidic chip (Lab-on-Chip) evaluation of oocytes and embryos

Rapid technological progress has resulted in the creation of a prototype device for an economic, rapid parametric evaluation of embryo quality on the basis of a fluidic microchip, the Lab-on-Chip (Kempisty et al. 2011b). The Lab-on-Chip is a miniature spectrophotometric system, the head of which consists of a silicon and glass chamber, and two optical fibres. The chamber has micro-grooves which create a fluidic channel. The device way is characterised by its small size and weight, is resistant to vibration and may be used outside of the lab successfully. A detailed description of the Lab-on Chip can be found in the papers of Szczepanska et al. (2009, 2010). The assessed embryo is located in the chamber by means of suction through a hole opposite to the inlet. Such a procedure guarantees movement of the embryo through the fluidic channel and its immobilization in the chamber.

Microfluidic chip measurement of oocyte-embryo quality based on their spectrophotometric characteristics is a new objective and a non-invasive method. The usefulness of this method was documented by Szczepanska et al. (2009), who assessed the spectral characteristics of porcine oocytes isolated from different sizes of follicles. They showed that oocytes collected from each class of follicular size after classic optical microscopy evaluation had similar morphology ("looked the same") in relation to morphological assessed factors described previously by Jackowska et al. (2009). However, the "ideal" group of gametes recovered from the same size follicles differed significantly with special respect to their transmittance, spectrum and absorption of specific wavelengths. These experiments showed that porcine oocytes which formed one morphological group of cells were highly heterogenous and could not be regarded to represent the same group of cells. Thus, following several steps of molecular analyses we have to focus our attention on "a real classification" of oocyte quality based in particular on its absorption characteristics or "pick of fluorescence". The microfluidic chip measurements of oocyte/embryo quality represent a new method and crucially they are non-invasive in character. This has partially been demonstrated by our non-published data, in which we showed that measurements of bovine oocytes using this chip had no influence on the *in vitro* fertilization (IVF) rate, as similar numbers of bovine blastocysts were obtained after IVF in the group of chip-measured oocytes and non-chip-measured gametes, used as controls. It was also shown in our experiments that after chip measurements of bovine morulas flushed out from donors and then transferred to the recipients, we obtained similar rates of pregnancy. These experiments demonstrated that the non-invasiveness of microfluidic chip measurements was manifested not only in IVF results but also in bovine pregnancy rates (our unpublished data).

6. CONCLUSIONS

There are several methods of oocyte/embryo quality assessment, based on morphology, as well as molecular, biochemical, cellular and metabolomical predictors. However, the methods (especially morphological evaluation) are highly subjective and, in addition, the analysed material is variable and highly heterogenous. In the past few years microfluidic chip measurements of oocyte/embryo quality were developed, based on oocyte/embryo transmittance, absorption, and spectral characteristics. This new method is objective and non-invasive. The Lab-on-Chip analysis of cell quality is related to oocyte/embryo physical properties, with special respect to the absorption of specific wavelengths. The pick of absorption or fluorescence is always specific for one analysed cell as well as being reproducible. The analysed material (oocyte or embryo) is not destroyed and, which is most important, no changes are noted in the physical and biological properties of the analysed cell. However, this non-invasive method requires future analysis which will prove its utility. Although its non-invasiveness has been proven in our experiments (our unpublished data), the next step should involve examination of the relationship between the physical characteristics of the oocyte/embryo and its biological and functional properties.

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