Protein content of cattle oocytes and embryos from the two-cell to the elongated blastocyst stage at day 16

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The protein content of cattle oocytes and preimplantation embryos produced *in vivo*, from the two-cell to the elongated blastocyst at day 16, was determined. From the oocyte to the expanded blastocyst stage (day 8), protein determination was carried out on zona pellucida-enclosed embryos. Protein content was measured by the Pierce Micro BCA protein assay. The mean protein content of oocytes was 0.126 µg, with no significant increase at the two-cell stage (0.132 µg). Protein content was higher at the morula stage (0.183 µg; $P < 0.05$) with a further increase at the expanded blastocyst stage (0.367 µg; $P < 0.05$). There was a 160-fold increase in protein content from the expanded blastocyst to the hatched day 13 stage. Spherical, ovoid and elongated blastocysts were collected on days 13 and 14. The mean protein content of day 13 (59.8 µg) and day 14 (92.4 µg) embryos was similar ($P > 0.1$), but the protein content of the elongated embryos was higher than that of ovoid or spherical embryos collected on the same day. Protein content of day 15 embryos (362.2 µg) was higher than that on day 14, with a further increase to 946.6 µg by day 16. The correlation between protein content and day of development contained both a linear and a quadratic component. Embryo length and width increased from day 13 (5.24 mm and 0.89 mm, respectively) to day 16 (51.6 mm and 1.82 mm, respectively). From day 13 to day 16, the protein content was correlated with both embryo length and width ($r^2 = 0.89$ and 0.51 , respectively; $P < 0.001$) and was highly correlated ($r^2 = 0.95$) with the product of embryo length by width, indicating that protein content increases as a function of surface area.

Introduction

A major problem faced by the cattle breeding industry is the high rate of embryo mortality which compromises reproductive efficiency, genetic improvement and the development and exploitation of embryo-related biotechnology. After a single insemination, fertilization rate is about 90%, but calving rate is only about 55%, with more than 30% of embryos lost by day 16 (Diskin and Sreenan, 1980). To address this problem of early embryo death and to enhance the supply of cattle embryos for *in vitro* fertilization it is vital to obtain a better understanding of normal embryo development. Knowledge of the protein content of embryos, from fertilization to development of the blastocyst, is essential for the interpretation of information arising from studies on parameters, such as energy metabolism (Rieger and Guay, 1988) and protein synthesis (Frei *et al.*, 1989), that are involved in the control of embryo development. However, while information is available on the protein content of the preimplantation embryos of mice (Brinster, 1967; Schiffner and Spielmann, 1976; Sellens *et al.*, 1981), rats (Schiffner and Spielmann, 1976), rabbits (Morgan and Kane,

1993) and pigs (Anderson, 1978; Wright *et al.*, 1981, 1983), such information has not been published for cattle embryos. We have therefore measured the protein content of cattle oocytes and of preimplantation embryos produced *in vivo* from the two-cell to the elongated blastocyst stage.

Materials and Methods

Embryos and oocytes

Hereford-cross heifers were used as donors of the embryos produced *in vivo*. Each heifer received 1500 iu of pregnant mares' serum gonadotrophin i.m. (Folligon, Intervet UK Ltd, Cambridge) during the mid-luteal phase of the oestrous cycle (days 10–14) and 500 µg cloprostenol (Estrumate, Coopers Animal Health Ltd, Berkhamsted) 48 h later to induce luteolysis. From 48 h after administration of cloprostenol the heifers were continuously checked for overt signs of oestrus and those observed at the onset of oestrus were artificially inseminated with semen from one sire. Embryo recovery was carried out during mid-ventral laparotomy performed under licence in accordance with the European Community Directive, 86-609-EC. Thiopentone sodium (5 g, i.v.; Rhone Merieux, Harlow)

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was used as anaesthetic followed by closed circuit anaesthesia with halothane (May and Baker Ltd, Dagenham) and oxygen. To obtain embryos at the two-cell stage, each oviduct was flushed with 20 ml medium between 44 and 54 h after the onset of standing oestrus. Morulae, mid-blastocyst and expanded blastocyst stages were flushed from the uterus at 6, 7 and 8 days after the observed onset of standing oestrus, while hatched and elongated blastocysts were flushed from the uterus at days 13, 14, 15 and 16. The composition of the flushing medium was 0.1% (w/v) polyvinyl alcohol, 139 mmol NaCl l⁻¹, 2.7 mmol KCl l⁻¹, 0.89 mmol CaCl₂ · 2H₂O l⁻¹, 1.47 mmol KH₂PO₄ l⁻¹, 0.49 mmol MgCl₂ · 6H₂O l⁻¹, 7.46 mmol Na₂HPO₄ · 2H₂O l⁻¹, 1 mmol glucose l⁻¹ and 0.5 mmol pyruvic acid (sodium salt) l⁻¹, pH 7.3. After recovery, embryos were graded for viability on a morphological scale from 1 (excellent) to 5 (degenerate) according to Hasler *et al.* (1987) for two-cell to expanded blastocyst stages. Viability of hatched and elongated blastocysts was based on morphological appearance and expected developmental stage for age. Only grade 1 and 2 embryos were included in the study. Length and width of the blastocysts at day 13 to 16 were measured using a graticule on a stereoscopic microscope for measurements under 2 mm or directly with a calibrated rule for larger embryos. Embryos were designated spherical when the length and width were approximately equal, ovoid when the length was more than 1.5 times the width but was less than 1 cm, or elongated when the length was more than five times the width and was longer than 1 cm. Surface area could not be estimated using the standard formula for a sphere or a cylinder, because from day 13 cattle embryos tend to be ribbon-like rather than perfect spheres or cylinders. However, length × width is always a component of total surface area of an object of general cylindrical, spherical or ovoid form and was used as an index of surface area. Only intact embryos were used in the study. Embryos were washed three times in the same flushing medium but from which the glucose was omitted to avoid interference with the protein assay. Embryos were transferred to 200 µl 95% ethanol (Merck, Darmstadt) in 1.5 ml conical plastic tubes (Safe-twist, Eppendorf, Hamburg) which were stored in liquid N₂ until assay. At least nine oocytes and pre-blastocyst stage embryos or four blastocysts or two expanded blastocysts were pooled for each determination to ensure enough protein to give an accurate reading. From day 13 onwards hatched embryos were assayed singly for protein content.

Oocyte collection was carried out by aspiration of follicles of 2–8 mm diameter from cattle ovaries obtained at a local abattoir. Ovaries were transported to the laboratory in saline (0.9% (w/v) NaCl) maintained at 30–35°C. Follicle aspiration was performed with a 10 ml syringe and an 18-gauge needle and the aspirated fluid was transferred to a small Petri dish and examined under a stereoscopic microscope (× 10) for oocytes. Denuded oocytes and those with expanded cumulus were discarded. Oocytes with at least three layers of cumulus cells were transferred to a 15 ml conical tube containing 2 ml flushing medium and vortexed for 5 min to remove cumulus cells. Cumulus-free oocytes were then selected, washed in the glucose-free medium and stored in the same way as embryos recovered *in vivo*. Samples of the final washing solution were also similarly stored for each group and used as blanks in the protein assay.

Protein determination

Embryos and oocytes were thawed at room temperature and sonicated in their storage tubes by exposure to sonication bursts of 6 s until visibly disrupted when examined under the microscope (× 10). Disruption required several sonication bursts for oocytes and zona-enclosed embryos, while one burst was sufficient for the hatched embryos. Oocyte and embryo tissue samples were then centrifuged at 12 000 g for 15 min. After centrifugation the supernatant was poured off and the pellet was washed once with 200 µl 95% ethanol and centrifuged again. Tubes containing blanks were treated and stored in a similar manner.

Protein in the pellet was estimated using the Pierce Micro BCA assay (Pierce and Warriner Ltd, Chester) as described by Morgan and Kane (1993). Protein standards containing 0.5–10 µg BSA per 0.5 ml distilled H₂O were prepared. Standards and oocyte and embryonic tissue samples were hydrolysed with 5 µl 10 mol NaOH l⁻¹, for 30 min at 56°C and neutralized with 10 µl 5 mol HCl l⁻¹. The hydrolysed material was diluted to a final volume of 500 µl. For stages from oocyte to expanded blastocyst the total volume was assayed, while for day 13–16 stages, aliquots of 5–100 µl were assayed in duplicate using the protocol supplied with the kit.

Statistical analyses

Data on the protein content of oocytes and early embryo stages up to the expanded blastocyst were analysed by analysis of variance (Proc GLM, SAS, 1988) followed by Duncan's multiple range test. The hatched embryos collected from day 13 onwards had a protein content more than 160 times that of the pre-hatched expanded blastocysts; therefore, the data were analysed separately. In this case, the protein content data were log transformed and analysed by analysis of variance followed by Duncan's multiple range test where appropriate. Day was fitted as a linear and quadratic term in the model. Results are presented as arithmetic means and ranges. The effect of recovery day (day 13–16) on embryo length and width and the relationship between embryo length, width and protein content were analysed by analysis of variance with linear and quadratic terms fitted where appropriate.

Results

Protein content was similar for oocytes and two-cell stages. It was higher ($P < 0.05$) at the morula and blastocyst stages, for which the results were similar, and was highest ($P < 0.05$) at the expanded blastocyst stage (Table 1).

Between days 13 and 16, the protein content increased in a linear ($P < 0.001$) and quadratic ($P < 0.001$) manner (protein content (µg per embryo) = 23 518 – 3508 (day) + 131 (day)²; $r^2 = 0.69$) (Table 2). Between days 13 and 16, embryo length also increased in a linear ($P < 0.001$) and quadratic ($P < 0.001$) manner (embryo length (mm) = 1109 – 166.86 (day) + 6.30 (day)²; $r^2 = 0.63$), while embryo width increased in a linear ($P < 0.001$) manner (embryo width (mm) = – 2.31 + 0.255 (day); $r^2 = 0.31$). The protein content increased linearly and quadratically ($P < 0.001$) with embryo length (protein content

Table 1. Protein content of cattle oocytes and pre-hatched embryos

Stage	Number of oocytes or embryos	Number of oocytes or embryos per replicate	Number of replicates	Protein (μg) per oocyte or embryo (mean $\pm$ SEM)
Oocytes	115	9–11	12	0.126 ± 0.0059^a
Two-cell (day 2)	67	8–14	5	0.132 ± 0.0196^a
Morula (days 6–7)	62	8–14	7	0.183 ± 0.0203^b
Blastocyst (days 7–8)	40	4–10	6	0.185 ± 0.0158^b
Expanded blastocyst (day 8)	12	2–4	5	0.367 ± 0.0277^c

Values with different superscripts are significantly different ($P < 0.05$).

Table 2. Protein content and size of hatched cattle blastocysts recovered on different days after oestrus

Day	Number of embryos	Length (mm)	Width (mm)	Protein (μg)
13	18	5.24 ± 0.869 (0.8–13.3)	0.89 ± 0.048 (0.58–1.3)	59.8 ± 8.19 (3.0–140.8)
14	37	7.84 ± 1.801 (0.8–55.0)	1.1 ± 0.037 (0.7–1.7)	92.4 ± 21.35 (5.7–701.4)
15	12	25.08 ± 3.636 (6.0–43.0)	1.28 ± 0.134 (0.5–1.8)	362.2 ± 73.16 (74.7–741.2)
16	5	51.6 ± 3.816 (40.0–60.0)	1.82 ± 0.296 (1.0–2.5)	946.6 ± 135.76 (613.2–1385.3)

Values are means $\pm$ SEM.

Figures in parentheses are the range.

(μg per embryo) = $9.65 + 8.35$ (embryo length mm) + 0.16 (embryo length)²; $r^2 = 0.89$). Protein content also increased linearly ($P < 0.001$) with embryo width (protein content (μg per embryo) = $-408.4 + 524.3$ (embryo width mm); $r^2 = 0.51$). The relationship between protein content, embryo length and width is described by the equation (protein content (μg per embryo) = $12.37 + 9.57$ (embryo length mm) (embryo width mm) and is graphically presented (Fig. 1). The coefficient of determination (r^2) was 0.95, indicating that only 5% of the observed variation in protein content was due to sources other than embryo length and width.

Within days 13 and 14 there was a large variation in size, shape and protein content of the embryos recovered (Table 3). Spherical, ovoid and elongated embryos were recovered on each of these days and there was a day $\times$ embryo developmental stage interaction on protein content; spherical embryos collected on day 14 had a higher protein content than did spherical embryos collected on day 13 ($P < 0.05$). Ovoid and elongated embryos collected on day 13 had similar protein contents to ovoid and elongated embryos collected on day 14 respectively ($P > 0.1$). Protein content was higher in ovoid

embryos (day 13 and day 14) than in spherical embryos and higher again in elongated embryos ($P < 0.05$).

Discussion

This is the first report of the protein content of cattle oocytes and embryos, from the two-cell stage to the elongated blastocyst stage at day 16. There is a relatively small increase in protein content from the oocyte to the blastocyst stage, while the zona-enclosed expanded blastocyst had a protein content approximately twice that of the blastocyst. There was a marked increase (160-fold) in the protein content of hatched blastocysts recovered on day 13 compared with the zona-enclosed expanded blastocyst. From day 13 to day 16 the protein content increased exponentially.

The protein content ($0.132 \mu\text{g}$) of the two-cell cattle embryos presented here is large compared with that reported for rats ($0.032 \mu\text{g}$; Schiffner and Spielmann, 1976) and mice ($0.026 \mu\text{g}$; Brinster, 1967; Schiffner and Spielmann, 1976), is similar to rabbits ($0.17 \mu\text{g}$; Morgan and Kane, 1993) and is

Fig. 1. Protein content of hatched cattle blastocysts recovered on days 13–16 expressed as a function of blastocyst length $\times$ diameter. Insert shows in detail results for smaller embryos ($< 150 \mu\text{g}$ protein).

Table 3. Protein content and size of cattle blastocysts recovered on days 13 and 14 by developmental stage

Day	Stage	n	Length (mm)	Protein (μg)
13	Spherical	4	0.89 ± 0.005	4.51 ± 0.661^a
	Ovoid	12	5.48 ± 0.612	66.08 ± 4.881^c
	Elongated	2	12.45 (11.6, 13.3)	132.7 (140.8, 124.6) ^d
14	Spherical	9	1.42 ± 0.159	12.47 ± 1.538^b
	Ovoid	20	4.04 ± 0.348	56.98 ± 5.731^c
	Elongated	8	24.58 ± 4.922	270.67 ± 67.884^d

Values given are means $\pm$ SEM.

Values with different superscripts are significantly different ($P < 0.05$).

lower than that reported for pigs ($0.273 \mu\text{g}$; Wright *et al.*, 1981). The significant increase in protein at the expanded blastocyst stage is similar to data reported for rabbits (Morgan and Kane, 1993) and pigs (Wright *et al.*, 1981) and is consistent with the increase in embryo size. The diameter of the expanded cattle blastocyst is about 1.2–1.5 times that of the blastocyst (Lindner and Wright, 1983) and contains about 160 cells compared with about 120 cells in the blastocyst (Mannaerts, 1986). The increase in protein content at this stage differs, however, from that of mice and rats, in which embryonic protein content decreases between the one–two-cell and morula–blastocyst stages (Brinster, 1967; Schiffner and Spielmann, 1976). The significant increase in protein content

from the two-cell to the morula stage, with a further increase at the expanded blastocyst stage, reported here is consistent with the increase in protein synthesis recorded at these stages by Frei *et al.* (1989). Increases in pyruvate uptake and ATP production (Thompson *et al.*, 1996) and glucose metabolism (Javed and Wright, 1991; Rieger *et al.*, 1992) are also consistent with the marked increase in protein content reported here, as they reflect the energy requirement of protein synthesis.

Between day 13 and day 16, embryo protein content increased in a linear and quadratic fashion, consistent with the linear and quadratic increase for embryo length, and the linear increase in embryo width recorded over this time. After hatching, cattle embryos begin a phase of rapid growth before implantation with a period of exponential growth between spherical blastocysts on day 13 and elongated blastocysts on day 16 (Betteridge *et al.*, 1980), and this is reflected in the linear and quadratic nature of the relationship between embryo protein content and day of development presented here. Implantation in cattle occurs later than in pigs and, therefore, the developmental stages are not equivalent. However, cattle embryos recovered on day 13 in the present study were similar in shape, size and protein content to pig embryos at day 10, while the results reported here for cattle embryos recovered on days 15–16 were similar to those reported for pig embryos recovered on day 11 by Anderson (1978).

There was significant within-day variation in the size, shape and protein content of the cattle embryos with spherical, ovoid and elongated embryos recovered at days 13 and 14. At days 15 and 16, the embryos were elongated but with great variation in length within days. This variation in size and shape

is not unique to superovulated embryos; a similar variation in the embryos of single-ovulating heifers has been reported by Betteridge *et al.* (1980). The variation in size and shape of embryos at the hatched stages is also consistent with the data on pigs reported by Anderson (1978).

The significant correlation between embryo length and protein content between day 13 and day 16 is similar to that reported by Anderson (1978) for pigs. However, Wright *et al.* (1983), in a subsequent study, failed to find a correlation between protein content and volume of pig blastocysts. In the study reported here, protein content increased with both embryo length and width, suggesting a positive correlation between embryo surface area and protein content. Some caution is required, however, in interpreting the data for width of day 13 to day 16 blastocysts, as the width is extremely difficult to measure accurately due to the folded nature of the elongated cattle blastocyst.

Proportionately more elongated embryos fragmented at days 15 and 16 than at days 13 or 14, indicating that the larger embryos have a greater tendency to fragment. The protein content of day 15 and day 16 embryos reported here is for intact embryos and may therefore represent the smaller embryos recovered on those days.

A knowledge of the protein content of normal preimplantation cattle embryos is necessary as a basis for interpretation of *in vitro* culture data, metabolic and protein synthesis studies. It is also an important parameter in the assessment of embryo growth and viability, particularly at the critical stage between blastocyst formation and elongation, when maternal recognition of pregnancy occurs and indeed when most embryo loss occurs.

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