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In Vitro Fertilization of Bovine Follicular Oocytes Obtained by Laparoscopy[1]

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ABSTRACT

Bovine follicular oocytes (n = 454), obtained after laparoscopy, were used to study in vitro capacitation, fertilization, and embryo development. Capacitation was accomplished by treating bovine spermatozoa with high ionic strength medium. Maturation, fertilization, and development studies were carried out in Brackett's defined medium or in Ham's F-10. In vitro fertilization rates, ranging from 14% to 55%, were found to be influenced by individual variations among males. Brackett's defined medium was found to be superior to Ham's F-10 for oocyte maturation, fertilization, and growth, these media giving cleavage rates of 60% and 32%, respectively. Oocytes with expanded cumuli at the time of recovery cleaved at a rate of 43%, which is significantly different from oocytes recovered without granulosa cells (22%) or oocytes with compact cumuli and corona cells (5%). The in vitro development pattern of the in vitro-fertilized embryos was found to be similar to that observed in vivo. Embryos were observed at the 2-cell stage 44.5 ± 6.3 h after in vitro insemination, 4-cell after 59.0 ± 9.4 h, 8-cell after 74.8 ± 12.7 h, and 16-cell after 96.2 ± 13.9 h (observations at 12-h intervals). The procedures described here resulted in cleavage rates of up to 60% using follicular oocytes embedded in expanded cumuli cells and semen samples from selected males.

INTRODUCTION

The new technologies in farm animal reproduction are greatly responsible for the rapid genetic progress observed in the last two decades. Artificial insemination and embryo transfer have resulted in an almost complete transformation of the breeding work in the bovine (Skjervold, 1982). In vitro fertilization is a relatively new approach with numerous applications (Brackett, 1981). In 1981, the first in vitro-fertilized calf was born (Brackett et al., 1982), and although additional such calves have been born since (Brackett et al., 1984), the technology used has been considered unsuitable for field application (Brackett, 1981; Wright and Bondioli, 1981). This is mainly because of the surgical procedures used and the subsequent infertility of the oocyte donors. To overcome these inconveniences, an operative laparoscopic technique was applied that permitted follicular aspiration and allowed the same animal to be used several times (Lambert et al., 1983). Using this technology, the objectives of this study were to evaluate the fertilizability of the oocytes recovered after laparoscopy, the variations in in vitro fertilization rates when using semen samples from different males, the effect of different media (Brackett's defined medium and Ham's F-10), and the development pattern of the in vitro-fertilized bovine embryos.

MATERIALS AND METHODS

Animals

Holstein-type heifers (27) and cows (2) were used in this study. Animals were kept on pasture (May to October) or in free-stabled housing. The females were monitored twice daily for estrus using a testosterone-treated cow. The hormonal stimulation was scheduled to begin 8 to 12 days after estrus and consisted of either a 4-day decreasing follicle-stimulating hormone (FSH) treatment (FSH-P, Schering Canada, Pointe-Claire, Québec, Canada; 28 mg total in 8 injections of 5.5, 4.4, 3.3, and 2.2 mg) or a single injection of pregnant mare's serum gonadotropin (PMSG) (Equinex, Ayerst Laboratories, Montreal, Canada). Pregnant

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mare's serum gonadotropin was administered in a single injection of 2000 IU given intramuscularly. Luteolysis was induced by a cloprostenol injection (Estrumate, ICI Pharma, Mississauga, Ontario, Canada; 500 μg) 48 h after the first gonadotropin injection. Starting at 36 h after prostaglandin (PG) injection standing estrus was verified at 4-h intervals (except from 0000 h to 0800 h) using a bull with a surgically deflected penis. Animals were allowed to reach estrus or were injected with 1500 IU of luteinizing hormone (A.P.L.; Ayerst Laboratories, Montreal, Canada) 44 h after the prostaglandin injection. Holstein bulls (4) were used to provide fresh semen.

Laparoscopy

Animals were deprived of food and water 12–18 h before laparoscopic surgery. The technique for ovarian observation and follicular aspiration has been described previously (Lambert et al., 1983), and was used throughout this study. Laparoscopies were performed 24–25 h after human chorionic gonadotropin (hCG) injection or estimated estrus time in order to obtain follicular oocytes as near as possible to ovulation. Some animals were used for laparoscopy up to 12 times during a 3-yr period. A minimum of 36 days (the time of one natural estrus) was maintained between any two surgeries on the same animal.

Semen Treatment

Fresh ejaculates were obtained from 4 different bulls using an artificial vagina. Semen samples were first incubated without dilution for 2–3 h at 34 ± 1°C with penicillin-G and streptomycin (Flow Laboratories, McLean, VA) at a final concentration of 100 IU/ml and 100 μg/ml, respectively. After this incubation, 0.2 ml was diluted in 3.8 ml of high ionic strength medium (defined medium with NaCl added to provide an osmolality of ∿380 mOsm/kg) as described by Brackett et al. (1980a), incubated 5 min at 37°C, and then centrifuged for 5 min at 250 × *g* (37°C). Supernatants were removed and spermatozoa were resuspended in 0.8 ml of defined medium (described in "Oocyte Culture") for an incubation of 3–20 h under a humid atmosphere of 5% CO_2/8% O_2/87% nitrogen (3×10^7 to 9×10^7 spermatozoa/ml). Spermatozoa were introduced in the fertilization drops at a final concentration of 1×10^6 cells per ml. At the time of insemination progressive motility ranged from 25% to 75%.

Oocyte Culture

Following follicular aspiration, the oocytes and follicular fluid were kept at 34 ± 1°C for transportation to the laboratory (20–30 min), and then observed under a dissecting microscope. Oocytes and part of their surrounding cells were transferred to the maturation medium and separated into three groups: 1) compact cumuli and corona, 2) without granulosa cells (denuded), or 3) expanded cumuli (see Figs. 1–3). No further contact was permitted between these groups and oocytes were incubated in vitro for 4–6 h before insemination. Maturation and fertilization media were either defined medium (DM, described by Brackett and Oliphant, 1975) or Ham's F-10. Defined medium was prepared for each experiment by adding 3 mg of bovine serum albumin (BSA), essentially fatty acid free (Fraction V; Sigma Chemical Co., St. Louis, MO), 2.5 mg of glucose (α-D(+) glucose, grade III; Sigma), 3.1 mg of sodium bicarbonate, and 0.137 mg of anhydrous sodium pyruvate (Sigma) to each ml of an acidic saline solution (stock) prepared in advance and kept at 4°C. Ham's F-10 (Flow Laboratories) with glutamine was prepared from powder by adding sterile distilled water, sodium bicarbonate (1.2 g/l), and BSA (3 g/l). Both media were sterilized by passing through a 0.45-μm membrane (Millipore Corporation, Bedford, MA), and gassed (1 min/ml) with a mixture of 8% O_2/5% CO_2/87% nitrogen before use. Osmolality and pH were adjusted to 280–290 mOsm/kg and 7.2–7.4, respectively.

Culture medium was prepared with either DM or Ham's F-10 (both without albumin) and contained 10% (v/v) heat-inactivated bovine serum (obtained from a female heifer 48 h after superovulated estrus). Antibiotics (penicillin-G, 50 IU/ml, and streptomycin, 50 μg/ml; Flow Laboratories) were added to all media and incubation mixtures were held under paraffin oil (Saybolt viscosity 125/135; Fisher Scientific, Fair Lawn, NJ) at 37°C and 5% CO_2 in moist air.

Embryo Evaluation

The embryos were allowed to cleave and observations were made at 12-h intervals to prevent excessive light exposure. In a few instances, cytogenetic studies (King, 1984) or DAPI staining (4′,6′-diamidino-2-phenylindole; Sigma) were performed before arrest of development. DAPI at a concentration of 0.01 mg/ml (Wagner et al., 1984) was used after treating the embryos with ethanol (25% v/v for 3 min). Four embryos were observed under the electron microscope after fixation, dehydration, and embedding (Brackett et al., 1980b) to verify the absence of cortical granules.

RESULTS

In this study, 110 laparoscopies were performed and more than 99% (n = 109) resulted in the recovery of at least one oocyte. FSH-P were found to be more effective than PMSG in stimulating follicular growth. Per laparoscopy, 7.3 ± 4.0 oocytes were obtained with FSH-P and only 3.6 ± 3.3 with PMSG ($P<0.05$, Chi-square test), but the recovery rates (no. of oocytes recovered/no. of aspirations) were the same (77% and 72%, respectively). Cleavage rates of embryos obtained from donors stimulated with FSH-P or PMSG were approximately the same (47% and 39%, respectively). The oocytes of each animal were then distributed between males (first part of the experiment) or between media (second part of the experiment).

After classification of cumulus-oocyte complexes, the results show (Table 1) that 68.4% of the oocytes were enclosed in an expanded cumulus, 22.3% were surrounded by compact cumulus and corona cells, and 9.3% were without any granulosa cells. No apparent

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TABLE 1. Influence of follicular cells on in vitro fertilization.[a]

Cumulus-oocyte complexes	Number of oocytes (%)	Number of cleaved[b] ova (%)
Oocytes with expanded cumulus	311 (68.4)	129 (42)[c]
Oocytes denuded	42 (9.3)	9 (22)[d]
Oocytes with corona and/or compact cumulus	101 (22.3)	5 (5)[e]
TOTAL	454 (100)	143 (32)

[a]Combined data from male and media experiments.

[b]To any stage from 2 to 16 cells.

[c-e]Different superscripts indicate statistically different subsets ($P<0.01$, Chi-square).

morphologic alteration of the cumulus complex was observed during the short (4–6-h) maturation period. Significantly different cleavage rates were observed for these three classes of morphologically different oocytes (Table 1). Because of these large differences, only oocytes with expanded cumuli (EC) were used to evaluate other experiments. In the first part of the experiment, 201 oocytes with EC were tested in Brackett's DM to show that one of the most important factors in in vitro fertilization is the male individual variation (Table 2). The rate of fertilization ranged from 14% to 46%, depending upon the individual, and was not related to the initial motility. Furthermore, semen from bull A yielded low percentages of fertilization and none of these fertilized ova cleaved or fragmented in culture. For this individual a longer incubation period was tried, but this did not improve the fertilization rate.

In the second part of the experiment, 181 oocytes with EC were used with the treated semen of bull C to determine whether the fertilization rates could be improved when Ham's F-10 is used in comparison with Brackett's defined medium for oocyte maturation, fertilization, and development. As shown in Table 3, DM is significantly more efficient ($P<0.05$) than Ham's F-10. Cleavage rates of 60% and 32% were obtained with DM and Ham's F-10, respectively, when used for all the steps involved.

Using the best experimental conditions (male C, defined medium, and oocytes in expanded cumuli), 6 oocytes out of 10 reached the 2-cell stage. The results of the in vitro development pattern of in vitro-fertilized embryos (Table 4) show that 58% of the 2-cell embryos reached the 4-cell stage, 35% of the 4-cell embryos reached the 8-cell stage, and 19% of the 8-cell embryos reached the 16-cell stage (see Plate I). Cytogenetic studies were performed on a few developing embryos and showed a number of nuclei equal to the number of cells (see Plate II). DNA fluorescent staining was also used, and all fluorescent embryos examined under ultraviolet light had a number of nuclei identical

TABLE 2. The effect of individual bull variation on in vitro fertilization.[d]

Bull of	No. exp.	Duration (h) of sperm incubation[a]	Number of oocytes with expanded cumulus (100%)	Number of oocytes fertilized (%)	Number of cleaved ova (%)
A	14	6–20	71	10 (14)[b]	0 (0)
C	16	5–10	44	14 (32)	14 (32)
D	23	7–13	65	30 (46)[c]	26 (40)
E	7	3–13	21	6 (29)	6 (29)

[a]Following high ionic strength defined medium treatment (380 mOsm/kg to 400 mOsm/kg).

[b,c]Different superscripts indicate statistically different subsets (Chi-square, $P<0.05$).

[d]Only DM medium was used in these experiments.

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TABLE 3. Bovine in vitro fertilization[a] using two different media.[b]

No. exp.	Maturation medium	Fertilization medium	Growth medium	No. of oocytes with expanded cumulus (A)	No. of oocytes fertilized	No. of embryos (2-cell) (B)	B/A ratio
16	DM	DM	DM-S[d]	62	41	37	0.60[e]
9	DM	DM	Ham's F-10-S[d]	24	12	11	0.46
10	DM	Ham's F-10	Ham's F-10-S	25	15	12	0.48
11	Ham's F-10[c]	Ham's F-10	Ham's F-10-S	25	11	8	0.32[f]
11	Ham's F-10	Ham's F-10	DM-S	25	10	7	0.28[f]
14	Ham's F-10	DM	DM-S	20	8	8	0.40

[a]Only bull C was used for this experiment.

[b]In all experiments two or more combinations were present.

[c]Supplemented with bovine serum albumin (3 g/l).

[d]Supplemented with 10% (v/v) serum from a superovulated female 2 days after estrus.

[e,f]Different superscripts indicate statistically different subsets (Chi-square, $P<0.05$).

to the number of cells (1- to 8-cell embryos). Electron microscopy (Plate II) showed the absence of cortical granules in blastomeres from 4-cell embryos.

DISCUSSION

The results demonstrate that laparoscopy is a very efficient way to obtain mature follicular oocytes for in vitro fertilization. Sixty-eight percent of the recovered oocytes are surrounded by expanded cumulus cells, which is a sign of maturity (Zamboni, 1971; Phillips, 1982). Of these, about 60% were fertilized and reached the 2-cell stage. Brackett et al. (1982) reported a 20% fertilization rate with follicular oocytes, but these oocytes were obtained by surgery under general anesthesia and neither male nor oocyte selection was made. Most of the results published concerning in vitro fertilization are shown as penetration rates after in vitro maturation (Sreenan, 1970; Iritani and Niwa, 1977; Iritani, 1980; Ball et al., 1983; Fukui et al., 1983; Sugawara et al., 1984). In this case, the oocytes were allowed to mature in vivo and, as in the human, they were then incubated in vitro for 4–6 h to permit final maturation (Trounson et al., 1982). The results presented here are in agreement with the theory that the last hours before ovulation are very important for the final maturation process (Donahue, 1971; Thibault, 1977: Dieleman et al., 1983; Ireland and Roche, 1983) and could explain our high cleavage rates.

The other barrier in bovine in vitro fertilization is the capacitation process. High ionic strength medium for bovine semen treatment has been found to be fairly successful (Brackett et al., 1982; Fukui et al., 1983; Westhusin et al., 1984), but male individual variations are

TABLE 4. In vitro development pattern of in vitro-fertilized embryos.

No. embryos	Developmental stage[a]	H postinsemination (mean)	Population standard deviation	Earliest time postinsemination	Time between divisions (h)
129	2-cell	44.5	± 6.3	33	–
75	4-cell	59.0	± 9.4	38	14.5
26	8-cell	74.8	± 12.7	59	15.8
5	16-cell	96.2	± 13.9	86	21.4

[a]Observations were made every 12 h beginning 24 h after in vitro insemination.

[b]Only bulls C, D, and E and combined data from DM and Ham's F-10 were analyzed.

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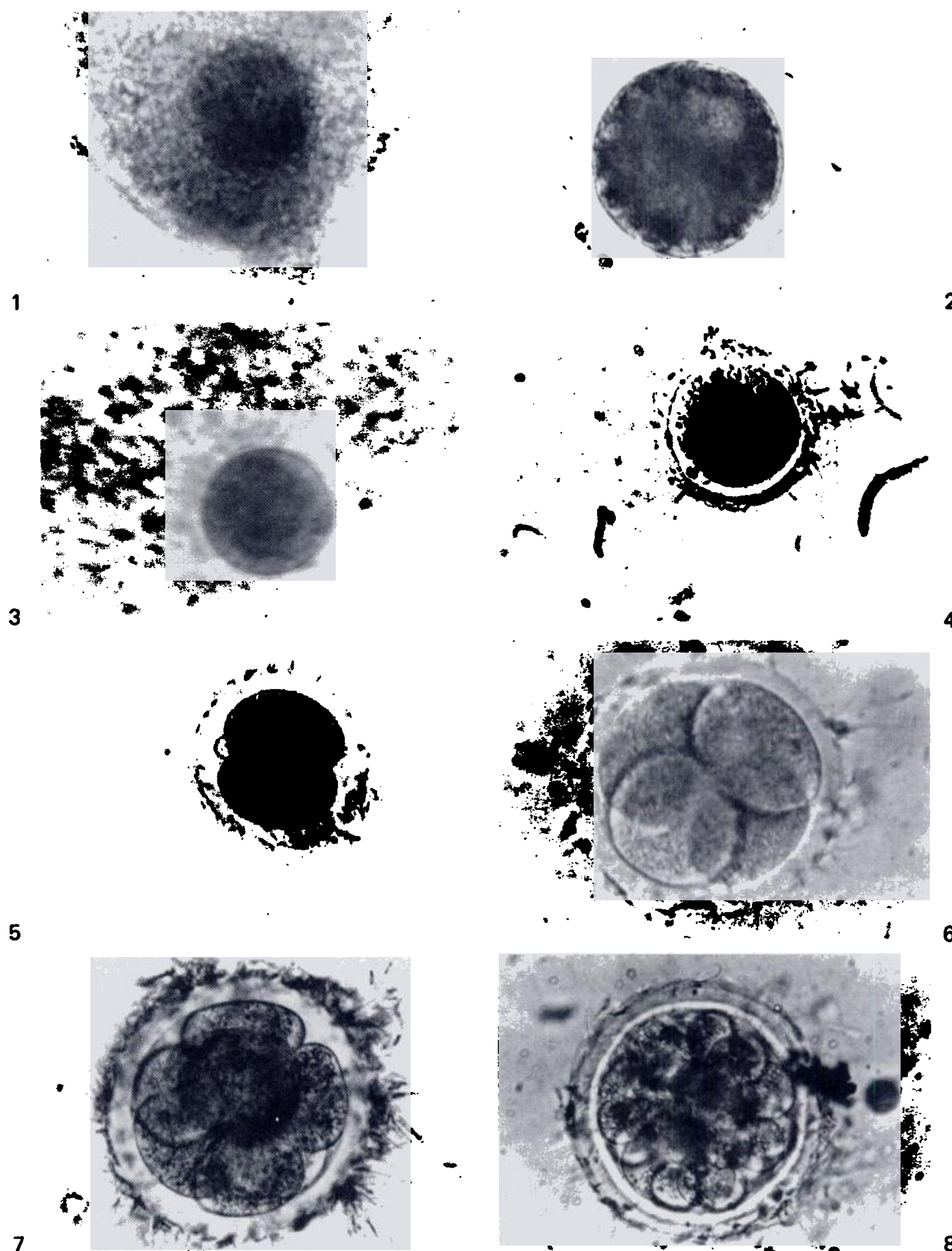

PLATE I
Fig. 1. Bovine oocyte with corona cells and compact cumulus cells (X250).
Fig. 2. Bovine oocyte without any cells as recovered after laparoscopy (X400).
Fig. 3. Bovine oocyte embedded in expanded cumulus preventing complete focusing (X250).
Fig. 4. Bovine oocytes with 2 polar bodies (*arrow*) immediately after the fertilization period. (X250).
Fig. 5. Two-cell bovine embryo, 38 h after in vitro insemination (X250).
Fig. 6. Four-cell bovine embryo, 45 h after in vitro insemination (X400).
Fig. 7. Eight-cell bovine embryo, 65 h after in vitro insemination (X400).
Fig. 8. Bovine morula (about 20 cells), 111 h after in vitro insemination (X400).

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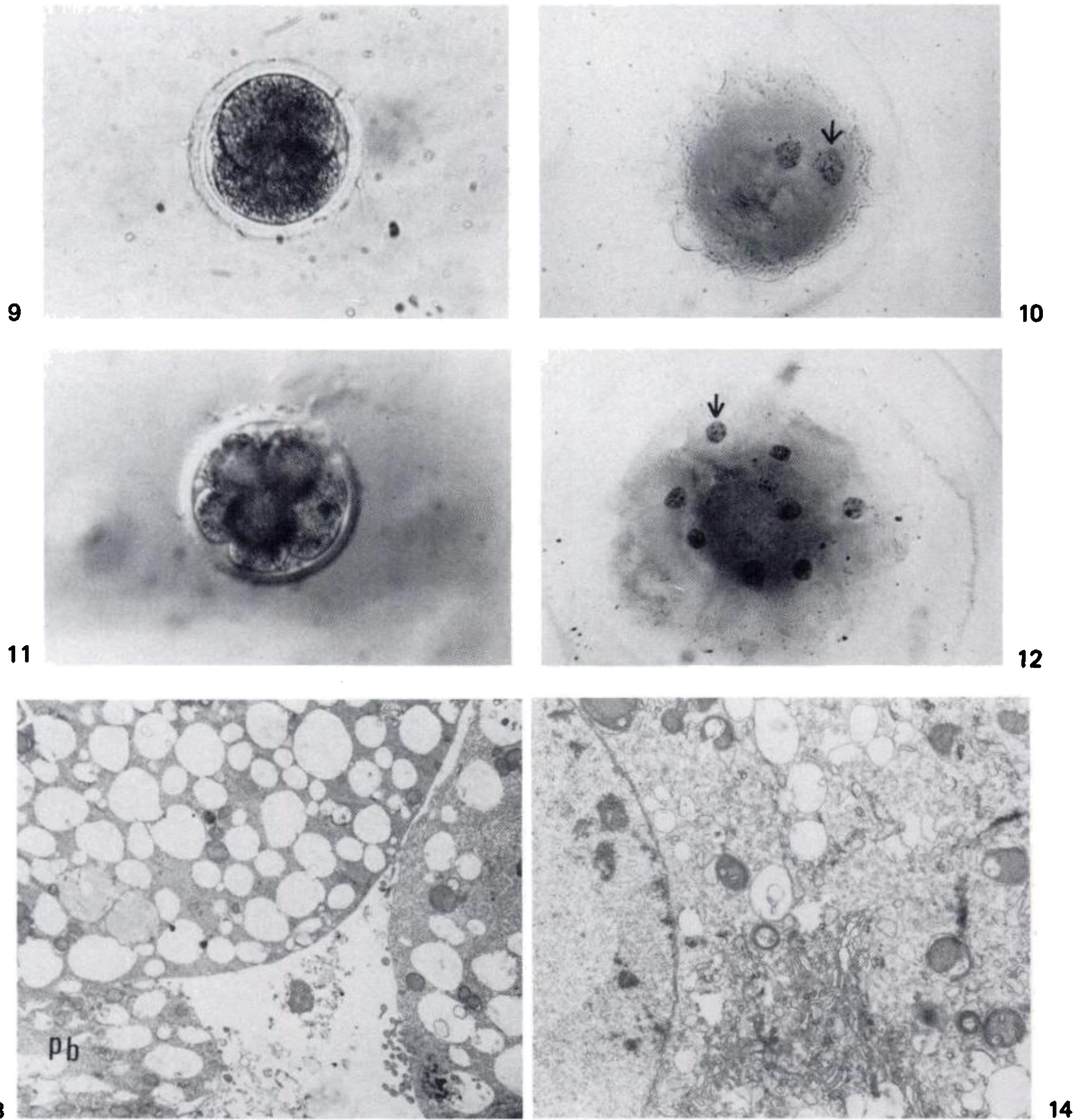

PLATE II

Fig. 9, 10. Bovine embryo at the 2-cell stage showing two nuclei (*arrow*) after fixation by the air-drying technique and staining with Giemsa 10% (X250).

Fig. 11, 12. Bovine embryo at the 8-cell stage showing eight nuclei (*arrow*) after fixation and Giemsa staining (10%) (X250).

Fig. 13. Electron micrograph of a 4-cell bovine embryo showing the absence of cortical granules and the remnant of one polar body (*Pb*) (X1500).

Fig. 14. Electron micrograph of the same embryo as in Fig. 12 showing parts of nucleus, Golgi apparatus, mitochondria, and numerous lipid enclosures (X5000).

quite important with this procedure (Brackett et al., 1982; Lambert et al., 1984; Sirard et al., 1984). The differences in cleavage rate observed for bull C in Tables 2 and 3 could be explained by the age of this bull [15–20 mo and 20–30 mo for the first part of the experiment (Table 2) and the second part (Table 3), respectively]. In this study, the cleavage rates were not statistically related to other measured factors such as motility, spermatozoa concentration

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(during incubation), or incubation length. How hypertonic medium induces complete or partial capacitation is not clear. However, it is known that the procedure removes sperm-bound proteins in the rabbit (Brackett and Oliphant, 1975). Recently, Cooper (1984) showed that a high ionic strength medium could promote hyperactivated motility in the mouse. In addition, Hyne et al. (1984) demonstrated that an increase in intracellular sodium has a functional role in acquisition of guinea pig sperm fertilizing ability. However, to date no specific data are available to explain the high sodium concentration effect of the medium on bovine spermatozoa.

Bovine in vitro development (Wright et al., 1976) and human in vitro fertilization have been accomplished in Ham's F-10 medium (Lopata et al., 1980). This medium is biochemically more complete than Brackett's defined medium and was thought to be more efficient for embryo culture. However, the results presented here show that Brackett's defined medium seems to be a more suitable medium for the bovine under the conditions used. In addition, the constitutents of the growth medium are very important. For example, a serum batch used for 35 experiments allowed 19 of 42 4-cell-stage embryos to reach the 8-cell stage. A different serum prepared under the same conditions, but from a different female, allowed only 2 of 20 embryos at the 4-cell stage to reach the 8-cell stage, even if the 2-cell stage was comparable. The cells of an adult female bovine produce and utilize metabolic products in a different manner than cells from humans or laboratory animals. It is therefore possible that culture media or coculture systems especially for the bovine embryo will have to be investigated if development is to be extended. The other factor to consider is the normality of the in vitro fertilized embryo. One way of estimating it is to assess the pattern of development, which in our study is similar to that observed in in vivo (Hamilton and Laing, 1946; Thibault, 1966, 1967) and in vitro studies (McGaugh et al., 1974; Wright et al., 1976). For the purpose of this evaluation, sperm penetration was considered to occur at the middle of the fertilization period. Cytogenetic studies, DNA staining, and electron microscopy are other means by which embryos can be evaluated. However, with these techniques no abnormality was found until cleavage had stopped. In the near future the fertilization process and the growth medium effect will be studied by transferring in vitro-fertilized embryos to the oviduct or the uterus of synchronized recipients.

In summary, the results presented here show that relatively high in vitro fertilization and cleavage rates could be obtained from bovine follicular oocytes collected by laparoscopy. Essentially, two conditions must be strictly followed: first, the follicular oocytes must be collected as near to ovulation as possible and oocytes in expanded cumuli must be used; and second, males must be selected for their semen responses to the capacitation procedure.

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