Male pronuclear formation and early embryonic development of hamster oocytes matured *in vitro* with gonadotrophins, amino acids and cysteamine

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Male pronuclear (MPN) formation in oocytes after *in vitro* maturation (IVM) was compared with that of matured follicular oocytes that had matured *in vivo* (controls). Cumulus–oocyte complexes were matured *in vitro* for 13 h in modified Tyrode's solution (TLP–PVA); cumulus-free oocytes were then incubated in 20% oviductal fluid for 3 h, and washed and capacitated spermatozoa were added. MPN formation was significantly lower ($P < 0.05$) in IVM oocytes 3 to 12 h after insemination (0 to 34%, respectively) than in control oocytes (range, 98–100%). Female pronuclear formation was 84–100% in controls and IVM oocytes, but spermatozoa incompletely decondensed in IVM oocytes. The addition of $10 \mu\text{mol l}^{-1}$ during IVM, significantly increased ($P < 0.05$) MPN formation (from 17% in the absence of cysteine to 47% in the presence of cysteine), but was lower than that in controls (88%). During IVM, the addition of 10% serum or gonadotrophins (FSH and LH) with or without amino acids did not support MPN formation without cysteamine, whereas the treatment with gonadotrophins and 11 amino acids plus $200 \mu\text{mol cysteamine l}^{-1}$ (82%) equalled controls (92%). Development of oocytes after IVM (in 0, 10, 20% serum) in TLP–PVA, gonadotrophins, 11 amino acids and $200 \mu\text{mol cysteamine l}^{-1}$ was compared with development in controls. Of the IVM treatments, 20% serum was inferior at fertilization, but yielded the highest percentage of fertilized oocytes developing to or beyond the four-cell stage (20% serum versus controls, respectively): fertilized oocytes, 75% versus 88%; $\geq$ four-cell embryo, 40% versus 53%; blastocyst, 8% versus 14%. It was concluded that during IVM, gonadotrophins plus 11 amino acids interacted with cysteamine, enhancing the decondensation of spermatozoa and MPN formation; oocytes matured in this medium with 20% serum were fertilized and some developed to the blastocyst stage.

Introduction

In vitro-matured (IVM) mammalian oocytes often have deficiencies in their cytoplasm, as indicated by their low frequency of male pronuclear (MPN) formation, low incidence of the first cleavage division after fertilization, and low developmental competence to blastocysts, compared with oocytes matured *in vivo* (Thibault, 1972; Thibault and Gerard, 1973; Motlik and Fulka, 1974, 1981; Rose and Bavister, 1992; Funahashi *et al.*, 1994b; Schramm and Bavister, 1994). Although IVM oocytes in numerous species will develop to offspring after transfer of eggs into foster mothers (Fleming *et al.*, 1985; Hanada *et al.*, 1986; Lu *et al.*, 1989; Yoshida *et al.*, 1993b), the mouse is the only species in which viability similar to that of oocytes matured *in vivo* has been achieved in IVM oocytes (Schroeder and Eppig, 1984). In addition, the zonae pellucidae

of IVM oocytes display deficiencies in their ability to be penetrated by capacitated spermatozoa (De Felici and Siracusa, 1982; Zhang *et al.*, 1991; Kito and Bavister, 1996a). Even though the capacity of the zona pellucida to be penetrated by spermatozoa can be acquired by changing maturation conditions, IVM oocytes often do not acquire full fertilization competence. Not only is MPN formation slow or low compared with oocytes matured *in vivo*, but also MPN and female pronuclear formation is asynchronous (Thibault, 1972; Motlik and Fulka, 1974, 1981; Leibfried and Bavister, 1983; Laurincik *et al.*, 1994). It has been suggested from these results that IVM oocytes lack a 'male pronuclear growth factor' (MPGF; Thibault, 1972).

The fertilization competence of IVM oocytes is often enhanced by adding gonadotrophins (hCG, pregnant mares' serum gonadotrophin (PMSG), FSH or LH singly, or a combination of hCG and PMSG or FSH and LH) to the maturation medium (Thibault, 1972; Shalgi *et al.*, 1979; Schroeder *et al.*, 1988; Younis *et al.*, 1989; Funahashi and Day, 1993; Schramm and Bavister, 1995). Recently, Yoshida *et al.* (1992, 1993a)

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showed that adding L-cysteine to simple maturation medium (Tyrode's solution) containing porcine follicular fluid enhances MPN formation by increasing the concentration of glutathione in pig oocytes. Glutathione is one of the important factors for decondensing spermatozoan nuclei after fusion of the spermatozoa with the egg (Perreault *et al.*, 1988; Perreault, 1990). In addition, agents that facilitate the transport of thiol groups into cytoplasm, such as mercaptoethanol or cysteamine (Issel *et al.*, 1988; Meister, 1991), enhance the fertilization and developmental competence of cow and porcine IVM oocytes (De Matos *et al.*, 1995; Grupen *et al.*, 1995).

In many studies, the contributions to maturation of various components of the complex tissue culture media used for IVM, including biological fluids such as serum or follicular fluid, have not been rigorously examined. Simple defined conditions are more useful for the systematic dissection of components specifically required for IVM (Bavister, 1995); these have been analysed only in mice, which have different requirements for oocyte maturation *in vitro* from other species (Biggers *et al.*, 1967; Bae and Foote, 1975; Kito and Bavister, 1997). More comparative data are needed to expand our knowledge of how oocyte maturation is regulated *in vitro*.

Using the hamster as a model, we examined MPN formation in oocytes matured in simple chemically defined medium. The hamster was selected to provide comparative information about requirements for IVM. Completion of nuclear maturation readily occurs in simple, defined culture medium (Leibfried and Bavister, 1983; Kito and Bavister, 1996a) and conditions for *in vitro* insemination of IVM hamster oocytes have been established (Kito and Bavister, 1996a). Subsequently, we investigated the effects of gonadotrophins (LH and FSH), amino acids, cysteamine and serum during IVM on the acquisition of oocyte competence for MPN formation. Finally, using the optimal conditions determined for MPN formation, the ability of hamster IVM oocytes to undergo fertilization, cleavage and development to the blastocyst stage was examined.

Materials and Methods

Culture media

Salts were purchased from Mallinckrodt Specialty Chemicals Co. (Paris, KY). Other chemicals were purchased from Sigma Chemical Co. (St Louis, MO), unless otherwise stated. All media were stored at 4°C without pyruvate, BSA, serum, cysteamine or amino acids for no more than one week. When necessary, glutamine, 11 amino acids, BSA or serum were added before equilibration of the media (see Table 1 for the 11 amino acids derived from hamster embryo culture medium 6 (HECM-6); McKiernan *et al.*, 1995). Pyruvate was added 1 h prior to experiments. Cysteine or cysteamine (2-mercaptoethylamine hydrochloride; Sigma Chemical Co.) was added at the beginning of oocyte maturation. The basic medium used for maturation (maturation medium) was Tyrode's solution containing 10.0 mmol lactate l⁻¹, 0.25 mmol pyruvate l⁻¹ and 0.1 mg polyvinylalcohol ml⁻¹ (TLP-PVA), as described by Bavister (1989) and Kito and Bavister (1996a, b). Maturation media were equilibrated for 8 h in 10% CO₂, 10% O₂ and 80% N₂ at 37°C under saturated

humidity. TLP-PVA containing 0.1 mmol pyruvate l⁻¹, 0.2 mmol L-glutamine l⁻¹ and 3 mg BSA ml⁻¹ (fatty acid-free Fraction V, code 82-002, lot 48, Pentex, Kankakee, IL), designated TALP-PVA, was used for capacitation of sperm (capacitation medium), and was equilibrated under 5% CO₂ in air at 37°C overnight. The sperm motility factors penicillamine (20 µmol l⁻¹), hypotaurine (100 µmol l⁻¹), and epinephrine (1 µmol l⁻¹) (that is, PHE; Bavister, 1989) were added to capacitation medium at the beginning of sperm capacitation. The medium used for cumulus removal, co-incubation with oviductal fluid and *in vitro* fertilization (fertilization medium) was TLP-PVA containing 0.2 mmol L-glutamine l⁻¹, as described by Kito and Bavister (1996a). Culture medium was equilibrated overnight under 10% CO₂, 10% O₂, 80% N₂ and high humidity at 37°C.

Collection of immature cumulus-oocyte complexes (COCs) and *in vivo* matured follicular COCs

Female hamsters bred in house were kept in a 14 h light:10 h dark cycle. Mature female hamsters (2–5 months old) were injected intraperitoneally with PMSG on the morning of the post-oestrous vaginal discharge (day 1 of the oestrous cycle). The dosage of PMSG varied between 10 and 25 iu depending on the weight of animals (Barnett and Bavister, 1992). Cumulus-oocyte complexes (COCs) with immature oocytes at the germinal vesicle (GV) stage were collected 56–58 h after PMSG injection, as described by Kito and Bavister (1996a, b). Briefly, antral follicles were punctured with a 26-gauge needle in prewarmed Hepes-buffered Tyrode's-lactate-polyvinylalcohol solution (TL-HEPES; Bavister, 1989). The liberated COCs were rinsed three times in 100 µl TLP-PVA. Only COCs with tightly attached cumulus cells were selected for IVM. The COCs from individual females were distributed in equal numbers into each treatment group. When COCs from two to three animals were matured together, controlled pooling (McKiernan *et al.*, 1991) was applied so that equal numbers of COCs from individual animals were distributed into each treatment group. COCs were matured in 100 µl TLP-PVA with various additives (see below), covered with 10 ml paraffin oil in 60 mm Petri dishes (Falcon Plastics, No. 1007, Beckton Dickinson and Company, Lincoln Park, NJ) for 13 h under 10% CO₂, 10% O₂ and 80% N₂ in saturated humidity at 37°C. Follicular COCs matured *in vivo* were used as controls, and collected from PMSG-primed animals 11.5 h after injection of hCG, as described by Kito and Bavister (1996a, b).

Collection of oviductal fluid and incubation of cumulus-free oocytes with oviductal fluid

The poor capacity for zonae pellucidae of hamster IVM oocytes and follicular oocytes matured *in vivo* to be penetrated by spermatozoa can be improved by incubating oocytes with 20% oviductal fluid for 3 h (Kito and Bavister, 1996a, b). The same procedure was applied in this study. Hamster peri-ovulatory oviductal fluid was collected as described by Kito and Bavister (1996b). Oviductal fluid was stored in 0.5 ml Eppendorf tubes at -20°C until use. COCs matured *in vitro* or *in vivo* and held in equilibrated TLP-PVA were denuded of

cumulus cells by vigorous pipetting, when cumulus expansion did not occur, or by using 1 mg bovine testis hyaluronidase ml^{-1} (type 1-S, Sigma Chemical Co.) containing 0.01 mg soybean trypsin inhibitor ml^{-1} (type 1-S, Sigma Chemical Co.), when cumulus expansion had occurred. Cumulus-free oocytes were washed four times in 100 μl equilibrated fertilization medium. Only oocytes with the first polar body (PBI) were selected for incubation with oviductal fluid. Cumulus-free oocytes were incubated at 37°C with 20% oviductal fluid in 20 μl drops of equilibrated fertilization medium for 3 h under a humidified atmosphere of 10% CO_2 , 10% O_2 and 80% N_2 (Kito and Bavister, 1996a). After incubation with oviductal fluid, oocytes were washed three times in 100 μl equilibrated fertilization medium and inseminated with capacitated spermatozoa (see below).

Preparation of spermatozoa

Collection and preincubation of epididymal spermatozoa were performed as described by Bavister (1989) and Kito and Bavister (1996b). Briefly, the distal cauda epididymal contents from mature male hamsters were expressed into paraffin oil; they were then diluted to approximately 1.0×10^6 spermatozoa ml^{-1} into capacitation medium (TALP-PVA) that contained 3 mg BSA ml^{-1} and PHE. Spermatozoa were capacitated for 3.5–4.0 h at 37°C under 5% CO_2 in air. One male was used on each experimental day.

In vitro insemination

Cumulus-free oocytes matured *in vivo* were inseminated with 1.0×10^4 capacitated spermatozoa ml^{-1} (Kito and Bavister, 1996b), and cumulus-free IVM oocytes were inseminated with 5.0×10^4 capacitated spermatozoa ml^{-1} (Kito and Bavister, 1996a) in fertilization medium (TLP-PVA containing 0.2 mmol L-glutamine l^{-1}), with PHE added just before the addition of spermatozoa. These concentrations of spermatozoa were chosen to provide equivalent penetrations of spermatozoa into eggs (Kito and Bavister, 1996a). The sperm motility factors (PHE) were added immediately before insemination with spermatozoa. Not more than 15 oocytes were inseminated in one 100 μl drop of fertilization medium. Spermatozoa and eggs were co-incubated under a humidified atmosphere of 10% CO_2 , 10% O_2 and 80% N_2 at 37°C.

Experiment 1: effects of IVM under protein-free, chemically defined conditions on subsequent MPN formation

The competence of oocytes matured in chemically defined medium (TLP-PVA) to form MPN was compared with that of oocytes matured *in vivo*. Cumulus-free oocytes matured *in vivo* or *in vitro* were divided into four groups and each group was incubated for to 3, 6, 9 or 12 h after insemination. Oocytes were rinsed by vigorous pipetting 3 h after insemination to remove adherent but non-penetrating spermatozoa. One group of oocytes was fixed at this time. The other three groups of oocytes were transferred to 100 μl drops of equilibrated HECM-6 (McKiernan *et al.*, 1995), and cultured under 10% CO_2 , 10% O_2 and 80% N_2 at 37°C for another 3, 6 or 9 h (total

of 6, 9 and 12 h after insemination, respectively). The eggs were fixed at the end of culture: eggs from all groups were mounted on glass slides, stained with aceto-orcein and evaluated using Nomarski interference or phase-contrast microscopy to score for zona penetration, extrusion of the second polar body (PBII) and MPN and female pronuclear formation, as described by Kito and Bavister (1996a, b). The stages of hamster sperm nuclear decondensation scored were as illustrated by Yanagida *et al.* (1991). Parthenogenetically activated oocytes were eliminated from the data because of their low incidence (< 3%).

Experiment 2: influence of cysteine on MPN formation of oocytes matured in chemically defined medium

To determine whether cysteine can enhance MPN formation, COCs were matured in the presence of 0, 10, 50, 100 or 500 (μmol L-cysteine l^{-1}). After IVM and co-incubation with oviductal fluid oocytes were inseminated as described above. After spermatozoa had been co-incubated with eggs at 37°C for 4 h, oocytes were fixed, stained and examined as in Expt 1.

Experiment 3: effects of gonadotrophins, serum and amino acids on MPN formation of IVM oocytes

In this experiment, amino acids, serum and gonadotrophins (FSH and LH), all of which have been shown to affect cumulus expansion, nuclear maturation and oocyte morphology in hamster COCs (Kito and Bavister, 1997), were added to maturation medium to investigate their effects on sperm penetration, MPN and female pronuclear formation and PBII extrusion of IVM oocytes. The serum used was bovine calf serum (BCS; number A-2111-D, HyClone Laboratory Inc., Logan, UT), preheated at 56°C for 30 min. Concentrations of gonadotrophins were 10 μg FSH ml^{-1} (NIDDK-oFSH-19-SIAFP, AFP-4117A, 2.4 iu μg^{-1}) and 10 μg LH ml^{-1} (NIDDK-oLH-26, AFP-5551B, 2.2 iu μg^{-1}). These concentrations and combinations were based on findings from another study (Kito and Bavister, 1997). The following maturation conditions were examined: (1) medium alone (TLP-PVA); (2) TLP-PVA + 0.2 mmol L-glutamine l^{-1} ; (3) TLP-PVA + 11 amino acids used in HECM-6 (McKiernan *et al.*, 1995); (4) TLP-PVA + 20 amino acids used in HECM-4 (McKiernan *et al.*, 1995); (5) TLP-PVA + 0.2 mmol L-glutamine l^{-1} + gonadotrophins; (6) TLP-PVA + 11 amino acids + gonadotrophins; (7) TLP-PVA + 20 amino acids + gonadotrophins; (8) TLP-PVA + 10% (v/v) BCS + gonadotrophins; and (9) *in vivo* matured oocytes (see Table 1 for the amino acids used). When 20 amino acids were added to the maturation medium, osmotic pressure was adjusted by reducing the NaCl concentration. After incubation at 37°C with oviductal fluid, cumulus-free oocytes were co-incubated with spermatozoa for 4 h, and were then fixed, stained and scored as in Expt 1.

Experiment 4: influence of cysteamine on MPN formation of hamster IVM oocytes

The effect of adding various concentrations of cysteamine to the maturation medium on MPN formation of IVM oocytes

Table 1. Composition of media used in the analysis of the effects of gonadotrophins, bovine calf serum (BCS) and amino acids on male pronuclear formation in hamster oocytes matured *in vitro* (Expt 3)

Amino acid	Concentration (mmol l ⁻¹) Treatments ^{ab}			
	1 and 8 ^{cd}	2 and 5 ^c	3 ^e and 6 ^{ce}	4 ^f and 7 ^{cf}
L-alanine	×	×	×	0.28
L-arginine	×	×	×	0.30
L-asparagine	×	×	0.01	0.50
L-aspartic acid	×	×	0.01	0.50
L-cysteine	×	×	0.01	0.01
L-glutamic acid	×	×	0.01	0.50
L-glutamine	×	0.20	0.20	0.20
Glycine	×	×	0.01	0.70
L-histidine	×	×	0.01	0.10
L-isoleucine	×	×	×	0.15
L-leucine	×	×	×	0.20
L-lysine	×	×	0.01	0.75
L-methionine	×	×	×	0.05
L-phenylalanine	×	×	×	0.30
L-proline	×	×	0.01	0.17
L-serine	×	×	0.01	0.24
Taurine	×	×	0.50	0.05
L-threonine	×	×	×	0.50
L-tryptophan	×	×	×	0.10
L-valine	×	×	×	0.20

^aAll treatments (maturation conditions) were performed in TLP-PVA (Bavister, 1989) containing 5.0 mmol glucose l⁻¹, 10 mmol lactate l⁻¹, 0.25 mmol pyruvate l⁻¹, and 0.35 mmol phosphate l⁻¹.

^bThe control, treatment 9, was *in vivo* matured ova.

^cTreatments 5, 6, 7 and 8 also contained FSH and LH (both at 10 µg ml⁻¹).

^dTreatment 8 also contained 10% BCS (v/v).

^eTreatments 3 and 6 also contained 11 amino acids used in hamster embryo culture medium 6 (HECM-6; McKiernan *et al.*, 1995).

^fTreatments 4 and 7 also contained 20 amino acids used in hamster embryo culture medium 4 (HECM-4; McKiernan *et al.*, 1995).

was examined. Oocytes were matured in the presence of 0, 25, 50, 100 or 200 µmol cysteamine l⁻¹ in TLP-PVA supplemented with 11 amino acids and gonadotrophins (10 µg FSH ml⁻¹ and 10 µg LH ml⁻¹) (see Table 1 for the 11 amino acids derived from HECM-6; McKiernan *et al.*, 1995). The spermatozoa were co-incubated with the eggs at 37°C for 4 h; eggs were then fixed, stained and scored as in Expt 1.

Experiment 5: requirements of gonadotrophins and amino acids for cysteamine-enhanced MPN formation by IVM oocytes

The influence of gonadotrophins and amino acids on cysteamine-enhanced MPN formation was examined in this experiment. Maturation conditions tested were: (1) medium (TLP-PVA) alone; (2) TLP-PVA + 200 µmol cysteamine l⁻¹; (3) TLP-PVA + 200 µmol cysteamine l⁻¹ + 0.2 mmol glutamine l⁻¹; (4) TLP-PVA + 200 µmol cysteamine l⁻¹ + 11 amino acids; (5) TLP-PVA + 200 µmol cysteamine l⁻¹ + 0.2 mmol glutamine l⁻¹ + gonadotrophins (10 µg FSH ml⁻¹ and 10 µg LH ml⁻¹); (6) TLP-PVA + 200 µmol cysteamine

l⁻¹ + 11 amino acids + gonadotrophins; and (7) *in vivo* matured oocytes (see Table 1 for the 11 amino acids derived from HECM-6; McKiernan *et al.*, 1995). Cysteamine (50 µmol l⁻¹) was added to culture medium for co-incubation with oviductal fluid, based on Expt 4. A lower concentration of cysteamine than that used for COCs was added because higher concentrations cause lysis of zonae pellucidae in cumulus-free oocytes. The spermatozoa were co-incubated with the eggs at 37°C for 4 h; oocytes were then fixed, stained and scored as in Expt 1.

Experiment 6: developmental competence of IVM oocytes, matured with and without BCS

The developmental competence of oocytes matured in optimized medium, with or without BCS, was compared with that of the control, mature follicular oocytes. The optimized IVM medium contained TLP-PVA, 10 µg FSH ml⁻¹, 10 µg LH ml⁻¹, 200 µmol cysteamine l⁻¹ and the 11 amino acids (from HECM-6; McKiernan *et al.*, 1995; see Table 1), with 0, 10 or 20% (v/v) preheated BCS. Maturation medium was equilibrated for 8 h at 37°C under 10% CO₂, 10% O₂ and 80% N₂. Gonadotrophins and amino acids were added before the medium was equilibrated, and pyruvate and cysteamine were added to the medium 1 h before the addition of COCs. COCs (10–15 per treatment drop) were matured for 13 h in 100 µl maturation medium, as described in preceding sections. Mature follicular oocytes were obtained as in preceding sections. After removal of cumulus cells, oocytes were incubated at 37°C for an additional 3 h in medium containing either BCS or oviductal fluid: oocytes matured in 10% or 20% BCS (v/v) were incubated with 10% or 20% BCS (v/v), respectively; while those matured without serum, and also the control follicular oocytes matured *in vivo*, were incubated with 20% oviductal fluid, as described in preceding sections. This modification was introduced because of the poor penetration of oocytes matured in BCS and then exposed to hamster oviductal fluid before insemination (see Expt 3). The TLP-PVA medium used for this conditioning step also contained 50 µmol cysteamine l⁻¹ and 0.2 mmol L-glutamine l⁻¹. Glutamine was added to the culture medium before equilibration. After this, all oocytes were washed four times in 100 µl of equilibrated, protein-free, TLP-PVA and inseminated with capacitated spermatozoa in fertilization medium (TLP-PVA), with PHE added just before adding spermatozoa. After co-incubating the oocytes with spermatozoa for 3 h at 37°C, oocytes were washed four times in equilibrated embryo culture medium (HECM-6; McKiernan *et al.*, 1995), while removing attached but non-penetrated spermatozoa by vigorous pipetting (Barnett and Bavister, 1992). All oocytes were cultured in 100 µl HECM-6 overlaid with paraffin oil in 60 mm Petri dishes. Embryo culture dishes had been pre-equilibrated for 8 h under 10% CO₂, 5% CO₂ and 85% N₂. Embryos were scored for the first cleavage division 24 h after insemination. Uncleaved oocytes were fixed, stained and scored for fertilization as described by Kito and Bavister (1996b). Oocytes fused with spermatozoa and those that had developed to the two-cell stage 24 h after insemination were scored as fertilized. In hamsters, oocytes that develop

to the two-cell stage are considered fertilized, because parthenogenetically activated oocytes do not undergo the first cleavage division (Yoo, 1990). Two-cell embryos that had developed 24 h after insemination were transferred into new culture drops of HECM-6 and cultured for another 72 h (total of 96 h after insemination). Embryo morphology 96 h after insemination (two-, four- or eight-cell embryos, morulae and blastocysts) was scored using a dissecting microscope. The experiment was replicated seven times.

Statistical analysis

Each experiment was replicated at least four times. All data were calculated as percentages of (1) inseminated oocytes, for zona pellucida penetration and fertilization, or (2) penetrated oocytes, for MPN and female pronuclear formation, or (3) fertilized oocytes, for cleavage development. All percentage data were transformed using arcsine transformation to control unequal variance (Tukey–Freeman transformation; Zar, 1984) and then analysed by two-way analysis of variance using the SAS program. Each experimental day (that is, each male) was designated as a block. The least significant difference (LSD) test was used for multiple comparisons, and a probability of $P < 0.05$ was considered as statistically significant.

Results

Experiment 1: effects of IVM under protein-free, chemically defined conditions on subsequent MPN formation

There were no significant differences in zona pellucida penetration (94–100%) and PBII extrusion (80–100%) between oocytes matured *in vivo* and *in vitro*, and no differences for each of these variables between the times examined (data not shown). At the times examined, almost all oocytes matured *in vivo* that were penetrated had male and female pronuclei (Fig. 1). After co-incubating the spermatozoa and eggs for 3 h, 80% of penetrated IVM oocytes had a female pronucleus, which was significantly different ($P < 0.05$) from oocytes matured *in vivo* at this time, whereas no MPN formation was observed. Six, 9 or 12 h after co-incubating the spermatozoa and eggs, the percentages of female pronuclear formation in IVM oocytes were not significantly different from those of oocytes matured *in vivo* (98–100%). In contrast, the maximum percentage of MPN formation in IVM oocytes was 34% 12 h after insemination, which was significantly lower ($P < 0.05$) than female pronuclear (100%) and MPN (100%) formation in oocytes matured *in vivo* and female pronuclear formation (100%) in IVM oocytes. When sperm nuclear transformation was examined carefully, nuclei were observed to have enlarged to some extent but had not fully decondensed even 12 h after insemination (Fig. 2a). In some cases, 12 h after insemination, a pronucleus-like structure with nucleoli was observed around the decondensed mid-region of the sperm head without decondensation of the anterior and posterior sperm head regions (Fig. 2b).

Fig. 1. Male and female pronuclear formation in cumulus-free hamster oocytes *in vivo* (□, male pronuclei; ○, female pronuclei) and *in vitro* (■, male pronuclei; ●, female pronuclei) 3, 6, 9 and 12 h after insemination with capacitated spermatozoa. Follicular oocytes matured *in vivo* were collected from ovaries 11.5 h after hCG injection, cumulus cells were removed, and oocytes were inseminated with 1.0×10^4 spermatozoa ml^{-1} after incubation with oviductal fluid for 3 h. Oocytes matured *in vitro* were matured in TLP-PVA for 13 h, cumulus cells were removed and oocytes were inseminated with 5.0×10^4 spermatozoa ml^{-1} after 3 h incubation with oviductal fluid for 3 h. Oocytes were washed 3 h after insemination, transferred into hamster embryo culture medium 6 (HECM-6) and cultured for another 3, 6 or 9 h (total of 6, 9 or 12 h after insemination, respectively). The numbers of oocytes matured *in vitro* examined were 44, 45, 41 and 43 at 3, 6, 9 and 12 h, respectively. The numbers of oocytes matured *in vivo* examined were 46, 48, 45 and 48 at 3, 6, 9 and 12 h, respectively. Significant differences between treatments were as follows:

	3 h	6 h	9 h	12 h
<i>In vitro</i> male pronuclear formation versus <i>in vivo</i> MPN	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
<i>In vitro</i> MPN versus <i>in vitro</i> female pronuclear formation versus	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
<i>In vitro</i> MPN versus <i>in vivo</i> FPN	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
<i>In vitro</i> FPN versus <i>in vivo</i> MPN	$P < 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
<i>In vitro</i> FPN versus <i>in vivo</i> FPN	$P < 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
<i>In vitro</i> FPN versus <i>in vivo</i> MPN	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$

Experiment 2: influence of cysteine on MPN formation of oocytes matured in chemically defined medium

There was a significant increase in MPN formation in oocytes matured in the presence of $10 \mu\text{mol cysteine l}^{-1}$ (47%) compared with those matured in medium alone (17%, $P < 0.05$, Table 2) 4 h after insemination. However, under these same conditions, MPN formation was still significantly lower in IVM oocytes than in oocytes matured *in vivo* (88%, $P < 0.05$). The incidence of MPN formation decreased as the concentration of cysteine increased. There was no effect of cysteine on female pronuclear formation (85–97%) nor on extrusion of the second

Fig. 2. Representative pictures (by Nomarski interference microscopy, $\times 400$) of nuclei of spermatozoa in hamster oocytes matured *in vitro*. Cumulus–oocyte complexes were matured for 13 h in TLP–PVA, then cumulus cells were removed. Cumulus-free oocytes were co-incubated with 20% oviductal fluid for 3 h and inseminated with capacitated spermatozoa. (a) Nucleus of a spermatozoon in the ooplasm 6 h after insemination. The mid-region of the head of the spermatozoon has undergone decondensation while the anterior (sa) and posterior portion (sp) of the head have not. (b) Nucleus of a spermatozoon in the ooplasm 12 h after insemination. The female pronucleus (fnp) has formed fully whereas the head of the spermatozoon has formed a pronucleus-like structure (pl) without decondensing the anterior and posterior portions of the head (arrows). Scale bars represent 10 μm .

Table 2. Effects of cysteine on the fertilization of hamster oocytes matured *in vitro* (Expt 2)

Site of maturation ^a	[Cysteine] ($\mu\text{mol l}^{-1}$)	N ^b	Number of eggs			
			Penetrated ^c	With MPN ^d	With FPN ^d	With PBII ^d
<i>In vitro</i>	0	51	72 $\pm$ 6 ^c	17 $\pm$ 8 ^{c,g}	97 $\pm$ 3	84 $\pm$ 8
<i>In vitro</i>	10	51	93 $\pm$ 3 ^{f,g}	47 $\pm$ 9 ^f	93 $\pm$ 3	93 $\pm$ 3
<i>In vitro</i>	50	56	92 $\pm$ 5 ^{f,g}	43 $\pm$ 13 ^{f,g}	97 $\pm$ 3	85 $\pm$ 3
<i>In vitro</i>	100	54	91 $\pm$ 5 ^g	40 $\pm$ 10 ^{f,g}	96 $\pm$ 2	96 $\pm$ 2
<i>In vitro</i>	500	52	100 $\pm$ 0 ^f	9 $\pm$ 5 ^e	96 $\pm$ 2	95 $\pm$ 4
<i>In vivo</i>	—	97	71 $\pm$ 3 ^{c,g}	88 $\pm$ 6 ^h	85 $\pm$ 5	80 $\pm$ 7

^a*In vitro*, 13 h of *in vitro* maturation; *in vivo*, 11.5 h after hCG injection; eggs from all sources were denuded of cumulus cells after maturation, further incubated with 20% oviductal fluid for 3 h, and then inseminated with capacitated spermatozoa (*in vitro* and *in vivo* matured oocytes were inseminated with 5.0×10^4 and 1.0×10^4 spermatozoa ml^{-1} , respectively).

^bThe total number of oocytes inseminated. (Only mature oocytes were inseminated: after 13 h of *in vitro* maturation, cumulus removal and 3 h co-incubation with oviductal fluid, oocytes with polar body I (PBI) were inseminated; 4 h later, eggs were fixed and stained for microscope observation of fertilization.)

^cValues are percentages of the total number of eggs inseminated.

^dValues are percentages of eggs penetrated.

^{e,f,g}Percentage values with different superscripts in a column are significantly different ($P < 0.05$).

Values are from a total of four replicates and are means $\pm$ SE.

Arithmetic means are shown but arcsine-transformed data were used for ANOVA.

MPN and FPN, male and female pronuclei, respectively; PBII, the second polar body.

Table 3. Effects of gonadotrophins, amino acids and bovine calf serum (BCS) on penetration and fertilization of hamster oocytes matured *in vitro* (Expt 3)

Amino acids ^a	Gonadotrophins ^b	N ^c	Number of oocytes			
			Penetrated ^d	With MPN ^e	With FPN ^e	With PBII ^e
None	—	78	77 ± 3 ^{f,g,h}	4 ± 2 ^{f,g}	70 ± 9 ^{f,g}	86 ± 4
Gln	—	62	88 ± 4 ^f	6 ± 4 ^{f,g}	70 ± 8 ^{f,g}	70 ± 5
11aa	—	51	69 ± 11 ^{f,g,h}	0 ± 0 ^{f,g}	95 ± 3 ^{h,i}	79 ± 12
20aa	—	62	79 ± 7 ^f	0 ± 0 ^g	76 ± 7 ^{f,g,h}	73 ± 7
Gln	+	82	56 ± 11 ^{g,h}	4 ± 4 ^{f,g}	85 ± 7 ^{f,g,h,i}	85 ± 7
11aa	+	80	74 ± 9 ^{f,g}	16 ± 10 ^{f,h}	84 ± 7 ^{f,h,i}	84 ± 7
20aa	+	31	52 ± 14 ^h	15 ± 10 ^h	89 ± 8 ^{f,g,h,i}	65 ± 15
BCS	+	77	21 ± 7 ⁱ	6 ± 6 ^{f,g,h}	56 ± 19 ^g	53 ± 17
Control oocytes (<i>in vivo</i>)		244	87 ± 3 ^f	92 ± 3 ⁱ	93 ± 3 ⁱ	80 ± 10

^aGln, 0.2 mmol glutamine l⁻¹; 11aa, 11 amino acids used in hamster embryo culture 6 (HECM-6; McKiernan *et al.*, 1995); 20aa, 20 amino acids used in hamster embryo culture 4 (HECM-4; McKiernan *et al.*, 1995); BCS is 10% (v/v).

^b10 µg FSH ml⁻¹ and 10 µg LH ml⁻¹.

^cThe total number of oocytes inseminated. (Only mature oocytes were inseminated: after 13 h of *in vitro* maturation, cumulus removal and 3 h co-incubation with oviductal fluid, oocytes with polar body I (PBI) were inseminated; 4 h later, eggs were fixed and stained for microscope observation of fertilization.)

^dValues are percentages of the total number of oocytes inseminated.

^eValues are percentages of penetrated oocytes.

^{f,g,h,i}Percentages with different letters within a column are significantly different ($P < 0.05$).

Values are from a total of five replicates and are means ± SE. Arithmetic means are shown but arcsine-transformed data were used for ANOVA.

MPN and FPN, male and female pronuclei, respectively; PBII, the second polar body.

polar body (PBII; 84–96%). Zona pellucida lysis occurred at high concentrations ($\geq 100 \mu\text{mol l}^{-1}$) of cysteine.

Experiment 3: effects of gonadotrophins, BCS and amino acids on MPN formation of IVM oocytes

Effects of gonadotrophins, BCS and amino acids on the penetration of zonae pellucidae and pronuclear formation were examined in this experiment (Table 3). Penetration was significantly reduced ($P < 0.05$) after IVM in the presence of BCS and gonadotrophins (21%) compared with that after IVM in medium alone (77%). MPN formation after IVM in the presence of 20 amino acids and gonadotrophins (15%) was significantly higher ($P < 0.05$) than after IVM in medium alone (4%), but not significantly different from that following IVM in the presence of gonadotrophins and either 11 amino acids (16%) or BCS (6%) (Table 3). MPN formation in oocytes matured *in vivo* (92%) was significantly higher ($P < 0.05$) than in IVM oocytes (0–16%, Table 3). In IVM oocytes, most of the head of the spermatozoa (that is, the nucleus) was incorporated into the ooplasm but the process of sperm nuclear decondensation was severely retarded. The percentage of female pronuclear formation in oocytes matured in the presence of BCS and gonadotrophins was significantly ($P < 0.05$) lower (56%) than in oocytes matured *in vivo* (93%), but was not significantly different from that of oocytes matured in medium alone (70%). There were no significant differences in PBII extrusion (53–86%) among treatment groups.

Experiment 4: influence of cysteamine on MPN formation of hamster IVM oocytes

Whether cysteamine can affect MPN formation of IVM oocytes was examined after maturation with 11 amino acids and gonadotrophins. The highest concentration of cysteamine, $200 \mu\text{mol l}^{-1}$, was selected because preliminary results showed that concentrations greater than $200 \mu\text{mol l}^{-1}$ caused zona pellucida lysis (data not shown). MPN formation was significantly ($P < 0.05$) improved in the presence of cysteamine (Table 4), and MPN formation in oocytes matured with $200 \mu\text{mol cysteamine l}^{-1}$ (82%) was not significantly different from that of *in vivo* matured oocytes (92%). There were no significant differences in female pronuclear formation (87–96%) and PBII extrusion (82–93%) among treatments (Table 4).

Experiment 5: requirements of gonadotrophins and amino acids for cysteamine-enhanced MPN formation by IVM oocytes

In this experiment, requirements for amino acids or gonadotrophins for the enhancement of MPN formation by cysteamine were investigated. Zona pellucida penetration (85–96%), female pronuclear formation (87–100%), and PBII extrusion (86–97%) were not significantly different among treatment groups (Table 5). The percentages of MPN formation in oocytes matured with 11 amino acids (35%) and with 11 amino acids and gonadotrophins (73%) were significantly higher ($P < 0.05$) than those of the other IVM oocyte treatment groups (0–4%). Although MPN formation in oocytes matured with 11 amino acids and gonadotrophins was highest among

Table 4. Effects of cysteamine on fertilization of hamster oocytes matured *in vitro* (Expt 4)

[Cysteamine] ($\mu\text{mol l}^{-1}$)	N ^a	Number of oocytes			
		Penetrated ^b	With MPN ^c	With FPN ^c	With PBII ^c
0	99	64 $\pm$ 7 ^d	19 $\pm$ 10 ^d	87 $\pm$ 6	82 $\pm$ 7
25	97	80 $\pm$ 5 ^{d,e,f}	60 $\pm$ 9 ^e	90 $\pm$ 6	89 $\pm$ 6
50	102	73 $\pm$ 5 ^{d,e}	64 $\pm$ 9 ^{e,f}	91 $\pm$ 5	87 $\pm$ 4
100	96	80 $\pm$ 5 ^{d,e,f}	60 $\pm$ 7 ^e	91 $\pm$ 2	84 $\pm$ 6
200	100	85 $\pm$ 3 ^{e,f}	82 $\pm$ 4 ^{f,g}	96 $\pm$ 2	93 $\pm$ 2
Control oocytes (<i>in vivo</i>)	307	89 $\pm$ 3 ^f	92 $\pm$ 3 ^g	97 $\pm$ 1	91 $\pm$ 2

^aThe total number of oocytes inseminated. (After 13 h of *in vitro* maturation in Tyrode's solution containing 10.0 mmol lactate l^{-1} , 0.25 mmol pyruvate l^{-1} and 0.1 μg polyvinylalcohol ml^{-1} (TLP-PVA), as well as 11 amino acids used in hamster embryo culture medium 6 (HECM-6; McKiernan *et al.*, 1995), gonadotrophins (10 μg FSH ml^{-1} and 10 μg LH ml^{-1}) and various concentrations of cysteamine, followed by cumulus removal and 3 h co-incubation with oviductal fluid, oocytes with polar body I (PBI) were inseminated; 4 h later, eggs were fixed and stained for microscope observation of fertilization.)

^bValues are percentages of the total number of oocytes inseminated.

^cValues are percentages of penetrated oocytes.

^{d,e,f,g}Percentages with different letters within a column are significantly different ($P < 0.05$).

Values are from a total of five replicates and are means $\pm$ se. Arithmetic means are shown but arcsine-transformed data were used for ANOVA.

MPN and FPN, male and female pronuclei, respectively; PBII, the second polar body.

Table 5. Effects of amino acids and gonadotrophins on cysteamine-enhanced fertilizability of hamster oocytes matured *in vitro* (Expt 5)

Amino acids ^a	Gonadotrophins ^b	Cysteamine ^c	N ^d	Number of oocytes			
				Penetrated ^e	With MPN ^f	With FPN ^f	With PBII ^f
None	—	—	100	85 $\pm$ 5	4 $\pm$ 3 ^g	90 $\pm$ 7	87 $\pm$ 8
None	—	+	95	85 $\pm$ 7	0 $\pm$ 0 ^g	87 $\pm$ 6	86 $\pm$ 5
Gln	—	+	90	88 $\pm$ 4	2 $\pm$ 1 ^g	95 $\pm$ 3	95 $\pm$ 3
11aa	—	+	93	83 $\pm$ 6	35 $\pm$ 9 ^h	93 $\pm$ 4	90 $\pm$ 5
Gln	+	+	97	88 $\pm$ 3	1 $\pm$ 1 ^g	98 $\pm$ 1	97 $\pm$ 1
11aa	+	+	99	93 $\pm$ 3	73 $\pm$ 5 ⁱ	100 $\pm$ 0	97 $\pm$ 2
Control oocytes (<i>in vivo</i>)			261	96 $\pm$ 2	95 $\pm$ 2 ⁱ	99 $\pm$ 1	97 $\pm$ 1

^aGln, 0.2 mmol glutamine l^{-1} ; 11aa, 11 amino acids used in hamster embryo culture medium 6 (HECM-6; McKiernan *et al.*, 1995).

^b10 μg FSH ml^{-1} and 10 μg LH ml^{-1} .

^cCysteamine concentration was 200 $\mu\text{mol l}^{-1}$.

^dThe total number of oocytes inseminated. (Only mature oocytes were inseminated: after 13 h of *in vitro* maturation, followed by cumulus removal and 3 h co-incubation with oviductal fluid, oocytes with polar body I (PBI) were inseminated; 4 h later, eggs were fixed and stained for microscope observation of fertilization.)

^eValues are percentages of total oocytes inseminated.

^fValues are percentages of penetrated oocytes.

^{g,h,i}Percentages with different letters within a column are significantly different ($P < 0.05$).

Values are from a total of six replicates and are means $\pm$ SEM.

MPN and FPN, male and female pronuclei, respectively; PBII, the second polar body.

IVM oocytes, it was significantly lower ($P < 0.05$) than that in oocytes matured *in vivo* (95%).

Experiment 6: developmental competence of IVM oocytes matured with and without BCS

Oocytes matured in serum-free medium as well as the control follicular oocytes matured *in vivo* were fertilized at a higher frequency (88% and 89%, respectively) than oocytes

matured in 20% BCS (75%, $P < 0.05$). Occurrence of first cleavage division of oocytes matured *in vitro* 24 h after insemination with or without BCS (47–57%) was significantly lower than that of follicular oocytes matured *in vivo* (81%, $P < 0.05$). From the four-cell stage onwards, IVM oocytes matured in 20% BCS showed numerically higher development than the other three IVM groups. For oocytes matured in the presence of either 10% or 20% BCS, development to the morula plus blastocyst stages 96 h after insemination was not

Fig. 3. Developmental competence of hamster oocytes matured *in vitro* (IVM), with or without bovine calf serum (BCS). Follicular oocytes were matured in the presence or absence of the indicated concentration of BCS (v/v). Cumulus cells were removed from oocytes matured *in vitro* and *in vivo*. Cumulus-free oocytes, either matured without serum (0%) or *in vivo*, were further incubated with 20% oviductal fluid for 3 h and then inseminated. Cumulus-free oocytes matured in the presence of serum were further incubated for 3 h with the same concentration of serum used during *in vitro* maturation and then inseminated. Co-incubation of oocytes with spermatozoa was for 3 h. Oocytes were scored for cleavage 24 h after insemination. Two-cell embryos were transferred to new drops of hamster embryo culture medium 6 (HECM-6; McKiernan *et al.*, 1995) and cultured for another 72 h. Uncleaved oocytes were fixed, stained and scored for fertilization. Stages of development: fertilized, observed at 24 h after insemination, expressed as a percentage of inseminated oocytes; two-cell embryo, observed 24 h after insemination, expressed as a percentage of fertilized oocytes 24 h after insemination; four-cell embryo and all later stages, observed 96 h after insemination expressed as a percentage of fertilized oocytes 24 h after insemination. Within developmental category, bars with no common superscript letters are significantly different ($P < 0.05$). Control oocytes were matured *in vivo*.

significantly different (9% and 24%, respectively) from that of oocytes matured in serum-free medium (12%, $P > 0.05$). Development to the morula plus blastocyst stages of follicular oocytes matured *in vivo* (42%) was significantly higher than in oocytes matured *in vitro* ($P < 0.05$). Of oocytes matured in the presence of 20% serum, 8% of the fertilized zygotes had developed to the blastocyst stage 96 h after insemination, and this percentage was not significantly different from that of the control follicular oocytes matured *in vivo* (14%, $P > 0.05$; see Fig. 3).

Discussion

Mammalian oocytes matured *in vitro* often have low competence for MPN formation (Thibault, 1972; Motlik and Fulka, 1974, 1981; Leibfried and Bavister, 1983; Laurincik *et al.*, 1994). This deficiency can be improved by adding various substances such as gonadotrophins, cysteine or cysteamine in the presence of serum or follicular fluid (Thibault, 1972;

Yoshida *et al.*, 1992; Funahashi and Day, 1993; De Matos *et al.*, 1995; Grupen *et al.*, 1995). In this study, a systematic investigation was conducted to examine the competence of MPN formation after *in vitro* insemination of hamster oocytes matured in simple, chemically defined culture medium (TLP-PVA). Once the ability to form MPN in oocytes matured in this medium had been determined, gonadotrophins, amino acids and cysteamine were added to TLP-PVA to examine specific regulators of oocyte maturation *in vitro*.

In the first experiment, we showed that MPN formation in oocytes matured in protein-free, chemically defined conditions was not only less than that of follicular oocytes matured *in vivo*, but was also asynchronous with female pronuclear formation in the IVM oocytes. This result is consistent with previous reports (Thibault, 1972; Motlik and Fulka 1974, 1981; Leibfried and Bavister, 1983; Laurincik *et al.*, 1994). In our study, only the mid-region of the sperm head was partially decondensed in the majority of spermatozoa incorporated into the ooplasm (stage 1, Yanagida *et al.*, 1991). In addition, some sperm nuclei showed unusual morphology in that an MPN-like

structure formed without full decondensation (Fig. 2b). These structures were similar to cases of abnormal MPN formation in hamster ova described by Yanagida *et al.* (1991). On the other hand, the female pronucleus had formed in almost all oocytes matured *in vitro* and *in vivo* by 6 h after insemination with spermatozoa (Fig. 1). Thus, while protein-free, chemically defined medium is sufficient for the completion of nuclear maturation (Kito and Bavister, 1996a) and for female pronuclear formation, this medium is insufficient for IVM oocytes to acquire competence for MPN formation.

The mammalian sperm nucleus contains arginine and disulfide-bond-rich proteins called protamines (Yanagimachi, 1994). For decondensation of the sperm nuclei, disulfide bonds in protamine molecules have to be reduced to -SH groups and protamines in the sperm nucleus have to be replaced with maternal histones (Perreault *et al.*, 1987; Zirkin *et al.*, 1989; Perreault, 1990). Glutathione is believed to play an important role in reducing the disulfide groups of protamines in spermatozoa (Perreault *et al.*, 1987, 1988; Yoshida, 1993; Yoshida *et al.*, 1993a; Funahashi *et al.*, 1994a). In hamster and pig oocytes, glutathione concentration is highly related to the ability of the egg to decondense sperm nuclei and to form MPN (Calvin *et al.*, 1986; Perreault *et al.*, 1987, 1988; Yoshida, 1993; Yoshida *et al.*, 1993a). Hamster oocytes matured *in vitro* have a significantly lower glutathione concentration compared with the concentration in oocytes matured *in vivo* (Perreault *et al.*, 1988), indicating that IVM oocytes may not have enough free thiol groups to decondense sperm nuclei. The abnormal MPN-like structures of IVM oocytes found in this study appeared to result from partial sperm nuclear decondensation, with only the decondensed portion able to interact with the maternal nuclear proteins to form a pronucleus. In normal fertilization in hamsters, male and female pronuclear formation in oocytes matured *in vivo* is tightly coordinated, and once sperm nuclear decondensation is complete, male and female pronuclear formation is regulated by similar mechanisms under the control of the maternal cell cycle (Wright and Longo, 1988; Longo, 1990). In hamster IVM oocytes, this process was asynchronous: female pronuclei formed in the absence of the male pronucleus, or delays occurred in sperm nuclear decondensation. Therefore, IVM conditions must be established that allow sufficient accumulation of free thiol groups (such as glutathione) needed for sperm decondensation before fertilization can be achieved in hamster IVM oocytes.

We postulated that sperm nuclear decondensation and subsequent MPN formation could be enhanced by IVM with amino acids, BCS, gonadotrophins or glutathione precursors. Cysteine, which enhances MPN formation after IVM of pig oocytes (Yoshida *et al.*, 1992), was tested in hamsters. Although MPN formation was significantly improved by the presence of 10 $\mu\text{mol cysteine l}^{-1}$, the frequency was still lower than in oocytes matured *in vivo*. One reason for low MPN formation in this series of our experiments may have been the lack of gonadotrophins or follicular fluid components used by others (Yoshida *et al.*, 1992). Another possibility is that cysteine may have been oxidized to cystine, which is not efficiently transported into the cytoplasm (Bannai, 1984; Issel *et al.*, 1988).

The effects of gonadotrophins, amino acids or BCS during IVM on MPN formation were examined. In a previous study

(Kito and Bavister, 1997) of hamster eggs that had not been inseminated, gonadotrophins (FSH and LH) inhibited nuclear maturation *in vitro* in the absence of BCS or amino acids, but were stimulatory in the presence of glutamine, 11 amino acids (from HECM-6) or BCS. MPN formation in IVM oocytes in the present study was not improved by gonadotrophins, amino acids or BCS (Table 3). The 11 amino acids (from HECM-6) and 20 amino acids (from HECM-4) contained 0.01 mmol cysteine l^{-1} and 0.01 mmol cysteine l^{-1} , respectively (see Table 1 for amino acids derived from HECM-4 and HECM-6), but in contrast to results from Expt 2, neither combination of amino acids supported MPN formation. In addition, the penetration of spermatozoa through the zona pellucida and signs of egg activation (that is, female pronuclear formation and PBII extrusion) were significantly depressed after oocyte maturation with only BCS and gonadotrophins. In our *in vitro* fertilization system, in which eggs were first co-incubated with 20% oviductal fluid and then washed and inseminated with spermatozoa, it is possible that bovine serum inhibited zonae pellucidae penetration by masking binding sites for hamster oviductal proteins on the zonae pellucidae (Boatman and Magnoni, 1995).

Because oocytes and somatic cells do not have a transport system to import glutathione directly into the cytoplasm (De Felici *et al.*, 1987; Meister, 1991), other compounds must be used to accumulate free thiol groups in the oocytes. Cysteamine and β -mercaptoethanol transport free thiol groups into the cytoplasm (Issel *et al.*, 1988; Meister, 1991), and may improve fertilization and the developmental competence of pig and cow IVM oocytes by increasing the glutathione concentration in the ooplasm (Takahashi *et al.*, 1993; De Matos *et al.*, 1995; Grupen *et al.*, 1995). When cysteamine was added to maturation medium with 11 amino acids and gonadotrophins, MPN formation in IVM hamster oocytes was significantly improved in a dose-dependent manner. Cysteamine at a concentration of 200 $\mu\text{mol l}^{-1}$ improved MPN formation in IVM oocytes to that observed in oocytes matured *in vivo*.

Cysteamine enhanced MPN formation only in oocytes matured in the presence of both 11 amino acids and gonadotrophins. Amino acids alone or glutamine and gonadotrophins alone did not support the acquisition of competence for MPN formation. Gonadotrophins alter metabolism and amino acid transport in COCs (Hillensjö *et al.*, 1975; Moor and Smith, 1979; Zuelke and Brackett, 1992, 1993). Amino acids (glutamine or 11 amino acids) in the presence of FSH and LH were required for completion of nuclear maturation, cumulus expansion and normal oocyte morphology in hamster IVM oocytes (Kito and Bavister, 1997). In our system, gonadotrophins may also have enhanced amino acid transport or changed the metabolic activity for the production of factors needed for sperm nuclear decondensation. The 11 amino acids included precursor amino acids (glycine, cysteine and glutamine) for glutathione synthesis (Meister, 1991). This may explain the higher frequency of MPN formation that occurred in this medium than was observed in the presence of glutamine alone.

Using our optimized IVM conditions (Expt 6) we have demonstrated, for the first time, that hamster oocytes matured *in vitro* are capable of completing preimplantation development to the blastocyst stage *in vitro*. The presence of BCS

during the maturation phase *in vitro* appeared to confer some developmental advantage to embryos from the four-cell stage onwards. However, neither the control follicular ova nor the IVM oocytes developed to the morula or blastocyst stages at high percentages (Fig. 3). This was consistent with the results of an earlier report from our laboratory, which showed that hamster oocytes that have either been fertilized *in vitro* or have been removed from the oviduct earlier than the afternoon following ovulation and activation by spermatozoa have poor capacity to develop to the blastocyst stage *in vitro* (Barnett and Bavister, 1992), whereas older one-cell and two-cell embryos develop to the blastocyst stage 70–90% of the time (S. H. McKiernan and B. D. Bavister, unpublished).

In summary, hamster oocytes matured in protein-free, chemically defined conditions without thiol-transporting agents did not acquire full competence for fertilization; competence for MPN formation was deficient in these IVM oocytes. In most cases, the sperm nuclei were arrested during the process of decondensation. Occasionally, the decondensed portion of the sperm nuclei formed pronucleus-like structures. Apparently, an inability to decondense the nucleus of the spermatozoon completely in the ooplasm prevented MPN formation in hamster oocytes. Cysteine, gonadotrophins or amino acids (glutamine alone or 11 amino acids formulated for HECM-6) added to maturation medium improved MPN formation but not to that of oocytes matured *in vivo*. However, when cysteamine was added to maturation medium with the 11 amino acids and gonadotrophins, the incidence of sperm decondensation and MPN formation was increased to that observed in oocytes matured *in vivo*. When this medium, optimized for MPN formation, was used for IVM in the presence of 20% BCS (v/v), many of the resultant IVM oocytes were subsequently fertilized and cleaved, forming the first reported IVM hamster blastocysts.

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