Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro

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The effect of inhibiting ATP production via oxidative phosphorylation during peri-compaction of in vitro produced bovine embryos was investigated. This was achieved by: (i) varying the atmospheric O₂ concentration (0, 1, 2, 4 and 7%); (ii) addition of oxidative phosphorylation inhibitors, NaN₃ and antimycin A; and (iii) addition of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation from electron transport. The development of embryos under various O₂ concentrations from day 5 to day 7 of development indicated that an optimal concentration occurred at about 2%. Addition of NaN₃ revealed that doses above 100 μmol l⁻¹ were toxic to embryo development, but that concentrations of 5–10 μmol l⁻¹ stimulated embryo development by 10–25%. A similar result was observed after addition of 2,4-dinitrophenol, whereas antimycin A was inhibitory at doses as low as 1 μmol l⁻¹. At concentrations of NaN₃ or 2,4-dinitrophenol that stimulated embryo development, the number of cells of the resulting blastocysts was also significantly increased. Addition of NaN₃ from day 1 of development inhibited subsequent development. Metabolic data of NaN₃-treated embryos revealed that O₂ uptake was significantly lower at inhibitory doses (100 μmol l⁻¹). A significant (P < 0.05) log linear increase in glucose uptake was measured between the three concentrations of NaN₃ (0, 10 and 100 μmol l⁻¹). These results demonstrate that ATP production via oxidative phosphorylation is essential for bovine embryo development in vitro. However, transient (subacute) inhibition appears to be beneficial to embryo development and the number of cells, perhaps by creating a more favourable intracellular environment.

Introduction

As with most cells, bovine pre-elongation embryos are highly dependent on oxidative phosphorylation as the primary energy production pathway (that is, ATP generating pathway; Thompson et al., 1996). This is particularly so during pre-compaction development, during which it is estimated that approximately 90% of all ATP is derived from oxidation (Thompson et al., 1996). During compaction and blastulation, the demand for ATP increases, to allow increases in protein synthesis (Thompson et al., 1996). For instance, the activity of the Na⁺–K⁺-ATPase (Leese, 1991) requires an osmotic potential across the trophectoderm, producing the blastocoele. The increased demand for ATP causes increases in consumption of the major substrates, including oxygen and pyruvate (Thompson et al., 1996), amino acids (Partridge and Leese, 1996) and glucose (Thompson et al., 1996). However, most of the glucose that is metabolized by ruminant embryos at the blastocyst stage is accounted for by glycolysis, and lactate is the end-product and is transported from the embryo to the surrounding medium. Only a small amount of glucose is oxidized, mostly via the pentose-phosphate pathway for ribose formation, rather than by the tricarboxylic acid cycle (Rieger and Guay, 1988; Thompson et al., 1991, 1996; Gardner et al., 1993). However, the increase in glucose consumption is such that the contribution of glycolysis alone to ATP production increases from approximately 4–8% to 15–18% between pre- and post-compaction stages, respectively, in an environment in which O₂ is abundant (Thompson et al., 1996). Recent studies have indicated that the O₂ tension of the reproductive tract decreases as an embryo passes from the oviduct to the uterine cavity (Brown and Mattner, 1984; Fischer and Bavister, 1993). Furthermore, of the little information available concerning the metabolism of inner cell mass (ICM) tissue, it appears that this is more glycolytic in activity than that of the trophectoderm (Hewitson and Leese, 1993). These findings indicate that there is a shift in the metabolic pathway preference for embryonic ATP production from oxidative phosphorylation to glycolysis, to correspond with development within the uterine cavity, an environment

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in which O₂ availability may be limited. Such a shift in pathway preference has been reported for mammalian embryos, such as human (Gott et al., 1990) and rat (Brison and Leese, 1991) embryos. The mouse embryo, the most examined model of early embryo development, is totally dependent on oxidative phosphorylation throughout development, but changes substrate preference at compaction from the tricarboxylic acids to glucose (Leese, 1991). In this species, oxidation of glucose after compaction occurs readily and glucose provides essential energy requirements during this period of development (Leese, 1991).

Using the rat embryo as a model, Brison and Leese (1994) demonstrated that embryos were not reliant on oxidative phosphorylation during compaction and blastulation. These authors used several oxidative phosphorylation uncouplers or inhibitors, including 2,4-dinitrophenol or cyanide, at relatively high concentrations (for example 1 mmol l⁻¹) to demonstrate that blastulation can take place in vitro in the presence of these compounds. Furthermore, these authors demonstrated that a compensatory rise in glycolysis and increased utilization of glucose occurred to generate sufficient ATP. Therefore, inhibition of oxidative metabolism was not detrimental to these embryos. In contrast, using similar concentrations of these compounds, Thomson (1967) demonstrated that inhibition of ATP production via oxidative phosphorylation was toxic to mouse embryo development, at either the two-cell or blastocyst stage. However, lower concentrations (for example 10–100 μmol l⁻¹) partially inhibited development in a dose-dependent manner (Thomson, 1967). Therefore, there appear to be two extremes of metabolic adaptability during peri-compaction development: rats, which are not reliant on oxidative phosphorylation, and mice, which are.

The aim of the present study was to inhibit oxidative phosphorylation by decreasing the atmospheric oxygen tension, or by the addition of an inhibitor or uncoupler, to elucidate whether bovine (in vitro produced) embryos behaved similarly to either rat or mouse embryos. All treatments were timed to occur during compaction.

**Materials and Methods**

**Production of embryos**

Ovaries were collected from an abattoir and transported to the laboratory in saline (approximately 35°C). The maximum interval from the time the animals were killed to oocyte collection was approximately 3 h. Cumulus–oocyte complexes (COC) were recovered by aspiration of follicles of 1–5 mm in diameter using an 18 g needle under vacuum (50 mm Hg). The COCs were collected into Hapes-buffered TCM 199 medium (with Earle’s salts; Life Technologies, Auckland) supplemented with 10 μg heparin ml⁻¹ (from pig intestinal mucosa; Sigma, St Louis, MO) and 0.4% (w/v) BSA (affinity column purified, ABRD; Immuno-Chemical Products, Auckland). Before in vitro maturation, COCs were assessed morphologically and only those that had a compact, non-ateletic cumulus oophorus-corona radiata and an oocyte with homogeneous cytoplasm were selected. All selected COCs were washed thoroughly in Hapes-buffered TCM 199 medium supplemented with 10% (v/v) fetal calf serum (FCS; Life Technologies), washed once in maturation medium, placed in 50 μl drops (ten per drop) of the same medium under oil and incubated for 24 h at 39°C under humidified 5% CO₂ in air. The medium used for maturation was TCM 199 supplemented with 10% FCS, 10 μg ovine FSH ml⁻¹ (Ovagen; Immuno-Chemical Products), 1 μg ovine LH ml⁻¹ (Immuno-Chemical Products), 1 μg oestradiol ml⁻¹ (Sigma) and 100 μmol cysteamine l⁻¹ (Sigma).

Spermatozoa were prepared from frozen-thawed semen obtained from a sire that had been characterized as suitable for in vitro fertilization in this laboratory. The contents of two 0.25 ml straws (each containing approximately 1 × 10⁸ spermatozoa ml⁻¹) were layered upon a Percoll gradient (45% : 90%) and motile spermatozoa were collected after centrifugation at approximately 1200 g for 20 min at room temperature. The motile fraction was washed once in Hapes-buffered Tyrode’s albumin lactate pyruvate medium (TALP) and then re-suspended to a final concentration at insemination of 2 × 10⁸ spermatozoa ml⁻¹ in fertilization medium, a modified TALP (Lu et al., 1987), supplemented with 0.01 mmol heparin l⁻¹, 0.2 mmol penicillamine l⁻¹ (Sigma) and 1 mmol hypotaurine l⁻¹ (Sigma). Insemination was performed in 50 μl fertilization medium in microdrops under oil (approximately five oocytes per drop) over a 24 h period under the same conditions as described for oocyte maturation.

**Experiment 1: effect of oxygen concentration**

After insemination, putative zygotes were removed and washed twice in a Hapes-buffered version of synthetic oviduct fluid (SOF) medium (Tervit et al., 1972), comprising 20 mmol Hapes l⁻¹, 5 mmol NaHCO₃ l⁻¹ and 3 mg BSA ml⁻¹ (fatty acid-free; Sigma) (H-SOF), and were placed in 20 μl microdrops of the modified SOF medium, SOFaaBSA, which includes MEM essential and non-essential amino acids (Life Technologies) and 8 mg BSA ml⁻¹ (Gardner et al., 1994), and incubated under humidified 5% CO₂, 7% O₂ and 88% N₂ at 38.5°C. Embryos were transferred to fresh media on day 5 of development (day 0 = day of insemination) and randomly allocated to one of five 5% CO₂ atmospheres which differed in the content of oxygen (and nitrogen, used to balance the atmospheres). The five atmospheres were: 0, 1, 2, 4 and 7% O₂. All embryos were removed from culture on day 7 of development and the stage of development (and quality of those that reached compacted morulae or blastocyst stages) were recorded after examination under a dissecting microscope. These experiments were conducted over five replicates using a total of 871 cleaved embryos. Stage 1 and 2 blastocyst stage embryos were removed for analysis of the number of cells. The number of cells was determined using fluorescence microscopy and image analysis (Video Pro, Adelaide) after fixation in acetic acid: ethanol:H₂O (3:2:1) and staining with 1% (w/v) propidium iodide.

**Experiment 2: addition of an oxidative phosphorylation inhibitor, NaN₃**

Embryos were produced and treated as in Expt 1, but at
day 5 of development, embryos were transferred to fresh medium containing one of four concentrations of NaN₃ (0, 10, 100 and 1000 μmol l⁻¹). Embryos were cultured in four-well plates, each well containing one of the four concentrations (20 μl medium). Each microdrop was overlaid with 0.5 ml mineral oil. Embryos were cultured under humidified 5% CO₂, 7% O₂ and 88% N₂ at 38.5°C. As with Expt 1, culture ended on day 7 and development was recorded after examination under a dissecting microscope. In this experiment, three replicates were conducted using a total of 335 cleaved embryos. On the basis of the results of the first experiment (that is, Expt 2a), a further dose–response trial was initiated. The concentrations of NaN₃ assessed were 0, 5, 10 and 20 μmol l⁻¹, over three replicates using 342 cleaved embryos (Expt 2b).

**Experiment 3: addition of NaN₃ from day 1 of development**

Embryos were produced using the same basic conditions described in Expts 1 and 2, with minor modifications. Firstly, the culture system was sequential (see for example Gardner, 1998), based on the principles outlined by Thompson (1996), and designated SOF-98 (AgResearch, Hamilton, New Zealand). In this system, embryos are cultured in an early development medium (eSOF-98) for 4 days and transferred to a peri-compaction and blastulation medium (LSOF-98) for 2 days. Secondly, this experiment was designed to assess the effect of NaN₃ from day 1 of development (first day of culture). Putative zygotes were cultured in eSOF-98 in either 0, 10 or 100 μmol NaN₃ l⁻¹ for 4 days and transferred into fresh drops of LSOF-98. Culture conditions and morphological observations were conducted as described earlier. Four replicates were performed using a total of 602 cleaved embryos.

**Experiment 4: addition of antimycin A**

Embryos were produced using the conditions described in Expt 3. This experiment examined the effect of antimycin A, another oxidative phosphorylation inhibitor (acts at cytochrome oxidase b). The concentrations assessed were 0, 1, 2, and 5 μmol l⁻¹, over five replicates (n = 1040 cleaved embryos).

**Experiment 5: metabolism of embryos after treatment with metabolic inhibitor**

Embryos were produced using the conditions described in Expt 3. The aim of this experiment was to determine whether the embryos of transferable quality derived from culture in medium containing different concentrations (0–100 μmol l⁻¹) of NaN₃ also differed in their metabolic profile. At the cessation of culture, embryos were graded morphologically and transferred to a modified late SOF medium; the modification was the replacement of 25 mmol NaHCO₃ l⁻¹ with 20 mmol Hepes l⁻¹ and 5 mmol NaHCO₃ l⁻¹ (this medium is referred to as transfer SOF; TSOF). In addition, TSOF also included the corresponding concentration of NaN₃ used for the culture of embryos. The metabolic profile of embryos was characterized by measuring the consumption of oxygen, glucose and pyruvate, and the production of lactate, performed using the techniques described by Houghton et al. (1996) and Thompson et al. (1996). After metabolic assays, the number of cells was determined as described earlier. Five replicates were conducted, using a total of 1192 cleaved embryos.

**Experiment 6: examination of embryo development after treatment with a metabolic uncoupler, 2,4-dinitrophenol**

This experiment was conducted to examine the effect of the addition of 2,4-dinitrophenol (0–1000 μmol l⁻¹) on day 5 of development. Embryos were produced using the techniques described in Expt 3. A total of ten replicates were conducted (n = 2233); embryos produced in the last five replicates were used for metabolic determinations. After the metabolic assays, the number of cells was determined using the techniques described earlier.

**Experiment 7: post-transfer survival of embryos after treatment with 2,4-dinitrophenol**

Embryos were produced using the conditions described in Expt 6. On day 5 of development, embryos were transferred into LSOF medium supplemented with 10 μmol 2,4-dinitrophenol l⁻¹. On day 7 of development, morphology was assessed and embryos of grade 1 and 2 (that is, transferable quality) were allocated to be transferred to suitably synchronized recipient parous cows (n = 24). All cows received an embryo ipsilateral to the functional corpus luteum. A further group of synchronized recipients (n = 45) were artificially inseminated using frozen semen from the same bull used to produce the embryos in vitro. On day 35 of pregnancy, all cows were scanned by ultrasonography to determine pregnancy status.

**Statistical analysis**

Unfertilized oocytes were excluded from all analyses. The proportional data for in vitro development of embryos were analysed, after logit transformation, using the generalized linear models (GLM) procedure in the Genstat statistical package. In addition to the main treatment groups, the data were also tested for random effects of day of oocyte collection. The number of cells and metabolic data were handled in a similar fashion, although transformation of the data using logs was only conducted when heterogeneity of variance was observed.

**Results**

**Experiment 1: oxygen concentration**

The results for embryo development are summarized (Fig. 1). A quadratic trend (P < 0.1) was observed for development...
to the compacted morulae and blastocyst stages and for the development of grade 1 and 2 embryos, indicating that development was improved with O₂ concentrations below 7% but above 0%. However, significance was only observed for the proportion of grade 1 and 2 blastocysts (P < 0.05) (Fig. 1).

The number of cells in blastocysts determined as grade 1 and 2 quality, derived from different oxygen concentrations, did not vary and contained on average 118 ± 5.0 cells per embryo.

Experiment 2: addition of NaN₃

The results of Expt 2a are presented (Table 1). Significant (log linear) differences were observed between the three NaN₃-supplemented solutions for the proportion of embryos developing to compacted morulae and blastocysts (P < 0.001) and grade 1 and 2 compacted morulae and blastocysts (P < 0.001). In all cases, the greatest development was achieved in 10 μmol NaN₃ l⁻¹ and least in 1000 μmol NaN₃ l⁻¹. No difference was observed in the development of embryos in the 0 (control) and 10 μmol NaN₃ l⁻¹ treatments.

The results for Expt 2b are summarized (Fig. 2). In contrast to Expt 2a, the average NaN₃ response was significantly higher than the control embryos for all stages of embryo development (P < 0.01). Furthermore, a significant linear decrease (P < 0.01) with increasing NaN₃ concentration was observed in the percentage of grade 1 and 2 compacted morulae and blastocysts.

In addition to the developmental data, visual assessment of the inhibitor-treated embryos indicated that they were also of superior quality to control embryos, in that the inner cell mass of the treated embryos appeared to be larger and ‘denser’.

Experiment 3: addition of inhibitor on day 1 of development

Addition of 10 μmol NaN₃ l⁻¹ on day 1 of embryo development, that is, the start of embryo culture, inhibited development significantly (P < 0.001). Furthermore, 100 μmol NaN₃ l⁻¹ was found to be highly toxic (Table 2).

Experiment 4: addition of antimycin A

Addition of antimycin A on day 5 of development significantly inhibited further development (P < 0.001), even at concentrations as low as 1 μmol l⁻¹ (Fig. 3).

Table 1. Development of bovine embryos incubated in SOFaaBSA medium supplemented with NaN₃ from day 5 to day 7 of development (Expt 2a)

<table>
<thead>
<tr>
<th>NaN₃ (μmol l⁻¹)</th>
<th>Compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
<th>Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>40 ± 5.4ᵃ</td>
<td>26 ± 4.8ᵃ</td>
</tr>
<tr>
<td>10</td>
<td>46 ± 5.3ᵇ</td>
<td>37 ± 5.1ᵇ</td>
</tr>
<tr>
<td>100</td>
<td>34 ± 5.3ᵃ</td>
<td>13 ± 3.7ᵇ</td>
</tr>
<tr>
<td>1000</td>
<td>5 ± 2.4ᵇ</td>
<td>1 ± 1.2ᵇ</td>
</tr>
</tbody>
</table>

Within columns, values with different superscripts are significantly different (P < 0.05).
Experiment 5: metabolism of embryos after addition of NaN₃

The data relating to development and the number of cells associated with the production of embryos cultured in NaN₃ is summarized (Table 3). There were significant \( P < 0.001 \) linear decreases (on a logarithmic scale) for both percentage compacted morulae and blastocysts and percentage grade 1 and 2 compacted morulae and blastocysts between the two concentrations of NaN₃ used. However, only for a proportion of grade 1 and 2 embryos was there evidence of an optimal dose \( (P < 0.05, \text{ that is, a quadratic function}) \). There was no significant difference in developmental rates between 0 (control) and 10 \( \mu \text{mol} \) NaN₃ l\(^{-1}\). However, the number of cells differed significantly among the three concentrations used; 10 \( \mu \text{mol} \) NaN₃ l\(^{-1}\) yielded expanded blastocyst stage embryos with the greatest number of cells (Table 3).

The effect of incubation in NaN₃ on embryo metabolism is summarized (Table 4). No difference was observed between 0 and 10 \( \mu \text{mol} \) NaN₃ l\(^{-1}\), except for a log linear increase in glucose consumption \( (P < 0.05) \). A similar increase in lactate production was also observed, but this was variable and not significant. Only at 100 \( \mu \text{mol} \) NaN₃ l\(^{-1}\) was a significant reduction \( (P < 0.05) \) in oxygen uptake observed.

Experiment 6: evaluation of 2,4-dinitrophenol

The development of bovine embryos and the resulting number of cells in expanded blastocyst stage embryos incubated in 2,4-dinitrophenol \( (0–1000 \mu \text{mol} \text{l}^{-1}) \) is summarized (Table 5). A highly significant \( (P < 0.001) \) log quadratic function was observed for total embryo development and development of grade 1 and 2 embryos. Optimal development appeared to lie between 10 and 100 \( \mu \text{mol} \) 2,4-dinitrophenol l\(^{-1}\). The number of cells increased significantly in the presence of 10 and 100 \( \mu \text{mol} \) 2,4-dinitrophenol l\(^{-1}\) compared with controls.

Experiment 7: embryo survival after treatment with 2,4-dinitrophenol

Of the 24 cows receiving a single grade 1 or 2 embryo by a standard non-surgical transfer technique, 12 (50%) became pregnant.

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### Table 2. Development of bovine embryos incubated in SOF-98 medium supplemented with NaN₃ from day 1 to day 5 of development (Expt 3)

<table>
<thead>
<tr>
<th>NaN₃ (μmol l(^{-1}))</th>
<th>Compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
<th>Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>54 ± 2.8</td>
<td>41 ± 2.8</td>
</tr>
<tr>
<td>10</td>
<td>39 ± 2.7</td>
<td>26 ± 2.5</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( P )</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

### Table 3. Development and number of cells of bovine embryos incubated in SOF-98 medium supplemented with NaN₃ from day 5 to day 7 of development (Expt 5)

<table>
<thead>
<tr>
<th>NaN₃ (μmol l(^{-1}))</th>
<th>Compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
<th>Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
<th>Number of cells (blastocysts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>57 ± 3.0(^a)</td>
<td>45 ± 3.0(^a)</td>
<td>103 ± 5.2(^a)</td>
</tr>
<tr>
<td>10</td>
<td>60 ± 2.9(^a)</td>
<td>50 ± 2.9(^a)</td>
<td>119 ± 5.2(^a)</td>
</tr>
<tr>
<td>100</td>
<td>24 ± 1.8(^b)</td>
<td>13 ± 1.4(^b)</td>
<td>73 ± 4.5(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Values with different superscripts are significantly different \((P < 0.001)\).

\(^b\)Values with different superscripts are significantly different \((P < 0.05)\).
pregnant, as determined by ultrasonography. This compares favourably with 49% (22/45) of cows that became pregnant after artificial insemination.

**Discussion**

The azide ion (N₃⁻) inhibits oxidative phosphorylation by inhibiting the electron transport cascade, specifically by inhibiting cytochrome oxidase a₃, as does cyanide. Therefore, both electron transport and oxidative phosphorylation are downregulated. There are several other inhibitors in this class, including rotenone and antimycin A, and these are distinct from those that inhibit oxidative phosphorylation itself, such as oligomycin. Uncouplers, such as 2,4-dinitrophenol, do not inhibit these pathways, but separate them. Therefore in the presence of uncouplers, electron transport can occur without concomitant ATP synthesis, a result which usually causes an increase in the activity of the tricarboxylic acid cycle and oxygen consumption, leading to an increase in the activity of the tricarboxylic acid cycle and oxygen consumption.

**Table 4.** Effect of NaN₃ in the culture medium on metabolism of bovine blastocyst stage embryos (Expt 5)

<table>
<thead>
<tr>
<th>NaN₃ (μmol l⁻¹)</th>
<th>O₂ uptake (pmol per embryo per h)</th>
<th>Pyruvate uptake (pmol per embryo per h)</th>
<th>Glucose uptake (pmol per embryo per h)</th>
<th>Lactate production (pmol per embryo per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1.1 ± 0.20*</td>
<td>5.2 ± 0.54</td>
<td>12 ± 3.2*</td>
<td>22 ± 6.0</td>
</tr>
<tr>
<td>10</td>
<td>1.1 ± 0.39*</td>
<td>5.2 ± 0.51</td>
<td>20 ± 3.1b</td>
<td>28 ± 5.7</td>
</tr>
<tr>
<td>100</td>
<td>0.4 ± 0.11b</td>
<td>5.4 ± 0.69</td>
<td>29 ± 4.5c</td>
<td>38 ± 7.7</td>
</tr>
</tbody>
</table>

Within columns, values with different superscripts are significantly different (*P* < 0.05).

**Fig. 4.** Carboxylic acid and glucose metabolic pathways of a mammalian embryo leading to the production of ATP. (a) Glucose is consumed and converted to a number of biosynthetic intermediates, including pyruvate. In most mammalian embryos at the blastocyst stage, glucose is stoichiometrically converted 1:2 to lactic acid; very little glucose-derived pyruvate is oxidized in the tricarboxylic acid cycle. For this reason, pyruvate, lactate and amino acids are added to culture medium to provide tricarboxylic acid cycle substrates. (b) Proposed model to describe the influence of NaN₃. An increase in glucose consumption provides increased biosynthetic precursors. However, pyruvate and lactate production remain constant and oxygen consumption (and electron transport) decreases.
Oxidative phosphorylation and bovine embryo development

The results of the present study provide evidence that partial (subacute) downregulation of mitochondrial ATP production during the compaction and blastulation stages of in vitro produced bovine embryos improves in vitro development. This was demonstrated in three ways: (i) by physiologically decreasing oxygen availability; (ii) by partially inhibiting electron transport using NaN₃; and (iii) by partially uncoupling oxidative phosphorylation from electron transport using 2,4-dinitrophenol. This finding differs from other species examined. For example, in rats, Brison and Leese (1994) demonstrated that inhibition of oxidative phosphorylation (using as high as 1 mmol cyanide l⁻¹) was only partially retarded at concentrations of 2–10 mmol l⁻¹, especially when included at day 3. It is concluded that ATP production via oxidative phosphorylation is an essential pathway for bovine embryo development at all stages of development, but that partial inhibition during the peri-compaction period is beneficial. The mechanism for this effect is unclear, but most likely involves the relative contribution of glycolytic ATP versus mitochondrial ATP production and could involve the establishment of an appropriate redox state which encourages increased glucose metabolism. Maintenance of an appropriate redox state was also suggested as a mechanism for the enhanced development of ovine embryos in vitro under pyruvate:L-lactate ratios of 1:5 (Thomson et al., 1993), a proposal supported by the observations of Edwards et al. (1997). One major difference between these studies on rodents and the present study is that in the rodent studies, in vitro derived embryos were used, which have an appropriate metabolic profile before in vitro culture. In contrast, the embryos used in the present study were produced in vitro, and abnormalities in metabolic profile may be inherent due to their non-physiological origins. It is possible that in vivo derived bovine embryos would provide different results from those described here.

The effectiveness of inhibitors and uncouplers varied. Sodium azide appears to be beneficial over a narrow range of concentrations (5–20 μmol l⁻¹). There was little effect on oxygen uptake at 10 μmol l⁻¹, but there was an increase in glucose consumption without a significant corresponding increase in lactate production, which supports the suggestion that partial inhibition of mitochondrial ATP production creates a more favourable redox state, and this in turn increases the availability of biosynthetic intermediates, potentially through the pentose phosphate or Embden–Meyerhoff (glycolytic) pathway. Further examination using radioisotope labelled substrates will be conducted to investigate this possibility. An increase in glucose consumption is correlated with increased embryo viability in post-hatching stage bovine blastocystcs (Renard et al., 1980). At higher NaN₃ concentrations, there is sufficient downregulation of electron transport and O₂ consumption to inhibit further development, although even at 100 μmol l⁻¹, some embryos do reach the blastocyst stage. This provides further evidence that mitochondrial ATP generation is still necessary for development to occur. Furthermore, the effect of NaN₃ was not always consistent among experiments. Although NaN₃ was significantly effective in the range 5–10 μmol l⁻¹ in Expt 2 (using SOFaBSA medium), this was not observed in Expt 5. It is suggested that the effect of NaN₃ may be conditional on the time of year, possibly mediated through the developmental competence of oocytes collected, which may be related to the condition of animals at the time they are killed.

Table 5. Effect of addition of 2,4-dinitrophenol on day 5 of development on subsequent development to day 7 of bovine in vitro produced embryos (Expt 6)

<table>
<thead>
<tr>
<th>2,4-Dinitrophenol (μmol l⁻¹)</th>
<th>Compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
<th>Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos (%)</th>
<th>Number of cells (expanded blastocyst)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>51 ± 2.0ᵇ</td>
<td>40 ± 1.9ᵇ</td>
<td>121 ± 3.7ᵇ</td>
</tr>
<tr>
<td>10</td>
<td>60 ± 1.9ᵇ</td>
<td>50 ± 1.9ᵇ</td>
<td>141 ± 4.9ᵇ</td>
</tr>
<tr>
<td>100</td>
<td>55 ± 2.3ᵇ</td>
<td>43 ± 2.3ᵇ</td>
<td>140 ± 4.4ᵇ</td>
</tr>
<tr>
<td>1000*</td>
<td>6 ± 1.4ᵇ</td>
<td>2 ± 0.7ᵇ</td>
<td>–</td>
</tr>
</tbody>
</table>

*Only the first five replicates used 1000 μmol l⁻¹, as it was found to be highly toxic.
†Within columns, values with different superscripts are significantly different (P < 0.05, "P < 0.01, "P < 0.001).
‡Within columns, values with different superscripts are significantly different (P < 0.001).

(Thompson et al., 1991).

The results of the present study provide evidence that partial (subacute) downregulation of mitochondrial ATP production during the compaction and blastulation stages of in vitro produced bovine embryos improves in vitro development. This was demonstrated in three ways: (i) by physiologically decreasing oxygen availability; (ii) by partially inhibiting electron transport using NaN₃; and (iii) by partially uncoupling oxidative phosphorylation from electron transport using 2,4-dinitrophenol. This finding differs from other species examined. For example, in rats, Brison and Leese (1994) demonstrated that inhibition of oxidative phosphorylation (using as high as 1 mmol cyanide l⁻¹) had little effect on blastulation rates, but these authors did not describe an increased developmental capacity. In contrast, 1 mmol l⁻¹ cyanide or 2,4-dinitrophenol is completely inhibitory to mouse embryo development when administered at either the two-cell or blastocyst stage (Thomson, 1967). However, in the same study, development was only partially retarded at concentrations of approximately 10–100 μmol l⁻¹, especially when included at day 3. It is concluded that ATP production via oxidative phosphorylation is an essential pathway for bovine embryo development at all stages of development, but that partial inhibition during the peri-compaction period is beneficial. The mechanism for this effect is unclear, but most likely involves the relative contribution of glycolytic ATP versus mitochondrial ATP production and could involve the establishment of an appropriate redox state which encourages increased glucose metabolism. Maintenance of an appropriate redox state was also suggested as a mechanism for the enhanced development of ovine embryos in vitro under pyruvate:L-lactate ratios of 1:5 (Thomson et al., 1993), a proposal supported by the observations of Edwards et al. (1997). One major difference between these studies on rodents and the present study is that in the rodent studies, in vitro derived embryos were used, which have an appropriate metabolic profile before in vitro culture. In contrast, the embryos used in the present study were produced in vitro, and abnormalities in metabolic profile may be inherent due to their non-physiological origins. It is
and the number of cells in resulting blastocysts. Examination of the metabolic profile of embryos treated with 2,4-dinitrophenol has yet to be completed, but O₂ uptake would be expected to increase with increasing concentrations of 2,4-dinitrophenol (Newsholme and Leech, 1983). Of significance was the high survival of embryos after transfer on day 7 after incubation in 2,4-dinitrophenol. This confirms that at such concentrations, these compounds do not appear to be detrimental to subsequent survival. However, the long-term survival to calving has still to be assessed.

The modulation of metabolic activity to improve embryo development has been observed in several other species. In vitro culture in the presence of physiological glucose concentrations stimulates glycolytic behaviour in different types of cell, including preimplantation embryos (reviewed by Leese et al., 1998). Such upregulation of glycolysis (that is, the ‘Crabtree effect’) during early cleavage is detrimental to embryo development in several species (Bavister, 1995) and has been controlled by lowering concentrations of, or completely removing, glucose (for example, mice: Chatot et al., 1990; hamsters: Seshagiri and Bavister 1989; sheep: Thompson et al., 1992; cattle: Rosenkrans et al., 1993) or the addition of EDTA (100 μM) (Abramczak et al., 1977). Gardner and Lane (1993) demonstrated that addition of EDTA inhibited glycolysis, most likely by chelating Mg²⁺ ions, which are an important co-factor for the activity of regulatory glycolytic enzymes. However, this is the first study to demonstrate that post-compaction metabolism can be manipulated, leading to improved development in a mammalian species. The significance of this is in the observation that the time to first cleavage of bovine embryos in vitro is correlated with successful development to the blastocyst stage (Holm et al., 1998, Rieger et al., 1999). However, in the present study common conditions for early cleavage were used and all cleaved embryos were randomly allocated to subsequent treatment groups on day 5 of development. Thus the effect of these compounds is to ‘rescue’ embryos that normally would not proceed.

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