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Assessment of preimplantation embryo development in vitro to determine viability

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Course in Reproductive Medicine 1995

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1. Introduction:

As in vitro fertilisation (IVF) becomes popular and expands to a routine procedure throughout the world, we tend to ignore the delicacy of the oocytes and embryos we are dealing with. Embryos are subject to many stress-factors that may be detrimental to their viability (Byatt-Smith 1991,Rieger 1992). It is important to understand that current technology is perhaps at the lower limit of effectiveness, and slight aberrations could jeopardize the normal growth of embryos in vitro. An embryo must feel in a IVF program just like a baby that you leave alone in the wilderness. After IVF only 10% of embryos are able to implant after transfer in utero (Steptoe 1986,Amso 1993) and it would be very helpful to know in advance which embryo is successful.

In the 1st part of this essay I will shortly discuss culture media of embryos. The question will be: How could culture technique be improved, so that the embryo would finally feel as comfortable as in utero? In the 2nd part of this essay we take a look at assessing embryos. Facing the lower limits of todays technology, the IVF practitioner wants to know, which embryo will finally implant and provide the parents with a baby.

2. Culture of preimplantation embryos

Human embryos can be cultured in vitro. It is absolutely fascinating, that the human embryo can develope from the 1st-cell to the blastocyst in vitro (Edwards 1970, Steptoe 1971). Unfortunately I never witnessed a preimplantation embryo growing in vitro, nevertheless I'm convinced that this very delicate procedure of culturing embryos should be done in an absolutely cautious manner. I'm tempted to raise the question, "if the stressful and hektic conditons in the IVF lab in Geneva, really help to increase the productivity?". Most gynaecologists are more accustomed to microsurgical techniques than to IVF procedures. Therefore it makes sense to underline, that both techniques follow the same basic principles. In both methods you can't emphasise enough how much attention has to be paid to details. The small droplets of media mentioned above must consist of detailed compositions in order to resemble the conditions found within the body. They are balanced with bicarbonate buffers against 5% CO_2, as in exhaled breath, to provide a correct pH and a buffering system (Edwards 1995). Examples of current culture media is shown in table 1.

Table 1: Culture medium for human preimplantation embryos (Bavister 1995)

m-Earle's BSS
Minimal essential medium (MEM)
Human tubal fluid (HTF)
Ham's F10 (1st medium to reach blastocysts in human)
Ham's F12

Most of the above mentioned culture media consit of a long list of different ingredients where each single one may be very important. A simple media consists of salt, energy sources (pyruvate, glucose, lactate), buffer and water. Complex media contain additionaly vitamins, fatty acids, amino acids and other substrates. Water is a good example to show, that even for a simple component, highest quality is required. It needs distillation and deionization. But millipore membranes can add emulsifiers to water and negativly affect IVF. If water is so difficult to obtain, what's about the other components? The very important question that hasn't yet been answered up till today is, which component, in which concentration, for which embryo-stage, is absolutely essential or ideal and which one is harmful? Unfortunately good research in this regard, leading to an answer, is lacking because of the following reasons: 1. Human embryos are difficult to obtain solely for research purpose. 2. In certain countries e.g. in switzerland, experiments with human embryos are not permitted. 3. Althought the implantation rate per single embryo is only 10% (see above), multiple transfers and multiple cycles lead to a cumulative success-rate as high as in normal fertile couples (Plachot 1992). If the success rate in IVF-programms can be as good as in normal fertile couples, then the queastion rises,"why should additional research be necessary?"

A common and simplistic approach to improve the quality of a media is the addition of blood serum on the assumption, that this mixture must contain all the needed components for the embryo. On the other hand this mixture can also consist of toxic substances for the embryo and therefore do more harm than good. To avoid the risks by adding serum to culture mediums, some IVF clinics use serum albumin instead. Therefore many components of serum that could be harmful are eliminated (Bavister 1995).

With the introduction of Co-cultures there was hope for further improvement. Various cell types were used to imitate the in vivo enviroment and to support the growth of human embryos. The use of embryonic tissue (trophoblast) to help the embryo through an autocrine effect. The use of female genital tract cells, to assist the embryo through a paracrine effect (Menezo YJ et al,1993). Unfortunately the hopes couldn't be fullfilled. Co-cultures provide no benefit for patients that are performing their initial treatment cycles in IVF (Sakkas 1994). The implimantation of Co-cultures systems implies similar problems as there are with serum components. Very little is known about the functions and the processes in a Co-Culture system, and they present for us rather black boxes than scientific achievements.

What are other possibilities to improve culture media?
An embryo is usually pushed and pulled down the fallopian tube by orchestrated cilia movements and tube peristaltic. These movements may help and improve the free exchange of metabolites between the enviroment and the embryo (Byatt-Smith 1991). Static cultures should may be changed into mobile cultures?

> In Vitro embryos are feasable to many stress-factors and their maternal mRNA is perhaps exhausted before the embryonic mRNA production starts. Could the injection of maternal mRNA by micromanipulation improve their survival?

Most students attending this course in Reproductive Medicine do come from developing countries. For a long time they received developing aid according to the ideas of the western world. But if you ask them, how to find out what people in their country really need, they will answer you: You must ask the people! Ham in 1982 understood, that this very same principle can be adapted to embryonic cells.

> A major key to progress is to forget what we think we know about cellular growth requierements and instead ask the cells we are studying to tell us at every step exactly what condition they consider to be optimal. The cells know far better than we do what their actual requirements are, and to the extent we impose our wills on them instead of listening to their needs, they will resist our efforts (Ham,1982)

The concept back to basics will probably give us more information about the real needs of embryos. Research is needed in culture media that do not rely on somatic cell contributions and that are chemically defined in order to avoid the introduction of foreign biological agents into the culture environment.

3. Methods of Assessing Embryo Viability:

Only a low percentage of embryos do lead to pregnancy, most embryos will die. On the other side, most mothers can by IVF finally achieve a pregnancy. The success-rate per single embryo is much lower than the success-rate per single mother. This facts lead me to the hypothesis, that the outcome is more determined by embryonic factors and less dependent from maternal parameters. This hypothesis is supported by the discovery of ped genes in the embryo, that control the cleavage rate independent from the uterine environment (Sakkas lecture).

The further a pregnancy progresses, the more accurate is the embryo assessment. In the extrem case, that's the golden standard, a live born baby is delivered and its viability will be known by 100% accuracy. The earlier an assessment is done, the less reliable its results tend to be. That explains the trend in IVF programms towards later uterine transfer in reason to get a more accurate embryo assessment. The blastocyst gave the best implantation rate per transfer (59%), when compared with morula (25%) or to early stages (12%)(Buster 1985).

Table 2 gives an overview about current methods in assessing preimplantation embryos. Methods such as staining blastomere nuclei (Hardy 1989) or electron microscopy (Motta 1988) can give us information about the viability of an embryo but at the same time destroying it. Therefore these methods are not applicable in

IVF programms. A further drawback is that because of a destroyed embryo these results can't be correlated to the true viability leading to a pregnancy (golden standard).

Table 2: Assessment of embryo viability
(modified from Plachot M(1992)

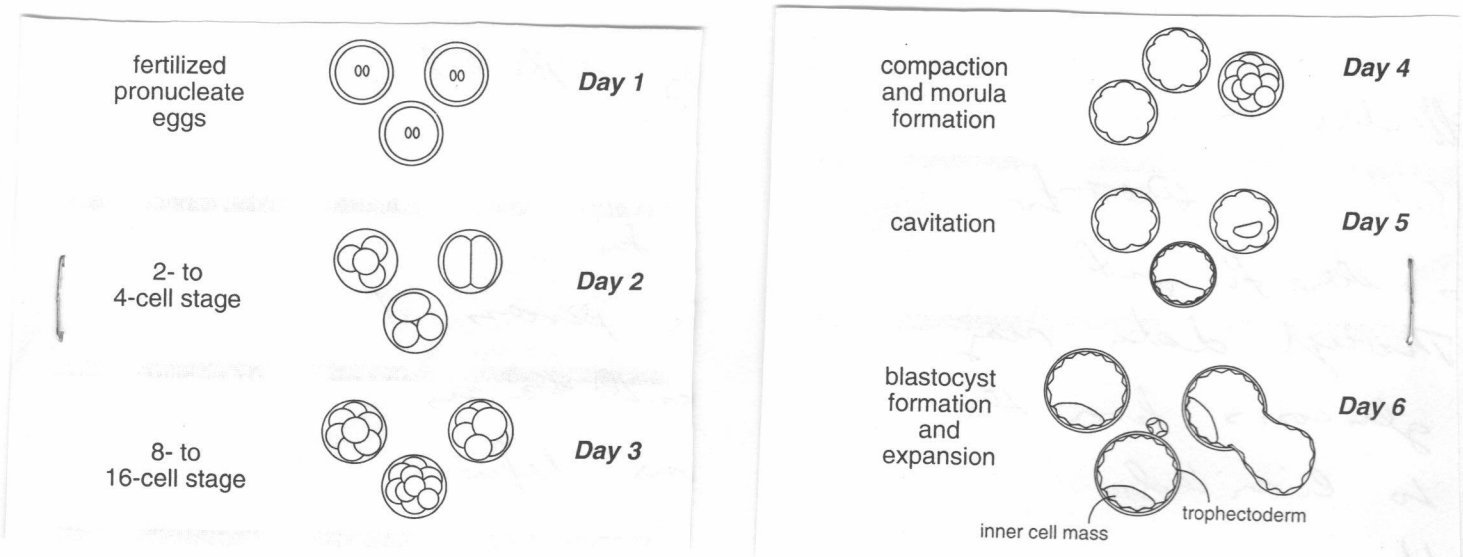
Non-invasive assessment of embryo viability		
	a) Physical methods	1)Cleavage rate
		2)Morphology
	b) Biochemical methods	1)βHCG production
		2)Platelet-activating factor
		3)Suppressor factors
		4)Oxygen consumption
		5)Glucose uptake
		6)Pyruvate uptake
Invasive assessment of embryo viability:		
	a)Embryo biopsy	
	b)Detection of mutations	
	c)Detection of chromosome abnormality	

The scope of this essay are the non-invasive assessments. A clinic usually has to compromise from the above mentioned methods and has to choose one. The embryos are usually assessed once daily with carefully maintaining the gas phase, temperature, osmotic pressure and sterility.

3a) Physical methods of scoring embryo viability

Around 32 hours after insemination, 98% of the fertilised eggs divide, leading to 2-cell embryos, followed about 14 hours later by a second division producing 4-cell embryos (Plachot 1990).

Figure 1: Preimplantation stages of human embryo development



As many clinics do intrauterine transfer on day 2 or 3, they wish for a quality control of these early embryos. Physical methods consist of a examination by stereomicroscope for a regular and well-timed cleavage and for morphologic scoring. The following question may be asked, with the answer below:

Is a "pretty" embryo
a viable embryo?
Payne D(1994)

The morphologic scoring-system (Plachot 1990) is widly used and ranges from grade 1 to grade 4. Grade 1 embryos are equally-sized blastomeres with no fragments (Fig. 2). Grade 2 display some fragments in the perivitellin space. Grade 3 are unequally-sized blastomeres and grade 4 look degenerated and fragmented (Fig. 3). Grade 1 - 3 do not discriminate actual quality because they all lead to similar implantation rates between 8.5% and 11.5%. Whereas no pregnancies occur with embryos grade 4.

Other morphological signs with good prognosis are adhesions between blastomeres and a minimal zona thickness (Cohen J 1989).

Figure 2:
A morphologically normal 4-cell embryo of Group 1 42h after insemination

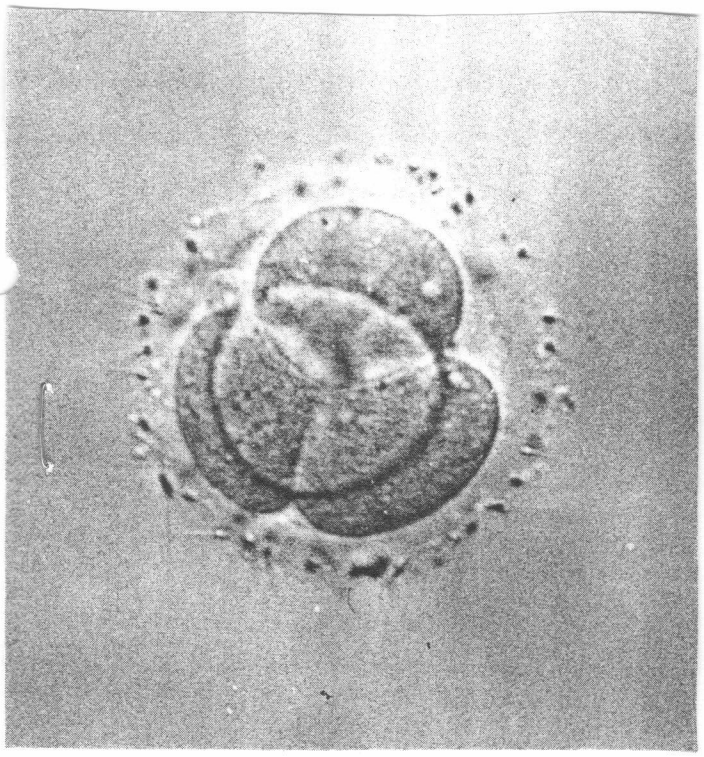
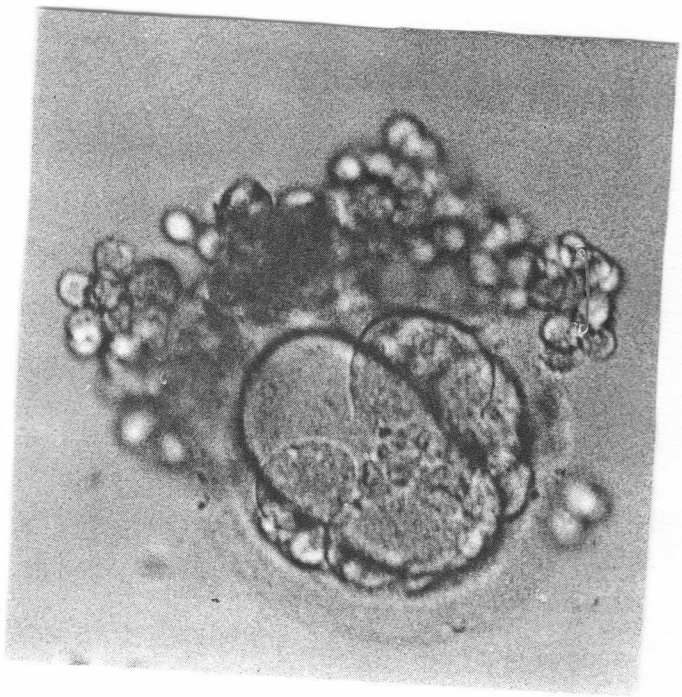


Figure 3:
An irregularly cleaved embryo of Group 4 with fragments in the peri-vitelline space 42h after insemination



Morphological methods are not free of artefacts. An embryo assessed exactly during cleavage can have a very irregular shape and therefore be classified as grade 4, while 30min later, after

completed cleavage, it may look perfectly normal.

Further parameters for embryo viability are cleavage arrest and cleavage delay. Cleavage arrest means no further viability. The significance for viability of cleavage delay is not that clear.

Cleavage arrest is common in 2-cell mouse embryos growing in vitro, while in human embryos development arrest is more common in late stages. The same time-gap is seen in genome activation, that is at the 2-cell stage in mice and at later stages in human embryos (Sakkas lecture). Genome activation is a complex process switching from maternal mRNA to embryogenic mRNA and probably in causative correlation to cleavage arrest. The non-viable embryo can not cope with this additional stress and goes into arrest.

The cleavage rate in rats is a good predictor for viability (McKiernan 1994) but not in human embryos. The capacity of 2-8 cell human embryos to implant is independant of their cleavage rate (Plachot M 1992,Testart 1986) and is therefore not a reliable index of viability. In IVF programms the embryos are usually inspected once every 24h. This leads to a further decrease in accuracy because it's not known if the last cleavage just happened 15min ago or 10h ago. This supports again the postponing of intrauterine transfer to a later stage in order to select the "strongest" embryos. But on the other hand, embryos long exposed to non-ideal culture environment are subject to further loss of viability and the discrepancy between fetal and maternal window increases as well.

Efforts to improve the reliability of the scoring-system led to a combination of the morphological features and the cleavage rate. Obviously this combined scoring-system (table 3) won't offer a much better index of viability, because the cleavage rate is a parameter with little additional value.

Table 3: Combined Score (Puissant, 1987)

No of blastomeres after 48h	<4	0 pts
	≥4	2 pts
size of blastomeres	typical	4 pts
	atypical	2 pts
fragments	10%	4 pts
(% of the embryo volume)	20-50%	2 pts
	>50%	0 pts
Total		2-10 pts

The assessment of the different scoring systems by different authors leads to the conclusion:

Embryo viability correlates with embryo-morphology and cleavage rates, althougt the relationship is not absolute.

It was hoped, that this relationship becomes more accurate if the embryos are scored at a later stage e.g. as blastocysts. In the mouse the transition to a blastocyst is called compaction and happens within hours by smoothing the outline of the blastomeres. This begins with the 8-cell embryo generating average of 9 "outside" and 7 "inside" cells (Winston 1991). The trophectodermal cells form into epithelial cells and start pumping fluid into the intracellular space. By this mechanism the 32-cell embryo will develop into a Blastocyst.

The practical assessment under the light microscope includes morphological differentiation, regular expansion of blastocele, presence/fusion/collaps of vacuoles, degeneration. These parameters were incorporated into a new scoring system especially designed for blastocysts grading from 1 - 3 (Dokras 1993). While blastocyst grade 3 failed to grow normally, there was no significant difference between grade 1 and 2.

In the future computarized scanning could enhance the assessment quality. Semi-computerised systems are on trial. Their quality will improve enormously in the next few years but today they can not detect subtile defects and are therefor of limited use in practice.

Unfortunatly there is no possibility to predict the viability of a "normal" looking embryo by physical methods alone. The light microscope can't detect metabolic pathologies or molecular changes. Biochemical methods are needed for an accurate measurement of these parameters. This unvisible parameters will provide more detailed information and therefore be increasingly important in the future.

3b) Biochemical methods of scoring Human embryos:

The metabolic conditions of an embryo are continously changed and adaption to new environmental and intracellular conditions are necessary. A few examples are the change from tubal environment to endometrial environment, maternal mRNA to embryogenic mRNA, preimplantation to postimplantation energy supply and many other unknown conditions. In the past it was thought, that glucose is an essential nutrients for all embryonic stages. But in early stages, energy supply is provided by pyrovate, lactate and glutamin and only after the 16-cell stage it swops to glycolysis. The idea that an embryo goes during life through the whole process of evolution may be an explanation why pyruvate utilisation as a more primitive energy supply comes in the embryogenesis before glycolysis (hypothesis). From the 16-cell stage the metabolic activity begins to rise sharply. This may be explained by increased numbers of cleaving cells and by a multiplied proteinsynthesis.

The metabolic intake or output is mainly controlled by the embryo. Sensitive microassays are required to measure these metabolic changes. 1-3 ml capacity cuvettes used in conventional

methods have been replaced by small droplets medium of 10nl-10µl in volume. The sensitivity of a test is also dependent on the relative decrease of nutrients in the media. Excessive pyruvate in media leads to only small differences, while a low pyruvate concentration gives higher concentration differences and therefore more sensitive results. The measurement of a nutrient e.g. pyruvate acid is linked to an oxidation of NADH in the following reaction:

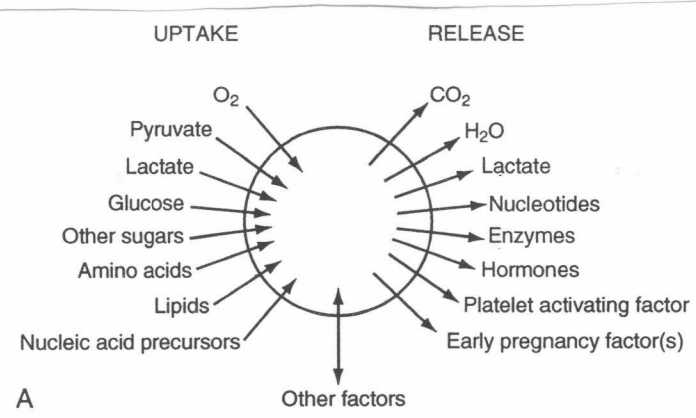
$$\text{pyruvate} + \text{NADH}^{*} + \text{H}^{+} \leftrightarrow \text{lactate} + \text{NAD}^{+}$$

* fluoresces by light at 340nm

If NADH fluorescence is high, then pyruvate concentration is also high and vice versa (Leese 1987).

A model of uptake and release of nutrients and other compounds from human embryos is shown in the model by below.

Figure 4: Model of uptake and release of nutrients and other compounds from human embryos in vitro (Leese 1987)



β-HCG production:

Known by every gynaecologist is the test for pregnancy with an βHCG assay. Although βHCG mRNA appears as early as the 6- to 8-cell stage, no secretion was identified before day 7 (Fishel et al,1985). Consequently this test is not useful to select embryos at day 2 or 3 after insemination, the usual time for embryo transfer. But early pregnancy factors can serve as markers in assessing embryo viability at later stages in cases complicated with vaginal bleeding during early pregnancy in order to differentiate threatened or missed abortion (Shahani SK et al,1994). In research βHCG-levels are measured in human blastocyst in order to qualify the culture media. A early rise in βHCG indicates a good media (Fishel 1985).

Pyruvate uptake:

Pyruvate uptake is relatively higher compared to glucose uptake in early embryos. A correlation exists between pyruvate uptake by embryos and their viability(Leese 1987,Hardy 1989). Since most IVF centers perform embryo transfer at day 2 or 3, i.e. from 2-8

cell stage, the pyruvate uptake test seems to be a good tool in assessing the viability of an embryo.

Glucose uptake:
Glucose seems not to be effective as an energy source in human before day 3 (Gott 1990). The measurment of glucose is therefore not practical in IVF-routine. But in mouse embryo lack of glucose, as early as from the 2-cell stage, can lead to limited viability (Sakkas 1993). Increasing sensitivity of glucose analysis could make the test applicable in the future.

Suppressive factors:
Embyos do produce so called suppressive factors. The antiproliferative activity of these factors could be directed against the maternal immune-system to protect against a horst-graft reaction. Clark et al (1989) could find a correlation between the suppression level and pregnancy rates, higher level = higher pregnancy rate. A similar correlation was found in interleukin 1-α, where higher levels are correlated with successfull pregnancies (Hamilton 1990).

In the 2-8 cell stages, only increased pyruvate consumtion and increased suppressive factors seem to be predictive for a higher viability. Changes in lactate and glucose concentrations can only be found in later stages. This is either done to non-metabolisme of the substrate in early embryos or to measurements below the sensitivity of the test.

Human embryo culture media contains a factor, that reduces the platelet count in splenectomized mice (O'Neill 1984). It's called platelet-activating factor (PAF) and could be responsible for the mild thrombocytopenia in early pregnancy. PAF may also play a role in severe thrombocytopenia in pregnancy, as the cause of HELLP-syndrom (haemolysis/elevated liver-encymes/low platelet) has not yet been established. The expression of PAS is positivly correlated to embryo viability

Oxygene consumption increases with viability as one would expect (Leese 1993).

ATP/ADP-ratio and steroids in follicular fluids are other new approaches in this field.

4. Conclusion:

Routine assessment of embryo viability is done with a light microscope by physical methods. Embryo-morphology and cleavage-rate correlate with embryo viability although a "pretty embryo" does not always lead to a successful pregnancy and vice versa.

Recently biochemical methods have been used for scoring human embryos. Uptake and release of embryo metabolites can be measured by sensitive microassays. These tests probably have a higher predictive value than morphology and cleavage-rate alone.

Increased pyruvate consumption and increased expression of suppressive factors correlate with good embryo viability.

Invasiv sampling of a few embryonic cells for further examination, e.g. genetic analysis, could be the method of the future. These test could provide most specific information about the embryo. Unfortunately some government policies strictly prohibit experiments with preimplantation embryos. It is not logic, that preimplantation embryo testing for genetic diseases should be restricted, while abortions for genetic diseases are supported.

5. Adnexe: (Edwards RG 1995)
Culturing and Examining Embryos:

Examining Cleaving Embryos

a) Assess embryos at a minimum of once daily until replacement.
b) Make assessments rapidly under low-power stereomicroscopy, avoiding loss of temerature or CO_2 tension.
c) Examen embryos for day 2 replacement 24 hours after scoring pronuclei.
d) Make frequent examinations when testing different media, novel forms of assessing growth, or new techniques.
e) Count and measure blastomere size.
f) Embryos should be 2-4 cell on day 2 postinsemination, and 8-16 cells on day3.
g) Classify fragments, debris, unusual conditions in the zona pelluzida, necrosis, or other pathological signs.

h) Rare embryos are still pronucleate on day 2; they might have matured or been fertilised later then predicted, or be arrested in growth.
i) Growth can be assessed in single embryos in 5µl microdrops by uptake of metabolites or secretion of various factors.
j) Assays for other metabolites, e.g., platelet activating factor (PAF), may be helpful.

Examining Morulae and Blastocysts

a) Morulae or early blastocysts form on day 4.
b) Score morulae for even shape, blurred outline of blastomeres, and initial
fluid secretion between cells.
c) Score inner cell mass and trophectoderm structure, size of the blastocelic cavity, and integrity of the zona pellucida in blastocyst.
d) Score blastocysts for irregularities, such as a small cavity, absent inner cell mass, structural damage, and necrosis.

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