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Fertilization and Early Development of Cow Ova

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ABSTRACT

Cow ova and embryos were recovered in 1–16-cell stages and studied by light and electron microscopy. Events associated with normal fertilization and early development in other species were documented by observations of acrosome-reacted sperm cells embedded in the matrix of the zona pellucida, presence of sperm remnants within ooplasm and disappearance of cortical granules, appearance of centrioles at the 8-cell stage, and changes in mitochondria with development. Few sperm cells, apparently limited in penetration to one-third the thickness of the zona, along with complete ovum penetration by only one sperm cell suggested a strong zona block to polyspermy. Sperm remnants were occasionally found in blastomeric cytoplasm of 2-cell stage ova. Prominent granules were seen in mitochondria of bovine ova before and after fertilization.

Efforts to achieve fertilization in vitro by combining ova, recovered near the expected time of ovulation from follicles or oviducts, with bull sperm treated with high ionic strength medium resulted in sperm penetration and development to 2- and 4-cell stage embryos judged normal by light and electron microscopy. Use of a different bull was associated with aberrant ovum activation with retention of cortical granules, thus emphasizing a need for further definition of conditions compatible with bovine fertilization.

INTRODUCTION

Cow ova and embryos have received sporadic attention since initial observations reported by Hartman et al. (1931). A better understanding of early embryonic development has followed clinical application of embryo transfer in cattle production during recent years (Betteridge, 1977). Although the chronological development of cow fertilization and cleavage is well known (Hamilton and Laing, 1946; Thibault, 1966, 1967), attention to morphological features has been limited. Blastocysts have been examined in the scanning electron microscope (Flechon and Renard, 1978) and transmission electron microscopy has been applied in studies of follicular oocytes (Senger and Saacke, 1970; Fleming and Saacke, 1972). Among the large domestic species, ultrastructural aspects of ova, fertilization and early development have been reported for the pig (Norberg, 1973a,b; Szollosi and Hunter, 1973, 1978) and sheep (Calarco and McLaren, 1976; Russe, 1975; Witenberger-Torres and Flechon, 1974). Progress has been made in efforts toward obtaining cow oocyte maturation in vitro (Edwards, 1965; Hunter et al., 1972; Jagiello et al., 1974; Iritani and Niwa, 1977; Leibfried and First, 1979; Newcomb et al., 1978; Shea et al., 1976; Sreenan, 1970; Satoh et al., 1977; Thibault et al., 1975, 1976; Trounsen et al., 1977). Only a few preliminary reports of in vitro fertilization of cow ova appear in the literature, including earlier reports from this laboratory (Brackett et al., 1977, 1978; Edwards, 1973; Iritani and Niwa, 1977; VonBregulla et al., 1974).

The purpose of work reported here was to investigate morphological and physiological aspects of bovine fertilization and early development and to initiate experiments toward the

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goal of developing a repeatable method for in vitro fertilization.

MATERIALS AND METHODS

Animals

Sixteen young cows of Angus (A), Hereford (H), Holstein (Hol), Jersey (J), and mixed breeds, including Angus × Guernsey (A×G) and Angus × Holstein (A×Hol), were observed for regular, approximately 21-day, estrus cycles prior to use. Estrus behavior was detected during approximately 30-min observation periods early in the morning, near mid-day and in the late afternoon or evening. When indicated by observations of nervousness or attempts to ride other cows late in the day, an additional observation for estrus, i.e., standing heat, was made near midnight. Several cows were used repeatedly in this study. Two young bulls, one Jersey and one Holstein, were used to supply fresh semen in this work.

Ovulation Induction and Artificial Breeding

Ova were ovulated in a normal cycle or following superovulation in response to treatments with follicle stimulating hormone and luteinizing hormone (FSH, "FSH-P" and LH, "PLH," respectively, Armour-Baldwin, Omaha, NE) or pregnant mare serum gonadotropin (PMS, "Gestyl," N.V. Organon, Oss, Holland) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, "Prostin," Upjohn Co., Kalamazoo, MI) as described by Elsden et al. (1976). The procedure adopted for superovulation in this work was initiated on cycle Days 9 to 11 with a single i.m. injection of 2000 to 3000 IU PMS followed by i.m. injection of 25 or 30 mg $PGF_{2\alpha}$ 48 h later, with the larger doses given to larger animals. Follicular development was monitored by rectal palpation and occasionally cows were excluded from the study due to inadequate responses. For in vivo fertilization, cows were artificially bred by deep cervical deposition of ∿1 ml of fresh semen diluted with Plus-X extender (∿10^8 sperm cells) or by semen that had been stored frozen in one instance. The sperm cells involved in observations of in vivo development were ejaculated by the same Jersey bull in each case.

Recovery of Ova and/or Embryos

For the in vivo studies, ova and/or embryos were recovered at intervals varying from 23 to 120 h after artificial breeding. In one normally cycling cow (J) inseminated with frozen stored semen, the embryo was recovered at necropsy. All other ovum recoveries were carried out surgically to enable repeated use of each animal.

Food was withheld for 36 to 48 h immediately prior to surgery. Animals were treated preoperatively with atropine and xylazine, and anesthesia was induced with thiopental sodium followed by intubation and maintenance with halothane. Preparation of the surgical site, draping, sterilization of instruments and other precautions for aseptic surgery were routine. The cow was positioned in dorsal recumbency with hind quarters slightly elevated so that viscera interfered less with manipulation of the genital tract. An ∿25-cm midline incision afforded entry as far posterior as possible without damaging the udder. The uterus was located and, by placing umbilical tape through the broad ligament and around the body of the uterus, the reproductive tract was elevated and secured to a stainless steel bar positioned transversely across the incision. Ovaries were examined for corpora hemorrhagica or corpora lutea. The fimbriated end of the oviduct to be flushed was cannulated with polyethylene or Silastic tubing of appropriate diameter, and the cannula was secured in place with fine silk or by manual pressure of an assistant. Flushings were retrograde with ∿50 ml of medium forced through a blunt 18-gauge needle introduced into uterine horn after clamping the horn near the cervix with forceps. The medium was Ham's F-10 (Grand Island Biological Co., GIBCO, Grand Island, NY) supplemented with 5% (v/v) heat-treated fetal calf serum (HTFCS, GIBCO) and antibiotics, 100 units penicillin and 100 μg streptomycin per ml ("Penicillin-Streptomycin Solution," GIBCO). Flushings were collected into large (50 ml) conical plastic test tubes. After a 30-min interval, ova and/or embryos were easily recovered by aspiration with a Pasteur pipette from the bottom of these tubes. For identification and manipulation of ova, surrounded by fluid, a low power (10-70X) dissecting microscope was employed. The postmortem flushing was carried out similarly.

In vitro Fertilization Experiments

Efforts were made to recover ova as near to the time of ovulation as possible for fertilization in vitro. Follicles greater than 8 mm in diameter were aspirated, and oviducts were flushed if ovulation had taken place following treatment of donor cows with PMS and $PGF_{2\alpha}$. Ovum donors were submitted to surgery for ovum recovery between 60 and 96 h after the prostaglandin treatment which preceded observed estrus behavior by around 40 to 48 h. Follicular aspiration was carried out by introducing a 20-gauge needle through the ovarian stroma and thus into the follicle at a point distal to the surface. Gentle and repeated aspiration and distention of the follicle using a 5 ml syringe (containing ∿1 ml of medium) was carried out to dislodge the ovum and to allow its entry into the syringe along with a gain in fluid volume of 0.5—4.0 ml. The medium for follicular aspiration and for flushing the oviducts was either Ham's F-10 modified as described above or heparinized saline, 5 IU heparin ("Panheprin," Abbott Laboratories, North Chicago, IL) per ml physiological saline ("Normal Saline, 0.9%," Abbott). When the former medium was employed for follicular aspiration, the syringe and needle were first lined with heparin. Ovulated ova were recovered by flushing oviducts retrograde as described above. All ova were held in large volumes (25—50 ml) of medium in conical 50 ml centrifuge tubes at 37—38°C for at least 1 h (1—2.7 h) following surgery. Upon recovery from the bottom of the large centrifuge tubes in a 38°C tissue culture room, ova were transferred to defined medium routinely used for rabbit in vitro fertilization (Brackett and Oliphant, 1975) and held in a small (30 mm diameter, 12 mm deep), glass, sealable-type, tissue culture dish under silicone oil ("200 Dielectric Fluid," 50 cs. viscosity, Dow Corning Corp., Midland, MI) for not more than 30 min prior to insemination.

Semen, collected with an artificial vagina, was held

in a tightly stoppered test tube at 37–38°C for 1–3.3 h in individual experiments. Sperm cells were then treated in a way known to allow in vitro capacitation of rabbit sperm (Brackett and Oliphant, 1975). Semen was diluted to 5.0 ml with defined medium prepared with 100 units penicillin and 100 μg streptomycin/ml. Sperm cells were washed by centrifugation (5 min at 734 X g), and diluted seminal plasma was removed by aspiration. Resulting packed sperm cells were resuspended in 2.0 ml high ionic strength medium (defined medium with NaCl added to provide an osmolality of ∿380 mOsm/kg) and incubated for 5 min. The cells were recentrifuged for 5 min and resuspended in 5.0 ml defined medium after supernatant fluid was discarded. Sperm cells were subjectively assessed microscopically for motility, and the concentration of sperm cells was determined following counting in a hemocytometer. Approximately 10^6 spermatozoa in a small volume of fluid (usually ∿10 μl) were added to 4.0 ml of defined medium containing ova. In two experiments, frozen semen stored in ampules was thawed according to directions (Atlantic Breeder's Service, Lancaster, PA), and the sperm cells were immediately treated and used for insemination as described above.

The ovum-containing solution was covered with silicone oil, and following insemination all air space within the dish was completely eliminated by additional oil and placement of the dish top. The closed dish was wrapped in foil to exclude light and incubated in a small chamber (Anaerobic Culture Apparatus, small size, Arthur H. Thomas Co., Philadelphia, PA). Media and oil were equilibrated with 5% CO_2: 8% O_2: 87% N_2 prior to use, and the same gas mixture, moistened by passage through a washing bottle, was continuously passed at a slow rate through the chamber. After initial observation at 20–24 h after insemination, some ova were transferred from the defined fertilization medium (Brackett and Oliphant, 1975) into Ham's F-10 modified as described above but with 10% (v/v) heat-treated fetal calf serum, a medium demonstrated to support early development for an extended culture interval (Wright et al., 1976). Composition of the atmosphere for embryo culture was not altered. All glassware, media and tubing contacting ova and/or sperm cells were sterilized as is routine: dry heat for glassware, Millipore filtration (0.45 μm pore size) for media, chemical sterilization with ethylene oxide for polyethylene or Silastic tubing.

Examination of Ova

For morphological studies, ova were transferred to microscope slides with small amounts of fluid and examined under cover slips supported by four small pillars of stopcock grease. The ova and any associated sperm cells were studied under phase contrast and interference contrast microscopy (12.5–1250×) and representatives were photographed in a Zeiss photomicroscope II. Ova were washed from microscope slides, collected and transferred for fixation in 1.0% gluteraldehyde in 0.1 M phosphate buffer (prepared at pH 7.2) for 30 min. When further processing for electron microscopic examination was to be delayed, ova were rinsed for 10 min in fresh 0.1 M PO_4 buffer and then submerged in fresh buffer for storage at 5°C for up to 10 days. For ultrastructural study, ova were postfixed in 1.0% OsO_4, dehydrated by passage through a series of alcohols, and individually embedded in Epon 812. All specimens were serially sectioned with a diamond knife on an LKB ultramicrotome initially at 1 μm thickness and stained with toluidine blue for light microscopic orientation, followed by thin sectioning (1 μm). Approximately 50 grids were prepared from each ovum. The grids were stained with saturated uranyl acetate in 0.1% acetic acid, followed by alkaline lead citrate (Reynolds, 1963). Samples were examined and photographed in a Hitachi 12B electron microscope.

RESULTS

In vivo Observations

Thirty-seven (71.2%) of 52 ovulated ova were recovered from five cows that were inseminated at variable intervals prior to ovum recovery (Table 1). The onset of estrus (standing heat) was observed 39–48 h after the $PGF_{2\alpha}$ injection in the superovulation treatments, and ovulation was well underway, if not completed, by 24 h after onset of estrus. These treatments clearly shortened the normally expected temporal relationship between onset of estrus and ovulation. Developmental stages from 1-cell to morula were obtained for morphological assessment (Table 1). Variability in development was probably due to variability in ovulation of individual ova, but hormonal influences on the temporal progression of fertilization and early developmental events cannot be completely ruled out. The former interpretation leads to the conclusion that ovulation in Exp. 34–66 (A×G) took place at least 20 h earlier for the 2-cell ovum, i.e., only 3 h after insemination, and quite recently for 6 ova still surrounded by cumulus cell masses. Even more remarkable was the finding of recently penetrated 1-cell stage and 8-cell stage ova 64 h after a single insemination (Exp. 34–53, A×Hol, Table 1). These data suggest that bull sperm can undergo capacitation within 3 h and maintain fertilizing ability in the female reproductive tract for ∿60 h.

From cow A×Hol (in Table 1) ova in four different (1-, 2-, 4-, and 8-cell) stages were found 64 h after artificial insemination. One of the 1-cell ova was characterized by severely degenerated ooplasm (Fig. 1). Since recovery was considered to be too soon after ovulation for aging within the oviduct to account for the condition of this ovum, it was concluded that one of 17 ova released from the ovaries in response to the PMS and $PGF_{2\alpha}$ treatment was incompetent prior to ovulation. An additional

TABLE 1. Observations, recovery and development of cow ova after artificial insemination.[a]

Exp. (Breed)[b]	Treatment	Time relationships		Ovum recovery				Cell-stage of development				
		Onset of estrus	Ovulation(s) detected[d]	AI	Estimated ovulation(s)[e]	n CH or CL	n Ova recovered	1	2	4	8	16
		(h after PG[c])		(h after)								
34–66 (AXG)	PMS + PG	39	69	23	<1–20	11	7	6	1			
34–57 (Hol)	FSH, LH + PG	48	72	42	24	12	1		1			
34–64 (A)	PMS + PG	40	64	42	24	11	11	6	5			
34–53 (AXHol)	PMS + PG	41	67	64	44	17	17	4	3	9	1	
34–39 (J)	None	...	...	112	88	1	1					1
Summary: All		39–48	64–72	23–112	<1–88	52	37 (71.2%)	16	10	9	1	1

[a]Cows were artificially inseminated 6–8 h after onset of estrus.

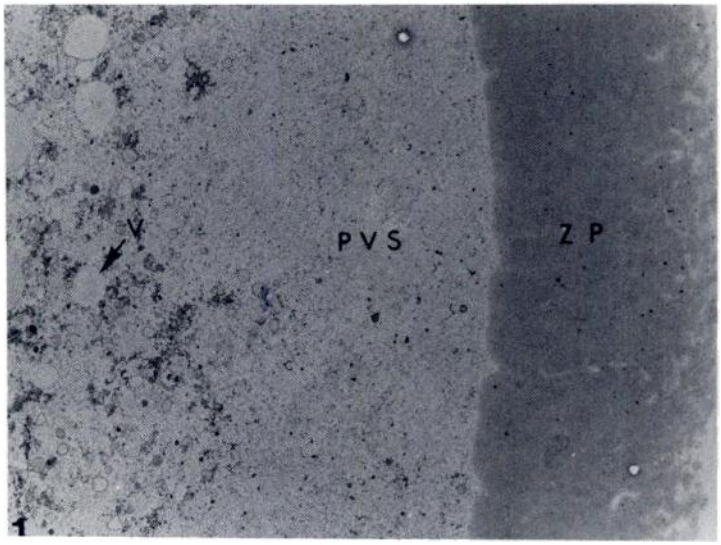
[b]A = Angus; G = Guernsey; Hol = Holstein; J = Jersey.

[c]PG = $PGF_{2\alpha}$.

[d]In Exp. 34–66 (Cow AXG), occurrence of ovulation was determined by direct inspection and ovum recovery by surgery. In other cases, a decrease in ovarian follicles palpable per rectum was detected at the indicated intervals; temporal limits, i.e., initial and final ovulations, were not defined, but in Exp. 34–64 (Cow A) all ovulations were noted to have occurred by the indicated time, i.e., 24 h after onset of standing heat.

[e]Hours (h) after estimated ovulations in Exp. 34–66 (Cow AXG) were based on developmental stages of ova found (see text). In other cases, additional hours (h) from times at which ovulation(s) were detected (or anticipated for the untreated cow) to the times of ovum recovery are given.

feature of this ovum in contrast to the others was a total absence of any evidence for interaction with sperm cells. The other uncleaved ova were mature as indicated by the presence of polar bodies within the perivitelline spaces of each. Evidence of fertilization was not obvious by light microscopy (see below), and the zonae pellucidae were free of surrounding cellular investments. When ovum recovery took place 23 h after insemination six 1-cell stage ova were recovered from oviducts of cow A×G, and these recently ovulated ova were still surrounded by cumulus oophorus cells, a feature not observed in other cows when ova were recovered at longer intervals after ovulation. Corona radiata cell processes deeply embedded in the matrices of zonae pellucidae were observed upon ultrastructural examination of the ova



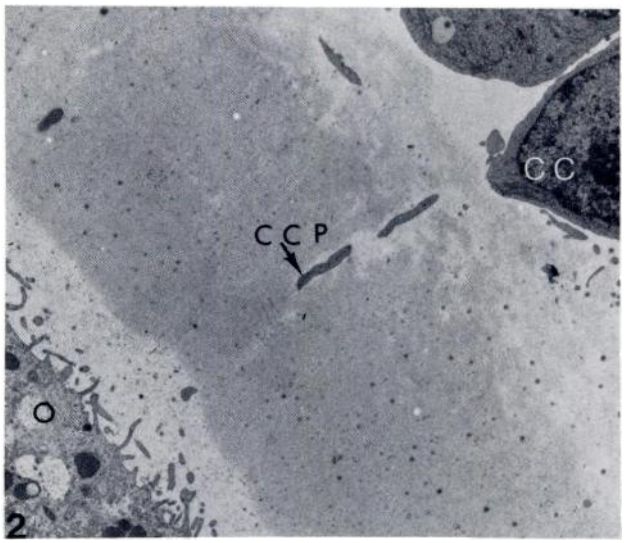


FIG. 1. Electron micrograph of an abnormal cow ovum ovulated in response to PMS and $PGF_{2\alpha}$ treatment. Note indefinite perivitelline space due to degenerate ooplasm. PVS = perivitelline space; V = vacuole (arrow) within degenerated vitellus; ZP = zona pellucida. × 2500.

FIG. 2. Ultrastructural relationship of corona cell to zona pellucida of a normal cow ovum recovered soon after ovulation. O = ooplasm; CCP = corona cell process (arrow) embedded within the matrix of the zona pellucida; CC = corona cell outside the zona pellucida. × 5200.

(Fig. 2), recovered within 36 h of the onset of estrus. Sperm penetration of the cow ovum with intact cellular investments was documented by the additional ultrastructural observation of an early stage of fertilization in one of these ova (Fig. 3). From adjacent thin sections of this ovum, another part of the sperm tail (not shown) was seen remaining in the perivitelline space. The nine outer fibers and mitochondria of the fertilizing sperm cell appeared somewhat degenerated (Fig. 3), and activation of the ovum was apparently well underway. The so-called "hooded" mitochondria, characteristic of oocytes of ungulates, represented a prominent ultrastructural feature. The abundance of mitochondria and the appearance of the mitochondrial cristae suggested increased metabolic activity within the ooplasm coincident with ovum activation. Ultrastructural features consonant with release of cortical granules (Fig. 4), associated with ovum activation, were observed in cow ova found to be in the early pronuclear stage by electron microscopy, although as mentioned above evidence for fertilization was not apparent by light microscopy. The ovum pictured in Fig. 4, recovered from Ax Hol 64 h after artificial breeding, contained a male pronucleus identifiable by its proximity to a sperm tail remnant within the ooplasm. Another ovum contained two well developed pronuclei that had already assumed a central position within the ooplasm.

Usually no more than three sperm cells were seen in association with zonae pellucidae of cow ova exposed to sperm in vivo. Such sperm cells were observed to have undergone the acrosome reaction, leaving the inner acrosomal membrane exposed. Penetration to approximately one-third the thickness of the zona pellucida, but no further, was a frequent observation (Fig. 5), suggesting a strong block to polyspermy. Pronounced dissolution of the matrix of the zona pellucida by a penetrating sperm cell was evidenced in the electron microscope (not shown) by a clear area (penetration slit) approximately 10 times the size of the sperm head.

A representative 2-cell stage cow embryo is shown in Fig. 6. This ovum was recovered from cow Ax Hol 64 h after artificial breeding. An

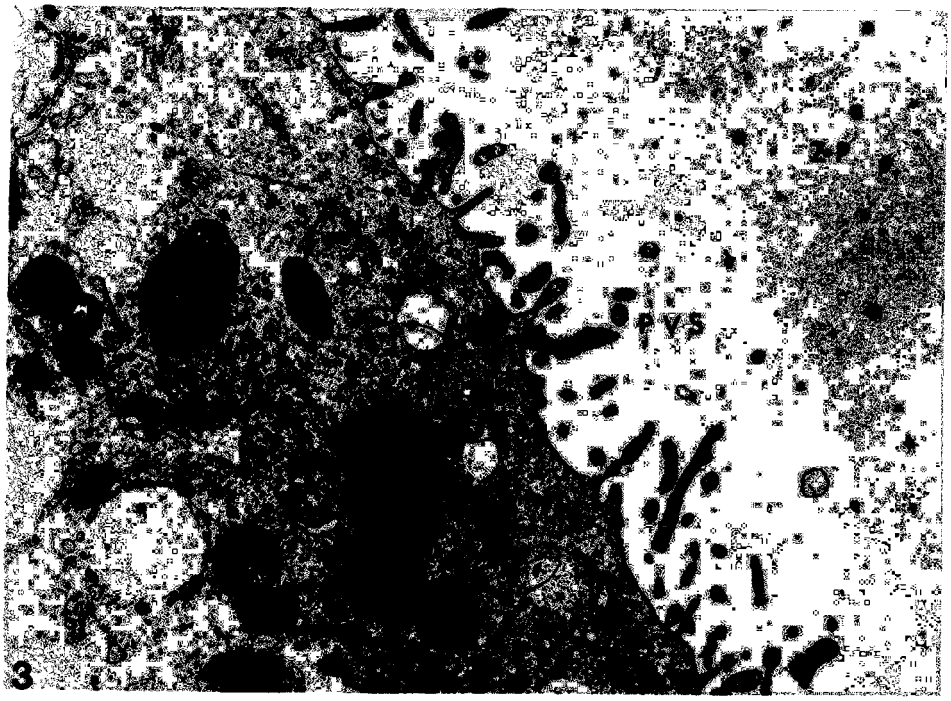


FIG. 3. Electron micrograph of a cow ovum undergoing fertilization as evidenced by presence of sperm cell within ooplasm, many apparently active mitochondria, depleted complement of cortical granules at periphery of ooplasm and prominent perivitelline space. M = mitochondrion with characteristic "hooded" appearance; SR = sperm remnant (arrow) within ooplasm; PVS = perivitelline space; ZP = zona pellucida. X 10,500.

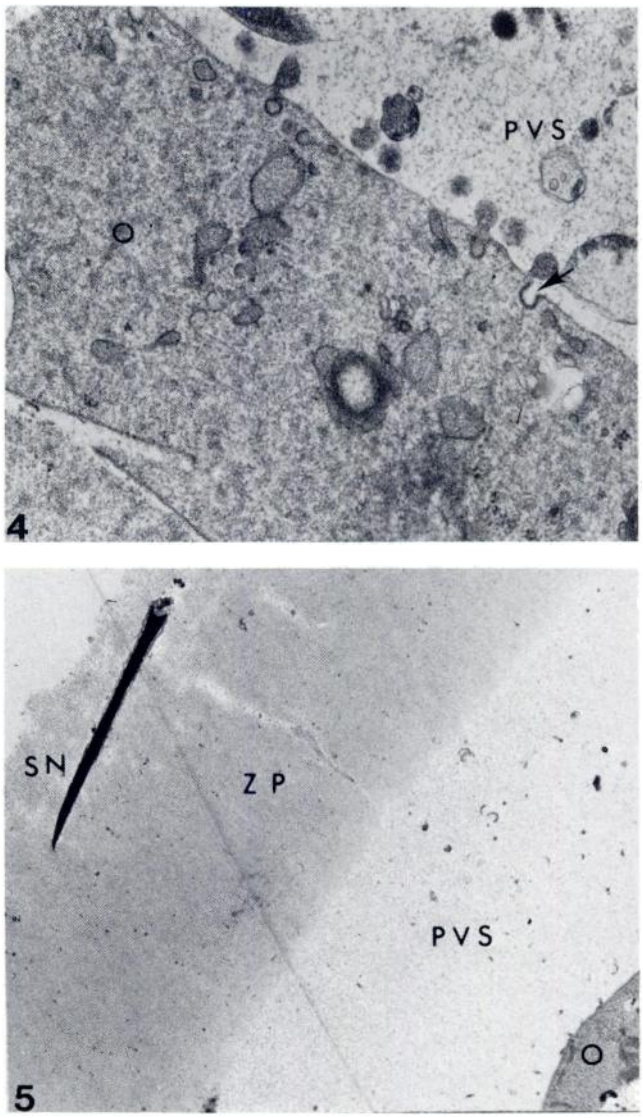


FIG. 4. Cow ovum in the early pronuclear stage with ultrastructural evidence of cortical granule breakdown at the periphery of the ooplasm (arrow). O = ooplasm; PVS = perivitelline space. × 2400.

FIG. 5. A representative electron micrograph showing an acrosome-reacted sperm cell that has apparently penetrated into the zona pellucida to the limit allowed by a strong block to polyspermy imposed by the fertilized cow ovum. SN = sperm nucleus; ZP = zona pellucida; PVS = perivitelline space; O = ooplasm. × 6000.

unexpected finding was the presence of sperm tail remnants within the blastomeric cytoplasm at this stage. This ultrastructural observation was made in two of the ten 2-cell stage embryos studied. Ooplasm of another 2-cell stage ovum from the same cow is shown in Fig. 7 to demonstrate typical intramitochondrial granules. These structures were occasionally observed in unfertilized as well as in fertilized ova. Usually only one granule was present, but sometimes two granules could be seen within the same mitochondrion. Impressions from this work were that these well developed granules are present in a small percentage of mitochondria and that the incidence increases soon after fertilization.

A representative 4-cell stage embryo, recovered from cow AxHol 64 h after artificial breeding, is illustrated in Fig. 8. The periphery of blastomeric cytoplasm of each cell was

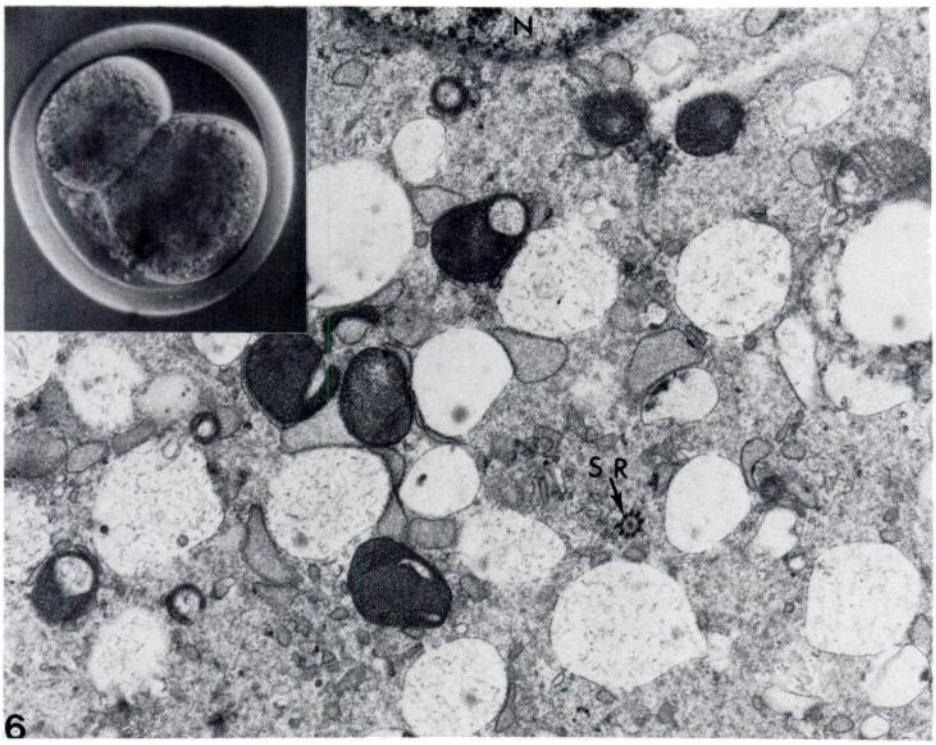


FIG. 6. A 2-cell stage cow embryo as observed by interference contrast microscopy (inset, X 240). Electron microscopic examination revealed remnants of the fertilizing sperm cell (arrow) within blastometric cytoplasm. N = nucleus of blastomere; SR = sperm remnant. X 15,000.

devoid of cortical granules as expected for normally fertilized ova. An 8-cell stage ovum, recovered from the same cow, afforded the first occasion during early bovine development in which the centrioles were identifiable (Fig. 9). Centrioles, found also in the morula stage, were found near the blastomeric nuclei.

Cortical granule breakdown appeared to be an ultrastructural feature of normal in vivo fertilization, and cortical granules were usually absent from the peripheral cytoplasm of each blastomere. Occasionally, associated with the Golgi apparatus or in peripheral areas of certain blastomeres, cleaved ova appeared to have more than a few remaining cortical granules. The morula, resulting from in vivo fertilization of a single ovum ovulated in the course of a normal estrus cycle, was characterized by the presence of a full complement of cortical granules in the peripheral ooplasm of a few of the approximately 16 blastomeres (Fig. 10).

In the morula stage, mitochondrial structure was different when compared with earlier stages (Fig. 11). The mitochondrial matrix appeared to become less electron dense. Also, mitochondria of the morula stage seemed to be characterized by an increase in peripherally arranged cristae.

In vitro Fertilization Experiments

For in vitro fertilization, 12 cows were treated with PMS and $PGF_{2\alpha}$ for ovulation induction in 16 estrus cycles. In four treated cycles of three different animals following repeated rectal palpation, the follicular response was judged inadequate to warrant surgery. Surgery was performed in 12 cases at intervals of 60 to 96 h after prostaglandin treatment, and 32 follicular and 42 tubal ova were recovered around the time of ovulation for in vitro insemination. Results of these experiments are shown in Table 2.

Several leads emerge from the data resulting from four series of experiments (Table 2). The superovulation procedure yielded recently ovulated and preovulatory ova between 67 and 76 h after the prostaglandin treatment, and best

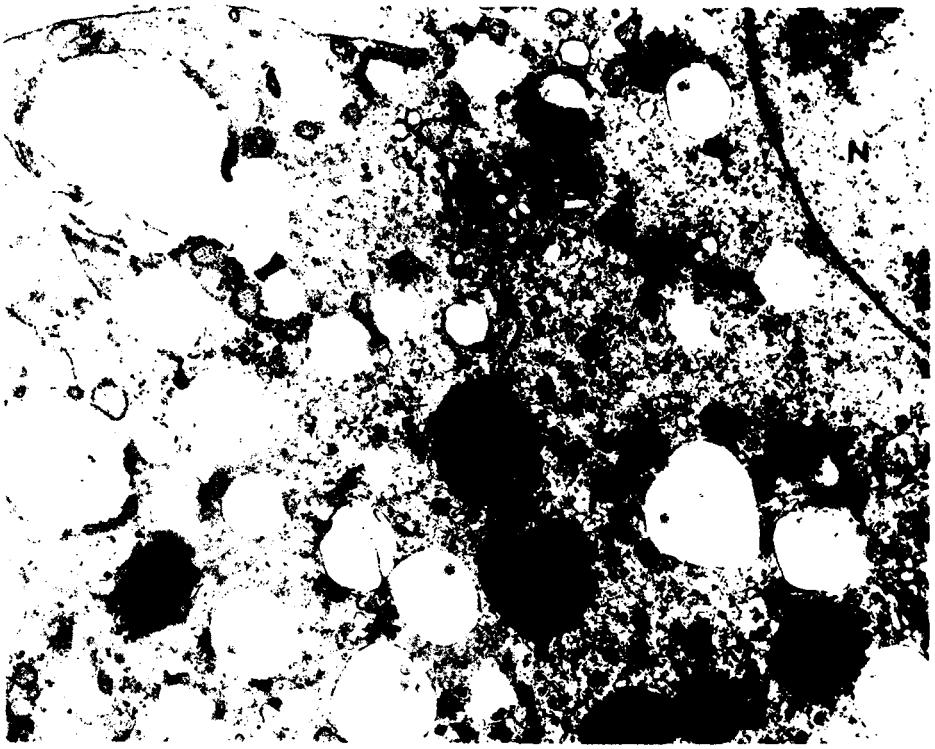


FIG. 7. Electron micrograph of part of a 2-cell cow embryo revealing intramitochondrial granules; these were seen in mitochondria of both unfertilized and fertilized cow ova. MG = mitochondrial granule; N = nucleus. X 15,000.

results were afforded when ova surrounded by a healthy complement of follicular cells, obtained within this interval, were inseminated in vitro.

In an initial series of experiments (Table 2) ova were recovered 86 to 96 h after prostaglandin treatment, and the absence of development after in vitro insemination might be attributable to excessive aging of oocytes prior to their recovery. This was suggested by an inadequately responding ooplasm which facilitated the detection of a sperm cell within the perivitelline space of one ovum. Although penetration of the zona pellucida might have been slower than normal, it was concluded that sperm capacitation and the acrosome reaction had taken place in vitro. Another series (two experiments) was carried out in which frozen semen was employed. Although ova were recovered at more appropriate intervals after prostaglandin treatment, no signs of activation or fertilization resulted. The most likely reason was the inadequate condition of sperm cells used.

The most promising results followed recovery of ova from treated donors between 67 and 76 h after prostaglandin treatment and the use of freshly ejaculated sperm from the Jersey bull. In four experiments, 5 of 12 preovulatory ova recovered from follicles and 9 of 16 recently ovulated tubal ova were apparently fertilized in vitro. The most convincing evidence of in vitro fertilization followed the demonstration by electron microscopy of an absence of cortical granules at the periphery of blastomeric cytoplasm in the 2-cell and 4-cell (Fig. 12) ova resulting from experiment 34–62 (Table 2).

A final series (3 experiments) was carried out with sperm from the young Holstein bull. The timing for ovum recovery seemed appropriate, but only 3 of 10 ova recovered from follicles and 2 of 16 ova recovered from the oviducts showed signs of fertilization as assessed by light microscopy. Ultrastructural examination of the pronuclear ovum from Exp. 40–50 (Table 2) revealed an absence of cortical granules at the periphery of the ooplasm but the presence of two or three clusters of cortical granules close to the vitelline membrane. Also,

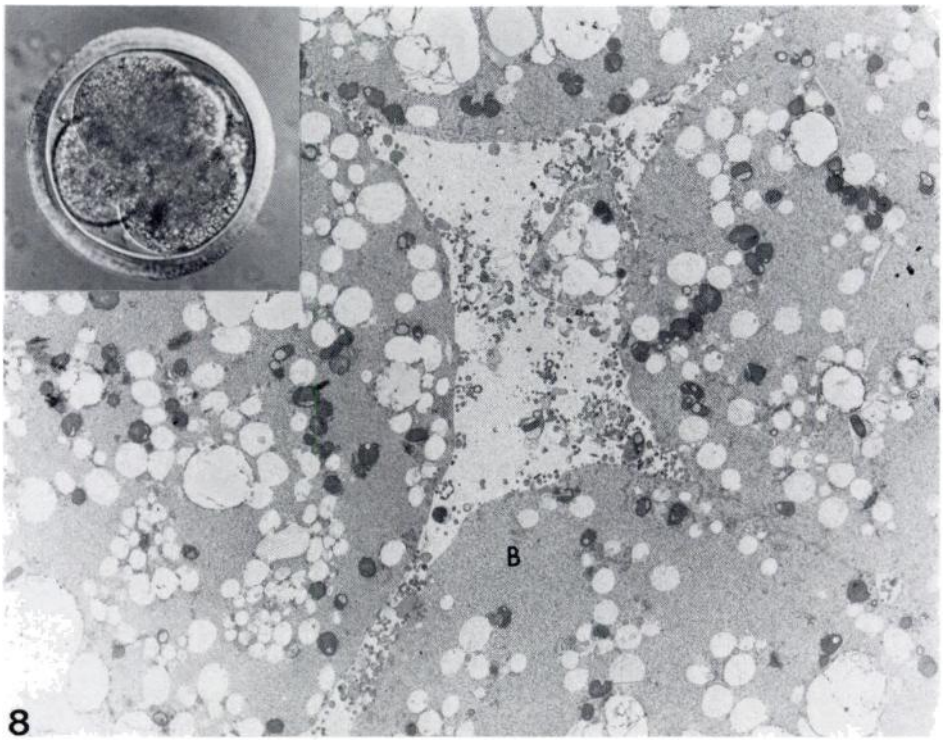


FIG. 8. A 4-cell stage cow embryo as observed by interference contrast microscopy (inset, × 200). The electron micrograph reveals absence of cortical granules in the peripheral cytoplasm of each blastomere. B = blastomere. × 4200.

no sperm remnant could be demonstrated within the cytoplasm, and by these more stringent criteria the ovum was judged not to be fertilized. The pronuclear ovum from Exp. 40–53 had many sperm attached to the zona pellucida, but cortical granules were present at the periphery of the ooplasm and no sperm tail remnant was seen within the cytoplasm. Although the organelles appeared healthy, this ovum failed the test for fertilization according to the accepted ultrastructural criteria. The two ova that developed to the 2-cell stage in Exp. 40–56 (Table 2) were examined ultrastructurally. Both had cortical granules present at the periphery of blastomeric cytoplasm. One appeared to be degenerated, while the other was apparently developing parthenogenetically.

DISCUSSION

Most cows remain in estrus (standing heat) 18 h (Trimberger and Davis, 1943), and ovulation usually occurs between 10 and 18 h after the end of estrus (Nalbandov and Casida, 1942). By comparison, the interval between onset of estrus and onset of ovulation in treated cows in the present study was usually shortened by several hours. The recovery of cow ova in pronuclear to 8-cell stages 64 h after artificial insemination (Exp. 34–53, Table 1) apparently reflects a prolonged interval over which ovulation takes place following PMS and $PGF_{2\alpha}$ treatment. The pronuclear ova were comparable to those found 5 to 12 h after ovulation (Thibault, 1967), and if the 8-cell stage was reached around 44 h after ovulation (Thibault, 1966; present data), the fertilized ova recovered from the A × Hol cow were ovulated over a 39 h period. Also, sperm cells capable of fertilizing ova were present within the oviducts during this 39 h interval. The degenerating 1-cell unfertilized ovum recovered at the same time (Fig. 1) was most likely abnormal and not merely degenerating as a result of aging following ovulation. The cow ovum is reportedly fertilizable for 24 h following ovulation (Thibault, 1967). Since the cow was bred while in estrus, at least 12 h before ovulation, and the interval

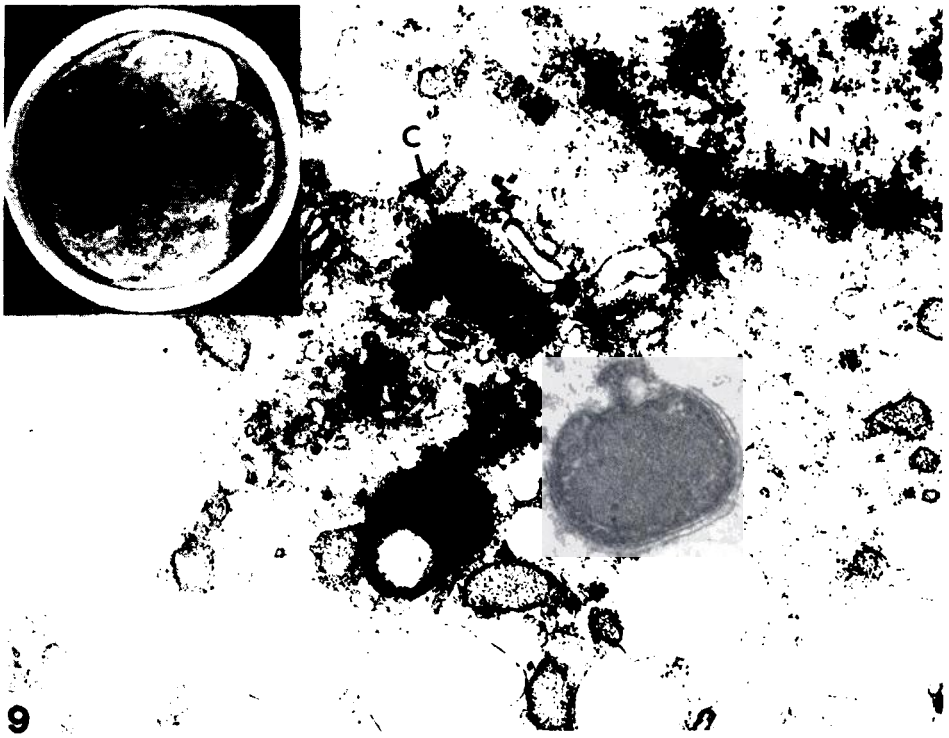


FIG. 9. A freshly recovered 8-cell stage cow embryo (inset, X 240). Centrioles become identifiable at this stage. C = centriole; N = nucleus. X 30,000.

necessary for bull spermatozoa to become capacitated is apparently brief (Mahajan and Menge, 1966), the nonfertile condition was most likely the result of the abnormal (degenerative) condition of this ovum at ovulation. Such a product of ovulation, resulting from unusual hormonal influences in this instance, might be a cause of occasional infertile cycles of subfertile cows.

Zonae pellucidae of cow ova not exposed to sperm cells were found to be free of follicular cells as early as 2–3 h after ovulation by Lorton and First (1979). In the present study, 6 ova recovered in the 1-cell stage 23 h after artificial insemination demonstrated retention of follicular cells possibly for several hours after ovulation. Corona radiata cell processes embedded within the zona pellucida, described in preovulatory and in very recently ovulated ova of several other species (Zamboni, 1970), were observed in cow ova recovered soon after ovulation (Fig. 2). Absence of an abrupt loss of follicular cells upon sperm penetration was documented in this work. The condition or maturity of individual ova might determine presence or absence of surrounding cellular investments.

Ultrastructural features of cow oocytes described previously (Fleming and Saacke, 1972; Senger and Saacke, 1970) were confirmed. The most striking of these is the characteristic appearance of the mitochondria previously described as "hooded" (Fig. 3). Acrosome-reacted sperm cells embedded in the substance of the zona pellucida (Fig. 5) were as described in the pig (Szollosi and Hunter, 1973). Disappearance of cortical granules coincident with activation by the penetrating sperm cell (Figs. 3, 4), first appearance of centrioles at the 8-cell stage (Fig. 9), and progressive changes in mitochondria with early in vivo development (cf. Figs. 3, 11) were comparable to ultrastructural events seen in early sheep development (Calarco and McLaren, 1976). Observation of few sperm cells penetrating approximately one-third the thickness of the zona pellucida (Fig. 5) and observation of no more than one sperm cell within any ovum suggested a strong and rapid block to polyspermy. Additional ultrastructural findings

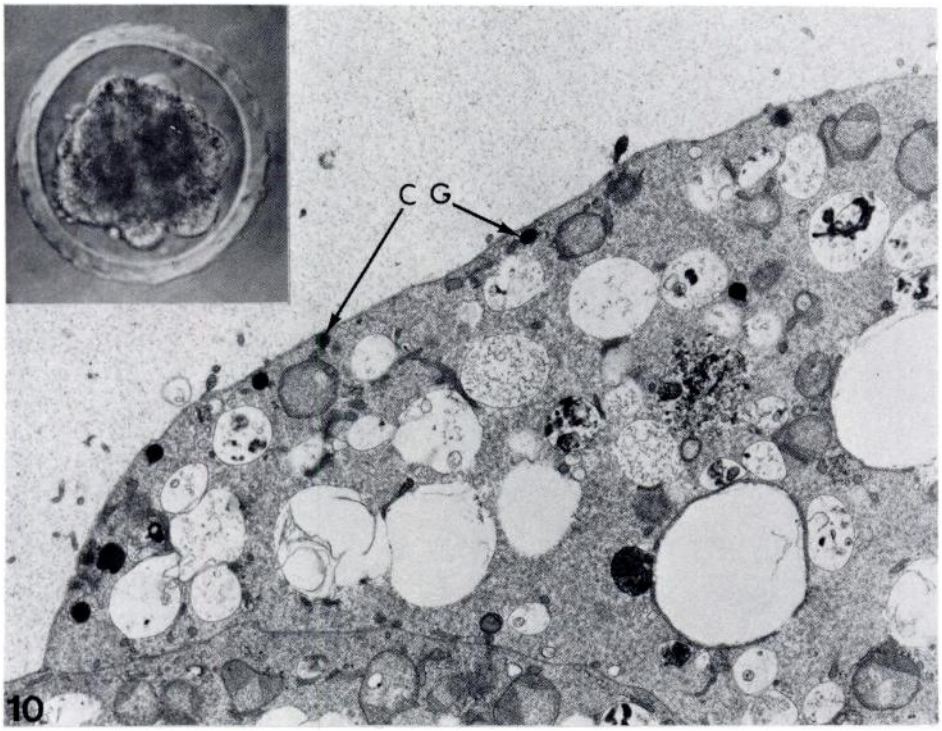


FIG. 10. A morula stage cow embryo exhibiting compaction of the blastomeres (inset, X 200). Although not representative of most blastomeres, the electron micrograph reveals peripheral cytoplasm of a blastomere with a full complement of cortical granules (arrows). CG = cortical granule. X 12,000.

included the identification of sperm remnants within blastomeric cytoplasm, but without any evidence of polyspermy or otherwise abnormal development in 2-cell stage ova (Fig. 6), and the presence of very prominent intramitochondrial granules (Fig. 7). The latter structures were found in all stages of bovine ova, but prominent intramitochondrial granules have not been noted in ova or embryos of other species. Mitochondrial granules found in chondrocytes are known to contain calcium and phosphorus, but functional roles remain speculative (Sutfin et al., 1971).

In contrast to the in vivo situation, many sperm cells were stuck to the zonae pellucidae after in vitro insemination. The acrosome reaction (Barros et al., 1967; Bedford, 1967) was completed in these spermatozoa that had at least started to penetrate the zona. Observation of a sperm cell within the perivitelline space of one ovum and normal ultrastructural development in two cleaved ova further evidenced the achievement of capacitation (Austin, 1951; Chang, 1951). Apparently, bull sperm can be capacitated in vitro by a treatment similar to that found effective for rabbit sperm (Brackett and Oliphant, 1975). Additional support for this follows work involving sperm-zona interaction (Gwatkin and Williams, 1978) and penetration of zona-free hamster ova by bull sperm (Lorton and First, 1979). Also, defined conditions similar to those developed in the present work consistently supported in vitro penetration by bull sperm and male pronuclear development in hamster zona-free ova, and when cow oocytes were inseminated in vitro with in vitro-capacitated spermatozoa from the same samples, sperm penetration through zonae pellucidae resulted (Brackett et al., 1980). Sperm penetration, normal temporal progression of initial cleavages, and absence of cortical granules from the peripheral blastomeric cytoplasm of 2- and 4-cell stage embryos represent documentation for in vitro fertilization in the present work.

The lowered incidence of presumptive evidence for fertilization and the presence of cortical granules in ova that showed signs of

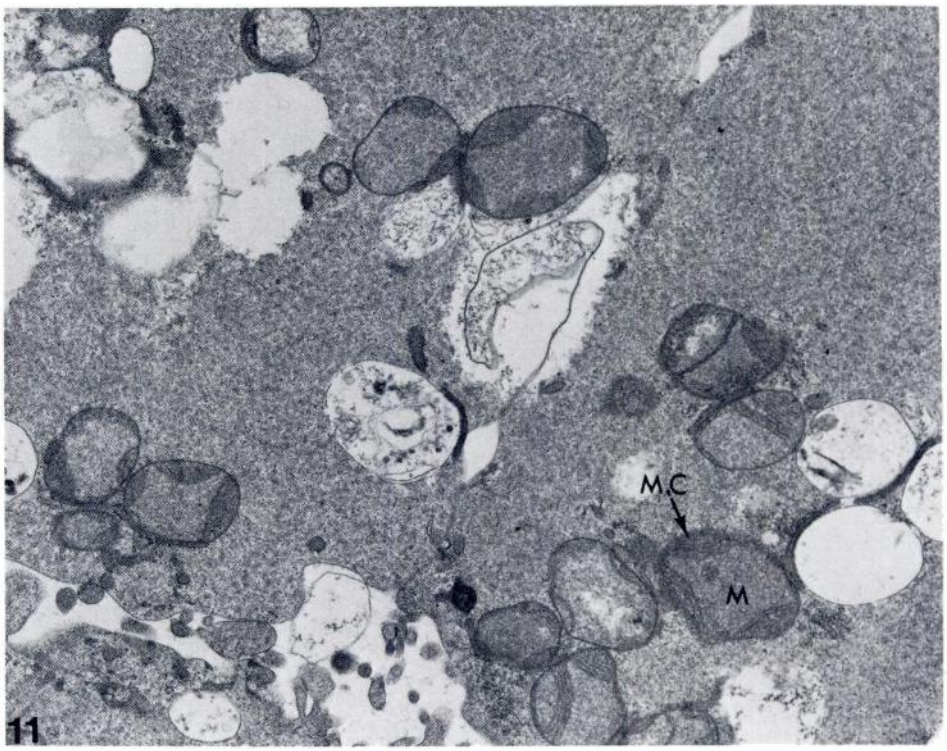


FIG. 11. Electron micrograph showing less electron-dense mitochondrial matrix with increased peripherally arranged cristae characteristic of the morula stage cow embryo. M = mitochondrial matrix; MC = mitochondrial cristae. × 11,200.

activation following insemination by spermatozoa from the young Holstein bull emphasize the need for additional work to define conditions that will yield repeatable results with bovine gametes. The role of sperm cells from the young Holstein bull in activating ova was not clear, although in the rabbit ovulated ova inseminated in vitro with immature epididymal sperm cells have been observed to undergo activation with retention of cortical granules (Brackett et al., 1978). Similar observations of retained cortical granules have been reported in cleaved rabbit ova after exposure to spermatozoa experimentally treated to achieve capacitation in vitro (Dandekar and Gordon, 1975) and in cleaved rhesus monkey ova resulting from superovulation and in vivo exposure to spermatozoa (Batta et al., 1978). The finding of many cortical granules in some blastomeres of a normally fertilized morula (Fig. 10) emphasizes the need for more observations relating cortical granule breakdown to the quality of early development.

In the absence of optimally conditioned sperm cells, exposure of bovine oocytes, especially of aged oocytes, to increased concentrations of calcium ions might initiate parthenogenetic development as described when calcium-deprived oocytes were treated with calcium (mouse and rat: Miyamoto and Ishibashi, 1975; mouse and hamster: Whittingham and Siracusa, 1978). In the mouse and hamster, release of cortical granules accompanied the observed activation, while the activation observed in cow ova inseminated in vitro involved retention of cortical granules as described for parthenogenetic activation of rabbit ova (Longo, 1975). Interestingly, cortical granules are apparently lost prematurely from oocytes of certain mouse strains that exhibit less efficient fertilization than of strains of mice having ova with larger complements of cortical granules (Wabik-Sliz, 1979). The premature loss of cortical granules may be a useful indicator but not the cause of infertility, since sperm penetration can follow experimentally provoked cortical granule loss in zona-free mouse ova (Wolf et al., 1979). Inefficient fertilization

TABLE 2. Results of efforts to fertilize cow ova in vitro.

Exp.	Cow[b]	Bull[c]	Ovum recovery (h after PG)[a]	n Ova inseminated in vitro	Tubal (T) or follicular (F)	n Ova apparently fertilized	Presumptive (light microscopic) evidence of fertilization		
							Pro-nuclear	2-Cell	4-Cell
34–27	Hol	J	96	4	T	0			
34–31	Hol	J	86	2	F	0			
				6	T	0			
34–61	A	J	89	2	F	1 (sperm cell within perivitelline space)			
34–29	Hol	Frozen semen	60	2	F	0			
40–46	H*	Frozen semen	75	4	F	0			
34–62	A×G	J	67	2	F	2		1	1
34–72	A×G	J	72	3	F	0			
40–28	A	J	74	3	F	1			1
40–36	H*	J	76	4	F	2		2	
				16	T	9	1	4	4
40–50	A×H**	Hol	76	4	F	1	1		
40–53	A	Hol	76	2	F	0			
				11	T	1	1		
40–56	A×H**	Hol	76	4	F	2		2	
				5	T	1	1		

[a]PG = $PGF_{2\alpha}$.

[b]Hol = Holstein; A = Angus; H = Herford; G = Guernsey.

[c]Two bulls were used: J = Jersey, Hol = Holstein.

*and ** Indicate repeated use of the same ovum donors.

resulting from asynchrony in molecular events of importance to normal gamete interaction might account, at least in part, for low incidences of successful fertilization after efforts to mature ova in vitro (Newcomb et al., 1978; Thibault et al., 1975, 1976; Trounsen et al., 1977). Similarly, lack of sperm receptivity by ooplasm allowed identification of a sperm cell within the perivitelline space in the present study. Optimal conditioning of both gametes must be correctly timed for normal fertilization to occur.

In addition to a requirement of calcium ions for ovum maintenance and fertilization, the sperm acrosome reaction is calcium-dependent (Yanagimachi and Usui, 1974). Uptake of calcium ions by bull sperm apparently links membrane alterations to activation of sperm motility and the acrosome reaction (Singh et al., 1978). A differential advantage of cauda sperm over ejaculated bull sperm in accumulating calcium ions is regulated by the surrounding concentration of a seminal plasma inhibitor (Babcock et al., 1979). This may account for the advantage in fertilization in vitro of cauda epididymal over ejaculated rabbit sperm following in vitro capacitation with high ionic strength medium (Brackett et al., 1978), as well as the achievement of the ability of bull sperm to fertilize in the present work. Removal of inhibitory influences by allowing the semen to become acidic during the initial prolonged incubation might have enhanced the acrosin activity, and an enhancing role of heparin in acrosin activation (Wincek et al., 1979) and/or in modulation of adenylate cyclase activity (Amsterdam et al., 1978; Salomon et al., 1978) might also have been operative. Additional research must focus on underlying mechanisms and on further definition of conditions to enable the development of a practicable procedure for in vitro fertilization.

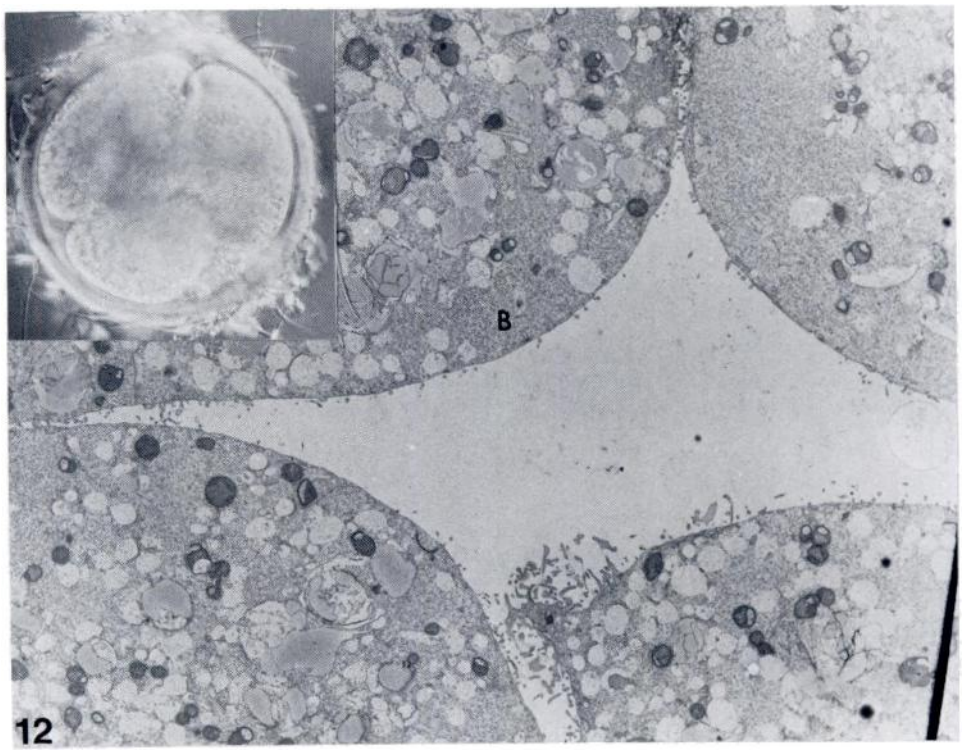


FIG. 12. A 4-cell stage cow embryo fertilized in vitro (inset, X 240). The electron micrograph reveals absence of cortical granules from the peripheral cytoplasm of each blastomere. B = blastomere. X 3800.

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REFERENCES

Amsterdam, A., Reaches, A., Amir, Y., Mintz, Y. and Salomon, Y. (1978). Modulation of adenylate cyclase activity by sulfated glycosaminoglycans, II. Effect of mucopolysaccharides and dextran sulfate on the activity of adenylate cyclase derived from various tissues. Biochem. Biophys. Acta 544, 273–283.

Austin, C. R. (1951). Observation of the penetration of sperm into the mammalian egg. Australian J. Res. B4, 581–596.

Babcock, D. F., Singh, J. P. and Lardy, H. A. (1979). Alteration of membrane permiability to calcium ions during maturation of bovine spermatozoa. Develop. Biol. 69, 85–93.

Barros, C., Bedford, J. M., Franklin, L. E. and Austin, C. R. (1967). Membrane vesiculation as a feature of the mammalian acrosome reaction. J. Cell Biol. 34, C1–C5.

Batta, S. K., Stark, R. A. and Brackett, B. G. (1978). Ovulation induction by gonadotropin and prostaglandin treatments of rhesus monkeys and observation of the ova. Biol. Reprod. 18, 264–278.

Bedford, J. M. (1967). Experimental requirement for capacitation and observations on ultrastructural changes in rabbit spermatozoa during fertilization. J. Reprod. Fert. Suppl. 2, 35–48.

Betteridge, K. J. (1977). Embryo Transfer in Farm Animals: A Review of Techniques and Application. Monograph 16, Canada Dept. of Agriculture, Ottawa. 92 pp.

Brackett, B. G., Evans, J. F. and Donawick, W. J. (1980). Interaction of bovine gametes in vitro. IXth International Congress of Animal Reproduction and Artificial Insemination, Madrid. In Press.

Brackett, B. G., Hall, J. L. and Oh, Y. K. (1978). In vitro fertilizing ability of testicular, epididymal and ejaculated rabbit sperm. Fertil. Steril. 29, 571–582.

Brackett, B. G., Oh, Y. K., Evans, J. F. and Donawick, W. J. (1977). Bovine fertilization and early development in vivo and in vitro. 10th Annual Meeting of Society for the Study of Reproduction, Austin, TX. pp 56–57, Abstr. 86.

Brackett, B. G., Oh, Y. K., Evans, J. F. and Donawick, W. J. (1978). In vitro fertilization of cow ova. Theriogenology 9, 89.

Brackett, B. G. and Oliphant, G. (1975). Capacitation of rabbit spermatozoa in vitro. Biol. Reprod. 12, 260–274.

Calarco, P. G. and McLaren, A. (1976). Ultrastructural observations of preimplantation stages of the sheep. J. Embryol. Exp. Morphol. 36, 609–622.

Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited in the fallopian tubes. Nature 168, 697–698.

Dandekar, P. V. and Gordon, M. (1975). Electron microscope evaluation of rabbit eggs exposed to spermatozoa treated with capacitating agents. J. Reprod. Fert. 44, 143–146.

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.

Elsden, R. P., Hasler, J. F. and Seidel, G. E., Jr. (1976). Non-surgical recovery of bovine eggs. Theriogenology. 6, 523–532.

Flechon, J. E. and Renard, J. P. (1978). Scanning electron microscopic study of the hatching of bovine blastocysts in vitro. J. Reprod. Fert. 53, 9–12.

Fleming, W. N. and Saacke, R. G. (1972). Fine structure of the bovine oocyte from the mature Graafian follicle. J. Reprod. Fert. 29, 203–213.

Gwatkin, R.B.L. and Williams, B. T. (1978). Bovine and hamster zona solutions exhibit receptor activity for capacitated but not for non-capacitated sperm. Gamete Res. 1, 259–263.

Hamilton, W. J. and Laing, J. A. (1946). Development of the egg of the cow up to the stage of blastocyst formation. J. Anat. 80, 194–204.

Hartman, C. G., Lewis, W. H., Miller, F. W. and Swett, W. W. (1931). First findings of tubal ova in the cow together with notes on estrus. Anat. Rec. 48, 267–275.

Hunter, R.H.F., Lawson, R.A.S. and Rowson, L.E.A. (1972). Maturation, transplantation and fertilization of ovarian oocytes in cattle. J. Reprod. Fert. 30, 325–328.

Iritani, A. and Niwa, K. (1977). Capacitation of bull spermatozoa and fertilization in vitro of cattle follicular oocytes matured in culture. J. Reprod. Fert. 50, 119–121.

Jagiello, G. M., Miller, W. A., Ducayen, M. B. and Lin, J. S. (1974). Chiasma frequency and disjunctional behavior of ewe and cow oocytes matured in vitro. Biol. Reprod. 10, 354–363.

Leibfried, L. and First, N. L. (1979). Characterization of bovine follicular oocytes and their ability to mature in vitro. J. Anim. Sci. 48, 76–86.

Longo, F. J. (1975). Ultrastructural analysis of artificially activated rabbit eggs. J. Exp. Zool. 192, 87–111.

Lorton, S. P. and First, N. L. (1979). Hyaluronidase does not disperse the cumulus oophorus surrounding bovine ova. Biol. Reprod. 21, 301–308.

Mahajan, S. C. and Menge, A. C. (1966). Influence of uterine environment on the fertilizing capacity of sperm in cattle. J. Anim. Sci. 25, 1083– 1086.

Miyamoto, H. and Ishibashi, T. (1975). The role of calcium ions in fertilization of mouse and rat eggs in vitro. J. Reprod. Fert. 45, 523–526.

Nalbandov, A. V. and Casida, L. E. (1942). Ovulation and its relation to estrus in cows. J. Anim. Sci. 1, 189–198.

Newcomb, R., Christie, W. B. and Rowson, L.E.A. (1978). Birth of calves following in vivo fertilization of oocytes removed from follicles and matured in vitro. Vet. Rec. 102, 461–462.

Norberg, H. S. (1973a). Ultrastructural aspects of the pre-attached pig embryo: Cleavage and early blastocyst stages. Z. Anat. Entwickl.-Gesch. 143, 94–114.

Norberg, H. S. (1973b). Ultrastructure of pig tubal ova: The unfertilized and pronuclear stage. Z. Zellforsch. 141, 103–122.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17, 208–212.

Russe, I. (1975). Unfertilized sheep eggs. J. Reprod. Med. 14, 200–204.

Salomon, Y., Amir, Y., Azulai, R. and Amsterdam, A. (1978). Modulation of adenylate cyclase activity by sulfated glycosaminoglycans, I. Inhibition by heparin of gonadotrophin-stimulated ovarian adenylate cyclase. Biochem. Biophys. Acta 544, 262–272.

Satoh, E., Iritani, A. and Nishikawa, Y. (1977). Factors involved in maturation of pig and cattle follicular oocytes cultured in vitro. Jap. J. Anim. Reprod. 23, 12–18.

Senger, P. L. and Saacke, R. G. (1970). Unusual mitochondria of the bovine oocyte. J. Cell. Biol. 46, 405–408.

Shea, B. F., Latour, J.P.A., Bedirian, K. N. and Baker, R. D. (1976). Maturation in vitro and subsequent penetrability of bovine follicular oocytes. J. Anim. Sci. 43, 809–815.

Singh, J. P., Babcock, D. F. and Lardy, H. A. (1978). Increased calcium-ion influx is a component of capacitation of spermatozoa. Biochem. J. 172, 549–556.

Sreenan, J. (1970). In vitro maturation and attempted fertilization of cattle follicular oocytes. J. Agr. Sci., Cambridge 75, 393–396.

Sutfin, L. V., Holtrop, M. E. and Ogilvie, R. E. (1971). Micro analysis of individual mitochondrial granules with diameters less than 1,000 angstroms. Science 174, 947–949.

Szollosi, D. and Hunter, R.H.F. (1973). Ultrastructural aspects of fertilization in the domestic pig: Sperm penetration and pronucleus formation. J. Anat. 116, 181–206.

Szollosi, D. and Hunter, R.H.F. (1978). The nature and occurrence of the acrosome reaction in spermatozoa of the domestic pig, *Sus Scrofa*. J. Anat. 127, 33–41.

Thibault, C. (1966). La culture in vitro de l'oeuf de vache. Ann. Biol. Anim. Biochem. Biophys. 6, 159–164.

Thibault, C. (1967). Analyse comparée de la fécondation et de ses anomalies chez la brebis, la vache et la lapine. Ann. Biol. Anim. Biochem. Biophys. 7, 5–23.

Thibault, C., Gerard, M. and Menezo, Y. (1975). Acquisition par l'ovocyte de lapine et de veau du facteur de décondensation du noyau du spermatozoide fécondant (MPGF). Ann. Biol. Anim. Biochem. Biophys. 15, 705–714.

Thibault, C., Gerard, M. and Menezo, Y. (1976).

Attempts to mature calf oocytes in culture. In: Egg Transfer in Cattle. (L.E.A. Rowson, ed.). Commission of the European Communities, Luxenbourg. pp. 175–184.

Trimburger, G. W. and Davis, H. P. (1943). Conception rate in dairy cattle by artificial insemination at various stages of estrus. University of Nebraska College of Agriculture, Agricultural Experiment Station, Lincoln. Res. Bull. 129, 1–14.

Trounson, A. O., Willadsen, S. M. and Rowson, L.E.A. (1977). Fertilization and development capability of bovine follicular oocytes matured in vitro and in vivo and transferred to the oviducts of rabbits and cows. J. Reprod. Fert. 51, 321–327.

VonBregulla, K., Gerlach, U. and Hahn, R. (1974). Versuche zur extrakorporalen Reifung, Befruchtung und Embryoenzucht mit Rinderkeimzellen. Dtsch. Tierarztl. Wochenschr. 81, 465–470.

Wabik-Śliz, B. (1979). Number of cortical granules in mouse oocytes from inbred strains differing in efficiency of fertilization. Biol. Reprod. 21, 89–97.

Whittingham, D. G. and Siracusa, G. (1978). The involvement of calcium in the activation of mammalian oocytes. Exp. Cell Res. 113, 311–317.

Wincek, T. J., Parrish, R. F. and Polakoski, K. L. (1979). Fertilization: A uterine glycosaminoglycan stimulates the conversion of sperm proacrosin to acrosin. Science 203, 553–554.

Wintenberger-Torres, S. and Flechon, J. E. (1974). Ultrastructural evolution of the trophoblast cells of the preimplantation sheep blastocyst from day 8 to day 18. J. Anat. 118, 143–153.

Wolf, D. P., Nicosia, S. V. and Hamada, M. (1979). Premature cortical granule loss does not prevent sperm penetration of mouse eggs. Develop. Biol. 71, 22–32.

Wright, R. W., Anderson, G. B., Cupps, P. T. and Frost, M. (1976). Successful culture in vitro of bovine embryos to the blastocyst stage. Biol. Reprod. 14, 157–162.

Yanagimachi, R. and Usui, N. (1974). Calcium dependence of the acrosome reaction and activation of the guinea pig spermatozoon. Exp. Cell Res. 89, 161–174.

Zamboni, L. (1970). Ultrastructure of mammalian oocytes and ova. Biol. Reprod. Suppl. 2, 44–63.