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Energy substrates and amino acids provided during *in vitro* maturation of bovine oocytes alter acquisition of developmental competence

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Summary

Energy substrates and amino acids were evaluated for supporting acquisition of developmental competence by bovine cumulus–oocyte complexes during *in vitro* maturation. The basic culture medium (Basic Medium-3) used for *in vitro* maturation of oocytes was modified to produce six media containing glucose or glutamine with lactate or pyruvate, or glucose + glutamine, or glucose + 11 amino acids; a seventh (control) medium was TCM199. All media contained polyvinyl alcohol, gonadotropins, epidermal growth factor and oestradiol. Following maturation, oocytes were incubated in medium TALP for fertilisation, then cumulus cells were removed and presumptive embryos cultured for 48 h in a chemically defined medium (HECM-6) followed by 120 h in medium TCM199 + bovine calf serum. Six substrate treatments yielded similar first cleavage responses (66–78%) at 72 h post-insemination; however, blastocyst development at 192 h varied significantly. Oocytes matured in medium with glucose + 11 amino acids gave the best blastocyst development: 21% of inseminated oocytes or 25% of 2-cell embryos. Cumulus expansion in HECM-6 required glucose with either glutamine, 11 amino acids or lactate, or glutamine + lactate. We conclude that (1) the type of energy substrate or nutrient supplied during *in vitro* maturation of oocytes profoundly affects subsequent developmental competence; (2) oocyte maturation in simple medium containing glucose with lactate or 11 amino acids or glutamine, or lactate + glutamine, can support development equally as well as the complex medium, TCM199; and (3) media supporting at least moderate cumulus expansion during oocyte maturation also support subsequent blastocyst development.

Keywords: Cleavage, Cumulus expansion, Embryo development, Fertilisation, Protein-deficient medium

Introduction

Successful oocyte maturation, defined as the ability to undergo normal embryo development following fertilisation, involves not only nuclear events (attainment of metaphase II), but also cytoplasmic changes (cytoplasmic maturation) that are poorly understood (Eppig *et al.*, 1994). The importance of proper cytoplasmic maturation is illustrated by the wide differences observed in blastocyst development after *in vitro* maturation (IVM) of oocytes under a variety of culture conditions (van de Sandt *et al.*, 1990; Rose & Bavister, 1992). It follows that better understanding of the factors such as energy substrates, nutrients and hormones that are important for oocyte maturation will lead to improved embryonic development of IVM oocytes. By better defining culture conditions that result in viable ova, inter- and intracellular mechanisms responsible for initiating and completing successful oocyte maturation can be more readily examined.

Basic studies on the factors controlling cytoplasmic oocyte maturation have been largely carried out with

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mice and cattle, although some recent studies have been reported using other species such as primates (Schramm & Bavister, 1995, 1996), pig (Mattioli *et al.*, 1991; Yoshida *et al.*, 1992; Funahashi & Day, 1993; Funahashi *et al.*, 1994) and hamster (Kito & Bavister, 1997). For a number of species, especially primates, the developmental competence of mature ova is a rate-limiting step in the *in vitro* production of embryos and increasing the yield of viable ova would significantly improve the efficiency of embryo production (Bavister, 1987; Bavister *et al.*, 1992; Brackett & Zuelke, 1993; Boatman *et al.*, 1993; Schramm *et al.*, 1994; Schramm & Bavister, 1995, 1996). More efficient production of mature ova would improve the outcome of assisted reproduction in humans and endangered species as well as benefiting transgenic biotechnology and basic research.

IVM of oocytes is generally performed in culture media containing serum or bovine serum albumin (BSA). Besides being a potential source of infectious particles, these undefined components (Bavister, 1995) make it difficult to determine basic metabolic and nutritional requirements of oocytes. Furthermore, the developmental competence of oocytes following maturation with individual substrates or combinations of several substrates is unknown. The present study was designed to evaluate the minimal substrate requirements for IVM of cumulus-enclosed bovine oocytes that impart the capacity for preimplantation embryonic development after *in vitro* fertilisation (IVF). Culture media during oocyte maturation contained polyvinyl alcohol (PVA) as a substitute for protein preparations. Media for sperm capacitation and IVF contained BSA while embryo development media were protein-free during the first 48 h and contained 10% fetal bovine serum during the remaining 120 h. These conditions were devised to optimise *in vitro* embryo development to the blastocyst stage (Pinyopummintr & Bavister, 1991, 1994, 1996*a*, *b*).

Materials and methods

Oocyte collection

Bovine ovaries were collected from a local abattoir and transported to the laboratory in thermos flasks containing 0.9% NaCl. Ovaries arrived at the laboratory at approximately 30 °C and were washed in the same temperature tap water. Oocytes were aspirated from 2–10 mm antral follicles with an 18–20 G needle and syringe and collected in a 50 ml centrifuge tube. After approximately 15 min the sediment was placed into 60 mm Petri dishes and cumulus–oocyte complexes (COCs) were isolated using a hand-held Hamilton syringe equipped with a flame-drawn Unopette tip. The COCs were washed twice in modified Basic Medium-3 with reduced $NaHCO_3$ supplemented with 10 mM Hepes buffer (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid; mBM-Hepes; see Table 1 for components) containing antibiotics / antimycotic (100 units / ml penicillin G (K salt), 100 µg / ml streptomycin and 0.25 µg / ml amphotericin B; = PSA).

In vitro maturation and fertilisation

Between 8 and 12 COCs were placed in 50 µl drops of medium covered with saline-washed and equilibrated paraffin oil in a 6 mm Petri dish (Falcon #1007) and matured for 24 h at 39 °C in 5% CO_2 and air with high humidity. The COCs were cultured in one of seven different culture media. Six of the maturation media contained the same basic salt components (modified Basic Medium-3 = mBM3; Table 1) as found in HECM-3 (McKiernan *et al.*, 1991) except without energy substrates. For each of these six media, mBM3 was supplemented with one of the following substrate combinations: 2 mM glucose + 2 mM glutamine; 2 mM glucose + 11 amino acids (includes glutamine; see Table 1 for components); 2 mM glucose + 0.25 mM pyruvate; 0.25 mM pyruvate + 0.2 mM glutamine; 4.5 mM lactate + 2 mM glucose; or 4.5 mM lactate + 0.2 mM glutamine. Lactate was DL-lactate (60% syrup; Sigma, St Louis, MO). The seventh maturation medium was TCM199 (Gibco Laboratories, Grand Island, NY) supplemented with 0.1 mg / ml PVA and 0.25 mM pyruvate. TCM199 was used as the control because it is commonly used for IVM of bovine oocytes to produce embryos, although typically it is supplemented with serum or BSA (reviewed in Bavister *et al.*, 1992; Bavister, 1995). All maturation media were additionally supplemented with 1 µg / ml oestradiol (β-oestradiol, Sigma; made up in 1000 × stock solution in absolute ethanol), 50 ng / ml epidermal growth factor (Sigma), 5 µg / ml NIAMDD ovine luteinising hormone (oLH) and 0.5 µg / ml ovine follicle stimulating hormone (USDA-oFSH-18). FSH and LH were prepared in stock solutions of NaCl containing 125 µg / ml BSA.

Following the IVM culture period, oocytes in all seven treatments were subjected to the same IVF and embryo culture procedures. Each treatment group of 8–12 COCs was evaluated for degree of cumulus expansion using a scale in which 0 denoted no expansion and 1–4 indicated progressively greater relative expansion. Each treatment group was then moved through three washes of mBM-Hepes-PVA (Table 1) and finally placed in separate 50 µl drops of IVF medium consisting of TALP (Bavister *et al.*, 1983) modified by omitting glucose and adding 0.25 mM pyruvate and 6 mg / ml of BSA (Fatty Acid Free, Sigma no. A7511, lot 50H9329). In addition, the medium contained 0.5 µg / ml heparin, 10 µM d-penicillamine,

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Table 1 Basic media formulations used for preparation and maturation of oocytes *in vitro* and for embryo culture

	Culture media		
	mBM3[a]	mBM3-Hepes[b]	HECM-6[c]
Component	mM	mM	mM
PVA	(0.1 mg / ml)	(0.1 mg / ml)	(0.1 mg / ml)
NaCl	113.80	113.80	113.80
KCl	3.00	3.00	3.00
$CaCl_2 \cdot 2H_2O$	1.90	1.90	1.90
$MgCl_2 \cdot 6H_2O$	0.46	0.46	0.46
$NaHCO_3$	25.00	2.00	25.00
HEPES (Na salt)	–	5.00	–
HEPES (acid)	–	5.00	–
HCl	(140 µl / 100 ml)	–	(140 µl / 100 ml)
Supplements[d]			
Glucose	2.00	–	–
Pyruvate	0.25	–	–
dl-Lactate	4.50	–	4.50
Eleven amino acids			
Asparagine	0.01	–	0.01
Aspartic acid	0.01	–	0.01
Cysteine	0.01	–	0.01
Glutamic acid	0.01	–	0.01
Glutamine	0.20	–	0.20
Glycine	0.01	–	0.01
Histidine	0.01	–	0.01
Lysine	0.01	–	0.01
Proline	0.01	–	0.01
Serine	0.01	–	0.01
Taurine	0.50	–	0.50

[a]mBM3 = Basic Medium-3 (McKiernan *et al.*, 1995) modified to contain no energy substrates. This medium, supplemented with different combinations of energy substrates and amino acids, was used for six of the seven oocyte maturation media.
[b]mBM3-Hepes = mBM3 medium supplemented with Hepes buffer.
[c]HECM-6 = hamster embryo culture medium (McKiernan *et al.*, 1995) used for the first 48 h following IVF of bovine eggs (Pinyopummintr & Bavister, 1994, 1996*a*, *b*).
[d]Different supplements used to produce different IVM treatments; see Materials and Methods.

50 µM hypotaurine and 0.5 µM adrenaline to promote sperm capacitation (Parrish *et al.*, 1988; Susko-Parrish *et al.*, 1990). The IVF medium drops were overlaid with paraffin oil. Incubation conditions for IVF were 39 °C in 5% CO_2 in air and high humidity for 24 h.

All experiments used frozen semen obtained from the same ejaculate of one bull packaged in straws at 50×10^6 sperm / ml (donated by American Breeders Service, DeForest, WI). For each replicate, one or two straws were thawed at 35 °C for 1 min. The IVF procedure used has been described previously (Pinyopummintr & Bavister, 1991; Rose & Bavister, 1992). Briefly, 250 µl of frozen-thawed semen was layered under 1 ml of TALP medium (modified to contain no glucose, 21.6 mM sodium lactate, 1 mM pyruvate, 10 mM Hepes, 6 mg / ml BSA (fraction V; Sigma, no. 7511, lot 50H9329) and 50 µg / ml gentamicin sulphate) contained in 12×55 mm test tubes and incubated at 39 °C for 1 h. The upper 800 µl of medium from each test tube was recovered and washed by resuspending the sperm twice in 3 ml of modified TALP medium and centrifuging at 100 *g*. The concentration of the final sperm pellet was determined with a haemocytometer and the fertilisation drops were inseminated at a final concentration of $0.7–1.0 \times 10^6$ sperm / ml.

In vitro embryo development and evaluation

After the 24 h IVF culture period, eggs were vortexed to remove cumulus cells and washed three times in

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mBM3-Hepes. Each oocyte maturation treatment group was cultured in separate 50 µl drops of HECM-6 medium containing 11 amino acids (see Table 1 for components) overlaid with 10 ml paraffin oil, at 39 °C in 5% CO_2, 10% O_2, balance N_2 in high humidity (Pinyopummintr & Bavister, 1995). A total of 728 presumptive embryos were cultured. At 72 h post-insemination (hpi) embryos were evaluated using both a dissecting microscope and an inverted microscope with phase and Nomarski interference contrast at ×40–100. All embryos ≥ 4 cells were placed into TCM199 with 10% BCS. At 192 hpi (day 8) embryos were re-evaluated and fixed in 1% formaldehyde. Embryos with a blastocoele cavity were classified as blastocysts and stained with Hoechst stain to assess the number of non-pyknotic nuclei (Pursel *et al.*, 1985).

Statistical analysis

A protected (Fisher's) analysis of variance (ANOVA) was performed on arcsin (square root)-transformed embryo development (percentage) data in a randomised block design where the block represented days. Cumulus expansion was analysed using non-transformed data and the same design. All treatments were replicated six times. Each replicate represented oocytes collected on a particular day with treatments duplicated (i.e. 12 samples per treatment were analysed) except in the case of three treatments where 11 samples were analysed. Treatment differences in mean number of nuclei per blastocyst were analysed using ANOVA. Significance level for all analyses was at $p \leq 0.05$. To determine whether a correlation existed between the degree of cumulus expansion and subsequent developmental competence a simple linear regression was performed.

Results

After 24 h of IVM there were significant treatment effects on cumulus expansion. Cumulus expansion was greatest in COCs matured in TCM199 (3.5 on a 4.0 scale; Table 2). The COCs cultured in mBM3 supplemented with pyruvate (i.e. pyruvate + glucose or pyruvate + glutamine) displayed minimal cumulus expansion (0.6 and 0.8 on a 4.0 scale). All other treatments supported intermediate cumulus expansion. Simple linear regression indicated a correlation (r^2 = 0.542) between cumulus expansion and oocyte developmental competence. Low cumulus expansion scores (< 1.0) were associated with very poor developmental competence (3% and 2% blastocysts for COCs matured in BM3 in combination with either pyruvate + glucose or pyruvate + glutamine, respectively; Fig. 1). At least moderate cumulus expansion, i.e. an overall treatment score ≥ 1.8, was associated with good developmental competence (Table 2; Fig. 1). However, there were no consistent differences in terms of developmental competence among oocyte maturation treatments that supported intermediate to high cumulus expansion (1.8 to 3.5).

Table 2 Degree of cumulus expansion after oocyte maturation in different culture media[a]

Maturation medium	Cumulus expansion[b]
Pyruvate + glucose	0.6 ± 0.2[c]
Lactate + glucose	2.1 ± 0.4[d]
Pyruvate + glutamine	0.8 ± 0.3[c]
Lactate + glutamine	1.8 ± 0.2[d]
Glucose + glutamine	1.8 ± 0.4[d]
Glucose + 11 amino acids	2.0 ± 0.4[d]
TCM199	3.5 ± 0.3[e]

[a]Oocyte maturation was carried out as described in Materials and Methods.
[b]After 24 h all cumulus–oocyte complexes cultured within a treatment were scored for overall degree of cumulus expansion: 0 = no expansion, 1 = minimal expansion, 4 = maximal expansion. Values are the mean ± SE.
Mean = numerical score of cumulus expansion averaged over all duplicates and replicates for a treatment.
SE = standard error.
[c–e]Different superscripts indicate significant differences ($p \leq 0.05$).

When evaluated at 72 hpi, embryo development to ≥ 2 cells was significantly less for COCs matured in mBM3 containing pyruvate + glutamine (54%) than in other oocyte maturation media (66–78%). A total of 75 blastocysts was obtained. The percentages of blastocyst development differed depending on the medium used for oocyte maturation. When expressed as a proportion of the total oocytes matured or of the 2-cell embryos produced, blastocyst development from COCs matured in mBM3 supplemented with glucose + 11 amino acids was significantly greater (21% and 25%, respectively) than for COCs matured in lactate + glutamine (9% and 12%, respectively), pyruvate + glucose (2% and 3%, respectively) and pyruvate + glutamine (2% and 2%, respectively). Blastocyst development from COCs matured in TCM199 or in mBM3 containing glucose + glutamine or lactate + glucose was not significantly different from blastocyst production obtained with COCs matured in mBM3 supplemented with glucose + 11 amino acids (see Figs. 1 and 2).

The mean number of non-pyknotic nuclei per blastocyst ranged from 52 to 88 among treatments and was not significantly different amount treatments. Of 25 expanded blastocysts from six replicates, 19 were derived from IVM in simple medium containing glucose + glutamine, glucose + 11 amino acids, lactate +

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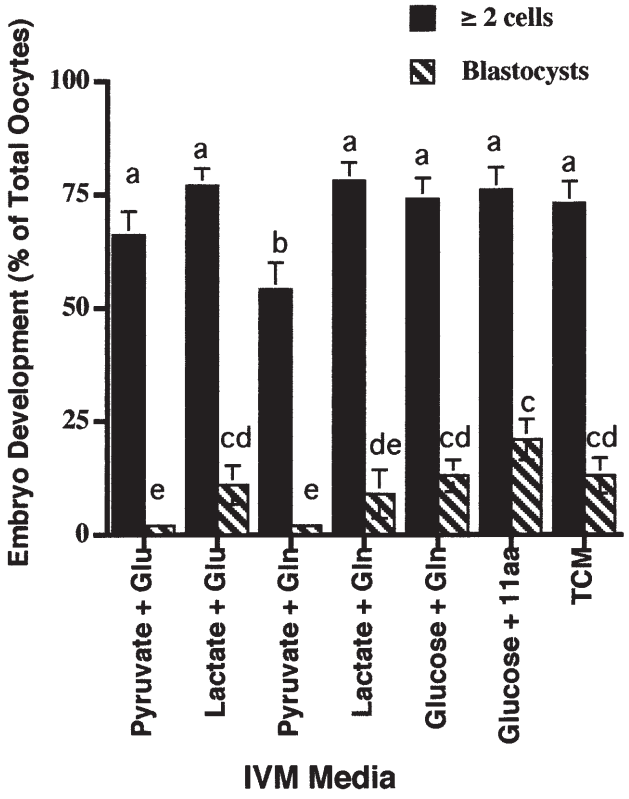

Figure 1 Embryo development *in vitro* from bovine oocytes matured in one of seven media supplemented with polyvinyl alcohol and subsequently fertilised *in vitro*. Percentage data are based on total oocytes placed in the maturation medium. Means and standard errors represent non-transformed data collected in six replicate experiments with duplication within replicates. [a–b]Statistically significant differences ($p \leq 0.05$) in percentages of ≥ 2 cells. [c–e]Statistically significant differences ($p \leq 0.05$) of blastocysts. Statistical analysis (Fisher's ANOVA) is based on arcsin (square root)-transformed data. Glu, glucose; Gln, glutamine; 11aa, 11 amino acids described in Table 1.

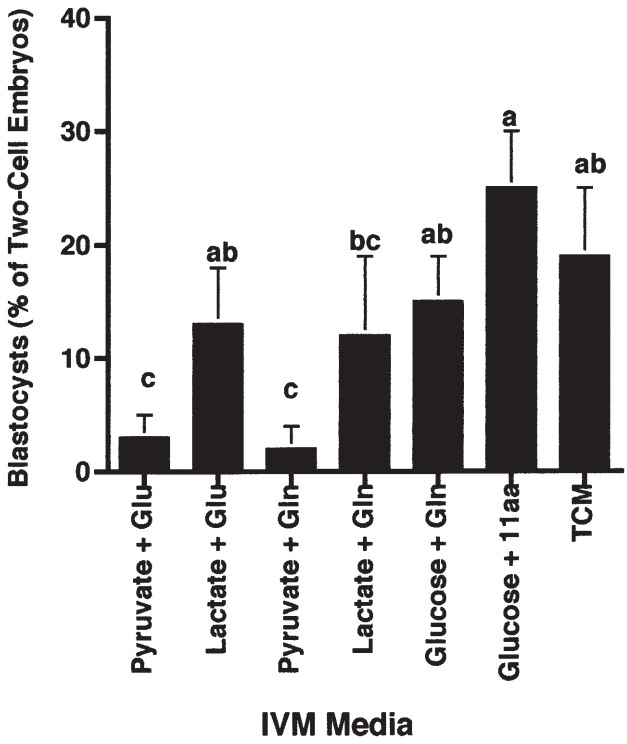

Figure 2 Blastocyst development *in vitro* from bovine oocytes matured in one of seven media supplemented with polyvinyl alcohol and subsequently fertilised *in vitro*. Percentage data are based on 2-cell embryos. Means and standard errors represent non-transformed data collected from six replicate experiments. [a–c]Statistically different ($p \leq 0.05$) in percentage of blastocysts. Statistical analysis (Fisher's ANOVA) is based on arcsin (square root)-transformed data. Glu, glucose; Gln, glutamine; 11aa, 11 amino acids described in Table 1.

glutamine or lactate + glucose, while 5 expanded blastocysts were obtained from IVM in TCM199 and only 1 expanded blastocyst from IVM in simple medium containing pyruvate. These expanded blastocysts contained 86 ± 5 (mean ± SE) nuclei per embryo. Seven hatched blastocysts contained 150 ± 11 nuclei per embryo. Hatched embryos were obtained from oocytes matured in TCM199, mBM3 containing glucose + glutamine, and mBM3 containing glucose + 11 amino acids.

Discussion

For many years, the traditional method of assessing oocyte maturation was attainment of metaphase II nuclear configuration (i.e. 'nuclear' maturation). *In vivo*, nuclear maturation is temporally linked with progression and completion of 'cytoplasmic' maturation as defined by the ability of the ovum to undergo normal fertilisation and embryo development. However, this relationship is not always maintained *in vitro* (Leibfried-Rutledge *et al.*, 1989*a*; Eppig *et al.*, 1994; Laurincik *et al.*, 1994; Schramm *et al.*, 1994). Not all oocytes exhibiting nuclear maturation are fertilisable using IVF procedures, and those that are penetrated by sperm may or may not be able to support sperm nucleus decondensation, pronuclear formation and syngamy (Leibfried & Bavister, 1983; Leibfried-Rutledge *et al.*, 1989*a*; discussed in Bavister, 1987; Kito & Bavister, 1997). Furthermore, fertilised ova may be incapable of normal embryo development, or may undergo the first cleavage division but exhibit varying abilities to reach the blastocyst stage depending on the conditions used for oocyte maturation (van de Sandt *et al.*, 1990; Rose &

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Bavister, 1992; present study). These graded levels of development indicate that cytoplasmic maturation occurs as a progressive series of events whereby oocytes that have completed the highest number of these steps possess the greatest potential for development (reviewed in Leibfried-Rutledge *et al.*, 1989*a*; Bavister *et al.*, 1992; Eppig *et al.*, 1994).

Oocyte maturation is influenced by non-hormonal components of the culture medium (Haidri & Gwatkin, 1973; Gwatkin & Haidri, 1973; van de Sandt *et al.*, 1990; Kim & Schuetz, 1991; Liebfried & Bavister, 1983; Rose & Bavister, 1992; Downs & Mastropolo 1994; Kobayashi *et al.*, 1994). Most studies on oocyte maturation *in vitro* have used culture media containing undefined supplements, i.e. serum or BSA preparations, that confound attempts to evaluate key factors regulating maturation and developmental competence (discussed by Bavister, 1995). More recent studies have eliminated use of these components during IVM (Harper & Brackett, 1993; Lonergan *et al.*, 1996; Keskintepe & Brackett, 1996). The present study used a simple, protein-deficient medium (mBM3; Table 1) to compare the ability of different combinations of substrates to support oocyte maturation, leading to acquisition of embryo development competence. Oocytes (COCs) matured under these conditions were compared with COCs matured in TCM199. The combinations of substrates and nutrients used in this study were selected because, in preliminary experiments, they supported oocyte nuclear maturation to metaphase II in medium BM3. However, when added as single substrates, only lactate was able to support nuclear maturation at a frequency similar to that in TCM199, but anomalies were frequent with lactate alone. Some oocytes did not exhibit a typical metaphase II configuration but contained two very condensed sets of chromosomes that suggested completion of metaphase I (data not shown). In addition to energy substrates, a group of 11 amino acids that stimulated hamster and bovine 1-cell embryo development (McKiernan *et al.*, 1995; Pinyopummintr & Bavister, 1996*b*) was used for these oocyte maturation studies.

Maturation of COCs in substrate/amino acid combinations of glucose + glutamine, glucose + 11 amino acids, glucose + lactate or lactate + glutamine supported cytoplasmic maturation of *in vitro* matured oocytes to the same extent as TCM199 (Figs. 1, 2). Except for lactate + glutamine, these treatments were significantly better than the other combinations of substrates (pyruvate + glutamine or pyruvate + glucose). Because there was no difference in response between glucose + glutamine and glucose + 11 amino acids (Figs. 1, 2), it appears that the beneficial effects of the 11 amino acids during maturation can be attributed to glutamine. These results suggest that acquisition of developmental competence by bovine COCs matured *in vitro* require glutamine in combination with glucose or lactate, or glucose + lactate. Further studies are needed to verify this; cytoplasmic oocyte maturation may be further enhanced by other combinations of amino acids – an approach already shown to enhance development of early cleavage stage embryos (Lane & Gardner, 1994; McKiernan *et al.*, 1995; Pinyopummintr & Bavister, 1996*b*; discussed in Bavister, 1995). Here we have observed that a simple salt solution containing appropriate substrates during IVM yields embryos capable of *in vitro* preimplantation development equally as well as a complex medium such as TCM-199.

COCs are made up of two distinct cell types: the somatic granulosa–cumulus cells and the oocyte. The presence of both components during oocyte maturation is required for acquisition of developmental competence (Liebfried-Rutledge *et al.*, 1989*a, b*; Buccione *et al.*, 1990; Zuelke & Brackett, 1992, 1993; Zhang *et al.*, 1995), so the metabolic and nutrient needs of both cell types must be met. The relative ability of pyruvate and inability of glucose to be metabolised by isolated mammalian oocytes devoid of cumulus cells has been demonstrated in several species, including mouse, rhesus monkey, cattle, human and rat (Brinster, 1971; Rushmer & Brinster, 1973; Tsutsumi *et al.*, 1990, 1992; Zuelke & Brackett, 1992, 1993; Rieger & Loskutoff, 1994; Saito *et al.*, 1994). Whether the beneficial effects on oocyte maturation of adding the different combinations of amino acids, glucose and lactate are mediated by the oocyte, the cumulus cells or both is uncertain and may be difficult to determine.

Glutamine is metabolised by bovine COCs and by denuded oocytes; however, there were pronounced differences in glutamine metabolism depending on whether oocytes were denuded before or after IVM (Zuelke & Brackett, 1993; Rieger & Loskutoff, 1994). Optimal metabolism of glutamine appears to require both cellular compartments (the oocyte and the cumulus cells) and is enhanced by LH in intact COCs (Zuelke & Brackett, 1993). The observation that cumulus cells can transfer amino acids to oocytes through gap junction channels (Moor & Smith, 1979; reviewed in Colonna & Mangia, 1983) suggests that glutamine moves into the oocyte via this mechanism. Nevertheless, glutamine improved nuclear maturation of denuded hamster oocytes (Gwatkin & Haidri, 1973). As the sole substrate, glutamine was able to support nuclear maturation of rabbit oocytes maturing *in vitro* and was oxidised by both cumulus cells and oocytes (Bae & Foote, 1975*a, b*). Glutamine is also metabolised by preimplantation bovine embryos (Rieger & Guay, 1988) and is important for early embryo development in hamsters and cattle (McKiernan *et al.*, 1991, 1995; Pinyopummintr & Bavister, 1996*a, b*). In addition, glutamine can serve as a major energy substrate in mitotic cells (reviewed by Zielke *et al.*, 1984). All these obser-

vations together with the present study indicate a primary role for glutamine in oocyte maturation.

Glucose was required by COCs for acquisition of developmental competence by the oocytes, but was not necessarily sufficient alone (Figs. 1, 2). We can deduce that this requirement was primarily by the cumulus cells because glucose metabolism by denuded cattle oocytes is either low (Rieger & Loskutoff, 1994) or absent (Zuelke & Brackett, 1992). It is interesting that glucose also fails to support cleavage of bovine embryos (Pinyopummintr & Bavister, 1996*a*), considering that these embryos may have similar metabolic and nutrient needs to oocytes because both are under maternal genomic control (Telford *et al.*, 1990). In addition, glucose effects on mouse oocyte maturation appear to require the presence of surrounding cumulus cells (Downs & Mastropolo, 1994). However, glycolysis did not increase in bovine cumulus cell complexes from which oocytes had been removed, indicating the metabolic integration of the somatic and gamete components of COCs (Zuelke & Brackett, 1992).

Lactate could partially replace glucose as IVM in lactate + glutamine gave intermediate embryo development results, equivalent to those with glucose + glutamine (Fig. 2). Lactate + glutamine support cleavage of bovine embryos (Pinyopummintr & Bavister, 1996*a*, *b*) and glutamine metabolism increases substantially during bovine oocyte maturation (Rieger & Loskutoff, 1994). Pyruvate could not replace glucose or lactate for IVM as evidenced by the developmental responses of oocytes matured with pyruvate + glutamine or pyruvate + glucose, which were significantly lower than with glucose + glutamine or lactate + glucose (Fig. 2). This is curious because cumulus cells produce pyruvate (Donahue & Stern, 1968; Leese & Barton, 1985; Zuelke & Brackett, 1993; Downs & Mastropolo, 1994) and pyruvate is required for nuclear maturation of denuded oocytes (devoid of cumulus cells) *in vitro* (Biggers *et al.*, 1967; Kennedy & Donahue, 1969; Brinster, 1971). Furthermore, pyruvate metabolism by bovine oocytes increases significantly during maturation (Rieger & Loskutoff, 1994). These data indicate that glucose conversion into pyruvate by cumulus cells for use by the oocyte is not the only role of glucose, because exogenous pyruvate did not stimulate cytoplasmic maturation in the present study. A significant proportion of glucose metabolism by bovine oocytes is via the pentose phosphate pathway. It is possible that exogenous pyruvate may not be as accessible to the oocyte as endogenously generated pyruvate.

Gonadotropin-induced cumulus expansion was observed to some degree in COCs matured in any of the maturation media (Table 2). However, the greatest expansion occurred in COCs matured in TCM199, followed by those matured in medium containing glucose + glutamine (or 11 amino acids) or glucose + lactate, or lactate + glutamine. This is consistent with data showing that substrates for mucopolysaccharide synthesis, including the combination of glucose + glutamine, are required for cumulus expansion of bovine COCs *in vitro* (Chen *et al.*, 1990; Ball *et al.*, 1980). Maturation of COCs in medium that resulted in at least moderate cumulus expansion (Table 2) also yielded equally developmentally competent embryos (Figs. 1, 2). The COCs with the poorest developmental competence that were matured in mBM3 containing pyruvate + glucose or pyruvate + glutamine had very little cumulus expansion. Although it can be concluded that cumulus expansion is associated with acquisition of developmental competence *in vitro*, our data do not indicate that the highest degree of cumulus expansion is required. Lonergan *et al.* (1994) previously concluded that cumulus expansion *per se* during IVM does not necessarily indicate or confer developmental capacity on oocytes.

It is clear that *in vitro* embryo development is a significantly better indicator of meiotic competence than nuclear maturation or oocyte fertilisability and/or first cleavage, because oocytes from six of seven oocyte maturation media were equally capable of being fertilised and cleaving to the 2-cell stage but then varied markedly in their ability to develop into blastocysts. The capacity of an oocyte to become a blastocyst after fertilisation apparently required further 'cytoplasmic' maturation processes in addition to those needed for successful fertilisation and cleavage to 2 cells, as indicated in our previous study (Rose & Bavister, 1992) and in a similar study with mouse oocytes (van de Sandt *et al.*, 1990). Using blastocyst development as a measure of oocyte competence, we have demonstrated that different combinations of pyruvate, glucose, lactate and amino acids alter the degree of cytoplasmic maturation during IVM.

In summary, the data from this study help to establish the optimal substrate requirements for IVM of bovine oocytes and acquisition of their developmental competence. More work is needed to determine the complete substrate and nutrient requirements of oocytes during maturation. Moreover, simplification of IVM culture media, especially removal of undefined biological components (serum and BSA), is a necessary step towards better understanding of the nutrient and regulatory requirements of isolated cumulus-enclosed oocytes and for the elimination of infectious agents. Studies are needed to establish defined culture conditions for IVM of oocytes from other species, including humans. The ability to support cytoplasmic maturation during IVM of human oocytes, consistently and routinely, could allow women undergoing radical treatments such as chemotherapy or radiation therapy to store oocytes for later restoration of fertility. IVM is also an approach for assisted reproduction in women

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exhibiting polycystic ovarian syndrome (Trounson *et al.*, 1994). When human oocyte IVM becomes a routine procedure, the necessity for ovarian stimulation might be eliminated altogether. This could not only avoid ovarian hyperstimulation syndrome (Navot *et al.*, 1992) but might also increase the success of assisted reproduction by avoiding disturbance of the menstrual cycle and disruption of the normal synchronisation of embryo development and endometrial differentiation (Paulson *et al.*, 1997; Kolb *et al.*, 1997).

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