Review Article

In vitro Human Embryo Morphology - An Odissey Outside the Oviduct

Hurtado R[1,2], de Lima Bossi R[2], Valle M[2], Sampaio M[2] and Geber S[1,2]*

[1]Department of Obstetrics and Gynaecology of the Medical School, Universidade Federal de Minas Gerais, Brazil

[2]ORIGEN, Center for Reproductive Medicine, Brazil

*Corresponding author: Selmo Geber, Department of Obstetrics and Gynaecology of the Medical School, Universidade Federal de Minas Gerais, Av Contorno 7747 Belo Horizonte, Minas Gerais, Brazil

Abstract

The authors describe the human embryo development *in vitro*, during the preimplantation stages, i.e., from the zygote until the blastocyst stage. They also describe the methods to select the most suitable embryo for transfer in cycles of *In vitro* fertilization treatment, performed by infertile patients, in order to increase implantation and pregnancy rates.

Introduction

Couples with the inability to conceive after one year of unprotected intercourse turn to infertility treatment and, in many cases, Assisted Reproductive Technologies (ART)/ *In vitro* Fertilization (IVF), a technique responsible for the birth of more than 8 million babies worldwide since 1978. Embryo early development and morphology hold important information gathered from basic science that can be extensively applied to ART and, therefore, optimize the outcomes.

The lack of treatment options for improving the quality of sperm and eggs is addressed by increasing gamete quantity. For women, multiple follicular developmet, to increase oocyte number, is induced by gonadotropins in a single menstrual cycle. Moreover, in order to increase pregnancy rates, more than one embryo is transferred for the uterus. Therefore, IVF might increase the rates of multiple pregnancies and premature delivery with consequences on public health [1]. There is no debate that a better embryo selection could downsize the number of transfered embryos per cycle, and therefore decrease the incidence of multiple pregnancies [2].

Even after decades of upcoming technologies for better evaluation of embryos, traditional morphology assessment through a binocular microscope is still the first-line method for embryo selection to transfer in IVF cycles, for none of the more recent technologies have been proven superior [3-5]. On the other hand, morphological assessment does not detect chromosomal abnormalities or defects in critical cellular processes and metabolism that could impact the viability of an embryo. For these purposes, the methods being used to evaluate chromosomal abnormalities of embryos are Pre-Implantation Genetic Testing (PGT) [6] after embryo biopsy or using a non-invasive technology; and for embryonic metabolism, technologies collectively called 'omics' which may include genomic, proteomic, transcriptomic and metabolomic profiling of the embryos [7]. Chromosomal analysis has been heavily criticized since its early years using Fuorescence *In-Situ* Hybridization (FISH) all the way down to modern PGT-A because it is very well documented that embryos are compartmentalized. This means that the DNA of an abnormal cell within an embryo could be present in one part of the

embryo and absent in the others [8]. Also, there is a possibility of an auto correction during development [9].

Prospective randomized trials, which would be the most reliable types of studies for generating evidence-based guidelines, are somewhat difficult to perform considering human embryos. This is the case for reasons highlighted by Matchinger in 2013: a) there is no way to establish a direct correlation between specific embryos transfered and viable ongoing pregnancies observed as result; b) Single-embryo transfers are still not widely used across fertility clinics; c) embryo grading criteria and patient selection may vary significantly from clinic to clinic and from study to study. The result for this situation is that most data concerning embryo assessment derives from retrospective and/or small-sampled studies. In recent years, several international scientific forums have been dedicated to standardize embryo quality assessments in order to promote better embryo selection, producing comparable results for different types of studies and, therefore more reliable guidelines.
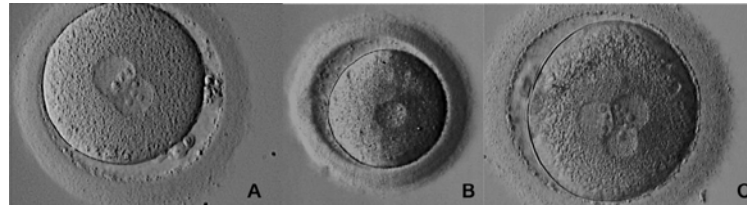
Morphological Parameters in Embryo Assessment

Currently, embryo selection is based on embryo morphology and the rate of embryo development in culture. This method is subjective because evaluations are based on the number of blastomeres in the embryo, symmetry of the blastomeres and degree of fragmentation. Also, human embryonic development follows a specific sequence of events where morphological characteristics are defined at determined points in time. In result, embryo evaluation can be carried out either by sequential assessments or a 'cumulative' one-time approach [10].

More recently a new emerging thechnology is being tested in order to evaluate embryo developement and improve selection and implantation. Although the use of time-lapse microscopic photography associated to Artificial Intelingence is increasing and gaining more attention there is no data suggesting an outvome improvement for ART.
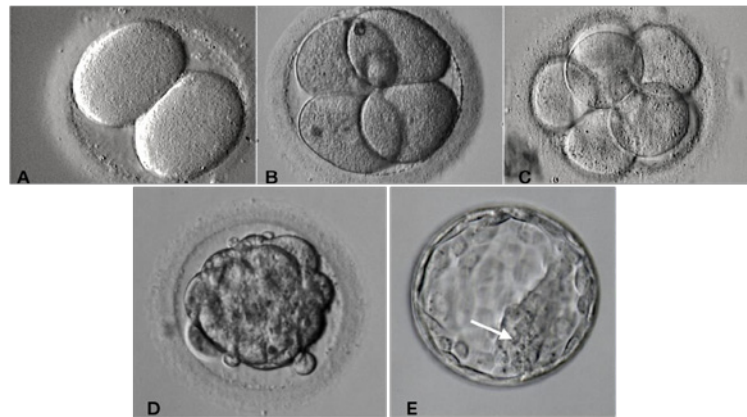
Zona Pellucida

Zona pellucia is a non-cellular glycoprotein layer surrounding

Figure 1: Fertilization and human Pro-nucleus formation.
A: Oocyte with normal fertilization exhibiting two pronuclei, in ooplasm, with nuclear precursor bodies aligned and two polar bodies in perivitelline space; **B:** Oocyte with abnormal fertilization, exhibiting one pro-nucleus in ooplasm; **C:** Oocyte with abnormal fertilization exhibiting three pronuclei in ooplasm.

Figure 2: Human embryo development from Day 2 to Day 5.
A,B,C: During embryo development from zygote through cleavage stage, mitotic divisions occur leading a rapid increase in cell number, from day 1 until day 3, without volume change. Embryo cells in that stage are called blastomeres. (A) the embryo have two blastomeres, (B) four blastomeres, (C) eight blastomeres. On day four, cell compaction leads to morula formation (D). Day five, two different cell types are present: outside the embryo the trofophectoderm and inside the embryo the inner cell mass cells (arrow).

the plasma membrane of oocytes and embryos, secreted during oogenesis. It has been speculated that both the thickness and the diameter of the Zona Pellucida in hatching embryos could be used as markers for embryo quality. It is known that it is easier for *in-vivo*-produced embryos to hatch in comparison to *in-vitro*-produced embryos. These embryos present their zona pellucida with smaller diameter and thickness. It is thought that this is due to a different molecular composition of the zona as a consequence to the exposure to the oviducts fluids, resulting in a more rigid structure that is more prone to crack [11].

Perivitelline space

Perivitelline space is located between the zona pellucida and the plasma membrane. Aside from some very scarce information concerning size and contents in *in-vivo* and *in-vitro* produced embryos, researchers have been paying very little attetion to the periviteline space [12] As pointed out by [13] , this space provides information related to culture conditions and therefore can suggest embryo quality. A reduction in periviteline space could translate as swelling of blastomeres, a marker for poor embryo quality directly related to less cell compaction at the morula stage [14].

Pro-nucleus

The first assessment of an embryo should be at the zygote stage for the appearance of two pronuclei (the first sign of successful fertilization). The Pronuclear scoring systems usually assess the number and relative position of the Nucleolar Precursor Bodies

(NPB), pronuclear size and aligment (similar size with central location) and appearance of the cytoplasm [15,16] (Figure 1). These features proved to have a predictive relation to blastocyst formation with increased implantation potential [17,18]. These studies were later challenged by robust data showing lack of consistency for pronuclear scoring systems as valid embryo selection criteria [19] (Table 1).
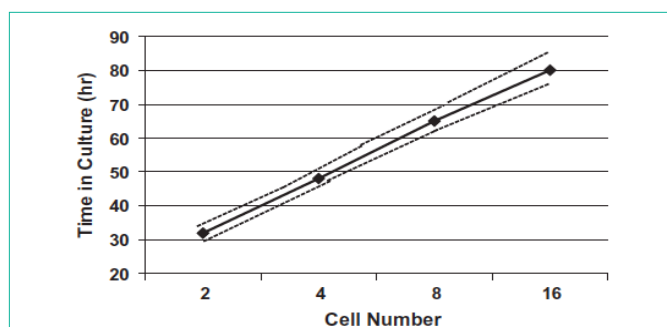
Embryo

The embryo is considered after pronuclei singamia and further successive cell divisions. Morphological scoring has been used to predict the highest implantion potential for embryos since the beginning of IVF [20]. Good quality parameters include the number and symmetry of blastomeres, the absence of multinucleation, early cleavage to the two-cell stage, and a low percentage of cell fragments in embryos. Some other factors found to increase pregnancy and implantation rates include the bastocoelic cavity expansion state and the cohesiveness and number of the inner cell mass and trophectodermal cells [21] (Figure 2).

Cleavage State

Several international forums have published cleavage-stage scoring systems to determine embryo viability such as the on from the Society for Assisted Reproductive Technology (SART) [22]. Another good example is the Istanbul international consensus panel gathered to come up with an embryo assessment score [23]. The following features are repeatedly present in scoring systems throughout history: the rate of division, symmetry of the blastomeres, multi-nucleation,

Table 1: Fertilization stage grading system (adapted from the Istanbul Consensus, 2011).

Grade	Rating	Description
1	Symmetrical	Equivalentto Z1and Z2
2	Non-symmetrical	Other arrangements, including peripherally sited pronuclei
3	Abnormal	Pronuclei with 0 or 1 NPB

Figure 3: Expected cell development for human embryos; dashed lines indicate 95% confidence limits (Edwards et al., 1981).

Figure 4: Different degrees of fragmentation in a cleavage stage human embryo.
During embryo mitotic cell divisions fragmentation may occur varying in position, degree and size among embryo blastomeres. A: Embryo with no fragmentation. B: Embryo with less than 10% of fragmentation; C: Embryo with 20-25% of fragmentation; D: Embryo with more than 30% of fragmentation.

and the degree of fragmentation. Cytoplasmic colour, the number of cells, and compaction are also frequently observed features.
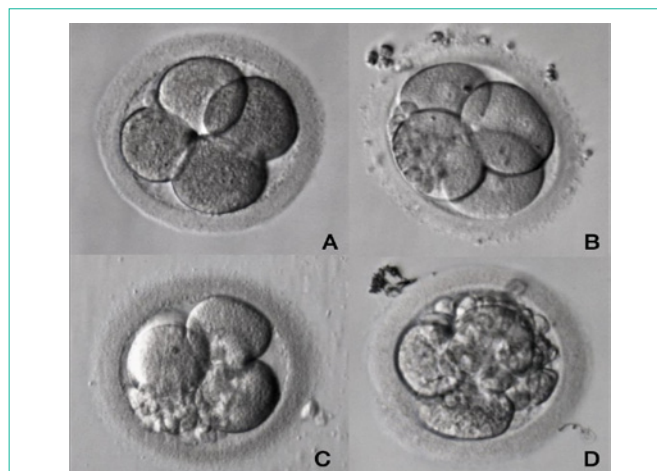
Colour of the Embryo

Cytoplasmic transluceny of human embryos make it fairly easy to observe important subcellular structures such as the nuclei and the nucleoli, which may be used for pronuclear scoring. In opposition, domestic animals such as pigs, cats and dogs' embryos have a large number of lipid droplets in the cytoplasm making it quite dark and opaque. Increased lipid content determines lower cryotolerance for embryos which translates as a lower score for embryo quality and transfer selection. A presence of a halo in the blastomeres and concentration of ooplasm in the central part may indicate embryos with a worse prognosis and more susceptible to degeneration. Vacuoles of different sizes and locations may appear in the ooplasm. It is believed that large quantities and large sizes can be harmful to embryo development [24-26].

Cell number

Cell number is still believed to be the single most important indicator for embryo viability [27]. Good quality human embryos evolve from the 1-cell stage to the 16-cell stage along a distinct timeline described by [20] (Figure 3). Embryos that go through cell divisions both ahead or behind the expected time frames are associated with compromised quality [26,28,29], although it has been shown that rapidly cleaving embryos are superior to the late-cleaving, in terms of morula and blastocyst formation [14]. It is also known now that embryos who start the first division between 24-27h are more likely to turn into ongoing pregnancies [30]. Several authors have correlated the number of cells (6 to 8) on day-3 embryos to higher blastocyst

formation [31], as well as higher implantation rates [32].

Fragmentation

The presence of fragmentation (anuclear membrane-bound extracellular cytoplasmic structure) has been related to abnormalities in cell metabolism that may result in apoptosis [33,34], anomalies in chromosomal segregation [35,36], and/or abnormalities of the oocyte membrane [37]. Moreover, the presence of fragments can prevent cell-cell interactions, making it difficult to compact, develop blastocele and differentiate in trophectoderm and Inner Cell Mass. There is also a possibility of releasing toxic substances that would compromise embryo development. The simplest scoring system for fragmentation indicates the percentage of the volume of the embryo occupied by fragments (e.g. score 0 = 0%; score 1 = <10%; score 2 = 10–25%; score 3 = >25%) which is negatively correlated with embryo developmental potential and implantation rate (Figure 4). When the embryo starts cleavage, the rate of division, symmetry of the blastomeres, multi-nucleation and the degree of fragmentation (Table 2) are assessed by the morphological criteria and scored.

Simmetry

Size and shape of the blastomeres during cleavage may be asymmetrical. This asynchrony in cell division may result from an uneven distribution of organelles, protein and RNA, between sister cells [38]. Embryos with significant asymmetry have been shown to have lower implantation rates [28,39] but not as clearly as compared with the number of cells or fragmentation (Figure 5).

Table 2: Cleavage stage grading system (adapted from the Istanbul Consensus, 2011).

Grade	Rating	Description
1	Good	<10% fragmentation, stage specific cell size, no multi-nucleation
2	Fair	Up to 25% fragmentation, stage specfic cellsize for majority of cells, no evidence of multi-nucleation
3	Poor	Severe fragmentation (>25%), cell size not stage specfic, evidence of multi-nucleation

Figure 5: Abnormal human embryo development.
A: Three-cell embryo exhibiting an irregularity in cells size B: Four-cell embryo with vacuoles inside the blastomeres; C: Five-cell embryo exhibiting blastomeres with multi nucleation (arrow).

Multinucleation

It has been shown that embryos with more than one nucleus in each blastomere are associated with development arrestment, lower implantantion and lower pregnancy rates due to an increased incidence of chromosomal abnormalities [40-42]. In some cases, however, it might be a transient cell stage self-corrected by the embryo during next cell divisions (Figure 5).

Compaction

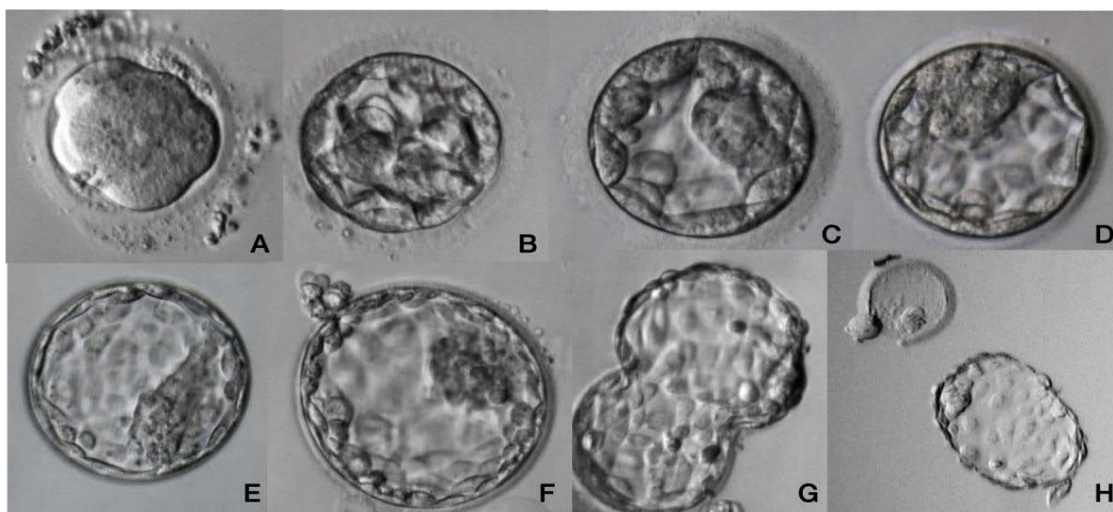Compaction is accompanied by polarization of the embryo's cells.

Nearby cells exchange gap junctions for tight junctions and begin to cluster as a result of activating the embryonic genome. A compaction on day 3 of development, in the 8-cell embryo, appears to be indicative of a good prognosis. A loss of maximal compaction, usually detected as an increase of up to 10% in the cell mass area, together with a shorter time period for blastulation are features closely related to aberrant allocation of cells to the inner cell mass and trophectoderm. This phenomenon is secondary to decreased expression of transcripts which are involved in the construction of tight junctions. Moreover, lack of compaction compromises cryosurvival of the subsequently formed blastocysts [43-48].

Blastocysts

Blastocyst stage embryos have two cell types, the Inne Cell Mass cells and the Trophectoderm, in addition to having a cavity called blastocoele. The cells of the ICM form the three embryonic leaflets endoderm, mesoderm and ectoderm and the cells of the trophectoderm will originate the extra-amniotic membranes and the placenta. As the blastocele fills with liquid, the blastocyst increases in size and at its maximum expansion it manages to leave the pellucid zone. The increase in the blastocele occurs due to the active increase in the concentration of salts within the embryo, which cause the liquid to penetrate into the cavity by osmosis. Blastocysts are classified according to their degree of expansion, quality of the trophoblast and quality of the MCI (Figure 6).

Morphological evaluation of blastocysts was first studied by Dokras in 1991 and 1993 but this initial assessment had no proven clinical utility. Later, a more practical score system was proposed by Gardner and Schoolcraft in 1999 and 2000 taking into consideration a specific morphological feature: the extent to which the volume of the embryo is occupied by the blastocoele (Table 3). It was noticed that the number and organization of cells in the inner cell mass and trophectoderm were also important.

More recently both the Society for Assisted Reproductive

Figure 6: Human blastocyst development.
Embryo compaction initiates on late Day 3 until Day 4 and culminates in morula formation (A). On day 5, cell differentiation occurs and an initial cavity – blastocoele - is observed (B). Blastocoele is fulfilled with water by osmosis meanwhile trophectoderm and inner cell mass are growing fast (C, D, E). When blastocyst is fully expanded some cells start the hatching process, to escape from zona pellucida (F, G). Blastocyst is ready for implantation when he was completely hatched from zona pellucida (H).

Table 3: Blastocyst grading system (Adapted from: Gardner and Schoolcraft, 1999).

Blastocyst stage	Grade	Characteristics
Early blastocyst	1	The blastocoele is less than half the volume of the embryo
Blastocyst	2	The blastocoele is greater than or equal to half of the volume of the embryo
Full blastocyst	3	The blastocoele completely fills the embryo
Expanded Blastocyst	4	The blastocoele volume is larger than that of the early embryo and the zona pellucida is thinning
Hatching blastocyst	5	The trophectoderm has started to herniate through zona pellucida
Hatched blastocyst	6	The blastocyst has completely escaped from the zona pellucida
Inner cell mass	A	Tightly packed, many cells
	B	Loosely grouped, several cells
	C	Very few cells
Trophectoderm	A	Many cells forming a tightly knit epithelium
	B	Few cells
	C	Very few cells forming a loose epithelium

Table 4: Blastocyst stage scoring system (adapted from the Istanbul Consensus, 2011).

Grade	Rating	Description
Stage of Development		
1		Early Blastocyst
2		Expanded
3		Hatched/Hatching
Inner Cell Mass		
1	Good	Prominent, easily discernible, with many cells that are compacted and tightly adhered
2	Fair	Easily discernible, with many cells loosely grouped together
3	Poor	Difficult to discern with few cells
Trophectoderm		
1	Good	Many cell forming a cohesive epithelium
2	Fair	Few cells forming a loose epithelium
3	Poor	Very few cells

Technology (SART) [22] and the Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011 developed standardized grading systems which included a more detailed scheme (Table 4).

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