Bovine blastocyst production *in vitro* after inhibition of oocyte meiotic resumption for 24 h

P. Lonergan, H. Khatir, C. Carolan and P. Mermillod*

Station de Physiologie de la Reproduction, INRA, 37380 Nouzilly, France

The aims of the present study were to assess the effect of various substances on meiotic resumption and subsequent development to the blastocyst stage of bovine oocytes. Immature cumulus–oocyte complexes were cultured for 24 h in (a) Medium 199 (M199) alone, or M199 supplemented with (b) 10% fetal calf serum (FCS), (c) 1 µg cycloheximide ml⁻¹, (d) 2 mmol 6-dimethylaminopurine (6-DMAP) l⁻¹, or (e) 0.1 mmol vanadate l⁻¹. After 24 h, groups (a) and (b) were inseminated with frozen–thawed spermatozoa and subsequently cultured, while groups (c–e) were washed and cultured for a second 24 h in M199 + FCS, after which they were inseminated and cultured. At all time points a representative sample of oocytes were fixed and stained with orcein to observe the nuclear status, while others were labelled with [³⁵S]methionine to study protein biosynthesis. Incubation with 6-DMAP, cycloheximide or vanadate completely blocked germinal vesicle breakdown with most oocytes remaining at the germinal vesicle stage after 24 h culture (89%, 100% and 85%, respectively). This inhibitory effect was fully reversible in the case of 6-DMAP and cycloheximide; after a second period of incubation, germinal vesicle breakdown occurred in almost all cases (99% and 100%, respectively), and most reached metaphase II (85% and 83%, respectively). In contrast, inhibition with vanadate was only reversible in 56% of oocytes, with only 6% reaching metaphase II. Cleavage rates at 72 h after insemination and blastocyst yields on day 8 of culture were, respectively: (i) M199, 72% and 34%; (ii) M199 + FCS, 80% and 45%; (iii) M199 + cycloheximide, 81% and 19%; (iv) M199 + 6-DMAP, 77% and 14%. 6-DMAP did not modify methionine incorporation. However, cycloheximide completely blocked protein synthesis when present during the period of labelling. Addition of epidermal growth factor to cycloheximide-inhibited oocytes was without effect. In contrast, epidermal growth factor overcame the effect of 6-DMAP in about 50% of oocytes, resulting in lower developmental rates after IVF. These results give an indication of the feasibility of *in vitro* meiotic inhibition as a tool in the study of the mechanisms involved in acquisition of competence.

Introduction

In recent years substantial progress has been made in the development of procedures for the *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of bovine embryos (for review see Gordon, 1994). However, further improvements are necessary to maximize embryo production. In most laboratories, the production of viable embryos reaches a plateau at 30–40% of inseminated oocytes, in spite of numerous variations on the basic technique. It would appear that oocytes are not fully capable of responding to the maturation conditions to which they are exposed. It is clear that as well as nuclear maturation (that is, progression from prophase I to metaphase II) normal cytoplasmic maturation must occur *in vitro* to produce a viable embryo. However, whether the cytoplasm of all oocytes submitted to IVM is ready to respond to the maturation conditions imposed on them is questionable.

The further along the developmental axis from immature oocyte to blastocyst that the oocyte/embryo is removed from the *in vivo* environment and placed *in vitro*, the better the final outcome. For example, oocytes from larger follicles are superior in terms of developmental potential than those from smaller follicles (Pavlok *et al.*, 1992, 1993; Lonergan *et al.*, 1994a). Also, zygotes recovered after *in vivo* maturation and fertilization develop better in culture than do zygotes produced totally *in vitro* (Marquant Le Guenne *et al.*, 1989; McCaffrey *et al.*, 1991). While oocytes for IVM are usually obtained from follicles 2–6 mm in diameter and at least 4–10 days away from any possible ovulation (Sirard *et al.*, 1992), oocytes resuming meiosis *in vivo* originate from dominant follicles of about 15 mm in diameter (Pavlok *et al.*, 1992). In addition, the IVM period lasts only 24 h, while the dominant follicle grows from 4 to 15 mm for approximately 5 days. Therefore, it is likely that developmental heterogeneity of chromosomally mature oocytes reflects intrinsic influences on the oocyte that occur differentially among follicles. It is well known that marked

*Correspondence.

Received 27 August 1996.

changes occur in oocyte nuclei during final growth and maturation as the follicle increases in diameter from 1 to 15–20 mm (Thibault *et al.*, 1987; Fair *et al.*, 1995, 1996). These changes may have a crucial effect on the developmental potential of the oocyte.

Despite endless modifications of the IVM medium, there may be a biological limit to the blastocyst yields achievable *in vitro*. It may well be that a prematuration treatment is necessary to allow the oocytes from smaller follicles, which constitute the majority of the surface visible follicle population (Lonergan *et al.*, 1994a) and of which the final phase of folliculogenesis has been artificially shortened by removal from the follicle, to 'catch up' with those from larger follicles or those matured totally *in vivo*. In support of this idea, the findings of Assey *et al.* (1994) indicate that bovine oocytes aspirated from dominant follicles before the preovulatory LH surge display alterations in their nuclear and cytoplasmic morphology. The authors suggest that these changes are a prerequisite for the oocyte to acquire full developmental competence. This would indicate that not only final oocyte maturation (that is, the processes occurring from LH surge to ovulation) is significant, but also the period preceding the LH surge may be important for the establishment of developmental competence. For *in vitro* embryo production, IVM is initiated shortly after removal of the immature oocyte from small antral follicles. Consequently, oocytes are not allowed the time to complete the changes described by Assey *et al.* (1994). This could in part explain the lower results in terms of development *in vitro*.

Under normal conditions, the chromosomes start to condense immediately after removal from the follicle, preventing almost all possible influence on oocyte chromatin during IVM. To understand the mechanisms responsible for the acquisition of developmental competence it is essential to simulate the latter stages of follicular development *in vitro*. The first step towards this objective requires a culture system that reproduces the ovarian follicular environment for the oocyte, in which nuclear maturation is prevented. Only when this is achieved can the effect of hormones and other ovarian factors on developmental competence be analysed under defined conditions. Several authors have reported on the inhibition of meiotic resumption in bovine oocytes using physiological and pharmacological methods (Sirard, 1990; Fulka *et al.*, 1991; Sirard *et al.*, 1992; De Loos *et al.*, 1994; Kotsuji *et al.*, 1994; Aktas *et al.*, 1996a, b; Richard and Sirard 1996a, b). However, few of these studies have reported on developmental rates of oocytes after periods of blockage. In addition, many of the products used are not compatible with the long-term survival of the oocyte and cannot therefore be used to enhance its developmental competence.

With this in mind the present study had these objectives: (i) to establish the kinetics of IVM in our defined system; (ii) to assess the effect of cycloheximide, a protein synthesis inhibitor; 6-dimethylaminopurine (6-DMAP), a phosphorylation inhibitor; and vanadate (NaVO_3), an inhibitor of protein tyrosine phosphatases on meiotic resumption in bovine oocytes; and (iii) to examine the developmental potential of such blocked oocytes after removal of the inhibitory conditions and reinstatement of conditions conducive to normal IVM. In addition, the effect of a putative stimulus, epidermal growth factor (EGF),

on blocked oocytes was examined, since it has been shown that EGF significantly increases the proportion of oocytes reaching metaphase II after 24 h as well as the proportion developing to the blastocyst stage (Lonergan *et al.*, 1996).

Materials and Methods

General procedures

Chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise indicated. Stock solutions of 50 mmol 6-DMAP l^{-1} , 50 mmol sodium metavanadate l^{-1} (NaVO_3) and 50 mg cycloheximide ml^{-1} were prepared in Medium 199 (M199).

The details of methods for oocyte recovery, *in vitro* maturation (IVM) and fertilization (IVF) and culture (IVC) have been described by Mermillod *et al.* (1993). Briefly, cumulus–oocyte complexes (COCs) were obtained by aspiration of 2–6 mm follicles of ovaries from slaughtered cows. After four washes in modified PBS (supplemented with 36 μg pyruvate ml^{-1} , 50 μl gentamycin ml^{-1} and 0.5 mg BSA ml^{-1} , Sigma, fraction V, A-9647), groups of up to 50 COCs were transferred to four-well plates (Nunc, Roskilde) containing 500 μl of maturation medium (see below) for 24 h culture at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. The interval between recovery of the COCs and onset of culture was approximately 60 min, during which COCs were left in follicular fluid.

For IVF, COCs were washed four times in PBS and once in fertilization medium before they were transferred in groups of up to 50 into four-well plates containing 250 μl of fertilization medium (TALP, containing 10 μg heparin-sodium salt ml^{-1} – 167 iu mg^{-1} , Calbiochem, San Diego, CA) per well. Motile spermatozoa were obtained by centrifugation of frozen–thawed spermatozoa on a Percoll (Pharmacia, Uppsala) discontinuous density gradient (2 ml at 45% over 2 ml at 90%) for 20 min at 700 g at room temperature. Viable spermatozoa, collected at the bottom of the 90% fraction were washed in TALP and pelleted by centrifugation at 100 g for 10 min at room temperature. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of TALP to give a concentration of 4×10^6 spermatozoa ml^{-1} ; 250 μl of this suspension was added to each fertilization well to obtain a final concentration of 2×10^6 spermatozoa ml^{-1} . Plates were then incubated for 20–24 h in 5% CO_2 in humidified air at 39°C.

Embryo culture took place in modified synthetic oviduct fluid medium (SOF), under paraffin oil in a humidified atmosphere of 5% CO_2 , 5% O_2 , 90% N_2 at 39°C (Carolan *et al.*, 1995). Twenty-four hours after insemination, presumptive zygotes were denuded by vortexing for 2 min in 2 ml PBS. The zygotes were subsequently washed twice in PBS and twice in SOF before being transferred in groups of 20–30 to the culture droplets (1 zygote per μl medium). Fetal calf serum (FCS) was added to the droplets (10%, v/v) 24 h after placement in culture (that is, 48 h post-insemination, hpi).

Cleavage was assessed 48 h after placement in culture, that is, 72 h after insemination (percentage non-cleaved, two–four cells, five–eight cells). The number of embryos developing to at

least the expanded blastocyst stage was assessed on days 6 and 8 of culture. Hatching was recorded on day 8 of culture and expressed as a percentage of day 8 blastocysts.

Experiment 1: kinetics of *in vitro* maturation

In order to establish the standard kinetics of *in vitro* maturation under these conditions, COCs were matured in either M199 alone ($n = 497$) or M199 + 10% FCS ($n = 510$). These two media represent the two controls used in our laboratory for all maturation experiments (see Lonergan *et al.*, 1994b). A sample of oocytes ($n = 125$) was fixed and stained immediately after removal from the follicle and represents 0 h. In addition, a sample of oocytes was removed from the maturation dishes at 4, 8, 12, 16, 20 and 24 h after the start of culture, denuded, fixed in acetic acid:ethanol (1:3), stained with orcein, and observed under the microscope. Oocytes were classified as germinal vesicle (GV), pro-metaphase I, metaphase I, anaphase I, telophase I or metaphase II.

Experiment 2: effect of different inhibitors on meiotic resumption

(a) COCs ($n = 442$) were incubated for 24 h in: M199, M199 + 6-DMAP (0.1, 1, 2, 4, 8 mmol l⁻¹), or M199 + 10% FCS. They were then denuded, fixed and stained as described above.

(b) COCs ($n = 305$) were incubated for 24 h in: M199, M199 + cycloheximide (0.1, 1, 10, 100 µg ml⁻¹), or M199 + 10% FCS. They were then denuded, fixed and stained as described above.

(c) COCs ($n = 264$) were incubated for 24 h in: M199, M199 + NaVO₃ (0.05, 0.1, 0.5, 1, 1, 2 mmol l⁻¹), or M199 + 10% FCS. They were then denuded, fixed and stained as described above.

A minimum of three replicates was performed for each inhibitor.

Experiment 3: reversibility of meiotic inhibition

(a) To test the reversibility of meiotic inhibition under the above conditions, after a primary incubation for 24 h in the presence of 6-DMAP (2 mmol l⁻¹; $n = 497$), cycloheximide (1 µg ml⁻¹; $n = 289$) or NaVO₃ (0.1 and 0.5 mmol l⁻¹; $n = 282$), COCs were washed four times in PBS for a total of approximately 5 min and twice in culture medium before they were cultured for a further 24 h in M199 + 10% FCS. They were then denuded, fixed and stained as above.

(b) To assess the developmental ability of oocytes after a period of 24 h meiotic inhibition, and to observe the effect of EGF on blocked oocytes, COCs ($n = 668$) were cultured for 24 h in (i) M199 alone, or M199 supplemented with (ii) 10% FCS, (iii) 10 ng EGF ml⁻¹ (Sigma), (iv) 1 µg cycloheximide ml⁻¹, (v) cycloheximide + EGF, (vi) 2 mmol 6-DMAP l⁻¹, (vii) 6-DMAP + EGF. After 24 h culture, groups (i–iii) were submitted to IVF, while groups (iv–vii) were further cultured for 24 h in M199 + FCS, after which they were inseminated and cultured for 8 days.

Experiment 4: protein synthesis patterns during inhibition

(a) To test whether maintenance of meiotic arrest using 6-DMAP affected oocyte protein biosynthesis, COCs were labelled at (i) 0 h; (ii) after 21 h maturation in M199; (iii) after 21 h culture in M199 + 2 mmol 6-DMAP l⁻¹ and; (iv) after 24 h culture in M199 + 2 mmol 6-DMAP l⁻¹ followed by a second 24 h incubation in M199 + 10% FCS.

(b) A similar experiment was carried out with cycloheximide. Oocytes were labelled at (i) 0 h; (ii) after 21 h culture in M199 alone; (iii) after 21 h culture in M199 + 1 µg cycloheximide ml⁻¹; (iv) after 24 h culture in M199 + 1 µg cycloheximide ml⁻¹ followed by a second 24 h incubation in M199 + 10% FCS.

For labelling, COCs were incubated for 3 h in PBS supplemented with 1 mCi [³⁵S]methionine ml⁻¹ (Express Protein Labelling Mix, NEN) at a ratio of 25–50 oocytes per 100 µl in a closed Eppendorf at 39°C. After the period of labelling, cumulus cells were removed by repeated pipetting and oocytes were washed twice in PBS (without BSA) and lysed in groups of ten in 15 µl of sample buffer (Laemmli, 1970). Samples were boiled for 3 min and stored at 20°C until electrophoresis.

Thawed samples were centrifuged (13 000 g for 5 min) and analysed on SDS-PAGE homogeneous slab mini gels (T = 10%; C = 2.6%). Ten oocytes were loaded on to each lane. Proteins of known molecular mass range (35–190 kDa, Sigma, SDS 7B) were run simultaneously as standards. Gels were treated with Amplify (Amersham) for 30 min, dried and exposed for 7 days (Hyperfilm-MP, Amersham) at room temperature.

Statistical analysis

Raw data were analysed by chi-squared analysis or Fisher's exact test where appropriate. $P < 0.05$ was considered significant.

Results

Experiment 1

Results of the kinetics of *in vitro* maturation in M199 and M199 + FCS are shown (Fig. 1). Oocytes isolated from follicles and immediately fixed contained a GV in all cases (125/125, 100%). The nuclear membrane was clearly visible and the nucleoplasm was finely granulated. GVBD occurred in most oocytes between 4 and 8 h of culture. By 8 h, GVBD had occurred in most oocytes (M199: 49/76, 64%; M199 + FCS: 51/69, 74%) and some oocytes had reached metaphase I (M199: 29/76, 38%; M199 + FCS: 40/69, 58%; $P < 0.02$). By 12 h, the majority of oocytes were at metaphase I (M199: 47/78, 60%; M199 + FCS: 76/82, 93%; $P < 0.0001$). It was also at 12 h that the first signs of cumulus expansion became apparent in the presence of FCS (there was no expansion in M199 alone even at 24 h). At 16 h, many had reached telophase and were in the process of extruding the first polar body. By 20 h, the majority had already reached metaphase II (M199: 45/86, 53%; M199 + FCS: 71/85, 84%; $P < 0.0001$). After culture for 24 h in M199 or M199 + 10% FCS, GVBD occurred in nearly all cases (M199: 79/96, 82%; M199 + FCS:

Fig. 1. Kinetics of *in vitro* maturation of bovine oocytes after culture in M199 in the presence ($n = 510$) or absence ($n = 467$) of 10% FCS. Cumulus–oocyte complexes (COCs) were removed from the dishes at 0, 4, 8, 12, 16, 20 and 24 h after the start of culture, fixed and stained with orcein to assess the nuclear status. Three replicates were performed. □, germinal vesicle; ▨, pro-metaphase I; ▩, metaphase I; ▤, telophase I; ■, metaphase II.

112/113, 99%; $P < 0.0001$). Most oocytes completed meiosis I and were arrested at metaphase II with the first polar body extruded (M199: 57/96, 59%; M199 + FCS: 99/113, 88%; $P < 0.0001$).

Experiment 2

(a) In the presence of 6-DMAP at concentrations of 2 mmol l^{-1} or above, GVBD was completely blocked with almost all oocytes containing a GV with distinct nuclear membranes (Fig. 2). The critical concentration to prevent GVBD was between 1 and 2 mmol l^{-1} . Therefore, 2 mmol l^{-1} was chosen for all further experiments.

(b) Cycloheximide completely inhibited meiotic resumption at concentrations of 1 $\mu g\ ml^{-1}$ or greater (100%, Fig. 3). At a concentration of 0.1 $\mu g\ ml^{-1}$, only 53% (24/45) of oocytes remained at GV. Therefore, a concentration of 1 $\mu g\ ml^{-1}$ was used for all further experiments.

(c) The presence of $NaVO_3$ completely inhibited GVBD at concentrations of 1 mmol l^{-1} or greater. At concentrations greater than 0.1 mmol l^{-1} , more than 80% of oocytes remained at GV (Fig. 4). Therefore, concentrations of 0.1 or 0.5 mmol l^{-1} were used for reversibility studies.

Experiment 3

(a) The inhibitory effect of 6-DMAP on meiotic maturation was fully reversible (Fig. 5). When oocytes were cultured in the presence of 2 mmol 6-DMAP l^{-1} for 24 h, washed several times and cultured in M199 + FCS for a further 24 h, GVBD

Fig. 2. Effect of 6-dimethylaminopurine (6-DMAP) on meiotic resumption in bovine oocytes after culture for 24 h in M199 alone or in M199 supplemented with 10% FCS or 6-DMAP at various concentrations from 0.1 to 8.0 mmol l^{-1} . A total of 442 cumulus–oocyte complexes were used over three replicates. □, germinal vesicle; ◇, metaphase I; ○, metaphase II.

occurred in almost all cases (104/105, 99%). The majority of these oocytes reached metaphase II (89/105, 85%). When control (that is, unblocked) oocytes were cultured for the first 24 h in M199 or M199 + FCS and for a second 24 h in M199 + FCS, the proportion at metaphase II increased (45/80, 56% versus 46/64, 72% for M199, $P < 0.05$ and 66/91, 72% versus 60/63, 94% for M199 + FCS, $P < 0.0002$, for 24 and 48 h, respectively).

Similarly, inhibition of meiotic resumption with 1 μg cycloheximide ml^{-1} was fully reversible (Fig. 6) with all oocytes (60/60, 100%) resuming meiosis after a second 24 h culture. The majority of these oocytes reached metaphase II (50/60, 83%).

In contrast to 6-DMAP and cycloheximide, inhibition of GVBD by 0.1 mmol vanadate l^{-1} was only reversible in 56% of oocytes (20/36) and of these very few reached metaphase II (2/36, 6%). Culture with 0.5 mmol vanadate l^{-1} was not reversible, with 98% (49/50) of oocytes remaining at GV stage (Fig. 7).

(b) The results of development after blockage with 6-DMAP and cycloheximide are shown (Table 1). Inhibition with 2 mmol 6-DMAP l^{-1} for 24 h had no effect on the cleavage rate (77%) compared with untreated oocytes cultured for 24 h in M199 (72%) or M199 + FCS (80%). The proportion of embryos at the five–eight-cell stage at 72 h after insemination, was significantly lower for 6-DMAP-blocked oocytes than for untreated oocytes cultured with FCS (33% versus 56%, respectively,

Fig. 3. Effect of cycloheximide (CX) on meiotic resumption in bovine oocytes after culture for 24 h in M199 alone or in M199 supplemented with 10% FCS or cycloheximide at various concentrations from 0.1 to 100.0 $\mu\text{g ml}^{-1}$. A total of 305 cumulus-oocyte complexes were used over three replicates. □, germinal vesicle; ◇, metaphase I; ○, metaphase II.

$P < 0.002$). Blastocyst yields were significantly reduced after inhibition, both at day 6 (22% and 37% versus 8% $P < 0.05$) and day 8 of culture (34% and 45% versus 14%, for M199, M199 + FCS and 6-DMAP, respectively; $P < 0.05$). There was no difference in hatching rate.

Similarly, oocytes inhibited with 1 μg cycloheximide ml^{-1} had similar rates of cleavage and progression to the five-eight-cell stage by 72 h after insemination to the two untreated groups. Blastocyst yields on day 6 of culture were significantly lower than untreated oocytes cultured in M199 + FCS (14% versus 37%). By day 8 of culture, the difference was significant compared with the two untreated groups (34% and 45% versus 19%, for M199, M199 + FCS and cycloheximide, respectively; $P < 0.05$). The hatching rate was significantly lower than that of the untreated controls ($P < 0.05$).

Addition of 10 ng EGF ml^{-1} to M199 significantly increased the cleavage rate (87% versus 72%, $P < 0.03$) and the proportion of oocytes reaching the five-eight-cell stage by 72 h after insemination (60% versus 43%, $P < 0.02$). In addition, blastocyst yields at day 6 were significantly higher than that of controls (40% versus 22%, $P < 0.02$). At day 8, while blastocyst rates were still higher with EGF (44% versus 34%) the difference was not significant. There were no differences between EGF and FCS for any of the parameters studied.

Addition of EGF to oocytes in the presence of cycloheximide had no effect on subsequent development. While EGF markedly stimulated cumulus expansion in the control, in the presence of cycloheximide or 6-DMAP there was no expansion

Fig. 4. Effect of sodium vanadate (NaVO_3) on meiotic resumption in bovine oocytes after culture for 24 h in M199 alone or in M199 supplemented with 10% FCS or NaVO_3 at various concentrations from 0.05 to 2.00 mmol l^{-1} . A total of 264 cumulus-oocyte complexes were used over three replicates. □, germinal vesicle; ◇, metaphase I; ○, metaphase II.

(data not shown). However, subsequent culture of cycloheximide-EGF-inhibited oocytes in M199 + FCS resulted in extensive cumulus expansion compared with those initially cultured in the absence of EGF. Addition of EGF to 6-DMAP-inhibited oocytes resulted in significantly lowered development compared with oocytes blocked in its absence (see Table 1).

It should also be noted that the results of a preliminary experiment demonstrated clearly that, while a 48 h maturation time significantly increased the proportion of oocytes at metaphase II, it had a deleterious effect on oocyte development in terms of both cleavage rate (M199: 24 h, 73%, 98/135 versus 48 h, 31%, 23/74, $P < 0.0001$; M199 + 10% FCS: 24 h, 68%, 107/158 versus 48 h, 26%, 15/57, $P < 0.0001$) and blastocyst yield after 8 days of culture (M199: 24 h, 18%, 24/135 versus 48 h, 0, 0/74, $P < 0.0001$; M199 + 10% FCS: 24 h, 25%, 39/158 versus 48 h, 0, 0/57, $P < 0.0001$).

Experiment 4

6-dimethylaminopurine. A representative gel is shown (Fig. 8). Comparison between protein profiles observed before maturation (0 h) and after 24 h maturation in M199 (lanes A and B, respectively) revealed both quantitative and qualitative changes. The quantitative changes were manifested by a decrease in the intensity of certain bands (45, 50, 69, 82 and 102 kDa) and an increase in the intensity of others (52 and

Fig. 5. Reversibility of 6-dimethylaminopurine (6-DMAP)-induced meiotic inhibition in bovine oocytes. Cumulus-oocyte complexes ($n = 497$) were cultured in M199 alone or in M199 supplemented with 10% FCS or 2 mmol 6-DMAP l^{-1} . After 24 h of culture, a representative number were fixed and stained to assess the nuclear status, while the remainder were washed and cultured for a further 24 h in M199 + 10% FCS, after which they were fixed and stained. Three replicates were performed. □, germinal vesicle; ▨, metaphase I; ■, metaphase II.

73 kDa). The qualitative changes were marked by the disappearance of certain bands during IVM (78 and 108 kDa) and the appearance of others (48 and 72 kDa).

Similarly, comparison between protein profiles observed before maturation (0 h) and after culture in the presence of 2 mmol 6-DMAP l^{-1} (lanes A and C, respectively) revealed both quantitative and qualitative changes. There was a decrease in the intensity of bands of 45, 78 and 102 kDa during culture, while that of bands of 52, 60, 76 and 82 kDa increased.

Oocytes cultured for 24 h in M199 (lane B) and those cultured for 24 h in the presence of 6-DMAP followed by 24 h in M199 + 10% FCS (48 h, lane D) exhibited similar patterns of proteins. The only major differences were a decreased intensity of bands of 50, 60 and 102 kDa and the absence of bands of 50, 74 and 200 kDa.

Cycloheximide. Representative gels are shown (Fig. 9). Comparing profiles before maturation (Fig. 9, lane A) and after maturation in M199/M199 + FCS (lanes B and C, respectively) certain bands increased in intensity (52, 54, 73, 90 and 220 kDa), some decreased in intensity (45, 57 and 82 kDa). Others bands appeared after maturation (48, 60, 72 and 105 kDa) while others disappeared (69, 78 and 108 kDa).

Comparing oocytes before maturation (0 h, lane A) and after 24 h culture in the presence of 1 μg cycloheximide ml^{-1} (lane D), the only major differences were the greater intensity of bands of 82 and 90 kDa at 24 h than at 0 h, while the band of 57 kDa decreased in intensity and that of 48 kDa appeared. No

Fig. 6. Reversibility of cycloheximide (CX)-induced meiotic inhibition in bovine oocytes. Cumulus-oocyte complexes ($n = 289$) were cultured in M199 alone or in M199 supplemented with 10% FCS or 1 μg cycloheximide ml^{-1} . After 24 h of culture, a representative number were fixed and stained to assess the nuclear status, while the remainder were washed and cultured for a further 24 h in M199 + 10% FCS, after which they were fixed and stained. Three replicates were performed. □, germinal vesicle; ▨, metaphase I; ■, metaphase II.

major differences were noted between oocytes cultured for 24 h in M199 (lane B) and those blocked for 24 h with cycloheximide and cultured for a further 24 h in M199 + FCS (lane E).

When cycloheximide was present during the period of labelling, protein biosynthesis was completely blocked (data not shown). When omitted from the labelling medium, oocytes resumed protein synthesis immediately in a manner similar to normal GV stage oocytes immediately after isolation from the follicle (Fig. 9, lane D). Oocytes cultured for 24 h in the presence of cycloheximide followed by 24 h in M199 + 10% FCS (Fig. 9, lane C) exhibited similar protein patterns to those cultured in M199 alone for 24 h (Fig. 9, lane B).

Discussion

Events before ovulation determine the ultimate fate of the oocyte. The use of defined morphological criteria in the selection of COCs has led to limited improvement in the identification of developmentally competent oocytes. It is probable that the intrafollicular environment to which the oocytes are exposed is a major cause of variability in developmental ability of oocytes (Callesen *et al.*, 1986).

There have been numerous and often conflicting reports on the inhibition of meiotic resumption in mammalian oocytes *in vitro*. In many reports the term 'reversibility' has been variously used as meaning ability to undergo GVBD after blockage

Fig. 7. Reversibility of sodium vanadate (NaVO_3)-induced meiotic inhibition in bovine oocytes. Cumulus-oocyte complexes ($n = 282$) were cultured in M199 alone or in M199 supplemented with 10% FCS, $0.1 \text{ mmol NaVO}_3 \text{ l}^{-1}$ or $0.5 \text{ mmol NaVO}_3 \text{ l}^{-1}$. After 24 h of culture, a representative number were fixed and stained to assess the nuclear status, while the remainder were washed and cultured for a further 24 h in M199 + 10% FCS, after which they were fixed and stained. Three replicates were performed. □, germinal vesicle; ▨, metaphase I; ■, metaphase II.

or ability to progress up to metaphase II. It should be emphasized that very few of these studies have reported on fertilization and developmental rates after periods of inhibition. While ability to progress to metaphase II is undoubtedly a prerequisite for further development, it should not be relied upon as the sole criterion. The term 'oocyte maturation' should be more specifically defined as the resumption and completion of the first meiotic division, subsequent progression to metaphase II and the accompanying cytoplasmic processes essential for fertilization and early embryo development. In this discussion we take reversibility to mean resumption of meiosis and progression to metaphase II at approximately normal rates.

The kinetics of IVM reported here are comparable with those reported for cattle (Sirard *et al.*, 1989; De Loos *et al.*, 1994), sheep (Moor and Crosby, 1986) and goats (Le Gal *et al.*, 1992). We have previously shown that oocytes matured in M199 alone reach metaphase II at a lower rate than those matured in the presence of FCS. Here we have shown that the difference in kinetics is already apparent by 12 h of culture, whereas in the presence of FCS, 93% of oocytes had reached metaphase I compared with 60% for M199 alone. This discrepancy between the two media was obvious for the remainder of the culture period with 84% of oocytes at metaphase II by 20 h in M199 + FCS compared with 53% in M199 alone. After

24 h culture, 18% of oocytes in M199 remained at GV compared with 1% in the presence of FCS.

Such kinetics have implications for the outcome of IVF. Various reports in recent years point to the fact that it is the fastest developing embryos *in vitro* that are most likely to be comparable with their *in vivo* counterparts. Oocytes that extrude the first polar body earlier during maturation are more likely to develop into blastocysts (Dominiko and First, 1992; Van der Westerlaken *et al.*, 1992). In addition, the earliest cleaving zygotes after IVF (within 40 h) are more likely to develop to blastocysts than are those that begin to cleave later (Loneragan *et al.*, 1992; Grisart *et al.*, 1994; Plante *et al.*, 1994).

All three potential inhibitors blocked meiotic resumption in a dose-dependent manner. However, different mechanisms are involved in each case. 6-DMAP, a puromycin analogue and inhibitor of protein kinase activity, inhibits the burst of phosphorylation that occurs just before GVBD, while cycloheximide is an established protein synthesis inhibitor (Maller *et al.*, 1977; Crosby *et al.*, 1984; Bornslaeger *et al.*, 1986; Kastrop *et al.*, 1990). Vanadate, an inhibitor of protein tyrosine phosphatases, reversibly blocks meiotic resumption in rat oocytes independently of cAMP (Goren and Dekel, 1994). At low doses of all inhibitors, the proportion of oocytes at metaphase I increased, indicating that at such doses the inhibitors were incapable of preventing GVBD but were capable of preventing the oocyte from completing meiosis.

Phosphorylations play a major role in regulating maturation. Characteristic changes in protein phosphorylation have been observed during maturation of mammalian oocytes. In all species studied so far, a large burst of phosphorylation occurs just before GVBD in conjunction with the appearance of maturation-promoting factor (MPF) activity (Maller *et al.*, 1977; Crosby *et al.*, 1984; Bornslaeger *et al.*, 1986; Moor and Crosby, 1986; Schultz, 1988; Kastrop *et al.*, 1990; Gall *et al.*, 1993). This burst of phosphorylation, inhibited by 6-DMAP, appears to be a necessary step for the reinitiation of meiosis.

The importance of protein synthesis in meiotic resumption has been shown in cattle (Hunter and Moor, 1987; Simon *et al.*, 1989; Kastrop *et al.*, 1991a, b), pig (Fulka *et al.*, 1986; Kubelka *et al.*, 1988), sheep (Moor and Crosby, 1986) and goat (Le Gal *et al.*, 1992) oocytes. New protein synthesis during the first 8 h of culture is indispensable for meiotic resumption in bovine oocytes (Kastrop *et al.*, 1991b; Tatemoto *et al.*, 1994). Similar results have been reported in goats where incubation in the presence of cycloheximide ($20 \mu\text{g ml}^{-1}$) prevented GV in all oocytes when present from 2 h from the start of culture (Le Gal *et al.*, 1992). This is in contrast to mice, where protein synthesis is not required for GVBD (Schultz and Wasserman, 1977; Motlik and Rimkevichova, 1990; Szollosi *et al.*, 1991).

In general, the pattern of protein biosynthesis observed was linked to the nuclear stage of the oocytes rather than to conditions of culture (although the latter dictated the former). 6-DMAP did not prevent the incorporation of [^{35}S]methionine into the total pool of newly synthesized proteins during maturation, in agreement with Rime *et al.* (1989). However, cycloheximide, an established protein synthesis inhibitor, completely blocked protein synthesis when present during the period of labelling. When cycloheximide was omitted from the labelling medium, blocked oocytes resumed protein synthesis in a manner similar to oocytes at the GV stage immediately

Table 1. Effect of meiotic inhibition of bovine oocytes using 6-dimethylaminopurine or cycloheximide on subsequent development

Treatment	n	Cleaved		5–8 cell		Day 6		Blastocyst yield		Day 8*	Hatching rate	
		n	(%)	n	(%)	n	(%)	n	(%)		n	(%)
24 h:												
M199	98	71	(72) ^a	42	(43) ^{ac}	22	(22) ^a	33	(34) ^a	(46) ^a	18	(55) ^a
M199 + FCS	93	74	(80) ^{ab}	52	(56) ^{ab}	34	(37) ^b	42	(45) ^a	(57) ^a	21	(50) ^a
M199 + EGF	75	65	(87) ^b	45	(60) ^b	30	(40) ^b	33	(44) ^a	(51) ^a	18	(55) ^a
48 h:												
M199 + 6-DMAP	104	80	(77) ^{ab}	34	(33) ^c	8	(8) ^c	15	(14) ^b	(19) ^b	7	(47) ^a
M199 + 6-DMAP + EGF	88	61	(69) ^a	13	(15) ^d	1	(1) ^d	2	(2) ^c	(3) ^c	0	(0) ^c
M199 + CX	105	85	(81) ^{ab}	48	(46) ^{abc}	15	(14) ^{ac}	20	(19) ^b	(24) ^b	3	(15) ^b
M199 + CX + EGF	105	84	(80) ^{ab}	48	(46) ^{abc}	15	(14) ^{ac}	19	(18) ^b	(23) ^b	8	(42) ^a

Mean of three replicates. *As a percentage of oocytes cleaved.
^{a,b,c}Values in the same column with different superscripts differ significantly (*P* < 0.05).
CX: cycloheximide, 1 µg ml⁻¹; DMAP: 6-dimethylaminopurine, 2 mmol l⁻¹; EGF: epidermal growth factor, 10 ng ml⁻¹.
The treatment with 6-DMAP or cycloheximide for 24 h was followed by a 24 h maturation in M199 + 10% FCS.
Day 6 and day 8 refer to days of culture.

Fig. 8. Effect of 6-dimethylaminopurine (6-DMAP) on protein biosynthesis in bovine oocytes. Cumulus–oocyte complexes were radiolabelled with [³⁵S]methionine for 3 h at the start of culture (lane A) or after culture for 21 h in M199 alone (lane B), M199 + 2 mmol 6-DMAP l⁻¹ (lane C) or after a primary incubation for 24 h in the presence of 6-DMAP and a subsequent 21 h culture in M199 + 10% FCS (lane D). Each lane represents ten oocytes.

after removal from the follicle, suggesting that the effect of cycloheximide was reversible even at the level of protein synthesis. The observed differences between untreated oocytes and those treated with inhibitors may go some way towards

Fig. 9. Effect of cycloheximide on protein biosynthesis in bovine oocytes. Cumulus–oocyte complexes were radiolabelled with [³⁵S]methionine for 3 h at the start of culture (lane A) or after culture for 21 h in M199 alone (lane B), M199 + 10% FCS (lane C), M199 + 1 µg cycloheximide ml⁻¹ (lane D) or a primary incubation for 24 h in the presence of cycloheximide and a subsequent 21 h culture in M199 + 10% FCS (lane E). Each lane represents ten oocytes.

explaining the differences in subsequent development to the blastocyst stage.
Both 6-DMAP- and cycloheximide-induced meiotic inhibition was fully reversible, and over 80% of blocked oocytes

progressed to metaphase II after a second 24 h culture in the absence of the inhibitor. This finding is consistent with other reports in which 6-DMAP specifically and reversibly inhibited meiosis reinitiation in starfish, sea urchin (Néant and Guerrier, 1988a, b), mouse (Rime *et al.*, 1989; Szollosi *et al.*, 1991) and cattle oocytes (Fulka *et al.*, 1991). In contrast, Gall *et al.* (1993) reported that the inhibitory effect of 6-DMAP on goat oocytes was only partially reversible, with no oocytes reaching metaphase II after blockage.

Le Gal *et al.* (1992) reported that cycloheximide inhibition of goat oocyte meiotic resumption was partly reversible; when present for 22 h, removal resulted in 85% of oocytes reaching metaphase I after 5 h. However, even after a second 24 h culture, no oocyte developed beyond metaphase I. Le Gal *et al.* (1992) also noted that GVBD was accelerated after cycloheximide removal, in agreement with observations in sheep (Osborn and Moor, 1983; Moor and Crosby, 1986), pigs (Kubelka *et al.*, 1988) and cattle (Simon *et al.*, 1989). This may be explained by the fact that chromatin condensation apparently occurs in the presence of cycloheximide, indicating that GVBD is partially engaged (pig: Kubelka *et al.*, 1988; cattle: Simon *et al.*, 1989).

In contrast to the effect of cycloheximide and 6-DMAP, the inhibitory effect of vanadate was not reversible, even at the lowest effective dose (0.1 mmol l^{-1}). The reasons for this species difference between cattle and rats are unclear. However, the mechanisms involved *in vivo* in meiotic inhibition appear to differ between rodents and larger mammals (Lévesque and Sirard, 1995). For example, in rodent oocytes, resumption of meiosis and progression can be inhibited *in vitro* by compounds that increase intracellular concentrations of cyclic adenosine monophosphate (cAMP), such as phosphodiesterase inhibitors and stimulators of the catalytic subunit of adenylate cyclase. In contrast, in bovine oocytes the effect of cAMP-related compounds is only transient (Sirard, 1990).

We have extended the results of others by demonstrating that oocytes maintained at GV by either 6-DMAP or cycloheximide remain capable of developing to the blastocyst stage, albeit at reduced rates. This has important implications since it demonstrates that at least a proportion of these oocytes retain their ability to develop to at least this stage. Having succeeded in reversibly blocking oocytes at GV, we then exposed them to putative stimuli without altering their nuclear status. EGF was chosen, since previous reports from our laboratory have shown that EGF, when present during IVM in M199, significantly increases the proportion of oocytes reaching metaphase II after 24 h as well as the proportion developing to the blastocyst stage (Lonergan *et al.*, 1996), even when prepubertal calf oocytes are used (Khatir *et al.*, 1996). In the present study these results were confirmed (Table 1). While addition of EGF to cycloheximide-inhibited oocytes was without effect, EGF overcame the effect of 6-DMAP in about 50% of oocytes (data not shown). Such oocytes lacked a visible nuclear membrane and displayed very condensed chromatin similar to that reported by Gall *et al.* (1993) in goats. This premature meiotic resumption could explain the lower development in the 6-DMAP + EGF group. This observation is perhaps not surprising as the receptor for EGF is a tyrosine kinase which, when activated, leads to rapid resumption of meiosis. 6-DMAP appears to

stimulate the tyrosine phosphorylation of P34, the catalytic subunit of MPF, thereby blocking MPF activity (Jessup *et al.*, 1991). It has been suggested that an EGF-like stimulation is involved in the post-translational modification of P34 (Lévesque and Sirard, 1995).

The use of somatic cells for meiotic inhibition would approximate more closely the situation inside the follicle. Indeed, follicular cells have been used to inhibit GVBD in bovine oocytes (Sirard and Bilodeau, 1990; Sirard *et al.*, 1992; Sirard and Coenen 1993; Kalous *et al.*, 1993; De Loos *et al.*, 1994; Kotsuji *et al.*, 1994; Richard and Sirard 1996a, b). The best results were obtained when the theca interna layer was used in association with granulosa cells in comparison with a hemisection consisting of all three follicular cell layers. The effect was shown to be mediated by the cumulus cells, since denuded oocytes failed to remain at GV stage. While in many of these studies the effect was apparently reversible in terms of oocytes reaching metaphase II, almost none has reported on the developmental rates of oocytes after the period of blockage. While such an approach to meiotic inhibition is promising and would be more physiological than the use of drugs, progress is still required to improve oocyte developmental competence. This is highlighted by Sirard *et al.* (1996), who reported that meiotic inhibition with cycloheximide is superior to that using thecal cell coculture, both in terms of cleavage rate after blockage and development to the > 32-cell stage.

In conclusion, while a certain proportion of early developmental failure *in vitro* may be attributable to suboptimal culture conditions, it is clear that germinal vesicle stage oocytes are not developmentally equivalent. Indeed, whether blastocyst yields in excess of 50% are realistically consistently achievable in practice could be questioned, given the morphological and qualitative heterogeneity of the raw material (that is, oocytes obtained from abattoirs). The results of this study show that it is possible to reversibly inhibit meiotic resumption in bovine oocytes using cycloheximide or 6-DMAP, and that over 80% of blocked oocytes reach metaphase II after removal of the inhibitory conditions and about 10–20% develop to the blastocyst stage. These results give an indication of the feasibility of *in vitro* meiotic inhibition as a tool in the study of the mechanisms involved in acquisition of competence. Improved knowledge of the mechanisms involved *in vivo* in the follicle will allow the establishment of techniques that better preserve the viability of the oocyte. Success in establishing a system of meiotic inhibition capable of reproducing the developmental rates observed in non-blocked oocytes will open up the exciting possibility of using this system on groups of less competent oocytes (for example, growing oocytes or those from prepubertal animals) in an effort to increase their developmental competence.

P. Lonergan was financed by a grant from the European Community (DG XII, grant BIO2-CT-94-7612). The authors thank O. Gerard of OGER and D. Bouttier of CIA-URCO, La Futaie, Rouillon, France for providing bull semen and to the staff at Tours abattoir for allowing access to ovaries. Thanks also to T. Fair and Y. Cognié for critically reading the manuscript and providing useful comments.

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