BIOLOGY OF REPRODUCTION 27, 147–158 (1982)

Normal Development Following In Vitro Fertilization in the Cow

BENJAMIN G. BRACKETT,[1,2,3] DANIEL BOUSQUET,[2,4] MELINDA L. BOICE,[2,5] WILLIAM J. DONAWICK,[2] JAMES F. EVANS[2,6] and MICHAEL A. DRESSEL[2]

Department of Clinical Studies, New Bolton Center[2]
University of Pennsylvania School of Veterinary Medicine
Kennett Square, Pennsylvania 19348
and
Department of Obstetrics and Gynecology[3]
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19174

ABSTRACT

A repeatable procedure for fertilization of bovine ova in vitro is described. Oocytes were recovered from ovarian follicles or from oviducts near the time of ovulation following treatment of donors with pregnant mare's serum gonadotropin (PMSG) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). For in vitro capacitation semen was incubated, then high ionic strength treated and subsequently incubated in defined medium prior to insemination of oocytes. In one experiment frozen bull semen was successfully used. In experiments with 4 bulls (B, C, D, F), 34 (43.6%) of 78 ova and 13 (19.7%) of 66 follicular oocytes were fertilized in vitro. In the last series (spermatozoa from Bull F) the fertilization of 22 (62.9%) of 35 tubal ova was achieved. In vitro development proceeded to the 8-cell stage.

No fertilization in vitro followed use of one male (Bull E), even though his spermatozoa could penetrate zona-free hamster ova in vitro, and higher than usual bacterial contamination of his semen was implicated as the probable cause. Findings suggested vigorous progressive sperm motility and acrosome integrity to be important features of good sperm samples.

In one experiment a 4-cell stage embryo was transferred with the result that the recipient gave birth to a normal bull calf on June 9, 1981. The first calf resulting from in vitro fertilization has been found to be completely normal.

INTRODUCTION

Prior to 1981, in vitro fertilization was accomplished in at least 14 mammalian species but documentation with resulting live offspring following embryo transfer was reported only for rabbits (Chang, 1959), mice (Whittingham, 1968), rats (Toyoda and Chang, 1974), and man (Steptoe and Edwards, 1978); (for additional references, see reviews by Bavister, 1981; Brackett, 1981; Wright and Bondioli, 1981). The process of sperm capacitation, which normally occurs in the female reproductive tract and renders sperm cells capable of fertilizing ova (Austin, 1951; Chang, 1951), has been a major technological barrier. Although in vitro fertilization was accomplished by sperm cells recovered from the uterine milieu of mated does as early as 1954 (Thibault et al., 1954), and birth of live offspring following in vitro fertilization was reported in 1959 (Chang, 1959) our understanding of the capacitation process remains incomplete. In spite of this, by 1975 it was clear that ejaculated rabbit spermatozoa could be in vitro capacitated to enable in vitro fertilization and development of resulting embryos into live offspring (Brackett and Oliphant, 1975). More recently, human offspring have also resulted from fertilization in vitro following in vitro insemination of ova with in vitro capacitated ejaculated, in contrast to epididymal, spermatozoa and progress has been made toward developing a clinically useful procedure (Edwards et al., 1980; Johnston et al., 1981; Lopata et al., 1980; Steptoe and

Accepted April 27, 1982.
Received April 12, 1982.

[1] Correspondence: Dr. Benjamin G. Brackett, University of Pennsylvania School of Veterinary Medicine, New Bolton Center, 382 W. Street Road, Kennett Square, PA 19348.

[4] Present address: Centre de Recherche en Reproduction Animale, Ecole de Medicine Veterinaire, Universite de Montreal, C. P. 5000 Saint-Hyacinthe, Quebec.

[5] Present address: School of Life and Health Sciences, University of Delaware, Newark, DE 19711.

[6] Present address: Genetics Unlimited, Inc., West Grove, PA 19390.

Edwards, 1978; Steptoe et al., 1980; Trounson et al., 1981; Wood et al., 1981). Although embryo transfer in cattle, involving transfer of embryos from the uterus of a valuable donor cow into uteri of less valuable recipients for development, has been widely practiced in veterinary medicine during the last decade (Seidel, 1981), efforts toward in vitro fertilization and embryo transfer in ungulates were termed "notoriously unsuccessful" in 1980 by Blandau, who cautioned against equating potential developmental risks of embryo transfer with those that remain largely unknown for in vitro fertilization and embryo transfer (Blandau, 1980).

Investigations of morphological and physiological aspects of bovine fertilization and early development, along with earlier in vitro efforts in this laboratory (Brackett et al., 1977, 1978b, 1980a,b), led to achievement of normal pregnancy following bovine fertilization in vitro (Brackett et al., 1981a). Goals of this research are to develop practical procedures for cattle breeding and, at the same time, to provide a useful model for more rapidly advancing our understanding of fertilization and development in man and other mammals. This report documents the development of a repeatable procedure for bovine fertilization in vitro and the birth and normal development of the first calf resulting from in vitro fertilization.

MATERIALS AND METHODS

Cattle

Twenty grade Holstein, and 2 Jersey (Exps. 49–1 and 49–8) heifers were used as ovum donors. Seven additional cows were used as recipients of in vitro fertilized embryos. Bull semen collected via an artificial vagina and frozen semen (Exp. 49–74a) were provided by Atlantic Breeders Cooperative of Lancaster, PA. At least one experiment was carried out with each of 4 Holstein bulls (B, C, D and F), and a Guernsey bull (E). Experiments with spermatozoa from Bulls B, C and D were carried out in the Fall of 1980, and those with spermatozoa from Bulls E and F were done in the Spring of 1981. All bulls were purebred, registered, with known blood types, and of superior type that led to their selection for use in artificial insemination (A.I.) programs. The bulls were also proven to be highly fertile through their A.I. usage.

Media and Conditions for In Vitro Fertilization

These experiments were carried out with the defined medium (DM) and in a manner similar to those previously reported from this laboratory (Brackett and Oliphant, 1975; Brackett et al., 1980b, 1981a). Media contained 50 IU sodium penicillin G per ml unless noted to the contrary. High ionic strength (HIS) medium was prepared with 100 IU penicillin and 100 μg streptomycin per ml; addition of 34 mg enzyme grade NaCl to 10.0 ml of DM resulted in a measured osmolality (Model 5130 Vapor Pressure Osmometer, Wescor, Inc., Logan, UT) of approximately 380 mOsm/kg for the HIS DM. Defined medium and HIS DM were usually equilibrated with 5% CO_2, 8% O_2, balance N_2; however, 5% CO_2, 5% O_2, balance N_2 was used in Exps. 49–45a and b; and 5% CO_2 in air was used in Exps. 49–54a and b, and 49–61. Incubations longer than 5 h were under paraffin oil (Paraffin Oil, Saybolt Viscosity 125/135, Fisher Scientific Co., Fair Lawn, NJ) and a moist 5% CO_2 atmosphere. For zygote culture the medium was 10% serum solution, composed of acidic saline (Chang, 1959; Hammond, 1949) supplemented with homologous luteal phase serum (heat-treated to destroy complement, 56°C for 30 min) from individual ovum donors and 2.5 mg glucose per ml, pH 7.1, under paraffin oil and an air atmosphere. On occasion, efforts were made to extend embryonic development in vitro by use of Ham's F-10 medium (Ham, 1963) supplemented with 10% (v/v) heat-treated cow serum (Wright et al., 1976a,b).

Preparation of Sperm and In Vitro Insemination

Following collection semen was incubated for up to 3 h. Within this interval it was transported by car (approximately 1 h during which the thermos temperature fell from 38°C to approximately 35°C) from the site of collection to the laboratory where incubation at 38°C was resumed. All subsequent steps were carried out in a 38°C room. An aliquot of 0.1 ml semen was diluted to 2.0 ml with HIS DM (for HIS treatment), incubated 5 min, then centrifuged at approximately 330 × *g* for an additional 5 min. The supernatant liquid was discarded and the sperm cells (approx. 3×10^7) were resuspended in 1.0 ml DM for an additional preincubation interval of 45 min to 5 h (under air). Following this an aliquot containing approximately 10^6 spermatozoa was transferred into 4.0 ml of DM containing freshly recovered oocytes for fertilization. This procedure was routinely carried out in experiments with semen from Bulls B, C and D. Alternatively, 2 to 7×10^6 sperm cells were diluted (following the HIS treatment) to 4.0 ml with DM, covered with paraffin oil, and incubated under a moist 5% CO_2 atmosphere for up to 23 h prior to addition of oocytes directly into such sperm suspensions. This procedure was followed in experiments with semen from Bulls E and F. Inseminations and all incubations of ova were carried out in small glass, sealable, tissue culture dishes (30 mm i.d., 12 mm deep).

Observations of Semen and Spermatozoa

All semen used was judged to be of good quality. Routine bacterial cultures to verify acceptable (low) levels of contamination of ejaculates were done. Increase in acidity of semen as a result of incubation was determined by pH meter. Sperm concentrations were routinely determined by counting in a hemacytometer.

Sperm motility was examined during the semen incubation, upon dilution with HIS DM and at the

time of insemination. Data included percentages of total sperm cell populations that were motile and percentages of sperm cells that were progressively motile. Attention was also paid to the character or vigor of motility observed. In some experiments attention was paid to acrosome morphology by phase-contrast and interference-contrast microscopy following fixation of sperm cells with buffered formalin (10% Buffered Formalin Phosphate, Fisher Scientific Co., Fair Lawn, NJ) and also after staining (Wells and Awa, 1970). Percentages of normal sperm cells with or without acrosomes and sperm cells with abnormal acrosomes were noted after examining 200 sperm cells in each instance. Additional studies included observations of the longevity of sperm motility and the functional ability to penetrate zona-free hamster ova, an approach originally used for study of human spermatozoa (Yanagimachi et al., 1976), following procedures recently developed for bull sperm (Bousquet and Brackett, 1982; Brackett et al., 1982).

Treatment of Ovum Donors

Ovum donors were selected between estrous cycle Days 8 and 13. Blood (approx. 50 ml) was taken from the jugular vein of each donor for pretreatment serum. Intramuscular injection of 1500 IU pregnant mare's serum gonadotropin (PMSG, Gestyl, Organon, Inc., West Orange, NJ) was followed 72 h later by an i.m. injection of 40 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse, The Upjohn Co., Kalamazoo, MI). Onset of behavioral estrus (standing heat) was recorded following routine observations at successive intervals of no more than 4 h beginning approximately 36 h after the prostaglandin injection. Surgery was scheduled for ovum recovery 20 to 30 h after the onset of estrus or, in the absence of standing heat, at an interval 65 to 70 h after prostaglandin injection.

Oocyte Recovery

Oocytes were recovered both from oviducts and from ovarian follicles. Upon inspection at surgery, if ovarian follicles were present and corpora hemorrhagica were absent, ovariectomy was performed. If ovulation had occurred, ovario-salpingectomy was carried out. Excised ovaries with or without oviducts (Fallopian tubes) were placed directly into 50 to 100 ml of warm saline in a plastic container, covered with a tight-fitting lid, and returned to a small water bath at 38°C for transport (5 min by car) to the 38°C tissue culture room where the experiments were carried out. Ovulated ova were obtained by flushing of oviducts at least 2 times with 10 ml of DM. Follicular oocytes were aspirated with heparinized DM (5 units heparin/ml DM). Upon identification under a dissecting microscope (at 10 to 70 X) oocytes were pooled for a brief interval in 4.0 ml DM under paraffin oil prior to insemination.

In Vitro Culture and Embryo Transfer

Oocytes were cultured with spermatozoa beginning within 80 min after ovariectomy and continuing for 18 to 24 h in DM under paraffin oil and a moist 5% CO_2 atmosphere. Observation for pronuclear stage development was routinely made, but only superficially, at the end of the initial incubation period. Ova were transferred from the sperm suspension into 10% serum solution under air for an additional interval, usually 22 to 24 h. Then surrounding sperm cells and remaining follicular cells were gently teased from zonae pellucidae under 10 to 70 X magnification to facilitate observation for cleavage. Decisions were made at this point regarding continued efforts to sustain in vitro development, or to transfer an embryo for continued in vivo development if an appropriately synchronous recipient cow was available. In some experiments, ova that did not cleave during the initial 40 h following insemination were carefully studied for possible sperm penetration and/or pronuclear development by light microscopy in the fresh state and after staining with 1.0% aceto-orcein following fixation with acetic acid-ethanol (1:3, v/v).

Surgical Procedures

Procedures involving surgery for ovum recovery were performed as previously described (Brackett et al., 1980b) but with ovaries, and usually oviducts also, removed with hemostasis obtained by a TA90 surgical stapling instrument. Two departures from this routine involved aspiration of follicles via standing laparotomy (Exp. 49–45a) and ovariectomy by the same approach (Exp. 49–54a). Routine physical examinations, hematological evaluations, and postoperative care were provided.

For embryo transfer, optimal abdominal access with appropriate reproductive tract exposure (Brackett et al., 1980b) was required. The oviduct that was ipsilateral to the developing corpus luteum was extended by the surgeon to enable the fimbriated end to encompass the tip of a finely drawn, fire-polished and siliconized Pasteur pipette containing the embryo in a small column of medium between air bubbles and additional small amounts of medium above and below the embryo. The pipette was inserted approximately 2.5 cm into the oviductal ampulla for deposition of approximately 50 μl of 10% serum solution containing the embryo along with a small amount of air. Closure and postoperative care were routine.

Observations of Recipient Cows

Beginning between 40 and 50 days after embryo transfer, recipient cows were examined per rectum for diagnosis of pregnancy. The pregnant recipient was thoroughly examined at monthly intervals throughout gestation to follow the progress of fetal development. During the last 2 weeks of gestation special housing was provided in an indoor box stall with an adjoining outisde pen and signs of imminent parturition were monitored with daily, then twice daily physical examinations. Delivery was assisted through provision of routine veterinary obstetrical care.

Observations of the Calf

The calf resulting from in vitro fertilization was observed for normal behavior at birth, including standing, walking and suckling. Intensive veterinary medical attention has been provided, and frequent observations of behavior, growth and sexual development (Curtis and Amann, 1981) have been made throughout neonatal and prepuberal stages. Blood testing for confirmation of genetic parentage was

performed by a commercial laboratory (Dr. Jerry Caldwell, Immunogenetics Laboratory, Dept. of Animal Science, Texas A&M University, College Station, Texas, in cooperation with the Holstein-Friesian Assoc. of America, Brattleboro, VT).

RESULTS

In Vitro Fertilization, Ovum Recovery, and Early Embryonic Development

Data involving oocytes from 15 cows are reported in categories according to the 4 bulls that provided semen (Table 1). Oocytes were from different cows in each experiment. Overall, 34 (43.6%) of 78 tubal ova and 13 (19.7%) of 66 follicular oocytes exhibited light microscopic evidence of fertilization in these experiments. At least one fertilization took place in 11 of 15 experiments and by this criterion 7 of 8 experiments, involving semen from 3 different bulls, were successful when ovulated (tubal) ova recovered from oviducts were inseminated in vitro. In the last series, with semen from Bull F, 22 (62.9%) of 35 ovulated ova from 4 cows were fertilized in vitro.

The onset of estrus for 9 of the 15 donors (Table 1) was between 36 and 48 h after $PGF_{2\alpha}$ injection, while for 2 cows this interval was extended to 58 and 68 h. Ova from one of the latter (Exp. 48–87) were found to have reached the 4-cell stage by 28 h after in vitro insemination, an earlier time than anticipated. Three ovum donors (Table 1) did not exhibit behavioral estrus and there was no evidence of ovulation between 65 and 70 h after their prostaglandin treatments. One cow that did exhibit behavioral estrus (Exp. 49–61) had not ovulated when surgery was performed 66 h after the prostaglandin treatment, or 26 h after onset of estrus. In another case (Exp. 49–54a) ovulation had occurred 20 h after onset of estrus, and it was not possible to recover tubal ova due to inability to remove the oviducts with ovaries via standing laparotomy. Two additional heifers that did not exhibit estrous behavior were determined unresponsive to the treatment, i.e. inactive ovaries palpated per rectum, and were not operated on.

Tubal ova were recovered from donors between 26 and 32 h after the onset of estrus. In two experiments follicular oocytes were obtained, apparently prior to ovulation, when surgery was performed at 22 to 24 h after the onset of estrus (Exps. 49–8, 49–45b). Data

TABLE 1. Bovine fertilization in vitro with in vitro capacitated spermatozoa.[a]

Bull and exp. no.	Oocyte donor's onset of estrus (h after $PGF_{2\alpha}$)	Duration (h) of sperm incubations[b]		No. tubal (T) follicular (F) oocytes inseminated	No. oocytes fertilized	No. observations of developmental stages (h post-insemination)			
		In semen	*In DM*			pn[c]	2-cell	4-cell	8-cell
Bull B									
48–57	38	3	4.5	6T	4	1(42)	3(42)	1(67)	1(67)
				2F	0				
48–67	36	3	1–2	7T	5	1(46)	4(46)	3(70)	
				2F	0				
48–72	No estrus	3	2.25	5F	3	2(20)	1(41)	1(47)[d]	
						1(47)	1(47)		
48–83	No estrus	2.25	1	2F	0				
48–87	58	3	1.25	3T	2			2(28)	
48–93	39	3	3	14T	0				
Summary: Oocytes from 6 cows and sperm from Bull B.				30T	11 (36.7%)				
				11F	3 (27.3%)				

Bull C									
49–8	43	2	3.5	7F	1				1(70)
			2.5	6F	0				
Bull D									
49–1	39	1–2	45 min–1.5 h	13T	1	1(52)			
				3F	0				
Bull F									
49–45a[e]	No estrus	1.5	2	12F	0				
49–45b	42	2.5		5F	0				
49–54a	48	2.5	2	2F	1				1(66)
49–54b	68	2.75	23	19T	16		1(45)	15(45)	11(54)
				2F	0				
49–61	40	2.25	2	12F	7	1(44)	1(21)	5(44)	
49–74a	39	2.5	3	6T	1		1(43)		
		1 min[f]	0.25	3F	0				
				5T	1		1(43)		
49–74b	39	2.5	5	5T	4	2(40)	2(40)		
				3F	1		1(68)		
Summary: Oocytes from 7 cows and sperm from Bull F.				35T	22 (62.9%)		1(40)		
				39F	9 (23.1%)				

[a]Fertilization was assessed by light microscopy of developmental stages indicated.

[b]*In semen* – interval prior to and *in DM* – interval following HIS treatment.

[c]*Pronuclear stage*, including penetrated ova with decondensing sperm heads detected after staining.

[d]Developed into normal bull calf (Virgil).

[e]*a* and *b* (following the same exp. no.) designate experiments in which ova were from different cows (as in other cases) but semen was from the same ejaculate.

[f]Frozen semen was used; the HIS treatment was begun within 1 min post-thaw.

indicating the ability of oocytes to undergo fertilization in vitro, coupled with the findings of tubal ova still surrounded by cumulus oophorus at 26 h following the onset of estrus (Exp. 49–1) and corpora hemorrhagica at 20 h (Exp. 49–54a), are consonant with the estimation of a range of 19 to 27 h after the onset of estrus for ovulations to begin in response to these treatments.

Approximately 65% of ovulated ova were recovered from tubal flushings as determined after counting corpora hemorrhagica. Approximately 75% of follicules aspirated yielded oocytes for study. Tubal ova were more readily fertilized in vitro than were follicular oocytes (Table 1). In Exp. 48–72, three additional follicular oocytes, devoid of surrounding cumulus oophorous cells, were recovered from small follicles 2 to 5 mm in diameter, and inseminated in vitro but no evidence of fertilization resulted. Since such oocytes were not considered candidates for normal fertilization, they were not accounted for in Table 1. Subsequent efforts to obtain normal fertilization in vitro included avoidance of obviously immature or atretic oocytes recovered from follicles of less than 8 mm in diameter. Procedural influences might also affect the potential for fertilization since ovulated ova were recovered from oviducts before follicular oocytes were aspirated. A beneficial influence offered by rapid ovum recovery was suggested by the fact that the 2 tubal ova that were not fertilized in Exp. 48–67 were found following the second flushes of oviducts.

Although precise timing for attainment of developmental stages was not defined, oocytes found in the pronuclear or sperm penetration stages at postinsemination intervals of 40 h and later could not be considered normal. Such an ovum (Exp. 49–61) was found to be penetrated by 2 sperm cells, one of which was apparently found in the process of penetration (Fig. 1).

Results demonstrate that bovine fertilization can occur under defined and repeatable conditions and that development can proceed normally in culture to the 8-cell stage before 70 h after insemination (Table 1). In Exp. 48–67 the 2-cell embryos were cultured in serum-supplemented Ham's F-10 medium. Devel-

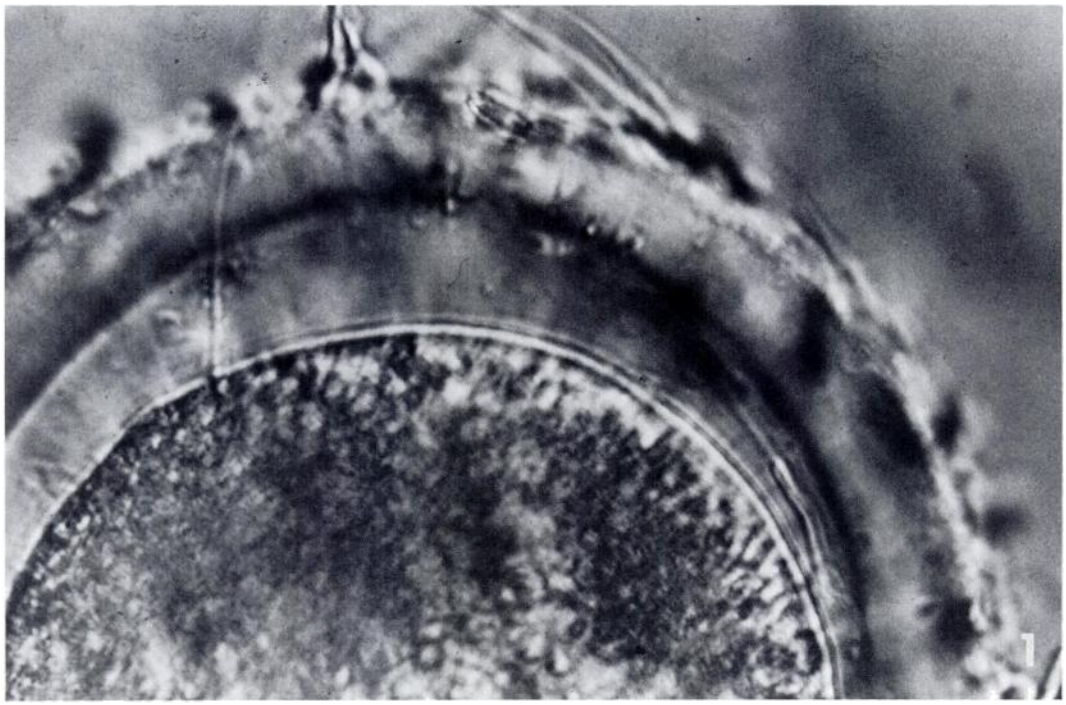

FIG. 1. An unusual cow oocyte found with penetrating sperm head attached to vitellus with tail still within the penetration pathway through the zona pellucida. Another sperm cell is inside the perivitelline space (from Exp. 49–61, photographed under interference-contrast at X550).

opment proceeded to the 4-cell stage but degeneration followed. In other experiments, 14 embryos reached the 8-cell stage (Fig. 2) when cultured in 10% serum solution. Additional efforts to sustain development of 8-cell embryos by subsequent culture in serum-supplemented Ham's F-10 were unsuccessful. Chromatin was demonstrable in each blastomere of 8-cell stage embryos stained following cessation of development in culture.

Observations of Semen and Spermatozoa

Variability in fertilization results was prominently associated with semen contributed by different bulls. Improvement in technique also contributed to better results obtained with spermatozoa of Bull F. The pH of semen fell from neutrality to somewhere between 5.5 and 6.0 during the initial incubations and occasionally a total absence of sperm motility was observed prior to dilution in HIS DM. After HIS treatment, motility improved and was subsequently maintained throughout the DM incubation. A high degree of vigor and characteristic progressive motility suggestive of greater propulsive thrust was also seen at the time of insemination. The ejaculate from Bull F that was used in Exps. 49–45a and b (Table 1) was suboptimal in that the sperm concentration was lower and sperm motility was less vigorous than usual. A comparison of motility (averages) at the time of insemination for sperm of Bull B and sperm of Bull F revealed a higher percentage of motile sperm cells (total motility) for Bull F (55.7% vs. 48.3%) but a somewhat lower percentage of progressively motile sperm cells for Bull F than for Bull B (33.3% vs. 44.3%).

Comparison of acrosome morphology re-

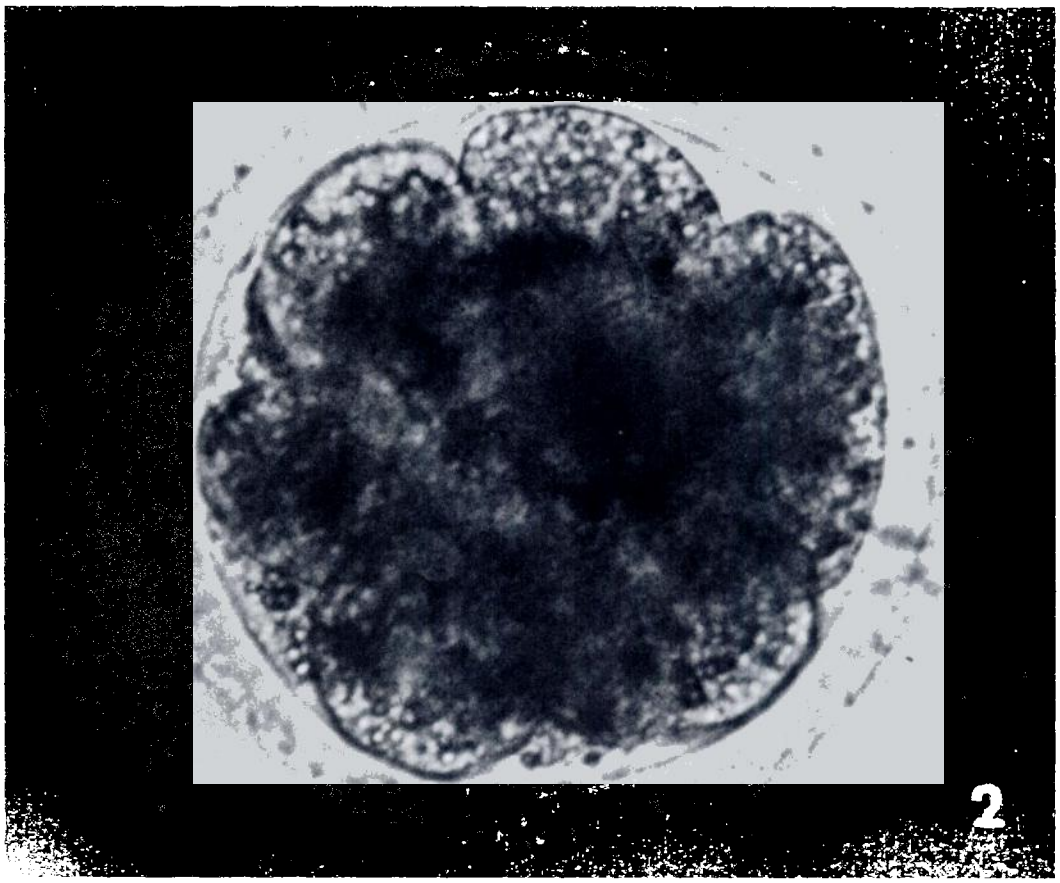

FIG. 2. An 8-cell stage bovine embryo fertilized in vitro. The 8-cell stage was first observed in this experiment (49–8) at 70 h after in vitro insemination (photographed at approximately X 200).

vealed higher percentages (averages) of normal sperm cells with acrosomes for Bull F than for Bull B. Thus, morphologically normal spermatozoa with intact acrosomes, with abnormal acrosomes, and without acrosomes after HIS treatment averaged 84%, 9% and 7%, respectively, for Bull F and 33%, 30%, and 37%, respectively, for Bull B; assessment of the same parameters at insemination revealed 62%, 13% and 25%, respectively, for Bull F and 20%, 39% and 41%, respectively, for Bull B. Higher percentages of morphologically normal sperm cells with intact acrosomes may be correlated with superior in vitro fertilizing ability.

In 3 experiments with semen from Bull E, carried out as for Bull F (Table 1), no fertilization followed in vitro insemination of 25 tubal and 12 follicular oocytes. The oocytes acquired shrunken vitelli with vesiculation and bacterial contamination was noted at the end of some of the in vitro cultures. Use of the zona-free hamster ovum test for bull spermatozoa revealed that semen incubation of 60–140 min, followed by HIS treatment and incubation in DM for another hour, led to sperm penetration of 13 (50%) of 26 hamster vitelli. However, when the DM incubation was extended to 22 h, spermatozoa from Bull E did not survive. An additional experiment revealed similar abilities of sperm samples from Bull E and from Bull F to interact with zona-free hamster ova when incubation intervals were limited to a few hours; 14 (87.5%) of 16 and 13 (76.5%) of 17 vitelli were penetrated by spermatozoa from Bull E and Bull F, repectively. However, after only 7 h in DM, following HIS treatment, a difference in motility became apparent and by 24 h all sperm cells from Bull E were dead, while those from Bull F were still vigorously motile and functional as evidenced by penetration of 6 (75%) of 8 zona-free hamster ova in another test. These observations led to the finding that higher, but still acceptable (for A.I. use), levels of bacterial contamination were associated with ejaculates obtained from Bull E than were associated with ejaculates from Bull F.

In Exps. 49–61 and 49–74b (Table 1) 11 (100%) of 11 and 16 (88.9%) of 18 zona-free hamster ova were penetrated, respectively, when spermatozoa from the same samples that fertilized high proportions of bovine ova were used.

Normal Development Following In Vitro Fertilization

In Exp. 48–72 (Table 1) 2 ova were in the pronuclear stage by 20 h and one of these reached the 2-cell stage by 41 h post-insemination. Both were transferred into freshly prepared 10% serum solution and at 47 h post-insemination the pronuclear ovum had cleaved and the 2-cell stage ovum had reached the 4-cell stage. The 4-cell stage embryo was transferred (at 1430 h on September 5, 1980) into the left oviduct of a recipient cow (No. 376, Penny) that had exhibited behavioral estrus 2 days earlier. This Holstein recipient had previously given birth to a Charolais calf by embryo transfer. She was diagnosed pregnant at 50 days and normal pregnancy was confirmed at 60 and 90 days. An uneventful pregnancy with normal fetal development followed.

Positioning of the fetus, progressive relaxation of the pelvic ligaments, udder and cervical changes of the surrogate dam developed normally. A 45 kg male calf (Fig. 3) was delivered with assistance at 1745 h on June 9, 1981. The calf was standing within 15 min and was consuming colostrum within the first hour after birth. Close observation during the neonatal and prepubertal months revealed no abnormalities in behavior or development. Measurements of weight gain and increases in scrotal circumference have been normal, i.e. similar to the averages reported for Holstein bulls.

Results of blood testing confirmed parentage of the calf to be compatible with the genetic combination resulting from in vitro fertilization of an ovum from the donor (No. 862) with a spermatozoon from the sire (Bull B) and incompatible with fertilization of an ovum from the recipient cow (No. 376, Penny) by a spermatozoon from Bull B.

Six additional embryo transfers were carried out. Although the recipients were within one day of precise synchrony with ovum donors the quality of the recipients, all of which were nonparous heifers, was considered poor since each had failed to become pregnant following previous transfers of in vivo fertilized bovine embryos into their uteri in the course of clinical embryo transfer procedures. Two embryos, one in the 4-cell stage and one in the 8-cell stage from Exp. 49–54b, one embryo in the 4-cell stage from Exp. 49–61, and three embryos in

FIG. 3. Surrogate dam (Penny) and the first calf resulting from in vitro fertilization (Virgil) just after birth.

the 2-cell stage from Exps. 49–74a and b were transferred into separate recipients, and although subsequent estrous behavior was not seen in some, none remained pregnant beyond 60 days.

DISCUSSION

Reports from other laboratories have described efforts to obtain in vitro fertilization in the cow (Bregulla et al., 1974; Iritani and Niwa, 1977), but development of a repeatable procedure for in vitro fertilization in this, or in any other large domestic animal species has not been forthcoming (Bavister, 1981; Brackett, 1981; Blandau, 1980; Wright and Bondioli, 1981). Normal development of 2- and 4-cell stage embryos after in vitro insemination of bovine ova with in vitro capacitated bull spermatozoa was documented by electron microscopy (Brackett et al., 1977, 1978b, 1980b). Similar treatments enabled bull spermatozoa to penetrate zona-free hamster ova (Bousquet and Brackett, 1982; Brackett et al., 1982) and bovine oocytes obtained at slaughter and at surgery following superovulation (Brackett et al., 1980a,b, 1981a). The methods for in vitro capacitation of bull spermatozoa in the present work represent an extension of these and earlier efforts in the mouse (Oliphant and Brackett, 1973a) and with rabbits (Brackett and Oliphant, 1975; Oliphant and Brackett, 1973b) through which it became apparent that treatment of spermatozoa with a medium of high ionic strength facilitated the removal of inhibitory or membrane stabilizing factors (Oliphant and Eng, 1981; Reyes et al., 1975) to allow spermatozoa to fertilize ova in vitro. This process was found to be more easily effected for cauda epididymal spermatozoa than for ejaculated spermatozoa of the rabbit as evidenced by higher proportions of ova fertilized and by more normal in vitro development of resulting embryos (Brackett et al., 1978a). In vitro fertilization in laboratory rodents involves use of epididymal spermatozoa which respond similarly to human ejaculated spermatozoa in that capacitation occurs more readily during preincubation in defined media (Yanagimachi et al., 1976) Now, preparation of bull spermatozoa for fertilization in vitro can also be

accomplished under defined and repeatable conditions.

Present data reflected variability in fertilization in vitro by different males. This was not surprising in light of previous findings in experiments with rabbit bucks (Brackett and Oliphant, 1975) and with bulls (Brackett et al., 1980a,b, 1982; Bousquet and Brackett, 1982). Efforts to tailor conditions for in vitro capacitation of spermatozoa from individual males through guidance afforded by ancillary studies, e.g. observations of sperm motility, longevity and ability to penetrate zona-free hamster ova and/or the zona pellucidae of follicular oocytes, may be necessary to assure the achievement of consistently high in vitro fertilization rates. Combinations of data including in vitro fertilization results should yield significant information for assessment of sperm fertilizing ability and, as applied in preliminary human work (Overstreet et al., 1980; Trounson et al., 1980), a better understanding of idiopathic infertility.

An earlier impression was that polyspermy in the cow was probably rare due to a strong zona block (Brackett et al., 1980b). Penetration of an oocyte by two sperm cells (Fig. 1) might have resulted from an unresponsive ooplasm or, alternatively, from a sperm cell able to penetrate but unable to initiate activation with the resulting zona reaction. Any advantage offered by reduced oxygen tension was not apparent in this study. Development to the 4-cell stage within 28 h after insemination (Exp. 48–87) was premature judging from earlier in vivo estimates (Hamilton and Laing, 1946), but in vitro fertilization provides a means for more accurately defining temporal development than has been possible previously. In light of encouraging results with culture, especially for later stages, of bovine embryos (Wright et al., 1976a,b) the inability of serum-supplemented Ham's F-10 to sustain in vitro development was disappointing. However, similar culture conditions to those found effective in early work of Chang (1959) that led to development of a repeatable procedure for rabbit in vitro fertilization (Brackett and Williams, 1965, 1968), i.e. acidic saline (Chang, 1959; Hammond, 1949) supplemented with homologous serum and glucose, proved effective in supporting bovine embryonic development to the 8-cell stage.

Beyond research and assessment of sperm fertilizing ability, additional refinements are needed for practical application of this new technology in animal breeding. Better means for recovery of oocytes, e.g. follicular aspiration by laparoscopy (Dukelow, 1978; Maxwell and Kraemer, 1980; Rioux et al., 1980), will be necessary. The possibility that normal oocytes might be routinely recovered from ovarian follicles with ensuing normal development gains support from the present work in which the bull calf resulted from in vitro fertilization of a follicular ovum recovered from a cow that did not exhibit behavioral estrus. Additional research is also indicated to develop culture conditions to sustain in vitro development beyond the 8-cell stage. However, uterine transfer may be possible with current technology since the first successful bovine embryo transfer involved recovery of an 8-cell stage embryo from the uterus and its transfer into a recipient's uterine horn near the uterotubal junction (Willett et al., 1951).

The further development and application of in vitro fertilization in cattle should provide information helpful in better management of problems related to human fertility and in efforts to develop procedures for human fertilization in vitro. The potential of the bovine model is supported by several considerations. Ejaculated spermatozoa can be capacitated in vitro to obtain fertilization of ova recovered from ovarian follicles. The cow has a nine-month gestation period and normally bears a single offspring. The long gestation might allow expression of teratology as a result of insults that would be reflected in increased embryonic wastage or fetal resorption in litter-bearing laboratory animals. Obviously, experimentation in the cow is associated with less formidable ethical barriers than are inherent in human research. The bioassay for endocrinological events associated with ovulation timing, provided by the onset of behavioral estrus in the cow, and the desirability for transfer of in vitro fertilized embryos into surrogate dams other than the ovum donor also favor the advancement of in vitro fertilization technology in the bovine species.

This technology potentially offers a new dimension in animal breeding beyond artificial insemination and embryo transfer procedures currently practiced (Brackett, 1981; Seidel, 1981). By in vitro fertilization and embryo transfer it should be possible to extend the fertile life of valuable animals from which normal ova can be obtained; these might include immature or very old animals or those

that are infertile as a result of pathological involvement of the reproductive tract that precludes in vivo fertilization and embryonic development. By in vitro fertilization each of several ova from a valuable cow might be fertilized by spermatozoa from different bulls to produce half-sibling embryos, and hence, half-sibling offspring after embryo transfer. In vitro fertilization also provides a means to maximize the efficient usage of semen from valuable bulls. By bringing fewer sperm cells into contact with many ova, the potential of a good bull to sire offspring might, in theory at least, be expanded 1000-fold over that afforded through artificial insemination. Hence, rather than 50,000 offspring (Foote, 1981), implementation of in vitro fertilization technology might enable a bull to sire 50 million calves in a single year! Other rapidly developing technologies (Brackett et al., 1981b), especially frozen storage of ova and embryos, fractionation of male- and female-producing spermatozoa, and a variety of approaches to genetic engineering can be anticipated to complement and extend the practical applications presently envisioned for in vitro fertilization in cattle.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Harry Roth, Floyd Wideler, and Charles Allen of Atlantic Breeders Cooperative, Lancaster, PA, and Nancy Ailes, Dennis Byrne, Tracy Higgins, Mark Hodgson, Lynne Klunder, Ronnie Krassenstein, Catherine Larmore, Bruce Leydig, Barbara Polin, Dawn Polin, Ginger Topkis, and Drs. G. Auman, T. Blanchard, J. Fetrow, L. Klein, R. McFeely, B. Reid, D. Richardson, A. Rushmer, L. Soma, R. Steele, and E. Tulleners of New Bolton Center for cooperation, assistance, and encouragement that made this research possible. This work was supported by NIH Grant HD09406 from the National Insitute of Child Health and Human Development and by the Medical Research Council of Canada (Fellowship to D. B.).

REFERENCES

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

Bavister, B. D. (1981). Analysis of culture media for in vitro fertilization and criteria for success. In: Fertilization and Embryonic Development In Vitro (L. Mastroianni, Jr. and J. Biggers, eds.). Plenum Press, New York, pp. 61–79.

Blandau, R. J. (1980). In vitro fertilization and embryo transfer. Fertil. Steril. 33:3–11.

Bousquet, D. and Brackett, B. G. (1982). Penetration of zona-free hamster ova as a test to assess fertilizing ability of bull sperm after frozen storage. Theriogenology 17:199–213.

Brackett, B. G. (1981). Applications of in vitro fertilization. In: New Technologies in Animal Breeding (B. G. Brackett, G. E. Seidel, Jr. and S. M. Seidel, eds.). Academic Press, New York, pp. 141–161.

Brackett, B G., Bousquet, D., Boice, M. L., Donawick, W. J. and Evans, J. F. (1981a). Pregnancy following cow in vitro fertilization. Biol. Reprod. 24 (suppl 1) 173:190A.

Brackett, B. G., Cofone, M. A., Boice, M. L. and Bousquet, D. (1982). Use of zona-free hamster ova to assess sperm fertilizing ability of bull and stallion. Gamete Res. 5:217–227.

Brackett, B. G., Evans, J. F., Donawick, W. J., Boice, M. L. and Cofone, M. A. (1980a). In vitro penetration of cow oocytes by bull sperm. Arch. Androl. 5:69–71.

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 Ann. Mtg. Soc. Study Reprod., Abstr. 86, 56–57.

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., Oh, Y. K., Evans, J. F. and Donawick, W. J. (1980b). Fertilization and early development of cow ova. Biol. Reprod. 23:189–205.

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

Brackett, B. G., Seidel, G. E., Jr. and Seidel, S. M., Eds. (1981b). New Technologies in Animal Breeding. Academic Press, New York, 268 p.

Brackett, B. G. and Williams, W. L. (1965). In vitro fertilization of rabbit ova. J. Exp. Zool. 160: 271–281.

Brackett, B. G. and Williams, W. L. (1968). Fertilization of rabbit ova in a defined medium. Fertil. Steril. 19:144–155.

Bregulla, K., Gerlach, U., Hahn, R. (1974). Versuche zur extrakorporalen reifung, befruchtung und embryonenzucht mit rinderkeimzellen. Tieraarztl. Wochenschr. 81:465–470.

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

Chang, M. C. (1959). Fertilization of rabbit ova in vitro. Nature (Lond.). 184:466–467.

Curtis, S. K. and Amann, R. P. (1981). Testicular development and establishment of spermatogenesis in Holstein bulls. J. Anim. Sci. 53: 1645–1657.

Dukelow, W. R. (1978). Laparoscopic research techniques in mammalian embryology. In: Methods in Mammalian Reproduction (J. C. Daniel, Jr., ed.). Academic Press, New York, pp. 437–460.

Edwards, R. G., Steptoe, P. C. and Purdy, J. M. (1980). Establishing full-term human pregnancy using cleaving embryos grown in vitro. Br. J. Obstet. Gynaecol. 87:737–756.

Foote, R. H. (1981). The artificial insemination industry. In: New Technologies in Animal Breeding. (B. G. Brackett, G. E. Seidel, Jr. and S.

M. Seidel, eds.). Academic Press, New York, pp. 13–39.

Ham, R. G. (1963). An improved nutrient solution for diploid Chinese hamster and human cell lines. Exp. Cell Res. 29:515–526.

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.

Hammond, J. (1949). Recovery and culture of tubal mouse ova. Nature (Lond.). 163:28.

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

Johnston, I., Lopata, A., Speirs, A., Hoult, I., Kellow, G. and duPlessis, Y. (1981). In vitro fertilization: the challenge of the eighties. Fertil. Steril. 36:699–706.

Lopata, A., Johnston, W.I.H., Hoult, I. J. and Spiers, A. L. (1980). Pregnancy following intrauterine implantation of an embryo obtained by in vitro fertilization of a preovulatory egg. Fertil. Steril 33:117–120.

Maxwell, D. P. and Kraemer, D. C. (1980). Laparoscopy in cattle. In: Animal Laparoscopy (R. M. Harrison and D. E. Wildt, eds.). Williams and Wilkins, Baltimore, pp. 133–156.

Oliphant, G. and Brackett, B. G. (1973a). Capacitation of mouse spermatozoa in media with elevated ionic strength and reversible decapacitation with epididymal extracts. Fertil. Steril. 24:948–955.

Oliphant, G. and Brackett, B. G. (1973b). Immunological assessment of surface changes of rabbit sperm undergoing capacitation. Biol. Reprod. 9:404–414.

Oliphant, G. and Eng, L. A. (1981). Collection of gametes in laboratory animals and preparation of sperm for in vitro fertilization. In: Fertilization and Embryonic Development In Vitro (L. Mastroianni, Jr. and J. Biggers, ed.). Plenum Press, New York, pp. 11–26.

Overstreet, J. W., Yanagimachi, R., Katz, D. F., Hayashi, K. and Hanson, F. W. (1980). Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: a study of fertile donors and infertile patients. Fertil. Steril. 33:534–542.

Reyes, A., Oliphant, G. and Brackett, B. G. (1975). Partial purification and identification of a reversible decapacitation factor from rabbit seminal plasma. Fertil. Steril. 26:148–157.

Rioux, J. E., Lambert, R. D., D'Amours, D., Soucy, B. and Brassard, A. (1980). Prelevement des ovules de bovins par laparoscopic: resultats preliminaires. L'Union Medicale du Canada 109:535–542.

Seidel, G. E., Jr. (1981). Superovulation and embryo transfer in cattle. Science 211:351–358.

Steptoe, P. C. and Edwards, R. G. (1978). Birth after the reimplantation of a human embryo. Lancet 2:366.

Steptoe, P. C., Edwards, R. G. and Purdy, J. M. (1980). Clinical aspects of pregnancy established with cleaving embryos grown in vitro. Br. J. Obstet. Gynaecol. 87:757–768.

Thibault, C., Dauzier, L. and Wintenberger, S. (1954). Etude cytologique de la fecondation in vitro de l'oeuf de la lapine. C. R. Soc. Biol., (Paris) 148:789–790.

Toyoda, Y. and Chang, M. C. (1974). Fertilization of rat eggs in vitro by epididymal spermatozoa and the development of eggs following transfer. J. Reprod. Fertil. 31:9–22.

Trounson, A. O., Leeton, J. F., Wood, C., Webb, J. and Wood, J. (1981). Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. Science 212: 681–682.

Wells, M. E. and Awa, O. A. (1970). New technique for assessing acrosomal characteristics of spermatozoa. J. Dairy Sci. 53:227–232.

Whittingham, D. G. (1968). Fertilization of mouse eggs in vitro. Nature (Lond.). 220:592–593.

Willett, E. L , Black, W. G., Casida, L. E., Stone, W. H. and Buckner, P. J. (1951). Successful transplantation of a fertilized bovine ovum. Science 113:247.

Wood, C., Trounson, A., Leeton, J., McTalbot, J., Buttery, B., Webb, J., Wood, J. and Jessup, D. (1981). A clinical assessment of nine pregnancies obtained by in vitro fertilization and embryo transfer. Fertil. Steril. 35:502–508.

Wright, R. W., Anderson, G. B., Cupps, P. T., Drost, M. (1976a). Blastocyst expansion and hatching of bovine ova cultured in vitro. J. Anim. Sci. 43: 170–174.

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

Wright, R. W., Jr. and Bondioli, K. R. (1981. Aspects of in vitro fertilization and embryo culture in domestic animals. J. Anim. Sci. 53:702–729.

Yanagimachi, R., Yanagimachi, H. and Rogers, B. J. (1976). The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. Biol. Reprod. 15:471–476.