Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos

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In vitro produced bovine zygotes show substantial variation in the time required to complete the first cell cycle and in their *in vitro* development potential. A number of reports have highlighted the fact that the fastest developing embryos *in vitro* are most likely to be comparable with their *in vivo* counterparts. At 24 h after IVF, presumptive zygotes were cultured in droplets of synthetic oviduct fluid medium. Droplets were examined at regular intervals and all cleaved embryos at each time point were transferred into new droplets and cultured separately for the duration of the experiment. All uncleaved zygotes were returned to the incubator and re-examined at the successive time points until 48 h after insemination, at which time the remaining uncleaved oocytes were retained as a group. A representative number of day 7 blastocysts from zygotes that had cleaved by 30 or 36 h were transferred to synchronized recipients and pregnancy was diagnosed by ultrasonography at day 35. Glucose and glutamine metabolism was examined in zygotes and blastocysts and compared retrospectively with time of first cleavage. A representative number of blastocysts from each of the cleavage groups was sexed using PCR. Data were analysed by chi-squared and regression analysis. Development to the blastocyst stage decreased as the time from insemination to first cleavage increased ($r = 0.97$, $P < 0.03$). There was no difference in blastocyst hatching, number of blastocyst cells or pregnancy rate between the 30 and 36 h groups. The overall sex ratio was 62% males ($n = 258$, $P < 0.0001$) and was not different in the 30 and 36 h groups (61%, $n = 155$ versus 63%, $n = 95$, respectively). These results indicate that although time of first cleavage has a major influence on the probability of an embryo developing to the blastocyst stage, once that stage is attained, subsequent developmental characteristics are unrelated to the time of first cleavage.

Introduction

The factors that determine whether an oocyte develops to the blastocyst stage after fertilization are not yet fully understood. Despite wide variation in the techniques of *in vitro* bovine embryo production, blastocyst rates tend to plateau at about 40% of oocytes submitted to *in vitro* maturation (Gordon, 1994). It is becoming increasingly clear that although suboptimal culture conditions undoubtedly contribute to this poor embryo yield, the developmental rates obtainable *in vitro* are also related to the intrinsic quality of the oocyte. There are several lines of evidence in support of this idea. For example, oocytes derived from large follicles (that is, in the terminal stages of follicular development) develop better in culture than those derived

from small follicles (Pavlok *et al.*, 1992; Lonergan *et al.*, 1994). In addition, zygotes obtained after *in vivo* maturation and fertilization are superior in terms of development in culture to their *in vitro* counterparts (Marquant-Le Guienne *et al.*, 1989; McCaffrey *et al.*, 1991).

In vitro produced bovine embryos show substantial variation in both the time required to complete the first cell cycle and in their *in vitro* development potential. Various reports have highlighted the fact that the fastest developing embryos *in vitro* are more likely to develop to the morula and blastocyst stages (Lonergan *et al.*, 1992; Van Soom *et al.*, 1992; Xu *et al.*, 1992; Yadav *et al.*, 1993; Grisart *et al.*, 1994; Plante *et al.*, 1994; Itagaki *et al.*, 1997; Kubisch *et al.*, 1998). Moreover, those blastocysts that form early appear to have a greater likelihood of providing live offspring after transfer (Hasler *et al.*, 1995). The mechanisms controlling this development are almost certainly in place in the oocyte before fertilization. For

example, oocytes extruding the first polar body earliest during *in vitro* maturation have a higher probability of reaching the blastocyst stage (Dominko and First, 1992; van der Westerlaken *et al.*, 1994). Thus, the speed of development is directly related to the development potential and may be an important parameter in selecting embryos that have the greatest likelihood of establishing and maintaining a pregnancy after transfer.

This situation is not unique to the bovine embryo. A relationship between the kinetics of early embryonic cleavage and subsequent development has been observed in mouse (Warner *et al.*, 1998), hamster (McKiernan and Bavister, 1994), rhesus monkey (Bavister *et al.*, 1983), buffalo (Totey *et al.*, 1996) and human (Lelaidier *et al.*, 1995; Sakkas *et al.*, 1998; Shoukir *et al.*, 1998) embryos. These kinetics of development have been related to the sex of the embryo and faster developing embryos tend to be males (Avery *et al.*, 1989, 1991; Xu *et al.*, 1992; Yadav *et al.*, 1993; Carvalho *et al.*, 1996), although this has not been observed in all cases (Grisart *et al.*, 1995; Lonergan *et al.*, 1995; Kaminski *et al.*, 1996). This difference in developmental rates between male and female embryos has been attributed to differences in metabolic activity in these embryos (Tiffen *et al.*, 1991; Ray *et al.*, 1995).

Although there is evidence of an effect of time of cleavage on subsequent development to the blastocyst stage in cattle, there is a lack of information on the ability of these blastocysts to establish and maintain a pregnancy. The objectives of this series of experiments were: (i) to examine the relationship between time of first cleavage and formation rates and sex of the resulting blastocysts; (ii) to examine the embryo metabolism rates according to cleavage times; and (iii) to determine pregnancy rates in synchronized recipients after non-surgical transfer of blastocysts originating from 'early cleaving' or 'late cleaving' embryos.

Materials and Methods

Oocyte collection and in vitro maturation (IVM), fertilization (IVF) and culture (IVC)

Chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise indicated. A stock solution of 10 µg epidermal growth factor (EGF) ml⁻¹ was prepared, aliquoted and stored at -20°C until use.

Cumulus-oocyte complexes (COCs) were obtained by aspiration of follicles 2–8 mm in diameter from the ovaries of cows killed at an abattoir. After four washes in modified PBS supplemented with 36 µg pyruvate ml⁻¹, 50 µg gentamycin ml⁻¹ and 0.5 mg bovine serum albumin (BSA) ml⁻¹ (Sigma fraction V, catalogue number A-9647), groups of approximately 50 COCs were placed in 500 µl maturation medium in four-well dishes (Nunc, Roskilde) and cultured for 24 h at 39°C in an atmosphere of 5% CO₂ in air with maximum humidity. The maturation medium was medium 199 supplemented with 10 ng EGF ml⁻¹ (Lonergan *et al.*, 1996).

For IVF, COCs were washed four times in PBS and then in fertilization medium before groups of 50 were transferred

into four-well plates containing 250 µl fertilization medium (Tyrode's medium with 25 mmol bicarbonate l⁻¹, 22 mmol sodium lactate l⁻¹, 1 mmol sodium pyruvate l⁻¹, 6 mg fatty acid-free BSA ml⁻¹ and 10 µg heparin-sodium salt ml⁻¹ (184 units heparin mg⁻¹), Calbiochem, San Diego, CA) per well. Motile spermatozoa (Dovea AI Station, Mallow, Ireland) were obtained by centrifugation of frozen-thawed spermatozoa on a discontinuous Percoll (Pharmacia, Uppsala) density gradient (2.5 ml 45% (v/v) Percoll over 2.5 ml 90% (v/v) Percoll) for 20 min at 700 g at room temperature. Viable spermatozoa collected at the bottom of the 90% fraction were washed in Hepes-buffered Tyrode's and pelleted by centrifugation at 100 g for 10 min. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of Tyrode's albumin lactate pyruvate (TALP) to give a concentration of 2 × 10⁶ spermatozoa ml⁻¹. A 250 µl aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1 × 10⁶ spermatozoa ml⁻¹. Plates were incubated for 24 h in 5% CO₂ in humidified air at 39°C. In the experiments described below, the time of addition of the spermatozoa to the oocytes is defined as the time of insemination.

Embryo culture was carried out in modified synthetic oviduct fluid medium (SOF) under mineral oil in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 39°C (Carolan *et al.*, 1995). At 24 h after insemination, presumptive zygotes were denuded of cumulus cells by vortexing for 2 min in 2 ml PBS. The zygotes were washed four times in PBS and then in SOF before they were transferred to 25 µl culture droplets. After 24 h in culture (that is, 48 h after insemination), 10% (v/v) fetal calf serum (FCS) was added to the droplets. Blastocyst rates were recorded at day 8 after insemination.

These conditions have routinely resulted in 90–95% maturation, 85–90% fertilization and 35–45% blastocyst formation for cattle oocytes (Carolan *et al.*, 1995; Lonergan *et al.*, 1996).

Experiment 1: effect of time of first cleavage on subsequent development

In a preliminary study (Expt 1a, three replicates), 577 COCs were used. After IVM and IVF, presumptive zygotes were cultured *in vitro* in droplets of SOF at 24 h after insemination. The drops were examined at 30, 36, 42 and 48 h after insemination. At each time point, all embryos with two or more distinct blastomeres were transferred into new IVC droplets and were cultured separately for the duration of the experiment. All uncleaved presumptive zygotes were returned to the incubator and re-examined at the successive time points until 48 h after insemination, at which time those still uncleaved were retained as a group.

For estimation of the total number of cells, blastocysts were placed on a slide, air-dried, and fixed in 100% ethanol overnight. They were subsequently stained using Hoechst 33342 (10 mg ml⁻¹ in 2.3% (w/v) sodium citrate) and visualized with an epifluorescence microscope (Zeiss, Oberkochen).

This experiment was repeated (Expt 1b, three replicates)

except that the zygotes ($n = 839$) were examined at more frequent intervals (27, 30, 33, 36, 42, 48 h after insemination) to examine the effect of time of cleavage in more detail. At each time point, embryos that had cleaved were removed and cultured separately for the remainder of the culture period as described for Expt 1a.

Experiment 2: measurement of embryo metabolism

Two separate metabolism studies were conducted. In Expt 2a (three replicates), presumptive zygotes were removed from culture at 21 h after insemination (that is, before the first cleavage division). Any remaining cumulus cells were removed and the zygotes were washed twice in metabolic measurement medium (see below) before they were subjected to the metabolic measurement procedure. After this procedure, the zygotes were cultured individually and evaluated for cleavage at 27, 30, 33, 36 and 42 h after insemination. The metabolic data were then analysed by time of cleavage.

In Expt 2b (two replicates), presumptive zygotes were separated by time of first cleavage (at 27, 30, 33, 36 and 42 h after insemination) and cultured in groups. On day 8, blastocysts from each group were removed from culture and washed twice in metabolic measurement medium before they were subjected to the metabolic measurement procedure.

The medium used during the measurement of embryo metabolism was SOF supplemented with 1.0 mmol glucose l^{-1} , 1.0 mmol glutamine l^{-1} , and 10 mmol Hepes l^{-1} . The technique used to measure embryo metabolism was as described by Rieger *et al.* (1992a), except for the composition of the medium. Mixtures of D-[5- ^3H]glucose (specific activity = 17.4 Ci mmol $^{-1}$, Amersham, Dublin) and L-[1- ^{14}C (U)]glutamine (specific activity = 277 mCi mmol $^{-1}$, Amersham) were dried under nitrogen and taken up in metabolic measurement medium to give concentrations of 0.25 $\mu\text{Ci } \mu\text{l}^{-1}$ of each radiolabelled substrate.

Individual embryos were taken up in 2 μl metabolic measurement medium and placed in the cap of a sterile 2.5 ml polypropylene screw-cap microtube with 2 μl radiolabelled substrate mixture (total culture volume of 4 μl) to yield final total concentrations (labelled plus unlabelled) of 1.0 mmol glucose l^{-1} and 1.5 mmol glutamine l^{-1} . The cap was fitted onto the tube quickly. The tubes had been loaded with 1.5 ml of 25 mmol NaHCO_3 l^{-1} equilibrated with a gas mixture of 5% O_2 , 5% CO_2 and 90% N_2 . Three sham preparations, containing all reagents but no embryo, were included for each preparation of labelled substrates. These served as controls for all non-specific counts due to machine background chemiluminescence, bacterial contamination, and spontaneous breakdown of the labelled substrate. After 3 h, the cap was removed and the bicarbonate was mixed with 15 ml scintillation fluid and counted for 5 min in a liquid scintillation counter. The total d.p.m. of labelled substrate was determined by mixing 2 μl labelled substrate solution with 1.5 ml of 25 mmol NaHCO_3 l^{-1} , and counting in the same way. The amount of each substrate metabolized by each embryo was calculated according to Rieger *et al.* (1992a).

Experiment 3: sex ratio of embryos

Two separate sexing experiments were carried out. In Expt 3a (three replicates), day 8 blastocysts ($n = 258$) originating from the different cleavage groups (30, 36 and 42 h after insemination) were sexed using the procedure described by Pegoraro *et al.* (1998), whereas in Expt 3b (three replicates), embryos were lysed at the two-cell stage ($n = 192$) as they cleaved (that is, at 30, 36, 42 and 48 h after insemination).

Briefly, embryos were lysed individually in an Eppendorf tube with 50 μl buffer containing 50 mmol KCl l^{-1} , 2.5 mmol MgCl_2 l^{-1} , 0.1% (v/v) Triton-X100, 150 μg proteinase K ml^{-1} and 15 mmol Tris-HCl l^{-1} , pH 8.9. Tubes containing lysed embryos were incubated at 37°C for 45 min, followed by incubation at 99°C for 10 min to inactivate proteinase K.

Two pairs of flanking primers were used: the first pair against an autosomal sequence of 443 bp, indicating the presence of bovine genomic DNA; the second detected a sequence of 148 bp specific to the bovine Y chromosome. Lysate (9 μl) was amplified with 2 iu Taq polymerase (Perkin Elmer, Courtaboeuf) in a final volume of 40 μl containing 10 mmol Tris-HCl l^{-1} , pH 8.3, 50 mmol KCl l^{-1} , 1.5 mmol MgCl_2 l^{-1} , 0.1% (w/v) gelatine, 0.2 mmol of each dNTP l^{-1} and 1 μmol of each Y chromosome sequence-specific primer l^{-1} and 0.08 μmol of each autosomal sequence-specific primer l^{-1} . The mixture was then amplified in a Perkin Elmer Cetus TC1 apparatus for 30 cycles; each cycle was composed of 20 s denaturation at 96°C, 20 s of hybridization and annealing at 60°C and 10 s elongation at 73°C.

After amplification, 3 μl gel loading solution (0.08% (w/v) Orange G, 30% (v/v) glycerol in 10 $\times$ TAE (4.84 g Tris l^{-1} , 1.142 ml acetic acid l^{-1} , 0.372 g EDTA l^{-1})) was added to the aqueous phase in each tube. Amplification products were resolved by electrophoresis on a 4.0% (w/v) agarose gel (Nusieve:Seakem, 3:1) prepared with a TAE buffer to which 0.3 μg ethidium bromide ml^{-1} was added. Gels were subsequently visualized under UV illumination and photographed.

Embryos were considered to be female when only a clear 443 bp product corresponding to the autosomic sequence was observed. When both the autosomic band and the band corresponding to the sequence of Y chromosome (148 bp) were observed or if a single clear 148 bp band was detected, the embryo was considered male. In all other cases (no visible bands, very weak signals, or bands in positions other than those expected), the sex of the embryo was not determined. Sex ratios of the various groups were compared with the expected ratio of 50%.

Experiment 4: embryo transfer

Day 7 blastocysts ($n = 107$, two replicates) originating from oocytes that had cleaved by 30 h after insemination ($n = 55$, 'early' group) or between 30 and 36 h after insemination ($n = 52$, 'late' group) were transferred to synchronized recipients (one blastocyst per recipient). Heifers were synchronized using a controlled internal drug releasing (CIDR) device (InterAg, Hamilton) for 10 days; 15 mg Luprostiol (Prosolvine; Intervet, Cambridge) was administered 2 days before CIDR device withdrawal. Pregnancy was diagnosed at day 35 by ultrasound scanning.

Table 1. Effect of time of first cleavage on development of bovine oocytes (Expt 1a)

Time of first cleavage (h after insemination)	Cleaved		Day 8 blastocysts			Hatched		Number of cells Mean ± SEM (n)
	n	Percentage of total	n	Percentage	Percentage of total	n	Percentage	
30	306	53.0	187	61.1 ^a	75.4	96	51.3	136 ± 6 (176)
36	170	29.5	59	34.7 ^b	23.8	35	59.3	120 ± 7 (59)
42	34	5.9	2	5.9 ^c	0.8	0	–	75 ± 15 (2)
48	19	3.3	0	–	–	–	–	–
NC	48	8.3	0	–	–	–	–	–
Total	577	100.0	248	43.0	100.0	131	52.8	131 ± 4 (237)

^{abc}Values in the same column with different superscripts are significantly different ($P < 0.0004$).
NC: not cleaved at 42 h after insemination.

Table 2. Effect of time of first cleavage after insemination on the number of bovine blastocyst cells

	Number of blastocyst cells Mean ± SEM (n)		
	30 h after insemination	36 h after insemination	42 h after insemination
Hatched blastocyst	189 ± 8 (87) ^{a1}	150 ± 8 (36) ^{a2}	–
Blastocyst	84 ± 2 (89) ^{b1}	72 ± 4 (23) ^{b2}	75 ± 15 (2)
Total	136 ± 6 (176)	120 ± 7 (59)	75 ± 15 (2)

^{a1}Values in the same column with different superscripts are significantly different ($P < 0.05$).
¹²Values in the same row with different superscripts are significantly different ($P < 0.05$).

Statistical analysis

Treatment effects on cleavage rate, blastocyst yield and blastocyst hatching rate were determined by examining totals of all replicates using chi-squared analysis. Blastocyst rates were analysed by regression analyses as a function of the time of first cleavage. The number of cells (Expt 1a) was expressed as mean ± SEM and totals were compared using the Mann–Whitney test. A P value < 0.05 was considered significant. Similarly, sex ratios were compared with the expected ratio of 50% using chi-squared analysis.

The effect of time of first cleavage on the metabolism of glucose and glutamine by individual zygotes was analysed by one-way analysis of variance, followed by Duncan's multiple-range tests to compare group means. The metabolism of glucose and glutamine by day 8 blastocysts was analysed by regression analyses of metabolism as a function of the time of first cleavage.

Results

Experiment 1a

When the presumptive zygotes were placed in culture at 24 h after insemination, none had cleaved, although several showed signs of imminent division. The number of zygotes that had cleaved at 30, 36, 42 and 48 h after insemination was 306 (53%), 170 (29%), 34 (6%) and 19 (3%), respectively; 48 (8%) remained uncleaved at 48 h after insemination (Table 1).

The overall cleavage rate assessed at 72 h after insemination was 94% (545/577), and 71% (410/577) of oocytes had reached the five- to eight-cell stages at this time. The overall blastocyst rate was 37% (213/577) at day 6 and 43%

(248/577) at day 8. The hatching rate at day 8 was 53% (131/248).

Development to the blastocyst stage decreased markedly as the time from insemination to first cleavage increased ($P < 0.0001$). Of the 306 zygotes that had cleaved by 30 h after insemination, 187 (61%) developed to the blastocyst stage, of which 96 (51%) hatched. In the 170 embryos that underwent cleavage between 30 and 36 h after insemination, 59 (35%) reached the blastocyst stage, of which 35 (59%) hatched. However, only two of the 34 (6%) embryos that cleaved between 36 and 42 h after insemination developed to the blastocyst stage and none hatched. None of the embryos that underwent cleavage 42 h after insemination developed to the blastocyst stage.

Data on the number of blastocyst cells are presented (Table 2). Irrespective of the time of first cleavage, hatched blastocysts had significantly more cells than non-hatched blastocysts (189 ± 8 versus 84 ± 2 , $P < 0.0001$, for oocytes that had cleaved at 30 h after insemination; 150 ± 8 versus 72 ± 4 , $P < 0.0001$, for embryos that cleaved at 36 h after insemination). In addition, hatched blastocysts originating from oocytes that cleaved at an earlier stage had a greater number of cells than those from late cleaving oocytes (189 ± 8 versus 150 ± 8 for oocytes that cleaved at 30 and 36 h after insemination, respectively; $P < 0.002$). The same observation was made in non-hatched blastocysts (84 ± 2 versus 72 ± 4 , for oocytes that cleaved at 30 and 36 h after insemination, respectively; $P < 0.01$).

Experiment 1b

The overall cleavage rate was 85% (716/839) and the overall blastocyst yield was 39% (328/839) on the basis of the

Table 3. Effect of time of first cleavage on development of bovine oocytes (Expt 1b)

Time of first cleavage (h after insemination)	Cleaved		Day 8 blastocysts		
	<i>n</i>	Percentage of total	<i>n</i>	Percentage	Percentage of total
27	350	41.7	204	58.3 ^a	62.2
30	200	23.8	89	44.5 ^b	27.1
33	76	9.1	23	30.3 ^c	7.0
36	26	3.1	5	19.2 ^c	1.5
42	51	6.1	6	11.8 ^c	1.8
48	13	1.5	1	1.6 ^c	0.3
NC	123	14.7	0	—	—
Total	839	100.0	328	39.1	100.0

^{abc}Values in the same column with different superscripts are significantly different ($P < 0.05$).

NC: not cleaved at 42 h after insemination.

Fig. 1. Effect of time of first cleavage of bovine embryos after insemination on blastocyst yield at day 8 (from Expt 1b). Values are mean $\pm$ SEM. The number of oocytes per group was 350, 200, 76, 26, 51 and 13 for zygotes that cleaved at 27, 30, 33, 36, 42 and 48 h after insemination, respectively.

number of oocytes inseminated (Table 3). In accordance with the results from Expt 1a, there was a significant correlation ($r = 0.97$, $P < 0.01$) between time of first cleavage and subsequent blastocyst yield, ranging from 58.3% in zygotes that cleaved by 27 h after insemination to only 7.7% in those cleaving between 42 and 48 h after insemination ($P < 0.0001$, Fig. 1).

Of the total number of blastocysts produced, 62.2% were derived from zygotes that cleaved by 27 h after insemination and 89.3% from zygotes that underwent the first cleavage division by 30 h after insemination.

Experiment 2

There was no effect of time of first cleavage on the metabolism of glucose ($P > 0.7$) or glutamine ($P > 0.7$) by presumptive zygotes from 21 to 24 h after insemination (Expt 2a, Table 4). There was a significant negative relationship between time of first cleavage and both glucose ($r = -0.363$, $P < 0.05$) and glutamine ($r = -0.393$, $P < 0.05$) metabolism by day 8 blastocysts (Expt 2b, Table 5).

Experiment 3

Results obtained from sexing the embryos in the different cleavage groups are shown (Table 6). From a total of 258 blastocysts (Expt 3a), 161 (62.4%) were male and 97 (37.6%) were female ($P < 0.001$). The male:female sex ratio of all groups was significantly higher than the expected ratio of 50% (30 h after insemination: 60.6%, $P < 0.01$; 36 h after insemination: 63.2%, $P < 0.025$; 42 h after insemination: 87.5%, $P < 0.05$). The sex ratio did not differ significantly among the different groups with respect to time of first cleavage.

At the two- to four-cell stages ($n = 192$), although the overall sex ratio differed significantly from the expected 1:1 (58.3% male, $P < 0.025$), the ratio within groups was not significantly different from 1:1. As in Expt 3a, the sex ratio did not differ among groups.

Experiment 4

After transfer of a single blastocyst per recipient, the overall pregnancy rate was 43% (46/107). There was no difference in pregnancy rates between the two groups (45% (25/55) versus 40% (21/52) for zygotes that cleaved by 30 and 36 h after insemination, respectively).

Discussion

The results of the present study demonstrate that early cleaving bovine embryos selected according to the time of completion of the first division have greater development potential than late cleaving embryos, as determined by the proportion reaching the blastocyst stage after 8 days in culture. The overall rates of cleavage ($> 85\%$), development to the blastocyst stage (40%) and number of cells are consistent with those in other studies using the same procedures for embryo production (Carolan *et al.*, 1995, Lonergan *et al.*, 1996). These findings indicate that the increased manipulation involved in the present study did not affect subsequent embryo development.

Table 4. Glucose and glutamine metabolism by bovine zygotes measured from 21 to 24 h after insemination (Expt 2a)

Time of first cleavage (h after insemination)	Number of zygotes	Glucose metabolism ^a	Glutamine metabolism ^a
27	3	2.0 ± 1.3	2.7 ± 0.6
30	26	1.6 ± 0.2	2.6 ± 0.2
33	12	1.7 ± 0.4	2.0 ± 0.2
36	4	2.2 ± 0.3	3.0 ± 0.4
42	7	1.5 ± 0.3	2.3 ± 0.4
NC	7	1.4 ± 0.2	2.5 ± 0.2
Total	59	1.6 ± 0.1	2.5 ± 0.1

^aValues are pmols per zygote per 3 h, mean ± SEM.

NC: not cleaved at 42 h after insemination.

Table 5. Glucose and glutamine metabolism by day 8 bovine blastocysts (Expt 2b)

Time of first cleavage (h after insemination)	Number of blastocysts	Glucose metabolism ^a	Glutamine metabolism ^a
27	9	162 ± 23	3.4 ± 0.5
30	9	140 ± 32	3.1 ± 0.8
33	9	99 ± 23	2.0 ± 0.6
36	5	100 ± 44	1.3 ± 0.6
42	1	36	1.7
Total	33	126 ± 14	2.6 ± 0.3

^aValues are pmols per embryo per 3 h, mean ± SEM.**Table 6.** Effect of time of first cleavage division on sex ratio of bovine embryos (Expt 3)

Time of first cleavage (h after insemination)	Sexed as blastocyst at day 8 (%)				Sexed as two- to four-cell embryo at 48 h after insemination (%)			
	<i>n</i>	Male	Female	Sex ratio	<i>n</i>	Male	Female	Sex ratio
30	155	94 (60.6)	61 (39.3)	1.54**	99	58 (58.6)	41 (41.4)	1.41
36	95	60 (63.2)	35 (36.8)	1.71***	69	39 (56.5)	30 (43.5)	1.30
42	8	7 (87.5)	1 (12.5)	7.00****	19	13 (68.4)	6 (32.6)	2.17
48	—	—	—	—	5	2 (40.0)	3 (60.0)	0.67
Total	258	161 (62.4)	97 (37.6)	1.66*	192	112 (58.3)	80 (41.7)	1.40***

Treatments in which the sex ratio differed significantly from 50% are indicated (chi-squared test).

P* < 0.001; *P* < 0.01; ****P* < 0.025; *****P* < 0.05.

The time points chosen were on the basis of previous studies indicating that the first cleavage of bovine zygotes *in vitro* occurs between 26 and 32 h after insemination (Barnes and Eyestone, 1990; Van Soom *et al.*, 1992; Yadav *et al.*, 1993). This was also the case under the conditions of the present study, in which no zygotes cleaved before 24 h after insemination. Approximately 42% of zygotes underwent the first cleavage division between 24 and 27 h after insemination. These represented the most competent oocytes in terms of developmental ability *in vitro*, and are comparable with the situation *in vivo* in which the first cleavage occurs at 24–28 h after ovulation in non-superovulated cows (Thibault *et al.*, 1987). More than 75% of zygotes underwent the first cleavage by 36 h after insemination, and these embryos accounted for more than 90% of all blastocysts produced.

The factors that determine the ability of a zygote to cleave

early rather than late are unclear. Although culture conditions influence the kinetics of early development (Pinyopummintr and Bavister, 1994; Van Langendonck *et al.*, 1997), the role of genetic factors should not be underestimated. In mice, a gene has been identified that controls the rate of preimplantation cleavage division and subsequent embryo survival (*Ped*: preimplantation embryo development) (for a review, see Warner *et al.*, 1998). Two alleles of the *Ped* gene have been identified: *Ped fast* and *Ped slow*. *Ped fast* mice have a faster rate of preimplantation development, larger litter sizes and are significantly larger at birth and at weaning. The Qa-2 antigen, the product of the *Ped* gene, is expressed in early mouse embryos, beginning at the two-cell stage, and is known to be present on the surface of mouse blastocysts (McElhinny and Warner, 1997). McElhinny *et al.* (1998) reported that *Ped fast* blastocysts contain a significantly greater number of cells in both the

inner cell mass and the trophoctoderm than *Ped slow* blastocysts, and that this phenotype is related directly to the expression of the Qa-2 antigen in both lineages. It is possible that such a gene is involved in the differential development rates in bovine embryos.

In addition to maternal influences transmitted via the oocyte, paternal influences may have an effect on development. It is well established that the bull can have a major effect on the outcome in an IVF programme (Hillery *et al.*, 1990; Marquant-Le Guienne *et al.*, 1990). The difference among bulls is apparent from the time of first cleavage (P. Lonergan, unpublished observation).

As well as positive influences, there may be negative influences involved in regulating the rate of development. Slimane *et al.* (1998) reported that 77% of two-cell bovine embryos that cleaved by 24–26 h after insemination were normal compared with only 44% of those that remained at the two-cell stage at 48 h after insemination, as determined by fluorescence *in situ* hybridization (FISH) with a probe specific for chromosome 1. It was suggested that monosomy and defective chromosome segregation at first mitosis are the main causes for delayed first cleavage. Similarly, Yadav *et al.* (1993) reported a higher incidence of chromosomal abnormalities in late cleaving compared with early cleaving bovine embryos. Other factors may also be involved, for example, Nolan *et al.* (1998) showed that factors such as nutritional treatment of the donor can influence the timing of blastocyst formation.

Significant changes in metabolic activity occur during development from the two-cell to the blastocyst stage in bovine embryos produced *in vitro*. Glucose metabolism is low until the 8- to 16-cell stages, and then increases continuously to the expanded blastocyst stage. In contrast, glutamine metabolism is high at the early cleavage stages, decreases to a minimum at the blastocyst stage and increases during blastocyst expansion (Rieger *et al.*, 1992a,b).

The overall mean metabolism of glucose by presumptive zygotes at 21–24 h after insemination (1.6 ± 0.1 pmols per 3 h) was consistent with other reports of glucose metabolism by mature oocytes (Rieger and Loskutoff, 1994) and two-cell embryos (Rieger *et al.*, 1992a,b). Mean glutamine metabolism by presumptive zygotes (2.5 ± 0.1 pmols per 3 h) was intermediate to previous measurements for mature oocytes (Rieger and Loskutoff, 1994) and two-cell embryos (Rieger *et al.*, 1992a,b). Although incubation in the metabolism medium for 3 h may have retarded the subsequent development of zygotes after they were returned to culture, there was no relationship between the metabolic activity of presumptive zygotes at 21–24 h after insemination and the time of first cleavage (27–42 h after insemination). This may indicate that the first cleavage is independent of the metabolic activity of the zygote, or that the metabolic requirements for first cleavage have been fulfilled before 21–24 h after insemination.

Mean glucose metabolism by day 8 blastocysts (126 ± 14 pmols per 3 h) was also consistent with other studies (Rieger *et al.*, 1992b), whereas mean glutamine metabolism by day 8 blastocysts (2.6 ± 0.3 pmols per 3 h) was lower than that observed in other studies (Rieger *et al.*, 1992b). The reasons for this are unclear, but may be related to differences in the source of oocytes or the embryo culture techniques. The

significant negative relationship between the time of first cleavage and both glucose and glutamine metabolism by day 8 blastocysts indicates that the mechanisms responsible for early first cleavage, or the increased time of development between first cleavage and day 8 can affect the metabolic activity of the blastocyst. However, the possibility that the observed differences were due to differences in the number of cells cannot be discounted.

Several studies demonstrated a relationship between development rate and sex of *in vitro* produced mammalian embryos, and male embryos developed faster than female embryos (mouse: Tsunoda *et al.*, 1985; Burgoyne, 1993; Valdivia *et al.*, 1993; Zwingman *et al.*, 1993; Peippo and Bredbacka, 1995; cattle: Avery *et al.*, 1989, 1991; Xu *et al.*, 1992; Yadav *et al.*, 1993; Dufour *et al.*, 1994; Carvalho *et al.*, 1996; sheep: Bernardi and Delouis, 1996; Catt *et al.*, 1997; pigs: Cassar *et al.*, 1994; human: Ray *et al.*, 1995). In contrast, Grisart *et al.* (1995) did not find an alteration in the sex ratio of bovine embryos cultured in serum-free oviduct cell-conditioned medium. Kaminski *et al.* (1996) concluded that pig embryonic development occurs at a rate determined by the uterine environment and not by the sex of the conceptus, which is in contrast to the results of Cassar *et al.* (1994). In the present study, significantly more male embryos were observed when sexing was carried out at the blastocyst stage (Expt 4a). This observation was not related to the time of first cleavage of the embryo. In contrast, when embryos were sexed at the two-cell stage, although the overall sex ratio was skewed, the deviation in sex ratio was not as marked as on day 8. This may indicate that differences between male and female embryos in terms of developmental ability are due to events that occur after cleavage. Similar results were reported by Dufour *et al.* (1994) in a study in which fast- and slow-cleaving embryos were separated at 48 h after insemination. There was no difference in sex ratio between the two groups at this stage. However, after 8 days of culture, fast-developing embryos showed a skewed sex ratio in favour of males, indicating that factors present after fertilization are involved.

Male and female embryos have different metabolic requirements and utilization rates *in vitro* (Tiffen *et al.*, 1991; Ray *et al.*, 1995). It has been proposed that these metabolic differences could be used to predict the sex and viability of embryos (Rieger, 1984). These differences may occur as early as the first cleavage in bovine (Dominko and First, 1993) and human (Ray *et al.*, 1995) embryos. Bredbacka and Bredbacka (1996) reported that male embryos develop faster than female embryos only in the presence of exogenous glucose and explained this fact by differential gene expression in male and female embryos.

Obtaining live offspring after embryo transfer is the ultimate test of a successful embryo production system. To date, the *in vivo* viability of embryos derived from oocytes that cleaved at different times has not been investigated in cattle. However, there is indirect evidence from the study of Hasler *et al.* (1995), in which higher pregnancy rates were observed for day 7 fresh blastocysts compared with day 8 (56% versus 43%). This difference was greater when frozen embryos were used (42 versus 20%), indicating that the fastest developing embryos were more tolerant of the cryopreservation procedures than the embryos that

developed more slowly. In addition, McKiernan and Bavister (1994) reported that one-cell hamster embryos that developed to the eight-cell stage by 58 h after egg activation produced almost twice as many day 14 fetuses (51%) as embryos that had reached the four-cell stage by 58 h (26%). It was proposed that faster developing embryos are more viable than embryos that develop more slowly. In the present study, no difference was observed in pregnancy rate between blastocysts derived from early cleaving and late cleaving zygotes. Embryos from the two extremes, that is, those cleaving by 27 h after insemination and those cleaving at 42–48 h after insemination, were not compared. However, most of the total number of blastocysts originated from oocytes that cleaved by 36 h after insemination (approximately 90%), and those derived from the 42–48 h group would not be selected for transfer in routine practice. This finding indicates that morphological grading at day 7 is an acceptable method of selecting embryos for transfer.

In conclusion, a non-invasive method of assessing oocyte and embryo quality would be invaluable in embryo transfer by allowing the selection of oocytes and embryos with the highest probability of producing live offspring after transfer. Kubisch *et al.* (1998) reported that interferon- τ production was lower in early forming bovine blastocysts compared with those that formed at a later stage. It was suggested that measurement of interferon- τ production coupled with visual assessment of morphology may provide a means of distinguishing between high and low quality blastocysts. The results of the present study show that the timing of early cleavage, particularly the time interval from insemination to first cleavage, is of critical importance in this respect, offering a non-invasive assessment of embryo viability before transfer or cryopreservation. This also has implications for human assisted reproduction in which embryos are usually transferred at the four- to six-cell stage. In such cases, considering the kinetics of cleavage could be invaluable in improving pregnancy rates.

The reasons why two morphologically identical two-cell embryos that differ only in the time between insemination and first cleavage, and which show apparently similar protein synthesis patterns and metabolism should display such a marked difference in development remain to be elucidated. These differences in developmental ability may provide a model for the study of developmental competence acquisition in mammalian oocytes and constitute a potentially useful tool for studying gene expression in early embryos.

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