Zygote **10** (November), pp 341–348. © 2002 Cambridge University Press
DOI: 10.1017/S0967199402004082 Printed in the United Kingdom

Cell allocation in bovine embryos cultured in two media under two oxygen concentrations

Amy Fischer-Brown, Rick Monson, John Parrish and Jack Rutledge
Endocrinology and Reproductive Physiology Program, Department of Animal Sciences, University of Wisconsin, Madison, Wisconsin, USA

Date submitted: 17.6.02. Date accepted: 25.7.02

Summary

Blastocyst development, total cell number and allocation to inner cell mass (ICM) and trophectoderm (TE) lineages was compared among day 9 hatched blastocysts from four culture treatments in a two-factor design. Two modified commercial media (KSOM and SOF) were used in atmospheres with two oxygen concentrations (5% and 20% O_2). No significant effect of medium on development was found, but 20% O_2 increased hatching ($p < 0.05$). There were more cells in hatched blastocysts cultured in KSOM than in SOF (181 vs 136, respectively; $p < 0.0001$); however, ICM/total cell ratio was not affected by medium. There was a trend suggesting that the proportion of cells allocated to ICM was lower in hatched blastocysts cultured under 5% O_2 compared with 20% O_2 (0.323 vs 0.380, respectively; $p < 0.1$). No significant interactions between medium type and oxygen concentration were found. These results indicate that culture components used in this study may affect cell proliferation without altering cell allocation, and that oxygen concentration may play a role in allocation of cells to ICM and TE lineages.

Keywords: IVC, Bovine, Cell number, Cell allocation, Differential staining

Introduction

In the continuing effort to identify ideal culture conditions for *in vitro* produced bovine embryos, endpoints of evaluation have evolved in recent years. In addition to blastocyst yield, timing of development and hatchability, many laboratories have turned to more invasive techniques, such as staining for total cell counts and cell allocation (Van Soom *et al*., 2001).

It is well documented that total cell number and number of cells allocated to the inner cell mass can be regarded as valuable indicators of cattle embryo viability (Jiang *et al*., 1992; Wurth *et al*., 1994; Gordon, 1994) and survivability following cryopreservation (Iwasaki *et al*., 1994; Deinnyés *et al*., 1996; Du *et al*., 1996; Kaidi *et al*., 1998). Cell allocation, which refers to the distribution between the inner cell mass (ICM) and trophectoderm (TE) lineages, is the first process of embryonic differentiation and establishes the basis for normal further development. In eutherian mammals, the TE gives rise to placenta and extraembryonic membranes and the ICM forms the three germ layers of the fetus as well as contributing to the extraembryonic membranes (Gilbert, 2000). An altered cell allocation that favours TE may lead to abnormal placental size and/or function, and possibly play a role in the large offspring syndrome (Walker *et al*., 1996; Leese *et al*., 1998), a condition in which placental abnormalities are frequently observed (for review see Young *et al*., 1998). Furthermore, early gestational fetal wastage characteristic of the large offspring syndrome may be of fetuses that develop from ICMs that are too small to progress beyond this stage of pregnancy (Walker *et al*., 1996). Those that do progress may go on to undergo compensatory growth later in gestation, as described by Bertolini & Anderson (2002).

Beyond causing a failed pregnancy, embryos with small ICMs that cease development can cause further complications with a delayed return to estrus. This arises since the TE cells secrete bovine trophoblast protein-1, also known as interferon τ, the signal for maternal recognition of pregnancy (Farin *et al*., 1990; Van Soom *et al*., 1997). A similar occurrence has been

All correspondence to: Amy Fischer-Brown, University of Wisconsin Department of Animal Sciences, 1675 Observatory Drive, Madison, WI 53706, USA. Tel: +1 (608) 265 6422. Fax: +1 (608) 262 5157. e-mail: afischer@students.wisc.edu

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press

suggested in humans regarding human chorionic gonadotropin levels (Hardy *et al.*, 1989).

Previous work has demonstrated that cell culture conditions can alter cell allocation (Van Soom *et al.*, 1996, 2002; Stojkovic *et al.*, 1998). In this study we compared four culture environments in a 2×2 factorial design. Two modified commercial media (potassium simplex optimized medium (KSOM) and synthetic oviductal fluid (SOF)) were used in atmospheres with two oxygen concentrations (ambient (20% O_2) and reduced (5% O_2)). Blastocyst yield, proportion of total blastocysts developed by day 7, hatchability, and total cell number and allocation of day 9 hatched blastocysts were evaluated.

Materials and methods

Unless otherwise specified, laboratory chemicals were purchased from Sigma Chemical Company, St Louis, MO.

In vitro maturation and fertilisation

Immature bovine oocytes were obtained via aspiration of predominantly Holstein abattoir-derived ovaries. They were matured for 20–22 h in bicarbonate-buffered TCM199 (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (Gibco BRL, Rockville, MD), 0.2 mM sodium pyruvate, and 1 µl estradiol, 0.01 NIH units FSH (Sioux Biochemical, Sioux Center, IA), 0.01 NIH units LH (Sioux), and 0.025 mg gentamicin sulfate (BioWhittaker) per millilitre. Oocytes and sperm were co-incubated for 20–22 h in IVF-TL (Specialty Media, Phillipsburg, NJ) supplemented with 0.2 mM sodium pyruvate and 3 mg fatty-acid-free BSA and 0.025 mg gentamicin sulfate per millilitre. Final IVF-TALP contained PHE (2 mM penicillamine, 1 mM hypotaurine, 0.25 mM epinephrine), 2 µg heparin and 1×10^6 Percoll separated frozen-thawed sperm per millilitre. Semen from two Holstein and two Angus bulls were used, with one bull per replicate. Presumptive zygotes were stripped of cumulus cells via vortex and randomly assigned to culture treatments. *In vitro* maturation and fertilisation were conducted in 50 µl droplets under mineral oil at 39 °C and 5% CO_2 in humidified air.

In vitro culture

A 2×2 factorial was used; factors were media (KSOM or SOF) and % O_2 (5% or 20%). Culture media were based on, but not identical to, original formulations (Tervit *et al.*, 1972; Erbach *et al.*, 1994; see Table 1). Both commercial media (Specialty Media) were supplemented with 0.4 mM sodium pyruvate, 20 µl MEM non-essential and 10 µl BME essential amino acids solutions per millilitre, and 0.025 mg gentamicin sulfate per millilitre. Fatty-acid-free BSA was added to achieve a final concentration of 8 mg/ml. Groups of 25 embryos were cultured in 50 µl drops under mineral oil at 39 °C in each treatment's corresponding gas atmosphere under high humidity. The 5% O_2 atmosphere was a mixture of 5% O_2/5% CO_2/90% N_2 and the 20% O_2 atmosphere was the standard incubation system of 5% CO_2 in air.

Table 1 Composition of modified KSOM and SOF

Components	KSOM	SOF
Inorganic salts (mM)		
$CaCl_2{\cdot}2H_2O$	1.71	1.17
KCl	2.50	7.16
KH_2PO_4	0.35	1.19
$MgCl_2{\cdot}6H_2O$		0.49
$MgSO_4$	0.20	
NaCl	94.97	107.7
$NaHCO_3$	25.00	25.07
Other components (mM)		
Na lactate (60% syrup)	21.9	3.3
Na pyruvate	0.6	0.4
BSA (mg/l)	8000	8000
EDTA (mg/l)	3	
D-glucose	0.2	
Penicillin (U/l)	100,000	
Streptomycin (U/l)	50	
Gentamicin sulfate (U/l)	500 000	500 000
Amino acids		
BME essential solution (ml/l)	10	10
MEM non-essential solution (ml/l)	20	20
L-glutamine (mg/l)	73.1	

On day 5 of culture, embryos were transferred to drops of fresh media. Embryos were evaluated for blastocyst development on days 6, 7, 8 and 9. On day 9 all hatched blastocysts were collected for differential staining.

Fifteen replicates of the above procedures were performed, utilising a total of 6941 oocytes.

Differential staining

The differential staining procedure was originally developed for mouse by Handyside & Hunter (1984) and later modified for cattle (Iwasaki *et al.*, 1990). With a few minor exceptions, we adhered to the further modifications for cattle described by Van Soom (1996). This approach is based on the permeation of outer cells by a species-specific complement-mediated reaction confirmed by nuclear staining with propidium iodide, a red DNA-specific fluorochrome that penetrates only permeated cells. Differential nuclear staining is accomplished simultaneously with bisbenzimide (Hoechst

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press

33342), a blue DNA fluorochrome that penetrates both intact and permeated cells. Successful staining therefore results in intact ICM cells staining blue (Hoechst), and permeated TE cells staining pink (Hoechst and propidium iodide).

The procedure is briefly as follows: Hatched blastocysts were incubated in 10 mM trinitrobenzenesulfonic (TNBS) acid in PBS containing 3 mg/ml polyvinylpyrrolidone (PVP-40, Sigma) on ice for 10 min. Embryos were washed three times in TL Hepes and incubated in a 30:70 dilution of rabbit anti-DNP-BSA (ICN Biomedicals, Aurora, OH) in TCM199 bicarbonate at 39 °C for 30 min. Embryos were washed three times in TL Hepes and incubated in a 1:4 dilution of guinea pig complement in TCM199 bicarbonate supplemented with 10 µg/ml propodium iodide at 39 °C for 30 min. Embryos were washed once in ice-cold TL Hepes with 10 µg/ml propidium iodide, and then fixed in ice-cold absolute ethanol and stained with 15 µg/ml Hoechst 33342 in absolute ethanol at room temperature. Embryos were kept in this solution for up to 3 days at 4 °C. Embryos were examined in glycerol whole mount under a fluorescence microscope (Nikon Microphot-FX) equipped with a UV filter. ICM and TE cells were counted and the data used to calculated total cell numbers and ICM proportions. In the event of unsuccessful differential staining, only total cell numbers were recorded.

Statistical analyses

The analysis of variance model included fixed effects of medium type, oxygen concentration and replicate; bull and replicate effects were partially confounded. All possible interactions among main effects were also included. The linear model was fitted using the GLM (general linear model) procedure (SAS Institute, Cary, NC). Percentage data were analysed with an arcsine square root transformation. Only replicates with data for all four treatments were included in the analysis.

Results

Effect of medium type and oxygen concentration on embryo development

Neither overall blastocyst yield nor the proportion of total blastocysts developed by day 7 were affected by treatment (Table 2). With very few exceptions, blastocysts were not detected on day 6. There was an effect of ambient oxygen concentration on hatching rate ($p < 0.05$), probably due to the difference observed between KSOM treatments. There was a trend suggesting a medium × O_2 concentration interaction ($p < 0.1$) in that fewer blastocysts hatched from the KSOM/5% O_2 treatment compared with the other treatments in which approximately 40% hatched.

Effect of medium type and oxygen concentration on cell number and allocation

Total cell numbers were affected by medium (Table 3, Fig. 1). There were significantly ($p < 0.0001$) more cells in blastocysts cultured in KSOM than in SOF. This relationship was maintained in both ICM and TE numbers,

Table 2 LS means for overall blastocyst yield (day 9), proportion of total blastocysts that were present on day 7, and proportion of total blastocysts that hatched[a]

Medium	Oxygen %	Oocytes	% blastocysts (*n*)	Day 7 blasts/ total (%)	% hatched
KSOM	5	1727	25.5 (460)	42.0	24.8
KSOM	20	1718	23.1 (407)	42.2	40.4
SOF	5	1789	21.5 (463)	31.8	40.4
SOF	20	1707	24.8 (482)	40.3	40.0

[a]Effects of medium, O_2 concentration and interactions not significant except for O_2 levels in % hatching.

Table 3 LS means ± SEM and range for total cell numbers of day 9 hatched blastocysts

Medium	Oxygen %	Embryos (*n*)	Total cells	Range
KSOM	5	73	185.6 ± 6.4[a]	99–293
KSOM	20	61	177.2 ± 7.4[a]	81–321
SOF	5	65	136.9 ± 8.0[b]	62–293
SOF	20	76	135.1 ± 7.7[b]	57–303

[a,b]Values with different superscripts within the same column are significantly different, $p < 0.05$.

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press

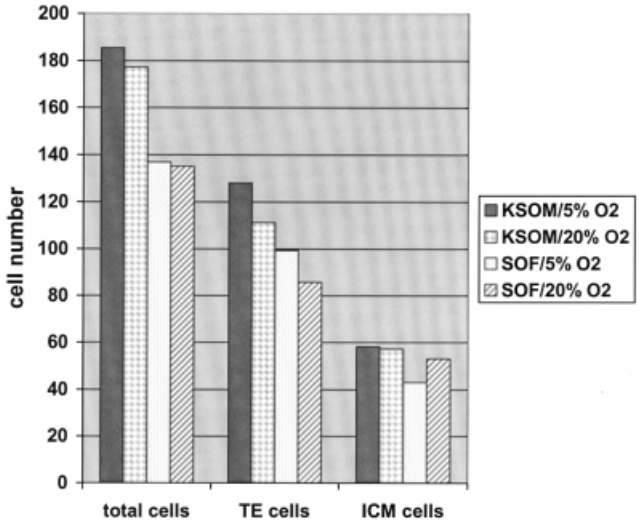


Figure 1 Total, TE and ICM cell numbers of day 9 hatched bovine blastocysts from four culture treatments in a 2×2 factorial design.

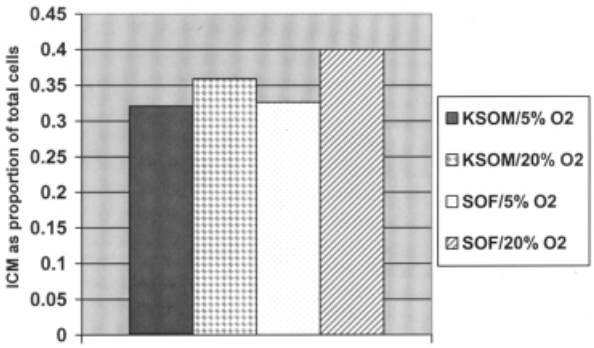


Figure 2 ICM as a proportion of total cells for day 9 hatched bovine blastocysts from four culture treatments in a 2×2 factorial design.

but the proportion of ICM cells was not significantly affected by medium type (Table 4, Fig. 2). Oxygen concentration had no significant effect on total, ICM or TE cell number. There was, however, a trend ($p < 0.1$) towards lower ICM proportions in the 5% O_2 treatments.

Effect of bull on development, cell number and allocation

Semen from four bulls was used in this study. Significant differences were found among bulls for number of total, ICM and TE cells, ICM proportion, and proportion of blastocysts developed by day 7 (Table 5). It must be noted that bull data are neither balanced nor contemporary with respect to replicate.

Discussion

A wide range of ICM numbers and ratios has been reported for *in vitro* produced bovine embryos (Table 6). Values for ICM numbers and ratios reported here fall within the higher end of that range. We attribute this observation to the semi-defined nature of the media (i.e. no serum or co-culture) and the fact that only those embryos that had reached the hatched blastocyst stage by day 9 were included, thus eliminating poorer-quality embryos with delayed development.

Table 4 LS means ± SEM and range for ICM number, TE number and proportion of ICM cells in day 9 hatched blastocysts

Medium	% O_2	*n*	ICM cells	Range	TE cells	Range	ICM/total	Range
KSOM	5	66	58.2 ± 2.7^{a}	20–122	128.1 ± 6.1^{a}	49–247	0.321 ± 0.017^{a}	0.105–0.637
KSOM	20	48	57.2 ± 3.5^{a}	23–98	$111.3 \pm 7.7^{a,b}$	32–247	$0.359 \pm 0.021^{a,b}$	0.106–0.692
SOF	5	53	$43.1 \pm 3.9^{b,c}$	17–127	$99.3 \pm 8.7^{b,c}$	33–253	0.326 ± 0.24^{a}	0.111–0.681
SOF	20	63	$53.0 \pm 3.4^{a,b}$	20–112	85.7 ± 7.5^{c}	24–231	0.400 ± 0.021^{b}	0.130–0.762

^{a,b,c}Values with different superscripts within the same column are significantly different, $p < 0.05$.

Table 5 LS means ± SEM for statistically significant endpoints among bulls

Bull	Breed	Reps.	Total cells (*n*)	ICM	TE	ICM/total	% day 7 blastocysts
A	Holstein	4	175.4 ± 4.6^{a} (118)	54.7 ± 2.1^{a}	115.8 ± 4.8^{a}	0.337 ± 0.013^{b} (107)	46.2^{a}
B	Angus	1	150.3 ± 10.3^{b} (19)	34.7 ± 4.6^{b}	$120.0 \pm 10.2^{a,b}$	0.247 ± 0.028^{a} (18)	$39.3^{a,b}$
C	Holstein	6	155.4 ± 5.7^{b} (90)	60.2 ± 2.9^{a}	91.3 ± 6.4^{c}	0.417 ± 0.017^{c} (67)	$40.2^{a,b}$
D	Angus	4	153.8 ± 7.7^{b} (48)	62.1 ± 3.5^{a}	$97.2 \pm 7.8^{b,c}$	0.405 ± 0.021^{c} (38)	30.8^{b}

^{a,b,c}Values with different superscripts within the same column are significantly different, $p < 0.05$.
Reps., replications.

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press

Table 6 Numbers of ICM cells, total cells, and ICM/total proportions for late-stage *in vitro* produced bovine embryos

Medium	% O_2	Serum	Co-culture	Day:stage* of blastocyst	ICM	Total	ICM/total	Reference
TCM199	20	+	+	E	11	77	0.149	Iwasaki *et al.* (1990)
				H	16	121	0.128	
				(dayNR)				
TCM199	20	+	+	9:H	84	224	0.37	Du *et al.* (1996)
MénézoB2	20	+	+	E	36.0	122.1	0.30	Van Soom *et al.* (1996)
				H	40.2	138.6	0.29	
				(dayNR)				
TCM199	20	+	+	E	34.9	105.4	0.34	
				H	25	93	0.27	
				(dayNR)				
TCM199	20	+	+	8:E	39.0	166.1	0.23	de la Fuente & King (1997)
				9:E	24.7	105.6	0.23	
MénézoB2	5	+	+	8:E&H	39.7	118.6		Stojkovic *et al.* (1998)
TCM199	5	+	+	8:E&H	25.9	83.8		
KSOM	20	–	–	8	NR	116	0.33	Van de Velde *et al.* (1999)
				(stageNR)				
SOF	5	+	–	8:U	26	90	0.28	Majerus *et al.* (2000)
				8:H	27	111	0.25	
SOF	5	+	–	9:H	51.4	233.8	0.225	Van Soom *et al.* (2002)
SOF	20	+	–	9:H	42.8	162.3	0.262	
SOF + cys	5	+	–	9:H	70.3	170.0	0.413	
SOF + cys	20	+	–	9:H	44.1	155.4	0.291	

*E, expanded; H, hatched; U, unhatched; NR, not reported.

Table 7 Numbers of ICM cells, total cells and ICM/total proportions for *in vivo* bovine embryos

Day:stage	ICM	Total	ICM/total	Reference
8:expanded	53.1	195.3	0.27	de la Fuente & King (1997)
Hatched (day NR)	57.9	171.4	NR	Van Soom *et al.* (1997*a*)
9:hatched	118	255	0.46	Du *et al.* (1996)
Hatched (day NR)	31	113	0.272	Iwasaki *et al.* (1990)
'late stage'	47.5	141.2	NR	Skrzyszowska & Smorag (1989)

NR, not reported.

Less information is available for *in vivo* bovine embryos; our data fall within reported ranges for ICM numbers and proportions and total cell numbers (Table 7).

Proportion of total blastocysts developed by day 7 and all categories of cell number were significantly affected by bull. The initial goal of this experiment was not to compare bulls; rather multiple bulls were used in order to enlarge the scope of inference and to ensure the results were not conditional upon a single bull. Since bull and replicate effects are confounded, these results should be interpreted with caution. This being said, it is not unlikely that there exists a sire influence on cell number and allocation, since other developmental parameters are influenced by sire, such as cleavage and development to morula and blastocyst (Eyestone & First, 1989; Shi *et al.*, 1990; Hillery *et al.*, 1990). Paternal influences exert effects on the S- and G1-phases of bovine zygotes, thereby affecting the timing of first cleavage (Eid *et al.*, 1994; Eid & Parrish, 1995; Comizzoli *et al.*, 2000), an endpoint used to distinguish bulls of high and low field fertility (Ward *et al.*, 2001). Number of ICM and TE cells has been shown to be affected by breed in preimplantation purebred pig embryos (Rivera *et al.*, 1996). The paternal genome is crucial for the proliferation of the extraembryonic tissues (Surani *et al.*, 1986), which derive mostly from the TE contribution (Gilbert, 2000).

Timing of development and degree of compaction affect cell allocation, since prolongation of the tight morula phase seems to allow more cells to be allocated to the ICM (Van Soom *et al.*, 1996, 1997*b*). The inclusion

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press

of serum in media has been implicated in perturbing both (Walker *et al*., 1992; for review see Thompson, 1979). The media in the present study did not contain serum, and there was no observed difference in timing of blastocyst development or degree of compaction among treatment groups. However, it must be noted that embryos were not monitored continuously, but at 24 h intervals, and that precise differences in timing of blastocoele formation remained undetected. While embryos cultured in KSOM had more total cells than those cultured in SOF (Fig. 1), the proliferation was in both ICM and TE lineages such that the proportion of ICM was not significantly altered (Fig. 2). This suggests that, although differences were observed in cell proliferation between the two media, the fundamental mechanism behind cell allocation was maintained. A mitogenic effect observed in both ICM and TE suggests that the increased cell division takes place prior to differentiation, i.e. the first 72 h of culture. Ethylenediaminetetraaceteic acid (EDTA), present in KSOM but not SOF (Table 1), is probably responsible for this effect. An identical pattern was previously reported by Gardner & Lane (1997), who observed that bovine blastocysts cultured in SOF with EDTA had significantly more total and ICM cells compared with embryos cultured without EDTA for the first 72 h, but that the ICM proportion was not different between treatment groups. EDTA optimizes energy metabolism via intracellular chelation of Mg^{2+}, a co-factor for several glycolytic enzymes. Culture-induced high glycolytic rates will compromise early development; thus the beneficial effect of EDTA is optimal in the first 72 h of culture (Thompson, 2000). EDTA also sequesters the toxic effects of other heavy metal cations. Since metal ions can activate superoxides to produce embryotoxic free oxygen radicals, heavy metal contamination from ingredients, media preparation equipment or mineral oil overlay can have detrimental effects on embryo development (Johnson & Nasr-Esfahani, 1994). Even modest increases in several essential metals can compromise blastocyst yield and cell number (Hanna *et al*., 1997; Ashworth & Antipatis, 2001). Therefore EDTA is a likely candidate to explain increased cell numbers observed in the KSOM-cultured embryos, though other factors may be involved.

It is generally thought that reduced oxygen tension yields better preimplantation development (for review see Bavister, 1995), yet not all studies confirm the beneficial effect (Fukui *et al*., 1991; Voelkel & Hu, 1992; Dumoulin *et al*., 1995). This suggests that different culture systems may require different oxygen concentrations for optimum results, due to differences between media components (serum or co-culture vs defined) and/or incubating conditions, such as the use of an oil overlay (Thompson *et al*., 1990). It has been suggested that the use of static microdrops with oil overlay may result in marginally hypoxic culture conditions at low oxygen concentrations, leaving the inner cells of larger embryos, such as human and the domestic species, in an anoxic environment (Byatt-Smith *et al*., 1991). In this experiment ICM proportions were lower for embryos cultured in a reduced O_2 atmosphere compared with ambient. Also there was a positive effect of ambient oxygen on hatching rate. Both media in this study contain compounds which provide some protection against free radical injury, including pyruvate and lactate (Herz *et al*., 1997) and BSA (Karrow & Critser, 1997). It is possible that protective effects of reduced oxygen tension are detected to a lesser extent in these media, perhaps even eliminated.

In conclusion, this experiment gives evidence that under semi-defined conditions, medium composition can affect cell proliferation without altering cell allocation, and oxygen concentration may affect TE growth and proportion of cells allocated to the ICM. These differences seem to occur with no subsequent impact on standard developmental endpoints, confirming the need for more detailed evaluation of *in vitro* culture systems.

Acknowledgements

The authors gratefully acknowledge Dr William Bement of the UW Zoology Department for fruitful discussion. We thank Nancy Jensen, Andrea Johannen, Jolene Fredrickson, Jessica Quam and Casey Jentz for technical assistance. This work was supported by the Babcock Institute for International Dairy Research and Development, the Association Liaison Office for University Cooperation in Development, and the United States Department of Agriculture (2001–52101–11318).

References

Ashworth, C. & Antipatis, C. (2001). Micronutrient programming of development throughout gestation. *Reproduction* **122**, 527–35.

Bavister, B. (1995). Culture of preimplantation embryos: facts and artifacts. *Hum. Reprod.* **1**, 91–148.

Bertolini, M. & Anderson, G. (2002). The placenta as a contributor to production of large calves. *Theriogenology* **57**, 181–7.

Byatt-Smith, J., Leese, H. & Gosden, R. (1991). An investigation by mathematical modeling of whether mouse and human preimplantation embryos in static culture can satisfy their demands for oxygen by diffusion. *Hum. Reprod.* **6**, 52–7.

Comizzoli, P., Marquant-Le Guienne, B., Heyman, Y. & Renard, J. (2000). Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes. *Biol. Reprod.* **62**, 1677–84.

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press

de la Fuente, R. & King, A. (1997). Use of a chemically defined system for the direct comparison of inner cell mass and trophectoderm distribution in murine, porcine and bovine embryos. *Zygote* **5**, 309–20.

Dinnyés, A., Carolan, C., Lonergan, P., Massip, A. & Mermillod, P. (1996). Survival of frozen or vitrified bovine blastocysts produced *in vitro* in synthetic oviduct fluid. *Theriogenology* **46**, 1425–39.

Du, F., Looney, C. & Yang, X. (1996). Evaluation of bovine embryos produced *in vitro* vs *in vivo* by differential staining of inner cell mass and trophectoderm cells. *Theriogenology* **45**, 211.

Dumoulin, J., Pieters, M., Vanvuchelen, R., Geraedts, J., Land, J. & Evers, J. (1995). Effect of oxygen concentration on *in vitro* fertilization and embryo culture in the human and the mouse. *Fertil. Steril.* **63**, 115–19.

Eid, L., Lorton, S. & Parrish, J. (1994). Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biol. Reprod.* **51**, 1232–7.

Eid, L. & Parrish, J. (1995). Duration of G2-phase and onset of M-phase during the first cell cycle of the bovine embryo is dependent on bull *in vivo* fertility. *Theriogenology* **43**, 205.

Erbach, G., Lawitts, J., Papaioannou, V. & Biggers, J. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol. Reprod.* **50**, 1027–33.

Eyestone, W. & First, N. (1989). Variation in bovine embryo development *in vitro* due to bulls. *Theriogenology* **31**, 191.

Farin, C., Imakawa, K., Hansen, T., McDonnell, J., Murphy, C., Farin, P. & Roberts, R. (1990). Expression of trophoblastic interferon genes in sheep and cattle. *Biol. Reprod.* **43**, 210–18.

Fukui, Y., McGowan, L., James, R., Pugh, P. & Tervit, H.R. (1991). Factors affecting the *in vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. *J. Reprod. Fertil.* **92**, 125–31.

Gardner, D., Lane, M. & 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.

Gilbert, S. (2000) *Developmental Biology*, 6th ed., pp. 358–61. Sunderland, MA: Sinauer Associates.

Gordon, I. (1994). *Laboratory Production of Cattle Embryos*, pp. 282–4. Wallingford, UK: CAB International.

Handyside, A. & Hunter, S. (1984). A rapid procedure for visualizing the inner cell mass and trophectoderm nuclei of mouse blastocysts *in situ* using polynucleotide-specific fluorochromes. *J. Exp. Zool.* **231**, 429–34.

Hanna, L., Peters, J., Wiley, L., Clegg, M. & Keen, C. (1997). Comparative effects of essential and nonessential metals on preimplantation mouse embryo development *in vitro*. *Toxicology* **116**, 123–31.

Hardy, K., Handyside, A. & Winston, R. (1989). The human blastocyst: cell number, death and allocation during late preimplantation development. *Development* **107**, 597–604.

Herz, H., Blake, D. & Grootveld, M. (1997). Multicomponent investigations of the hydrogen peroxide and hydroxyl radical scavenging antioxidant capacities of biofluids: the role of endogenous pyruvate and lactate. *Free Rad. Res.* **26**, 19–35.

Hillery, F., Parrish, J. & First, N. (1990). Bull specific effect on fertilization and embryo development *in vitro*. *Theriogenology* **33**, 249.

Iwasaki, S., Yoshiba, N., Ushijima, H., Watanabe, S. & Nakahara, T. (1990). Morphology and proportion of inner cell mass of bovine blastocysts fertilized *in vitro* and *in vivo*. *J. Reprod. Fertil.* **90**, 279–84.

Iwasaki, S., Yoshikane, Y., Li, X., Watanabe, S. & Nakahara, T. (1994). Effects of freezing of bovine preimplantation embryos derived from oocytes fertilized *in vitro* on survival of their inner cell mass cells. *Mol. Reprod. Dev.* **37**, 272–5.

Jiang, H., Wang, W., Lu, K., Gordon, I. & Polge, C. (1992). Examination of cell numbers of blastocysts derived from IVM, IVF and IVC of bovine follicular oocytes. *Theriogenology* **37**, 229.

Johnson, M. & Nasr-Esfahani, M. (1994). Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos *in vitro*? *Bioessays* **16**, 31–8.

Kaidi, S., Donnay, I., Van Langendonckt, A., Dessy, F. & Massip, A. (1998). Comparison of two co-culture systems to assess the survival of *in vitro* produced bovine blastocysts after virtrification. *Anim. Reprod. Sci.* **52**, 39–50.

Karow, A. & Critser, J. editors (1997). *Reproductive Tissue Banking: Scientific Principles*, p. 197. San Diego, CA: Academic Press.

Leese, H., Donnay, I. & Thompson, J. (1998). Human assisted conception: a cautionary tale. Lessons from domestic animals. *Hum. Reprod.* **13**, 184–202.

Majerus, V., Lequarré, A., Ferguson, E., Kaidi, S., Massip, A., Dessy, F. & Donnay, I. (2000). Characterization of embryos derived from calf oocytes: kinetics of cleavage, cell allocation to inner cell mass and trophectoderm, and lipid metabolism. *Mol. Reprod. Dev.* **57**, 346–52.

Rivera, R., Youngs, C. & Frod, S. (1996). A comparison of the number of inner cell mass and trophectoderm cells of preimplantation Meishan and Yorkshire pig embryos at similar developmental stages. *J. Reprod. Fertil.* **106**, 111–16.

Shi, D., Lu, K. & Gordon, I. (1990). Effects of bulls on fertilization of bovine oocytes and their subsequent development *in vitro*. *Theriogenology* **33**, 324.

Skrzyszowska, M. & Smorag, Z. (1989). Cell loss in bisected mouse, sheep and cow embryos. *Theriogenology* **32**, 115–22.

Stojkovic, M., Büttner, M., Zakhartchenko, V., Brem, G. & Wolf, E. (1998). A reliable procedure for differential staining of *in vitro* produced bovine blastocysts: comparison of tissue culture medium 199 and Ménézo's B2 medium. *Anim. Reprod. Sci.* **50**, 1–9.

Surani, M., Barton, S. & Norris, M. (1986). Nuclear transplantation in the mouse: heritable differences between parental genomes after activation of the embryonic genome. *Cell* **45**, 127–36.

Tervit, H.R., Whittingham, D. & Rowson, L. (1972). Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fertil.* **30**, 493–7.

Thompson, J., Simpson, A., Pugh, P., Donnelly, P. & Tervit, H.R. (1990). Effect of oxygen concentration on *in vitro* development of preimplantation sheep and cattle embryos. *J. Reprod. Fertil.* **89**, 573–8.

Thompson, J. (1997). Comparison between *in vivo*-derived and *in vitro*-produced pre-elongation embryos from domestic ruminants. *Reprod. Fertil. Dev.* **9**, 341–54.

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press

Thompson, J. (2000). *In vitro* culture and embryo metabolism of cattle and sheep embryos: a decade of achievement. *Anim. Reprod. Sci.* **60–61**, 263–75.

Van de Velde, A., Liu, L., Bols, P. & Ysebaert, M.T. (1999). Cell allocation and chromosomal complement of parthenogenetic and IVF bovine embryos. *Mol. Reprod. Dev.* **54**, 57–62.

Van Soom, A., Boerjan, M., Ysebaert, M. & de Kruif, A. (1996). Cell allocation to the inner cell mass and the trophectoderm in bovine embryos culture in two different media. *Mol. Reprod. Dev.* **45**, 171–82.

Van Soom, A., Boerjan, M., Bols, P., Vanroose, G., Lein, A., Coryn, M. & de Kruif, A. (1997*a*). Timing of compaction and inner cell allocation in bovine embryos produced *in vivo* after superovulation. *Biol. Reprod.* **57**, 1041–9.

Van Soom, A., Ysebaert, M. & de Kruif, A. (1997*b*). Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in *in vitro*-produced bovine embryos. *Mol. Reprod. Dev.* **47**, 47–56.

Van Soom, A., Vanroose, G. & de Kruif, A. (2001). Blastocyst evaluation by means of differential staining: a practical approach. *Reprod. Dom. Anim.* **36**, 29–35.

Van Soom, A., Yuan, Y., Peelman, L., de Matos, D., Dewulf, J., Laevens, H. & de Kruif, A. (2002). Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. *Theriogenology* **57**, 1453–65.

Voelkel, S. & Hu, Z. (1992). Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. *Theriogenology* **37**, 1117–31.

Walker, S., Heard, T. & Seamark, R. (1992). *In vitro* culture of sheep embryos without co-culture: successes and perspectives. *Theriogenology* **37**, 111–26.

Walker, S., Hartwich, K. & Seamark, R. (1996). The production of unusually large offspring following embryo manipulation: concepts and challenges. *Theriogenology* **45**, 111–20.

Ward, F., Rizos, K., Corridan, D., Quinn, K., Boland, M. & Longergan, P. (2001). Paternal influence on the time of first embryonic cleavage post-insemination and the implications for subsequent bovine embryo development *in vitro* and fertility *in vivo*. *Mol. Reprod. Dev.* **60**, 47–55.

Wurth, Y., van der Zee-Kotting, W., Dieleman, S., Bevers, M. & Kruip, T. (1994). Presence of mitotic cells: a parameter of embryo quality. *Anim. Reprod. Sci.* **35**, 173–82.

Young, L., Sinclair, K. & Wilmut, I. (1998). Large offspring syndrome in cattle and sheep. *Rev. Reprod.* **3**, 155–63.

https://doi.org/10.1017/S0967199402004082 Published online by Cambridge University Press