In vitro culture and embryo metabolism of cattle and sheep embryos — a decade of achievement

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Abstract

At the beginning of the 1990s, co-culture of cattle and sheep embryos was the most favoured method to support embryo development, but the use of this system has hampered progress in raising the efficiency of embryo production. Furthermore, little was known of the requirements of embryos and the biochemistry of early embryo development. As the decade progressed, energy metabolism studies improved our understanding of the energy substrate requirements for embryo development. Furthermore, an appreciation of the reproductive tract environment increased. This resulted in more “defined” systems, which have evolved further in the development of “sequential” media systems, where components change in accordance to the needs of the embryo. Nevertheless, wholly defined systems, such as the replacement of albumin with PVA, are less able to support similar levels of development as protein-containing medium, and the resulting embryos are metabolically compromised. This highlights the nutritive role of albumin. One area in which much work has been conducted, but yet no unifying theory has emerged, is that of the interactive roles of growth factors (including autocrine/paracrine), cytokines and extra-cellular matrix molecules in the development of a viable embryo. A new concept is that of regulation of energy metabolism. Compounds such as ethylenediamine tetraacetic acid (EDTA), NaN₃ and 2,4-dinitrophenol have been shown to increase embryo development and quality of resulting embryos. This demonstrates that the process of ATP production is a key regulator of in vitro embryo development. © 2000 Elsevier Science B.V. All rights reserved.

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1. Overview of the development of cattle and sheep embryo culture systems

At the beginning of the last decade of the 2nd millennium, very little was known about the metabolic requirements of ruminant embryos. There was a general assumption that they were similar to the metabolic patterns of other mammalian embryos, especially the mouse. The pioneering work of Brinster, Biggers, Wales, Whittingham and others...
had shown that early development (1- to 8-cell stage) in the mouse was supported by the oxidation of carboxylic acids pyruvate and lactate, especially pyruvate. Glucose was poorly utilised, which was attributed to a block to a regulatory glycolytic enzyme, phosphofructokinase (Barbehenn et al., 1974). During progression through compaction and blastulation, this block was removed and glucose contributed increasingly to ATP production via oxidation, so that mouse morula development to the blastocyst stage could be supported by glucose alone. Formulations, such as M16 (Whittingham, 1971), which included glucose, pyruvate and lactate, were found to be effective in the development of 2-cell embryos of most mouse strains (and 1-cell embryos from hybrid strains).

Against this background, the pioneers of ruminant embryo culture struggled to develop comparably successful systems (e.g. Wright and Bondioli, 1981). Tervit et al. (1972, 1974) described ‘‘Synthetic Oviduct Fluid’’ (SOF), a culture system that produced viable blastocysts from early cleavage stage embryos produced in vivo from both cattle and sheep. However, little uptake of this system was initially attempted, with the exception of Walker et al. (1989). Indeed, for ruminant embryos, there were conflicting reports as to the appropriate in vitro requirements. For example, Betterbed and Wright (1985) advocated the use of concentrations of glucose of 5.6 mM and an atmospheric oxygen concentration for the in vitro development of sheep embryos, whereas Tervit et al. (1972) had advocated a low O₂ tension and energy substrate levels based on oviduct concentrations. Underpinning these conflicting results was the inability to reliably culture ruminant embryos through the so-called ‘‘8- to 16-cell developmental block,’’ a culture-induced phenomenon which was irreversible but did not result in immediate embryonic death (Wright and Bondioli, 1981; Eyestone and First, 1991). Due to a general lack of understanding as to what caused the block to development and the few but highly variable results obtained with SOF and other simple media, an alternative strategy was sought which led to the advent of co-culture systems (Gandolfi and Moor, 1987; Eyestone and First, 1989). Co-culture significantly advanced the application of in vitro embryo production, in as much as viable blastocysts were obtained. Nevertheless, in terms of our understanding of factors influencing embryo development, much less information has been generated using co-culture systems compared to defined systems (Bavister, 1995). Furthermore, positive factors influencing embryo development, such as a low O₂ environment and low glucose levels, which have been attributed to the success of co-culture conditions (Watson et al., 1994b; Edwards et al., 1997), were first identified using defined systems.

2. Oxygen, carboxylic acids, carbohydrates and lipids

Utilising both radioisotope-labelled substrates (e.g. Rieger and Guay, 1988) and non-invasive quantitative fluorescence-linked substrate uptake/production assays (e.g. Gardner et al., 1993), the rates of oxygen uptake, uptake/production and utilisation of carboxylic acids and glucose have been well documented over the past 10 years. As a brief synopsis, Fig. 1 represents the relative contributions of glycolysis and oxidative phosphorylation to ATP production in early and post-compaction stage cattle and sheep.
embryos. In general, embryos throughout pre-elongation development are reliant on oxidative phosphorylation (via oxidation of pyruvate and amino acids) for the generation of ATP for embryo development (Javed and Wright, 1991; Rieger et al., 1992a,b; Gardner et al., 1993; Thompson et al., 1996, 2000). However, there is a switch to an increased contribution of glycolysis during compaction and blastulation (Gardner et al., 1993; Thompson et al., 1996, 2000). Failure to depress glycolysis during pre-compaction is one factor associated with the “8- to 16-cell” developmental block (Gardner et al., 1997; Thompson et al., 1992a). Therefore, culture media should be designed to suppress glycolysis during pre-compaction development followed by removal of the suppression during post-compaction development. The former is often confused with complete removal of glucose from culture medium throughout pre-elongation development. However, this is unlikely to benefit the embryo, as glucose plays other roles including ribose and NADPH production through the pentose-phosphate pathway. In particular, ribose synthesis is important for the embryo, as this molecule is a precursor for DNA
and RNA synthesis, which is essential for embryonic development. Such metabolic intermediates have been detected in sheep embryos following incubation with [U-14C]-glucose, as well as incorporation into non-glycogen macromolecules (Thompson et al., 1995a).

There remains one major question regarding the metabolism of these substrates. Why is glucose poorly oxidized through the tricarboxylic acid cycle, when exogenous pyruvate appears to be readily oxidised via this pathway? The current and yet inadequately tested hypothesis is that there is a block to the NAD+/NADH shuttle mechanism across the mitochondrial membrane. Thus, to maintain an appropriate redox equilibrium, cytoplasmic NADH must be oxidised in order to maintain glycolytic flux (Thompson et al., 1993; Edwards et al., 1997). Thus, glucose is nearly 100% converted to L-lactate, especially at the blastocyst stage (Gardner et al., 1993; Thompson et al., 1996). A possible explanation for this phenomenon is that there is substrate compartmentalisation within the cytoplasm of the developing embryo. Glucose-derived pyruvate is almost entirely converted to lactate, possibly through a mechanism such as substrate channelling.

Another major question still unresolved within ruminant embryos is the role of intracellular lipid in ATP production and other cellular functions. There is ample evidence that lipid is present in reproductive tract secretions (e.g. Grippo et al., 1994), as there is evidence that lipid can accumulate within embryos cultured in vitro (e.g. Thompson et al., 1995b; Ferguson and Leese, 1999). More recently, attempts have been initiated to characterise the lipid content of cattle and sheep embryos (e.g. Ferguson and Leese, 1999). It is hoped that further analysis and improved understanding will answer the question of lipid metabolism.

3. Amino acids

If one had to choose the most significant “new” medium component affecting ruminant embryo development in vitro identified this decade, then the addition of pooled amino acids would rate very highly. This is because their impact has been the removal of serum from culture systems while maintaining high levels of development (e.g. Rosenkrans and First, 1994; Gardner et al., 1994). It was the pioneering work of Bavister and colleagues in the hamster (e.g. Bavister and Arlotto, 1990) that first showed the benefits of amino acids during in vitro development. The significance of pooled groups of amino acids, especially the non-essential amino acids was demonstrated in sheep embryos by Thompson et al. (1992b) and then more thoroughly by Gardner et al. (1994). Furthermore, the latter authors, based on their experience with mouse embryo development in vitro, demonstrated that embryos were sensitive to the production of ammonia, generated from the spontaneous degradation of amino acids during culture (especially glutamine) and amino acid metabolism (Gardner et al., 1994). This led to the strategy of replacing media with fresh every 2nd day, to optimise development rates (Gardner et al., 1994). The importance of removing ammonia is significant, as it is a primary candidate for the induction of “fetal oversize syndrome” induced during embryo culture (McEvoy et al., 1997, 1999).
A clear understanding of the metabolism of amino acids is still lacking, although slow progress is taking place. Glutamine is known to contribute to ATP production (Rieger et al., 1992b). Uptake studies of pooled amino acids have revealed that uptake levels and preferences change during development (Partridge and Leese, 1996). However, it is recognised that the amino acid concentrations are likely to differ between those commercially supplied as “pooled” preparations and that found within the oviduct and uterine secretions. Indeed, culture in “in vivo-like” concentrations of amino acids has been shown to improve development (Moore and Bondioli, 1993; Hill et al., 1997). Some of this may be attributed to the intracellular osmolyte function of those amino acids found in relatively high concentration in vivo, such as glycine (e.g. Dawson and Baltz, 1997; Dawson et al., 1998).

4. Growth factors, cytokines and extra-cellular matrix molecules

During the 1980s, co-culture, especially with primary cultures of oviduct epithelial cells, emerged as the most effective method for “overcoming” the so-called 8- to 16-cell development block (Gandolfi and Moor, 1987). These observations stimulated the search for embryotrophic factors that were provided by the epithelial cells. Indeed, one such factor was identified, a tissue inhibitor of metalloproteinase (TIMP-1, Satoh et al., 1994). Nevertheless, the focus of research was chiefly directed towards secreted growth factors (Gandolfi et al., 1989, 1992). Furthermore, it became evident that culturing in “groups” of embryos under serum-free conditions, in relatively small volumes of defined medium, conferred benefits to embryo development (e.g. Gardner et al., 1994; Keefer et al., 1994). Only under conditions of serum supplementation can single culture systems perform as well as group culture (Hagemann et al., 1998). Thus, the importance of autocrine/paracrine growth factors has emerged. For example, by use of antibodies and single or group culture, PDGF was shown to act as a paracrine growth factor (Thibodeaux et al., 1995). Furthermore, a number of mRNAs for several growth factors and their receptors are expressed during cattle and sheep embryo development (Watson et al., 1992, 1994a). More recently, attention has focussed not so much on mitogenic/differentiation properties, but rather that of anti-apoptotic, or “survival factors” (Conlon and Raff, 1999). For example, it has been found that transforming growth factor β (Brison and Shultz, 1997) and platelet activating factor (PAF, O’Neill, 1998) act as autocrine/paracrine survival factors for mouse embryos, by reducing levels of apoptosis within the inner cell mass. Cytokines, such as leukemia inhibitory factor (Fry et al., 1992), granulocyte-macrophage colony stimulating factor (de Moraes and Hansen, 1997) and extracellular matrix molecules such as hyaluronic acid (Furnus et al., 1998) also appear to influence embryo development. However, most in vitro studies performed are restricted to examining the action of a single factor. Furthermore, to avoid the confounding influence of growth factor-contaminated biologicals, the medium is usually protein-free (which adds its own complications to interpretation of data, see Section 5). Much of the research ignores the interplay between these classes of molecules, as found within the reproductive tract (extensively reviewed by Kane et al., 1997). Furthermore, such factors exert their influence following activation of the embryonic genome, the excep-
tion being those which act as survival factors (Brison and Shultz, 1997; O’Neill, 1998).
To date, there is no unifying model that describes the various roles of growth factors, cytokines and extra-cellular matrix molecules that contribute to the development of a viable embryo. Indeed, the growth factor cocktail that exists within serum, remains a powerful promoter of peri-compaction embryo development in vitro (e.g. Hagemann et al., 1998), despite its non-uniform constitution, adverse effects on embryo morphology, biochemistry and post transfer consequences (see review by Thompson, 1997). This has led some investigators to study signal transduction mechanisms and their influence on embryo development (e.g. Fahy and Kane, 1993; Grealy and Sreenan, 1999). This approach should assist our understanding of the complexities of embryo–growth factor interactions.

5. The need for albumin

Biologically derived media components, such as serum albumin and serum itself, have been widely used throughout the history of the development of embryo production systems. However, due to the undefined nature and variability in composition, the often less-than-desirable effects on embryos, plus the risk of infection from contaminated biological fluids, much research has been conducted into embryo culture using “defined” media (Bavister, 1995). In particular, the use of polyvinyl alcohol (PVA) has been mooted as a preferred additive, especially as this provides a surfactant activity similar to albumin. However, albumin is the most prevalent protein in the mammalian reproductive tract, and evidence from the mouse suggests that albumin may have intracellular roles, as well as the additional extracellular support roles that it is thought to play (Dunglison and Kaye, 1993; Dunglison et al., 1995). However, albumin is not an absolute requirement for cattle embryo development in vitro (Keskintepe et al., 1995). Nevertheless, apart from the excellent results achieved by the laboratory of Brackett and colleagues (e.g. Keskintepe et al., 1995), most others have reported that levels of development are generally lower following in vitro development in PVA — than in albumin-supplemented medium (Bavister, 1995). An examination of the total protein content of in vitro derived embryos (Thompson et al., 1998) revealed that during early cleavage, protein content decreases. This is followed by an increase in content during compaction and blastulation. Hence, protein degradation exceeds protein synthesis during early cleavage. Furthermore, total protein content was lower in blastocysts cultured in PVA-supplemented medium, compared to those produced in vivo or in culture medium supplemented with either BSA or fetal calf serum (Fig. 2; Thompson et al., 1998). This was not due to a difference in protein synthesis rates but the action of protein uptake via pinocytosis (Thompson et al., 1998). Furthermore, a profound effect on energy substrate utilisation was found in blastocyst stage embryos cultured in PVA medium compared to BSA medium. In PVA-medium, pyruvate uptake was considerably higher than in BSA medium (Eckert et al., 1999) although oxygen uptake was reduced. This would normally be regarded as contradictory, because increased pyruvate uptake would normally be associated with increased oxygen consumption. However, Lee et al. (1998) found that oxidation of
Fig. 2. Log¹⁰ plot histograms of protein content (±S.E.M.) of Day 7 blastocyst stage embryos produced in vitro and incubated in medium supplemented with either: polyvinyl alcohol (PVA); bovine serum albumin (BSA); fetal calf serum (FCS-D1), BSA from Days 1–5 of development and FCS for Days 5–7 of development. A further group, Day 7 blastocysts derived in vivo (following superovulation and embryo collection) were also included (in vivo). Different superscripts signify significant differences (P < 0.001). (Reprinted with permission from Wiley, New York.)

Pyruvate was significantly lower in blastocysts derived from culture in PVA medium compared to BSA medium, in agreement with the oxygen uptake data of Eckert et al. (1999). Thus, despite an increase in pyruvate uptake, oxidation of this substrate was decreased. Therefore, it appears that pyruvate plays other roles in blastocysts derived from PVA media. One such role is possibly the amination of pyruvate to alanine in the detoxification of NH₃, generated by endogenous protein metabolism, which may occur in the absence of exogenous protein, such as albumin. Based on the data from the mouse (Dunglison and Kaye 1993; Dunglison et al., 1995) and from our own observations, it seems likely that albumin has a significant nutritive role to play during embryo development, especially post-compaction. It is clear that blastocysts derived from PVA-supplemented medium have an altered metabolic profile compared to those cultured in the presence of albumin, or those derived in vivo. Whether other protein sources can replace albumin during in vitro development has yet to be established. In any case, the trend towards “defined” systems must be tempered with the physiological requirements of the embryo during development.

6. Sequential media, perfusion and metabolic regulation

As reviewed by Thompson (1996) and Gardner and Lane (1997), our understanding of the requirements of early embryos as they progress through development and the temporal relationship between the reproductive tract fluid milieu and embryo development, has led to the concept that media components and physical conditions should be altered during culture to achieve improved development. This concept has subsequently been termed sequential media systems (Gardner and Lane, 1997) and has led to at least two human embryo culture systems now commercially available (G1/G2, Scandinavian IVF and Cook human embryo culture medium, Cook Australia). For cattle embryos, our Ruakura laboratory has developed a sequential media system for fertilisation and culture of IVP embryos, known as “SOF-98” (AgResearch, Hamilton, New Zealand). A further
development of this concept is the use of perfusion culture as the vehicle to introduce changes in media composition (Thompson, 1996). Work on the development of the equipment necessary for perfusion culture is well underway (Lim et al., 1996; McGowan and Thompson, 1997). The introduction of perfusion culture should enable more elegant experimentation on altering concentrations and introducing new components into media, while causing minimal disturbance to embryos during development.

Metabolic regulation is a relatively new concept, but has its origins before the beginning of this decade. Perhaps, the best known example of metabolic regulation is the use of ethylenediamine tetraacetic acid (EDTA), a non-selective chelator of divalent cations during embryo development. Several studies have shown that when in vitro glucose concentrations are higher than in vivo, early embryo development is retarded or even blocked (Seshagiri and Bavister, 1989; Chatot et al., 1989; Thompson et al., 1992a). This has been likened to the “Crabtree-effect”, whereby oxidative phosphorylation is depressed due to an abnormally high glycolytic rate (Seshagiri and Bavister, 1989). Until recently, this was generally controlled by reducing the concentration of, or even removing, glucose within the culture medium (e.g. Chatot et al., 1989). However, such conditions are unphysiological, as glucose is found in lumen fluids of the reproductive tract (Gardner, 1998). A previously unrelated observation is that EDTA addition during mouse in vitro embryo development overcame culture-induced development (“2-cell”) blocks (Abramczuk et al., 1977; Mehta and Kiessling, 1990; Nasr-Esfahini et al., 1992). It is believed that EDTA sequesters the toxic effects of contaminating heavy metal cations, most likely by inhibiting the production of reactive oxygen species, catalysed by ions such as Fe$^{2+}$ and Cu$^{2+}$ (reviewed by Johnson and Nasr-Esfahini, 1994). However, Lane and Gardner (1997) have demonstrated that, at a cellular level, EDTA depresses glycolytic rates within pre-compaction mouse embryos, a result also demonstrated for cattle embryos (Gardner et al., 1997). These authors believe that in the mouse, at least, the mechanism of EDTA is via the intracellular chelation of Mg$^{2+}$, a necessary co-factor for several glycolytic enzymes (Lane and Gardner, 1997). The beneficial effect of EDTA is optimal only when introduced during early development, when culture-induced high glycolytic rates will compromise development. Thus EDTA is used to manipulate the metabolic profile of embryos during stage-specific developmental points, in order to optimise energy metabolism, leading to improved development. We have also used metabolic manipulation strategies during peri-/post-compaction development to significantly improve cattle embryo production yields. This has been achieved by inducing the “opposite” effect of EDTA, i.e. stimulating glycolysis by partially inhibiting oxidative phosphorylation during post-compaction development (Thompson et al., 2000). Non-toxic concentrations of metabolic inhibitors such as NaN$_3$ and 2,4-dinitrophenol (5–10 μM) present in SOF-98 medium from Day 5–7 of development have been found to stimulate embryo development, increasing the proportion of embryos developing to compact morulae and blastocysts from 50% to 60%, with a similar increase in transferable quality embryos (40% to 50%) (Thompson et al., 2000). Furthermore, both cell number and glucose uptake increase in NaN$_3$-treated embryos (Thompson et al., 2000). The exact mechanism by which such compounds increase development has yet to be determined. Nevertheless, previously it was thought that the rate of early cleavage is associated with successful embryo development (e.g.
Van Soom et al., 1997). However, use of peri-/post-compaction metabolic regulation can also “rescue” embryos that normally would not undergo further development.

7. Concluding remarks and the future

Our understanding of the requirements for the development of cattle and sheep embryos in vitro to the blastocyst stage from in vitro or in vivo produced zygotes has progressed enormously in this final decade of the 2nd millennium. Nevertheless, there remains a clear distinction between the embryos derived in vivo and those produced in vitro. In particular, subtle differences exist at the cellular level in terms of metabolic profiles and morphology (Thompson, 1997; Leese et al., 1998). Of great interest has been the demonstration that gene expression differences exist between in vivo and in vitro derived embryos (Wrenzycki et al., 1996) and between different methods of in vitro culture (Wrenzycki et al., 1999). The array of post-transfer abnormalities, such as fetal oversize syndrome (e.g. Kruip and den Daas 1997) and the high incidence of late embryo-early fetal loss (e.g. Reichenbach et al., 1992) require further understanding of the relationship between development and sub-optimal culture environments. However, there is also many other puzzling questions and challenges confronting embryologists in this field. First, embryos of other ruminants do not necessarily develop in systems developed for the cattle or sheep. The red deer (Cervus elaphus) embryo, for example, is extremely difficult to culture to the blastocyst stage in vitro (Berg et al., 1995). Second, an inability to support normal development post-hatching, and even the processes of hatching, are areas of significant interest. Understanding the role of intracellular pH and cell volume regulation, signal transduction mechanisms and mRNA transcription regulation in embryo development should provide answers to many of these questions. Of particular interest will be a greater understanding of the regulation of expression of those genes related to “stress” responses (Leese et al., 1998) and blastocyst formation (Watson et al., 1999). With advances in technology, it has become increasingly possible to investigate many of these facets within individual developing embryos. However, just as Brinster, Biggers and company found with the mouse embryo in the 1960s and early 1970s, the key regulator of cattle and sheep in vitro embryo development is the way in which the embryo produces ATP.

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References

Gardner, D.K., Lane, M.W., Lane, M., 1997. Bovine blastocyst cell number is increased by culture with EDTA for the first 72 hours of development from the zygote. Theriogenology 47, 278.


acetic acid and oxygen tension on the concentration of reactive oxygen species and on development of the mouse preimplantation embryos in-vitro. J. Reprod. Fertil. 96, 219–231.


