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The Culture of Bovine Oocytes to Obtain Developmentally Competent Embryos[1]

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ABSTRACT

Bovine cumulus-oocyte complexes (COC) (n = 4230) were used in this study to assess the effects of culture method, hormonal supplementation, and cumulus cell concentration on maturation, fertilization and development of resulting embryos. Five treatments were evaluated. 1) 10 COC/50-μl drops under oil in TCM 199 supplemented with 10% heat-treated fetal calf serum, follicle-stimulating hormone (0.5 μg/ml), luteinizing hormone (5 μg/ml), and estradiol-17β (1 μg/ml); 2) as in 1 without hormones; 3) as in 1 but in 3 ml TCM-199 in petri dishes without paraffin oil; 4) as in 2 but only 1 COC/50-μl drop; and 5) as in 1 but with denuded oocytes. After 24 h maturation, the frequencies of oocytes reaching metaphase II were 98, 84, 92, 93, and 87%, respectively, for the five treatments. In the same order, percentages of normal fertilization were 73, 70, 62, 81, and 62%, and the frequencies of embryos containing two or more blastomeres at 65 h postinsemination were 69, 82, 66, 51, and 43%. The same five treatments were used in a second study in which 3199 oocytes were fertilized, allowed to cleave in vitro to the 2- to 3-cell stage (42 h postinsemination), and transferred to oviducts of sheep (one treatment/oviduct) for 4 days. The frequencies of morulae or blastocysts obtained were 28, 18, 23, 24, and 11% for the five treatments, respectively. After nonsurgical transfer to bovine recipients (n = 8) using fresh or frozen-thawed embryos, three pregnancies past 50 days were obtained. Only one went to term with the birth of a live heifer calf.

INTRODUCTION

In vitro experimentation with gametes and embryos is now a tool rather than an objective in many species. In farm animals, progress has been moderate compared to that in laboratory animals, mainly because of limited access to material but also for reasons such as the inability to capacitate sperm and an insufficiency of high quality female gametes. With the early work of Brackett et al. (1980, 1982, 1984) and the recent work of Parrish et al. (1984, 1985, 1986a,b,c), sperm capacitation is now an easy task in cattle even if many conditions must be respected for the procedure to be successful. At present, the major limitation to the use of in vitro fertilization in this species is oocyte availability.

Most pregnancies or calves after in vitro fertilization have resulted from in vivo-matured eggs obtained by laparotomy (Brackett et al., 1982, 1984) or by laparoscopy (Sirard and Lambert, 1985, 1986; Lambert et al., 1986; Sirard et al., 1986; for a review, see: First and Parrish, 1987). However, three calves are reported from bovine oocytes matured entirely in vitro (Newcomb et al., 1978; Critser et al., 1986a; First and Parrish, 1987) with material collected randomly after slaughter. These reports demonstrate that bovine primary oocytes from smaller (immature) antral follicles, collected regardless of the stage of the donors' estrous cycles, can be matured in vitro with ensuing developmental competence. The efficiency of systems using randomly collected bovine oocytes is lower than is obtained using certain murine oocytes (Schroeder and Eppig, 1984) or ovine oocytes from gonadotropin-treated donors (Crosby et al., 1981; Staigmiller and Moor, 1984). With improvements in culture systems, a local abattoir could provide an abundant and economical source of bovine oocytes.

Recently, Leibfried-Rutledge et al. (1987), using in vitro- or in vivo-matured eggs, reported that fertilization (28% for in vitro, 57% for in vivo, follicular) and in vitro cleavage (3% and 41% for in vitro and in vivo, respectively) differed between these two groups. More importantly, no morulae or blastocysts were obtained with the in vitro group compared to 25 to

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45% of embryos obtained in the in vivo group. Subsequently, the addition of granulosa cells taken from follicles of estrous, gonadotropin-treated cows was shown to improve developmental competence of oocytes taken from 1- to 5-mm follicles and matured in vitro, resulting in 36% of the oocytes developing to morulae or blastocysts (Critser et al., 1986a). Using modifications of the granulosa coculture approach, Lu et al. (1987) and Xu et al. (1987) reported preliminary results of 73% and 38% success, respectively, in obtaining advanced stage bovine embryos after starting with randomly collected oocytes. A similar approach had been used previously in sheep by Staigmiller and Moor (1984) and they, too, reported an increase in the number of fertilized eggs reaching the blastocyst stage. Critser et al. (1986b) also published results concerning maturation of bovine oocytes without the granulosa cell component. Such a system could be combined more conveniently with other gamete technologies. However, in this case, the frequency of development to blastocyst stage was much lower: 13% in the best group.

The rationale for trying to mature oocytes without the addition of granulosa cells is to avoid the problems and expense of obtaining a readily available and standardized suspension of granulosa cells from estrous, gonadotropin-treated cows. Use of culture-maintained granulosa does not seem feasible because attachment causes possible luteinization of the granulosa cells (Staigmiller and Moor, 1984) and perhaps a change in cellular function. Therefore, this study was undertaken to assess the possibility of improving the culture of isolated cumulus-oocyte complexes (COC) without added granulosa cells while maintaining high levels of fertilization and acquisition of developmental competence. Since the addition of granulosa cells has a positive effect (Critser et al., 1986a), it is possible that the concentration of cumulus cells during incubation could affect later development as demonstrated in preliminary work by Critser et al. (1986b) in cattle and by Staigmiller and Moor (1984) in sheep. The incubation conditions were also studied to assess the importance of hormones (estradiol, follicle-stimulating hormone [FSH] and luteinizing hormone [LH] during maturation. In addition, a few transfers to the uteri of bovine recipients were made to evaluate the viability of the embryos produced.

MATERIALS AND METHODS

Oocyte Recovery

Bovine ovaries were obtained from a slaughterhouse and transported at 37°C in saline (0.9% NaCl in distilled H_2O) to the laboratory (2 h). Follicles ranging from 1 to 5 mm in diameter were aspirated using an 18-gauge needle and a vacuum pump connected to a fluid trap (50 ml) as used for laparoscopic aspiration (200 mm Hg; Sirard and Lambert, 1985). COCs were removed from follicular fluid and washed three times in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Tyrode's medium (T-H; TALP-HEPES, Bavister et al., 1983) adjusted to pH 7.3–7.4. This medium was modified as follows: bovine serum albumin (BSA) was replaced by 10% fetal calf serum (heat-treated, Gibco, Grand Island, NY; HTFCS), pyruvate was increased to 0.25 mM, and gentamycin (50 μg/ml) was also added. Only oocytes with an intact, unexpanded cumulus were used for culture (Leibfried and First, 1979). Where specified, oocytes were completely denuded of somatic cells by vortex agitation for 2 min in T-H after washing.

Oocyte Maturation

Maturation in vitro was accomplished in tissue culture Medium 199 (TCM-199) supplemented with 0.2 mM pyruvate, 10% HTFCS (one lot was selected and used after storage at −70°C) and gentamycin (50 μg/ml, Sigma Chemical Co., St. Louis, MO). Oocytes were cultured in 50-μl drops of medium under 10 ml parraffin oil in 60-mm petri dishes or in 3 ml of medium in 35-mm petri dishes. Three hormones (estradiol-17β, 1 μg/ml diluted 1:1000 from ethanol stock solution kept at −20°C; FSH, 0.5 μg/ml, National Institute of Arthritis, Metabolism and Digestive Diseases [NIAMDD]-oFSH-015; LH, 1.0 μg/ml NIAMDD-oLH-024) were added where specified. The gonadotropins were divided into aliquots, lyophilized, and stored at −20°C before use. The oocytes were incubated at 39°C under an atmosphere of 5% CO_2 and 95% air with high humidity. The maturation period lasted for 24 h, after which the oocytes not used to assess nuclear maturation were used for fertilization.

In Vitro Fertilization

The matured COCs were washed three times in glucose-free T-H as described above and transferred to fertilization medium; a modified Tyrode's medium supplemented with 0.2 mM pyruvate, 6 mg/ml fatty acid-free BSA (Sigma Chemical Co., 1 μM epinephrine, 10 μM hypotaurine (Leibfried and Bavister, 1982; Bavister et al., 1983) and 0.2 μg/ml heparin (Parrish et al., 1984). Sperm/egg incubations were in 50-μl drops of medium under 10 ml of paraffin oil (60-mm disposable petri dishes) for 18 h at 39°C in 5% CO_2 and 95% air with high humidity.

Frozen bovine semen from a pool of five bulls, donated by American Breeders Service (DeForest, WI), was packaged at 5×10^7 spermatozoa per 0.5-ml straw. The use of five bulls as a pool resulted in a number of straws sufficient to last through these studies. For each replicate, two straws were thawed in a 35°C water bath for 1 min and swim-up-separated as previously described (Parrish et al., 1986b). The final concentration of bovine sperm was 1×10^6 cells/ml and five ooctyes were used for each 50-μl drop as described by Leibfried-Rutledge et al. (1987). Motility for each sperm preparation was not determined but was generally 70-90% after swim-up separation (Parrish, 1983).

In Vivo and In Vitro Development

At the end of the fertilization period, the oocytes were washed once for 2 h in Ham's F-10 supplemented with 0.2 mM pyruvate, 50 μg/ml gentamycin and 10% HTFCS (same lot as for maturation) under 5% CO_2 atmosphere. The oocytes were then pipetted to remove most of the cumulus cells and transferred to 100-μl drops of Ham's F-10 (modified as above) under paraffin oil in groups of 25—40 per drop and incubated at 30°C (as above) for 24 h. After this in vitro incubation, all cleaved eggs were separated from the uncleaved. Portions of the cleaved eggs from each treatment were used for a cytological assessment of the cleavage stage. The remaining embryos were then transferred to the ligated oviducts of sheep under general anesthesia, (Eyestone et al., 1985), with one oviduct used for each treatment but without agar embedding. After 4 days, excised oviducts were flushed with T-H. Recovered embryos were washed once in the same medium and classified before a further incubation in Ham's F-10 (modified as above) for 24 h. During the last 6 h of incubation, 0.05 mg/ml colcemid was added (Sigma Chemical Co.), and embryos containing more than 8 cells were fixed for cytogenetic studies as described by King et al. (1979). Prior to colcemid addition, some embryos were transferred nonsurgically to synchronized Holstein heifers. A few embryos were stored frozen (Ware, 1986) and transferred nonsurgically when recipients became available. Statistical computations were made using Chi-square (χ^2) analysis (Snedecor and Cochran, 1980).

Experimental Design

Experiment 1. Five different maturation treatments were evaluated by microscopic examination for their ability to allow completion of meiosis I (24 h after beginning of incubation oocytes were denuded by vortex agitation for 3 min). Oocytes were mounted on slides with coverslip, fixed 24 h in ethanol:acetic acid (3:1) and stained with 1% aceto-orcein for examination by light microscopy at 200 × and 500 ×. The treatments were 1) 10 COCs per 50-μl drop of medium plus hormones (FSH, LH, and estradiol as described above); 2) same as 1 without hormones; 3) approximately 100 COCs in 3 ml of medium without paraffin oil but with hormones; 4) one COC per 50-μl drop without hormones; and 5) 10 denuded oocytes per 50-μl drop with hormones. The same five treatments were also evaluated for normal fertilization (fixation as above at the end of the fertilization period) and in vitro development (fixation as above 65 h after in vitro insemination). Normal fertilization was assessed by the presence of two pronuclei 18 h postinsemination. Three replicates using all five treatments simultaneously were performed to assess maturation, fertilization, and in vitro development.

Experiment 2. The treatments described in Experiment 1 were evaluated for developmental ability by surgical transfer of embryos (20—40 in an oviduct) to ligated sheep oviducts after cleavage in vitro as described. Treatment 1 was used in every sheep to compare with one of the other treatments in the contralateral oviduct. One sheep was used for each replicate of the noncontrol treatments, making a total of 12 sheep for the entire experiment.

RESULTS

The results of Experiment 1 are summarized in Table 1. The treatments with 10 COC/50-μl drop without hormones and denuded oocytes resulted in lower maturation (84% and 87%, respectively)

TABLE 1. In vitro maturation, fertilization, and development of bovine eggs using different maturation conditions.

Treatment[a]	N	Maturation Met II (%)	N	Fertilization: Unfertilized or only one pronucleus	Fertilization: Polyspermic	Fertilization: Normally fertilized (%)	N	In vitro development: Number of embryos with ≥2 nuclei (%)	In vitro development: Mean number of nuclei
1	68	67 (99)	67	13	5	49 (73)	78	69 (88)	5.36
2	67	56 (84)[b]	69	15	6	48 (70)	96	82 (85)	4.98
3	66	61 (92)	71	24	3	44 (62)	95	66 (69)[c]	4.79
4	54	50 (93)	52	5	5	42 (81)	67	51 (76)	4.65
5	54	47 (87)[b]	53	16	4	33 (62)	74	43 (58)[c]	3.70
Total	309	281	312	73	23	216	410	311	4.8

[a]Treatments (all treatments include three replicates):
1) 10 COC/50-μl drops + follicle-stimulating hormone (FSH, 0.5 μg/ml), luteinizing hormone (LH, 5 μg/ml) and estradiol-17β (E_2, 1 μg/ml).
2) 10 COC/50-μl drops, no hormone.
3) 100 COC/3-ml + FSH (0.5 μg/ml), LH (5 μg/ml) and E_2 (1 μg/ml).
4) 1 COC/50-μl drops, no hormone.
5) Naked oocytes, 10 oocytes/50-μl drops + FSH (0.5 μg/ml), LH (5 μg/ml), and E_2 (1 μg/ml).

[b]Statistically different from Treatment 1 (χ^2, $p<0.05$).

[c]Statistically different from Treatment 1 (χ^2, $p<0.01$).

compared to 10 COC/50-μl plus hormones (99%). As is also shown in Table 1, no statistical differences were observed in fertilization percentages between the five treatments, but more denuded oocytes were degenerate (15%) than in other treatment groups (3–4%, results not shown). A total of 410 oocytes were allowed to develop in vitro until 65 h after fertilization, and 311 had two or more nuclei at this time. The number of nuclei observed per embryo in each treatment is shown in Table 1. The use of denuded oocytes was consistently found to produce a lower number of developing embryos compared to the treatment with 10 COC/50-μl with hormones (χ^2, $p<0.01$). In addition, in this treatment, a form of nuclear fragmentation (22/74) difficult to distinguish from polyspermia was observed. This was not seen in other treatments. The mean number of nuclei per embryo is also shown in Table 1 and indicates similar trends in the five treatments.

Table 2 illustrates the results obtained in the second experiment in which the five treatments were evaluated for their capacity to influence developmental competence in vivo. Statistical comparisons were made of each treatment and its corresponding

TABLE 2. In vivo development of bovine embryos following in vitro maturation, fertilization, and development to the 2- to 3-cell stage.

Treatment[a]	No. oocytes	Replicates	No. of oocytes that cleaved[b] in vitro (%)	Development in vivo (transfer to the sheep): No. of embryos transferred	No. of embryos recovered (%)	Morula or blastocyst (%)	Mean number of nuclei embryo ± SD
1	1417	12	864 (61)	424	380 (90)	108 (28)	62.6 ± 22.3
2	385	3	225 (58)	130	80 (62)	14 (18)	62.9 ± 21.0
3	437	3	253 (58)	114	82 (72)	19 (23)	51.0 ± 15.0
4	410	3	253 (62)	69	54 (78)	11 (20)	56.5 ± 19.5
5	550	3	219 (40)[c]	98	79 (81)	9 (11)[c]	41.0 ± 12

[a]Treatments:
1) 10 COC/50-μl drops + follicle-stimulating hormone FSH, 0.5 μg/ml), luteinizing hormone (LH, 5 μg/ml) and estradiol-17β (E_2, 1 μg/ml).
2) 10 COC/50-μl drops, no hormone.
3) 100 COC/3 ml + FSH (0.5 μg/ml), LH (5 μg/ml) and E_2 (1 μg/ml).
4) 1 COC/50-μl drops, no hormone.
5) Denuded oocytes, 10 oocytes/50-μl drops + FSH (0.5 μg/ml), LH (5 μg/ml), and E_2 (1 μg/ml).

[b]Cleavage was evaluated with a stereoscope at 25X, 42 h after insemination.

[c]Statistically different from Treatment 1 (χ^2, $p<0.05$).

replicate with the control treatment (10 COC/50-μl plus hormones). At 42 h postinsemination, only denuded oocytes were found to be different in cleavage frequency from the control treatment. Denuded oocytes were also different from those in the control treatment (χ^2, $p<0.05$) in regard to the frequency of morulae or blastocysts obtained (11% and 28%, respectively). The number of nuclei in each advanced embryo (more than 32 nuclei) is listed in Table 2, but statistical analysis was not done due to the low number of embryos available.

Seventeen embryos were transferred nonsurgically to recipient heifers (n = 8). Each heifer received two embryos, except in one case in which three were transferred. Four animals received embryos produced from the control treatment (10 COC/50-μl with hormones), two animals received frozen-thawed embryos from the control treatment, one animal received embryos from the denuded treatment, and the last animal received embryos produced from oocytes incubated in 3 ml medium plus hormones. Three pregnancies (past Day 50) resulted, two from the control treatment (fresh embryos) and one from the 3 ml culture. The latter animal aborted a fetus at Day 150. One recipient aborted twin bull calves with bacterially caused nephritis at Day 250. All aborted calves had normal development for their gestational age. The last recipient gave birth to a normal heifer calf at Day 285.

DISCUSSION

The data from this research demonstrate that a static culture system without supplementary granulosa cells can support complete maturation of bovine oocytes. Following in vitro fertilization and development to the morula/blastocyst stage in sheep oviducts, normal pregnancies and birth can result. The results presented here, obtained by using a static granulosa-free system for maturing bovine oocytes, yield a slightly lower frequency of development (28%) than the results published from studies using granulosa cells (36%; Critser et al., 1986a), but the culture conditions have been simplified to a point where highly consistent results can be achieved (28 ± 9%, 12 replicates with 10 COC/50-μl with hormones). The results presented here are also lower than those obtained by Lu et al. (1987) or Xu et al. (1987) who utlized granulosa cocultures, but used granulosa flushed from medium-sized follicles from animals in random stages of the estrous cycle. These coculture systems also differ in using 20% blood serum from cows in estrus as protein supplement and no exogenous hormonal supplementation. Since other details of methodology are different, no explanation for differences in developmental success can be offered.

Inability to demonstrate a hormonal requirement for acquisition of developmental competence was unexpected. In the sheep, steroids and gonadotropins affect maturation, and steroid synthesis inhibitors can induce abnormal fertilization following in vitro maturation (Moor et al., 1980). It is also known that developmental capacity of sheep oocytes cultured within the follicle in vitro, or as isolated complexes, is hormone-related (Moor and Trounson, 1977; Staigmiller and Moor, 1984). Published results by Fukushima and Fukui (1985), using xenogenous fertilization in rabbit oviducts, indicated that LH, with or without estradiol or FSH, could increase fertilization frequency in cows but that the same hormones had no effect on maturation frequency. Differences in frequency of fertilization were less pronounced after in vitro fertilization when maturation was in the absence or presence of hormones (20–29%) (Fukui et al., 1983). In interpreting the results presented here, it must be recognized that the fetal calf serum often used as protein supplement does contain gonadotropins and steroids. Furthermore, the phenol red included in culture media as a pH indicator also has estrogen-like effects on pituitary cells (Hubert et al., 1986) and may exert an estrogenic effect on oocytes and embryos.

Under the experimental conditions used in this study, the number of cumulus cells per unit of volume was found to be without significant effect on later development. However, as judged by the number of nuclei per embryo, the fastest cleaving embryos are in treatments with 10 COC/50-μl with or without hormones added. According to an in vivo study by Gayerie de Abreu et al. (1984), 71% of Day 7 embryos contain 32 to 128 cells, which is similar to the results obtained here (62 nuclei per morula after 1 day of maturation, 42 h fertilization, and in vitro development followed by 96 h in the sheep oviduct and 18 h in vitro, total incubation = 180 h = 7.5 days). The limited number of available recipients prevented a further analysis of embryo quality according to the different treatments. Removal of corona and/or cumulus cells around individual oocytes before maturation did have adverse effects on

maturation and development in vitro (Table 1) and in vivo (Table 2). Therefore, a possible role for the cumulus in oocyte maturation cannot be totally discounted.

In a previous study from this laboratory, only COC in coculture with granulosa cells resulted in development to blastocysts after fertilization (Critser et al., 1986a). The contrasting success in the present experiments using isolated complexes cannot be explained. Culture conditions did differ and later results using more similar methods are comparable (Critser et al., 1986b).

The results presented in this paper raise an intriguing question. Why are only a limited number of oocytes capable (with the conditions used here) of developing to at least the morula/blastocyst stage? COCs were selected from 1- to 5-mm follicles and, according to Motlik and Fulka (1986), meiotic competence is acquired in oocytes from follicles of 0.5–3 mm in diameter. Meiotic resumption and completion of the first reduction division was not a problem in this study. It is possible that the capacity to acquire developmental competence is related to follicle maturity, as in the human (Veeck, 1984), or to stage of atresia. Choudary et al. (1968) reported that 23.7% of the antral follicles on bovine ovaries from cycling dairy cows and heifers are normal. Perhaps the maximum success possible using the present culture system has been obtained (Leibfried and First, 1979). In order to improve developmental capacity of in vitro-mated bovine oocytes, perhaps it will be necessary to narrow the range of donor follicular size, develop biochemical selection criteria for COCs (perhaps to detect early atresia), and/or mimick in vivo maturation more closely.

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