VIABILITY OF IN VITRO PRODUCED CATTLE EMBRYOS

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1. SUMMARY

Embryo transfer is being increasingly used in the cattle industry. As well as direct embryo transfers, many embryo-based biotechnologies have the potential to improve cattle production efficiency through enhanced breeding strategies, by facilitating the introduction of desirable traits such as disease resistance and through the production of desirable medical or pharmaceutical products in the milk. These biotechnologies are, however, dependent on a supply of viable *in vitro* produced (IVP) embryos. While the *in vitro* fertilization rate is high (80%) in cattle, only about 30 transferable embryos, or blastocysts, are produced from every 100 fertilized oocytes. A major factor affecting the viability of IVP embryos is their failure, in a high proportion of cases, to undergo normal development to the blastocyst stage in the manner of *in vivo* embryos. The major problem relates to a failure of the cells of IVP embryos to form a compact cell mass when they are 5 - 6 days old. This ultimately leads to developmental problems and compromised viability. Cell compaction is recognized as a critical event in early embryo development and has been associated with marked changes in protein synthesis and phosphorylation in the embryos of some species. This report is the first, to our knowledge, to describe the rate and pattern of protein synthesis and phosphorylation before, during and after compaction in both *in vivo* and in IVP cattle embryos. The main results are summarised below.

Compaction and protein synthesis

- Protein synthesis increased in both *in vivo* and IVP embryos from the 8-16-cell stage to the blastocyst stage, at one week old.

- Protein synthesis was similar in both *in vivo* and IVP embryos at the blastocyst stage but was higher in IVP embryos at all other stages.

- Only minor differences were evident in the pattern of proteins synthesised at the different developmental stages within *in vivo* and IVP embryo categories.

- A major difference, however, between *in vivo* and IVP embryos up to the blastocyst stage was the increased synthesis by IVP embryos of a protein with an apparent molecular mass of 60kDa. By the time embryos had reached the

blastocyst stage, however the pattern of protein synthesis was similar between *in vivo* and IVP embryos.

Compaction and phosphorylation

- Protein phosphorylation increased from the 8-16-cell and compacting stages to the compacted and blastocyst stages and remained at a similar level at the blastocyst stage in both *in vivo* and IVP embryos.

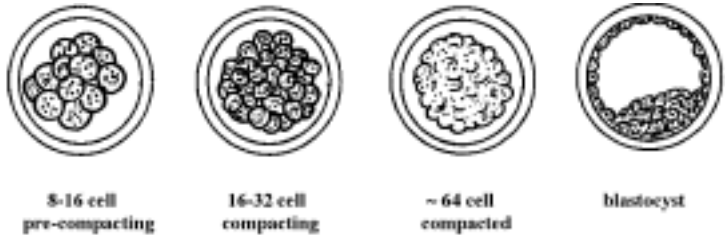
- There was no difference in the rate of phosphorylation between *in vivo* and IVP embryos at any of the developmental stages studied.

- The pattern of protein phosphorylation indicated the phosphorylation of one particular protein with an apparent mass of 65kDa at all *in vivo* and IVP stages studied. It appears that this may be due to the synthesis and phosphorylation of a newly synthesised protein rather than phosphorylation of a constitutive protein.

- The pattern also indicated the phosphorylation of two proteins with an apparent molecular mass of 40 and 26kDa, present in both *in vivo* and IVP embryos and which first appear at compaction.

2. INTRODUCTION

Embryo transfer is being increasingly used in the cattle industry. According to the International Embryo Transfer Society more than 500,000 *in vivo* produced cattle embryos were transferred to recipients world-wide in 2000 and the number of transfers is increasing each year. As well as direct embryo transfers, many embryo-based biotechnologies have the potential to improve cattle production efficiency through enhanced breeding strategies, by facilitating the introduction of desirable traits such as disease resistance and through the production of desirable medical or pharmaceutical products in the milk. These biotechnologies are, however, dependent on a supply of viable IVP embryos. While the *in vitro* fertilisation rate is high (80%) in cattle, only about 30 transferable embryos, or blastocysts, are produced from every 100 fertilised oocytes. A major factor affecting the viability of IVP embryos is their failure, in a high proportion of cases, to undergo normal development to the blastocyst stage in the manner of *in vivo* embryos. The major problem relates to a failure of the cells of IVP embryos to form a compact cell mass when they are 5 - 6 days old. This ultimately leads to developmental problems and compromised viability. Compaction is recognised as a critical event in early embryo development. Following fertilisation the embryo divides approximately once every 24 hours and at 4 days old individual cells or blastomeres number approximately 16 and are distinct and separate. From the 16-cell stage onwards, in normally developing embryos, the blastomeres begin to pack together and form a compact cell mass. At this time intracellular junctions called "tight junctions" are formed between individual blastomeres. When the compaction phase is complete the embryo then forms a cavity in the compact mass of cells, termed the blastocoele cavity and the cells or blastomeres differentiate into



Different stages of cattle embryo development from the pre-compacting 8-16 cell to the blastocyst stage

inner cell mass cells, which becomes the foetus and trophectoderm cells which become the placenta. Tight junction formation is necessary for intracellular communication and ultimately for normal embryo development to proceed successfully. Failure of this junction formation and cell compaction in early embryos results in reduced viability, A high proportion of IVP embryos do not compact properly but "jump" from the 16 cell non-compacted stage directly to the blastocyst stage.

Cell compaction has been associated with marked changes in protein synthesis and phosphorylation in the embryos of some species. Tight junction formation, necessary for compaction, requires the synthesis and phosphorylation of specific proteins. However, to-date there is no published information on the occurrence of protein synthesis and phosphorylation during compaction in cattle embryos. In this project which was part of an EU funded transnational program to study cattle embryo compaction and viability we have characterised embryo development with regard to the rate and pattern of protein synthesis and phosphorylation, before, during and after compaction in both *in vivo* and in IVP embryos.

Objectives

To measure the rate and pattern of protein synthesis and phosphorylation by *in vivo* and IVP cattle embryos before, during and after compaction. These objectives are addressed in a number of studies, the results of which are summarised in this report.

3. METHODOLOGY

Oocytes, or immature ova prior to fertilisation were collected by aspiration of follicles from cattle ovaries. IVP embryos were produced using standard serum-free and cell-free embryo culture conditions. *In vivo* produced embryos at various stages post fertilisation were recovered from crossbred heifers. All embryos produced were graded for normality and for presumed viability on a morphological scale from 1 (excellent) to 5 (degenerate). Only grade 1 and 2 embryos were included in the study. Embryos were washed in standard media and prepared as appropriate for the various measurements and, or, assays. In some situations embryos were snap frozen in liquid nitrogen and stored at -80^{O}C prior to assay.

Incorporation of ^{35}S-methionine into embryonic protein during a 4-hour culture period was used as an index of *de novo* protein synthesis, while incorporation of ^{32}P-orthophosphate was used to measure protein phosphorylation. *De-novo* protein synthesis and phosphorylation was determined in *in vivo* and IVP embryos of 4 to 7 days old. The rate of protein synthesis and phosphorylation was measured following a 4-hour incubation with ^{35}S methionine or ^{32}P orthophosphate. Total uptake and incorporation into TCA insoluble material was measured by liquid scintillation spectrometry. Samples of labeled embryos were also subjected to 1-D PAGE and phosphor imaging to determine the pattern of *de novo* protein synthesis and phosphorylation.

4. PROTEIN SYNTHESIS AND PHOSPHORYLATION DURING COMPACTION

A total of 137 *in vivo* and 242 IVP embryos from the 8-cell (pre-compaction) to the expanded blastocyst stage (post compaction) were cultured in the presence of ^{35}S methionine (n=238) or ^{32}P orthophosphate (n=141). The incorporation of ^{35}S methionine into TCA insoluble material at >70% and ^{32}P orthophosphate at >6% allowed us, in the case of the expanded blastocyst, to subject the lysate of a single embryo to electrophoresis on 1-D gels. In the case of the 8-16 cell pre-compaction stage it was necessary to pool up to 15 embryos for 1-D analysis.

Compaction and protein synthesis

^{35}S methionine uptake increased in both *in vivo* and IVP embryos from the 8-16-cell stage to the blastocyst stage. Uptake at the blastocyst stage was similar for IVP and *in vivo* embryos, however, uptake at all other stages was higher for IVP embryos ($P<0.05$). Uptake by *in vivo* and IVP blastocyst stage embryos was also higher ($P<0.05$) than that of any other respective stage. The incorporation of radiolabel into TCA insoluble material (Fig. 1) mirrored the pattern of uptake.

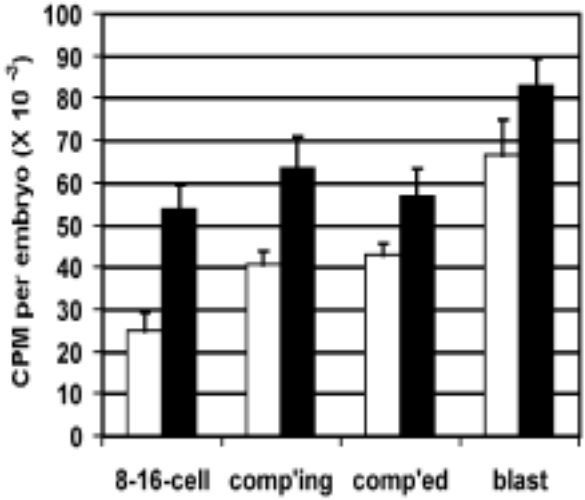


Figure 1. Incorporation of ^{35}S methionine by in vivo (❑) and IVP (■) embryos

Incorporation increased ($P<0.05$) from the 8-16-cell stage to the blastocyst stage in both *in vivo* and IVP embryos. Incorporation, however, was not different between the 8-16-cell, compacting and compacted stages. Incorporation was similar for *in vivo* and IVP blastocysts and was higher in IVP embryos at all other stages ($P<0.05$). Following 1-D SDS-PAGE of ^{35}S methionine labeled embryos and phosphor imaging, only minor differences in the pattern of *de novo* protein synthesis were evident between the different stages within *in vivo* and IVP embryo categories (Fig. 2).

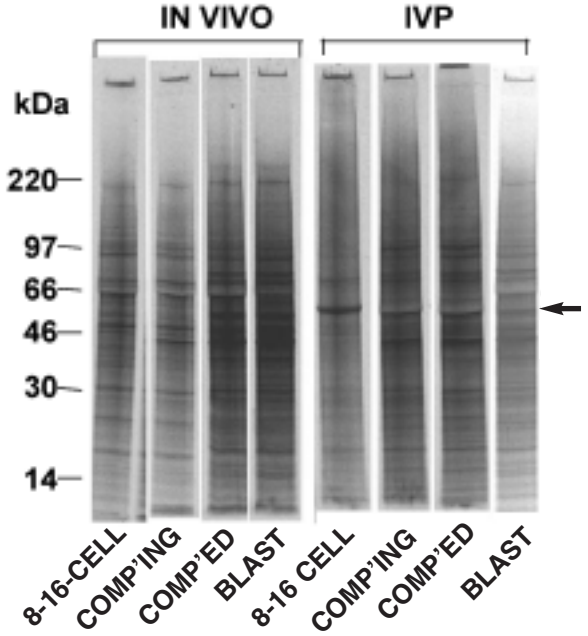


Figure 2. Pattern of de novo protein synthesis by in vivo and IVP embryos

A major difference, however, evident between the *in vivo* and IVP category embryos at the 8-16-cell, compacting and compacted stages was the increased synthesis by IVP embryos of a protein with an apparent molecular mass of 60kDa. By the time embryos reached the blastocyst stage, however the pattern of protein synthesis was similar between *in vivo* and IVP embryos.

Compaction and phosphorylation

Uptake of ^{32}P orthophosphate in *in vivo* and IVP embryos increased numerically from the 8-16-cell and compacting stages to the compacted and blastocyst stages, but these increases were not significant ($P > 0.05$). Uptake was not different between *in vivo* and IVP embryos at any of the developmental stages studied. The pattern of ^{32}P orthophosphate incorporation into TCA insoluble material (Fig. 3), was similar to that of uptake.

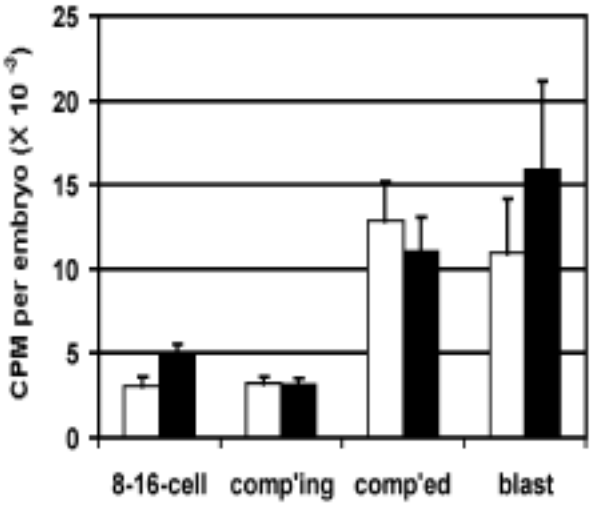


Figure 3. Incorporation of 32P orthophosphate by in vivo (❑) and IVP (■) embryos

The percentage incorporation, however, tended to be higher in *in vivo* compared to IVP embryos. Incorporation of ^{32}P orthophosphate via ATP into TCA insoluble material was not different between 8-16-cell and compacting stage embryos between *in vivo* and IVP embryos at any of the developmental stages studied, however, it increased at the compacted stage ($P < 0.05$) and remained at a similar level at the blastocyst stage in both *in vivo* and IVP embryos. Neither was there any difference between *in vivo* and IVP embryos at any of the developmental stages studied. SDS-PAGE of embryos labeled with ^{32}P orthophosphate indicated the phosphorylation of one particular protein with an apparent mass of 65kDa phosphorylation at all stages studied (Fig. 4).

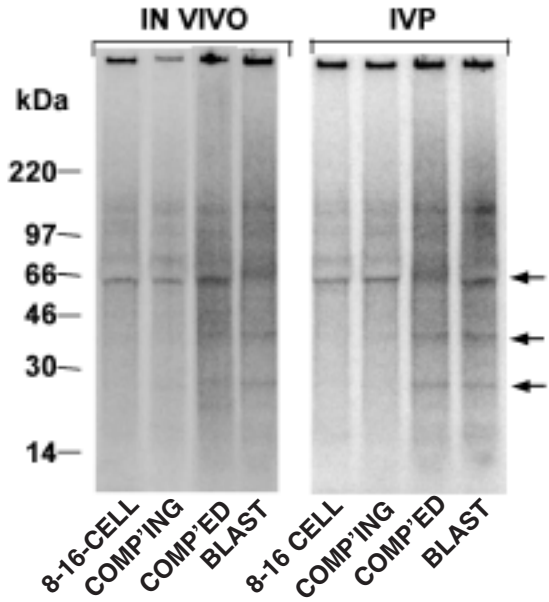


Figure 4. Pattern of protein phosphorylation by in vivo and IVP embryos

When this pattern was compared to embryos labeled with ^{35}S methionine it appears that these changes may be of a synthetic nature rather than phosphorylation of a constitutive protein. SDS-PAGE also indicated the presence of two prominently labeled bands appearing at compaction with an apparent molecular mass of 40 and 26kDa and present in both *in vivo* and IVP embryos.

5. CONCLUSIONS

These results indicate that while protein synthesis increases from the 8-16-cell stage to the blastocyst stage only minor increases occur during compaction itself. The apparent increase in *de novo* synthesis by IVP embryos before blastocyst formation may indicate that IVP embryos are attempting to 'catch up' synthetically. The increased synthesis of a protein with an apparent molecular mass of 60kDa by IVP embryos suggests that this is possibly a heat shock protein (HSP60) and that at least some IVP embryos may be responding to the culture conditions. The similarity of the synthetic pattern between *in vivo* and IVP embryos at the blastocyst stage suggests that by the time IVP embryos have blastulated they are developmentally similar to in vivo embryos. The increase in phosphorylation and the appearance of two newly phosphorylated proteins at compaction could be the first biochemical indicators of compaction.

6. PUBLICATIONS ARISING FROM THIS PROJECT

Morris, D.G. Diskin, M.G. and Sreenan, J.M. 2001. Viability of *in vitro* produced cattle embryos, In:*Proceedings of the Agricultural Research Forum, Tullamore,, 3rd-4th September,* p73, and abstract in *Irish Journal of Agricultural and Food Research 39 (3):465.*

Morris, D.G. Diskin, M.G. and Sreenan, J.M. 2001. De-novo protein synthesis and phosphorylation by *in vivo* and IVP cattle embryos around compaction. In: Proc. *Association Europenne De Transfert Embryonaire, Lyon, France, p152.*

Morris, D.G., Diskin, M.G. and Sreenan, J.M. 2001. Biotechnology in Cattle Reproduction. Teagasc ie 26pp.

Sreenan, J.M., Diskin, M.G. and Morris, D.G. 2001. Embryo survival in cattle: a major limitation to the achievement of high fertility. In: *Fertility in the high producing dairy cow BSAS Occasional Publication, Vol 1, pp 93-105.*

Morris, D.G., Humpherson, P., Leese, H.J. and Sreenan, J.M. 2001. Protein and energy metabolism in the pre-implantation cattle embryo. In: *Fertility in the high producing dairy cow BSAS Occasional Publication Vol 2, pp 443-446.*

Morris, D.G. and Sreenan, J.M. 2001. Biotechnology in Cattle Reproduction. Farm and Food 11: No 1, p12-15.

Carolan C., Sheth, B., Morris, D.G. and Kane, M.T. 2000. Immunolocalization of E-cadherin and ZO1a+ in bovine *in vivo* and *in vitro* produced (IVP) embryos Journal of Reproduction and Fertility Abstract Series 25, 75.

Morris, D.G., Diskin, M.G. and Sreenan, J.M. 2000. Protein synthesis and phosphorylation by elongating 13 to 15-day old cattle blastocysts. *Reproduction Fertility and Development 12, 39-44.*

Morris, D.G., Humpherson, P and Sreenan, J.M. 2000. Amino acid metabolism by elongating cattle embryos. In:*Proceedings of the Agricultural Research Forum, University College Dublin, 14th-15th March,* pp19-20, and abstract in *Irish Journal of Agricultural and Food Research 39 (3):465*.

Morris, D.G. and Sreenan, J.M. 2000. Protein synthesis and phosphorylation by elongating day 13 to day 15 cattle embryos In: *Proceedings of the Annual Conference of the International Embryo Transfer Society, Maastricht, The Netherlands 9-11th January In. Theriogenology 53, p 280*

Sreenan, J.M, Leese, H.J., Morris, D.G. and Dunne, L.D. 1999. Early embryo development and survival in cattle. *Cattle Practice* 7:267-270