IN VITRO OVUM MATURATION AND ANESTRUS OVULATION INDUCTION IN SHEEP

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This is to certify that the

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David Andrew Snyder

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IN VITRO OVUM MATURATION AND ANESTRUS OVULATION INDUCTION IN SHEEP

By

David Andrew Snyder

A DISSERTATION

Submitted to

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ABSTRACT

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Experiments were conducted to determine the effect of various endogenous, cyclic reproductive patterns on the ability of sheep follicular oocytes to mature in vitro and to determine the ability of FSH, HCG, testosterone, and estradiol to induce estrus and ovulation in the anestrous ewe. A total of 103 ova, which were recovered from ovaries collected at slaughter and stored for approximately three hours during transport to the laboratory, were used to determine the most efficient means of storage. Percents ova maturing were 16.7, 50.0, and 37.8 for ovaries transported at 30-37°C, 21°C, and 1°C, respectively. Ova recovered from ovaries that were stored at 21°C were more normal in appearance than those from ovaries that were stored at 1°C. For subsequent studies, ovaries were stored at approximately 21°C. To determine the effect of 10 or 20% Fetal Calf Serum (FCS) added to Hams F_{10} culture media for ovum maturation, 162 ova were recovered from anestrous ewes at laparotomy and at slaughter. Very little differences were found among the culture media (57.6, 51.3, and 53.1% maturation for Hams F_{10}, Hams F_{10} with 10% FCS, and Hams F_{10} with 20% FCS, respectively). For subsequent studies, Hams F_{10} alone was used

because of its highly defined nature. The time of ovum maturation *in vitro* was determined using 77 ova recovered from anestrous ewes at slaughter. The time sequence of maturation beginning with removal of the ovum from its follicular environment was similar to that observed *in vivo* following the endogenous LH surge. However, 25% of the ova were atretic upon recovery and an additional 20.5% became atretic during the first 12 hours of culture. An additional 319 ova were recovered at laparotomy and slaughter from cycling, anestrous, pregnant, and prepuberal ewes. No consistent differences in maturation rate were found among ova recovered from ewes under various endogenous hormonal influences or from CL bearing or opposite ovaries. There were also very little differences between large (> 2 mm) or small (< 2 mm) follicles of origin. Ovulation was induced in anestrous ewes with 5 mg FSH or 5 mg FSH and 100 iu HCG. However, coincident estrus was not exhibited. Estradiol and testosterone (1 mg/da each) were not effective in the induction of estrus without progesterone pretreatment. Following a five day progesterone pretreatment (20 mg/da), ovulation was inhibited with estradiol but not with testosterone.

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INTRODUCTION

The mammalian ovary is a seemingly endless storehouse of genetic material capable of being transmitted to the next generation. Most of the reproductive potential of the ovary is never realized due to wastage through various natural causes. Although the ovary contains many oocytes that have the ability to mature and ovulate at any particular time, only a few will do so and only at specific times. Two methods of increasing the utilization of the ovaries reproductive potential would be to induce ovulation of viable oocytes at times other than those normally dictated by the animal or to place oocytes into an artificial environment that would preclude natural degeneration and allow maturation with continued development.

The sheep ovary is in a dynamic state of follicle growth and atresia throughout its reproductive life. Oocytes within those follicles represent a ready source of genetic material that can be harvested with relative ease. One of the objectives of the present study was to determine the effects of the endogenous hormonal state of the ewe on subsequent *in vitro* development of follicular oocytes.

Through environmental influences the ewe undergoes a period of seasonal anestrus. This can be interrupted with appropriate hormonal treatments allowing the ewe to conceive at a time other than the normal breeding season. In this study, the efficiency of low doses of gonadotropins and steroids for the induction of estrus and ovulation

was determined in anestrous ewes.

IN VITRO MATURATION OF FOLLICULAR OOCYTES

Literature Review

Oocytes within the ovaries of most mammals initiate meiosis in pre-natal or early post-natal life (Thibault, 1972). Development is arrested at the dictyate stage of prophase in meiosis I until a few hours before ovulation when an endogenous gonadotopic surge initiates the resumption of maturation to metaphase of meiosis II (Donahue, 1972 and Thibault, 1976). The following is a review of some of the factors that control oocyte maturation. When possible, species differences will be indicated but with the present state of knowledge these have not been fully elucidated. Oocyte maturational stages have generally been evaluated using the chromatin configuration. For the purposes of this review the following abbreviations (Baker and Neal, 1972) will be used:

GV	oocyte in the dictyate stage of Prophase I with a germinal vesicle present.
GVBD	germinal vesicle breakdown indicating resumption of meiosis beyond the dictyate stage.
Met I	oocyte in which maturation has progressed beyond the dictyate stage but not to the point of extrusion of the first polar body.

This will include oocytes in metaphase I, anaphase I and telophase I.

Met II — oocyte with chromatin in metaphase configuration with a polar body present.

Early work has shown that explantation of the oocyte from its follicular environment into a simple culture medium is sufficient to cause the resumption of meiosis up to Met II (Pincus and Enzmann, 1935; Pincus and Saunders, 1939; Chang, 1955; Edwards, 1965a, b; and Foote and Thibault, 1969). It was further demonstrated by these investigators, and confirmed by others (Jagiello, Miller, Ducayen, and Lin, 1974; Shea, Latour, Bedirian, and Baker, 1976) that the rate of maturation *in vitro* was very similar to the rate of intra-follicular maturation *in vivo* following the endogenous LH surge or adequate stimulation with an exogenous LH source. Experimental evidence has shown that cow and pig oocytes cultured in direct contact with granulosa cells do not resume meiosis *in vitro* (Foote and Thibault, 1969; Tsafriri and Channing, 1975). In this same study, follicular fluid was also found to be inhibitory to oocyte maturation, however, this inhibition was overcome by the addition of 5 ug/ml LH to the culture media (Tsafriri, Pomerantz, and Channing, 1976). A 50:50 mixture of Tyrodes solution plus 1 mg/ml BSA and homologous follicular fluid supported *in vitro* maturation of bovine follicular oocytes only to Met I (Hunter, Lawson, and Rowson, 1972). This was attributed to inadequacies in the medium. However, in light of the findings of Tsafriri, *et al*. (1976), there appears to be an inhibition of maturation by bovine follicular fluid. These studies indicate that a substance associated with the follicular environment is responsible

for the prolonged prophase arrest observed in mammals.

Some of these early workers used a variety of crudely prepared media for oocyte culture: sterile rabbit blood plasma (Pincus and Enzmann, 1935); human serum (Pincus and Saunders, 1939); a 50:50 mixture of rabbit serum and Ringer-Lock's solution (Chang, 1955); or human follicular fluid (Edwards, Bravister, and Steptoe, 1969). Resumption of meiosis with maturation to Met II was achieved in each of these media. However, results were quite variable due to their undefined nature. The use of chemically defined media facilitated physiological studies with in vitro cultured oocytes. Some of the most common commercial media are shown in Table 1. Addition or deletion of various constituents to or from these media make comparisons between studies and, in some cases, within studies, difficult.

One of the most common additions to a basic nutrient media is a source of large molecular weight protein. This is usually in the form of serum or bovine serum albumin (BSA). A lower rate of oocyte degeneration was found with the addition of 1% BSA to a Kreb's bicarbonate media which would indicate that the macromolecule provides either a protective or supportive function.

Some discrepancy is found in the literature regarding the importance of the macromolecular source and the macromolecular source that yields the best results. Shea, Baker, and Latour (1976) found a greater number of rabbit oocytes undergoing GVBD and maturing to Met II in TCM 199 plus 10% rabbit serum (92% and 82%, respectively) than in TCM 199 plus 3% BSA (73% and 54%, respectively). In contrast, Cross and Brinster (1970) found no difference in mouse oocytes maturing to Met II in either TCM 199 plus 15% calf serum (86.7%) or TCM 199 plus

TABLE 1. Commonly used tissue culture media.

	GH_1[a]	GH_2[a]	$BMOC_3$[b]	Hams F_{10}[c]	TCM 199[d]
Balanced Salt Solution	+	+	+	+	+
Energy Sources					
Pyruvate	+	+	+	+	
Lactate	+	+	+		
Glucose	+	+	+	+	+
Macro Molecules					
BSA		+	+		
PVP	+				
Amino Acids					
Alanine		+		+	+
Arginine		+		+	+
Asparagine				+	
Aspartic acid				+	+
Cysteine		+		+	+
Cystine					+
Glutamic acid				+	+
Glutamine	+	+		+	+
Glycine				+	+
Histadine		+		+	+
Hydroxyproline					+
Isoleucine	+	+		+	+
Leucine		+		+	+
Lysine				+	+
Methionine	+	+		+	+
Phenylalanine	+	+		+	+
Proline		+		+	+
Serine		+		+	+
Threonine				+	+
Tryptophan				+	+
Tyrosine				+	+
Valine		+		+	+

[a] Haidri and Gwatkin, 1973.
[b] Grand Island Biological Company, 1976-1977 catalog, p. 123.
[c] Ham, 1963.
[d] Morgan, Morton, and Parker, 1950.

3% BSA (96.7%). No difference was found by Kennedy and Donahue (1969) in the ability of human oocytes to mature to Met II in either TCM 199 with serum (51%) or Hams F_{10} without serum (63.2%). Hams F_{10} alone or with 0.4% or 3% BSA were equally effective in supporting 22% maturation of human follicular oocytes to Met II (Zamboni, Thompson, and Smith, 1972). McGaughey and Polge (1971) demonstrated greater maturation of pig follicular oocytes in medium 199 with 1-10 mg bovine plasma albumin (BPA) per ml, compared to Hams F_{10} with 1-10 mg BPA per ml. The maturation of bovine ova was no different in SOF medium with 1% BSA or with 3% BSA (Pope and Stephens, 1974). When polyvinylpyroladine (PVP) replaced BSA as a macromolecule source there was no decrease in maturation of mouse follicular oocytes (Haidri, Miller, and Gwatkin, 1971). However, in subsequent studies (Haidri and Gwatkin, 1973) involving maturation of oocytes from large hamster follicles, it was not possible to replace BSA with PVP.

Energy requirements for maturation of oocytes must be supplied by the culture media. The addition of pyruvate, lactate, and glucose to Krebs-Ringer solution was as effective in supporting <u>in vitro</u> maturation of human oocytes as Hams F_{10} which contains glucose, pyruvate, and glutamine as energy sources (Kennedy and Donahue, 1969). Maturation of rat oocytes in balanced salt solution (31%) was shown by Zeilmaker and Verhamme (1974) to increase slightly with the addition of pyruvate (39%), and more so with the addition of lactate (47%). The addition of pyruvate, lactate, and insulin to TCM 199 (which contains glucose and glutamine) with 15% pig serum resulted in a higher incidence of maturation in pig oocytes (Tsafriri and Channing, 1975b).

Gwatkin and Haidri (1974) found that with the addition of several amino acids (GH-2 media, Table 1), BSA, pyruvate, lactate, and glucose could be eliminated from the culture media. This may reflect the ability of the ovum to metabolize amino acids for macromolecule and energy sources.

The nutritional role of granulosa cells in oocyte maturation has been investigated. No difference was found between denuded or cumulus covered oocytes from rabbits in their ability to utilize glutamine (Bae and Foote, 1975) or from rats in their ability to utilize glucose, lactate, pyruvate or oxalacetate (Hillensjo, Hamberger, and Ahren, 1975) as energy sources. Intact cumulus cells were necessary for the incorporation of uridine into the nucleus of mouse follicular oocytes for RNA Synthesis (Wassarman and Letourneau, 1976). McGaughey (1977) showed approximately 50% lower maturation of pig oocytes without granulosa cells compared to those with the granulosa cells intact.

The mammalian ovary is in a dynamic state of follicle growth and atresia with changing luteal and other hormonal influences, all of which may effect the ability of follicular oocytes to undergo maturation. Early work with the rabbit (Chang, 1955) and mouse (Edwards, 1962) showed no differences between small and large follicles of origin or between pregnant and pseudopregnant animals in subsequent oocyte maturation in vitro. When human oocytes were collected from polycystic ovaries, ovaries at various stages of the menstrual cycle, during pregnancy, or several years after hysterectomy, no correlation was found with maturation in vitro (Edwards, 1965b). This was confirmed by Shea, Baker and Latour (1975) in the human and by Suzuki and Mastroianni (1966) in the Rhesus monkey. Oocytes from immature

rats were also shown to mature in vitro (Niwa and Chang, 1975). In contrast, Szybek (1972) demonstrated an increase in the diameter of follicular oocytes from 11 to 17 days of age in mice with a concurrent increase in in vitro maturability. Mouse oocytes within medium sized antral follicles respond to HCG both in vivo and in vitro with the resumption of meiosis. However, maturation is arrested at Met I while those in large antral follicles mature to Met II (Neal and Baker, 1973). Lee (1976) demonstrated a four-fold increase in HCG binding sites in granulosa cells from small (1-2 mm) to medium (3-5 mm) pig ovarian follicles and a 26-fold increase in cells from small to large (6-12 mm) follicles. GH-1 medium (Table 1), with pyruvate, lactate, and glucose as energy sources, supported the maturation of hamster oocytes from small follicles, but the addition of BSA and eight more amino acids (GH-2, Table 1) was necessary to support maturation of oocytes from large follicles (Haidri and Gwatkin, 1973).

A correlation between follicle size or oocyte diameter and the subsequent ability of the oocyte to mature in vitro was also demonstrated in the hamster (Iwamatsu and Yanagimachi, 1975). Oocytes from follicles 126-166 um in diameter or oocytes of 70-78 um in diameter showed no in vitro maturation while 70-93% of those from 202-710 um follicles or 79-91 um in diameter were in Met II after 12 hours. Prepubertal hamsters less than 22 days of age had only follicles less than 150 μ in diameter and showed no in vitro oocyte maturation. Oocytes from small (1-2 mm) pig follicles had a lower total rate of GVBD and a lower rate of maturation to Met II than oocytes from medium (3-5 mm) or large (6-12 mm) follicles (Tsafriri and Channing, 1975b). Oocytes from medium sized follicles also had a lower rate of maturation to

Met II than oocytes from large follicles, but this difference was overcome by increasing the pig serum content of the culture medium from 15% to 50%.

Although Thibault, Gerard, and Menezo (1976) found no difference in _in vitro_ oocyte maturation between follicle sizes in the calf and rabbit, they demonstrated a definite correlation in the monkey. Oocytes from small follicles (less than 1 mm) failed to mature and those from medium sized follicles (1 mm to 1.4 mm) matured only to Met I while those from large follicles (greater than 1.5 mm) matured to Met II. The inhibitory effects of granulosa cells on oocyte maturation were found to be correlated with follicle size of origin (Tsafriri and Channing, 1975a). Granulosa cells from small follicles were ten times more potent than granulosa cells from large follicles. These studies clearly indicate an effect of follicle size of origin on subsequent _in vitro_ maturation of oocytes. However, they also show that more complete media can mask these endogenous effects. The factors responsible for this correlation between oocyte maturation and follicle size remain to be determined.

Changes in intrafollicular protein composition, in relation to follicle development, have been demonstrated. Andersen, Kroll, Byskov, and Faber (1976) found a correlation between increases in follicle size and increases in the concentration of αmacroglobulin in cow follicles. Slow migrating α-globulin was shown to increase with early oocyte maturational stages in the pig (McGaughey, 1975). Intracellular protein synthesis is necessary for _in vitro_ maturation of mouse oocytes (Wassarman, 1974; Wassarman, Josefowicz, and Letourneau, 1976). Proteins synthesized early in maturation are concentrated in the

nucleus and, although not required for GV breakdown, are necessary for completion of meiosis I and progression to Met II (Wassarman and Letourneau, 1976a; Schultz and Wassarman, 1977).

The necessity of 5-10% O_2, in the culture atmosphere, was demonstrated for maximum in vitro maturation of oocytes from mice (Haidri, Miller, and Gwatkin, 1971) and from hamsters (Gwatkin and Haidri, 1974). This requirement, however, was found necessary only for initiation of GVBD in rat oocytes, which would continue maturation when transferred to an O_2 free atmosphere (Zeilmaker and Verhamme, 1974). Pig oocytes matured best in an atmosphere with 20% O_2, but 45% and 95% O_2 atmospheres were also capable of supporting maturation (Tsafriri and Channing, 1976b).

The mechanism of gonadotropic stimulation of oocyte maturation in vivo has been investigated using the in vitro system. Early workers added pituitary extracts to their culture media and demonstrated the lack of a direct stimulatory effect on the resumption of meiosis (Pincus and Enzmann, 1935; Chang, 1955). It has been postulated that gonadotropins exert their effect through intermediates such as steriods, c AMP, or prostaglandins. Meiotic division of rat oocytes within intact follicles in vitro is increased by the addition of LH or prostaglandin E_2 to the culture medium or by microinjection of c AMP (Tsafriri, Lindner, Zor, and Lamprecht, 1972). This effect was not seen with prolactin, progesterone, 20 α dihydroprogesterone, oestradiol -17β, linolenic acid or adenosine -5' - monophosphate. These findings suggest that a multistep process is involved in the stimulation of oocyte maturation with c AMP being an intrafollicular messenger. Weiss, Seamark, McIntosh, and Moor (1976) demonstrated a rapid increase

in c AMP production within sheep follicles after treatment with LH, HCG, PGE_2 or noradrenalin. In the pig, increased c AMP in response to HCG was correlated with follicle size (Lee, 1976). Cyclic AMP showed a 1.5, 3.7, and 13-fold increase for small (1-2 mm), medium (3-5 mm), and large (6-12 mm) follicles, respectively, as compared to untreated controls. Hillensjo (1975), using follicle enclosed rat oocytes, found an increased GVBD response to LH when a phosphodiesterase inhibitor was also added to the medium. Jagiello, Ducayen, Miller, Graffeo, and Fang (1975) added c AMP to oocyte cultures and stimulated human and monkey oocyte maturation in McCoy's medium, whereas maturation of cow and sheep oocytes was inhibited in Diploid medium. Differences in effect may be the result of dosage differences since Hillensjo (1975) demonstrated stimulation of GVBD in follicle enclosed rat oocytes in response to 1 mM dibutyryl c AMP, but no effect with 5-10 mM dibutyryl c AMP.

In contrast to this work, Stern and Wassarman (1973), and Wassarman (1974) have demonstrated the ability of 0.4 mM dibutyryl c AMP to block maturation of mouse oocytes prior to GVBD. The enhancement of this inhibition by disulfide reducing agents led these investigators to conclude that c AMP acts to prevent dissolution of the nuclear (germinal vesicle) membrane (Wassarman and Turner, 1976). The suggestion has been made that fluctuating levels of intrafollicular c AMP may be active in controlling oocyte maturation in the mouse (Wassarman, Josefowicz, and Letourneau, 1976).

Intrafollicular progesterone levels vary in a specific pattern throughout the menstrual cycle in humans (McNatty, Hunter, McNeilly, and Sawers, 1975). During the follicular phase, these investigators

demonstrated highest progesterone levels in the large follicle population, while during the luteal phase the small follicle population had the highest progesterone. Rush, Tibbits, and Foote (1973) found no effect of LH and FSH, progesterone, or LH, FSH, and progesterone on the in vitro maturation of oocytes from puberal heifers.

Progesterone (10 ug/ml) added to Hams F_{10} or medium 199, each with 10% fetal calf serum, had no effect on germinal vesicle breakdown in human oocytes (Shea, et al., 1975). However, a significant increase in maturation of prepubertal monkey oocytes to Met II was achieved with the addition of 25 ug/ml progesterone with or without 8 ug/ml c AMP to Hams F_{10} with 15% fetal calf serum (Gould and Graham, 1976). No increase in maturation rate was found with c AMP alone.

Progesterone (32 ug/ml) added to a basic salt solution containing pyruvate or glutamine as energy sources, decreased the time of maturation of rabbit oocytes from large follicles (Bae and Foote, 1975). However, no effect of progesterone was seen on oocytes from small follicles or with LH on oocytes from large or small follicles. No effect on in vitro maturation of cow oocytes resulted from the addition of progesterone or progesterone and estradiol to BMOC-3 media (Table 1) compared to BMOC-3 media without steroids (McGaughey, 1977). Addition of estradiol -17B also had no effect on pig oocytes with granulosa cells intact. However, maturation of denuded oocytes was inhibited.

LH or PGE_2 caused an increase in both GVBD and glycolysis in rat Graafian follicles in vitro (Hillensjo, 1975). LH was shown to overcome the inhibitory action of follicular fluid on oocyte maturation (Tsafriri, et al., 1976). Addition of LH to a balanced salt media,

containing pyruvate or oxalacetate as energy sources, decreased the oxygen uptake of rat oocytes (Hillensjo, et al., 1975). LH and prostaglandins of the E series were shown to increase sheep and monkey oocyte maturation in vitro (Jagiello, Ducayen, Miller, Graffeo, and Fang, 1975). However, this study was complicated by high concentrations of steroids in the serum used as a supplement and by differences in the basic culture media, making it difficult to reach conclusions on the mechanisms of maturation.

Most studies of in vitro ovum maturation involve ova that are collected at laparotomy or immediately after ovariectomy or death of the donor. However, in some studies, especially with ova from domestic animals, ovaries are collected in a commercial abattoir and must be transported to a laboratory for ovum recovery and culture. Jagiello, et al., (1975) collected sheep and cow ovaries at slaughter, washed them with sterile saline, and transported them in dry containers for about 30 minutes. They achieved maturation rates up to 100% using this system. With pig ovaries transported in sterile saline with penicillin and streptomycin at room temperature for up to five hours, Tsafriri and Channing (1975b) achieved up to 82% subsequent ova maturation. Shea, Latour, Bedirian, and Baker (1976) transported cow ovaries with no precautions to maintain body temperature and found no difference in GVBD between one and two hour storage times (88% vs 86%, respectively). In another study (Shea, Baker, and Latour, 1976) a significant decrease in GVBD in rabbit ova occurred between 0.5 hour and two hour storage times (88% vs 12%, respectively).

Materials and Methods

Sheep ovaries were to be collected for much of this research along the slaughter line at Wolverine Packing Company, Detroit, Michigan. Storage time for transport to the laboratory was two to four hours depending on conditions. Because most studies in the literature involve only 30 or 40 minute storage times, little information was available on the transport and storage of ovaries. A preliminary study was therefore undertaken to determine the optimum storage temperature for the purposes of *in vitro* ova maturation studies.

Sixteen pairs of ovaries were collected along the slaughter line. Upon removal from the carcass, each pair of ovaries was sutured together, rinsed with sterile saline containing 100 iu penicillin/ml, and placed in one of three flasks containing sterile saline and penicillin. Flask 1 was in a waterbath at 37^{o} and was stored in a styrofoam box with warm water bottles to maintain the temperature during transport to the laboratory. Flask 2 was a thermos bottle and was transported with no attempt to regulate the external temperature. Flask 3 was transported in a styrofoam box filled with ice. Approximately 30 minutes were required to collect the ovaries and storage time was three hours. Upon arrival at the laboratory, the temperature of the storage saline was $30^{o}C$, $21^{o}C$, and $1^{o}C$ in flasks 1, 2 and 3, respectively.

Ovaries were removed from the storage solution in a warm room and all visible follicles were aspirated with a 25 ga needle and a 1 ml syringe. Aspirates were placed in the chambers of Lab-Tech tissue culture chamber slides in 0.25 ml of Hams F_{10} with 100 iu penicillin/ml, 6.7 iu heparin/ml, and either 10% fetal calf serum (FCS), 20% FCS,

10% FCS plus 10 iu human chorionic gonadotropin (HCG)/ml or 20% FCS plus 10 iu HCG/ml. These were cultured in an atmosphere of 5% CO_2 in air at 37°C for 24 to 48 hours. Following culture, cumulus cells were removed by treating the ova with 10% hyaluronidase for five minutes, followed by repeated passage through a drawn pipette. Ova were mounted on glass slides, fixed in 10% neutral formalin for 20 minutes, stained with 1% orcein in 45% acetic acid, and examined by light microscopy. Maturational stages were determined by chromosomal configuration as described by Donahue (1972). Experimental variables involved in this study are shown in Table 2.

TABLE 2. Effects of storage temperature, culture media, and ovary of origin on subsequent *in vitro* ovum maturation: experimental variables.

Storage Temperature	Culture Media	Ovary
30° - 37°C	Hams F_{10}[a] + 10% FCS[b]	Corpus Luteum Bearing
21°C	Hams F_{10} + 20% FCS	Opposite
1°C	Hams F_{10} + 10% FCS + 10 iu/ml HCG[c]	
	Hams F_{10} + 20% FCS + 10 iu/ml HCG	

[a]Also contains 100 iu penicillin/ml and 6.7 iu heparin/ml.
[b]Heat inactivated Fetal Calf Serum, (GIBCO), % by volume.
[c]Human Chorionic Gonadotropin (Ayerst).

Effects of Various Media

The purpose of this experiment was to compare the ability of Hams F_{10}, alone or with two levels of fetal calf serum added, to support *in vitro* maturation of sheep follicular oocytes. To reduce the variables involved, only ova from anestrous ewes were used in

this study.

In the first portion of this study ova were collected from anestrous ewes at laparatomy. To detect estrus a vasectomized ram, with oil containing colored dye applied to his brisket, was penned continuously with a flock of 25 ewes. Ewes were examined daily for fresh breeding marks. Only ewes which had not shown estrus for 25 days and that did not have evidence of luteal tissue on their ovaries were used for this study. Ova were collected from 15 ewes.

Mid-ventral laparotomy was performed and the ovaries exposed for follicle aspiration with a 1 ml syringe and a 25 ga needle. Aspirates were grouped by follicle size (greater than 5 mm, 2-5 mm, or less than 2 mm) and ovary of origin, and placed into the chambers of an 8 chamber tissue culture chamber slide (Lab-Tech) in 0.25 ml of media (Hams F_{10}, Hams F_{10} + 10% FCS, or Hams F_{10} + 20% FCS randomized separately for left and right ovaries). Chamber slides were placed immediately in a desicator jar maintained at 37°C in a waterbath until completion of surgery, at which time they were transferred to an atmosphere controlled incubator. Incubation was for 36 hours, at 37°C in a saturated 5% CO_2 in air atmosphere.

After culture ova were recovered from the culture chambers under a dissecting microscope at 10-15X. Those ova with portions of the cumulus oophorus intact were placed in depression slides in a fresh 1% hyaluronidase solution for three to five minutes and then cleaned mechanically by repeated passage through a drawn pipet.

Ova were then placed on the center of a glass slide and a cover slip with a spot of petroleum jelly on each corner was applied and depressed until the ovum was slightly squashed. Mounted ova were fixed

in 10% neutral formalin for at least 20 minutes, stained with 0.5% aceto orcein for 10 minutes, and examined by light microscopy. Ova were classified as atretic, germinal vesicle, metaphase I, or metaphase II based on general morphology and chromatin configuration (Donahue, 1972).

Ovaries were also collected from ewes at slaughter at a local slaughter house. Forty pairs of ovaries were collected on two separate days. These were immediately placed in sterile saline with 100 iu penicillin/ml and maintained at room temperature for the duration of transport. The minimum transport time to the laboratory was two hours. At the laboratory follicles were aspirated and ova were cultured and examined as described above. Results of this experiment were analyzed by Chi square. Maturation stages were analyzed by two methods. Percent maturing indicates any ova which have resumed meiosis beyond the dictyate stage while metaphase II indicates only those ova classified as metaphase II. Experimental variables for this study are shown in Table 3.

TABLE 3. Effects of various culture media on in vitro ovum maturation: experimental variables.

Culture Media	Follicle Size
Hams F_{10}[a]	greater than 5 mm
Hams F_{10} + 10% FCS[b]	2-5 mm
Hams F_{10} + 20% FCS	less than 2 mm

[a]Also contains 100 iu penicillin/ml and 6.7 iu heparin/ml.
[b]Heat inactivated Fetal Calf Serum (GIBCO) % by volume.

Time of Maturation

For this study, sheep ovaries were collected along the slaughter line at a local slaughter house. Only sheep with no indication of cyclic activity on either ovary were used. Left and right ovaries were pooled, placed into sterile saline with 100 iu penicillin/ml, and transported to the laboratory in a styrofoam box at room temperature. Transport time was between two and three hours. Two replicates were carried out with 21 pairs of ovaries used in the first replicate and 10 pairs in the second.

All visible follicles on each ovary were aspirated as previously described and the aspirates were grouped by follicle size (greater than 5 mm, 2-5 mm, and less than 2 mm) and placed in the chambers of an 8 chamber tissue culture chamber slide (Lab-Tech). Chamber slides were randomized for ovum examination at 0, 12, 24, 36, 48, or 60 hours from the beginning of culture. Culture was in an atmosphere of 5% CO_2 in air at 37°C. Upon recovery ova were cleaned, fixed and examined as previously described. Experimental variables for time of maturation are given in Table 4.

Estrous Cycle Effects

In the first portion of this study, eleven cycling ewes were used. For the detection of estrus, a vasectomized ram was run continuously with the ewes. Fresh colored dye mixed with motor oil was applied to his brisket daily and ewes were observed for fresh breeding marks. The first day of estrus was designated day 0. Ewes were randomly assigned to ovum collection on day 5, 10, or 15. Ova were collected by follicle aspiration at mid-ventral laparotomy as previously described. Aspirates were grouped by follicle size and

TABLE 4. Time sequence of *in vitro* ovum maturation: experimental variables.

Replicate	Culture Time (hr)	Follicle Size
1	0	greater than 5 mm
2	12	2-5 mm
	24	less than 2 mm
	36	
	48	
	60	

ovary of origin, and placed in the chambers of Lab-Tech tissue culture chamber slides in 0.25 ml of Hams F_{10} media. Culture was for 36 hours in a saturated atmosphere of 5% CO_2 in air at 37°C. Upon recovery, ova were prepared and examined as previously described. Experimental variables for estrous cycle effects: I, are given in Table 5.

TABLE 5. Effects of ovum collection on various days of the estrous cycle and ovary of origin on *in vitro* ovum maturation: experimental variables.

Day of the Estrous Cycle[a]	Ovary
5	Corpus luteum bearing
10	Opposite
15	

[a]Day 0 = first day of estrus.

Other portions of this study involved ovaries collected at three separate times along the slaughter line at a local slaughter house. These were stored in sterile saline with 100 iu penicillin/ml and transported to the laboratory at room temperature. Ovaries were collected from 17 old ewes in the second portion, from 6 cycling and 13 prepuberal lambs in the third portion, and from 4 prepuberal,

2 anestrous, 26 cycling, and 3 pregnant ewes and lambs in the fourth portion of this study. Follicle aspiration, ova culture, recovery and examination were performed by previously described methods. Ova were cultured in Hams F_{10} for 36 hours. Experimental variables for these studies are given in Tables 6, 7, and 8, respectively.

TABLE 6. Effects of the luteal state and ovary of origin on in vitro ovum maturation: experimental variables.

Luteal State	Ovary
Early luteal	Corpus luteum bearing
Mid luteal	Opposite
Late luteal	
Anestrus	

TABLE 7. Effects of puberty, ovary of origin, and follicle size on in vitro ovum maturation: experimental variables.

Luteal State	Ovary	Follicle Size
Cycling lambs	Corpus luteum bearing	2-5 mm
Prepuberal lambs	Opposite	less than 2 mm

TABLE 8. Effects of luteal state, ovary of origin, and follicle size on in vitro ovum maturation: experimental variables.

Luteal State	Ovary	Follicle Size
Pregnant	Corpus luteum bearing	greater than 5 mm
Early luteal	Opposite	2-5 mm
Mid luteal		
Late luteal		
Anestrous		
Prepuberal		

Results

Results of the first study are given in Table 9. A total of 103 ova were recovered from ovaries collected at slaughter. Hams F_{10} with 10% FCS supported a greater percent maturation of oocytes recovered from CL bearing ovaries that were stored at 21°C than from those CL bearing ovaries stored at 30-37°C ($P< 0.1$) or at 1°C ($P< 0.05$). No differences in percent maturation among storage temperatures were demonstrable among ova from opposite ovaries.

Differences among media were only apparent among ova from ovaries that were transported at 1°C. Ova from CL bearing ovaries showed a greater percent maturation when 10 iu HCG was added to either Hams F_{10} with 10% FCS or Hams F_{10} with 20% FCS ($P< 0.05$). However, ova from opposite ovaries had a higher percent maturation without the addition of HCG.

Ova from CL bearing ovaries had a lower percent maturation compared to opposite ovaries ($P< 0.05$) when the ovaries were stored at 21°C and ova were cultured in Hams F_{10} with 20% FCS or when ovaries were stored at 1°C and ova were cultured in Hams F_{10} with either 10% or 20% FCS. However, when ovaries were stored at 1°C and ova were cultured in Hams F_{10} with 10% FCS and 10 iu HCG/ml, ova from CL bearing ovaries had a greater ($P < 0.05$) percent maturation than opposite ovaries.

In Tables 10, 11, and 12, the data from Table 9 are grouped by main effects. Although accurate analysis of the main effects is not possible because of interactions, some inferences can be made. Maturation was inhibited in oocytes from ovaries that were stored at 30-37°C. There was no difference in maturation among oocytes from ovaries stored at 22°C or at 1°C. However, disruption of the cumulus

TABLE 9. Effects of storage temperature, culture media, and ovary of origin on subsequent in vitro ovum maturation.

Storage Temperature	Media Hams F_{10}[1] with	% Maturation Corpus Luteum Bearing Ovary		Opposite Ovary	
30-37°C	10% FCS[2]	0	(2)[4]	42.9	(7)
	20% FCS	0	(4)	33.3	(3)
	10% FCS + 10 iu HCG[3]	0	(7)	0	(2)
	20% FCS + 10 iu HCG	14.3	(7)	25.0	(4)
22°C	10% FCS	71.4	(7)[a]	33.3	(3)
	20% FCS	0	(2)	100.0	(2)
	10% FCS + 10 iu HCG	0	(0)	16.7	(6)
	20% FCS + 10 iu HCG	50	(6)	75.0	(4)
1°C	10% FCS	0	(5)[c]	60.0	(5)[a]
	20% FCS	14.3	(7)[bc]	100.0	(3)[a]
	10% FCS + 10 iu HCG	100	(1)[ab]	0	(5)[b]
	20% FCS + 10 iu HCG	75	(4)[ab]	42.9	(7)

Values with different superscripts within columns are different at $P < 0.05$.

[1]Also contains 100 iu penicillin/ml and 6.7 iu heparin/ml.
[2]Heat inactivated Fetal Calf Serum (GIBCO), by volume.
[3]Human Chorionic Gonadotropin (Ayerst).
[4]Number in parenthesis = number of ova.

TABLE 10. Summary of the main effect of ovary storage temperature on subsequent in vitro maturation of follicular oocytes.

Storage Temperature	% Maturation
30-37°C	16.7 (36)[a]
22°C	50.0 (30)[b]
1°C	37.8 (37)[b]

Values with different superscripts are different at $P < 0.05$.
Number in parenthesis = number of ova.

TABLE 11. Summary of the main effect of culture media on *in vitro* ovum maturation.

Media F_{10} With	% Maturation
10% FCS	41.4 (29)[a]
20% FCS	33.3 (21)[b]
10% FCS + HCG	9.5 (21)[b]
20% FCS + HCG	43.7 (32)[a]

Values with different superscripts are different at $P < 0.05$.
Number in parenthesis = number of ova.

TABLE 12. Summary of the main effect of ovary of origin on subsequent *in vitro* maturation of follicular oocytes.

Ovary	% Maturation
CL Bearing	26.9 (52)
Opposite	41.2 (51)

Number in parenthesis = number of ova.

cell layer was apparent in oocytes from ovaries that were stored at 1°C. Ovum maturation was inhibited in Hams F_{10} with 10% FCS and HCG compared to Hams F_{10} with 10% FCS, Hams F_{10} with 20% FCS and HCG ($P < 0.05$) and Hams F_{10} with 20% FCS (P approaching 0.05). There was no difference in maturation between CL bearing and opposite ovaries.

Effects of Various Media

For purposes of analysis, it was found necessary to combine ova from greater than 5 mm follicles and those from 2-5 mm follicles into one category (large follicles) for comparison with ova from small follicles (less than 2 mm). A total of 39 ova were recovered from ewes at laparotomy and 123 ova from ovaries collected at slaughter

on two separate days. Using the Chi-square test for independence, a time effect was found among the three portions of this study: ova collected at laparotomy, ova collected at slaughter the first day, and ova collected at slaughter the second day. This made separate analysis of ova cultured from each collection time necessary. Results of this study are given in Table 13.

In the first portion of this study, ova from small follicles initiated maturation and matured to Met II more readily in Hams F_{10} with 20% FCS than in Hams F_{10} alone. There was a tendency ($P < 0.1$) for ova from large follicles to mature more readily in Hams F_{10} alone than in Hams F_{10} with 10% FCS. When Hams F_{10} was the culture media, the percent of ova initiating maturation was greater for ova from large follicles compared to those from small follicles.

No differences were found among culture media for ova collected at slaughter. For ova collected at the first slaughter time and cultured in Hams F_{10} with 10% FCS, there was a tendency ($P < 0.1$) for ova from small follicles to have a greater percent maturation than those from large follicles. However, at the second slaughter time, Hams F_{10} with 20% FCS supported greater ($P < 0.05$) percent maturation of ova from large follicles compared to small follicles.

In Table 14 the main effects of media and follicle size from the three ova collection times are summarized from Table 13. Although statistical analysis cannot be performed on the data in this table, very little differences can be seen.

TABLE 13. Effects of various culture media on *in vitro* ovum maturation.

Ova Collection	Media	Follicle Size[3]	Total Ova	% Maturing[4]	% Met II[5]
1	Hams F_{10}[1]	large	8	87.5[a]	62.5[a]
		small	8	37.5[b]	0
	with 10% FCS[2]	large	3	33.3	0
		small	4	50.0	0
	with 20% FCS	large	6	83.3	50.0
		small	10	90.0[a]	50.0[b]
2	Hams F_{10}	large	6	66.7	16.7
		small	12	75.0	25.0
	with 10% FCS	large	4	25.0	0
		small	11	72.7	36.4
	with 20% FCS	large	2	100.0	50.0
		small	16	43.75	31.3
3	Hams F_{10}	large	9	55.6	22.2
		small	16	37.5	18.8
	with 10% FCS	large	7	42.9	14.3
		small	10	50.0	10.0
	with 20% FCS	large	8	75.0[a]	25.0
		small	22	22.7[b]	0.1

Values with different superscripts within ova collection times and within columns are different at $P < 0.05$.

[1]Also contains 100 iu penicillin/ml and 6.7 iu heparin/ml.
[2]Heat inactivated Fetal Calf Serum (GICBO).
[3]Large = greater than 2 mm, small = less than 2 mm.
[4]Includes ova in Met I, Ana I, Tel 1, or Met II.
[5]Includes only ova with Metaphase chromosomes and a polar body.

TABLE 14. Summary of the main effects of culture media and follicle size of origin on *in vitro* ovum maturation.

	Total Ova	% Maturing
Hams F_{10} alone	59	57.6
with 10% FCS	39	51.3
with 20% FCS	64	53.1
Follicle size		
large	53	64.2
small	109	49.5

Time of Maturation

Results of this study are given in Table 15. The two replicates were found to be independent and were combined for further analysis. Also, for purposes of analysis, ova from greater than 5 mm follicles and ova from 2-5 mm follicles were combined into one category (large follicles) for comparison with less than 2 mm follicles (small follicles). A total of 77 ova were used in this study.

A greater ($P < 0.05$) percent of ova contained GVs at 0 hours compared to those ova cultured for 12 or more hours. No difference in percent of ova initiating maturation were found between ova from large or small follicles at 0, 12, 24, 36, 48, or 60 hours. At 36 hours, however, there was a greater number of ova from large follicles matured to Met II compared to ova from small follicles. There was a progressive increase in ova initiating maturation from 0 hours to 24 hours in culture. However, only at 0 hours were there significantly fewer ova initiating maturation. If ova from all follicle sizes were combined, the percent of ova maturing to Met II was constant among ova cultured for 24, 36, 48, or 60 hours with a significant increase between 12 and 60 hours.

Estrous Cycle Effects

Results of this study are given in Tables 16 through 21. In the first portion, no differences were found among ova collected by laparotomy on day 5, 10, or 15 of the estrus cycle or among ova from CL bearing or opposite ovaries for percent maturing or percent Met II.

Ova from cycling lambs showed no differences in percent maturing among CL bearing or opposite ovaries of origin. There was a tendency

TABLE 15. Time sequence of *in vitro* ovum maturation.

Culture Time (hr)	0	12	24	36	48	60
% GV	(12) 66.7[a]	(13) 8.3[b]	(7) 0[b]	(15) 0[b]	(16) 6.25[b]	(14) 0[b]
% Maturing[1]						
large follicles[2]	(4) 0[a]	(8) 50.0	(1) 0	(5) 80.0[b]	(3) 33.3	(6) 66.7[b]
small follicles[3]	(8) 12.5[a]	(5) 40.0	(6) 66.7[b]	(10) 50.0	(13) 61.5[b]	(8) 37.5
combined	(12) 8.33[a]	(13) 46.2[b]	(7) 57.1[b]	(15) 60.0[b]	(16) 56.3[b]	(14) 50.0[b]
% Met II[4]						
large follicles	(4) 0	(8) 0[a]	(1) 0	(5) 40.0[x]	(3) 0	(6) 50.0[b]
small follicles	(8) 12.5	(5) 0	(6) 16.7	(10) 0[y]	(13) 23.1	(8) 12.5
combined	(12) 8.33	(13) 0[a]	(7) 14.3	(15) 13.3	(16) 18.8	(14) 28.6[b]

Number in parenthesis = number of ova.
Values with different superscripts within rows are different at $P < 0.05$.

[1] Includes ova in Met I, Ana I, Tel I or Met II.
[2] Follicles greater than 2 mm in diameter.
[3] Follicles less than 2 mm in diameter.
[4] Includes only ova with Metaphase chromosomes and a polar body.

TABLE 16. *In vitro* maturation of ova collected at laparotomy on various days of the estrous cycle and from CL bearing or opposite ovaries.

Day of the Estrous Cycle	Ovary	Total Ova	% Maturing	% Met II
5	CL bearing	4	25	25
	opposite	1	100	0
10	CL bearing	3	67	0
	opposite	4	50	50
15	CL bearing	2	0	0
	opposite	5	20	20

TABLE 17. *In vitro* maturation of ova collected at slaughter from cycling and prepuberal lambs.

	Total Ova	% Maturing	% Met II
Cycling lambs	29	48.3	
Prepuberal lambs	54	29.6	
Cycling lambs arranged by ovary			
CL bearing	19	52.6	
opposite	10	40.0	
Prepuberal lambs arranged by follicle size			
large	14	64.3^{a}	35.7^{a}
small	40	17.5^{b}	2.5^{b}

Values with different superscripts within columns are different at $P < 0.05$.

TABLE 18. *In vitro* maturation of ova collected at slaughter from ewes in various luteal states.

Luteal State	Ovary	Total Ova	% Maturation	% Met II
early luteal	CL bearing	9	77.8^{ab}	33.3
	opposite	12	33.3^{c}	8.3^{a}
mid luteal	CL bearing	12	50.0^{bc}	16.7^{a}
	opposite	7	100.0^{a}	71.4^{b}
late luteal	CL bearing	14	57.1^{bc}	35.7
	opposite	11	63.6	18.2^{a}
anestrous		4	50.0^{bc}	25.0

Values with different superscripts within columns are different at $P < 0.05$.

TABLE 19. *In vitro* maturation of ova collected from cycling and anestrous ewes and lambs.

		Follicle Size		
Luteal State	Ovary	Large	Small	Combined
early luteal	CL bearing	(7) 57.1	(21) 23.8^{a}	32.1
	opposite	(2) 50.0	(16) 62.5^{b}	61.1^{a}
mid luteal	CL bearing	(6) 16.7	(12) 25.0^{a}	22.2^{b}
	opposite	(1) 0	(10) 50.0	45.5
late luteal	CL bearing	(3) 0 ^{a}	(14) 28.6	23.5^{b}
	opposite	(1) 100.0^{b}	(6) 50.0	57.1
anestrous		(2) 50.0	(5) 40.0	42.9

Values with different superscripts within columns are different at $P < 0.05$.

TABLE 20. *In vitro* maturation of ova collected from pregnant, cycling, anestrous and prepuberal ewes and lambs.

Luteal State	Total Ova	% Maturing	% Met II
pregnant	21	33.3	14.3
early luteal	46	43.5	19.6
mid luteal	29	34.6	13.8
late luteal	24	33.3	12.5
anestrous	7	42.9	0
prepuberal	21	57.1	28.6

TABLE 21. *In vitro* maturation of ova collected from pregnant, cycling, anestrous and prepuberal ewes and lambs, arranged by ovary of origin.

Luteal State	Ovary	Total Ova	% Maturing	% Met II
pregnant	CL bearing	10	30.0	10
	opposite	11	36.4	18.2
cycling	CL bearing	63	27.0^{a}	11.1
	opposite	36	55.6^{b}	25.0
anestrous		7	42.9^{ab}	0
prepuberal		21	57.1^{b}	28.6

Values with different superscripts are different at $P < 0.05$.

($P < 0.1$) for ova from cycling lambs to have a higher percent maturing than those from prepuberal lambs. Additionally, ova from large follicles from prepuberal lambs had a higher percent maturing and percent Met II than ova from small follicles. No differences in percent ova maturing or in percent Met II were found among ova collected from large (greater than 2 mm) or small (less than 2 mm) follicles or from older ewes during early luteal, mid luteal, late luteal or anestrus states. Ova collected from CL bearing ovaries during the early luteal stage of the estrous cycle had a tendency ($P < 0.1$) for a greater percent maturing than ova from the opposite ovaries. However, ova from mid luteal, CL bearing ovaries had a decreased percent maturing and percent Met II compared to those from mid luteal opposite ovaries. Ova from mid luteal opposite ovaries had a greater percent maturing than ova from early luteal opposite ovaries, late luteal, CL bearing ovaries, and anestrous ovaries, and a greater percent Met II than ova from early luteal, opposite ovaries and late luteal opposite ovaries.

There was no difference in percent ova maturing or percent Met II among ova from pregnant, early luteal, mid luteal, late luteal, anestrous, or prepuberal ewes, or among large (greater than 2 mm) or small (less than 2 mm) follicles or origin. Ova from CL bearing ovaries from cycling ewes had a decreased percent maturing compared to ova from opposite ovaries from cycling ewes, or from prepuberal ewes.

Discussion

The preliminary study was undertaken to provide information for the possible use of a potential source of large numbers of sheep ova. As a result, many variables were incorporated into a single study, making accurate analysis of each quite difficult. Several important findings are, however, apparent. The major objective of this study was to determine the optimum temperature for ova transport. Storage of ovaries at 21°C yielded a large number of normal appearing ova with a high rate of maturation *in vitro*. A comparison of maturation rates of ova collected at laparotomy and from ovaries after transport in the media test study reveals very little difference in maturation rates. This finding is also apparent among the various parts of the study of estrous cycle effects. Maturation rates were similar to those achieved by Shea, Latour, Bedirian, and Baker (1976) after storage of cow ovaries at room temperature for one to two hours. These findings indicate that storage and transport of sheep ovaries in sterile saline at room temperature for up to four hours has no detrimental effect on subsequent *in vitro* maturation of follicular oocytes.

The addition of BSA or FCS to culture media results in a variety of responses depending upon the study that is cited. This can probably be attributed to the variability in product as well as the variability in culture conditions among studies. Jagiello, *et al.* (1975) and Tsafriri and Channing (1975b) found relatively high amounts of both steroids and gonadotropins in their FCS supplies. This effectively reduces any physiological inferences that can be made because of unknown interactions and variations that may exist. To

determine the effects of endogenous hormonal changes on subsequent *in vitro* ovum maturation, we needed a culture media with as few unknown or variable ingredients as possible. Finding no differences among media with 10% or 20% FCS in the preliminary study led to the media test study for comparison among media with 0%, 10% or 20% FCS. Again no consistent differences were found so we chose the most highly defined media, Hams F_{10} with no FCS added, for subsequent studies.

In contrast to work reported by Edwards (1965) and Jagiello, *et al*. (1974), who found germinal vesicles in 100% of sheep oocytes upon removal from the follicle, the present study revealed that 25% of the sheep follicular oocytes were atretic upon removal from the follicle. All visible follicles were aspirated in the present study with no attempt to eliminate atretic ova prior to examination. All oocytes aspirated were included in culture, whereas Tsafriri and Channing (1975b) suggest that only oocytes with intact cumulus should be utilized to avoid oocytes from atretic follicles.

A further, though not significant, increase in atretic oocytes occurred at 12 hours (45.5%), and remained fairly constant for the remainder of the culture times (42.1, 40, 43.7, and 50% for 24, 36, 48, and 60 hours, respectively). The data suggest that only a portion of the oocytes that contained a GV were capable of resuming meiosis in the present *in vitro* system.

Using HCG to mimic the endogenous LH surge, McGovern, *et al*. (1969) found ovulation in 24-28 hours. Dziuk (1965), also using HCG stimulated ewes, showed a time sequence of *in vivo* ovum maturation. Germinal vesicles remained for 10 hours followed by Met I between 12 and 24 hours, and Met II between 22 and 24 hours. Ovulation occurred

between 22 and 26½ hours after HCG. Using an *in vitro* system, Edwards (1965) found Met I at 12.5 hours and Met II by 46 hours. He did not observe oocytes between these times. Jagiello, *et al.* (1974) found GV breakdown beginning at 12 hours with Met I between 16 and 24 hours, and Met II between 23 and 26 hours.

The time sequence of maturation in the present study was similar to those reported. Ova initiated maturation by 12 hours and matured to Met II by 24 hours. One ova was matured to Met II prior to removal from the follicle. This ova could have been stimulated prior to slaughter since the estrous status was not known for these animals.

The fact that no statistical differences were found among groups in the laparotomy study is partially a result of the low numbers of sheep available and the low rate of ovum recovery. With the presence of an intact blood supply, follicles were not as visible as those from ovaries collected at slaughter. Because of these factors (limited numbers of sheep and poor ovum recovery), the collection of ova at laparotomy was discontinued. However, comparison of Table 16 with Tables 18 through 21 reveals very little difference in maturation rates among ova placed in culture immediately and those transported for two to four hours.

The size of ovarian follicle from which an ovum is obtained for *in vitro* studies may affect its ability to mature *in vitro*. Smeaton and Robertson (1971) found that follicular growth and atresia was constant throughout the estrous cycle in sheep. This would provide follicles in any size category at any given time during the cycle. Studies on *in vitro* maturation of sheep follicular oocytes have not distinguished between follicle sizes of origin. No differences were

found among ova from large or small rabbit (Chang, 1955) and mouse (Edwards, 1962) follicles in their ability to mature in vitro. In the pig (Tsafriri and Channing, 1975b) there is an increase in the in vitro maturation rate with increasing follicle size. The macaque also showed an increase in maturation rate with increasing follicle size of origin (Thibault, Gerard, and Menezo, 1976). In the rabbit, Thibault, Gerard, and Menezo (1975) found no difference in the maturation rate among oocytes from small (less than 1 mm) or pre-ovulatory follicles, whereas Bae and Foote (1975) demonstrated greater response of follicle enclosed oocytes to progesterone stimulation when large follicles were used compared to small follicles. In the present study differences among follicle sizes were only demonstrable in one case (Table 15). With the methods of aspiration, it was impossible to distinguish very small (less than 1 mm) follicles. It may be that if these very small follicles had been isolated using a dissecting microscope, differences in the in vitro maturation rate may have been apparent. However, the data suggest that there is no difference in maturation rate among ova from follicles greater than 1 mm in diameter.

Preliminary data suggested that a difference in the in vitro maturation rate may exist between ova collected from the CL bearing or opposite ovary (Table 9). Dufour, Ginther, and Casida (1971, 1972) demonstrated greater follicular growth in the CL bearing ovary compared to the opposite ovary. Because of some inconsistencies in the present study, no definite conclusions can be made regarding ova from CL or opposite ovaries. It is likely that local progesterone levels would be highest in the CL bearing ovary. Progesterone decreased the time of maturation of oocytes from large rabbit follicles. However, in

the present study (Tables 19 and 21), ova from opposite ovaries had a higher rate of maturation.

In agreement with work on the rabbit (Edwards, 1962), human (Edwards, 1965; Shea, Baker, and Latour, 1975) and monkey (Suzuki and Mastroianni, 1966), no differences in maturation rate were found among ova collected at various stages of the estrous cycle, or from pregnant, anestrous, or prepuberal sheep.

INDUCTION OF ESTRUS AND OVULATION IN ANESTROUS EWES

Literature Review

Most ewes undergo a period of anestrus which is controlled by seasonal environmental changes and varies with specific breed differences and management practices. In Michigan, this period is generally from early April until late August and severely limits the times at which an ewe can be bred. It would be advantageous, both in numbers of lambs produced and in the ability to regulate lambing times, to breed ewes during the anestrous period. An extensive review of research on the use of gonadotropins and steroids to induce estrus and ovulation in anestrous ewes has been published (Snyder, 1974). The present review will deal mainly with research since 1974 and research especially pertinent to the present study.

The most common source of gonadotropin used for ovulation induction has been pregnant mare serum (PMS). In research previously reviewed (Snyder, 1974) dosage levels ranging from 600 iu to 800 iu PMS were used with resulting ovulation rates of 1-4 per ewe ovulating. Keane (1975a, b) found no difference in ovulation rate following treatment of non-cyclic lambs with 300, 500, or 600 iu PMS in combination with progestin treatment. Our preliminary studies (Snyder and Dukelow, 1974) showed that ovulation rates of 3.0 could be achieved with gonadotropin doses as low as 5 mg FSH and 100 iu HCG given 48 hours apart.

Generally, gonadotropin induced ovulation is not accompanied by estrous behavior without some type of steroid pretreatment. Progestins, administered for periods of time ranging from three to 17 days and followed by gonadotropin treatment, have been shown by several investigators to induce ovulation with coincident estrus. Freidrick, Davant, and Burfening (1974) found no difference in ovulation rates among 5, 9, or 13 day progesterone treatment by subcutaneous implant when 1 mg estradiol -17B was administered on day 1 and 600 iu PMS was administered at the time of implant removal. Keane (1975a, b) showed 60-100% estrus induction in non-cyclic lambs following 12 or 13 day MAP treatment. However, Saba, Cunningham, Symons, and Millar (1975) induced estrus in only 10% of ewes treated with 500 ug estradiol followed by 100 mg progesterone implants for ten days.

The administration of estradiol prior to the progesterone treatment has generally been associated with improved estrous response but lower ovulation rates (Hunter, 1968; Burfening and Van Horn, 1970; Hulet and Stormshak, 1972; Keane, 1975a). Radford, Wallace, and Wheatly (1970) administered two 25 ug estradiol injections 36 hours apart after a 12 day progestin regimen and observed ovulatory LH peaks 24 hours after the last injection. Other investigators (Jackson and Thurmon, 1974; Beck and Reeves, 1973; Symons, Cunningham, and Saba, 1973) have demonstrated LH peaks 12-18 hours after estradiol treatment. Reeves, Beck, and Nett (1974) also showed FSH peaks coincident with LH peaks induced by estradiol. In the ovariectomized ewe, estradiol administration resulted in a decrease in baseline LH levels within two hours with a subsequent rise to preovulatory levels beginning 11 hours after treatment (Goding, Blockey, Brown, Catt, and Cumming, 1970).

Immunization of ovariectomized ewes against estradiol blocked the estrous response to a progesterone and estradiol treatment regimen (Scaramuzzi, 1975). These studies suggest that estradiol may play a role in controlling the preovulatory LH surge and may be useful in estrus and ovulation induction in the anestrous ewe.

Concentrations of androgens in utero-ovarian venous blood of sheep throughout the estrous cycle is closely correlated with estradiol concentrations (Baird, Land, Scaramuzzi, and Wheeler, 1976). The increase in LH observed in response to estradiol administration to anestrous ewes is delayed or abolished by immunization against androstenedione (Martensz, Baird, Scaramuzzi, and VanLook, 1976). These observations suggest that androgens may play a role in estrous behavior and ovulation, and may be useful during the anestrous period. Following a progesterone regimen, Freidrick, et al. (1974) achieved an equal estrous response with testosterone or PMS; however, the number of ewes ovulating was greater with PMS (88% vs 42%).

Materials and Methods

As a follow-up study to research previously reported (Snyder, 1974; Snyder and Dukelow, 1974), a study was designed to test the effectiveness of low doses of FSH and HCG on ovulation induction in mid-anestrous ewes. Twenty ewes which had not shown estrous behavior for at least 20 days and had no visual evidence of luteal tissue on their ovaries at the time of initial ovarian examination, were assigned to two treatment groups: control, FSH and HCG. Ten ewes each were assigned to control and FSH-HCG treatment. These were then separated into two replicates to facilitate handling of the animals. After

completion of replicate 1, it became apparent that a third treatment, FSH alone, should be included. Three additional ewes and three ewes that had been used as controls in the first replicate were assigned to FSH treatment at the time of the second replicate. FSH-HCG treated ewes received 5 mg FSH intramuscularly followed 48 hours later with 100 iu HCG administered intravenously. Time 0 was the time of HCG administration. Ewes in the FSH group received 5 mg FSH intramuscularly 48 hours prior to time 0. The extent of ovulatory response was determined by ovarian examination at 12, 24, 48, and 72 hours with a follow-up examination at approximately seven days. Examination was by midventral laparoscopy as described by Snyder and Dukelow (1974).

To compare the effectiveness of testosterone and estradiol -17B for the induction of behavioral estrus during the anestrous season, two studies were designed. All ewes assigned to these studies had not shown estrus for at least 20 days and showed no evidence of luteal tissue on their ovaries at the time of initial examination before assignment to groups. In the first study 12 ewes were assigned to three treatment groups: control, testosterone, and estradiol -17B. Testosterone and estradiol -17B treatments were administered as a daily subcutaneous injection of 1 mg of steroid in peanut oil vehicle for six days. Control ewes received the vehicle only. Three rams were introduced at 12 hour intervals throughout the treatment period for the detection of estrous. Following treatment, a raddled, vasectomized ram was continuously with the ewes and fresh breeding marks were recorded daily.

The second study was designed to determine the effect of progesterone pretreatment on estrus induction with estradiol -17B and

testosterone. Twenty ewes were randomly assigned to five treatment groups: control, injected control, progesterone, progesterone and estradiol -17B, and progesterone and testosterone. Progesterone was administered subcutaneously in peanut oil at doses of 20 mg/day for five days. Testosterone and estradiol were also administered subcutaneously in peanut oil at doses of 1 mg/day for three days beginning the day following the last progesterone treatment. Estrus was determined every 12 hours with the introduction of three active rams. Ewes underwent laparoscopic examination 48 hours after the beginning of estrus or after the last treatment to determine the extent of ovulatory response.

Results of this study were analyzed by Chi-square comparison.

Results

Results of ovulation induction in anestrous ewes with FSH and HCG are given in Table 22. There was no time effect among replicates making it possible to combine them for analysis of FSH-HCG treated and control groups. Because the FSH treatment was administered at about the same time as the other treatments and the ewes were handled in a manner identical to control and FSH-HCG treated ewes, this treatment group was included in the analysis. The number of ewes ovulating was greater ($P \leq .05$) for FSH and FSH-HCG treated groups than for controls. There was no diffence between FSH and FSH-HCG groups in the number of ewes ovulating. The number of ovulations per ewe ovulating was not different among groups. More than 50% of the ovulations for the FSH-HCG and the FSH groups occurred prior to 24 hours (8 of 11 and 5 of 8, respectively).

TABLE 22. Ovulation induction with FSH and HCG.

Treatment	No. of Ewes	Ewes Ovulating	Ovulations Per Ewe Ovulating
Control	10	1[a]	2.0
FSH	6	4[b]	2.2
FSH-HCG	10	5[b]	2.0

Values with different superscripts are different at $P \leq 0.05$.

Ewes treated with 1 mg testosterone or 1 mg estradiol -17B for six days did not show estrous behavior during the time of treatment or for at least seven days following treatment (Table 24). However, when ewes were pretreated with progesterone for only five days (Table 25), a significant increase in estrous activity was observed. Progesterone, 20 mg/day for five days, alone induced estrus in only one ewe and was not different from control groups. Progesterone followed by estradiol, 1 mg/day for three days, or testosterone, 1 mg/day for three days, caused a significant increase in estrous activity over controls. Induction of estrus with progesterone and testosterone was only slightly greater (approaching significance at $P < 0.1$) than with progesterone alone. The induction of ovulation with progesterone and estradiol was slightly depressed ($P \simeq 0.1$) compared to other groups.

Discussion

Ovulation in anestrous ewes can be induced with low levels of gonadotropins. The time of ovulation following HCG administration has been reported as being about 24 hours (McGovern, Williams, and Hancock, 1969). However, in the present study using FSH and HCG, 73%

TABLE 23. Time of ovulation after FSH and HCG.

Treatment	Examination Time 12	24	48	72	Follow Up	Total
Control				2 (1)[a]		2 (1)
FSH	8 (3)[a]		1 (1)[b]	2 (2)[a]		11 (5)
FSH-HCG	4 (2)	1 (1)[b]	1 (1)[b]		2 (1)	8 (4)

[a]One ewe had four ovulations prior to 12 hours and one between 48 and 72 hours.
[b]This was the same ewe.

TABLE 24. Induction of estrus with testosterone and estradiol.

Treatment	No. of Ewes	Ewes in Estrus
Control	4	1
Testosterone	4	0
Estradiol	4	0

TABLE 25. Induction of estrus and ovulation with progesterone, estradiol, and testosterone.

Treatment	No. of Ewes	Ewes in Estrus	Ewes Ovulating
Control	4	0^{a}	2
Injected control	4	0^{a}	2
Progesterone	4	1^{ab}	2
Progesterone and Estradiol	4	4^{c}	0
Progesterone and Testosterone	4	3^{bc}	2

Values with different superscripts are different at $P < 0.05$.

of the ovulations occurred within 12 hours after HCG. Because of this discrepancy, a FSH only group was added. Induced ovulations in the FSH group were as high as the FSH-HCG group indicating that the ewes were ovulating in response to FSH. The time that ovulations occurred suggests that additional ovulations were not induced by HCG treatment. The FSH preparation used was found to contain about 10% LH activity (Armour-Baldwin, personal communication) which may account for the ovulation induction observed.

In agreement with previous work, estrus was not induced in the present study without pretreatment with progesterone. It was also noted that as few as five days of progesterone is sufficient to induce estrus, in agreement with Freidrick, et al. (1974) and in contrast with Robinson (1962).

In all portions of this study, spontaneous ovulations occurred in control groups. Ovulation in anestrous ewes may be induced by spontaneous LH release which occurs periodically during the anestrous period (Martensz, et al., 1976). Inhibition of ovulation as a result of estradiol administration (Table 4) is in agreement with previous studies (Hunter, 1968; Burfening and Van Horn, 1970; Hulet and Stormshak, 1972; Keane, 1975a, b). However, the failure of testosterone to inhibit ovulation combined with its ability to induce as high a rate of estrus as estradiol may indicate its usefulness in ovulation induction in the anestrous ewe.

SUMMARY AND CONCLUSIONS

The effect of various endogenous, cyclic reproductive patterns on the ability of sheep follicular oocytes to mature *in vitro* and the ability of FSH, HCG, testosterone, and estradiol to induce estrus and ovulation in anestrous ewes was determined. A total of 103 ova, which were recovered from ovaries collected at slaughter and transported for approximately three hours, were used to determine the most efficient storage temperature for transport. Percents ova maturing were 16.7, 50.0, and 37.8 for ovaries transported at 30°-37°C, 22°C, and 1°C, respectively. Ova recovered from ovaries that were stored at 22°C were more normal in appearance than those from ovaries that were stored at 1°C. For subsequent studies, ovaries were stored at 22°C for transport to the laboratory. To determine the effect of 10 or 20% Fetal Calf Serum (FCS) added to Hams F_{10} culture media for ovum maturation, 162 ova were recovered from anestrous ewes at slaughter. Little difference was found among the culture media (57.6, 51.3, and 53.1% maturation for Hams F_{10}, Hams F_{10} with 10% FCS, and Hams F_{10} with 20% FCS, respectively). For subsequent studies, Hams F_{10} alone was used because of its highly defined nature. The time of ovum maturation *in vitro* was determined using 77 ova recovered from anestrous ewes at slaughter. The time sequence of maturation from removal of the ovum from its follicular environment was similar to that observed *in vivo* following the endogenous LH surge. However, 25% of the ova were atretic upon recovery, and an additional 20.5% became atretic during the first 12 hours of culture. An additional 319 ova were recovered at laparotomy and slaughter from cycling, anestrous, pregnant, and

prepuberal ewes. No consistent differences in maturation rates were found among ova recovered from ewes under various endogenous hormonal influences or from CL bearing or opposite ovaries. There was little difference among large (> 2 mm) or small (< 2 mm) follicles of origin. Ovulation was induced in anestrous ewes with 5 mg FSH and 100 iu HCG. However, coincident estrus was not exhibited. Estradiol and testosterone (1 mg/da each) were not effective in the induction of estrus without progesterone pretreatment. Following a five day progesterone pretreatment (20 mg/da), ovulation was inhibited with estradiol but not with testosterone.

APPENDIX

APPENDIX

Sheep and Swine Surgery Table

Ovarian examination in the sheep and pig is generally accomplished by means of laparotomy or laparoscopy. To facilitate examination by either procedure, it is advantageous for the animal to be in a dorsal recumbent position with the posterior portion elevated at a 30-40 degree angle. Lamond and Urquhart (1961) designed a stationary laparotomy cradle for use in sheep. Hulet and Foote (1968) modified this cradle, making it possible to secure the ewe in a supine position, then increase the angle to that desired for examination. With some minor modifications, this table was used for laparoscopic ovarian examination in the goat (Dukelow, Jarosz, Jewett, and Harrison, 1971), the pig (Wildt, Fujimoto, Spencer, and Dukelow, 1973), and the sheep (Snyder and Dukelow, 1974). This table was stationary and had no adjustment in the angle at which the animal was positioned necessitating much physical movement of anesthetized animals. Examination of larger pigs and sheep required the assistance of several people.

This communication describes an examination table that was designed to facilitate loading of large pigs and sheep at the place of initial anesthesia, movement to the surgery area, and easy adjustment of the angle used for examination. A schematic diagram is presented in Figure 1.

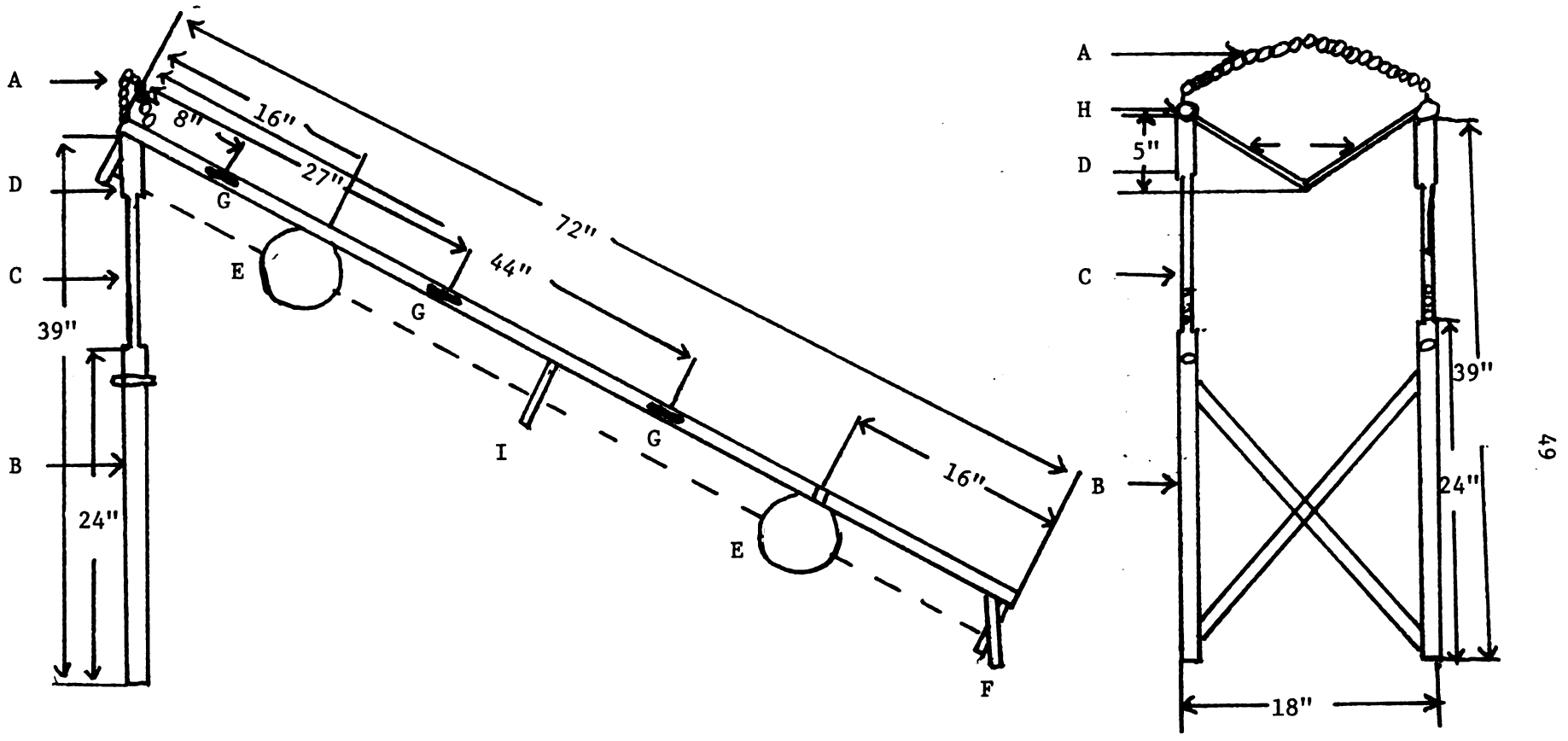

FIGURE I. Sheep and swine surgery table.

A. 1/4" chain for attaching hoist for raising table.
B. Floor stand, made with 1-1/2" pipe.
C. Adjustable legs, 1" pipe, adjust by sliding inside floor stand.
D. 1-1/2" x 5" pipe leg holders.
E. 5" wheels.
F. 1" pipe stabilizer legs.
G. Rope ties for securing animal.
H. 1" pipe side rails.
I. 3/4" conduit center rails and cross bars.

Basic construction is of galvanized tubing to allow for strength and ease of cleaning. In the loading-unloading position the table is low to the floor to facilitate handling of large animals and rests on five-inch wheels so that large animals can easily be moved from holding pens into the surgery area while under general anesthesia. A hoist attached to the ceiling of the surgery room is used to raise the surgery table to the height desired for surgery. Removable and adjustable legs are attached to the posterior end of the table for stability. This surgery table has been used for fast and efficient laparotomies, laparoscopies, and other surgical procedures in sheep and pigs of various sizes.

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