IV) DISCUSSION

In this study, a model system to investigate a question relevant to human ART used bovine embryos because of the lack of availability of human material, and so this work would still provide an indication of the impact of early egg morphology on developmental pattern in another mammalian species, in which we have better opportunities to investigate. “Bovine ova and embryos may be the best available non-primate models for human embryos” (Bavister, 1995).

The preliminary experiment of this study was aimed at finding out the exact timing when the bovine model used would best correspond to the human situation in terms of prevalence of 4-cell embryos. That is because many clinics transfer eggs on day 2 in human ART, when the human embryos would then be around the 4-cell stage, the mean time for the second cleavage division being 45.7 hrs after insemination (Steer *et al*, 1992). The best time for scoring the bovine embryos in the Experiment 1 replicates in the present study was found to be a little later, between 48 and 52 hours post-insemination (43.1% and 32.3% at 3-4 cell stage). For determining this, SOFaaBSA was the medium used consistently as the initial medium in Experiment 1. The same medium also was used for early embryos in Experiment 2.

In Figure III.1A in the Results Section, it can be seen that more blastocysts developed and they emerged faster in sequential media: SOFaaBSA/SOF+ABS (23.3% of blastocyst formation) than in a simple medium like Treatment 1 (only SOFaaBSA, 6.15% of blastocyst formation). This would support the view, as stated in the literature review, about the convenience of sequential media for blastocyst culture (Bongso, 1999). According to Gardner and Lane (1997), the latter medium in sequential culture should also be generally complemented with serum. That recommendation was made 6 years ago, during the earlier stages of the serum controversy which then related almost solely to ruminant ART. Meanwhile, the reservations expressed by scientists at this laboratory (McEvoy and others) have been extended to include human ART considerations. As seen, the newer human IVF

media (G1.2/G2.2) tend to avoid the use of serum because of its lack of definition and the possibility of disease transmission, as well as its implications for foetal oversize in animals (McEvoy *et al*, 2000). Serum is still used in bovine embryo production systems because, according to other authors, its addition after early cleavage and before morula and blastocyst formation can lead to improved embryo development (Thompson and Peterson, 2000). Olson and Seidel (2000) reported that more embryos expanded to blastocysts and had larger surface areas when vitamin E was present in serum-supplemented culture medium. Vitamin E protects polyunsaturated fatty acids in the membranes, whose peroxidation can produce structural damage, affecting function and permeability of membranes, eventually resulting in irreversible cell injury and death. Oxidative damage happens to cells *in vivo* and *in vitro* by attack from free radicals generated by exogenous agents (radiation, chemicals, hyperoxia). Early mammalian embryos can suffer severe damage from reactive oxygen species and increase their production of free radicals *in vitro*. Vitamin E also protects ovine embryos from reactive species (Reis *et al*, 2003). For these reasons and to counteract the possible oxidising effects of serum, vitamin E was added to the culture medium in the 5 replicates in Experiment 2.

It is also interesting to note that Treatment 2 (SOFaaBSA + lipoprotein) gave a higher yield of blastocysts (9.09%) than when SOFaaBSA was used on its own. This suggests that provision of fatty acids may have been beneficial. One of the reasons Bavister (1995) thought BSA was important was because it has fatty acid components, which help the development of the embryo to the morula stage, even though the specific roles of lipids are still largely ignored. However, a point could be made that some embryos develop slower than others but "catch up" at some stage. Figure III.1.B in the Results section illustrates the rate at which blastocysts developed in Experiment 1. Treatment 1 and 2 were similar (21.43% and 20.83% of the blastocysts had appeared at 140 hrs vs 13.3% for treatment 3) but although blastocyst formation on Treatment 3 started more slowly, it caught up more rapidly in the end (28.57%, 25% and 46.67% of blastocysts had appeared by 152 hrs). This shows how some "slower" embryos can also reach the blastocyst stage, with this being dependent on individual genotype, on the medium and on the time they are left in culture. Maybe there is an adaptation period when embryos are shunted from the first medium of the sequential series to the next. It has been indicated (Mc Evoy *et al*, 2000) that certain

batches of serum decrease the number of blastocyst cells and increase apoptotic activity, and therefore it is reasonable to presume that a small shock for the embryos occurs before they become properly adapted to the new sources of nutrients. A great deal of scientific research and analysis has been dedicated so far to the development of media that will be able to support the development of human embryos. However, no one knows about the long-term effects of human blastocyst culture on future development (Butterworth, 2000). As sequential media are not yet perfect (Martin, 2000), one cannot discard the possibility of negative effects on those children that, at the embryonic stage of their existence, have been exposed for longer than 48 hours to *in vitro* conditions.

The metabolic requirements of mammalian embryos are not easy to understand. It is clear that blastocysts have to increase their protein synthesis, therefore needing histones, proteins and antigens (Martin, 2000). Thus the relevance of sequential media in blastocyst culture is clear and, in Experiment 1, embryos had a better development with serum present. Prepared and filtered heat-inactivated maternal serum has in the past been added to IVF culture media. Then it was substituted by albumin and later, PVA (synthetic polymer). It is true that these also have good implantation rates (Elder and Dale, 2000). In spite of everything that has been said about the risks of the chemically undefined serum (and the problems in bovine, Mc Evoy *et al*, 2000), it has given good results in humans, and it was used for some time in clinics. The new generations of artificial polymer media have non-physiological compounds that could turn out to have unexpected consequences in the long term. Thus serum is not the sole source of possible adverse effects and, for best practice, all constituents of culture media should be screened fully and used cautiously until proven to be safe.

The aim of the main study (Experiment 2) was to test the validity of the day-2 (post-insemination) selection of embryos in IVF clinics and to determine whether the selection was associated with embryos that continued to be viable, in opposition to the unselected group.

On day 2, all 5-8 cells embryos in the present study were “selected”. This reflects the fact that priority was given to rate of cleavage sometimes even over grade. This is in agreement with the idea that the faster the cleavage, the more possibilities of

pregnancy (Steer *et al*, 1992). The "selected" cohort also had a high prevalence of grade 1, which was to be expected. This is the usual scenario in an IVF clinic. If transfer will take place on day 2, the most advanced embryos with the better morphology will be selected for transfer, the ones that still would be viable but are not "as good" would be cryopreserved for a future embryo transfer and the rest discarded. However another question arises: if, as in the preliminary results (Experiment 1), blastocysts behaved differently according to time of emergence (sometimes the ones that cleaved more slowly at the beginning cleaved faster in the end), what would happen if the slower embryos were allowed to "catch up" with the rest?

Results on day 4, after the genome has been activated (it becomes activated around the 4-cell stage) and two days after the initial selection in Experiment 2, indicated that the most slowly cleaving embryos hadn't caught up (see Table III.2.3f). Maybe the slower cleaving embryos came from poorer oocytes and so the embryos derived from them were also poor (Gardner *et al*, 2000b). Replicate number 2 on day 4 yielded no "selected" embryos with 9 to 16 cells, and so all of the embryos had less than 8 cells; this replicate also had an intermediate cleavage rate (78%). At day 6, a different one of the replicates, number 5, was poorer than the rest (Table III.2.4.e) and had fewer morulas from "selected" embryos; it also was one of the replicates with an intermediate cleavage rate (77%). Variability in oocyte competence may have contributed to such variation during subsequent development, since gamete's quality (probably the reflection of its genetic condition) can affect subsequently embryo's viability.

The number of grade 1-2 blastocysts obtained on day 7 was also significantly different between "selected" and "unselected" cohorts. This finding supports the initial hypothesis. However, if considering cell counts and diameters of blastocysts obtained on day 7 from both the selected and the unselected group, it is evident that the difference is not significant between the "survivors" in selected and the unselected group. This means that even though fewer blastocysts were obtained from unselected eggs, some of the unselected embryos were capable of reaching the blastocyst stage with appropriate rates of development and appropriate cell numbers. This may reflect the fact that in the present study not all "selected" eggs were inferior. The design applied a rigid 5:5 segregation policy rather than a flexible good:poor separation.

Even so, this indicates that any policy which restricts embryo selection (e.g a limit on numbers of eggs to be spared) could be condemning viable embryos to destruction.

As observed in the literature review (see Fig. I.1.f), the early embryo classification systems are all based on the same criteria: fragmentation, rate of cleavage, symmetry of blastomeres. Fragmentation doesn't allow good communication between blastomeres (Hartshorne, 2000) and unevenness of daughter cells can be caused by abnormal chromosomal function (Scott *et al*, 2000). Indeed, irregularity in blastomeres has been found to lower pregnancy rates (Giorgetti *et al* 1995). The importance of cell cleavage speed as a major quality marker has been considered elsewhere (Bongso 1999). These are all then justified and seemingly logical criteria for embryo selection, even though, as in this study, they have been partial rather than perfect predictors of subsequent viability. Thus, even if most of the embryos rejected on these criteria degenerate, it is likely that some of the embryos thereby discarded on day 2 were nevertheless inherently viable embryos after all. Since the implantation rate of an early cleavage embryo is about 20%, 80% of the embryos selected for transfer will not develop. Graham *et al* (1999, cit by Butterworth, 2001) revealed that 48% of embryos that would have been chosen for transfer on day 3 developed to become blastocysts. The rest didn't. Apparently, and as confirmed by this study, morphological criteria for selection on day 3 are not entirely reliable. One problem is that some abnormal embryos can still develop in culture (Munne and Cohen, 1998). At the moment, other parameters to assess embryos as polarity and morphology of the pronuclei are being researched, as well as information about cleavage symmetry (Butterworth, 2001).

What certainty can a clinic ("that transfers embryos on day 2") have of not discarding viable embryos? There is, as already said, no point in extending the culture if the implantation and pregnancy results are not good enough following day 2-3 transfer. But what about the moral uncertainty of discarding viable embryos? What would the impact on the patients' psychology be then? It must be remembered, that very few embryos have a perfectly "normal" appearance (many blastomeres show multinucleation as a normal part of their development (Kligman *et al*, 1996)) and many embryos that have seemed unpromising have implanted and subsequently developed into a healthy baby. However, it must also be recognised that merely by

culturing to blastocyst, we won't necessarily obtain a better embryo. Blastocyst culture contains an increased risk of failure: you can end up having nothing to transfer, which would both affect the couple psychologically (Scott, 2002) and would also affect the finances of the clinic. Transferring at day 3 would mean lower culture media costs, more possibility of transfers (satisfied patients) and less extra time and expense of the working team (Dr. Maureen Wood, personal communication). If the embryos of a couple don't make it to blastocyst stage, then there is no transfer, which means one less procedure for the woman but also the loss of many hopes. This is very difficult for patients to accept. There are usually questions and doubts as to whether the embryos wouldn't have developed better in the uterine environment had they been transferred on day 2. No matter how improved an *in vitro* environment is, it can hardly ever be as perfect as healthy *in vivo* conditions. On the other hand, it can also give information to couples as to whether their embryos are ever able to reach the blastocyst stage and so time, financial and emotional costs at least are minimised if not eliminated and the couple for whom IVF is not feasible can start searching for alternative options to become parents.

In a clinical ART setting, the risk of having nothing to transfer should be balanced against the risk of rejecting viable embryos. Should then more embryos be cryopreserved after the transfer, just in case? In the early days of cryopreservation, many "poor" embryos tended to be cryopreserved, but as time has elapsed, and noticing that this procedure is quite harsh on the embryo (Shaw *et al*, 2000), the tendency nowadays is to cryopreserve mostly the "good quality" ones (Dr. Lidia Cantu, Montevideo Fertility Center, personal communication).

Blastocyst culture and transfer is indicated in human ART to avoid multiple pregnancies and so only one or two such embryos will be transferred. It also reduces the risk of tubal pregnancies. Now that PGD (preimplantation genetic diagnosis) has been developed, blastocyst culture can be used to select the "genetically" viable embryos. Even though aspects can be improved, the implantation rate reported after blastocyst culture and transfer is promising (40-50%) (Gardner *et al*, 1998, Behr *et al*, 1999). Maybe, since most selection in human clinics is done from the morphological point of view, in the future it could be possible to identify and select the best embryos in the earlier stages of development, transfer those promptly, and give the remaining

embryos extra time to “self-select” naturally against chromosomal abnormalities or unviable traits by extending the culture before cryopreserving healthy survivors. If viable oocytes could be spotted, then maybe the selection follow-up wouldn’t have to go as far as the blastocyst stage and early stage embryos could be selected for transfer and cryopreservation on a stronger basis. Selection would then be based on at least two criteria at two very distinct stages and so hopefully be more accurate.

FINAL CONCLUSIONS

Overall, the proportion of newly fertilised human embryos capable of implantation remains low, whatever scoring criteria are applied. This is undoubtedly a very upsetting feature of human ART and it indicates low fertility in our species. Maybe all IVF is about is selecting the best embryos a couple has, as identified at whatever stage a particular clinic believes to be most appropriate. As seen with this study, controversy on the best time for embryo transfer is still present. There still need to be explanations as to why sperm samples have so many abnormal spermatozoa, why oocytes and embryos have so many chromosomal anomalies and why the latter have such low implantation statistics, when a woman only has about 500 cycles in her whole life, each time ovulating a single egg. Should one be looking at obtaining the best egg instead of the best embryo? (Dr. Maureen Wood, personal communication). Are we the only species to display such a loss of reproductive potential? It has been said that “perhaps cranial size and prolonged parent-child bonding have modified evolutionary pressures and restricted human reproduction to a few births.” (Edwards and Beard, 1999).