Visual evaluation of early (~ 4-cell) mammalian embryos. How well does it predict subsequent viability?

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I) LITERATURE REVIEW

INTRODUCTION AND BACKGROUND

Implantation rate, a limiting factor in human assisted reproductive technologies (ART), has changed very little since the birth of Louise Brown in 1978, in spite of many advances in the field. Increasing implantation rates will lead to a higher overall efficiency of human ART. In human *in vitro* fertilisation (IVF), the embryos are routinely transferred to the uterus on day 2 or 3 of development, when they have between 4 and 8 cells. As the embryonic genome is fully activated after the 8-cell stage, it may be beneficial to delay embryo transfer until after the transition from maternal to embryonic genome, identifying thus the embryos with poor developmental potential.

A detailed proposal for a move to blastocyst culture and transfer as a means of increasing implantation rates in clinical IVF has been put forward (Gardner and Lane, 1997). However, many clinics are still not convinced about this change in embryo culture. Although many alternatives to assessing embryo viability have been taken into consideration, the reality remains that most embryologists still rely on morphological criteria. If morphology is our main weapon, when is then the best stage to use it? Two schools of thought have emerged, one in favour of early selection and one in favour of late selection at the blastocyst stage. Are blastocysts better? Transfer of blastocysts can improve embryo selection and cumulative pregnancy rates, but at the cost of time, expense and requirement for expertise, as well as an increase in risk. "The Holy Grail of IVF continues to be the ability to transfer the "right" embryo" (Brison, 2000)

I.1) Factors influencing embryo selection prior to transfer in human ART

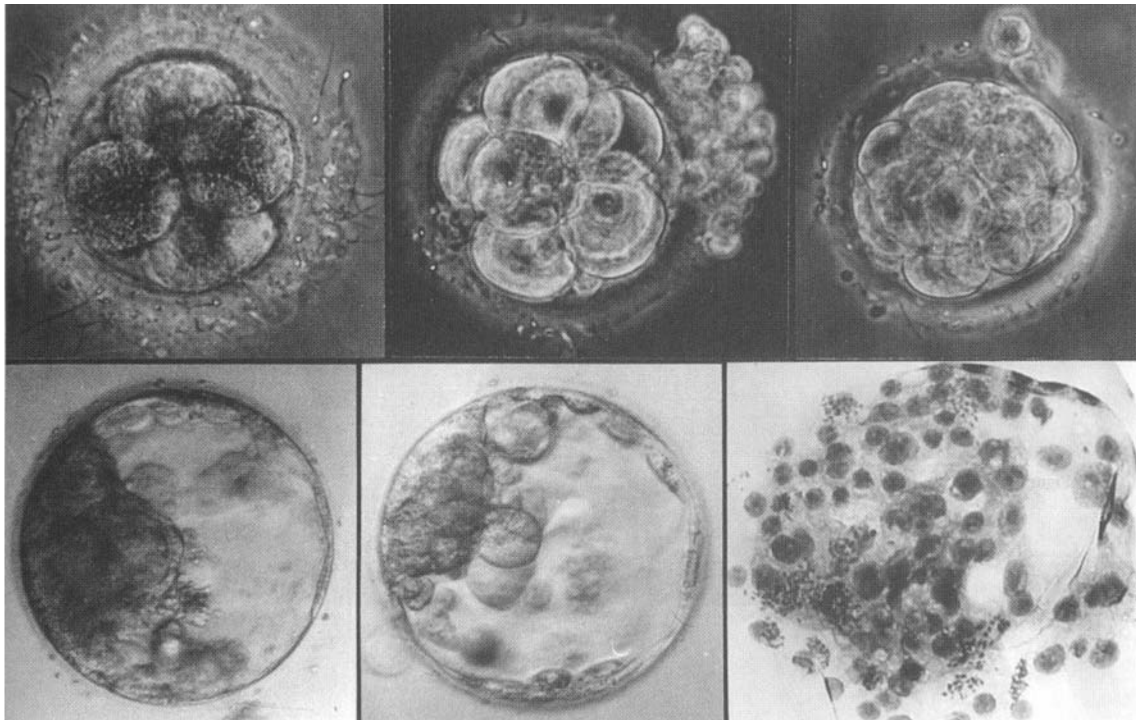
First of all, it is good to have an idea of the characteristics of the different stages a human embryo goes through before being able to score it (see Fig. I.1.a and I.2.b).

Figure I.1.a: Embryonic stages (human) between day 2 and 6 (Bongso, 1999)

Day (am)	**Embryonic stage**	**Description**
2	Cleavage stage	2-4 cells
3	Cleavage stage Compacting	6-10 cells Blastomeres compacting
4	Compacting Compacted Early cavitating	Blastomeres compacting Blastomeres compacted First signs of blastocoele
5	Late cavitating Early blastocyst Expanding blastocyst	Distinct blastocoele ICM and TE not laid down Distinct ICM, TE and blastocoele Embryo diameter same as day 4 or slightly larger Distinct ICM, TE and blastocoele. Thin zona pellucida. Substantial increase in embryo diameter but not fully expanded
6	Fully expanded blastocyst Hatching blastocyst Hatched blastocyst	Distinct ICM, TE and blastocoele. Thin zona pellucida. Fully expanded diameter (approx. 215 ⊠m) ICM and TE hatching out of zona pellucida ICM and TE completely hatched out from zona pellucida Empty zona pellucida.

ICM = Inner Cell Mass
TE = Trophectoderm

Figure I.1.b: Successive stages of growth of human embryos *in vitro*. Upper left: 4 cell embryo (see sperm still attached to its zona pellucida from the *in vitro* fertilisation procedure). Upper centre: 8-cell embryo. Upper right: human morula. Lower left and centre: blastocysts 4-5 days after insemination. Lower right: day 5 blastocysts prepared for cytogenetic examination (Edwards, 2000).



I.1.A) Early Embryo Scores

Steer *et al* (1992) combined embryo quality with number of embryos transferred to establish the cumulative embryo score (CES).

CES = (E.G. x CN)a + (E.G. x CN)b + (E.G. x CN)c + ….

Where E.G. is the embryo grade and CN the cell number for each of the transferred embryos. Pregnancy rate increases significantly with CES (Visser and Fourie, 1993).

A score that relies on a combination of several early characteristics of the early embryo is the GES (Graduated Embryo Scoring). See Fig. I.1.c

Figure I.1.c Graduated Embryo Scoring of cleavage-stage embryos (Fisch *et al*, 2001)

Evaluation	**Hours after insemination**	**Developmental milestone**	**Score**
1	16-18	Nucleoli aligned along pronuclear axis	20
2	25-27	Cleavage regular and symmetrical Fragmentation: Absent <20% >20%	30 30 25 0
3	64-67	Cell number and grade: 7,I; 8,I; 9,I 7,II; 9,II; 10,I; 11,I; compacting I	 20 10
Total score			100

Note: embryos with a total score of 70 or more are selected to be transferred (or cryopreserved).

I.1.B) Pronuclear scoring

There are countries where embryos are replaced at the pronuclear (PN) stage for religious and ethical reasons. Many authors argue that pronuclear scoring is one of the best predictors of future embryos quality (Balaban *et al*, 1998, Garello *et al*, 1999). The markers at the PN stage for the best embryos (Bongso, 1999) include:

1) Proximity of the two pronuclei
2) Alignment in vertical rows of the nucleoli of each pronucleus
3) Appearance of a cytoplasmic clear halo around the pronuclei
4) Early appearance of the first cleavage (2-cells stage) at around 24-25 hours after insemination.

Tesarik and Greco (1999) determined that a single static observation of pronuclear morphology could predict viability. Many authors have come up with different scoring systems (See Figure I.1.d for detailed patterns of pronuclear morphology). Basically, good quality embryos display the following features:

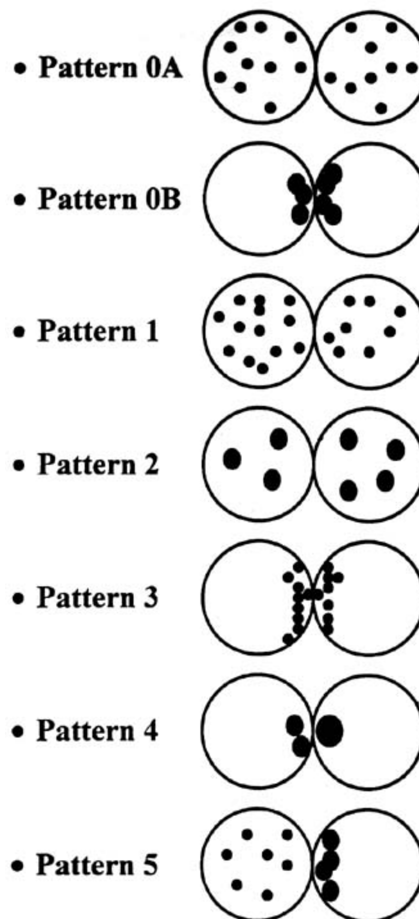
1) Number of nucleoli in each pronucleus does not differ by more than three.

2) When the number of nucleoli is less than 7, they are always polarised

3) When they are greater than 7 they are never polarised

4) Nucleoli are all polarised or all non-polarised, not both.

Figure I.1.d Different patterns of pronuclear morphology show one possible classification system (Montag and Van der Ven, 2001)



Other groups have graded embryos based on first polar body characteristics, distance between polar bodies… (cit: www.ivf.net/cgi-bin/webbbs/amy/config.pl?read=10)

I.1.C) Day 2 embryo quality

Day 2 cleavage stage embryos (2 to 4 cells. See Fig. I.1.e) are usually scored on the basis of:

1) Size and regularity of the blastomeres

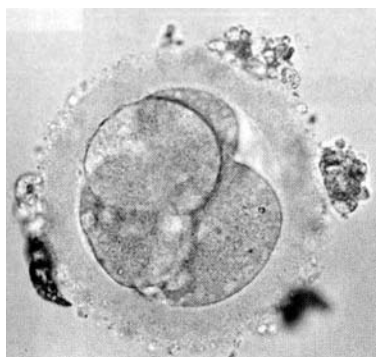
2) Presence or absence of cytoplasmic fragments

3) Rate of cleavage: this is a useful parameter to predict embryo viability. At 44-48 hrs post-insemination, embryos should have 4-5 cells. If there are still 2-cell embryos, these should be given lower priority for transfer.

4) Presence of multinucleated blastomeres

Another secondary factor also observed is the granularity of embryonic cells' cytoplasm.

Figure I.1.e: Four-cell human embryo 40 hrs after ICSI. Two blastomeres have a visible nucleus, two blastomeres are small in size and there is <10% cytoplasmic fragmentation (Hartshorne, 2000).



Grading system for early cleavage embryos recommended by Bongso (1999):

Grade 3: Good - Equal sized blastomeres; No fragments

Grade 2: Fair - Equal sized blastomeres; Moderate fragments

Grade 1: Poor - Unequal or equal sized blastomeres; many fragments.

Some embryos can be classified as in between the whole-number grades (1.5 or 2.5).

Bongso and Gardner (1999) amplified the previous system to a 4-grading scale according to Bolton *et al* (cit: Bongso and Gardner, 1999):

Grade 4: Regular, spherical blastomeres with no extracellular fragmentation

Grade 3: Regular, spherical blastomeres with some extracellular fragmentation

Grade 2: Blastomeres slightly irregular in size and shape with considerable extracellular fragmentation

Grade 1: Barely defined blastomeres with considerable extracellular fragmentation.

Finally, the following table is a summary of different aspects of embryo morphology that different authors take into account for their research relating to egg quality. (See Fig. I.1.f)

Figure I.1.f: Different criteria applied by various authors to evaluate cleavage-stage embryos

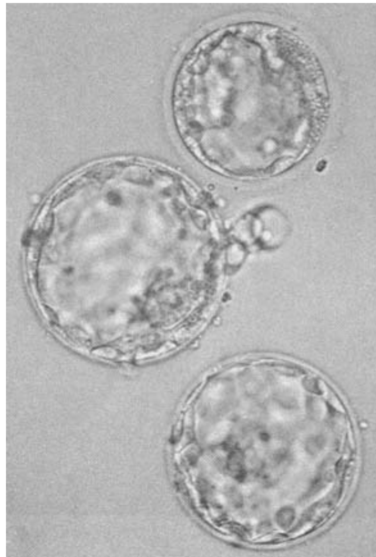
	Ertzeid *et al* 1999	**Laverge *et al* 2001**	**Rijnders and Jansen, 1998**	**Ziebe *et al* 1997**	**Rienzi *et al* 2002**	**Plachot *et al* 2000**
Equality of blastomere size	+	+		+	+	+
Anucleate fragments	+	+	+	+	+	+
Cleavage speed					+	
Multinucleated blastomeres					+	
Number of blastomeres						+
Granularity						+

I.1.D) Blastocyst quality

There are many morphological markers that identify the best blastocysts to transfer (See Fig. I.1.g). The best blastocysts (and the more likely to implant) will be cavitated or expanded on Day 5, have a distinct inner cell mass (ICM), a well laid down trophectoderm (TE) with sickle-shaped cells, a thin zona pellucida and ideally a high total cell number (TCN). Good quality blastocysts should have 50-150 cells on day 5, so TCN can be taken into account as an important value for blastocyst quality. TCN has been considered to be the most sensitive indicator of embryo viability and it can be for example, measured via fluorescence (in case of research, that embryo cannot be transferred afterwards) or non-fluorescent staining. The values for good blastocysts are significantly higher than those for poor blastocysts (See Fig. I.1.h). It is also a good option to transfer blastocysts that have

already begun to hatch (so we know hatching will be carried out). Those blastocysts that show all these good qualities on day 6 can also be transferred (if ever the good quality Day 5 blastocysts are not enough). Otherwise, they can be cryopreserved (unhatched).

Figure I.1.g: Different stages of embryonic development after 5 days of embryo culture. Three blastocysts showing varying amounts of inner cell mass and different size according to the stage of expansion and beginning of hatching (Plachot, 2000).



Possible markers for blastocysts quality (Bongso 1999):

- Morphological characteristics:
    - ICM size
    - Thin zona
    - Single large blastocoele
    - "Sickle-shape" cells in TE
- Cleavage speed
    - Cavitated, expanding - day 5 (am)
    - Fully expanded – day 5 (pm)
    - Hatching – day 6 (am)
- No collapse of blastocoele
- Glucose or pyruvate uptake
    - Conclusive in animal studies

- TCN of blastocysts

Figure I.1.h: TCN (total cell number) of human blastocysts in sequential culture media (Bongso, 1999)

Blastocyst (day/stage)	Good (Mean±SEM)	Poor (Mean±SEM)
Day 6 (144 hs)		
Expanded	168.3+/-14.1	64.3+/-6.0
Hatching	173.0+/-13.5	82.0+/-5.8
Hatched	166.5+/-16.0	
Day 7 (168 hrs)		
Expanded	227.1+/-17.1	88.9+/-7.2
Hatching	286.0+/-13.5	85.3+/-6.2
Hatched	284.0+/-17.7	

A morphological grading of developing human blastocysts was detailed by Dokras *et al* (1993). Blastocysts were graded from 1 to 3 based on cleavage time and morphology. Good blastocysts (BGI) had an early cavitation, well-laid TE and distinct clear ICM on day 5. Grade II (BG2) blastocysts were called "transitional" and had cavitation and looked vacuolated on day 5 with transition to BGI on day 6. The BG3 blastocysts cavitated on day 6, with defects in the ICM and collapse of the blastocoele within 24 hours. Probably, the most complete blastocyst scoring system was developed at the Colorado Centre for Reproductive Medicine, and this takes into account blastocyst expansion, zona thinning, as well as inner cell mass and trophectoderm development.

The following is the scoring system for human blastocysts from Gardner and Lane (1999), David Gardner being a key figure in blastocyst culture development. Initially blastocysts are given a numerical score from 1 to 6 based upon their degree of expansion and hatching status (see Figure I.1.i):

1) Early blastocyst; the blastocoele being less than half the volume of the embryo
2) Blastocyst; the blastocoele being greater than half of the volume of the embryo
3) Full blastocysts; the blastocoele completely fills the embryo

4) Expanded blastocyst; the blastocoele volume is now larger than that of the early embryo and the zona is thinning.

5) Hatching blastocyst; the trophectoderm has started to herniate through the zona

6) Hatched blastocysts; the blastocyst has completely escaped from the zona.

Then, for blastocysts graded 3 to 6, look at (x 320 on inverted microscope) ICM grading:

A) Tightly packed, many cells

B) Loosely grouped, several cells

C) Very few cells

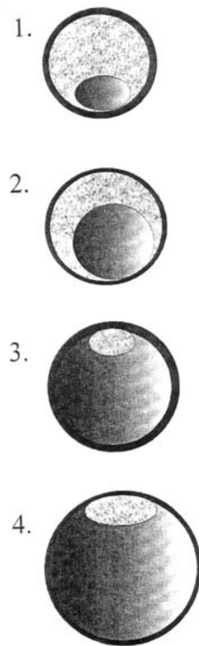
And trophoectoderm grading:

A) Many cells forming a tightly knit epithelium

B) Few cells

C) Very few cells forming a loose epithelium.

Figure I.1.i: Different maturation stages shown in volume proportions in early human blastocysts: 1- Early blastocyst, the blastocoele is less than half the volume of the embryo. 2- Blastocyst, the blastocoele is greater or equal to half the volume of the blastocyst. 3- Full blastocyst, the blastocoele completes the cavity. 4- Expanded blastocyst, zona is thinning and volume of blastocyst is greater than that of earlier embryo. Hatching and hatched blastocysts are not represented as they are easier to classify since, in the former, the trophoectoderm has begun to herniate through the zona and, in the latter, it has completely escaped the zona (Gardner, 2000).



The grading systems mentioned so far are the more common or "en vogue" ways to score embryos viability. Other authors encourage a combination of systems (Wittemer *et al*., 2000; Salumets *et al*, 2001) or grading at other stages like the morula stage (Tao *et al*, 2002). Many clinics use combinations of these or similar scoring systems. In Appendix 2, there is, for reference, a copy of the embryo score system used in the Aberdeen Fertility Centre. It cannot be forgotten though, that, as important as it is to be able to grade the embryos, this might mean too much time out of the incubator, hence involuntary harm might be inflicted on them (Dr. Maureen Wood, Aberdeen Fertility Centre, personal communication).

I.2) Strengths and weaknesses of current procedures

A key question in embryo scoring is whether embryo morphology on day 2 to 3 is predictive of further development to the blastocyst stage.

I.2.A) Early embryo transfer

Some authors like Lundin *et al* (2001) and Bos-Mikich *et al* (2001) have found that a high number of early-cleaving embryos became good quality embryos, as well as giving significantly high pregnancy, implantation and birth rates. Their results imply that good cleavage is indeed a good biological indicator of embryo potential. Embryos having fewer than four cells at day 2 or fewer than six cells at day 3 have significantly lower pregnancy and implantation rates than faster-cleaving embryos (Van Royen *et al*, 1999). According to some authors (Sakkas *et al*, 1995, Shoukir *et al*, 1997, Lundin *et al*, 1998, Sakkas *et al*., 2001), the rate of embryo development appears to be a more reliable indicator of embryo viability than morphology and it can be established in the early embryo.

Giorgetti *et al* (1995) found that 4-cell embryos transferred on day 2 implanted twice as often as embryos with more or fewer cells. They also found a decreased pregnancy rate when transferring embryos having irregular-sized blastomeres. When transferring two or three good quality embryos of different cleavage stages, Staessen *et al* (1992) found that pregnancy rate increased proportionally to the number of 3-4 cells embryos. However, if we look at the implantation rates when transferring only one embryo (on day 2), single embryo transfers being a goal that most embryologists would like to reach in order to avoid multiple pregnancies, the implantation rates decrease (Ziebe *et al*, 1997). A 4-cell cleavage stage embryo (implantation rate of 23% according to Ziebe *et al*), even with the presence of minor amounts of fragments, should be preferred for the transfer rather than a 2-cell embryo with no fragments (12 % implantation rates according to the same authors).

Since it has been reported that the overall pregnancy rates are similar after transfer on either day 3 or day 5, it could be that the benefit obtained by a better selection method does not outweigh the disadvantage of exposing embryos for longer to artificial culture conditions.

Furthermore, if the available number of embryos is small, there is no advantage in a long culture period (Rijnders and Jansen, 1998).

Because the clinical and laboratory procedures related to blastocyst formation are more expensive to the patient than the procedures related to early embryo transfer and storage, day 2 or 3 transfer policy, is, in many cases clinically more efficient and more cost-effective as compared with day 5 transfers (Sakkas *et al*, 1998, Rienzi *et al*, 2002).

I.2.B) Blastocyst transfer

The potential advantages of the uterine transfer of blastocysts include synchronisation of the embryos with the uterine endometrium and selection of the best quality embryos with high implantation potential, thus limiting the number of embryos transferred and minimising the risk of multiple pregnancy (Plachot *et al*, 2000, Balaban *et al*., 2001, Kausche *et al*, 2001). Blastocyst formation on day 5 offers more information about the implantation potential compared to embryo morphology on day 3 (Rijnders and Jansen, 1998). It has been reported that day 3 embryo morphology alone is a poor predictor of blastocyst quality. More than 50% of embryos derived from IVF patients with a poor prognosis are genetically abnormal and as many as 40% of blastocysts exhibits some chromosomal mosaicism in the inner cell mass. This high rate of genetic dysfunction can be related (and maybe partly responsible) to previously reported blastocyst production rates of barely 50% as well as with the clinical observation that approximately half of the embryos seen on day 3 will not survive to day 5 (Behr *et al*, 1999).

Development of embryos to the blastocyst stage with sequential media is possible and the implantation rate of blastocysts is at least as good as that of cleavage stage embryos or even better. Implantation rates of approximately 40% per blastocyst transferred have been reported (Gardner *et al*, 1998). The incidence and quality of blastocysts depend on several factors: intrinsic embryo quality, culture conditions, batch of medium. However, it is important to ask: “Of those embryos that fail to reach the blastocyst stage *in vitro*, how many would have been able to give rise to offspring if transferred earlier?” (Tsirigotis, 1998). Some authors argue that in human IVF, many embryos are transferred on day 2 and

3, when the embryo has between 4 and 8 cells and the embryonic genome might not have been activated yet. Though some paternally-derived genes are already activated in the 3 to 4-cell stage embryo, the overall genome gets activated at the 8-cell stage. The predictive value of embryo morphology on day 3 for subsequent blastocyst formation is limited (Rijnders and Jansen, 1998). It is beneficial to transfer embryos after the genome has been activated as this allows embryologists to identify embryos with poor developmental potential. Fewer embryos are necessary then for the transfer. As is explained in Section I.5.B, synchronisation between embryo development and the surrounding environment is also better. The implantation rate after blastocyst transfer is influenced by blastocyst quality. It appears however, to be useless to wait until day 6 if blastocyst quality is not ideal on day 5 (Rienzi *et al*, 2002).

Blastocyst culture could also have scientific benefits in clarifying the processes of implantation. If the embryos have survived 5 days, they have a proven quality. Why then do more than 50% of them fail to implant when they are the survivors of an even greater number of fertilised oocytes? One of the causes could be aneuploidy. Non receptive endometrium could be another factor. It seems that similar failure rates occur *in vivo* (Edwards and Beard, 1999). A blastocyst is an indicator of successful development up to that point but not a guarantee of ability to continue normal development (Mc Evoy *et al*, 2000)

However, extended culture has many potential drawbacks. Not all laboratories are equipped to perform blastocyst culture. Extended embryo culture requires a specialised sequential media system, which makes the success rates of IVF programs even more susceptible to variations in culture media quality. Increased incubator space is needed to accommodate extended culture. Prolonged culture is known to induce abnormalities in animals e.g. large calves and although not yet demonstrated there is a fear that the same could be true for human (Mc Evoy *et al*, 2000)

Interestingly, Menezo *et al* (1999, cit. Gardner, 2000a) reported a shift in the sex ratio of children born after the transfer of blastocysts produced in co-culture media (58.0 % boys

and 41.7% girls). However, this seems to have disappeared with the introduction of sequential media.

The drawback from the extended culture to blastocyst could be the decrease in the number of pregnancies resulting from the transfer of cryopreserved and thawed blastocysts due to a lower number of embryos suitable for cryopreservation (as compared to day 3) and a decreased survival rate of the blastocyst after thawing, The ability to cryopreserve blastocysts successfully is a prerequisite for the overall success of extended culture in ART. In spite of the encouraging implantation and pregnancy rates obtained with cryopreserved blastocysts (Langley *et al*, 2001), values are still below those for transfers using fresh blastocysts. This may be due to the preferential transfer of the highest quality blastocysts within a cohort, resulting in the cryopreservation of lower scoring blastocysts, or to the slow freezing procedures (Gardner, 2000a). The blastomeres have formed tight junctions at this stage, hence enabling cellular signalling mechanisms, and these more advanced embryos may be more sensitive to the destructive forces of cryopreservation (Rijnders and Jansen, 1998). There is also the thorny issue of not having surviving embryos on the fifth day. That is why also, many programmes that perform blastocyst transfer have criteria such as 4 or more 8-cell embryos on day 3 for recommending extended culture (Milki *et al*, 1999). For this, it is also better to culture embryos separately instead of in groups to be able and follow the developmental progression of individual embryos (Fisch *et al*, 2001).

It has been found that around half of the pregnancies achieved by blastocyst transfer are twins when only two blastocysts are transferred. The only effective way of eliminating dizygotic twins is to transfer a single embryo. It is therefore necessary to develop the most effective way to identify the most viable embryo in a cohort (Gardner 2000, b). If the quality of individual human blastocysts can be quantitated by systems such as the three-part scoring system of Gardner, a substantial number of patients could benefit from the implantation potential of a single top-quality blastocyst.

It is clear that there is a distinct group of patients that would benefit from prolonging the embryo culture period. Those patients producing embryos on day 2 to 3, which are hard to

select because of the presence of a large number of embryos with approximately the same morphology, may have several good blastocysts by day 5 and as a result, have a greater chance to achieve pregnancy despite previous failures using less advanced embryos (Rijnders and Jansen, 1998). Blastocyst culture also allows for assessment of embryo viability and trophectoderm biopsy to be performed as extra tests of embryo viability, although these are not very common. Other patients who can benefit from blastocyst transfer would be those with a high risk of multiple gestation or with probability of gynaecological risks if having a multiple pregnancy.

I.2.C) Blastocysts and ICSI

It is an ability to select highly viable embryos non-invasively that is crucial when approaching blastocyst culture. This can be a great benefit when using assisted reproduction techniques (ART) that can compromise the quality of the embryo obtained. One example of this is ICSI (intracytoplasmic sperm injection). The use of blastocyst culture can then have a direct applied benefit to human assisted reproduction. There has been discussion of whether ICSI increases the development of abnormal offspring (Sakkas, 1999). ICSI does not allow embryologists to be discriminatory with the quality of paternal nuclear material they introduce in the egg. Sperm with defective chromatin packaging, damaged DNA and others that have not entirely completed spermatogenesis are currently used in infertility treatments for men (http://gateway2.ovid.com/ovidweb.cgi). Sperm defects could not only affect the whole fertilisation process but also the quality of the embryos (Sakkas, 1999). Abnormal sperm can carry genetic abnormalities that can be transferred to the offspring, that is why we must still search for the best methods to select the sperm to be injected.

In in-vitro fertilisation (IVF), where the standard parameters of sperm evaluation are good, there is a relationship between sperm quality and embryo development. Cleavage rate does not show any correlation with further embryonic development in cases with poor motility spermatozoa. Poor quality spermatozoa lead to poor rates of blastocyst formation, but once the blastocyst stage is obtained, the developmental future is equal whatever the sperm quality at the origin (Parinaud *et al*, 1993). In the human, it has recently been reported that

ICSI patients have a lower percentage of embryos that form blastocysts compared with patients that undergo routine IVF (43.1% vs 55.8%) (Huang *et al*, 2000, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12426195&dopt=Abstract)
How can the probability of ICSI offspring inheriting a paternal defective genome be decreased?

The options are, either to preselect the best spermatozoon to use for ICSI (new suggestions and techniques are always coming up for this issue, like the idea of screening sperm via PCR before injection (Science Update, http://www.nature.com/nsu/030804/030804-14.html), or to use a selection process to identify the embryo that is more likely to develop into a normal offspring. Using extended culture to blastocyst stage offers the second possibility to ICSI patients.

It would be unwise to suggest that blastocyst culture and transfer represents a panacea for all clinics and patients. It is probably ideal for certain patients, specially those that have enough day 3 embryos to venture extended culture with a low risk of ending up with no embryos to transfer at all on day 5. Before extended culture is carried out, all clinical and laboratory aspects need to be optimised. If problems are present either in patient stimulation protocols or within the laboratory, extended culture will only exacerbate the situation.

I.3) Novel approaches to improve efficiency in selecting "viable" embryos

New IVF techniques allow not only the treatment of infertility but also a deeper study of pre-implantation embryos (and gametes). Nowadays, genetic analysis can be performed on a single blastomere from a day 3 embryo so the genetic profile of the originating embryo can be assessed. There are two ways of analysing genetic material in *in vitro*-embryos. One is to aspirate the first and the second polar bodies of the embryos (in which case we only analyse the mother' s genetic material). The other way is to remove one or two blastomeres from, as already mentioned, a day 3 embryo (approximately 8 cell-embryos) by embryo biopsy to diagnose its genetic constitution (preimplantation genetic diagnosis). Even though this might be seen as quite an invasive method, the embryos' plasticity is such at this stage (we must remember it can still divide and produce twins) that the procedure does not affect the capacity of the embryo to evolve correctly or to implant (Gianaroli, 2000). The genetic screening then takes place through polymerase chain reaction (PCR), for single gene defects, or through fluorescent in-situ hybridization (FISH) for aneuploidy. The first is based on the amplification of short sequences of DNA so as to detect even single base mutations. The latter labels chromosome-specific probes fluorescently which allows the enumeration of different chromosomes (Gianaroli, 2000). Blastocyst biopsy has been suggested but since the blastocyst has a higher degree of chromosomal mosaicism and since trophoectoderm cells could be taken out by mistake (can differ chromosomally from inner mass cells), then the day-3 biopsy proves more accurate (Harper and Delhanty, 1996).

I.3.A) PGD (preimplantation genetic diagnosis) and blastocyst culture

We must also remember that preimplantation genetic diagnosis (PGD) is another indication for blastocyst transfer, which is carried out in cases of repeated abortions, "aged" women, or indications such as sex-linked diseases, monogenic diseases and aneuploidy screening (Gianaroli *et al*, 1997). Since most centres carry out the biopsy on day 3, if the embryos are then cultured to blastocyst stage, developmental arrest due to the biopsy can more easily be seen and the arrested embryos would not be transferred (Hardy *et al*, 1990).

I.3.B) Polar body biopsy

The first polar body is the result of the extrusion of surplus chromosomes after the oocyte's first meiotic division. It can be removed approximately 4 hours after oocyte retrieval. The oocyte is held by the holding pipette and the polar body is kept at the 12 o'clock position. A slit is drilled mechanically in the zona pellucida and the polar body is aspirated through a thin polished glass needle. The oocyte can then be inseminated by ICSI (intracytoplasmic sperm injection) through the hole in the zona. This slit will also be used later on to remove the second polar body (Verlinsky *et al*, 1990). This technique is advantageous because it maintains the embryo's viability and integrity. Nevertheless, it does not include in its diagnosis paternally-derived defects or those originated after fertilisation or cleavage (Gianaroli, 2000).

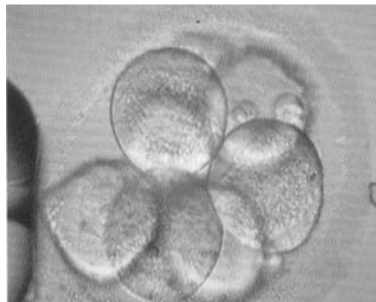
I.3.C) Embryo biopsy

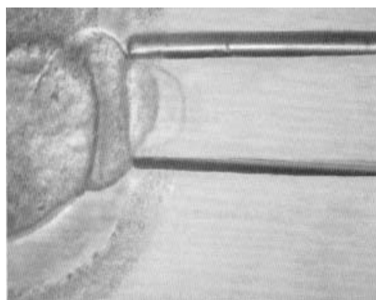
The embryo biopsy consists in the extraction of one or two blastomeres from the preimplantation embryo. The incidence of embryo damage as a result of the procedure is very low. The procedure is usually carried out 62-64 hrs after fertilisation, when compaction begins but hasn't completely settled yet, since there can be damage if the junctions between the cells have become too tight. To solve this problem, embryos are usually incubated in media with low divalent cation (Ca^{2+}, Mg^{2+}) concentrations. For the biopsy, there is a hole of approximately 20 ⊠m that is made in the zona pellucida with laser, mechanically or chemically, and the blastomere is gently aspirated and released into the medium. The embryo is then incubated and the blastomere goes for genetic analysis.

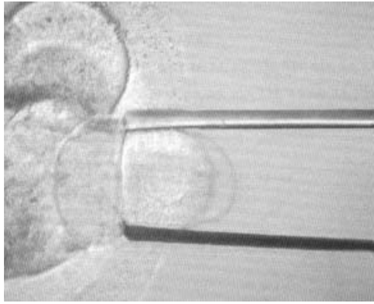
There are reports that show that one out of four embryos transferred after PGD is capable of implantation (Gianaroli, 2000). So, despite the invasiveness of this technique, it does not destroy the embryo's capability to form a blastocyst, to hatch and to implant, giving way to normal offspring. The morphological criteria for embryo transfer are now supported by a genetic basis. There still is room to improve the accuracy and reproducibility of the diagnostic procedures, but through this examination, embryologists have a way to recognise the embryos that are capable of full development. The understanding of the genetic defects

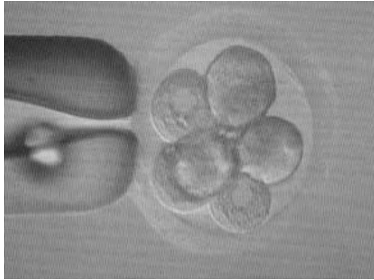
and the evaluation of the embryo's morphological appearance can be another step towards understanding the first stages of human life. See Fig. I.3.a

Figure I.3.a: Blastomere biopsy from 8-cell embryo (X40). Sequence: embryo maintained by holding pipette while zona drilling needle with Tyrode's solution makes a hole in zona pellucida. Biopsy needle then aspirates a blastomere through the hole created. Blastomere is released into the medium. Nucleus is evident (Gianaroli, 2000).









I. 4) Evaluation of the biological basis / validity for decision-making during embryo selection

As mentioned already, after more than 20 years of experience of *in vitro* fertilisation techniques, the implantation rates per embryo are still very low (sometimes as low as 20%). The key point is not so much achieving fertilisation as achieving implantation. Information varies but some reports indicate, whereas implantation rates for day 2 or day 3 transfer are low, for blastocyst transfer on days 5 or 6, the implantation rate can get as high as 40% (Gardner *et al* 1998).

As already discussed, the advantages of transferring blastocysts are multiple and they include reducing the number of embryos that are transferred and the number of embryos cryopreserved for indefinite amounts of time. However, blastocyst culture and transfer should not be seen as a universal remedy for all the problems of IVF. The culture systems and the laboratory are just one aspect; it must always be remembered that good oocytes, from appropriate stimulation regimes, can give rise to good embryos. It is not possible to obtain good embryos from poor oocytes (Gardner *et al*, 2000b)

I.4.A) Zona anomalies

The zona pellucida is formed mainly by the oocyte, early in follicle growth. Sometimes unusual forms occur: various shapes, thickness, colours. These may reflect an abnormality and affect sperm penetration (Hartshorne, 2000).

I.4.B) Early zygote classification

In the fetus, some oogonia give rise to oocytes when they enter the first meiotic division. Many oogonia also die at this period, but after birth, all the remaining oogonia have entered the first meiotic division and are arrested at the dictyate stage. At this time, the gonad's somatic cells surround each oocyte forming the primordial follicles. During reproductive life when these are recruited into growth, the granulosa cells proliferate, the follicle develops an antrum, forming a Graafian follicle just before ovulation. The metabolism of the oocyte changes with its growth, nucleoli increase in size (indication of rRNA synthesis)

and ribosomes and mitochondria multiply. The cortical granules move to the subcortical region of the growing oocytes. Once fully grown, oocytes can resume meiosis and go from the first meiotic prophase to the second meiotic division. Oocyte maturation with germinal vesicle breakdown occurs when meiotic arresting substances decrease their passage via the gap junctions between granulosa cells and the oocytes. During the oocyte´s growth, both nuclear and cytoplasmic maturation occur in a co-ordinated manner. Some of the aberrant events in zygote development may be directly dependent on an asynchrony between these maturation occurrences (Scott *et al* 2000).

While the oocyte is growing, its blood supply increases to allow it access to a higher oxygen concentration. A decrease in blood flow can give rise to increased spindle and chromosome defects and decreased fertilisation, as well as disorganised cytoplasm and lower ATP content (Gregory and Leese, 1996).

Following insemination in the afternoon, two pronuclei confirming normal fertilisation are usually visible in the next morning, approximately 16 hrs later (in humans). Sometimes pronuclei appear as early as 12 to 14 hrs or as late as 20 to 22 hrs (Bongso and Gardner, 1999).

Many scoring systems for human zygotes are based on the alignment of the pronuclei, which is caused by the centriole (sperm-derived structure) and the microtubules (they bring the male and female pronuclei together). The embryos that cannot attain this stage usually fail to progress and never produce blastocysts. It is also important to look at the sizes of pronuclei: the female one is usually larger but if the difference is too evident, that is usually the evidence of some kind of aneuploidy (Munne and Cohen, 1998). However, since many of these embryos still can get to the blastocyst stage, if they have not been checked at the pronuclear stage, they can still be among those transferred, which would lessen the chances of pregnancy.

Many zygote scoring systems (see Section I.1), rely on the size, number and distribution of nucleoli (Tesarik *et al*, 2000). The nucleoli are active sites in the female and male pronuclei

where rRNA is synthesized, which is essential for the meiotic mechanism. The oocytes inside Graafian follicles have very well defined nucleoli, at the time of ovulation, rRNA synthesis decreases (nucleoli are small) and at the time of fertilisation, nucleoli reform and grow and rRNA synthesis starts again. Around the 4-8 cell stage, the human genome becomes active, and the embryo starts depending on its own synthesised rRNA instead of the maternal version (Gregory and Leese, 1996).

Thus, the morphology of the human zygote at 16-18 hrs post-fertilisation can be used as a means of preselecting embryos for transfer (Scott and Smith, 1998).

I.4.C) Preimplantation embryos

Several key events control pre implantation development (Hartshorne, 2000):

- Cleavage of the fertilized egg through about five mitotic cell divisions
- Switch of control from maternal to embryonic genome
- Differential expression of imprinted genes (depend on whether maternal or paternal)
- Initial steps in differentiation (blastomere orientation and fate)
- Expression of key molecules mediating communication among blastomeres.

I.4.D) Cleavage rates and timing

In humans, following insemination, two pronuclei should be visible around 16 hours later. Embryos which do not cleave after 24 hours are said to be arrested. This can reflect (if not a problem with culture medium) a gross nuclear anomaly, failure of embryonic genome activation or cytoplasmic deterioration.

When examining embryo growth, it should be remembered that some authors have claimed that male embryos grow faster than female: Menezo *et al* in 1995 (cit. Butterworth, 2001) reported a sex ratio of approximately 1.5 males: females.

Asynchrony in daughter cells is a result of abnormal chromosomal function (Scott *et al*, 2000).

By days 2 and 3, the embryos are expected to be at the 2- to 4-cell and 8-cell stages respectively. The mean time for the first three cleavage divisions is 35.6, 45.7 and 54.3 hrs after insemination (Hartshorne, 2000). Compaction can begin to be seen at the 8-cell stage. Full expansion of the blastocyst takes place on day 5 and hatching between late day 5 and day 6 (completed on days 6 and 7). Medium composition has a significant effect on embryo cleavage rates. The faster these are, the more likely embryo transfer will result in pregnancy (Steer *et al*, 1992).

I.4.E) Chromosomal Disorders and Multinucleation

Chromosomal disorders during syngamy or during the early stages of human development can result in embryos with limited developmental ability. In embryo cultured *in vitro*, these abnormalities can only be screened with an embryo biopsy and preimplantation genetic diagnosis. There is a broad relationship between embryo morphology and chromosomal status but this connection can be ambiguous. However, some specific chromosomal abnormalities can be encountered morphologically. Polyspermy, producing multi-pronuclear zygotes, results in embryos that are chromosomal mosaics. Rougier and Plachot (1995, cit. Kligman *et al*, 1996) found a high incidence of mosaicism when performing karyotypes of single blastomeres from abnormal embryos after IVF. Other studies have found chromosomal mosaicism in apronuclear, unipronuclear and polypronuclear human embryos using fluorescent in-situ hybridisation techniques (FISH). Cell size and distribution also reflect the chromosomal complement. Embryos with a larger cell surrounded by fragments and embryos developing from oocytes larger than normal were found to be polyploid and triploid. (Munne *et al*, 1994, cit., Kligman *et al*, 1996).

Another morphological abnormality that can be found in *in vivo* and *in vitro* cleaving embryos is multinucleation. Multinucleation incidence is slightly higher when accompanied by other chromosomal abnormalities, and there is a correlation between anucleate and multinucleate cells and morphology (Jackson *et al*, 1998, Pelinck *et al*, 1998). Even though it can appear at the 2-cell stage, multinucleation is most common at the 8-cell stage. It is also unclear whether the chromosomal aberration caused by multinucleation is shared by all

the cells or belongs only to one. Embryos with many multinucleated blastomeres (MNB) usually fail to develop. Some studies have found that multinucleation, if limited to one or two cells per embryo (at 8-cell stage or later) is a benign morphological abnormality. Others argue that there are two kinds of multinucleation: one occurring on day 2 or 3, producing multinucleated and chromosomally abnormal embryos and the other on day 4 or later, generating mostly binucleated cells and chromosomally normal embryos (Kligman *et al*, 1996). Despite many descriptive reports of MNBs in early embryos, little is known about how they form. Some possibilities include karyokinesis without cytokinesis, partial fragmentation of nuclei or defective migration of chromosomes at mitotic anaphase, possibly induced by changes in temperature or suboptimal culture conditions (Pickering *et al*, 1995).

I.4.F) Cytoplasmic anomalies

The cytoplasm of the embryos should be pale and finely granular. Unusual colours, texture and vacuoles can happen and their importance changes with interpretation by different individuals, the assessment of cytoplasmic changes being subjective (Hartshorne, 2000).

I.4.G) Fragmentation.

Fragmentation occurs in many cleaving embryos. Proportion and distribution of fragments is variable. The impact of fragmentation on the embryo can depend on its obstruction of communications between blastomeres. Fragment removal has been considered but never taken into common practice (Hartshorne, 2000).

I.4.H) Apoptosis

It is important to remember, however, that various degrees of cytoplasmic fragmentation and a high incidence of developmental arrest characterise preimplantation human embryos before the blastocyst stage (Antczak and Van Blerkom, 1999). Apoptosis is a kind of cell death that characteristically affects single cells in isolation, with no associated inflammation. It has a part in early human development and embryonic loss but also in normal development. Embryos examined 24 h or more after arrest often show characteristic features of apoptosis including cytoplasmic, nuclear and DNA fragmentation.

In contrast, embryos of good morphology that appear to be "healthy" show no evidence of apoptosis before compaction. However, at the morula and blastocyst stages, scattered cells with fragmented nuclei and DNA characteristic of cells undergoing apoptosis are common features. Apoptosis may result from suboptimal culture conditions or show the lack of growth or survival factors (Hardy, 1999). It may also be involved in the elimination of abnormal cells, those with no developmental potential and those bearing chromosomal and nuclear abnormalities. However, the causes, role and regulation of apoptosis in the human preimplantation embryo remain to be determined: whether they involve the activation of an endogenous programme or are induced by stimuli. During early rodent development, apoptosis plays a crucial role in amniotic cavity formation soon after implantation. It appears that cell death may also play a role before implantation, as soon as the totipotent cells start to differentiate (Hardy, 1999).

I.5) *In vitro* embryo production options

The transfer of a blastocyst to the uterus resembles more the *in vivo* situation from the physiological point of view than the transfer of an earlier embryo to the uterus. This is probably what Robert Edwards and Patrick Steptoe were thinking when, by blastocyst transfer, they achieved the first human pregnancy following *in vitro* fertilisation (Butterworth, 2001).

Early culture systems and culture media were not good enough. The co-culture of embryos on a monolayer of cells increased implantation rates to 23% (Menezo *et al*. 1995, cit Butterworth, 2001). Recently, new sequential, serum-free culture media have been created to accompany the embryo's different needs in its growth.

I.5.A) Culture options

I.5.A.1) Co-culture

Co-culture implies having both embryos and helper cells; both homologous and heterologous co-culture systems have been used to culture embryos in the presence of a layer of "feeder cells".

It has been found that co-cultured embryos have a higher number of cells and a more fully cohesive inner cell mass when compared with embryos cultured in simple media. This improvement in development can be due to four major reasons (Elder and Dale, 2000):

1) "Metabolic locks": co-culture cell layers provide small molecular weight metabolites that simple culture media lack. This can help keep up the metabolism necessary for genome activation.

2) The feeder cells can provide growth factors (Bavister and Boatman, 1997)

3) Cell metabolism produces toxic compounds that can be removed by the feeder cells (for example, feeder cells' metabolic cycle recycles urea and ammonium).

4) Free radicals are prevented from being formed thanks to the action of reducing agents synthesised by feeder cells.

It has been suggested that for certain patients (repeated IVF failure, advanced reproductive age, poor stimulation with poor follicular response, poor embryonic development), co-culture can improve prognosis by providing a different culture environment (Weimer *et al*, 1998; Langley *et al*, 2001).

The extent to which a co-culture system can help embryonic development is only as good as the cells that form the monolayer. Co-culture systems use mainly foetal calf endometrial fibroblasts, human ampullary and endometrial cell lines, granulosa cells and Vero (African Green Monkey Kidney) cells. In spite of having many advantages, this system also has disadvantages:

1) Since the feeder cell medium tends to be rapidly metabolised, pH can change very rapidly and embryos might not tolerate it.
2) Reproducibility is not easy to obtain
3) Establishing cell monolayers takes time and lots of patient work (subculture of explants, cell morphology and growth patterns that change with time).
4) Bacteria, mycoplasm and viruses easily contaminate cell layers. Dr. Maureen Wood (Aberdeen Fertility Centre, personal communication) stated: " My greatest fear about it (co-culture) has always been the risk of accidentally transmitting an infection particularly when animal cells are used." There is then, a risk of infection to the embryo, so screening is essential in that both cells and medium must be checked for infection (Ertzeid *et al*, 1999).

Co-culture has played an important part in research of preimplantation embryos' development and metabolism. The data obtained helped in obtaining better media. Embryologists are still searching for the perfect media (Bongso *et al*, 2000). If it existed, discussion about co-culture would be over (Tom Mc Evoy, personal communication). Meanwhile, co-culture in spite of having its advantages, is not being used routinely, (it was initially used to facilitate blastocyst transfer but implantation rates were never better than those obtained on day 3 (Ertzeid *et al*, 1999)), for fear of its potential for the transmission of infectious disease from animals to humans. The U.S. Food and Drug Administration

(FDA) cautions that "co-culture of human embryos with non human animal cells raises health concerns for the recipients of such embryos, the offspring resulting from such embryos, and the general public." (FDA, 2003, http://www.fda.gov/cber/infosheets/humembclin.htm)

I.5.A.2) Perifusion culture

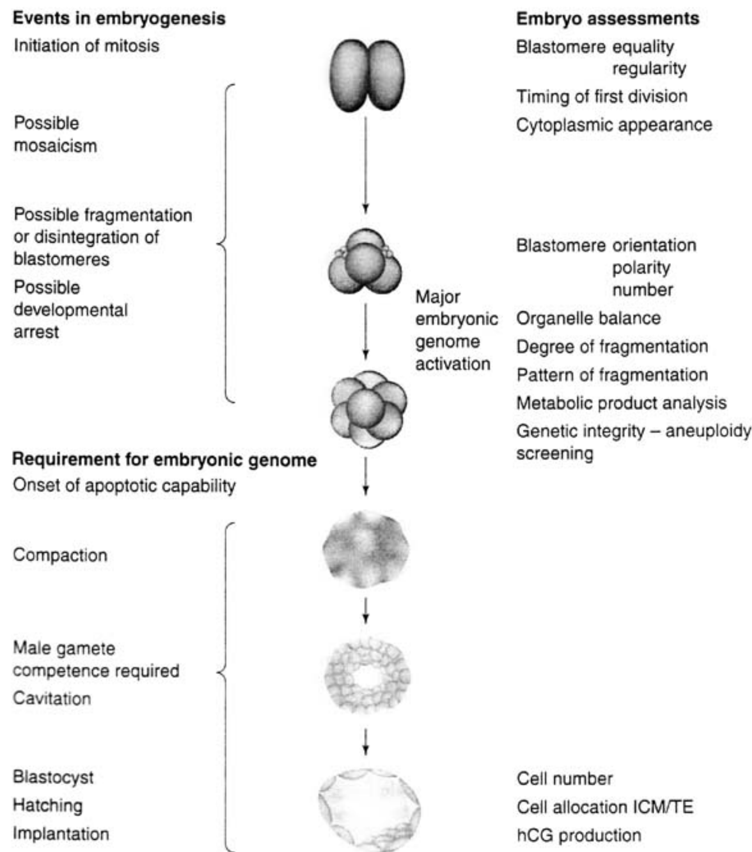
This culture system consists basically in a pipeline that brings medium to the dish while another one takes it out. Medium flows and washes the egg (sitting in the dish) constantly. A dynamic (continuous flow) culture system can have many advantages compared with a static embryo culture system: growth factors or other embryotrophic substances can be added in a continuous or pulsatile manner, and potentially toxic metabolites are continually removed from the embryos (Thompson and Peterson, 2000). Attempts to use perifusion culture systems have been used in equine, bovine and murine embryos. However, viability greatly decreased after culture. This may be due to suboptimal pH conditions (hard maybe to keep the CO_2 constant). It also may be due to continuous flow of perifusion medium (preventing a favourable micro-environment) and maybe unsuitable composition of the medium or absence of helper cells (Lim *et al*, 1996). This system requires a lot of expense in terms of medium and maintenance, so many scientists are waiting for a simpler version.

I.5.A.3) Biological Basis for Sequential Media

The embryo's life begins as a zygote, it then cleaves repeatedly and becomes on day 5 or 6 after fertilisation, a blastocyst. In the meantime, the embryo proliferates, compacts and then cavitates and differentiates into two kinds of cells: the inner cell mass and the trophoectoderm. In the beginning, the embryo does not have any notable transcriptional activity, there is no mRNA synthesis by the embryonic genome, and the maternal genome is still in control. The early embryo uses a limited amount of glucose as an energy source and has a low respiratory rate. At the four-to eight-cell stage, the human genome is activated and the genes of the embryo start to work. Respiratory capacity and demands for glucose increase at this stage (Gardner and Lane, 1997). The embryo becomes sensitive to a whole new set of factors (has new receptors for growth factors, new membrane structures, etc). Growth factors can stimulate blastocyst formation, rescue the embryo from apoptosis,

allow changes in protein synthesis and encourage the hatching process (Kane *et al*, 1997). See Fig. I.5.a.

Figure I.5.a: Preimplantation assessment of viability in human embryos and some of the key events during this period. (Hartshorne, 2001)



I.5.A.4) Nutritional and energetic metabolism of the preimplantation embryo

There are two main ways in which mammals can generate ATP: by the oxidation of energy substrates to CO_2 and H_2O and by the conversion of glucose to lactic acid by glycolysis. Preimplantation embryos in the early stages, normally spent in the oviduct, are mainly under autonomous control. As the blastocyst stage is reached, there is an increase in the dependence on maternal factors. It is the beginning of true growth. In all mammalian species studied *in vitro*, in the initial stages of preimplantation development, the embryo generates ATP by oxidising pyruvate, lactate, amino acids and fat. As the blastocyst stage

is reached, glucose consumption increases sharply and it is mostly converted to lactate (Gregory and Leese, 1996).

Pyruvate

Pyruvate is metabolised via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the generation of energy (ATP) and as such is an important oxidizable energy substrate for human embryos and those of other mammalian species. Up to 56% of the pyruvate consumed is oxidised and the remaining converted to lactate (Hardy *et al*, 1989). This conversion to lactate would probably be important to regulate intracellular pH. Pyruvate also protects the embryo against homeostatic stress, degrading hydrogen peroxide (Elder and Dale, 2000).

Glucose

The human embryo has a dynamic requirement for glucose, which increases with development. There has been confirmation of glucose consumption with blastocysts formation in human and bovine (Gott *et al*, 1990).

Some studies have reported an inhibitory effect of glucose on the development of early cleavage stage embryos *in vitro* (Leppens-Luisier and Sakkas, 1997). Glucose was also seen to slow the embryo development during cleavage stage, if included in simple culture media, by diminishing their oxidative capacity (Quinn, 1995). However, apparently, the inhibitory effect of glucose is an artefact induced *in vitro* and is alleviated by the inclusion of amino acids and vitamins in the culture medium (Gardner and Lane, 1996). In spite of this, glucose has still tended to be lowered in culture media (there is none in P1 – first medium in a sequential media system, see Appendix 3) because there still is a tendency to believe that the cleavage stage embryo seems to use amino acids and carboxylic acid instead of glucose as energy source (Bavister, 1995 cited the replacement of glucose by glutamine in mouse embryo culture). Apparently, *in vivo*, the oocyte and the zygote are exposed to a low concentration of glucose in their environment: low glucose in tubal fluid and low metabolic activity of cumulus cells that during early cleavage stages use glucose and release pyruvate and lactate (Gardner *et al*, 1996). However, glucose has a very

important part to play in the embryo's metabolism after its early cleavage and before its compaction stage, when the embryo's genome is activated. A study following the metabolic fate of radio-labelled glucose in human embryos showed that glucose consumption and metabolism via glycolysis become more important with the development of the embryo to blastocyst stage, whereas earlier cleavage embryos rely more on oxidative metabolism (Wales *et al*, 1987). After the early stages, the embryo *in vivo* is no longer surrounded by cumulus cells and is exposed to higher glucose concentrations. Moreover, with the activation of the embryo's genome, the biosynthetic activity of the embryo increases and it will need glucose to synthesise certain nucleic acids (Reitzer *et al*, 1980). It is also essential for blastocyst hatching, when there is an absolute requirement for C-5 sugars in the pentose phosphate pathway.

Lactate

Lactate is added to embryo culture media for most mammalian species. It regulates pyruvate metabolism and maintains redox potentials (NAD+/NADH) (Bavister, 1995).

The embryo can encounter changes in concentrations as it travels along the reproductive tract: from the Fallopian tube to the uterus, it encounters an increasing concentration of glucose and a decreasing concentration of lactate and pyruvate. The changing requisites for substrates and metabolites are satisfied *in vivo* by the surrounding environment (Gregory and Leese, 1996). This requirement has been achieved *in vitro* by using at least two sequential media that vary in compositions and concentration of nutrients for different stages of embryo development (Martin, 2000)

Growth factors, antioxidants, antibiotics and other additives

Media containing various additives such as hyaluronate and vitamins still give low incidences of cleavage-stage mammalian embryos developing to blastocysts (Bavister, 1995). IGF-I, IGF-II, EGF, TGF-alpha, TGF-beta, PDGF-A, FGF-4 and LIF have all been shown to stimulate either cleavage, amino acid transport, protein synthesis, blastocoele formation or inner cell mass development. There appeared recently an optimistic study on blastocyst culture in which heparin-binding epidermal growth factor (Hb-EGF) was added to embryo cultured from day 2-7 post insemination. The stimulatory effect of Hb-EGF was only apparent from the 8-cell stage onwards (Martin *et al*, 1998). Some authors agree to the

benefits of also adding antioxidants to the medium, especially to reduce the oxidative stress created by high oxygen tension, exposure to light and the presence of transitional metals in the medium. The antioxidant taurine is beneficial for blastocysts development while hypoxanthine is detrimental. EDTA is also an important component of embryo culture in the early stages, since it chelates the free radicals created by copper and iron cations that arrest embryo development (Elder and Dale, 2000). Traditionally, antibiotics such as penicillin, streptomycin or gentamycin have always been included in embryo culture media (Gardner and Lane, 1999).

Salts and ions

Many studies have attempted to define the optimal concentrations for salts and other metabolites in culture medium, but it has been difficult to define the stage-specific requirements of the embryo because of all the different biochemical pathways' interactions that take place (Lawitts and Biggers, 1991).

Amino acids

Another issue concerning blastocyst culture media is what amino acids to include. Amino acids can give an embryo a higher capacity for development. Yves Menezo (Elder and Elliot, 1999) stated that there still is a knowledge gap about what amino acids are essential to humans, since no deprivation tests are available because of ethics. Human and bovine embryos cultured in a mixture of amino acids deplete individual amino acids at different rates at different stages of development. Amino acids are traditionally classed into one of two groups: I) essential amino acids (not synthesised by the body, have to be included in the diet) and non-essential amino acids (synthesised by pathways in the body). A very low concentration of essential amino acids, except for glutamine, has been reported in the oviduct of female mice. Essential amino acids added to the culture media before the 8-cell stage diminish the viability of embryos (Martin, 2000). However, after the 8-cell stage, essential amino acids help increase the development of the inner cell mass. The non-essential amino acids have been shown to have accelerate development, increasing the viability of 8-cell embryos and stimulating the formation of the blastocoele and the inner cell mass (Lane and Gardner, 1997). Late morula and blastocyst stage embryos use

essential and non-essential amino acids because transcription begins on day 3 and these will be necessary for further protein synthesis. In this way, the embryo culture media should have non-essential amino acids and glutamine in the first hours of culture, followed by a medium that will have both essential and non-essential amino acids.

The main problem with amino acids seems to come from their transport into the embryo. Probably some amino acids are competitive in the way that they block the entry of other essential amino acids by occupying common molecular transport systems. It is preferable to grow the media with the full panel of amino acids as we do not know specific needs. David Gardner (Elder and Elliot, 1999) recommends: ala, asp, asn, glu, gly, pro and ser. Methionine is used for transmethylation reactions involving proteins, phospholipids and nucleic acids. Via cysteine, methionine is used for taurine and hypotaurine synthesis. Cysteine is a precursor of cysteamine and glutathione, and redox coupling between these amino acids helps the embryo to maintain its redox potential and prevent damage from peroxidative reactions, therefore offsetting oxidative stress. Taurine can neutralise toxic aldehyde by-products of peroxidative reactions. Glycine is an energy source, has an osmoregulatory role, and chelates toxic divalent cations. As stated, some amino acids can be used as oxidizable energy substrates for the generation of ATP, they protect the embryo from free oxygen radicals, they recycle ammonia (alanine), they help maintain homeostasis (pH) and they are also important precursors for the synthesis of proteins and bases for nucleic acid synthesis (Gardner and Lane, 1996). However, when adding amino acids to culture media there is the problem of ammonia production: amino acids are metabolised (by the embryo and in the culture drop itself) to ammonia, which is harmful for embryos (Elder and Elliot, 1999).

Fatty acids

In several mammalian species there is evidence that exogenous fatty acids may be used as energy substrates or incorporated into lipid by the preimplantation embryo. Limited data indicate an increase in fatty acid utilisation during the preimplantation phase (Martin, 2000). Lipids can be synthesised (through C-2 condensation reactions), accumulated from

the surrounding medium or carried with albumin. Cholesterol synthesis is possible but slow (if it is inhibited, the embryo arrests and dies).

Other culture conditions

Finally, when culturing embryos, it is important to remember the requirement of using a buffer system (bicarbonate/CO_2, for example) that will maintain a physiological pH of between 7.2 to 7.4 in the medium (an oil overlay also slows pH changes when dishes are taken out of the incubator). Optimal culture conditions for the mammalian embryo also include the appropriate oxygen and carbon dioxide concentrations and the prevention of temperature fluctuations and exposure to strong lights (disrupts completion of meiosis and fertilisation). Some clinics prefer to culture their embryos in groups: the embryo produces factors capable of stimulating itself and the surrounding embryos, stimulating blastocyst formation, cell allocation to the inner cell mass and cleavage rate in mouse embryos (Ahern and Gardner, 1998). Other centres prefer to do it individually, since it becomes easier to track the embryo's individual development (Dr. A. Srikantharajah, Aberdeen Fertility Centre, personal communication), which is probably a better option in human ART, since it facilitates embryo selection for transfer.

I.5.A.5) Sequential media: what should be in it?

During the first phases of embryo culture, just one culture medium can support development. This is usually a simple medium with a basal salt solution (e.g. Earle´s balanced salt solution, EBSS), an energy source (pyruvate, glucose or lactate) and a protein source (e.g. human serum albumin). Embryos can use either exogenous energy substrates or endogenous lipids (Butcher *et al*, 1998). After the genome has been activated, the embryo has new needs and a second more complex medium should then come into use. Whereas later cleavage embryos require plenty of glucose, the relative pyruvate requirements are vice versa. Prior to genome activation, pyruvate and lactate seem to be good energy substrates (pyruvate would be better, since it removes toxic ammonium ions via transamination to alanine). This new medium for later stage embryos has to contain glucose as the main energy source, as well as amino acids, vitamins, hormones, growth factors,

nucleic acid precursors and was generally complemented with serum in the past or alternatives to serum in latter years (Gardner and Lane, 1997).

For optimal blastocyst development, it has been suggested that early-cleavage stage embryos be grown in simple media and from day 3 onwards, when the genome is being activated, the embryos should be transferred to complex media (Bongso, 1999).

I.5.A.6) Different options in sequential media

Recently, sequential media have been developed which take into account the changes in embryo physiology and requirements of the embryo, as it becomes a blastocyst. Some of the most famous sequential media include G1/G2, G1.2/G2.2, Universal IVF medium/M3, P1/Blastocyst medium (Irvine Scientific, USA) (Butterworth, 2001).

IVF 50 (simple)-S2 (complex) sequential combination from Scandinavian IVF Science (Sweden) has been reported to support 65 to 70% blastulation rates of good quality on day 5 post-insemination. Other observations, on G1.2 (complex)-G2.2 (supercomplex) sequential media combination, also from IVF Scandinavian Science (Sweden) show also equally good results: 43 % implantation rates for blastocyst transfer (Van Langendonckt *et al*, 2001), even though implantation rates of up to 70% have been reported by Gardner *et al* (2000 b) for transfer of two top-scoring blastocysts.

Recently, the developments of sequential culture media without serum that are quite similar to the female genital tract environment have given good results. With G1 being used from the zygote stage to the 8-cell stage and then G2 from 8-cell stage to blastocyst, the rates of blastocyst formation have been 66%, implantation rates 45% and pregnancy rates 63% (Gardner *et al*, 1998). These media expose the early embryo to lower concentrations of glucose and non-essential amino acids as well as higher concentrations of pyruvate, as we can see from the composition of G1/G2 in the Appendix 3. The older embryo (after 8 cells) is exposed to higher concentrations of glucose (and lactate) and a combination of essential and non-essential amino acids. Interestingly, taurine (has an important action as osmolyte and energy source), which is important for blastocyst formation, is only seen in G1, when it

might be important in G2 as well. These media are not supplemented with serum (G1 has HSA), which leaves part of contamination and abnormalities risks behind (Thompson, 1995).

In other sequential media, like P1/Complete Blastocyst Medium with SSS (Synthetic Serum Substitute) from Irvine Scientific, USA (see Appendix 3), the early medium has pyruvate and lactate as energy sources (but glucose is absent, maybe due to its negative effects) and the latter medium has more glucose and still some lactate in it. Interestingly, as in the G1/G2 case, only the early medium has taurine, while the latter (G2) has a whole range of amino acids, iron ions and vitamins in it. These media are recommended to be cultured with 10 % SSS (Irvine Scientific, 2003 http://www.irvinesci.com/web1/website/techinfo/index.cfm).

Throughout the years, a better understanding of the embryonic environment *in vivo* has taken place and has been translated into improvements in technology and media preparations. For instance, there are differences between G1/G2 and the more modern G1.2/G2.2 (see Appendix 3) released by the same company, Vitrolife, Goteborg, Sweden, (a new version of the sequential media, G3 has appeared but the company won't disclose its composition details). In the new generation of media, NaCl levels have increased. pH reasons could be the explanation. Na_2HPO_4 has been replaced by NaH_2PO_4 in a lower concentration in the new version, this could be explained by the better adequacy of this salt buffer, a decrease in the importance given to phosphate or it might just be a matter of keeping the right pH and osmolality in the final media. One hint could be that "One criticism that can be levelled at KSOM and G1/G2 is the presence of phosphate and glucose together. This may necessitate the presence of a mixture of amino acids whereas in phosphate-free medium (eg Basal XI HTF and P1), a single amino acid will suffice" (Patrick Quinn 9/1997, Embryo Mail http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl).
The issue of phosphate and its possible association with glucose does not seem to be that clear.

Another striking finding from the new generation of these media is the association of glutamine with alanine and also the fact that it appears in a lower proportion in the first medium (G1.2). Glutamine was found in the oviduct and seemed to have mostly a positive effect, but: "As far as amino acids are concerned, glutamine is not the only effective amino acid. Blocks to development (produced by increased osmolarity) are relieved by glutamine, but also by glycine, proline, beta-alanine and hypotaurine, among others." (Jay Baltz 9/97, EmbryoMail http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl). According to Gardner (Embryo mail 8/1997 http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl), glutamine is not as essential as it seems: "Our data indicate that glutamine is not as important as the literature would lead one to believe. This is probably because glutamine has been added as a sole amino acid in the mouse and human studies. Although glutamine is clearly beneficial, other amino acids are also as important (certain of the non-essential ones for example)."
Other authors believe glutamine is a major culprit in ammonia generation. Interestingly, there is no glutamine in P-1 (See Appendix 3) (Rusty Pool 9/97, Embryo Mail http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl).

Whereas the original G1/G2 did not contain serum as such, but did contained HSA (Human Serum Albumin), the new version does not. Actually, the original version contained BSA (Bovine Serum Albumin), but it was later changed. It is true that it is always more appropriate to have a human component than a bovine component in human egg culture media, but some diseases, like AIDS and so many human infections could be a risk with HSA (Tom Mc Evoy, Scottish Agricultural College, personal communication) as "there is a risk, no matter how small, when using blood products" (David Gardner, cit Elder and Elliot, 1999). Some authors argue that proteins are not important: "Our study shows that a chemically defined, protein-free culture medium supports the development and viability of cleavage stage human embryos equally well as standard medium containing proteins. This offers a means to avoid potential contamination with pathogenic organisms derived from

serum products.” (Barry Bavister 2/98, Embryo Mail http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl)

Patrick Quinn recently wrote (2/98, Embryo Mail http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl) Albumin still has supporters as being the “real thing”, after all, every time there is a scare, media components tend to be completely banned, even when, as in this case, they might have benefits. The scientific community and the public see them black or white and forget the grey: “Let's face it, the real reason albumin has been slammed recently is the scare of CJD contamination in blood donors (and lot to lot variation, eg unbound stabilisers, etc), but, I believe the chances of CJD actually being present in new lots of commercial HSA are "vanishingly small". This is especially so with the new, more stringent screening process of blood donors, as has been pointed out extensively by Rusty Pool in recent times. Cynically, I believe it is the same type of legal biased approach that has made us get rid of Percoll. So, why use protein for embryo culture? Granted, protein is not necessary but as I have said, let's try to mimic nature as close as possible and therefore add albumin (plus growth factors, fatty acids, etc, etc).” (Patrick Quinn, 2/98, Embryo Mail http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl)

Another component that has disappeared in the new series of media is phenol red. There were indications that it might have an estrogenic effect, but this could be an exaggerated idea, as when thoroughly tested in a number of estrogenic sensitive cell lines, it was found that the concentration of phenol red used in a typical culture medium was not sufficient to cause estrogenic effects. (Patrick Quinn 6/03, Embryo Mail http://www.lpsi.barc.usda.gov/scripts/odbic.exe/embryomail/boolean_search_output.tpl)

The development of sequential media has allowed an improvement in blastocyst transfer and therefore a higher control over the rate of multiple pregnancies, but certain factors about gametes and embryos are still unknown, therefore, cannot yet be corrected by modifying media.

I.5.A.7) Serum issues

Offspring of blastocyst culture in other mammalian species

Gardner and Lane (1993) showed that the presence of amino acids in the culture medium resulted in the production of ammonium, inducing neural tube birth defects after transfer in mice. However, if the medium is renewed every 48 hrs, ammonium concentration does not reach toxic level. In ruminants, there is a phenomenon called large calf/lamb syndrome. This syndrome appears after *in vitro* maturation and co-culture of embryos with somatic cells and serum. These pregnancies give rise to abnormally large offspring, many of which die (Behboodi *et al*, 1995; Mc Evoy *et al*, 2000). The problem seems to be the inclusion of serum in the culture medium as macromolecule, which overstimulates the embryo (Thompson and Peterson, 2000). Mammalian embryos are not exposed to serum *in vivo* (neither in the oviduct nor in the uterus). Serum seems to be an extremely variable pathological fluid that is also a reservoir for growth factors. It induces premature blastulation, changes in embryo morphology and perturbations in ultrastructure and energy metabolism. Ruminant embryos cultured to the blastocyst stage in the presence of serum, possessed mitochondria with an abnormal folding of the cristae, possibly associated with reduced oxidative capacity (Walker *et al*, 1992). There appears to be a link between the concentration of ammonium in the serum and the induction of foetal oversize (Sinclair *et al*, 1998)

If any abnormalities were to arise after extended culture in clinical ART, they might be expected to have arisen after the use of co-culture and serum, as was observed in sheep and cattle. However, there have been studies with co-culture and 15% serum and no birth abnormalities or difference in gestation length were reported (Menezo *et al*, 1999. cit. Gardner *et al*., 2000b). It could be that *in vitro* culture-induced large offspring syndrome is only found in ruminants. Serum is also reputed for facilitating hatching and avoiding the attachment of cells to plastic, as well as having antioxidant properties. However, it is strongly recommended that human embryos should not be exposed to whole serum at any time. Supplementation of culture media with serum for improving culture conditions may contribute to contamination and toxicity. A culture containing serum cannot be specifically chemically defined since trace elements change with every batch. The desired goal for IVF

is the culture of human embryos in a serum-free chemically defined medium (Ertzeid *et al*, 1999)

Alternatives to serum

Some alternatives to serum e.g. serum albumin (each batch has to be screened for quality control) have been developed. Many low-molecular weight potentially harmful components of serum do not appear in HSA. HSA would then be more controllable than serum. Recombinant human serum albumin has become available recently, eliminating part of the problems associated with blood derived products and variability. Another example would be PVA (PolyVinyl Alcohol), that has been used extensively by Bavister' s laboratory and could be a substitute for both albumin and serum (Mc Evoy *et al*, 2000). There also exists a synthetic serum substitute: SSS, which contains 84% Human Serum Albumin+16% alpha and beta globulins (Gardner and Lane, 1999).

I.5.B) Uterine environment

Some authors (Gardner *et al*, 1996) indicated that embryos change environments as they travel through the reproductive tract and so if early cleavage embryos are prematurely transferred to the uterus instead of the Fallopian tubes, this could account for implantation failure. The oviductal fluid is more alkaline and the uterine more acidic. The acidic environment of the uterus cannot be buffered by the early embryo, which may affect its metabolism. However, other authors (Bavister and Boatman, 1997) argue the human uterus is an excellent incubator for 4-cell and 8-cell embryos.

Only embryos with sufficient viable cells in the trophoectoderm, expressing the correct communication signals, will have the ability to implant and produce enough quantities of human chorionic gonadotrophin (hCG) to prevent luteolysis and menstruation. At the blastocyst stage, most cells are allocated to trophectoderm, showing how important it is. During implantation, the trophectoderm has to be working correctly and the mother has to give an adequate response to the implanting embryo. However, the inner cell mass also has to have a minimum size, since it will be the body of the embryo. Most anomalies that affect

embryo competence in humans seem to appear before the time of implantation (Hartshorne, 2001).

Another issue concerning blastocyst transfer is how the cervix changes between days 2 and 5. Since progesterone levels increase between days 2 and 5, the cervix loses its inner lubricating mucoid secretion. Mucus can act as a barrier between the embryos and the endometrium as well as interfere with the liberation of embryos in the uterus during the transfer procedure, blocking the catheter's opening, so the procedure might have to be repeated, resulting in violence to both the embryo (which is out of incubator) and to the uterine environment (Milki *et al*., 2000).

On the other hand, the endocervix contracts on day 5, so the lumen narrows and so even if there is less mucus, the passage of the catheter can become more difficult. But there also are less uterine contractions at day 5, therefore less chance for embryonic expulsion and loss (Gardner *et al*, 2000a)

I.5.C) Embryo metabolism and viability

An understanding of embryo metabolism has led to the development of tests for assessing embryo viability. These may be alternatives to the assessment of morphology already mentioned. These include (cited by Shoukir *et al*, 1997, except as indicated):

- Dye exclusion: survival of cells related to their ability to exclude the trypan blue dye.
- Fluorescence of degenerate cells: using dyes that have a great affinity for DNA (like DAPI: 4',6'-diamindino 2-phenylindole) that stain only the nuclei of degenerate blastomeres
- Production of fluorescent metabolites: the viability of preimplantation embryos can be assessed since poor embryos lose the ability to accumulate intracellular fluorescein.
- Production of human chorionic gonadotrophin (hCG): hCG production reflects embryonic health. The human blastocyst releases hCG into its surroundings and its quantification can be used as a measure of its viability

- Enzyme leakage: leakage of specific cytosolic enzymes (such as lactate dehydrogenase, LDH) into the surrounding medium is an indicator of embryo viability (plasma membrane integrity).
- Oxygen uptake measurements: a good measure of energy production is the consumption of oxygen, along with the glucose consumed and converted to lactate.
- Amino acid turnover: depletion/appearance of amino acids (Houghton *et al*, 2002) by liquid chromatography.
- Nutrient uptake measurements: using microfluorometric or microchemical techniques, you are able to find out the glucose uptake of blastocysts.
- Fluorometric assays: variety of procedures that can be used to assess embryo metabolism by the generation or consumption of molecules (like NADH and NADPH or ATP and ADP while consuming/producing glucose, pyruvate, glutamate)
- Nutrient uptake and metabolite release by single human embryos can also be obtained through metabolite (pyruvate, glucose, lactate) assays (Conaghan *et al*, 1993). For example, through ultramicrofluorescence methods (Gardner *et al*, 2001) or radio-labelling of pyruvate or glucose and measurement of how much has been used by the embryo. Embryos, which develop to the blastocyst stage, have consistently higher pyruvate uptake than those that arrest, indicating that this might form the basis for a quantitative biochemical criterion for embryo selection prior to transfer. Of greater significance is the work of Lane and Gardner (1996) who used glucose uptake together with lactate production to prospectively select mouse blastocysts for transfer.

There has always been a clinical need for tests of embryo viability, which can be applied at the blastocyst stage, when the embryo genome has been activated. This would hopefully let the embryology world switch to single blastocyst transfers with resultant pregnancy rates higher than 50% (Gardner and Leese, 1999).

I.5.D) Clinical aspects

One of the major concerns in IVF centres is pregnancy rates. These rates increase as the number of transferred embryos increases but so also do the rates of multiple gestations. The latter can lead to problems in costs, maternal morbidity, foetal problems, prematurity,

financial stress on both the marriage and the health care system, etc. The ideal all clinicians aim for is the perfect single embryo transfer (Salumets *et al*, 2003). It has been shown that the comparison between day 3 transfer and blastocyst transfer shows a higher pregnancy and implantation rate for the blastocyst transfer patients (Milki *et al* 2000). However, a higher incidence of monozygotic twinning has been reported in blastocyst transfers (higher obstetrical risk), the cause of which is not well known. Maybe atypical hatching separates two half-blastocysts or the zona pellucida is more fragile at the transfer moment and the blastocyst gets traumatised, leading to its division (Van Langendockt *et al* 2000).

Also, there is a decreased ectopic pregnancy rate if the embryo transfer is postponed to day 5. Blastocysts are capable of implantation soon after transfer, whereas early cleavage stage embryos are probably moving around the uterus for 2 or 3 days before implanting (Lesny *et al*, 1998).

I.5.E) Laboratory

To have the best results in cultivating blastocysts, the laboratory has to be in optimal conditions, with special attention paid to temperature, pH and sterility.

Not all laboratories are ready to take up blastocyst culture. Longer-term culture also demands more of embryologists (Dr. Tom Mc Evoy, Scottish Agricultural College, personal communication).

The laboratory requirements for successful blastocyst culture include:

- Evaluation skills: it is easier to spot something wrong in a 4-cell stage embryo (less evaluation parameters, cells are more distinguishable) than in a blastocyst. Early evaluation is more straightforward but later evaluation depends on better training (Dr. Tom Mc Evoy, Scottish Agricultural College, personal communication).
- More attention paid to sterility since there will be more activities in the lab,
- Intensive record keeping,

- Careful distribution of incubator space (no embryos should be "mixed", patients' embryos should be kept in different clear areas or shelves). Since culturing to blastocysts will require more days of culture, more incubator space is needed (always try to avoid crowded incubators to avoid mistaking different patients' dishes),
- Sequential media have to be bought (an extra cost)
- More time from the embryologists will also be required.

The cost:benefit ratio has to be then assessed. To take care of blastocysts, the lab will probably have to be working 7 days a week (many labs work 5 or 6 days), that means paying extra time to embryologists, clinicians and nurses.

Finally, it is also important that good cryopreservation of blastocysts is assured, especially since the number of embryos transferred in the fresh cycle is decreasing (Butterworth, 2001).

CONCLUSIONS

The success rates of human IVF remain relatively low when the number of pregnancies is considered in proportion to the number of embryos transferred. This leads to the transfer of more than one embryo, which increases the number of multiple pregnancies. A more efficient and rigorous procedure for embryo selection previous to transfer is needed. The reality is embryos are chosen mostly on a morphological basis. There are many options in terms of scores and different rates of development to perhaps help choose the best embryos. Blastocyst transfers have good implantation rates (40%) but, as discussed, culturing for so long is hard sometimes on the patients and the clinics. Many fertility clinics transfer their embryos on day 2 or 3, which also means more embryos tend to be preserved for future transfers. A good scoring system that allowed precise prediction of the embryo's viability at

earlier or later stages would be a crucial step forward. Questions that arise include: How to choose the best embryos in future? How accurate are the procedures used nowadays?

The following sections of this dissertation include a study which evaluated the assessment of embryo morphology at early preimplantation stages as a means to determine whether the assessment was predictive of development to blastocysts when bovine embryos were grown in sequential media.