Morphological Assessment of Oocytes, Pronuclear and Cleavage Stage Embryos

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The efficiency of human-assisted reproductive techniques (ART) is still low and most of the embryos transferred fail to implant. As a consequence, the policy of simultaneously replacing multiple embryos has been adopted but unfortunately this practice has led to an undesirable increase in multiple pregnancy rate [1]. The ability to objectively assess gametes and embryos with the most developmental potential is challenging and would also represent a tool for troubleshooting. In fact, it would help reduce the number of embryos transferred without affecting the overall pregnancy rate but lowering, at the same time, multiple gestations. To date, the evaluation of embryo quality relies mainly on morphology and routine inverted microscopic investigations are performed at predetermined checkpoints [2]. A number of different grading systems have been described in the literature but there are some concerns regarding the predictive value of these parameters. Recently, the Alpha Scientists in Reproductive Medicine and the ESHRE Special Group of Embryology stated that an international consensus on oocyte and embryo morphological assessment is needed. In fact, the standardization would help to validate the morphological criteria as end-points in clinical trials and other studies that assess the effect of new technologies and new products, improving the safety and efficacy of IVF treatments [3]. The document produced by the two societies in collaboration will be referred to as the "Consensus document" and will be discussed in this chapter [3].

Morphological Assessment of Oocytes

Morphological evaluation of oocytes has been superficial, at least in the last few years. In fact the general approach is the postponement of the problem: all the available mature oocytes are inseminated and the selection is done at the preimplantation embryo level. However, since the embryo competence is likely due to oocyte quality, the introduction of earlier evaluations, in conjunction with the well-known cleavage stage embryo assessment, may be fundamental for the improvement of the in vitro fertilization techniques. In fact it could help reducing the number of oocytes inseminated, and thus the number of supernumerary embryos produced. "Good quality" oocytes should be preferred to "bad quality" ones for insemination in order to avoid the production of potentially abnormal embryos. Moreover, when possible, this earlier selection could help in the identification of an appropriate number of oocytes to be used in egg donation programs. Unfortunately, the literature on oocyte morphological assessment and the impact of certain egg

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features on embryo development and clinical results is still controversial, and this is probably due to the complex picture depicted [2].

Cumulus-Corona-Oocyte Complex Evaluation

Cumulus-corona-oocyte complex (CCOC) assessment generally represents the first-line evaluation in the everyday work of an average IVF laboratory. Cumulus cells surround the oocyte and establish a bidirectional communication that is fundamental for the growth of both the egg and the follicle [4–7]. The innermost layer of the cumulus oophorus is called "corona radiata." The cumulus-corona mass of fully mature oocytes is expected to be highly expanded as a consequence of the secretion of hyaluronic acid that interposes among the cells and separates them conferring to the complex a fluffy appearance [8]. However, in stimulated cycles the CCOC morphology is not strictly connected to the oocyte maturity; this seems to be due to a different sensitivity to the drugs used, with the subsequent lack of synchrony between the expansion of cumulus and the egg maturation process [8–11]. To date there is little evidence of a clear relationship between the aspect of the CCOC and the oocyte/embryo developmental competence [2]. However, given the fundamental metabolic connection between cumulus cells and eggs, the aspect of CCOC should predict the intrinsic quality of gametes and their further developmental potential [12, 13]. Therefore, an appropriate CCOC scoring system should be a tool for troubleshooting. Apart from the different grading systems described so far, recently the Consensus document suggested the use of a simple binary score (0 or 1) to describe "poor" CCOC or "good" CCOC on the basis of cumulus expansion and corona radiation [3, 10, 12–15].

Nuclear Maturity Evaluation

The removal of the cumulus-corona cell mass allows for a more accurate evaluation of oocyte morphology and more importantly nuclear maturity. Nuclear maturity is morphologically defined as the presence of the first polar body (IPB) in the perivitelline space (PS). In this condition the egg is at the resting stage of metaphase II (MII), characterized by the alignment of the homologous chromosomes on the spindle equatorial plate during the second division of meiosis. It is generally recognized that in about 85 % of the cases the retrieved oocytes display the IPB and are classified as MII, whereas 10 % present an intracytoplasmic structure called the "germinal vesicle" (GV), characteristic of the prophase of the first meiotic division. Approximately 5 % of the oocytes have neither visible GV nor IPB extruded: these oocytes are generally classified as metaphase I (MI) [16].

Additional information on oocyte nuclear status can be obtained with the use of polarized light microscopy combined with software for image processing for the noninvasive visualization of the meiotic spindle (MS) and other oocyte birefringent structures. The MS is a microtubular structure involved in chromosome segregation, and thus is crucial in the sequence of events leading to the correct completion of meiosis and fertilization. Parallel-aligned MS microtubules are birefringent and able to shift the plane of polarized light inducing a retardance; these properties enable the system to generate contrast and image the MS structure [17] (Fig. 1a). This technique allows both a qualitative and a quantitive analysis. First of all, MS presence gives more accurate information about the nuclear stage. In particular, some oocytes were found to be clearly immature (at the stage of early telophase I) when observed with the polarized light microscopy. At this stage, there is continuity between the ooplasm and the cytoplasm of the forming IPB and the MS is interposed between the two separating cells (75–90 min). These oocytes would have been classified as "mature" when assessed by routine light microscopy based solely on the presence of the IPB. Moreover, the MS has been found to disappear in late telophase I, reforming only 40–60 min after [18, 19]. However, it must be underlined that other factors can contribute to MS absence. For example, suboptimal culture

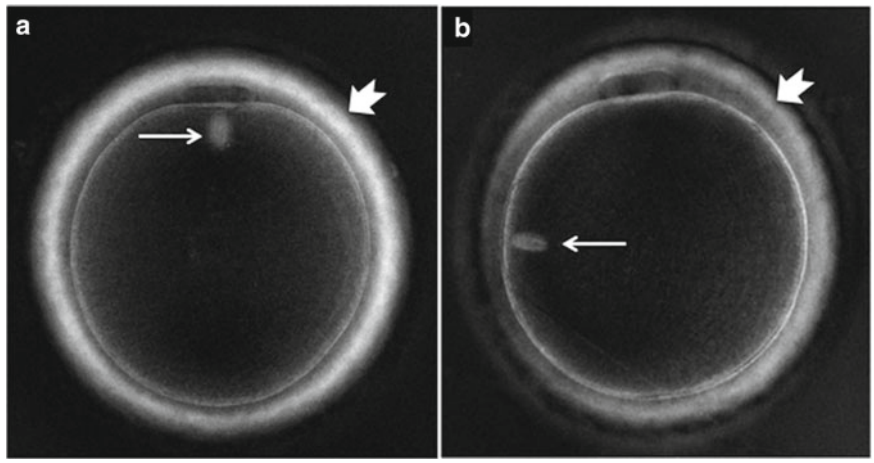

Fig. 3.1 Metaphase II oocytes observed by polarized light microscopy. Meiotic spindles (MS) (*long arrows*) and ZP inner layers (*short arrows*) are clearly visible. (**a**) The MS is aligned under the first polar body (IPB); (**b**) the MS is dislocated and placed at about 90° from the IPB

conditions, such as temperature fluctuations and chemical stress during manipulation, can account for MS disassembly [20–25]. Moreover, the MS may be artifactually not visualized if not correctly orientated during the analysis [26, 27]. Finally, the percentage of oocytes with detectable MS is related to the time elapsed from HCG administration and is higher after ≥38 h post-administration of HCG [28]. This finding may be explained by the fact that more oocytes are still in pro-metaphase II at time intervals closer to HCG administration [19]. Data regarding the correlation between the presence of MS and IVF/ICSI outcomes are controversial. In fact, although MS presence is generally correlated to higher fertilization rates and better embryo development, pregnancy and implantation rates seem not to be significantly affected [29].

Besides its role played in chromosome segregation, the MS is also a key organelle in the creation of the IPB and IIPB. Its position at the very periphery of the cell, attached to the oolemma cortex, is believed to determine the true animal pole and the plane of the first cleavage and thus the PB extrusion site [27, 30]. However, the IPB has been found to be frequently dislocated from the MS location and the manipulation required for cumulus-corona cells removal has been identified as the cause of this artifactual displacement [26, 31, 32] (Fig. 1b). The software associated with polarized light microscopy is able to calculate the deviation degree and it has been found that mechanical stress-induced IPB dislocation superior to 90° correlates with lower fertilization ability [26]. Another possible drawback of IPB displacement is the potential injury of MS during the microinjection procedure. In fact, a displaced IPB may be a false indicator of the real position of MS, which can then be disturbed by an ICSI micropipette [16, 33].

Finally, since the degree of MS birefringence is directly proportional to the molecular organization of the structure and the density of microtubules, it has been postulated that higher spindle retardance may correlate with oocyte competence and better embryo development and clinical results [34–38]. However, the literature on this correlation is still controversial and some authors failed to find any statistical significant correlation between the degree of birefringence and IVF/ICSI outcomes [39]. Instead, there should be a relationship between spindle retardance and maternal age [37, 39]. However, it must be kept in mind that spindle retardance seems to be MS orientation-dependent and probably correlated with the time of investigation, thus its reliability as a new marker of oocyte quality should be questioned [19].

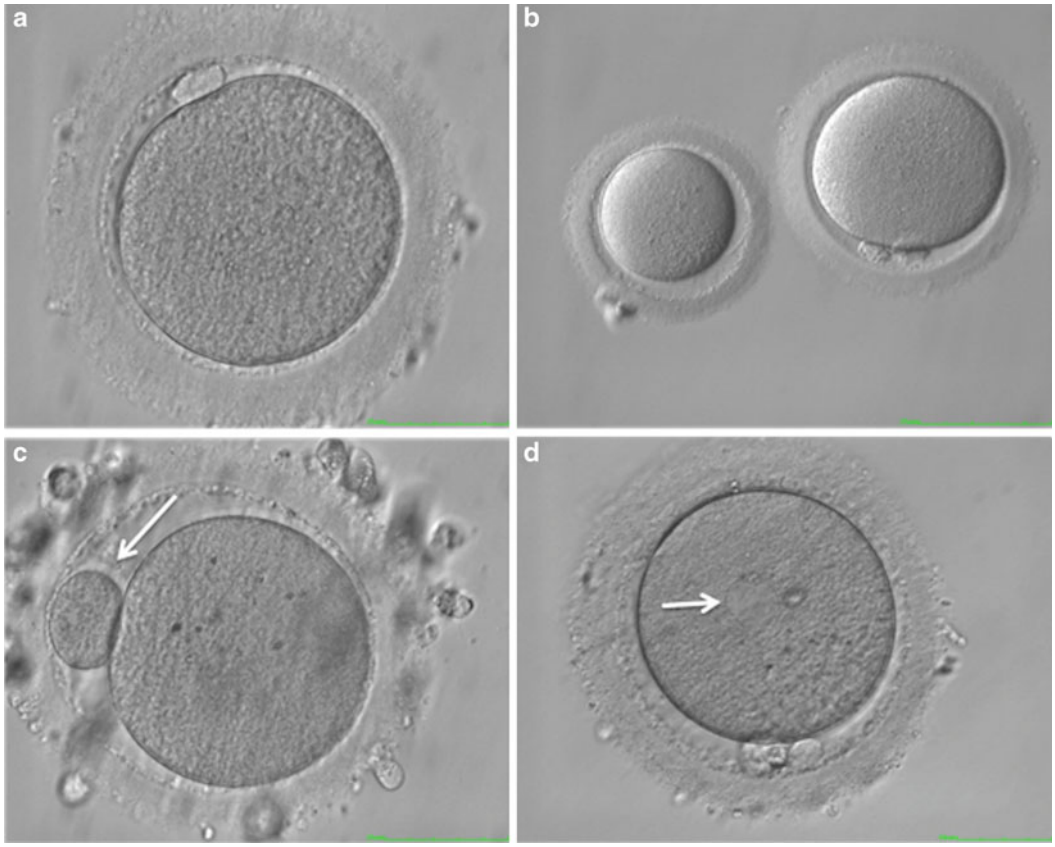

Fig. 3.2 Metaphase II oocytes with different morphological characteristics. (**a**) Normal appearing MII oocyte; (**b**) giant MII oocyte compared to a normal sized MI oocyte; (**c**) MII oocyte with giant IPB (*arrow*); (**d**) MII oocyte presenting SER aggregations (*arrows*)

Metaphase II Oocyte Morphological Evaluation

It has been recognized that oocytes undergo both nuclear and cytoplasmic maturation. This latter comprises the events that prepare the oocyte for activation, fertilization, and further development. Since these processes can be desynchronized, the simple assessment of nuclear maturity is highly unlikely to indicate the proper developmental competence of oocytes [40]. Therefore, a variety of cytoplasmic/extracytoplasmic factors have also been recently taken into account.

The normal morphology of a metaphase II oocyte consists of a spherical structure enclosed by a uniform zona pellucida and a small perivitelline space containing one single unfragmented polar body. The cytoplasm is translucent and moderately granular, with no inclusions [3] (Fig. 2a). However the majority of oocytes present the so-called one or multiple morphological abnormalities that have been investigated in order to find a correlation with the IVF/ICSI outcomes. Although many papers have been written on this subject, a recent systematic review of the literature found out that none of the features investigated so far was unanimously correlated with normal or compromised development and slight deviations should be considered as expression of phenotypic variance [2, 3]. As a consequence, the Alpha and ESHRE Scientists noted that a prolonged evaluation of gametes doesn't necessarily lead to knowledge improvement. Therefore, the costs vs. benefits of a more detailed morphological classification should be considered in order to avoid an excessive stress to the oocytes [3].

The oocyte shape is of biological interest only if exceptionally large (Fig. 2b). In fact the so-called

giant oocytes may contain additional set of chromosomes and two distinct MS are present [41, 42]. These oocytes should not be inseminated in order to avoid the risk of aneuploidy [3]. As to the ovoidal shape, some authors found a correlation with abnormal cleavage and delay in in vitro parameters [43].

The Zona Pellucida (ZP) is a trilaminar glycoprotein structure that plays a crucial role in the protection of oocytes and embryos and in the fertilization process. Each ZP layer is characterized by different molecular arrangements and exhibits different birefringence patterns: the inner and the outer layers are constituted respectively by radiant and tangentially oriented filaments and appear birefringent if visualized with the light microscopy (Fig. 1). On the contrary, the middle layer has minimal birefringence, probably caused by a random orientation of the filaments [44]. The darkness, thickness, and the retardance of ZP, in particular those of the inner layer, have been suggested by many authors as new markers of oocyte competence [37, 44–46]. However, literature results are still controversial and the Alpha Scientists in conjunction with the ESHRE Special Interest Group of Embryology stated that, to date, there's no clear evidence of the reliability of ZP characteristics, at least thickness measurement, as markers of oocyte quality [2]. However, it was also noted that there could be patient-specific effects and thus exceptional observations should be performed [3].

The perivitelline space (PS) interposes between the ZP and the oolemma and accomodates the polar bodies. The presence of inclusions is considered anomalous but not sufficient to support any negative correlation with IVF/ICSI outcomes; similarly, contradictory results were obtained regarding the effect of large PVS on further developmental potential [2, 3]. However, an exceptionally large PVS may reflect an overmaturity of the ooplasm and a note should be made [3, 47].

The IPB can appear smooth ("normal" morphology), fragmented, degenerated or large. The evaluation of polar body appearance and consequences on further development has been troublesome. For example, early IPB division and disintegration (prior to 20 h after extrusion) may be a sign of disrupted events in meiosis and decreased development and implantation ability [48–52]. However, fragmented IPB has been associated with the time elapsed from denudation and ICSI and correlated to post-ovulatory aging instead of being considered a proper marker of oocyte quality [53, 54]. Similarly, a degenerated IPB may reflect asynchrony between nuclear and cytoplasmic maturation and be the result of overmaturity of the oocytes but its impact on embryo development has not been clearly demonstrated [2, 54]. It has been concluded that only large IPB (Fig. 2c) should be noted; in fact, large IPB may indicate an inability of the MS to migrate correctly at the very periphery of the cell and thus may account for oocyte aneuploidy [3, 39, 55].

The cytoplasmic texture includes different characteristics such as diffuse or centrally located granularity and presence of inclusions. Among these features, nonhomogeneous cytoplasm is of unknown biological significance whereas the presence of translucent smooth endoplasmic reticulum clusters (SER) is troublesome [3] (Fig. 2d). In fact, it seems that SER aggregation is associated with an abnormal calcium surge and pathway and is correlated with lower chances of successful pregnancy, early fetal demise, and certain fetal anomalies [56–58]. Given the severity of impaired outcomes, it has been strongly recommended not to inseminate this type of oocytes [3]. As to the vacuoles, only large ones (>14 μm) seem to be associated with fertilization failure [3].

Unfortunately, it is clear that these evaluations are not sufficient to select between normal appearing oocytes the one with higher developmental potential. Thus far, later morphological analysis, such as pronuclear and cleavage stage embryo assessment, is still essential in routine clinical applications in order to gain reliable information about embryo implantation fate.

Morphological Assessment of Pronuclear Stage Embryos

During the natural fertilization process, a spermatozoon passes the cellular investments, contacts the zona pellucida, undergoes the acrosomal reaction, and attaches to the oolemma for its

subsequent incorporation into the oocyte. A complex intracellular cascade of events, "oocyte activation," is then initiated and comprises the calcium surge, cortical granule release, cytoplasm rearrangement, and the resumption of meiosis. The oocyte completes the second division of meiosis with the split of the chromosomes at the centromeres and the separation of the sister chromatids to the oocyte or a second polar body. In this manner, the egg contributes a haploid number of chromosomes and amount of nuclear DNA. Within a few hours male and female pronuclei are formed: this stage is termed "pronuclear stage." It has been suggested that the morphology of pronuclear stage embryos may be correlated with further development, thus it could be an additional valuable selection criterion for embryo transfer and cryopreservation [31, 59–67].

As to the timing of fertilization assessment, it would be advisable to take into account the different insemination methods used (standard IVF or ICSI): in fact, since ICSI bypasses some time-consuming processes, the ICSI-derived zygotes may be early [68]. Therefore, fertilization check is normally performed 16 h after ICSI and 18 h after standard IVF [3, 69]. The first sign of fertilization is the presence of two pronuclei (PN) in the ooplasm and two polar bodies (PB) extruded in the perivitelline space. Actually, this simple first-line evaluation may be troublesome: in fact, PN appearance can be asynchronous and PBs may have disintegrated before the fertilization check. In these cases, time-lapse recordings should be helpful in the recognition of correctly fertilized oocytes. However, it is likely that better embryos develop from zygotes with shorter intervals between the appearance of the two pronuclei [31].

The PN should be juxtaposed, centrally located, and evenly sized. Male PN appear in the center of the ooplasm while the female PN in the cytoplasmic region near the IIPB. The female PN then moves towards the male PN until the two abut [31]. The lack of correct apposition and localization of the pronuclei in the cytoplasm can be caused by the abnormal function of the sperm-derived centriole and microtubules which are responsible for PN alignment, and this is correlated with poor developmental potential and implantation [60, 62, 66, 70–72]. Therefore, the configuration of widely separated pronuclei is considered severely atypical and zygotes of this kind should not be transferred [3]. Similarly, pronuclear stage embryos with PN of unequal size or fragmented have an increased incidence of chromosomal abnormalities and, if possible, should not be used [3, 72–75]. The failure of PN growth may be the consequence of fertilization with immature sperm cells [76, 77].

Pronuclear orientation relative to the PBs might be an additional feature to relate to embryo development. In fact, the oocyte seems to establish polarity by pronuclear rotation towards the IIPB after fertilization [78]. This alignment is fundamental because the IIPB extrusion site defines the polar axis of the first cleavage division [79]. A misalignment of the PN and PBs has been related to decrease morphologically quality and chromosomal abnormalities of the early embryo [72, 80].

The central point of pronuclear scoring is the evaluation (in terms of number, size, and distribution) of the nucleolar precursor bodies (NPBs) present within the nuclei. NPBs are the precursors of nucleoli, which are the sites of ribosomal gene transcription and are thus essential for protein synthesis. Nucleoli are formed by a dense fibrillar component (DFC) required for rDNA transcription, a fibrillar center (FC), a structural part that acts as storage for inactive transcription factors, and a granular component (GC), which consist in a collection of maturing preribosomes [81, 82]. Nucleoli are first seen in oocytes in antral follicles, where they are completely formed and synthesize rRNA. During the maturation process that leads to ovulation, rRNA synthesis decreases and nucleoli appear small and dissociated, composed only by the FC; they are known as "nucleolar precursor bodies" [83]. At fertilization, the subsequent increase of rRNA synthesis, sees them grow and coalesce to reach their final form at the time of embryonic genome activation [81, 84–87].

A variety of scoring systems have been proposed through the years but the most popular

ones are those used by Tesarik and Scott [59–63]. These grading systems are very similar to each other and differ only in the terminology adopted. In both cases, much attention has been paid to the identification of any asynchrony between the two pronuclei (different NPBs' number and/or size and/or distribution). In fact, asynchronous pronuclear stage embryos have slower development, undergo abnormal cleavage more frequently leading to more fragmentation, and result in lower blastocyst and implantation rates [59, 60, 62–66]. Notwithstanding, the prognostic value of pronuclear classification is still controversial and this is probably due to different experimental designs and terminology used [88, 89]. Recently, Alpha Scientists in collaboration with the ESHRE Special Interest Group in Embryology found a consensus suggesting the allocation of NPBs' patterns into only three categories: symmetrical (equal number/size/distribution of NPBs), asymmetrical (other arrangements, including peripherally located PN), and abnormal (pronuclei with 0 or 1 NPBs) [3].

Another level of pronuclear stage embryo assessment relies on the cytoplasmic appearance. Some authors found a positive correlation between the presence of a cytoplasmic halo and day-3 embryo grading, blastocyst rate and implantation rate [59, 90, 91]. The "halo" effect is described as the microtubule-mediated redistribution of organelles, in particular mitochondria, from the cortex to the perinuclear region, the most metabolically active site of the cell [31, 59, 92]. Moreover, it could be an indicator of the rotational effect of the male pronucleus during PN alignment in order to place the centrosome and complete fertilization and subsequent mitotic division [66]. However, to date there's insufficient evidence of a prognostic value of the halo appearance in embryo selection [3].

The prognostic value of pronuclear score alone is still a matter of debate but it seems to be more informative if used in conjunction with the cleavage stage embryo grading [67, 93–95]. Therefore, the combination of these morphological evaluations may lead to the optimization of the selection of embryos for transfer.

Morphological Assessment of Cleavage Stage Embryos

In most of the cases, pronuclear stage embryos divide mitotically (cleavage) into daughter cells, called "blastomeres," without a discernible increase in overall size. Different morphological criteria for cleavage embryo assessment have been described through the years in the literature and a variety of characteristics have been proposed as indicative of embryo viability: early cleavage, cleavage rate, blastomere size, presence of multinucleation, extent of fragmentation, and distribution of fragments [59, 96–117].

Lately, it has been suggested that the embryo's capacity to reach the blastocyst stage could have an additional prognostic value, and an increase in pregnancy and implantation rates has been reported after fresh or cryopreserved blastocyst transfers [118–122]. However, data are still controversial, since some authors found comparable results after cleavage embryo transfers [93, 123, 124]. Moreover, most embryos fail to develop to the blastocyst stage in extended in vitro culture and it's not possible to know how many of these embryos would have implanted if they had been replaced earlier [93, 125].

One of the most critical factors in the evaluation of cleavage stage embryos is the strict timing for the assessment. In fact, this is the only way for a comparative embryo selection [126]. For standardization, it has been agreed to perform the 2- and 3-day evaluation, respectively, at 44 ± 1 and 68 ± 1 h post-insemination. Syngamy and embryo cleavage assessment should be done, respectively, at 23 ± 1 h post-insemination, and 26 ± 1 h after ICSI or 28 ± 1 h after standard IVF [3]. Early cleavage in two daughter cells has been associated with higher development and pregnancy and implantation rates; therefore, it could be used as a valuable additional embryo selection criterion at the discretion of the laboratory [3, 59, 96, 98–101].

The Alpha and ESHRE Scientists agreed that the expected stage of development for a 2- and 3-day embryo is, respectively, 4 cells and 8 cells [3].

Too slow or too fast cleavage embryos have little developmental potential and present a high degree of chromosomal abnormalities [103–106, 117, 127, 128]. For embryos with 2, 4, and 8 cells, the blastomeres should have equal or very similar size. In fact, embryos with uneven sized blastomeres seem to have lower developmental capacity [104, 105, 107]. This impairment may be due to the unequal distribution of proteins, mRNA, mitochondria, and other organelles between the sister cells as well as the disruption of the polarization of certain proteins and gene products within the oocyte [78, 116]. Furthermore, the genetic analysis conducted on unevenly cleaved embryos revealed a higher degree of multinucleation and chromosomal abnormalities [107]. As to the other stages (5, 6, 7 cells), uneven blastomere size may be simply the effect of asynchronous cleavage rather than abnormal cytoplasmic distribution [3, 69].

A central point in the cleavage embryo assessment is the correct identification and differentiation of blastomeres and fragments. These latter indicators are defined as anucleate membrane-bound cytoplasmic structures <45 μm diameter in day-2 embryos and <40 μm diameter in day-3 embryos [3]. The cause of cell fragmentation is unknown and its impact on embryo development uncertain [69]. Fragmentation is considered "mild" when <10 %, moderate if 10–25 %, and severe when >25 % [3]. Mild and moderate fragmentation has not been associated with impaired IVF/ICSI outcomes [107, 115, 129]. Finally, fragment localization has been proposed as a new marker of embryo quality; however, this pattern is difficult to evaluate since these structures are dynamic and may vary or disappear during culture [3, 116].

Another very important morphological parameter in embryo selection is multinucleation, namely the presence of more than one nucleus (micronuclei included) in a single blastomere [3]. The evaluation of multinucleation should be done on day-2 because of the greater cell size and the better optical accessibility that facilitate the analysis [3]. Multinucleation rates vary greatly in the literature [109, 112, 130]. Culture media composition and improper temperature control have been proposed as possible factors that affect multinucleation rate and the underlying mechanisms include (1) karyokinesis in the absence of cytokinesis; (2) partial fragmentation of the nuclei; and (3) defective chromosome migration at the mitotic anaphase [20, 131, 132]. Multinucleation has been linked to chromosomal abnormality as well as to uneven cleavage and cleavage rate and fragmentation degree [107, 108, 112]. It is thus not a surprise that multinucleated embryos have lower developmental potential and lead to an increased risk of abortion [3].

Other morphological features, such as cytoplasmic texture, have been suggested to be related to the embryonic developmental potential. The embryo cytoplasm is considered normal if pale and clear or finely granular in appearance, although the definitive norm has yet to be established. In fact, some authors found increased cytoplasmic granularity and tiny pits in some day-3 embryos and suggested that these could be early signs of cytoplasmic activity correlated to a better development to the morula and blastocyst stages [106, 133–135]. However, a more recent study failed to find any significant correlation between cytoplasmic texture and fertilization rate, embryo quality, and pregnancy, implantation, and miscarriage rates [136]. To date there are insufficient evidences to support the prognostic value of the blastomere's cytoplasmic appearance and more research is required [3]. Similarly, even if early compaction on day 3 is atypical, it is still of unknown biological significance [3].

A great number of scoring systems that include all the features pointed as markers of embryo viability have been proposed [69, 137–140]. However, at present the lack of standardization (in the nomenclature used as well as the number of characteristics considered and the calculated threshold values) is an obstacle for an easy and univocal interpretation of the different results. Therefore, it has been suggested to use simple categories named "good," "fair," and "poor" as related to embryo quality. A "good" cleavage embryo is characterized by mild fragmentation (<10 %), stage-specific cell size, and absence of multinucleation, whereas a "fair" embryo is defined by the presence of moderate fragmentation

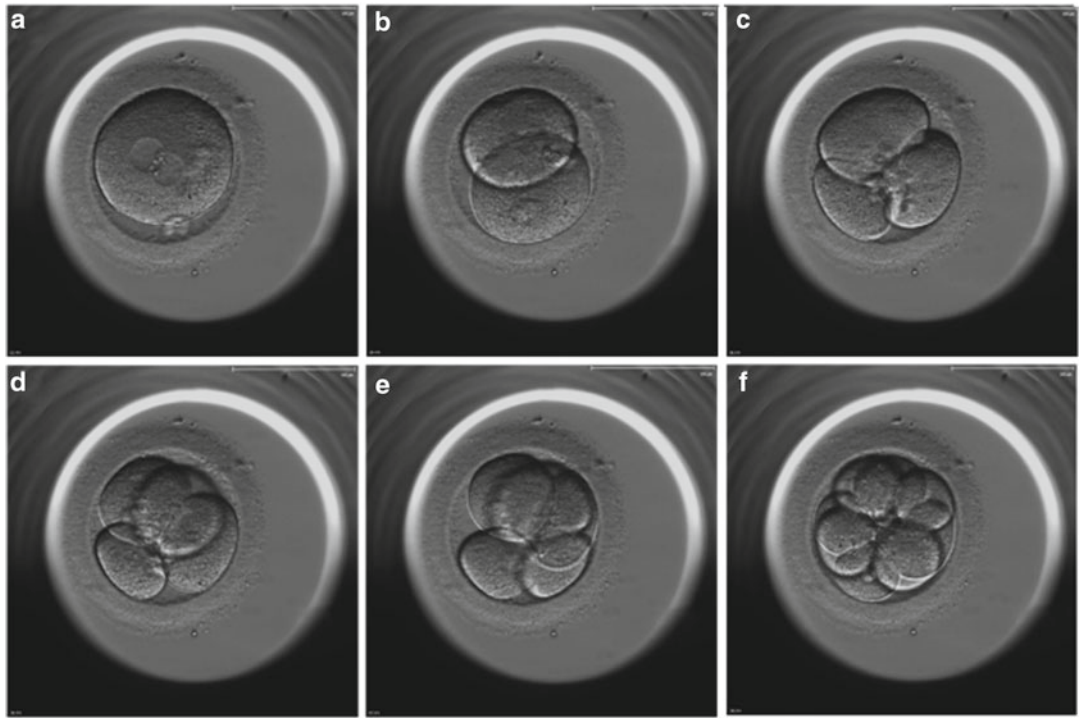

Fig. 3.3 Sequence of events recorded in time-lapse cinematography from 2PN formation (**a**) to 2-cell cleavage stage embryo (**b**), 3-cell cleavage stage embryo (**c**), 4-cell cleavage stage embryo (**d**), 5-cell cleavage stage embryo (**e**), and 8-cell cleavage stage embryo (**f**)

(10–25 %), stage-specific cell size for the majority of blastomeres, and no evidence of multinucleation. Finally, a "poor" embryo presents severe fragmentation (>25 %), cell size not stage-specific, and evidence of multinucleation [3].

One major limit relative to classic embryo selection based on morphology is the static evaluation (that means only few observations at specific time points) regardless of the dynamic nature of the human embryo. Continual monitoring by means of time-lapse cinematography leads to a more complete picture of embryo morphological changes and permits a better correlation of morphokinetics with further development and clinical fate (Fig. 3). Indeed, timing of different embryonic developmental events post-insemination has been proposed as an additional criterion in embryo selection [141–143]. Early disappearance of pronuclei, onset of the first cleavage division, and synchrony in the reappearance of the nuclei after the first cleavage have been shown to be correlated to the embryo cleavage status and pregnancy rate [141]. Moreover, blastocyst rate seems to be affected by the duration of the first cleavage division and the time required for the division of 2-cell and 3-cell embryos, respectively [142]. A more recent study revealed that an optimal time range (time window) exists for every early cell division, supporting the hypothesis that viable embryos undergo tightly regulated cellular events [143]. In particular, the time required by a 4-cell embryo to divide into 5 cells (t5), the duration of the period as a 3-cell embryo (s2), and the duration of the second cell cycle (cc2) are strong predictors of the further implantation fate and may be combined with the morphology score in the identification of the embryos with the higher developmental potential [143].

Conclusions

Morphological assessment of gametes and embryos is still the key-point in the everyday work of an IVF laboratory. In fact, it is simple, noninvasive, and cost-effective. The combination

of different morphological criteria, from the oocyte to the pronuclear stage and the cleavage embryo, has proven to be predictive for embryo developmental potential, even if the overall efficacy of ART is still low, considering that most of the embryos produced fail to implant. The improving knowledge about gametes and embryo physiology would allow the identification of novel markers of embryo quality to be used as additional selection criteria. The recent "-omics" technology (metabolomics, trascriptomics, and proteomics) is promising in widening the horizon of human-assisted reproduction and will probably be an additional tool for improvement. Furthermore, preimplantation genetic screening may help in the determination of embryonic "health" through the screening of the genetic constitution of the embryo. Unfortunately, even if their promise is powerful, they are still far from routine introduction in the IVF clinic and further investigation is needed to ensure the reliability and sensitivities of these methods.

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