FOLLICULAR OOCYTE MATURATION FOR USE IN BOVINE XENOGENOUS AND IN VITRO FERTILIZATION

By

Vernon D. Dooley

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ABSTRACT

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A series of experiments was conducted to morphologically characterize follicular oocytes from bovine ovaries collected at slaughter, to define a culture system for consistent in vitro maturation of these oocytes, and to assess the feasibility of using xenogenous and in vitro fertilization to produce early embryos from in vitro matured oocytes.

There was no difference between oocytes from cull cows or heifers, or between oocytes ipsilateral and contralateral to the most recent corpus luteum. Follicles larger than 3 mm contained a higher proportion of degenerate oocytes than smaller follicles. Of the 12.5 oocytes collected per ovary throughout the series of experiments, 20 to 30% were degenerate. Germinal vesicles were found in 76.9% of the remaining oocytes. The vital dye, trypan blue, indicated 83.3% of such oocytes were viable. Trypan blue exclusion and fluorescein diacetate uptake were correlated with each other, and with morphological classifications. No oocytes with severely degenerate ooplasm (score 3) and only 2% of oocytes devoid of surrounding cumulus cells

matured during 27 to 30 hours of culture. The percentage of oocytes maturing was 25.6 and 13.8 for ovaries transported to the laboratory at 37°C and 0°C, respectively, despite similar oocyte appearance at collection.

Oocyte maturation was not enhanced by lactate (50 or 100 mM) in medium containing glucose and pyruvate. Neither the proportion of oocytes remaining viable (survival), nor maturation, differed between a phosphate buffered saline and a TC199-based medium. Addition of FSH (10 μg/ml) tended to reduce maturation. Reducing the atmospheric oxygen content from 20% to 5% did not alter maturation. Increasing culture temperature from 37° to 39° did not affect survival but tended to increase maturation. Estradiol (1 μg/ml) or progesterone (1 μg/ml) in the culture medium, alone or in combination with FSH (10 μg/ml), reduced survival and did not alter maturation. Fertilization (2 pronuclei or cleavage) was obtained in a pseudopregnant rabbit oviduct (xenogenous fertilization) in 51.8% of oocytes expected to have matured at 37°C under 20% O_2. Percents fertilized were 70.8 and 52.7 following maturation under 5% O_2 at 37°C and 39°C, respectively. After adjustment for the normal maturation rate, <u>in vitro</u> fertilization (sperm penetration and one pronucleus) was achieved in 51.0% of oocytes and 40.0% of fertilized ova formed two pronuclei. No differences were found among media.

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INTRODUCTION

Mammalian ovaries harbor a large supply of genetic material which is not fully utilized. The number of ova ovulated spontaneously at estrus is a minute portion of the oocytes in the ovaries at birth. The remainder degenerate within the ovary through follicular atresia. Recovery of oocytes from bovine vesicular follicles could greatly increase the germ cell population available for fertilization and production of embryos. However, before fertilization can occur, quiescent follicular oocytes must resume meiosis to metaphase II, i.e. mature. The primary objective of the present study was to determine the culture conditions necessary to preclude degeneration and induce maturation of bovine oocytes, so that oocytes liberated from developing follicles could be utilized for embryo production.

Alternatives to natural fertilization can provide rapid, relatively inexpensive methods of producing embryos for morphological, biochemical, or teratological analyses. In addition, these have clinical and laboratory significance as a means of diagnosing some infertility problems, to extend usage of valuable semen, to assess the functional performance of male and female gametes, and to provide synchronously developing zygotes for research on such new technqiues as nuclear transfer and gene injection.

In vitro fertilization of laboratory animals has resulted in detailed morphological descriptions of fertilization as well as an increased understanding of biochemical events involved and environmental conditions required for fertilization (Stambaugh, 1978; Bavister, 1981). Species differences exist and the optimal conditions for *in vitro* fertilization of domestic animal oocytes are not well defined. Xenogenous fertilization, the fertilization of ova by homologous sperm in the oviduct of a heterologous species, provides a natural fertilization environment and more closely controls the culture conditions for the researcher. Fertilization of *in vitro* matured ova was tested using both *in vitro* and xenogenous systems in this study. The objective was to determine the effects of various environmental conditions on maturation and fertilization of bovine oocytes. This specifically included the atmospheric environment, temperature, and media. Additionally, the feasibility of using xenogenous and *in vitro* fertilization techniques to produce early bovine embryos was to be determined.

II. LITERATURE REVIEW

In Vitro Maturation

Gametogenic cells of female mammals initiate meiosis during late stages of fetal development (Erickson, 1966a; Thibault, 1976). Meiotic progression is arrested, however, and at birth bovine ovaries contain several thousand immature oocytes with their nuclei in prophase of meiosis I. These nuclei are surrounded by a nuclear membrane, termed a germinal vesicle (GV) (Erickson, 1966b; Baker and Franchi, 1967). Follicular cells maintain the oocyte in this arrested state by producing a maturation inhibiting substance(s) or by withholding essential nutrients. The oocyte in turn can prevent luteinization of granulosa and thecal cells (Mauleon and Mariana, 1977). These primordial follicles therefore represent a resting pool of genetic material from which small numbers of oocytes will be recruited for further growth. Development into a vesicular follicle is characterized by formation of a fluid-filled antrum, thecal and granulosa cell hyperplasia, and differentiation of the granulosa cell layer into a zone surrounding the oocyte, the cumulus oophorus, and a zone lining the remainder of the follicle (Chang et al., 1978). A layer of more densely packed cells, the corona radiata, develops within the cumulus oophorus and in direct contact with the oocyte. Vesicular bovine follicles near ovulation are 10-20 mm in diameter (Staigmiller and

England, 1982). A few hours before ovulation an endogenous luteinizing hormone (LH) surge induces follicular changes which allow resumption of meiosis (Donahue, 1972; Thibault et al., 1975). The actual mechanism of this LH action on meiosis remains obscure. Once initiated, meiosis proceeds through the germinal vesicle breakdown (GVBD) stage, metaphase I (met I), anaphase I, and telophase I (Fleming and Saacke, 1972). Oocytes in these stages are considered to be maturing in the present study. Oocytes that complete the first meiotic reductional division extrude the first polar body into the perivitelline space between the cell membrane and zona pellucida and progress to metaphase II (Met II) (Donahue, 1972). These oocytes are meiotically mature and prepared for ovulation and penetration by spermatazoa. Hormonal cyclicity results in release of one such oocyte from the ovary of a mature, nonpregnant cow approximately every 21 days.

A variety of techniques have been used in attempts to harvest the large number of follicular oocytes that would normally remain arrested in ovaries. Yet the optimum conditions for collection and maturation of these oocytes remains largely obscure. Early work showed explantation of an oocyte from its follicular environment into a simple culture medium is sufficient to stimulate resumption of meiosis through Met II (Pincus and Enzmann, 1935; Edwards, 1965; Foote and Thibault, 1969). These investigators and others further demonstrated the rate of maturation in vitro was similar to that obtained in vivo following an endogenous LH surge. Ovulation of a mature bovine oocyte usually occurs 24-28 hr after the LH peak (Hafez et al., 1963; Christenson et

al., 1974). Progression to Met II occurs when bovine follicular oocytes have been cultured for 24-30 hr (Sreenan, 1970; Thibault, 1972; Shea et al., 1976).

Most laboratory animal and primate in vitro oocyte maturation studies have involved oocytes collected at laparotomy, laparoscopy, or immediately after ovariectomy. In domestic animals, ovaries have been collected at commercial abattoirs and transported to a laboratory for ovum recovery and culture. Comparable maturation rates have been observed for bovine ova when ovaries were transported at 30-37°C (Fukui and Sakuma, 1980), at ambient temperature (Newcomb et al., 1978), or at 0°C (Kruip and Vernooy, 1982). Shea et al. (1976) found no difference in GVBD between oocytes from ovaries stored at ambient temperature for one or two hours. Snyder (1977a) studied the effects of transport temperature on ovine oocyte maturation. No detrimental effect on maturation was reported following ovary storage for up to four hours at 22° or 1° compared to 30-37°C. This study further found similar maturation rates for oocytes placed in culture immediately following laparotomy and those from ovaries transported from an abattoir. Crosby et al. (1971) found no advantage by removing ovine ovaries in a room adjacent to the oocyte recovery laboratory to ensure a minimal time interval between collection and start of culture.

The mammalian ovary is in a dynamic state of follicle growth and atresia under changing hormonal influences, which in turn affects the ability of oocytes to mature after liberation from the follicle. Oocytes from small (1-2 mm) porcine follicles had a lower rate of GVBD, as well as a lower rate of maturation to Met II, than oocytes

from medium (3-5 mm) or large (6-12 mm) follicles (Isafriri and Channing, 1975a). Oocytes from medium sized porcine follicles also had a lower rate of maturation than those from large follicles, but the difference was overcome by increasing the serum content of the culture medium. Snyder (1977b) reported no consistent differences between ovine oocytes from follicles greater than 2 mm diameter and those from smaller follicles. Moor and Trounson (1977) classified ovine follicles on the basis of their opacity, vascularization, and the integrity and uniformity of the granulosa cell layer. Following culture of oocytes within intact, nonatretic follicles, they recovered greater numbers of mature oocytes from 3-5 mm follicles than from smaller follicles. In contrast, culture of follicles considered atretic resulted in maturation rates similar to 3-5 mm nonatretic follicles regardless of size. Size of follicle from which an oocyte is recovered does not influence in vitro maturation of bovine oocytes (Thibault et al., 1976a; Leibfried and First, 1979; Fukui and Sakuma, 1980). However, a higher proportion of apparently degenerating oocytes was obtained from follicles greater than 3-5 mm in diameter. This is consonant with studies of bovine folliculogenesis. Several investigators have reported faster turnover rates in large bovine follicles (Ireland et al., 1979; Matton et al., 1981; Staigmiller and England, 1982). Thus, one could expect a higher proportion of large follicles to be in various stages of atresia as compared to their smaller counterparts. Choudary et al. (1968) reported the mean diameters of the largest healthy follicles and largest atretic follicles

were 4.1 mm and 9.5 mm, respectively, during the majority of the bovine estrous cycle.

Marion et al. (1968) and Rajakoski (1960) utilized micromorphological characteristics observed in thin paraffin embedded ovary sections to classify follicles as normal or in early or late atresia. The relative proportions of each follicle class did not differ between follicular and luteal phase ovaries. Several other reports, based on macromorphology of ovine and bovine ovaries, concluded that vesicular follicle development is largely continuous and independent of estrus and ovulation (Kammalade et al., 1952; Bane and Rajakoski, 1961; Smeaton and Robertson, 1971; Brand and de Jong, 1973).

The percentage of ovine follicular oocytes that matured in vitro was not affected by stage of estrous cycle (Snyder, 1977b). Fukui and Sakuma (1980) investigated the relation of bovine oocyte maturation to stage of the cycle. There was no difference in in vitro maturation of oocytes from ovaries with a corpus luteum compared with those having a corpus albican (49.6% vs. 45.9%). Leibfried and First (1979) used characteristics of corpora lutea to divide ovary pairs into three classes: early luteal, luteal, and follicular. They then examined the appearance of recovered oocytes as well as in vitro maturation in relation to cycle phase. In one experiment they found a slightly higher proportion of degenerate chromatin configurations from luteal phase ovaries. In a second trial, however, oocyte morphology, including post-culture chromatin configuration, was more normal in luteal phase oocytes. Thus, the stage of the estrous cycle would not appear to influence the in vitro maturation rate of the oocytes from a particular ovary pair.

Evaluation of oocytes upon liberation from follicles reveals considerable variation in amount of adhering cumulus. The presence of cumulus cells surrounding the oocyte appears to improve maturation *in vitro* in porcine (Tsafriri and Channing, 1975a; McGaughey, 1977), ovine (Crosby *et al.*, 1981), and bovine material (Bedirian and Baker, 1975; Fukui and Sakuma, 1980). Studies designed to investigate the requirement for cumulus cells for oocyte maturation has often involved mechanical removal of adhering cells by repeated aspiration and expulsion through small-bore pipettes. Kennedy and Donahue (1969) provided three possible explanations for fewer oocytes reaching Met II when divested of cumulus cells: (1) removal of cumulus cells may damage the oocyte, (2) cumulus cells may be required to supply required substances to the oocyte, and (3) cumulus cells may detoxify substances in the medium inhibitory to maturation of the denuded oocyte. The first reason can be eliminated by studies utilizing oocytes lacking cumulus when collected. However, the presence of cumulus may be more consistent with descriptions of *in vivo* matured ova (Dziuk, 1965; Lobel and Levy, 1968; Marion *et al.*, 1968). During the preovulatory period, cohesiveness of the cumulus cells is disrupted, but the corona radiata remains anchored in the zona pellucida (Lorton and First, 1979; Cran *et al.*, 1980). Nude oocytes, those lacking cumulus cell investment, were probably recovered from follicles in advanced stages of atresia (Henricson and Rajakoski, 1963; Hay *et al.*, 1976; Hay and Cran, 1978).

Numerous vesicles or globules within the ooplasm are characteristic of normal bovine (Fleming and Saake, 1972) and ovine oocytes (Russe, 1975; Cran et al., 1980). This presents a granulated appearance. Uneven granulation or formation of large cellular inclusions are characteristic of degenerating oocytes from follicles in late or tertiary atresia (Peluso, 1978; Leibfried and Frist, 1979).

Little information is available in the literature on the use of vital dyes to assess oocyte viability. In general, the procedures used for other cell cultures can be adapted to ova and embryos. Correlations with morphological criteria or ensuing development, as well as toxicity evaluations, have been performed with embryos for trypan blue (TpB) (Thadani et al., 1982) and fluorescein diacetate (FDA) (Mohr and Trounson, 1980; Looney et al., 1982). TpB is an indicator of membrane integrity (Tennant, 1964). Cells with a disrupted membrane permeability are unable to exclude the dye. Conversely, normal cells are permeable to the nonfluorescent compound, FDA (Whittingham, 1978). The acetate groups of FDA are cleaved by non-specific esterases inside the cell, releasing free fluorescein intracellularly. Intact plasma membranes are impermeable to this polar compound. The resulting green fluorescence under high-energy blue or ultraviolet light has been correlated with embryo development (Church and Raines, 1980; Renard et al., 1982).

Oocytes selected for culture must be placed in a medium that will support development. A variety of commercial media have been tested for bovine oocyte maturation (Table 1). This variation, coupled with individual modifications of media or culture environment, makes

TABLE 1

Summary of *In Vitro* Maturation Studies with Bovine Oocytes

Reference	Incubation Time (hr)	Culture Atmosphere	Medium	Maturation Percentage
Pope and Stephens, 1974	24-28	5% O_2:5% CO_2:90% N	SOF[1] + 1% BSA[2]	42
			SOF + 3% BSA	42
Pope and Turman, 1974	24-28	5% O_2:5% CO_2:90% N	SOF	77
			SOF + 10% FCS[3]	89
			SOF + Eagles aa	68
			SOF + FCS + aa	82
Shea *et al.*, 1976	22-25	5% CO_2 in air	Ham's F10[4] + 10% FCS	34
	26-28			60
	29-30			73
Iritani and Niwa, 1977	20-24	5% CO_2 in air	mKRB[5] + 4% BSA	61
Trounson *et al.*, 1977	24	air	FCS	65
		5% CO_2 in air		60
		5% O_2:5% CO_2:90% N		73
Leibfried and First, 1979a	24-26	5% CO_2 in air	TC199[4] + 15% BS[6]	71
Leibfried and First, 1979b	24-26	5% CO_2 in air	EBM[4] + 10% FCS	64

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TABLE 1 (continued)...

Reference	Incubation Time (hr)	Culture Atmosphere	Medium	Maturation Percentage
Leibfried and First, 1980a	24-26	5% CO_2 in air	EBM + 10% FCS	71
			EBM + 50% FCS	71
			EBM + 50% PS[7]	76
			TC199 + 10% BS	60
			TC199 + 50% BS	56
Fukui and Sakuma, 1980	24-33	5% CO_2 in air	TC199 + 3% BSA	54
Fukui et al., 1982	27-30	---	TC199	32
			TC199 + 20% FCS	46
			Ham's F12[4]	49
			Ham's F12 + 20% FCS	46
			BMOC-3[4]	17
			BMOC-3 + 20% FCS	44
			mKRB	22
			mKRB + 20% FCS	53
Kruip and Vernooy, 1982	24	5% CO_2 in air	Ham's F12 + 1% BSA	65

[1]Synthetic oviductal fluid (Tervit et al., 1972)
[2]Bovine serum albumin
[3]Fetal calf serum
[4]Grand Island Biological Company 1982 Tissue culture products catalog GR-1-3/82, pp. 138-185.
[5]Modified Krebs-Ringer Bicarbonate solution
[6]Bovine serum
[7]Porcine serum

comparisons between studies and, in some cases, within studies, difficult to interpret.

Quirke and Gordon (1971) reported negative results in a study on in vitro maturation of ovine oocytes in TC199 medium, TC199 supplemented with 15% fetal calf serum (FCS) or sheep serum. Bovine follicular fluid supported minimal meiotic resumption while 50% resumed meiosis in ovine follicular fluid. This team of researchers reported markedly superior results (91% maturation after 40 hours) in a synthetic growth medium. Crosby and Gordon (1971) confirmed successful maturation in this growth medium. This culture medium also supported GVBD of bovine oocytes similar to that observed in bovine follicular fluid (Sreenan, 1970). However, Met II was reached twice as often in growth medium (59.3%) as in follicular fluid (27.6%).

One of the most common additions to a basic culture medium is a source of large molecular weight protein, generally serum or bovine serum albumin (BSA). Some discrepancy is found in the literature regarding the importance of the macromolecular source. Jagiello et al. (1975) increased ovine oocyte maturation from 60.0 to 77.7% in McCoys medium with the addition of 22% ovine serum. Replacement of ovine serum with FCS provided similar maturation (77.4%). These workers also reported 64.9% Met II in Diploid medium containing 12% ovine serum and 83.3% in Diploid medium with 22% FCS.

Maturation of bovine ova was no different in synthetic oviductal fluid (Table 1) with 1% BSA or with 3% BSA (Pope and Stephens, 1974). However, in another study, maturation was enhanced by adding 10% FCS (Pope and Turman, 1974). Leibfried and First (1980) found no differences bewteen 10% FCS, 50% FCS, or 50% porcine serum addition to

Eagle's Basal Medium. Use of 3 mg/ml BSA in TC199 for bovine oocytes (Fukui and Sakuma, 1980) was inferior to serum addition in one report (Leibfried and First, 1979a) but not in a second (Leibfried and First, 1980). Fukui et al. (1982) found significantly greater maturation in a modified Krebs-Ringer Bicarbonate solution (KRB) when 20% FCS was included (22% vs. 53%). However, an earlier study had obtained 71% in vitro maturation using a KRB medium without FCS (Iritani and Niwa, 1977). FCS addition improved results significantly in Brinsters medium for ovum culture #3 (BMOC-3), but no improvement was observed with Ham's F12 medium (Fukui et al., 1982). Other workers (Kruip and Vernooy, 1982) obtained better maturation in Ham's F12 with 1% BSA (65%) than Fukui et al. (1982) reported for Ham's F12 with 20% FCS (46%).

Energy requirements for maturation of oocytes must be supplied by the culture media. Glucose, pyruvate and glutamine are included in most media. Few studies have examined the importance of individual culture components. The addition of pyruvate, lactate and insulin to TC199 (which contains glucose and glutamine) with 15% pig serum resulted in a higher incidence of pig oocyte maturation (Tsafriri and Channing, 1975a). In contrast, McGaughey (1976) reported porcine ovum maturation with glucose as the only energy source (58%) was not statistically different from controls (68%) with glucose, lactate and pyruvate.

Hormones have been added to culture media in an effort to mimic in vivo oocyte maturation. Peripheral gonadotropins are known to surge shortly before ovulation (Schams et al., 1977; Rahe et al.,

1980). Follicle stimulating hormone (FSH) has been measured in bovine follicular fluid and no correlation has been found between FSH concentration and follicle size (Henderson et al., 1982). However, concentrations of LH fell significantly as follicle size increased, perhaps indicating utilization of fluid LH by follicular cells. LH has been postulated as the key to in vivo resumption of meiosis by overcoming the action of an oocyte maturation inhibitor (OMI) (Tsafriri et al., 1982). Spontaneous maturation of porcine oocytes has been inhibited, presumably through OMI action, when cultured with follicular hemisections (Leibfried and First, 1980b), granulosa cells (Foote and Thibault, 1969; Tsafriri et al., 1975b), medium in which granulosa cells had been cultured, or follicular fluid (Tsafriri et al., 1976; Stone et al., 1978). The inhibition could, however, be overcome by LH addition. In contrast, Jagiello et al. (1977) showed no effect on maturation of ovine, porcine and bovine oocytes cultured with granulosa cell monolayers. Others have failed to demonstrate OMI activity in follicular fluid or granulosa cells on porcine (Racowsky and McGaughey, 1982) or bovine oocytes (Leibfried and First, 1980a).

Addition of LH to culture media has resulted in increased in vitro maturation rates of ovine oocytes from 60% to 72% (Jagiello et al., 1975). However, the stimulatory effect was not observed when LH was added to media containing FCS or ovine serum. Bovine oocyte maturation was inhibited by ovine LH in either McCoy's or Diploid medium containing 22% FCS. Fukui et al. (1982) reported no significant effect on bovine oocyte maturation when 10 μg/ml ovine LH or 2 i.u./ml human chorionic gonadotropin (HCG) was added to various media containing FCS, although some variability was noted. Percent of

oocytes maturing was not increased by LH addition to TC199. Rush et al. (1973) also reported no effect of either LH or FSH on bovine oocyte maturation. Addition of HCG to TC199 increased the proportions of matured porcine oocytes in one study (Meinecke and Meinecke-Tillmann, 1979a), while others reported porcine oocytes were not affected by gonadotropin addition (Hillensjo and Channing, 1980).

The effect of gonadotropins is possibly not exerted directly on the oocyte, but through the mediation of granulosa and cumulus oophorus cells. One intermediate associated with gonadotropin action is cyclic AMP (cAMP) (Lindner et al., 1974; Moor et al., 1980). Weiss et al. (1976) has demonstrated a rapid increase in cAMP within ovine follicles after LH treatment. Increased follicular cAMP in response to HCG was correlated with porcine follicle size, with the greatest response in large (6-12 mm) follicles (Lee, 1976).

Direct addition of cAMP to oocyte culture media has resulted in reduced maturation rates (Racowsky, 1983; Rice and McGaughey, 1981). Maturation of cumulus-enclosed porcine oocytes fell from 74% to 58% with the addition of 1 mM cAMP derivative, N^6,O^2-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (dbcAMP) (Rice and McGaughey, 1981). This inhibition was reversible. Maturation levels of oocytes cultured in dbcAMP-containing medium for 24 hours, then transferred to control medium for 24 hours, was not different than that for oocytes in control medium for 24 hours (54.4% and 69.0%, respectively). Inhibited meiosis was also reported for ovine oocytes with 1 or 2 mM cAMP, and bovine oocyts with 2 mM cAMP (Jagiello et al., 1975). Therefore, cAMP at the level of the oocyte appears to inhibit meiosis resumption. Its

action within the follicle may, however, be mediated by other cells. Tsafriri et al. (1982) theorized that cAMP, the physiological inhibitor of meiosis, is transmitted from cumulus cells through gap junctions and that gonadotropins induce maturation by disrupting oocyte-cumulus cell junctions. This hypothesis was not supported by the finding that GVBD precedes demonstrative disruption of oocyte-cumulus cell communication (Moor et al., 1980). Moor et al. (1981) demonstrated induced maturation without affecting oocyte-cumulus cell coupling by adding low levels of LH to the culture system. They further stated that low levels of FSH suppressed intracellular coupling without inducing meiosis in ovine oocytes. Stimulation of cumulus expansion with FSH has also been reported in porcine and bovine oocytes (Ax and Ryan, 1979; Lenz et al., 1982; Hensleigh and Hunter, 1983). Furthermore, the FSH response was mimicked with dbcAMP addition (Schweitzer et al., 1981; Bellin and Ax, 1981). The importance of cumulus expansion is not clear but it is known to be temporally related to nuclear maturation (Thibault et al., 1975; Baker and Polge, 1976; Ainsworth et al., 1980).

The preovulatory gonadotropin surge induces marked changes in follicular steroidogenesis (Linder et al., 1974; Eiler and Nalbandov, 1977). This suggested that steroids are involved in the regulation of meiosis resumption. Prior to the gonadotropin surge, developing follicles produce primarily estradiol (Moor, 1973; Edwards, 1974; Webb and England, 1982). Estrogen levels fall abruptly in the preovulatory follicle at the first detection of elevated serum LH (Moor, 1974; England et al., 1981; Murdoch and Dunn, 1982). Androgen

concentrations in follicular fluid remain stable (Ainsworth et al., 1980) or decline shortly after the LH peak (Baird et al., 1981; Murdoch and Dunn, 1982). Progesterone levels, increasing during follicle growth, tend to plateau concomitant with the gonadotropin surge, only to increase again before ovulation (Bjersing et al., 1972; Wheeler et al., 1975; Moor et al., 1975). Thus, GVBD occurs in follicular fluid characterized by declining estrogen levels and relatively high and constant levels of progesterone and androgen. Gerard et al. (1979) studied oocyte maturation by culturing whole porcine follicles in vitro. Exposure of follicles (>6 mm diameter) to HCG, FSH, or LH resulted in the expected drop in estrogen and rise in progesterone levels in follicular fluid and resumption of oocyte meiosis. However, while gonadotropins were mildly effective in inducing maturation of oocytes enclosed in <6 mm follicles, the characteristic inversion of the estrogen-progesterone ratio did not occur in these small follicles in vitro.

Direct addition of steroids to culture medium has also given variable results. Inhibition of steroidogenesis did not prevent the LH-induced resumption of meiosis in ovine follicle-enclosed oocytes, but did block meiosis at the Met I stage (Moor and Warnes, 1978). Addition of estradiol to such cultures, together with the gonadotropin, prevented this block and improved fertilization of the oocytes. Jagiello et al. (1975) was not able to demonstrate increased maturation rates in ovine oocytes with the addition of estradiol-17β (1000 pg/ml) followed 0 to 6 hours later with the addition of LH (30 ng/ml). Neither progesterone nor estradiol-17β had a significant effect on maturation of cumulus-clad porcine oocytes (McGaughey, 1977). He did

report inhibited maturation of denuded oocytes with estradiol at 1.0 or 10.0 μg/ml, but this inhibition was overcome by including progesterone in the medium or removing oocytes to steroid-free medium. This reversible inhibition by estradiol led to speculation that this is the mechanism by which follicles maintain oocytes in arrested meiosis. Testosterone was shown to inhibit oocyte maturation (Richter and McGaughey, 1979; Rice and McGaughey, 1981), and since granulosa cells aromatize androgens to estrogen (porcine: Bjersing and Cartensen, 1967; ovine: Moor, 1977; bovine: Lacroix et al., 1974), estradiol from granulosa cells may mediate the inhibition in small follicles known to contain high androgen levels (Carsen et al., 1981; Henderson et al., 1982).

An increased incidence of chromosomal abnormalities was observed in oocytes cultured in progesterone or estradiol containing medium (McGaughey, 1977). However, oocytes cultured in media containing both steroids exhibited a higher incidence of normal telophase I and Met II chromosomes than did control oocytes.

Fukui et al. (1982), studying bovine oocyte maturation, reported that the effect of added hormones differed slightly according to the media tested. No significant effect was found for hormonal treatments in TC199 or modified KRB but was evident in Hams-F-12 and BMOC-3. Addition of either estradiol alone or estradiol and LH in combination to the culture medium significantly increased maturation rates. Proportions of matured oocytes were 47.8%, 61.7%, 61.0% and 55.2% for no additions, estradiol, estradiol + LH, and progesterone, respectively. Estradiol has also been reported to reduce maturation of cumulus

enclosed bovine oocytes to 12% compared to 60% for controls (Robertson and Baker, 1969). These workers reported that adding progesterone to medium TC199 increased GVBD as well as first polar body formation in denuded bovine oocytes. No effect of progesterone addition was noted by others (Rush et al., 1973; Fukui et al., 1982).

There is some evidence that the hormones in follicular fluid, or culture media, play a role in maturation beyond the nuclear changes normally monitored. Cytoplasmic and membrane maturation are poorly understood and generally assessed by development following fertilization. Nevertheless, the rate of amino acid uptake, rate of amino acid incorporation into proteins, and resulting patterns of protein synthesis in oocytes maturing in vitro have been induced by gonadotropins to approximate those of in vivo matured ovine oocytes (Warnes et al., 1977; Moor and Smith, 1978, 1979; Crosby et al., 1981; Moor et al., 1981).

Other important aspects of the culture environment include pH and osmolality of the medium, and culture atmosphere. Very little research has been done to define optimum values of these components for oocytes from domestic animals. Studies with mice (Brinster, 1965) and rabbits (Kane, 1974) indicated development can occur over a wide range of pH values (6.0 to 7.8). Shea et al. (1976) stated that bovine oocytes matured in media with pH values from 6.7 to 7.6, but the highest proportion of GVBD (80%) and Met II (69%) was obtained at a pH range from 7.0 to 7.3. This also appears to be the pH range of follicular fluid (Edwards, 1974; Knudsen et al., 1978).

Commonly used culture media range in osmolality from 270 to 316 mOsm (Wright and Bondiol, 1981). Bae and Foote (1980) matured rabbit oocytes in medium ranging from 250 to 310 mOsm. Best results were obtained in 270 mOsm medium. Similarly, porcine oocytes matured in media with calculated osmolalities of 265 to 305 mOsm (McGaughey, 1977b). However, poor maturation rates were obtained in the higher ranges, and 285 mOsm was considered optimal. This is approximately the osmolality of plasma, and similar to that of follicular fluid (Olds and VanDemark, 1957; Edwards, 1974).

Two culture atmospheres, 5% CO_2 in air and 5% CO_2, 5% O_2, 90% N_2, have been routinely used for _in vitro_ culture of mammalian oocytes and embryos. The reduced oxygen atmosphere of this second mixture was beneficial to development of early bovine (Tervit _et al._, 1972), ovine (Trounson and Moore, 1974; Wright _et al._, 1976a) and porcine (Wright, 1977) embryos. Contradictory evidence was reported by Wright _et al._ (1976b), who were unable to demonstrate a beneficial effect of lower oxygen concentrations on bovine embryos cultured in medium under paraffin oil. The oil cover may mask the difference by restricting oxygen exchange (Gwatkin, 1972). While granulosa cells may best be sustained by a gas phase rich in oxygen (Moor, 1973), mammalian oocytes appear to require an oxygen concentration of less than 20%. Survival and maturation were sharply improved by reducing oxygen concentration in hamster oocyte cultures (Gwatkin and Haidri, 1973; 1974). Porcine oocytes have been demonstrated to be less sensitive to the oxygen content during culture (Tsafriri and Channing, 1975a). The

percentages of oocytes with one polar body were 52.7, 43.6 and 37.5 in 20%, 45% and 95% oxygen, respectively. Nearly twice as many degenerated oocytes were found in 95% O_2. Trounson et al. (1977) reported superior bovine oocyte maturation under a reduced oxygen atmosphere (Table 1).

A variety of culture vessels have been used for bovine oocyte culture, including stoppered test tubes (Pope and Turman, 1974; Trounson et al., 1977; Fukui and Sakuma, 1980), media droplets under oil (Hunter et al., 1972; Iritani and Niwa, 1977; Liebfried and First, 1979b), and covered petri dishes (Shea et al., 1976; Newcomb et al., 1978). Studies within which comparisons were made reported no differences (Leibfried and First, 1980a; Enright, 1982).

In Vitro Fertilization

Mammalian fertilization has been thoroughly reviewed (Chang and Pincus, 1951; Bedford, 1970; Stambaugh, 1978) and no attempt will be made here to describe the physiological events involved. In vitro fertilization (IVF) has also been the subject of reviews, including recent books (Mastroianni and Biggers, 1981; Hafez and Semm, 1982).

Following recognition of the need for sperm to be capacitated in a female reproductive tract prior to fertilization (Austin, 1951; Chang, 1951), several reports of IVF in laboratory animals appeared. Significant among these was documentation of live rabbit offspring resulting from IVF and transfer of a 4-cell embryo (Chang, 1959). Offspring resulting from IVF have since been reported for mice (Whittingham, 1968), rats (Toyada and Chang, 1974), humans (Steptoe and Edwards, 1978), and cattle (Brackett et al., 1982).

Attempts at IVF of large domestic animals have largely been disappointing when compared to successes in laboratory species. Baker and Polge (1976) observed no penetration by sperm *in vitro* using porcine oocytes recovered from follicles 1-3 hours before ovulation or ova recovered from oviducts 1-4 hours after ovulation. The sperm added to the cultures were washed ejaculated sperm, epididymal sperm or sperm recovered from the tract of inseminated estrous gilts. Sperm penetration did occur if the oocyte and sperm mixture was transferred after 4 hours of *in vitro* incubation to oviducts of unmated estrous recipients. They suggested that the failure of sperm penetration *in vitro* resulted from lack of sperm capacitation (Polge, 1977). Iritani *et al*. (1975) determined the developmental time-course following porcine IVF. Ovaries were obtained at slaughter and follicular oocytes aspirated and cultured for 26 to 30 hours to maturity. When combined with collected semen preincubated in modified KRB, the oviduct, or the uterus of an estrous sow, fertilization rates of 7.3%, 5.2% and 11.5%, respectively, resulted. Sperm penetration into the periviteline space was observed three to five hours after insemination with penetration into the vitellus after five to ten hours. The second meiotic division, with extrusion of a second polar body, was observed at 10 to 16 hours. Two pronuclei were seen at 16 to 24 hours and cleavage at 16 to 30 hours. Using similar oocyte and semen treatments, ejaculated and epididymal sperm were then compared (Iritani *et al*., 1978). No fertilization was reported in this study following sperm preincubation in KRB. Penetration after capacitation of epididymal sperm was better than that of ejaculated sperm (23% *vs*. 13%).

Formation of morphologically normal male and female pronuclei was low (27.8%). A delay in the transformation of the sperm head into a pronucleus was frequently observed. Similar observations in the rabbit lead to the suggestion that in vitro oocyte maturation was incomplete. Synthesis of a male pronucleus growth factor (MPGF) in the egg cytoplasm during in vivo maturation may not accompany in vitro maturation (Thibault and Gerard, 1973; Thibault et al., 1975b, 1976b). A role of ovarian steroids in this cytoplasmic maturation has been postulated (Thibault, 1977; Moor, 1978). Failure of normal fertilization and development in swine induced to ovulate with HCG while follicular estrogens were low supports this theory (Hunter et al., 1976). Leman and Dziuk (1971), however, reported some normal embryonic development following transfer of oocytes collected from the ovaries of HCG-treated gilts 12 hours before the expected time of ovulation. Normal fertilization has also been obtained following transfer of in vitro matured porcine oocytes to oviducts of inseminated sows (Motlik and Fulka, 1974).

Thibault and Dauzer (1961) found that none of 41 ovine ova from seven ewes were fertilized following in vitro insemination with ejaculated ram semen. These studies used ovulated ova. Four ova developed to the second polar body or 2-pronuclei stage following IVF of 78 ova from 38 ewes with sperm recovered from the uterus 12 to 24 hours after coitus. Using similar conditions (naturally ovulated ova and oviductal-incubated semen), Kraemer (1966) reported that four of 23 ova formed a second polar body, but only one developed two pronuclei. Tubal ova, collected by flushing ewe oviducts, were successfully

fertilized in synthetic oviductal fluid (SOF) and Minimal Essential Medium (MEM) by *in vitro* capacitated sperm (Bondioli and Wright, 1980). Sperm treatment involved a 3 hour preincubation of ejaculated semen in the culture medium at 37°C before addition of ova. Sperm penetration was achieved in 9.5% of 294 ova, and cleavage in 53.6% of fertilized ova. No difference was found in the ability of SOF or MEM with no protein, 3% BSA, or 20% lamb serum to support fertilization. A high incidence of fragmentation (30 to 60%) was reported in all media. Four of six follicular oocytes from gonadotropin treated lambs extruded a second polar body in a preliminary trial of IVF following *in vitro* maturation of ovine oocytes (Dahlhausen *et al.*, 1980). Ram sperm capacitated in a rabbit uterus was capable of fertilizing *in vitro* matured oocytes, as well as ovulated ova *in vitro* (Bondioli and Wright, 1981). Penetration was observed in 22% of the oocytes and cleavage in 10% following IVF.

Other groups have had limited success in obtaining fertilization following transfer of *in vitro* matured ovine oocytes to the oviduct of previously inseminated ewes (Crosby *et al.*, 1971: 5% 2-pronuclei; Quirke and Gordon, 1971: 25% 2-pronuclei). Woody *et al.* (1961) transferred follicular oocytes to mated recipient ewes without *in vitro* culture. While 25% of ova recovered 24 to 48 hours later had formed two pronuclei, none had cleaved. Conversely, 67% of ovulated ova cleaved in recipient ewes, and one pregnancy resulted following retransfer (Woody and Ulberg, 1963). Moor and Trounson (1977) reported encouraging results after oocyte maturation through follicular organ

culture. Normal blastocysts developed from less than 5% of ovine oocytes matured in follicles and then transferred to recipient ewes for 7 days when either no hormone or only FSH and LH were included in the culture medium. However, follicle-enclosed oocytes cultured 24 hours in medium containing 1 μg/ml estradiol-17β, 2 μg/ml FSH and 1 μg/ml LH before transfer resulted in 40% development to blastocysts. Only one of 61 ova cultured outside the follicle before transfer developed into a blastocyst, and four developed to the morula stage. Further studies confirmed adding estradiol to the medium during *in vitro* maturation within the follicle reduced fertilization and cleavage abnormalities in the ovine (Moor, 1978). Crosby *et al.* (1981) also reported fertilization of *in vitro* matured ovine oocytes in ewe oviducts following transfer. Normal blastocysts developed from 4% of cumulus-enclosed oocytes matured outside the follicle in the absence of gonadotropins. The proportion of blastocysts increased to 30% with LH addition (10 μg/ml) during maturation. Cumulus cells were essential for development. Only 3% of denuded oocytes cleaved during the 10 to 12 days in recipients. Removing cumulus cells after the first eight hours of the 24 hour maturation period before transfer resulted in 31% cleavage and 5.4% blastocyst development.

IVF of bovine oocytes has received considerable attention in the last decade (Table 2). In several studies follicular oocytes have been recovered and transferred to oviducts of previously inseminated recipients. Hunter *et al.* (1972) reported 51.9% fertilization of *in vitro* matured bovine oocytes by this method. Formation of two pronuclei was complete in 68.8% of the fertilized ova recovered 22 hours

TABLE 2

Summary of Fertilization Studies Using Bovine Oocytes

Authors	Oocyte Source[1]	Sperm Source and Capacitation	Fertilization Site[2]	Incubation Conditions[3]	Results[4]
Hunter et al. (1972)	FO	---	ov	Tyrodes + 50% ff + 1% BSA; 37°C; 20% O_2	11/29 pn
Newcomb et al. (1978)	FO	---	ov	Hams F10 + 20% HTS; 37°C; 5% O_2	23/60 $\geq$ 2-cell
Shea et al. (1983)	FO[5]	---	ov	---	84/450 fertilized
Sreenan et al. (1970)	FO	ejaculated; 4 h in growth medium	iv	ff or growth medium 37°C; air	0/107 penetrated
Bregulla et al. (1974)	FO	frozen; uterus or uterine secretions	iv	TC199 + 10% HTS; 37°C; 20% O_2	22/1977 2 pb or beyond
Baker and Polge (1976)	FO[6]	ejaculated; uterus or *in vitro*	iv	various media + ff	0 penetration
Menezo et al. (1976)	FO[5]	ejaculated; various *in vitro* treatments	iv	5% CO_2 in air	2/18 penetrated
Iritani and Niwa (1977)	FO	ejaculated; (1) mKRB 12-14 h (2) rabbit uterus 12-14 h; (3) isolated cow tract 3-4 h	iv	mKRB + 4% BSA; 37°C; 20% O_2	(1) 0/62 penetrated (2) 11/56 penetrated, 6 pn (3) 10/47 penetrated, 6 pn

CONTINUED ON NEXT PAGE....

TABLE 2 (continued)....

Authors	Oocyte Source[1]	Sperm Source and Capacitation	Fertilization Site[2]	Incubation Conditions[3]	Results[4]
Brackett et al. (1978)	FO[5] or OO	ejaculated; HIS medium[7]	iv	---	14/25 fertilized; 10/14 2-cell; 4/7 4-cell
Brackett et al. (1980)	(1) FO[5] (2) OO	ejaculated; HIS medium		DM; 37-38°C 8% O_2	(1) 9/24 pn or beyond (2) 11/42 pn or beyond
Brackett et al. (1982)	(1) FO[5] (2) OO	ejaculated; HIS medium	iv	DM; 8% O_2 or 5% O_2	(1) 13/66 pn or beyond (2) 34/78 pn or beyond
Enright (1982)	(1) FO[5] (2) OO (3) FO	ejaculated; HIS medium	iv	DM; 37°C; 8% O_2	(1) 0/2 fertilized (2) 5/21 fertilized (3) 12/55 pn
Ball et al. (1983b)	FO	epididymal TALP + HEPES	iv	TALP + 6% BSA; 20% O_2; 39°C	33/64 penetrated; 27/64 pn
Hensleigh and Hunter (1983)	FO	---	iv	MEM + 10% FCS	24/194 2-cell

[1]FO = follicular oocytes; OO = ovulated ova

[2]ov = oviduct of recipient; iv = in vitro

[3]ff = follicular fluid; BSA = bovine serum albumin; HTS = heat-treated homologous serum; mKRB = modified Krebs-Ringer bicarbonate solution; DM = defined medium; TALP = modified Tyrodes medium; FCS = bovine fetal calf serum.

[4]pn = pronuclear stage, i.e.: male and female pronuclei present; pb = polar body.

[5]Females treated with gonadotropins to effect in vivo maturation.

[6]Preovulatory follicles from untreated donors

[7]HIS medium = high ionic strength medium

after transfer to recipients. Newcomb et al. (1978) recovered ova from recipient heifers 7 days after insemination and reported a 38.3% fertilization rate. However, development was severely retarded in several cases, as only eight (12.7%) late morula or blastocysts were recovered. Nonetheless, this study did show that in vitro matured follicular oocytes could develop normally. The birth of a calf followed transfer of one of the recovered blastocysts.

Results of early experiments in which fertilization was attempted entirely in vitro were less encouraging (Table 2). Sreenan (1970) added sperm suspensions, made with bovine follicular fluid or growth medium, to oocyte cultures either immediately or after 4 to 4 1/2 hours of culture. Though maturation rates were high, no oocytes showed evidence of fertilization. Baker and Polge (1976) also observed no penetration after culturing preovulatory follicular oocytes with ejaculated sperm or sperm obtained from the uterus of a mated cow. Iritani and Niwa (1977) capacitated sperm in the isolated oviduct or uterus of an estrous cow, the uterus of an estrous rabbit, or in a KRB solution. Respective fertilization rates of the in vitro matured oocytes were 20.7%, 18.5%, 21.3% and 0%. Brackett et al. (1978) capacitated bull sperm in vitro using a high ionic strength treatment (defined medium with NaCl added to provide an osmolality of 380 mOsm) involving centrifugation and incubation at 38°C for 3 hours. Sperm treated in this manner fertilized 56% of oocytes which had either been ovulated or collected just prior to ovulation from hormonally induced donors. Furthermore, development progressed to the 2-cell stage in ten of the 14 fertilized ova and the 4-cell stage in

four of seven ova cultured for 48 hours. Subsequent work by this group resulted in the birth of the first calf resulting from bovine *in vitro* fertilization in 1981 (Brackett *et al.*, 1982). IVF of *in vitro* matured bovine oocytes has recently been reported using epididymal sperm (Ball *et al.*, 1983a,b). Oocytes were recovered from 1-5 mm follicles on ovaries collected at slaughter, and cultured 24 to 28 hours for maturation. Sperm were washed by centrifugation (200 x g for 10 minutes), then added to oocyte cultures to yield a concentration of $4x10^6$ sperm/ml. Presence of cumulus cells did not affect penetration rates but increased frequencies of ova with male and female pronuclei were found when cumuli were present. Improved oocyte penetration and formation of two pronuclei followed inclusion of FSH or cAMP during the maturation culture. Although significant replicate differences and a 9% polyspermy incidence were reported, these efforts do provide evidence an entirely *in vitro* system is possible. The most successful experimental treatments resulted in 41.9% development of two pronuclei (Ball *et al.*, 1983b).

The question of cytoplasmic maturation in bovine oocytes has been addressed by IVF of zona-free oocytes (Fulka *et al.*, 1982). Oocytes were recovered from 3-5 mm follicles and matured 22-24 hours prior to enzymatic removal of their zonae pellucidae and *in vitro* insemination with epididymal sperm. Of the 575 oocytes examined 12 to 14 hours after insemination, 88.7% were penetrated and 45.4% had formed normal male and female pronuclei. The investigators concluded that a cytoplasmic substance responsible for sperm head decondensation and male

pronucleus formation is present in about half of *in vitro* matured bovine oocytes.

Ball *et al.* (1982) has reported improved maturation rates with increased incubation temperature. Oocytes were *in vitro* matured at 39°C in their study before coculture with sperm. Number of oocytes fertilized/oocytes cultured at 35°, 37°, 39° and 41°C was 4/88, 4/54, 28/53 and 20/50, respectively.

Embryo Culture in Foreign Tract Environment

Until recently, attempts to store developing embryos *in vitro* had met with little success (Brackett, 1981; Wright and Bondioli, 1981). The ability of the female genital tract to support development of embryos of other species provides an alternative means of culturing embryos. Reciprocal transfers between sheep and goats demonstrated that early embryonic development can occur in a heterologous species (Warwick and Berry, 1949).

The most commonly used recipient for short-term foreign tract culture of domestic animal embryos has been the rabbit. A summary of these investigations is presented in Table 3. Polge *et al.* (1972) studied porcine embryo development in oviducts of estrous or pseudo-pregnant rabbits ligated at the tubo-uterine junction. Embryos of four different developmental stages were maintained in rabbits for either 1, 2 or 3 days. Irrespective of initial stage of development, embryos developed normally until recovery from the rabbits. After examination, the embryos were transferred to recipient gilts and recovered one week later. Continued development in recipients

TABLE 3

The Culture of Domestic Animal Embryos in the Rabbit Oviduct

Embryo Species	Authors	Developmental Stage at Transfer	Time in Rabbit	Resulting Developmental Stage
Equine	Allen et al. (1976)	32 cell to blastocyst	40-49 h	early blastocysts to hatched blastocyst[1]
Porcine	Polge et al. (1972)	1 cell to morula	1-3 days	8 cell to blastocysts
Ovine	Averill et al. (1955)	2-12 cell	4-5 days	morula to blastocysts
	Adams et al. (1961)	1-8 cell	4-5 days	morula to blastocysts[1]
	Hunter et al. (1962)	2-8 cell	102-108 h	morula to blastocysts[1]
	Adams et al. (1968)	8-32 cell	4-7 days	blastocyst[1]
	Lawson et al. (1972a)	2-16 cell	3-7 days	morula to blastocyst[1]
Bovine	Hafez and Sugie (1963)	1-8 cell	2 days	2-16 cell
	Adams et al. (1968)	8-32 cell	3-4 days	morula to blastocyst
	Sreenan and Scanlon (1968)	5-16 cell	4 days	blastocyst
	Sreenan et al. (1968)	2-10 cell	46-95 h	10-64 cell
	Seidel et al. (1971)	1-2 cell	2 days	8-10 cell
	Lawson et al. (1976)	1-8 cell	2-4 days	morula to blastocyst[1]
	Trounson et al. (1976)	8-32 cell	48-96 h	blastocyst

[1]Retransfer after embryo recover from rabbit resulted in pregnancy and live birth.

declined with longer periods of incubation in the rabbit. This effect was most dramatic in older embryos. Survival of 1- to 2-cell embryos placed in rabbits for 2 days and then transferred as 8- to 16-cell embryos was equal to results following routine embryo transfer in this laboratory.

Ovine embryos have developed to the early blastocyst stage in the uterus and oviduct of pseudopregnant rabbits (Averill et al., 1955; Adams et al., 1961). Further, normal 22-day fetuses and pregnancies were reported following retransfer to ewes. Adams et al. (1968) used older embryos and increased storage time in the rabbit. Two births occurred following transfer of hatched ovine blastocysts. These had developed from 8- to 16-cell embryos held in ligated rabbit oviducts six days. Neither the initial cleavage stage of ovine embryos transferred to rabbits, nor the hormonal state of the doe (estrous or pseudopregnant) affected embryo development (Lawson et al., 1972a). One-cell zygotes have developed to blastocysts within the oviducts of estrous rabbits. Lawson et al. (1972a) did suggest, however, that the oviduct in pseudopregnancy provided a better environment for development than that afforded by the estrous doe. Cleavage rates of ovine embryos were slightly retarded in oviducts of estrous rabbits. These researchers further discovered that viability of ovine embryos declines rapidly after more than five days in the rabbit.

In addition to nurturing embryos to later stages of development, rabbit oviducts have served as incubators during transport. Hunter et al. (1962) transported sheep embryos from England to South Africa in

the ligated oviduct of a pseudopregnant doe. Six pregnancies and four live lambs resulted from transfer of the 16 embryos. Allen et al. (1976) transported equine embryos from England to Poland in the ligated estrous rabbit oviduct. Pregnancy resulted in three of four mares receiving embryos.

In the earliest report of bovine embryo culture in pseudopregnant rabbit oviducts (Hafez and Sugie, 1963), five of 142 embryos cleaved. The authors concluded bovine embryos do not generally survive in rabbit reproductive tracts. Contradictory evidence is offered by more successful later experiments. Adams et al. (1968) noted development in 16 of 17 bovine embryos recovered from follicular phase rabbit oviducts. However, six embryos appeared degenerate and no pregnancies resulted from transfer of some blastocysts. Continued development has been reported for 51% to 89% of early bovine embryos in pseudopregnant rabbit oviducts ligated at the tubo-uterine junction (Sreenan and Scanlon, 1968; Sreenan et al., 1968). These results are comparable to those in sheep, despite evidence that early bovine embryos are particularly sensitive to manipulations (Johnson et al., 1966). Lawson et al. (1972b) recovered 85.4% of 48 bovine embryos placed in ligated oviducts of rabbits. Normal development had occurred in 34 embryos. Thus, more advanced stage embryos were obtained from 70.8% of the embryos placed into rabbits. One cell embryos developed into 8- to 16-cell embryos and most 4-cell embryos reached the 16 cell stage during the 3 day culture. Eight cell embryos developed to morulae and blastocysts after 2 or 4 days, respectively. Fifteen embryos were transferred, following recovery from rabbits, to eight synchronized

heifers. Eleven embryos resulted in calves being born from the six pregnant recipients.

Trounson et al. (1976) utilized subsequent development within the rabbit oviduct to assess affects of different in vitro storage temperatures on viability of bovine embryos. This exemplifies another use for this culture system, short-term development studies following other experimental treatments.

Xenogenous Fertilization

Attempts to fertilize ova from one species in the reproductive tract of another (xenogenous fertilization) seems a logical extension of the knowledge that both sperm capacitation (Bedford, 1983) and early embryonic development (Hunter, 1980) can take place in a heterologous species. It should be noted, however, the first report of xenogenous fertilization precedes (Umbaugh, 1949) either of these discoveries. This early researcher mixed bovine ova and semen in vitro before transfer to rabbit oviducts. Two 2-cell embryos were flushed from the rabbit 20 hours later.

Attempts to fertilize human ova in oviducts of estrous rabbits and rhesus monkeys have failed (Edwards et al., 1966). However, the pseudopregnant rabbit oviduct has been found capable of supporting squirrel monkey and golden hamster fertilization (DeMayo et al., 1980). Saling and Bedford (1981) used mice, rats, hamsters and rabbits to test fertilization in 11 different combinations of gametes and hosts. Ovum penetration by sperm was reported in 10 of the combinations. Interestingly, the only xenogenous combination that did

not support fertilization in that study was hamster gametes in an estrous rabbit oviduct. As noted earlier, xenogenous fertilization of hamsters in a pseudopregnant rabbit has been successful (DeMayo et al., 1980).

Most of the research concerning xenogenous fertilization has dealt with bovine gametes. This literature is summarized in Table 4. Sreenan (1970) reported fertilization of in vitro matured bovine oocytes within the oviduct of estrous ewes. Semen was deposited in ewe oviducts one to seven hours prior to oocyte transfer. Half the fertilized ova cleaved, and some developed to the 8-cell. No fertilization occurred when bull semen was extended with egg yolk citrate and stored for 24 hours before inseminations. No evidence of fertilization was seen when pseudopregnant rabbits were used as hosts.

Another group using in vitro matured bovine oocytes and pseudopregnant rabbits recovered ova 12 hours to seven days after transfer (Trounson et al., 1977). Although 17 nucleated 2- to 4-cell ova were recovered at 24 to 48 hours after insemination, the authors suggested the development was parthenogenic. The development was consistent with that found in vivo 24 to 48 hours post-fertilization (Betteridge, 1977), however, two such ova were also recovered from does which had not been inseminated. Development did not progress beyond the 8-cell stage in any rabbits. Successful fertilization of bovine ova in the pseudopregnant rabbit oviduct has recently been reported (Hirst et al., 1981). This study used follicular oocytes and both fresh and frozen semen.

TABLE 4

Summary of Xenogenous Fertilization Studies in the Bovine

Authors	Recipient Oviduct	Recovery Rate	Recovery Time	Fertilization Results
Umbaugh (1949)	rabbit	26/59	18-72 h	2 2-cells
Sreenan (1970)	estrous rabbit	---	---	0 fertilized
	estrous ewe	198/375	---	8 pronuclear, 9 cleaved
Bederian et al. (1975)	prepuberal gilt	27/70	16-18 h	6 pronuclear
Shea et al. (1976)	cycling ewe	16/24	15-30 h	0 penetration
	prepuberal gilt	29/67	15-30 h	5 pronuclear
Trounson et al. (1977)	estrous rabbit	337/491	12 h-7 days	25 2- to 8-cell
Meinecke and Meinecke-Tillman (1979b)	rabbit	68/120	36-48 h	no penetration
	sow	46/123	24-30 h	no penetration
	ewe	21/42	30-48 h	no penetration
Hirst et al. (1981)	pseudopregnant rabbit	261/582	40-75 h	35 pronuclear
Enright (1982)	ewe	20/35	3 days	3 penetrated

Bedirian et al. (1975) obtained oocytes from gonadotropin-primed cows. Oocytes with an expanded cumulus were considered to have been stimulated, and thus mature. Those surrounded by a compact cumulus mass were considered unstimulated. Both groups were transferred to gilt oviducts following tubal insemination with bull semen. No fertilization occurred in the unstimulated oocytes but 67% of recovered oocytes from the stimulated group had formed pronuclei and contained sperm remnants within the cytoplasm.

Shea et al. (1976) also obtained fertilization in porcine oviducts, but not in ovine oviducts, using in vitro matured oocytes. Similar percentages (50 and 55) of ova had sperm attached to the zona pellucida when recovered from ewes and gilts that had been tubally inseminated. Some success was reported by Enright (1982) in ewe oviducts. In vitro matured oocytes were transferred to ewes simultaneously with fresh and in vitro capacitated bull sperm. Upon recovery and staining, sperm were noted in the cytoplasm of 15% of the ova. One ovum had formed two pronuclei.

MATERIALS AND METHODS

Oocyte Collection

Ovaries were collected 20 minutes after stunning and exsanguination of cattle at two local abattoirs. Cattle were predominantly Holstein or Holstein crosses. Ovary pairs were placed in plastic bags containing 0.15 M NaCl. These bags were either immersed immediately into a thermos flask containing water at 37°C, or, in one study, maintained at 0°C on ice. Only ovaries from nonpregnant cows and ovaries showing normal follicular development were chosen. The period from ovary recovery to oocyte recovery in the laboratory varied from one to five hours.

Notable structures on the ovarian surface were recorded, including number of follicles and their surface diameters, presence of a corpus luteum (CL) or corpus albicans (CA), and appearance of the CL. This information was used to estimate the stage of estrous cycle (Table 5), based on reports of ovarian morphology (Choudary et al., 1968; Ireland et al., 1979). Oocyte collection and handling were performed at 20°C. Oocytes were obtained by aspirating follicular contents using a 22 gauge needle and 3 cc syringe containing 0.2-0.3 cc of medium. All visible follicles on the ovarian surface were aspirated in initial experiments; follicles less than 8.1 mm diameter were aspirated in later trials. Follicular contents were expelled

TABLE 5

Changes in Ovarian Structures During the Bovine Estrous Cycle

Estimated Days of Cycle	Corpus Luteum Appearance	Follicle Size
1-4	New: 5-15 mm, red, point of rupture apparent Old: 6 mm or less, white, hard and fibrotic	less than 10 mm
4-10	16-20 mm, red to brown, vasculature around periphery point of rupture covered	8-10 mm
10-17	18-25 mm, tan to orange, vasculature over apex	6-12 mm
17-21	10-12 mm, yellow, vasculature absent	12-15 mm

into a sterile watch glass and viewed under a stereomicroscope (20X). Oocytes were removed using a micropipette (SMI micro/pettor A, Scientific Manufacturing Industries, Emeryville, CA) and transferred to a spot depression slide containing 0.5 ml medium to await further processing. The basic media used are listed in Tables 6 and 7. Medium was sterilized by filtration (0.45 μm pore size, Millipore Corp., Bedford, MA) and stored in sterile vacutainers at 4°C. Media were supplemented with 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO) and 1 unit/ml heparin (sodium salt, The Upjohn Co., Kalamazoo, MI) for follicle aspiration. Osmolarity of these media, as determined by a vapor pressure osmometer (Wescor Inc., Logan, UT), ranged between 285 mOsm and 295 mOsm. The pH was adjusted to 7.1-7.2 before use.

Oocytes were evaluated under the stereomicroscope using a classification scheme (Table 8) adapted from Soupart and Morgenstern (1973) and Leibfried and First (1979). Numerical values of one were assigned for characteristics typical of oocytes from nonatretic, vesicular follicles as described by Rajakoski (1960) and Marion et al. (1968). Further oocyte processing varied with the experimental design.

In some cases, oocytes were transferred in groups of five to 10 into 10 μl of 15 μM fluorescein diacetate (FDA) in PBS and held three minutes at room temperature. Oocytes were then examined for 10 seconds under a fluorescence microscope (Leitz BG12 and BG38 exciter filters and a K510 long bypass barrier filter, The Microscope Co., New Castle, PA). Oocytes were classified as either positive (bright

TABLE 6

The Basic Contents of TC199 Medium for Gamete Handling and Culture

Ingredient	Amount	Source
TC199[a]	80%	GIBCO Laboratories Grand Island, N.Y.
GG Free Fetal Bovine Serum[b]	20%	GIBCO Laboratories Grand Island, N.Y.
Sodium Pyruvate	115.2 μg/ml	Sigma Chemical Co. St. Louis, MO
Gentamicin	0.1 mg/ml	Schering Corp. Kenilworth, N.J.

[a]Medium 199 with 25 mM HEPES buffer, Earle's salts and L-glutamine.

[b]Heated at 56°C for 30 minutes.

TABLE 7

The Basic Contents of PBS Medium for Gamete Handling and Culture

Ingredient	Amount	Source
Dulbecco's Phosphate Buffered Saline - 1X	100 ml	GIBCO Laboratories Grand Island, N.Y.
Bovine Serum Albumin	4 mg/ml	Sigma Chemical Co. St. Louis, MO
Sodium Pyruvate	36 µg/ml	Sigma Chemical Co. St. Louis, MO
D-Glucose	1 mg/ml	Mallinckrodt, Inc. Paris, KY
Gentamicin	0.1 mg/ml	Schering Corp. Kenilworth, NJ

TABLE 8

Classification System for Oocyte Evaluations

Component	Description	Numerical Score
Cumulus Investment	cumulus oophorus complete, compact, and more than 3 layers thick	1
	not completely surrounding oocyte or less than 3 layers	2
	nude; cellular investment absent	3
Ooplasm	fine, even granulation; fills zona pellucida evenly	1
	granules clumped or unevenly distributed - withdrawn to center leaving clear periphery or coalescing into black bodies; fills zona pellucida	2
	shrunk away from zona pellucida; vacuolated or fragmented; not evenly filling zona pellucida	3

fluorescence) (FDA+) or negative (faint or no fluorescence) (FDA-). These were then rinsed in medium.

A second viability assay used was Trypan Blue dye (TpB) exclusion. Oocytes were transferred to a 0.2% solution in PBS for 1 minute at room temperature. They are then washed twice and examined for exclusion (TpB-) or uptake (TpB+). A total of 54 TpB- oocytes, representing various investment and ooplasm conditions, were fixed in Bouin's fluid, embedded in paraffin, and serially sectioned at 5 μm. Sections were then mounted on glass slides, stained with Harris haemotoxylin-acid fuchsin, and examined under a compound microscope to evaluate nuclear status.

Oocyte Culture

Oocytes selected for culture were deposited into an 8-chamber tissue culture slide (Lab-Tek, Miles Laboratories Inc., Naperville, IL) containing 0.4 ml medium. Additions to the basic media for culture included gonadotropins and steroids. Commercial follicle stimulating hormone (FSH) (FSH-P, Burns-Biotec Lab., Omaha, NE) was diluted to obtain a final concentration of either 1 μg/ml or 10 μg/ml. Absolute ethanol was used to dissolve estradiol-17β (E_2) and progesterone (P) (Nos. E8875 and P-0130, respectively, Sigma Chemical Co., St. Louis, MO). This solution was diluted to obtain final concentrations of 1 μg/ml E_2 or P and 0.2% ethanol in the culture medium. The culture period prior to fertilization trials was 24 to 26 hours. Oocytes were cultured 27 to 30 hours during *in vitro* maturation

trials. Incubation was at 37°C or 39°C and in a 5% CO_2 in air or 5% CO_2:5% O_2:90% N_2 humidified environment depending on the experimental design.

After culture oocytes were removed from culture chambers and evaluated under the dissecting microscope (50-70X). Viability was again assessed using vital dyes. Oocytes selected for xenogenous fertilization were placed into a depression slide to await transfer. For evaluation of maturation, oocytes with portions of the cumulus oophorus intact were placed in a fresh 1% hyaluronidase solution for five minutes and then cleaned mechanically by repeated passage through a finely-drawn pipet. All oocytes were examined under 100X magnification with an inverted microscope and the presence of polar bodies was recorded. Oocytes were then transferred to a fixative (4 methanol:1 glacial acetic acid:5 distilled water by volume) for 5 minutes. Oocytes were mounted on a glass slide in a drop of final fixative (3 methanol:1 glacial acetic acid, v/v), and a cover slip supported at the corners with spots of petroleum jelly was applied. The slide was then emersed in the final fixative for 24 hours prior to staining with 2% giemsa (GIBCO, Grand Island, NY). Examination was then made by light microscopy and the nuclear stage was recorded.

Xenogenous Fertilization

Host animals for these experiments were adult New Zealand White rabbits. Rabbits were housed singly in stainless steel cages at 20°C during winter months and ambient temperature during the summer. Laboratory Rabbit Chow^R (Ralston Purina Co., St. Louis, MO) and water

were provided ad libitum. Pseudopregnancy was induced one day prior to surgery by i.v. administration of 100 IU HCG (A.P.L., Ayerst Laboratories, Montreal). Rabbits were anesthetized with approximately 60 mg/2.5 kg body weight of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) administered through the marginal ear vein. A surgical plane of anesthesia was maintained by ether inhalation. The abdominal area was shaved and scrubbed with an iodine solution, and the rabbit restrained on an inclined surgery table. Finally, the reproductive tract was exposed through a mid-ventral incision. Surgical instruments were previously cold sterilized in a benzalkonium chloride solution (Zepharin Chloride, Winthrop Laboratories, NY).

Frozen bull semen was obtained in ampules or straws from the Michigan Animal Breeders' Cooperative and Michigan State University. Semen was thawed, diluted 3:1 with 37°C medium and maintained in a 37°C waterbath. A visual estimate of motility was made and approximately $3x10^6$ sperm in 0.05 ml volume of medium were deposited 1- to 2 cm into the rabbit oviduct through the infundibulum. The inseminating device consisted of a 0.25 cc glass TB-syringe and 20 gauge 1.5" needle to which 5 cm polystyrene tubing (PE 90 Intramedic, Clay Adams, Parsippany, NJ) was affixed.

Immediately following insemination, a known number of oocytes, ranging from 5 to 15, were placed into the infundibulum using the micropipette. The tubal-uterine junction was then ligated with 00 chromic gut suture, and the incision closed.

Rabbits were sacrificed by cervical dislocation, 24 to 48 hours after surgery, for embryo recovery. The oviducts and small portions of the uterus were excised and the tubal-uterine ligature removed. A blunted 25 gauge needle was introduced through the uterus and tubal-uterine junction into the oviduct and 2 cc medium followed by 1 cc air was flushed through the oviduct. The flushed medium was collected in a watch glass. Embryos were located with a stereomicroscope and transferred to a depression slide for examination under an inverted microscope. Occurrence of polar bodies or cleavage were recorded. Embryos were then fixed as previously described and stained with giemsa to assess nuclear stage. Cleaved ova, as well as ova with 2 pronuclei and a sperm tail, were considered fertilized.

Two rabbit oviducts, containing a total of 33 ova, were inseminated with dead sperm as a control for parthenogenic activation.

In Vitro Fertilization

All IVF experiments were conducted at 39° under a 5% O_2 atmosphere. Oocytes involved in IVF trials were not evaluated following in vitro maturation culture. To reduce any deleterious effects of handling, oocytes remained in the same culture chamber throughout the maturation and fertilization period. After 24 hours of oocyte culture to affect maturation, frozen bull semen was thawed and diluted as previously described. The semen solution was then centrifuged 5 minutes at 2200 rpm (equivalent to 731XG; relative centrifugal force = (1.119×10^{-5}) (RPM^2)(radius in cm)). The supernatant was decanted off and discarded. Packed sperm cells were resuspended in 1.5 ml medium, and the procedure was repeated. Following the second

resuspension, motility was visually estimated. Ten microliters of this suspension were added to each culture chamber to yield a final concentration of 1 to $3x10^6$ sperm/ml. The culture medium was replaced approximately 20 hours after insemination. This was done by aspirating most of the medium in a chamber, without disturbing the cumulus-oocyte complexes, and immediately refilling the culture chamber with fresh media. Oocytes were removed from culture 40 to 46 hours after insemination, stripped of adhering cumulus, and examined for evidence of fertilization in a manner previously detailed.

A total of 86 oocytes in 3 different media were cocultured with dead sperm to assess parthenogenic activation. Evidence of fertilization consisted of 2 pronuclei or a single pronucleus and penetrating sperm present in the cytoplasm.

Statistical Analyses

Distribution-free nonparametric statistics were used for analyses of all data. Proportions were arranged into categorical contingency tables and tested for independence using the Chi-square statistic. Where no interaction was found, main effects were evaluated using the Chi-square test of goodness of fit.

RESULTS

Results of a preliminary trial to determine the effect of the age of the ovary donor on oocyte collection are shown in Table 9. A total of 22 cows and 73 heifers were included. The number of total follicles per ovary were similar for cows and heifers (15.34 and 15.17, respectively), and the mean $\pm$ SE for recovery rate was 70.17$\pm$3.68 for both age groups. No differences were detected between mean investment scores for follicle size or age grouping. There was a tendency ($p<.1$) for more oocytes from small follicles to have a complete, compact cumulus investment when the data were summarized for both heifers and cows (Table 10).

The relationship between presence of a corpus luteum and follicle or oocyte characteristics is shown in Tables 11, 12 and 13. There were 196 ovaries collected at 20 sampling times included in this trial. No difference in size distribution of follicles was observed between ovaries containing a CL and those containing a CA (Table 11). The mean numbers of small ($\leq$3 mm) and large ($\geq$3 mm) follicles for ovaries with a CL and with a CA were 13.10$\pm$4.12, 1.27$\pm$0.56, 14.33$\pm$ 4.23 and 1.21$\pm$0.78, respectively. Oocytes were recovered from 78.65% of the follicles. Corpus luteum presence had no effect on the cumulus investment surrounding recovered oocytes (Table 12). The mean investment scores were 2.20 and 2.18 for ovaries with CL and CA,

TABLE 9

Subclass Frequencies of Oocytes with Various Investment Scores within Follicle Size on Cow or Heifer Ovaries

	Investment Score	Follicle Diameter (mm)			Row Sum	(%)
		<3	4-8	>8		
Cows	1	52	2	0	54	(10.95)
	2	273	16	3	292	(59.23)
	3	143	4	0	147	(29.82)
	Column Sum	468 (2.19)[1]	22 (2.09)	3 (2.00)		
Heifers	1	170	3	1	174	10.76
	2	906	49	14	969	59.92
	3	443	22	9	474	29.31
	Column Sum	1519 (2.18)[1]	74 (2.26)	24 (2.33)		

[1]Numbers in parenthesis are mean investment scores for the respective follicle sizes within an age group. $\chi^2 = 0.134$, n.s.

TABLE 10

Summary of Follicle Size Effects on Investment Scores Across Age Groups

Investment Score	Follicle Diameter (mm)			
	<3		>3	
	n	(%)	n	(%)
1	222	(11.17)	6	(4.88)
2	1179	(59.34)	82	(66.67)
3	586	(29.49)	35	(28.45)

$\chi^2 = 5.331$, $p<.1$

TABLE 11

Proportions of Follicles in Each Size Group and Relation to Ovarian Activity

Ovarian Activity[1]	(n)	Follicle Size (mm)			Total	(n)
		<3	4-8	>8		
CL	(88)	90.4	8.2	1.3	42.2	(1275)
CA	(114)	91.3	7.3	1.4	57.8	(1737)
overall		90.9	7.7	1.4	100.0	(3012)

[1]CL = Corpus lutea present; CA = Corpus albicans present.

χ^2 = .8893, n.s.

TABLE 12

Proportions of Oocytes in Each Investment Classification and Relation to Ovarian Activity

Ovarian Activity[1]	(n)	Oocyte Investment Score			Total	(n)
		1	2	3		
CL	(88)	10.7	58.1	31.2	47.7	(1012)
CA	(114)	10.5	61.0	28.5	57.3	(1357)
overall		10.6	59.7	29.7	100.0	(2369)

[1]CL = Corpus lutea present; CA = corpus albicans present

$\chi^2 = 2.2783$, n.s.

TABLE 13

Proportions of Oocytes in Each Ooplasm Classification and Relation to Ovarian Activity

Ovarian Activity[1]	(n)	Ooplasm Score			Number of Oocytes
		1	2	3	
CL	(16)	32.62	49.73	17.65	187
CA	(20)	32.50	47.50	20.00	240
overall		32.55	48.48	18.97	427

[1]CL = Corpus luteum present; CA = Corpus albicans present.

χ^2 = .4153, n.s.

respectively. Quality of ooplasm was similarly unaffected by corpora lutea presence on the ovary (Table 13). Mean ooplasm scores were 1.85 for ovaries with a CL and 1.88 for ovaries with a CA. In either ovary group, nearly 20% of recovered follicular oocytes had ooplasm classified as degenerate. Approximately one-third of the oocytes had ooplasm considered typical of a healthy oocyte from nonatretic follicles.

Results of an expanded examination of follicle size effects on oocyte characteristics are shown in Table 14. This experiment included 158 ovaries collected at 18 samplings. There was an association ($p<.05$) between follicular diameter and investment scores of oocytes. As noted for an earlier experiment, smaller follicles tended to have more oocytes scored at 1. Mean investment scores were 2.19 and 2.27 for follicles less than and greater than 3 mm diameter, respectively. Follicle diameter significantly affected ooplasm scores ($p<.01$, Table 14). Degenerate ooplasm (score 3) were found in one-third of the oocytes recovered from larger follicles, while 23% of oocytes from smaller follicles were given a score of 3. Mean ooplasm scores for small ($\leq$3 mm) and large ($\geq$3 mm) follicles in this trial were 1.87 and 2.22, respectively.

The effect of the time required after slaughter to complete oocyte processing was evaluated (Table 15). Oocytes recovered more than 4 hours after a cow was killed did not differ from those recovered earlier, as shown by both mean scores and percentage of oocytes in each classification. More than one-third of the oocytes

TABLE 14

Frequency of Oocytes in Each Classification and Relation to Follicle Size

		Follicle Diameter (mm)			
		<3		>3	
Cumulus Investment Score[2]	1	257	(10.88)[1]	9	(3.64)
	2	1407	(59.57)	106	(66.06)
	3	698	(29.55)	50	(30.30)
Oocyte Cytoplasm Score[3]	1	330	(35.99)	17	(10.76)
	2	377	(41.11)	89	(56.33)
	3	210	(22.90)	52	(32.91)

[1]Numbers within parenthesis are percentages within a criteria and follicle size.

[2]$\chi^2 = 8.890$, $p<.05$

[3]$\chi^2 = 39.3141$, $p<.01$, $C = .1878$

TABLE 15

Effect of Interval from Slaughter to Oocyte Culture on Proportion of Oocytes in Each Classification

Score	Slaughter to Culture Time	
	<4 hours	>4 hours
Cumulus Investment		
1	11.56	10.95
2	60.26	60.10
3	28.18	28.95
mean	2.17	2.18
(n)	(692)	(1172)
Ooplasm		
1	33.54	36.78
2	45.83	36.36
3	20.62	26.86
mean	1.87	1.90
(n)	480	726

processed at either time interval were classified with ooplasm with a score of 1, and 71% had some cumulus cell investment.

A relationship between ooplasm classification and viability, as assessed by vital dyes, is shown in Figure 1. A total of 891 oocytes were evaluated in 12 trials. TpB exclusion and FDA uptake were highly correlated ($r = .987$). Fewer oocytes were FDA+ than were TpB- in all categories.

Oocytes fixed immediately after collection were predominantly in the germinal vesicle stage (66.67%, Table 16). The percentage of oocytes with an intact GV appeared to decline with higher scores, although the number of oocytes examined was small and differences were not significant.

The number of oocytes in each investment and ooplasm category are shown in Table 17 for collections from ovaries transported to the laboratory at two temperatures. There was a tendency for more degenerate ooplasm in oocytes collected after transport on ice. No difference was found between temperatures for investment scores. Subsequent culture of the oocytes revealed transport temperature effects (Table 18). Survival (proportion which remain TpB-) during *in vitro* culture was superior ($p<.01$) when ovaries had been kept close to 37°C. There was also a trend for more oocytes to mature to Met II after warm transport ($p<.10$). Changes in mean investment and ooplasm scores during culture were small (warm transport; -0.33, -0.34; cold transport, -0.29 and -0.37 for investment and ooplasm scores, respectively).

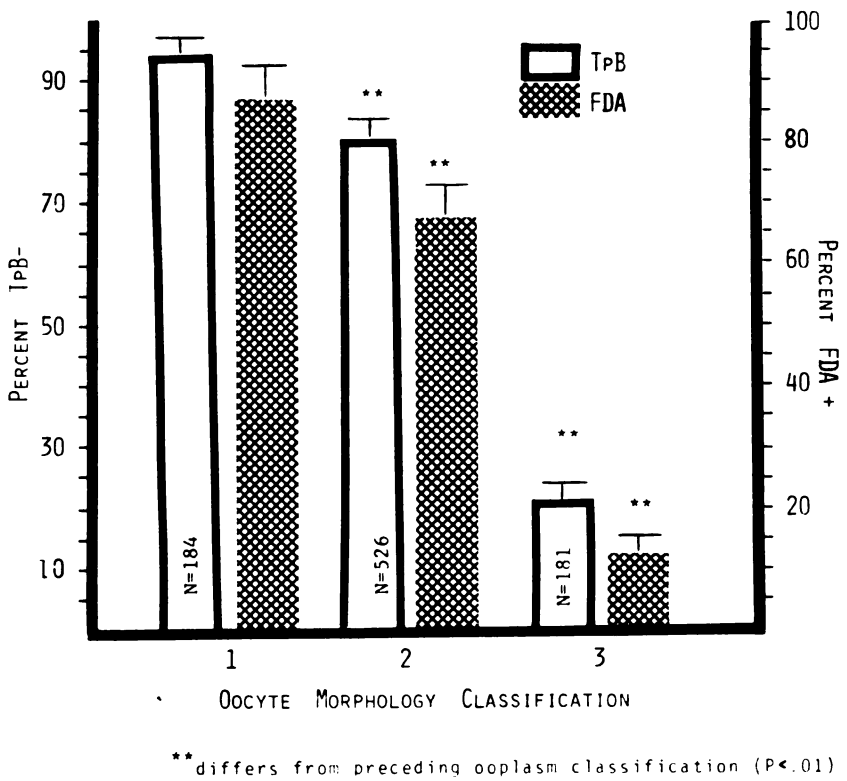

Figure 1. Oocyte viability assessments and relationship to ooplasm quality score at collection.

TABLE 16

Meiotic stage of Follicular Oocytes at Collection

Ooplasm Score	Cumulus Enclosed		Nude	
	1	2	1	2
Number with GV[1]	5	18	5	8
%	83.33	75.00	66.67	53.33
Maturing[2]	1	2	3	5
%	16.67	11.11	33.33	33.33
Unidentifiable	---	4	1	2
Total	6	24	9	15

[1] $\chi^2 = 3.200$, n.s.

[2] $\chi^2 = 4.69$, n.s.

TABLE 17

Effects of Transport Temperature on Oocyte Characteristics at Follicle Aspiration

Transport[1]	Investment Score[2]					
	1	(%)	2	(%)	3	(%)
Warm	11	(6.92)	102	(64.15)	46	(28.93)
Cold	14	(8.19)	97	(56.72)	60	(35.09)

Transport[1]	Ooplasm Score[3]					
	1	(%)	2	(%)	3	(%)
Warm	97	(61.01)	57	(35.85)	5	(3.14)
Cold	105	(61.40)	54	(31.58)	12	(7.02)

[1] warm = transport in an incubated container at 32-37°C; cold = transport on ice near 0°C.

[2] $\chi^2 = 1.901$

[3] $\chi^2 = 2.848$, $p<.1$

TABLE 18

Effects of Transport Temperature on Oocyte Characteristics After *In Vitro* Culture

Transport[1]	n	TpB-[2]	(%)	Mature[3]	(%)
Warm	90	79	(87.78)	23	(25.56)
Cold	80	55	(68.75)	11	(13.75)

[1] warm = 32-37°C; cold = 0°C.

[2] $\chi^2 = 9.19$, $p<.01$

[3] $\chi^2 = 3.69$, $p<.10$

Maturation ratios (number mature/number cultured) for investment-ooplasm combinations are shown in Table 19. Oocytes devoid of cumulus cells (score 3) matured less often than those with surrounding cells (score 1 or 2). No maturation occurred in oocytes with score 3 ooplasm.

Table 20 shows the maturation ratios for oocytes that were tested with TpB and FDA prior to culture. Significantly poorer maturation was again detected in oocytes with the poorest ooplasm rating. This difference was not found, however, among oocytes selected on the basis of TpB or FDA tests.

Addition of lactate to TC199 medium was not beneficial for oocyte culture (Table 21).

A significant interaction was found between medium and atmospheric environment during oocyte culture (Table 22). Addition of FSH tended to benefit survival when the basic medium was TC199 and the oocytes were incubated in 5% CO_2 in air ($p<.10$). No benefit was seen with PBS. No differences between media were detected in a 5% O_2 environment. Survival was higher in the lower O_2 environment in all cases, though statistical differences were found only for TC199 and PBS + FSH. Effects of these culture conditions on maturation can be seen in Table 22. The percentage of O_2 in the atmosphere did not alter maturation ratios. The only media effect found was with FSH addition to PBS which resulted in fewer mature ova ($p<.05$). Maturation in the other 3 media was 21.84%.

TABLE 19

Proportion of Follicular Oocytes within an Investment-ooplasm Rating that Matured In Vitro

		Cumulus Investment Score						Totals	(%)
		1		2		3			
		Rate	(%)	Rate	(%)	Rate	(%)		
Ooplasm Score	1	$8/25^{ac}$	(32.00)	$21/112^{ac}$	(18.75)	$1/28^{ad}$	(3.57)	30/165	(18.18)
	2	$2/16^{ac}$	(12.50)	$26/146^{ac}$	(17.81)	$1/54^{ad}$	(1.85)	29/216	(13.42)
	3	$0/3^{b}$	(0.0)	$0/10^{b}$	(0.0)	$0/17^{a}$	(0.0)	0/30	(0.0)
Totals		10/44	(22.73)	47/268	(17.54)	2/99	(2.02)		

^{ab}Ratios within a column with different superscripts differ ($p<.05$).

^{cd}Ratios within a row with different superscripts differ ($p<.05$).

$\chi^2 = 24.094$, $p<.01$

TABLE 20

Effect of Preselecting Oocytes by Vital Dyes on *In Vitro* Maturation Rates

Preselection	Ooplasm Score			Overall	(%)
	1	2	3		
None	22/181[a]	25/254[a]	0/86[b]	47/521	(9.02)
TpB	41/286[a]	44/408[a]	0/8[a]	85/702	(12.11)
FDA	41/275[a]	44/380[a]	0/8[a]	85/663	(12.82)

$\chi^2 = 19.615$, $p<.05$.

[ab]Ratios within a row with different superscripts differ ($p<.01$).

TABLE 21

Effect of Lactate Addition on Oocyte Characteristics After *In Vitro* Culture

Lactate Level	Investment Score Change[1]	Ooplasm Score Change[1]	TpB-[2]	(%)	Mature[3]	(%)
0	0.38	0.76	47/66	(71.21)	11/66	(16.67)
50 mM	0.12	0.08	17/25	(68.00)	4/25	(16.00)
100 mM	0.08	0.62	17/24	(70.83)	2/24	(8.33)

[1] Score change is the difference between preculture mean and postculture mean.

[2] $\chi^2 = .092$, n.s.

[3] $\chi^2 = 1.008$, n.s.

TABLE 22

Effects of Culture Media and Atmospheric Environment[1] on Survival and Maturation of Oocytes During Culture

Media	5% CO_2 in Air		90% N_2:5% O_2:5% CO_2		Total	
	Survival	Met II	Survival	Met II	Survival	Met II
TC199	118/212[ac]	31/118	43/59[d]	6/32	161/271	37/150[a]
TC199 + FSH[2]	64/97[bc]	16/64	23/30[c]	5/29	87/127	21/93[a]
PBS	63/94[bc]	11/63	74/100[c]	14/74	137/194	25/137[ab]
PBS + FSH[2]	80/122[bc]	8/80	62/79[d]	9/62	142/201	17/142[b]
	325/525	66/325	202/268	34/197		

survival interaction $\chi^2 = 21.323$, $p<.01$

maturation interaction $\chi^2 = 10.653$, n.s.; media main effect $\chi^2 = 8.451$, $p<.05$

[1]survival = TpB- after culture/TpB- before culture.

[2]10 µg/ml FSH-p

[ab]Ratios within a column without a common superscript differ ($p<.05$).

[cd]Survival ratios within a row without a common superscript tend to differ ($p<.10$).

Oocyte survival rates at two incubation temperatures are shown in Table 23. The increased temperature did not affect survival; 76.56% of cultured oocytes remained TpB-. Survival rates can be seen to vary among the media used in this trial, however, differences were not significant. Table 23 contains the maturation results of this trial. Again, variation among media is evident, but the interaction of medium and incubation temperature was not significant. The number of oocytes that matured in TC199 was not affected by including FSH. Maturation in PBS with added FSH was not statistically different than that in other media but was lower at both temperatures. The difference approached significance when averaged over temperature ($\chi^2 = 2.13$). There was a trend for increased maturation at 39° ($p<.10$). This effect was consistent for all media. Further media trials were conducted at 39°C in 90% N_2:5% O_2:5% CO_2.

Results of two modifications to the basic culture medium are shown in Table 24. The first was elimination of HEPES buffer from TC199 medium. A second was addition of heat-inactivated fetal calf serum to PBS medium. A comparison of the modified media shows no difference in oocyte maturation. However, both media supported a higher maturation percentage than observed without modification (Table 25). Oocyte survival was better in the PBS medium than TC199 medium (Table 24).

Addition of steroids to the culture media did not increase the number of oocytes completing meiosis (Table 25). In addition, the number of oocytes excluding TpB was reduced by including either progesterone or estrogen during culture.

TABLE 23

Effect of Two Different Incubation Temperatures on Survival and Maturation of Oocytes in Several Media[1]

Media	37°C		39°C		Total	
	Survival	Met II	Survival	Met II	Survival	Met II
TC199	43/59	6/32	21/34	4/18	64/93	10/50
TC199 + FSH[2]	23/30	5/29	36/42	8/36	59/72	13/65
PBS	74/100	14/74	44/53	14/44	118/153	28/118
PBS + FSH[2]	62/79	9/62	17/21	3/17		
	202/268	34/197	118/150	29/115		

Survival: contingency $\chi^2 = 8.54$, n.s.; temperature $\chi^2 = .58$, n.s.; media $\chi^2 = 4.63$, n.s.

Maturation: contigency $\chi^2 = 5.41$, n.s.; temperature $\chi^2 = 2.85$, $p<.10$; media $\chi^2 = 2.15$, n.s.

[1]Survival = TpB- after culture/TpB- before culture; 90% N_2:5% O_2:5% CO_2 atmosphere.

[2]10 µg/ml FSH-p

TABLE 24

Comparisons Between Modified Media in Ability to Support *In Vitro* Maturation Under 5% O_2 at 39°

Media	Survival[1]	(%)	MET II	(%)
TC199 (-h)	38/50[a]	(76.0)	11/38	(28.9)
TC199 (-h) + 1[3]	15/21[a]	(71.4)	6/15	(40.0)
TC199 (-h) + 10[4]	34/46[a]	(73.9)	12/34	(35.3)
PBS-FCS[5]	62/69[b]	(89.8)	22/62	(35.48)
PBS-FCS + 1[3]	29/31[b]	(93.5)	9/29	(31.03)
PBS-FCS + 10[4]	45/50[b]	(90.0)	13/45	(28.89)
	χ^2=13.17, p<.05		χ^2=1.26, n.s.	

[1]Survival = TpB- after culture/TpB- before culture.

[2]TC199 (-h) = TC199 without HEPES buffer.

[3]1 µg/ml FSH added.

[4]10 µg/ml FSH added.

[5]PBS-FCS = PBS with 20% fetal calf serum.

[ab]Ratios lacking a common superscript differ (p<.05).

TABLE 25

Effects of Steroid Addition to PBS Medium on Oocyte Viability and Maturation

Added Hormone[1]	Survival[1]	(%)	MET II	(%)
None	44/53[a]	(83.02)	14/44	(31.82)
E	31/45[bc]	(68.89)	7/31	(22.58)
E + FSH	21/32[bc]	(65.62)	3/21	(14.29)
P	28/36[b]	(77.78)	7/28	(25.00)
P + FSH	19/35[c]	(54.28)	6/19	(31.58)

[1]E = 1 μg/ml estradiol, FSH = 10 μg/ml FSH, P = 1 μg/ml progesterone.

[2]Survival = TpB- after culture/TpB- before culture. Survival $\chi^2 = 9.84$, $p<.05$.

[3]Maturation $\chi^2 = 2.79$, n.s.

[abc]Ratios lacking a common superscript differ ($p<.05$).

Xenogenous fertilization was attempted using the information gained through *in vitro* maturation experiments. The first trial was an attempt to demonstrate the benefit of a culture period prior to attempted fertilization in the rabbit oviduct (Table 26). No differences were detected, using four to five rabbits for each culture group, in the number fertilized. Higher recovery rates of oocytes were experienced with longer culture times.

Having determined the maturation percentage in various media, information was sought on fertilization rates following culture. Two to three replicates were performed for each of the media in Table 27. No statistical differences were detected among media in recovery or fertilization of ova. Failure to obtain fertilization in oocytes after 24-hour culture in FSH-containing medium seems noteworthy. Three media were tested in an atmosphere of 5% O_2 at 37°C (Table 28). Again, there were no differences among media. Fertilization (10%) was achieved after *in vitro* maturation in an FSH-containing medium. A comparison of data in Table 30 with that in Table 29 indicates a benefit to fertilization following culture in 5% O_2 rather than 18%. *In vitro* maturation at 39°C rather than 37°C appeared to improve subsequent xenogenous fertilization percentages (Table 29). All tested media supported fertilization equally well. Oocyte recovery was complete from the two rabbits used to test fertilization after culture in mPBS with FCS.

Parthenogenesis was not induced in the XF system following *in vitro* maturation at 37° or 39°C. One oocyte (3.0%) inseminated with dead sperm formed two polar bodies but lacked pronuclei or evidence of

TABLE 26

Effects of In Vitro Culture for Varying Lengths of Time on Subsequent Xenogenous Fertilization

Hours in Culture	Recovery Ratio	(%)	Fertilization Ratio	(%)
0	48/114[a]	(42.10)	3/48	(6.25)
24	128/237[b]	(54.01)	22/128	(17.19)
36	64/85[c]	(75.29)	10/64	(15.62)
	χ^2=21.90, p<.01		χ^2 = 3.43, n.s.	

[abc]Ratios with different superscripts differ (p<.05).

TABLE 27

Xenogenous Fertilization Ratios of Ova *In Vitro* Matured in Various Media at 37°C Under 5% CO_2 in Air

Media	Recovery Ratio	(%)	Fertilized Ratio	(%)	Percent Fertilized[1] of Mature
TC199	105/152	(69.1)	11/105	(10.5)	39.9
TC199 + FSH[2]	18/28	(64.3)	0/18		
PBS	18/23	(78.3)	2/18	(11.1)	63.6
PBS + FSH[2]	21/28	(75.0)	0/21		
	$\chi^2 = 1.58$, n.s.		$\chi^2 = 4.49$, n.s.		

[1]Extrapolating previously determined maturation rates to this sample of oocytes incubated under the same conditions.

[2]10 μg/ml FSH-p

TABLE 28

Xenogenous Fertilization Ratios of Ova In Vitro Matured in Various Media at 37°C under 5% O_2:5% CO_2:90% N_2

Media	Recovery Ratio	(%)	Fertilized Ratio	(%)	Percent Fertilized[1] of Mature
TC199	61/69	(88.4)	9/61	(14.7)	78.7
TC199 + FSH[2]	20/26	(76.9)	2/20	(10.0)	58.0
TC199 (-h)[3]	14/19	(73.7)	2/14	(14.3)	76.1
	95/114	(83.3)	13/95	(13.7)	
	$\chi^2 = 3.50$, n.s.		$\chi^2 = 0.29$, n.s.		

[1]Extrapolating previously determined maturation rates to this sample of oocytes cultured under the same conditions.

[2]10 µg/ml FSH-p

[3]mTC199 without HEPES buffer.

TABLE 29

Xenogenous Fertilization Ratios of Ova In Vitro Matured in Various Media at 39°C under 5% O_2:5% CO_2:90% N_2

Media	Recovery Ratio	(%)	Fertilized Ratio	(%)	Percent Fertilized[1] of Mature
TC199	41/48[a]	(70.7)	7/41	(17.1)	76.8
TC199 (-h)[2]	46/57[a]	(80.7)	9/46	(19.6)	43.2
PBS	32/36[a]	(88.9)	4/32	(12.5)	35.71
PBS-FCS[3]	44/44[b]	(100.0)	5/44	(11.4)	55.1
	163/185	(88.11)	25/163	(15.34)	
	χ^2=9.28, p<.05		χ^2=1.46, n.s.		

[1]Extrapolating previously determined maturation rates to this sample of oocytes cultured under the same conditions.

[2]TC199 without HEPES buffer.

[3]PBS with 20% FCS

[ab]Ratios without a common superscript differ (p<.01).

sperm penetration. Similarly, parthenogenesis was not found during IVF experiments. Two oocytes (2.3%) were activated to form two polar bodies. However, no oocytes formed pronuclei.

Data on *in vitro* fertilization of *in vitro* matured oocytes are presented in Table 30. All media supported fertilization and no differences were found. The mean fertilization percentage was 14.46. Forty percent of penetrated ova developed to the 2 pronuclei stage. Variation in the proportion forming 2 pronuclei can be seen among the tested media, but differences were not statistically significant. One ovum completed mitosis to form a 2-cell following maturation and fertilization in mTC199 with 10 μg/ml FSH.

TABLE 30

Effect of In Vitro Maturation Media on Subsequent Fertilization In Vitro

Media	Fertilized	(%)	2 Pronuclei	(%)
TC199	5/38	(13.2)	1	(20.0)
TC199 + FSH[1]	5/36	(13.9)	3	(60.0)
PBS	6/44	(13.6)	2	(33.3)
PBS-FCS[2]	8/35	(22.8)	2	(25.0)
TC199 (h-)	5/42	(11.9)	1	(20.0)
TC199 (h-) + FSH[1,3]	6/47	(12.8)	5	(83.3)
	35/242	(14.46)	14	(40.00)
	χ^2 = 2.41, n.s.		χ^2 = 8.06, n.s.	

[1]10 µg/ml FSH-p

[2]PBS-FCS = PBS with 20% fetal calf serum.

[3]TC199 (h-) = TC199 without HEPES buffer.

DISCUSSION

The oocyte recovery rate of 70.2% obtained in this study was higher than that reported by others (Leibfried and First, 1979a; Fukui and Sakuma, 1980). The ability to recover oocytes by follicle aspiration is undoubtedly related to the expertise of the technician, which is in turn, developed through experience. Recovery rates improved rapidly during the early portions of this study.

Size of the follicle population varies with the age of the cows (Betteridge, 1977), nutritional status (Hill et al., 1970), and breed (Choudary et al., 1968). Thus, the lack of a difference between heifers and cows in this study was unexpected. The heifers were predominantly of beef breeding and had been on a higher plane of nutrition than had the cows, which were primarily cull dairy animals.

A local effect of corpora lutea on follicular development has been postulated (Dufour et al., 1972; Dailey et al., 1982), however, no effect was found in the present study on follicle size or oocyte characteristics. Similar results were reported by Fukui and Sakuma (1980). The effect of the CL may vary during the estrous cycle (Matton et al., 1981). Thus, one would not anticipate its effect to be evident in a sample of ovaries if the stage of the cycle is random.

A higher proportion of oocytes showing morphological signs of atresia was obtained from follicles with greater than 3 mm diameters. This finding has been reported previously (Leibfried and First, 1979a; Staigmiller and England, 1982) and can be related to high turnover rates in large follicles (Ireland et al., 1979). With the exception of the three days prior to ovulation, the larger follicles are destined to undergo atresia (Dufour et al., 1972). Therefore, in any sample of ovaries collected without regard to stage of cycle, the large follicles are likely in various stages of atresia. Despite reports that the oocyte morphology may degenerate late in stages of follicular atresia (Miller and Jagiello, 1973; Uilenbroek et al., 1980; Kruip and Dielman, 1982), a high incidence of atresia in large follicles will logically result in a greater proportion of degenerate oocytes. Accordingly, subsequent experiments involved oocytes from the more abundant small follicles only. Irrespective of follicle diameter, about 30% of all collected oocytes were nude. These oocytes are considered to have originated from atretic follicles (Rajakoski, 1960; Ingram, 1962).

When the blood supply to the ovary is discontinued, follicular atresia begins in all follicles (O'Shea et al., 1978; Hay et al., 1977). Knudsen et al. (1979) reported marked postmortem changes in porcine follicular fluid as a result of anoxia and acidosis which quickly follow ischemia. The effects of these changes on the cumulus-oocyte complex are unknown. Since collection and transport of ovaries to the laboratory required a minimum of 2 hours in initial experiments, oocyte degeneration during ovary storage was a possibility.

Oocytes collected after 4 hours did not show any morphological signs of deterioration during storage. Similarly, Shea et al. (1976) did not affect bovine oocyte maturation by storing ovaries for 2 hours before oocyte collection. Bovine follicular fluid possibly does not undergo the dramatic changes seen in other species upon ovary removal. Or, more likely, the oocyte is resistant to the toxic effects of follicular fluid in isolated ovaries. Ovaries were stored up to 7 hours after slaughter of cattle before oocyte collection in this experiment.

The significant differences in viability between classification categories indicate that the subjective evaluation of cytoplasm is allowing oocytes to be sorted into functional groups. However, the viability stains, TpB or FDA can be used to further identify nonviable oocytes that are present in all categories. FDA is a more sensitive test than TpB, since the former requires a functional enzyme system in addition to an intact cell membrane to give a viable-reading.

The percentage of oocytes with intact germinal vesicles at recovery in this study (66.7%) was lower than the 93.2% reported by Hunter et al. (1972) or 84.4% by Leibfried and First (1979a). Oocytes were classed as unidentifiable if chromosomes could not be located or if a meiotic stage was not clearly identified. The later group represents degenerated oocytes, derived from late atretic follicles (Marion et al., 1968).

As stated above, intrafollicular degeneration is a major concern when lag times are involved before oocytes can be recovered. Maintaining ovaries on ice may slow the degenerative process. However,

oocytes are vulnerable to temperature fluctuations (Brackett, 1981). A comparison of warm and cold transport temperatures in this study did not reveal differences in oocytes at collection. However, oocytes did not survive *in vitro* culture nor mature as well after being exposed to low temperatures. This is in contrast to work with sheep ovaries which demonstrated greater maturation after transport at 1° or 22°C than after transport at 35°C (Snyder, 1977a). The optimal transport and storage temperature, to achieve *in vitro* maturation in a high proportion of collected oocytes, has not yet be ascertained.

Oocytes assigned a score of 3 for either cumulus investment of ooplasm exhibited very poor maturation ratios. No oocytes with the poorest cytoplasm score matured to Metaphase II in culture, although the number of score 3 oocytes was small. The percent maturation following culture of nude oocytes was 2.02. These oocytes were probably from follicles in advanced atresia, and may have lost the ability for spontaneous nuclear maturation. Oocytes given a score of 1 for both components fitted the description of a bovine oocyte from a normal vesicular follicle (Marion *et al.*, 1968). While the maturation rate was high in this group, it was not statistically greater than that in oocytes given a score of 2. This confirms reports that oocytes retain full developmental capacity until late in atresia (Moor and Trounson, 1977; Hay and Cran, 1978). Discarding the poorest oocytes (score 3 investment or ooplasm) leaves 72.7% of the recovered oocytes and 19.06% maturation. A lower percentage maturation was achieved in the study designed to examine the use of vital dyes. Although not statistically significant, an improved incidence of

maturity was seen following TpB use. This higher maturation rate was achieved by eliminating nonviable oocytes before culture. The ease of the TpB test, coupled with the observation of nonviable oocytes in all morphological categories, makes this screening method of value to reduce the number of cultured oocytes. Only TpB- oocytes with ooplasm and investment ratings of 1 or 2 were chosen for subsequent studies.

Several additions to the basic medium were tested in an attempt to find an optimal medium for bovine oocyte culture. Lactate is an important energy substrate for mouse oocytes (Brinster, 1965, 1967), but its importance to porcine oocytes has been questioned (McGaughey, 1976). Results of this trial with 115 bovine oocytes indicate no advantage for inclusion of lactate in a medium containing glucose and pyruvate. This is similar to work showing lactate and pyruvate did not support 1- to 2-cell ovine or bovine embryos better than either substrate alone (Boone et al., 1978).

Despite efforts to culture a homogeneous collection of oocytes, considerable variation was observed in response to medium and culture conditions. More oocytes remained viable under 5% O_2 than under 20% O_2, particularly in TC199 and PBS + FSH. The nitrogen component of the gas mixture is considered to be inert. The 5% CO_2, in combination with bicarbonate in TC199, is used to regulate pH. Thus, any differences between oocytes cultured in the two gas mixtures is probably the result of O_2 concentration. Components of the media may, however, play a role in oocyte response.

Increasing the incubation temperature from 37° to 39°C did not affect oocyte survival. This was a concern, since 1-cell rabbit

embryos exposed to 40.3°C exhibited decreased RNA synthesis and increased mortality following transfer to recipient does (Alliston et al., 1965; Criblez and Alliston, 1974). The same group of researchers had earlier reported a detrimental effect on ovine embryos: 9.5% of day-2 embryo transfers were successful when donors and recipients were maintained at 21° and 32°C, respectively (Alliston and Ulberg, 1961; Woody and Ulberg, 1964). A 24% pregnancy rate was achieved in the reciprocal transfers. Conversly, Ball et al. (1982) reported significantly higher fertilization rates at 39°-41°C than at 35°-37°C. Maturation rates were slightly but consistently improved in the present experiment by increased culture temperature. These findings suggest that incubation temperatures should perhaps be adjusted to approximate the body temperature of the species studied.

FSH, included at 10 μg/ml, had variable effects. Survival was generally higher in FSH-containing medium. Maturation, in contrast, was slightly inhibited, or unaffected. These results are consistent with Rush et al. (1973) and Fukui et al. (1982). Specific effects on cumulus cell expansion were not recorded although FSH reportedly stimulates this process.

FSH added in combination with steroids reduced survival of cultured oocytes from 73.3% to 60.0%. Maturation rates did not differ between media containing E_2, P, or a combination of FSH and either steroid. The overall maturation rate for treated groups (23.4%) was similar to mean rates in previous trials using 5% O_2 at 39°C. Evidence can be found to support (Rush et al., 1973; Jagiello et al., 1975; McGaughey, 1977a) or refute (Robertson and Baker, 1969; Fukui et

al., 1982) these findings. The hormonal milieu in follicular fluid prior to oocyte recovery may influence the ability of the oocyte to respond to hormone action and thus contribute to the variable responses reported. There are marked differences in the follicular fluid profile of follicles differing in size, stage of the estrous cycle, or stage of atresia (Hay et al., 1979; Merz et al., 1979; Kruip and Dielman, 1982; Ireland and Roche, 1983a,b). While neither follicle size nor stage of the estrous cycle have had demonstrable effects on oocyte in vitro maturation, the stage of follicle maturation may influence the oocyte response to hormone addition to culture media. Therefore, more detail is needed on the follicles from which oocytes are derived and the culture medium adjusted to complement the oocyte source.

Fertilization must occur after oocyte maturation and before degeneration. Therefore, higher percentages of fertilization are expected following a period for in vitro maturation. The media employed for this maturation period did not appear to affect the xenogenous fertilization rate. A notable exception was the first six attempts to fertilize oocytes after culture in FSH-containing media. Fertilization was obtained in later trials after in vitro incubation in TC199 with added FSH. Following culture in PBS or TC199 at 37°C under 20% O_2, 10.6% of the recovered ova showed evidence of fertilization. Data from the previous maturation experiments showed, however, that a small percentage of these ova would have been at Metaphase II when transferred to the rabbit. The fertilization rate was adjusted for the number of mature ova by multiplying the number

transferred into rabbit oviducts by previously determined *in vitro* maturation percentages. While this adjustment does not allow for oocytes that complete maturation in the oviduct, it provides an indication of the fertilization rate based on mature oocytes. Fertilization percentages calculated in this way averaged 51.8% following culture in 20% O_2 at 37°. Fertilization increased to 70.8% when oocytes were *in vitro* matured under 5% O_2. The number fertilized was again increased by maturing oocytes at 39°C. However, due to generally higher maturation ratios achieved in this culture environment, the percentage of mature ova that were xenogenously fertilized fell to 52.7% in the final experiment. These results are comparable to recent reports of bovine fertilization in rabbit (Hirst *et al.*, 1981) and ewe (Enright, 1982) oviducts.

Results of the *in vitro* fertilization experiments are similar to those of Iritani and Niwa (1977). Other researchers have reported higher fertilization rates using *in vitro* matured oocytes (Ball *et al.*, 1983b; Hensleigh and Hunter, 1983). A portion of the difference could be attributable to use of frozen semen. The generally lower motility and shorter lifespan of frozen-thawed sperm (Brown *et al.*, 1982) may require closer synchrony between insemination and oocyte maturation, as well as higher concentrations of sperm in the fertilization culture. Frozen semen has been successfully used for *in vitro* fertilization of *in vivo* matured (Brackett *et al.*, 1982) and *in vitro* matured ova (Bregulla *et al.*, 1974), although fertilization rates were low. The present experiment confirms other reports that bull sperm

can be capacitated *in vitro* without the use of a specialized medium (Bondioli and Wright, 1982; Ball *et al.*, 1983b).

The present IVF results are contrary, however, to a report of significantly higher sperm penetration following *in vitro* maturation with FSH in comparison to controls (Ball *et al.*, 1983). No differences in fertilization rate were found among media in the present studies. There does appear to be greater developmental capacity in oocytes matured with gonadotropins. Although the number of oocytes was small, a greater percentage of fertilized ova formed pronuclei if they had been matured in an FSH-containing medium (71.6% *vs.* 24.6%). There was no advantage to adding FCS to the BSA-containing PBS medium. As discussed with reference to xenogenous fertilization, fertilization rates should properly be based on number of mature ova. Applying this adjustment to the IVF data, fertilization ranged from 36.2% to 64.4%, with a mean of 51.0% of mature ova. While this percentage was similar to that achieved through xenogenous fertilization, the oviducts were clearly superior in supporting development. One cleaved zygote was observed following IVF (2.8%). A total of 43.1% of xenogenously fertilized ova cleaved with development reaching the 4-cell stage. Deficiencies in the *in vitro* system are emphasized by this disparity. In addition, culture conditions are more sensitively controlled in the rabbit than under *in vitro* conditions. Xenogenous fertilization does, however, generally involve loss of a small proportion of the ova due to incomplete recovery.

SUMMARY AND CONCLUSIONS

This series of experiments resulted in morphological characterization, *in vitro* maturation, and xenogenous and *in vitro* fertilization of bovine follicular oocytes. No differences were found between oocytes from heifers and cows, or oocytes from follicles ipsilateral and contralateral to the most recent corpus luteum. Oocytes were recovered by follicle aspiration from 70 to 78% of the 10 to 20 follicles on each ovary. Classification schemes defining the extent of oocyte degeneration, based on amount of cumulus cell investment and morphological condition of the ooplasm, were verified using vital dyes. A larger percentage of oocytes assigned lower scores were capable of excluding trypan blue or taking up fluorescein diacetate. Based on a total of 411 oocytes, maturation rates were 22.7, 17.5 and 2.0 for investment categories of 1, 2 and 3, respectively. Maturation rates for the three ooplasm categories were 18.2%, 13.4% and 0.0%. Follicles less than 4 mm in diameter contain a higher percentage of oocytes with low scores, i.e., less degeneration. Oocytes collected from ovaries up to 7 hours after slaughter had characteristics similar to those collected within 4 hours of slaughter. Appearance of oocytes at recovery was not altered by the transport temperature. However, oocyte survival and maturation during *in vitro* culture were greater

when ovaries were kept at 37°C rather than 0°C prior to follicle aspiration. Ovaries were transported in a warm insulated container for in vitro maturation studies. Follicles less than 4 mm were aspirated and oocytes with cumulus cells, having ooplasm scores less than 3, and capable of excluding trypan blue were selected. Approximately 60% of collected oocytes were cultured. Based on an examination of 30 oocytes in this selected group, germinal vesicles are present in 76.9% of the oocytes prior to culture.

In vitro maturation was not affected by reduced oxygen concentration in the culture atmosphere (5% compared to 20%) or increased culture temperature (39° compared to 37°C). A modified tissue culture 199 medium and modified phosphate buffered saline medium supported maturation equally well. FSH addition was not beneficial for in vitro maturation. There was some evidence of reduced maturation rates in FSHcontaining media compared to the same media without gonadotropin. More oocytes remained viable in the control medium than in medium containing estrogen, progesterone, or either steroid together with FSH. The percentage of oocytes completing meiosis was nonsignificantly lower in media containing steroids. Maturation was completed by 20% to 25% of cultured oocytes.

Either xenogenous or in vitro techniques can be used to fertilize in vitro matured follicular oocytes. The choice of in vitro maturation media did not alter the frequency with which oocytes where penetrated by sperm and formed pronuclei or cleaved in the rabbit oviduct. When adjustments are made for maturation rates, 51.8% to 70.8% of the

recovered oocytes were fertilized, and 43.1% of fertilized ova had cleaved. *In vitro* fertilization rates were variable but averaged 51.0%. Pronuclei formation was achieved in many of these fertilized ova, but cleavage was observed only once. Thus, if the objective is to obtain early embryos, the xenogenous system is clearly preferable. The extra cost and labor involved are justified by the results. Alternatively, if the objective is to define components involved in fertilization and early embryogenesis, the *in vitro* system is more desirable. Much remains to be learned about oocyte maturation and the effects of oocyte quality on fertilization. The ability to *in vitro* fertilize *in vitro* matured oocytes allows the study of these phenomena.

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APPENDIX A

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APPENDIX B

Vita

NAME:	Vernon Dale Dooley
BORN:	January 26, 1956 Huron, South Dakota
FORMAL EDUCATION:	Huron High School Huron, SD, 1970-1974
	South Dakota State University Brookings, SD, 1974-1980
	Michigan State University East Lansing, MI, 1981-1983
DEGREES RECEIVED:	Bachelor of Science South Dakota State University
	Master of Science South Dakota State University
SOCIETY MEMBERSHIP:	American Society of Animal Science Society for the Study of Reproduction International Embryo Transfer Society