Fertilization and development capability of bovine follicular oocytes matured *in vitro* and *in vivo* and transferred to the oviducts of rabbits and cows

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Summary. Follicular oocytes were cultured for 24 h *in vitro* or obtained 6–24 h after HCG injection from cows pretreated with PMSG, and transferred to the oviducts of oestrous rabbits or heifers, inseminated with bull spermatozoa, to determine their capability for normal development.

More than 65% of oocytes cultured in fetal calf serum, equilibrated with 5% CO₂ + 5% O₂ + 90% N₂, matured to metaphase II within 24 h. Fertilization was not obtained in the rabbit oviduct but about 8% of oocytes matured *in vitro* underwent parthenogenetic cleavage. Sperm penetration was observed in 49% of oocytes in the cow oviduct, but although 47% had undergone development 96 h after transfer few developed to morulae and blastocysts, confirming the impaired ability of oocytes matured *in vitro*, as assessed by nuclear change, to develop normally.

Oocytes obtained from heifers slaughtered 24 h after HCG developed normally when transferred to the oviduct of inseminated heifers. Of the oocytes with an activated cumulus, 39% developed to blastocysts and of 16 blastocysts transferred to suitable recipients, 13 developed to normal fetuses at 13–17 weeks gestation.

Introduction

Pincus & Enzmann (1935) first reported the resumption of meiosis in oocytes liberated from the follicle and cultured *in vitro*. However, in the rat (Noyes, 1952) and rabbit (Chang, 1955a, b) it was found that fertilization and normal development could only be obtained if oocytes had reached at least the tetrad stage of the first meiotic division *in vivo* and that the proportion which developed normally was substantially increased if oocytes had undergone the second meiotic division *in vivo*. Subsequent experiments have largely confirmed these observations, although small proportions of young have been obtained from mouse oocytes matured *in vitro* (Cross & Brinster, 1970; Mukherjee, 1972). There have been few reports of assessment of the viability of oocytes obtained from the follicles of cows, although nuclear maturation of cow and calf oocytes *in vitro* has often been reported (Edwards, 1965; Foote & Thibault, 1969; Sreenan, 1970; Hunter, Lawson & Rowson, 1972; Jagiello, Miller, Ducayen & Lin, 1974; Jagiello, Ducayen, Miller, Graffeo & Fang, 1975). The *in-vivo* fertilization and cleavage of cow oocytes matured *in vitro* was achieved by Hunter *et al.* (1972) and Sreenan (1970) observed apparently normal cleavage.

The purpose of the present experiments was to examine the capability of bovine follicular oocytes matured *in vivo* and *in vitro* for fertilization and normal development in the reproductive tracts of rabbits and heifers.

Materials and Methods

The ovaries, from animals of mixed breeds, were obtained aseptically immediately after slaughter and kept in modified Dulbecco's phosphate-buffered saline (PBS: Whittingham, 1971) at room temperature.

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Culture procedures

Oocytes were aspirated from follicles of 2–5 mm diameter and the follicular contents mixed with PBS. Oocytes with degenerate cytoplasm or surrounded by degenerating cumulus with large atretic bodies were discarded and the remainder were washed twice in PBS and placed in culture medium within 1½–2 h of slaughter. Preliminary experiments had shown that heat-treated (56°C for 30 min) fetal calf serum (FCS: obtained from slaughtered 6-month fetuses) was a particularly reliable medium for obtaining nuclear maturation of cow oocytes and this medium was used in the present experiments. Oocytes were cultured at 37°C in 1–2 ml FCS, equilibrated with air, 5% CO₂ + 95% air or 5% CO₂ + 5% O₂ + 90% N₂ in small glass test-tubes sealed with silicone rubber bungs. At the end of culture, oocytes were transferred to recipient rabbits or heifers or were mechanically denuded of cumulus and corona cells, fixed in acetic-ethanol and stained with 1% Lacmoid for nuclear evaluation by phase-contrast microscopy. Nuclear maturation was assessed according to the criteria of McGaughey & Polge (1971).

Collection of oocytes matured in vivo

Donor heifers were given an i.m. injection of 2000 i.u. PMSG (Folligon: Intervet, Cambridge, U.K.) during the luteal phase of the oestrous cycle, followed 48 and 96 h later by 750 µg cloprostenol (Estrumate: I.C.I., Macclesfield, U.K.) (i.m.) and 2500 i.u. HCG (Gonadotrophon L.H.: Paines & Byrne, U.K.) (i.v.) respectively. Donors were slaughtered 6–30 h after the HCG injection. The ovaries were removed and follicles > 5 mm in diameter were dissected free and opened with fine scissors. The cumulus-surrounded oocyte was removed, washed in PBS and kept at room temperature until transfer to recipient rabbits or heifers.

Oocyte transfers

Rabbits in the follicular phase were given an i.v. injection of 50 i.u. HCG 9–12 h before transfer of oocytes. Recipient does were inseminated directly into the uterus, 0–4 h before oocyte transfer, with 0.1–0.2 ml freshly ejaculated bull semen or with washed bull spermatozoa. Control does were not inseminated. The spermatozoa were washed by centrifugation (1000 g for 3 min) of ejaculated semen, removal of the supernatant and a further two resuspensions and centrifugations in five times the volume of PBS before resuspension in a volume equal to that of the original ejaculate. Spermatozoa were examined for motility before insemination and only samples with active spermatozoa were used. Oocytes transferred to the oviducts were recovered from 12 h to 7 days later by flushing the excised oviducts and uteri with PBS.

Recipient heifers were inseminated at the onset of oestrus and again 24–30 h later at the time of transfer of oocytes to the oviducts. The transferred oocytes were recovered 18 h, 96 h or 7 days later by flushing the uteri and oviducts with PBS. To account for the natural ovulation of the recipient animal, a single normal embryo was deducted from the total obtained in the flushings from the tract ipsilateral to the ovary containing the corpus luteum.

The ova obtained from recipient does and heifers were fixed, stained and examined by phase-contrast microscopy. Ova were classified as fragmented if cleavage had occurred but nuclei were absent or grossly abnormal. Cleaved ova with apparently normal nuclei, from experiments in which recipient animals were not inseminated or where there was no evidence of sperm penetration of any oocytes within 24 h of insemination, were termed parthenogenetic (Pl. 1, Fig. 1).

Results

Experiment 1: nuclear maturation of oocytes cultured in vitro

After culture of 181 oocytes for 24 h in FCS equilibrated with air, 5% CO₂ + 95% air or 5% CO₂ + 5% O₂ + 90% N₂, 26/40 (65%), 30/50 (60%) and 66/91 (73%) matured to the metaphase II stage, respectively. The proportion of oocytes undergoing maturation to metaphase II in the three gas

Table 1. Development of cow oocytes cultured for 24 h *in vitro* and transferred to the rabbit oviduct

Insemination	Time between transfer and recovery of oocytes	No. of oocytes							
		Transferred	Recovered	Degenerate (1-cell)	Metaphase II (1-cell)	Fragmented (2-8-cell)	Nucleated		
							(1-cell)	(2-4-cell) (6-8-cell)	
No	48 h	42	37	21	6	3	5	2	0
	7 days	100	89	75	—	1	7	4	2
Yes, 0-4 h	12 h	60	44	9	34	0	1	0	0
before	24 h	107	90	44	43	1	0	2	0
transfer	48 h	209	136	57	49	7	7	15	1
	7 days	115	67	48	—	5	7	6	1

Table 2. Development of cow oocytes cultured for 24 h *in vitro* and transferred to the oviducts of inseminated heifers

Time between transfer and recovery of oocytes	No. oocytes transferred/recovered	Development of oocytes <i>in vivo</i>									
		Penetration by sperm.	No. degenerate (1-cell or fragmented)	No. at MI	No. at MII	No. at AII-TII	No. with pronuclei	No. of nucleated ova			
								1-cell	2-4-cell	5-7-cell	8-16-cell Blastocysts
18 h	53/45	No	3	7	13	0	0	0	0	0	0
		Yes	0	2	6	7	6	0	1	0	0
96 h	58/54	—*	19	0	4	0	0	4	9	9	0
168 h	131/93	—*	88	—	—	—	—	0	0	0	1

MI, metaphase I; MII, metaphase II; AII, anaphase II; TII, telophase II.

* Not classified because sperm filaments cannot be identified with confidence.

mixtures tended to be different ($\chi^2 = 6.09$; $P < 0.05$) although there was no marked advantage of any single gas mixture. Another 13/40 (33%), 13/50 (26%) and 18/91 (20%) oocytes had resumed meiosis in the respective gas mixtures but had not matured beyond telophase I. Culture of oocytes for 48 or 72 h did not increase the proportion at metaphase II (43/59, 73%; 14/26, 54%). The apparent reduction in the proportion of oocytes at metaphase II after 72 h culture may be due to the difficulties in distinguishing between metaphase I and II in ageing oocytes, as similar proportions had undergone resumption of meiosis in both groups (49/59, 83%; 20/26, 77%, respectively). After culture for 24, 48 and 72 h, a further 4, 8 and 12% of oocytes, respectively, had developed one or more nuclei but had not cleaved.

Experiment 2: oocyte transfer to rabbits

A total of 633 oocytes, cultured for 24 h in FCS equilibrated with 5% CO₂ + 5% O₂ + 90% N₂, was transferred to does inseminated with bull spermatozoa or not inseminated (Table 1). There appeared to be no effect on maturation of oocytes *in vivo* of inseminations, at 0, 2 or 4 h before oocyte transfer, with washed or unwashed bull spermatozoa or with washed spermatozoa preincubated for 1 or 4 h at 37°C. There was some reduction in the percentage of ova with spermatozoa on the zona (100, 85 and 54%) after insemination at 0, 2 and 4 h respectively before transfer of oocytes. In recipients that were not inseminated 8% of oocytes cleaved parthenogenetically. There was no evidence of sperm penetration in oocytes recovered from inseminated does, indicating that the limited development obtained was almost certainly parthenogenetic. Re-transfer of 12 2- to 4-cell ova, obtained after 48 h *in vivo*, to the ligated oviducts of follicular-phase rabbits for a further 48 h resulted in 5 ova developing to the 8-cell stage but no further.

Experiment 3: oocyte transfer to heifers

A total of 242 oocytes, cultured for 24 h in FCS equilibrated in 5% CO₂ + 5% O₂ + 90% N₂, was transferred to inseminated heifers and recovered 18 h, 96 h or 7 days later (Table 2). After 18 h *in vivo*, sperm penetration was confirmed in 22/45 oocytes. In most penetrated oocytes at metaphase I to metaphase II, there was little swelling of the sperm head (Pl. 1, Fig. 2) and in those at anaphase II to telophase II apparently normal male pronuclei and earlier stages of decondensation of the sperm head were observed (Pl. 1, Figs 3 and 4).

Thirteen 4- to 16-cell ova recovered after 96 h *in vivo* (Table 2) were re-transferred to the ligated oviduct of a follicular-phase rabbit for a further 72 h. Two blastocysts (> 80 nuclei), a retarded blastocyst (32-cells), a morula (24-cells), 2 16- to 32-celled ova and 7 degenerate 8- to 12-celled ova were obtained. The low capacity for normal development of cultured oocytes was confirmed by analysis of the 93 oocytes recovered after 7 days *in vivo* (Table 2).

EXPLANATION OF PLATE

Fig. 1. Parthenogenetic 7-cell egg with 3 of 6 apparently normal nuclei visible. Oocyte matured *in vitro* and transferred to oviduct of rabbit. Lacmoid stain, $\times 490$.

Figs 2-4. Stages of fertilization of oocytes matured *in vitro* and transferred to oviducts of inseminated heifers for 18 h. Lacmoid stain.

Fig. 2. Filament and head of penetrated spermatozoon detached within oocyte cytoplasm. No swelling of the sperm head. $\times 1580$.

Fig. 3. Decondensing head and tail of penetrated spermatozoon within oocyte cytoplasm. $\times 975$.

Fig. 4. Male and female pronuclei in oocyte penetrated by a spermatozoon. Filaments of sperm tail out of focus. $\times 975$.

Fig. 5. Day 7 blastocyst of normal appearance obtained after removal of oocyte from follicle 24 h after HCG injection and transferred to an inseminated heifer for maturation. Lacmoid stain, $\times 550$.

Table 3. Development of cow oocytes obtained 24 h after HCG injection and transferred to the oviducts of inseminated heifers for 7 days

Source of oocytes	State of cumulus	No. of oocytes		Development of oocytes <i>in vivo</i>		
		Transferred	Recovered	No. degenerate (1-8-cell)	No. retarded (16-32-cell)	No. normal (> 64-cell)
Follicular	Non-activated	17	15	13	0	2
Follicular	Activated	83	71	38	5	28
Ovulated	Absent	7	7	3	0	4

Experiment 4: in-vivo oocyte maturation and transfer to rabbits

Oocytes (27, 41 and 23) obtained 6, 24 and 30 h, respectively, after HCG injection were transferred to rabbits which had been inseminated 2 h previously with washed bull spermatozoa. After 48 h, 50 oocytes were recovered: only 3 were nucleated 2- to 4-cell stages and there was no evidence of sperm penetration.

Experiment 5: in-vivo oocyte maturation and transfer to heifers

One hundred oocytes obtained 24 h after injection of HCG and 7 recently ovulated eggs that were devoid of all but a few corona cells were transferred to the oviducts of inseminated heifers and recovered 7 days later (Table 3). Follicular oocytes in which the cumulus had not shown the characteristic dissociation of 'activated' oocytes at ovulation had a relatively low potential for normal development compared with follicular oocytes with 'activated' cumulus and with ovulated oocytes (2/15, 13%; 28/71, 39%; 4/7, 57%: respectively), although the proportions between groups did not differ significantly ($\chi^2_2 = 4.26$; $0.25 > P > 0.1$). The proportion of normal embryos recovered from 4 recipient heifers within the group to which oocytes with an 'activated' cumulus had been transferred varied considerably (4/11, 27%; 14/32, 44%; 10/14, 71%; 0/10, 0%).

After transfer and maturation of follicular oocytes *in vivo*, 16 blastocysts (Pl. 1, Fig. 5) were transferred to 8 recipient heifers that had been in oestrus 7 days earlier. Three animals had normal twin fetuses and two had normal single fetuses at autopsy 12-16 weeks after transfer. The other 3 animals have been diagnosed as pregnant by rectal palpation and 2 are carrying twin fetuses and the other a single fetus. The overall embryo survival rate compares favourably with the results of transfer of blastocysts from ovulated eggs (33/56, 59%: A. O. Trounson, A. Baker and L. E. A. Rowson, unpublished data).

Discussion

It is apparent that bull spermatozoa cannot penetrate cow oocytes in the reproductive tract of the rabbit, under the conditions employed in the present experiments. Sreenan (1970) reported that cow oocytes failed to cleave in oestrous rabbits inseminated 4-10 h before transfer, but in similar experiments with cow oocytes transferred to the oviducts of inseminated sheep apparently normal cleavage was obtained. However, the evidence in the present experiments indicated that cow oocytes matured *in vitro* readily undergo parthenogenetic development to at least the 8-cell stage. Thibault (1973) also reported parthenogenetic activation of calf oocytes cultured in follicular fluid *in vitro*. Early cleavage is clearly not a reliable indication of normal embryonic development of oocytes matured *in vitro*.

The very limited potential for normal development of oocytes matured *in vitro* and transferred to the oviducts of inseminated cows is similar to the results obtained in other species (rat: Noyes, 1952; rabbit: Chang, 1955a; mouse: Cross & Brinster, 1970; Mukherjee, 1972) and emphasizes that nuclear maturation alone is not an adequate indication of normal maturation. Thibault & Gérard (1973) postulated the absence of a male pronucleus growth factor in rabbit oocytes matured *in*

vitro because of the failure of normal formation of the male pronucleus. Various stages of decondensation of sperm heads and apparently normal male pronuclei were found in the present experiments 18 h after transfer of oocytes to heifers, suggesting that in some cow oocytes matured *in vitro* male pronucleus formation is also inhibited or delayed. However, it has now been shown that normal maturation, leading to live young, can be obtained in the rabbit (Thibault, Gérard & Menezo, 1975) and sheep (Moor & Trounson, 1977) for about 50% of oocytes cultured within the intact follicle *in vitro*. Maturation of cow oocytes by using these methods may yield better results than does culture of the liberated oocyte, but such attempts have not so far been successful (Thibault *et al.*, 1976).

As reported for other species (rat: Noyes, 1952; rabbit: Chang, 1955b; Brackett, Mills & Jeitles, 1972; hamster: Barros & Austin, 1967; pig: Leman & Dziuk, 1971; man: Edwards, 1973), cow follicular oocytes are capable of normal development after transplantation provided they have undergone at least the first meiotic division within the follicle *in vivo*. In the present experiments normal development of viable embryos was obtained when oocytes were removed from the follicle 24 h after HCG. At this time cow oocytes are at the first to second meiotic division, providing the cumulus cells have undergone the characteristic activation process (M. F. Hay & A. O. Trounson, unpublished data). It is not possible to determine why such large variations in the proportion of oocytes which develop normally were found between individual replicates of the experiment. There may have been unnoticed differences in the technique of obtaining embryos or differences between recipients or donors; the proportion of normal embryos obtained from individual cows induced to superovulate varies very considerably (Newcomb, Rowson & Trounson, 1976). Until methods are developed for the consistent maturation of cow oocytes *in vitro*, the use of oocytes matured *in vivo* by the methods described in this paper appear to be appropriate for studies requiring normal follicular oocytes.

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References

- BARROS, C. & AUSTIN, C.R. (1967) *In vitro* fertilization and the sperm acrosome reaction in the hamster. *J. exp. Zool.* **166**, 317–324.
- BRACKETT, B.G., MILLS, J.A. & JEITLES, G.G. (1972) *In vitro* fertilization of rabbit ova recovered from ovarian follicles. *Fert. Steril.* **23**, 898–909.
- CHANG, M.C. (1955a) The maturation of rabbit oocytes in culture and their maturation, activation, fertilization and subsequent development in the Fallopian tubes. *J. exp. Zool.* **128**, 379–405.
- CHANG, M.C. (1955b) Fertilization and normal development of follicular oocytes in the rabbit. *Science*, **N.Y.** **121**, 867–869.
- CROSS, P.C. & BRINSTER, R.L. (1970) *In vitro* development of mouse oocytes. *Biol. Reprod.* **3**, 298–307.
- EDWARDS, R.G. (1965) Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature, Lond.* **208**, 349–351.
- EDWARDS, R.G. (1973) Physiological aspects of human ovulation, fertilization and cleavage. *J. Reprod. Fert., Suppl.* **18**, 87–101.
- FOOTE, W.D. & THIBAUT, C. (1969) Recherches expérimentales sur la maturation *in vitro* des oocytes de truie et de veau. *Annls Biol. anim. Biochim. Biophys.* **9**, 329–349.
- HUNTER, R.H.F., LAWSON, R.A.S. & ROWSON, L.E.A. (1972) Maturation, transplantation and fertilization of ovarian oocytes in cattle. *J. Reprod. Fert.* **30**, 325–328.
- JAGIELLO, G.M., MILLER, W.A., DUCAYEN, M.B. & LIN, J.S. (1974) Chiasma frequency and disjunctional behaviour of ewe and cow oocytes matured *in vitro*. *Biol. Reprod.* **10**, 354–363.
- JAGIELLO, G., DUCAYEN, M., MILLER, W., GRAFFEO, J. & FANG, J.S. (1975) Stimulation and inhibition with LH and other hormones of female mammalian meiosis *in vitro*. *J. Reprod. Fert.* **43**, 9–22.
- LEMAN, A.D. & DZIUK, P.J. (1971) Fertilization and development of pig follicular oocytes. *J. Reprod. Fert.* **26**, 387–389.
- MCGAUGHEY, R.W. & POLGE, C. (1971) Cytogenic analysis on pig oocytes matured *in vitro*. *J. exp. Zool.* **176**, 383–396.
- MOOR, R.M. & TROUNSON, A.O. (1977) Hormonal and follicular factors affecting maturation of sheep oocytes *in vitro* and their subsequent developmental capacity. *J. Reprod. Fert.* **49**, 101–109.
- MUKHERJEE, A.B. (1972) Normal progeny from fertilization *in vitro* of mouse oocytes matured in culture and spermatozoa capacitated *in vitro*. *Nature, Lond.* **237**, 397–398.
- NEWCOMB, R., ROWSON, L.E.A. & TROUNSON, A.O. (1976) The entry of superovulated eggs into the uterus. In *Egg Transfer in Cattle*, pp. 1–15. Ed. L. E. A. Rowson. Commission of the European Communities. Luxembourg.
- NOYES, R.W. (1952) Fertilization of follicular ova. *Fert. Steril.* **3**, 1–12.

- PINCUS, G. & ENZMANN, E.V. (1935) The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. 1. The activation of ovarian eggs. *J. exp. Med.* **62**, 665–675.
- SREENAN, J. (1970) *In vitro* maturation and attempted fertilization of cattle follicular oocytes. *J. agric. Sci., Camb.* **75**, 393–396.
- THIBAUT, C. (1973) *In vitro* maturation and fertilization of rabbit and cattle oocytes. In *Regulation of Mammalian Reproduction*, pp. 231–240. Eds S. K. Crozier, P. Corfman & P. K. Condliffe. C. C. Thomas, Springfield, Illinois.
- THIBAUT, C. & GÉRARD, M. (1973) Cytoplasmic and nuclear maturation of rabbit oocytes *in vitro*. *Annls Biol. anim. Biochim. Biophys.*, Suppl. **13**, 145–156.
- THIBAUT, C., GÉRARD, M. & MENEZO, Y. (1975) Acquisition par l'ovocyte de lapine et de veau due facteur de décondensation due noyau du spermatozoïde fécondant (MPGF). *Annls Biol. anim. Biochim. Biophys.* **15**, 705–715.
- THIBAUT, C., GÉRARD, M. & MENEZO, Y. (1976) Attempts to mature calf oocytes in culture. In *Egg Transfer in Cattle*, pp. 175–184. Ed. L. E. A. Rowson. Commission of the European Communities, Luxembourg.
- WHITTINGHAM, D.G. (1971) Survival of mouse embryos after freezing and thawing. *Nature, Lond.* **233**, 125–126.

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