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Date July 21, 1988

IN VITRO MATURATION OF GERMINAL VESICLE STAGE OOCYTES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

John David Wininger

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ABSTRACT

Many reproductive procedures require large numbers of mature oocytes, which are fertilized for embryo production, or are used in various fertility evaluations. The number of oocytes obtainable by the usual method of superovulation and oviduccal flushing, is a limiting factor in these procedures. A method is described for isolating large numbers of immature germinal vesicle stage oocytes from ovaries and culturing these ovarian oocytes to the same maturational stage as oviductal oocytes.

In the present study, bovine and murine ovaries were macerated and cumulus-free, germinal vesicle stage oocytes isolated and placed in culture for maturing. Oocytes were randomized into various treatment groups, including controls, and incubated with one or more of the following hormonal supplements: (1) 17-Estradiol-3-Benzoate, (2) Pregnant Mare's Serum Gonadotrophin, (3) Human Chorionic Gonadotrophin, or (4) Ovine Prolactin.

While all experimental groups exihibited increased maturation

rates when compared to controls, groups treated with prolactin alone had the highest rates. Bovine and murine oocytes which were cultured with prolactin only had mean maturation rates of 27.2% and 41.1%, respectively. These data suggest that prolactin treatment alone is sufficient for increasing the rate of maturation of germinal vesicle stage oocytes and producing large numbers of oocytes at the second metaphase of meiosis. Implementation of this oocyte isolation procedure in conjunction with in vitro maturation with prolactin has the potential of greatly increasing the number of fertilizable oocytes which can be obtained from a single ovary.

TABLE OF CONTENTS

SECTION	PAGE
1. INTRODUCTION	1
Overview	1
Ovarian Morphology	2
The Fetal Ovary	4
The Prepubertal Ovary	5
The Postpubertal Ovary	6
2. MATERIALS AND METHODS	14
Oocyte Isolation	14
Culture Conditions	16
Experimental Group I	18
Experimental Group II	18
Experimental Group III	18
Experimental Group IV	19
Experimental Group V	19

Control Groups 19

3. EXPERIMENTAL RESULTS 21

Oocyte Isolation 21

Indicators of Maturation 21

Control Groups 24

Experimental Group I 26

Experimental Group II 26

Experimental Group III 29

Experimental Group IV 29

Experimental Group V 32

4. DISCUSSION 34

5. CONCLUSION 41

LIST OF REFERENCES 42

VITA 46

LIST OF TABLES

TABLE PAGE

1. Effect of HCG and E2 on Maturation of Bovine Oocytes..... 27
2. Effect of PMSG, HCG, and E2 on Maturation of Bovine Oocytes..... 28
3. Effect of Increasing Prolactin Concentrations on the Maturation of Murine Oocytes in the Presence of 1.0 ug/ml E2..... 30
4. Effect of Increasing Prolactin Concentrations on Maturation of Murine Oocytes..... 31
5. Effect of Increasing Prolactin Concentrations on Maturation of Bovine Oocytes..... 33

LIST OF FIGURES

FIGURE PAGE

1. Diagrammatic Representation of Follicular Growth..... 7
2. Pre-ovulatory Maturation of the Oocyte..... 11
3. Cross Section of the Mammalian Ovary Showing Follicles at Various Stages of Development..... 13
4. Double-filtering Process..... 15
5. Bovine Oocytes at Various Stages of Development which were Isolated from the Ovary by Maceration..... 22
6. Germinal Vesicle Stage Murine Oocyte..... 23
7. Germinal Vesicle Stage Bovine Oocyte Following DAPI Staining..... 23
8. *In Vitro* Matured Murine Oocyte showing First Polar Body..... 25
9. *In Vitro* Matured Bovine Oocyte following DAPI Staining..... 25
10. The Effect of Increasing Prolactin Concentrations on the Maturation of Murine Oocytes..... 37

1. INTRODUCTION

Overview

Many reproductive procedures require a supply of mature oocytes, which are used for research, fertility evaluations, or are fertilized for embryo production. Although methods such as superovulation and uterine flushing of embryos are available, the implementation of these methods is both time consuming and expensive. A far more practical approach for obtaining large numbers of mature oocytes would be an *in vitro* maturation system. Under proper culture conditions, immature primary oocytes will resume meiosis and mature to secondary oocytes.

A major motivation for the development of an *in vitro* maturation system is the need for mature oocytes which can be fertilized *in vitro* for embryo production. Routinely, techniques such as hormonal superovulation and follicular aspiration are used

for oocyte collection (Leibfried-Rutledge et al.1986). As an alternative to these methods, large numbers of immature oocytes may be easily isolated from the ovary and matured *in vitro* to a point where fertilization can occur.

Oocyte maturation is defined as those events that enable the oocyte to become fertilized and begin preimplantation embryonic development. The first stages of maturation are those events associated with germinal vesicle breakdown and completion of the first meiotic division. Experiments presented here involve isolation of germinal vesicle stage oocytes from ovaries and their subsequent maturation *in vitro* by subjecting them to various hormonal treatments.

Ovarian Morphology

The mammalian ovary consists of three distinct regions: an inner hilum at the point of attachment of the ovary to the mesovarium, a central medulla, and an outer cortex. The hilum contains nerves, blood vessels, supporting connective tissue, and

steroid hormone-producing cells, called hilar cells. The medulla consists of a heterogeneous collection of cells that remain after the oocyte is removed as follicular complexes mature. The cortex contains the immature germ cells, which are enclosed in cellular complexes embedded in stroma (the follicles), and is covered with coelomic epithelium, called germinal epithelium (Yen and Jaffe. 1986).

Follicular complexes are structurally the most conspicuous and functionally the most important elements in the ovarian cortex. Changes in the microscopic appearance of the follicle, along with rearrangement of internal components, coincide with follicular development during fetal and postnatal life. The terms "primordial ," "primary," "secondary," and "Graafian" are used to describe follicular complexes at sequential stages of development. For a more comprehensive description of ovarian morphology, see: Yen and Jaffe. Reproductive Endocrinology; Chpt 4 (1986).

The Fetal Ovary

Following migration of germ cells into the presumptive gonads, which occurs in the first trimester in mammals, the ovary rapidly takes on the form of the adult organ. The ovarian germ cells rapidly become transformed into oogonia. The population of oogonia increases greatly in a relatively short period of time. Following this period of oogonial mitoses, the oogonia become transformed into primary oocytes and enter prophase of the first meiotic division. From this time onwards, there is no increase in the numbers of oocytes, estimated to be 500,000 (Ball et al. 1984). The fixed population of germ cells can only be reduced with age, due to ovulation and/or atresia. This is in contrast to the male germ cells, the spermatagonia, which remain mitotically active throughout adult life.

The nucleus and chromosomes of the primary oocyte remain in the diplotene stage of the first meiotic prophase until the time of ovulation, following the pre-pubertal period. The primary oocyte is characterized by the presence of a large nucleus, the germinal

vesicle. The germinal vesicle contains chromosomes which bear lateral projections in the form of branches and loops which actively replicate ribonucleic acid (RNA). These chromosomes closely resemble the lampbrush chromosomes which are found almost universally in eggs of lower vertebrates and some invertebrates (Austin and Short. 1972).

The Prepubertal Ovary

Follicular maturation begins during late fetal life and continues throughout the reproductive life of the animal. The oocytes become surrounded by a single layer of flattened epithelial cells soon after they are formed, and the primordial follicle is established. As follicular growth occurs, the surrounding cells become cuboidal in shape and begin to proliferate through mitosis. The spaces between the cells subsequently become filled with fluid and the follicle is now said to be vesicular. Vesicular follicles are constantly forming from primordial follicles, but only with the establishment of the correct hormonal balance and reproductive cycles at the time of

puberty, is the process of follicular growth permitted to culminate in ovulation (Austin and Short. 1972). The majority of vesicular follicles which are formed before puberty become atretic and degenerate.

Early signs of follicular growth in primordial follicles are: (1) an increase in the size of the oocyte, (2) a change in shape and increase in numbers of the granulosa cells, and (3) the formation of the zona pellucida. The dividing granulosa cells are subsequently invaded by blood capillaries and an outer vascular layer, the theca interna, is formed. This layer, along with the theca externa, is the major source of nutrients to the granulosa cells and oocyte. Figure 1 gives a diagrammatic representation of follicular growth which can be used for a wide range of species.

The Postpubertal Ovary

Following the onset of puberty, several of the growing follicles are stimulated to undergo further growth and maturation during each reproductive cycle. The number of follicles which

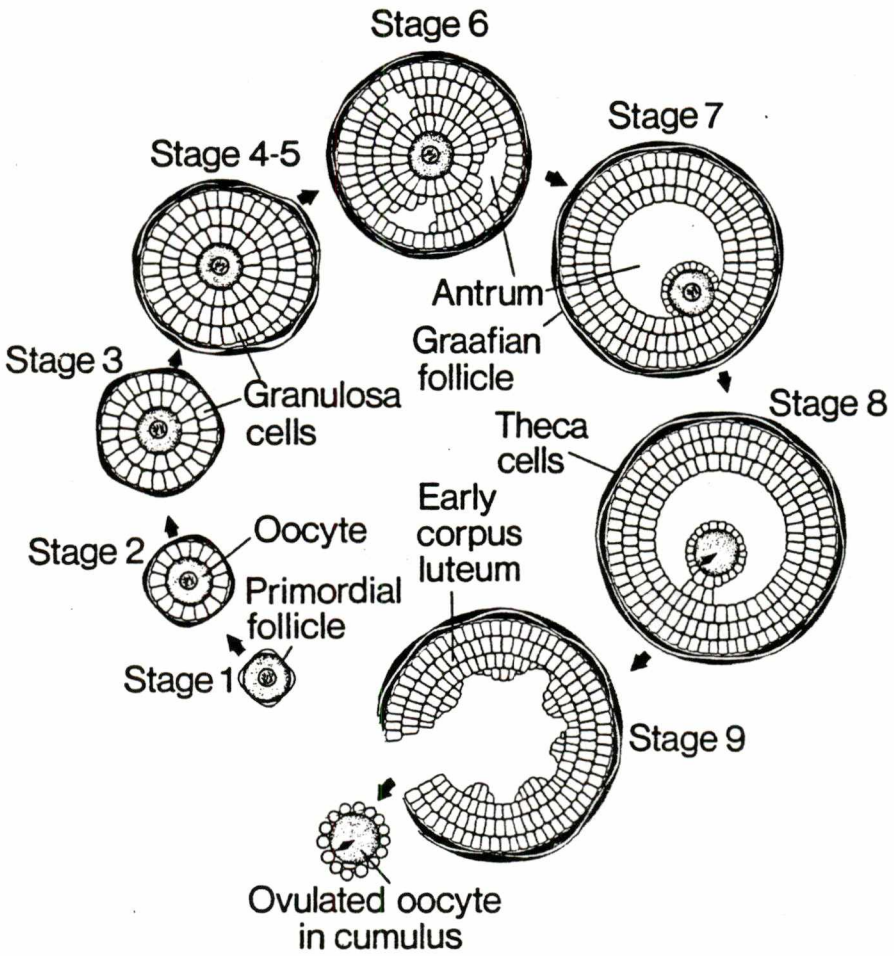


Figure 1. Diagrammatic representation of follicular growth.

Source: Austin and Short, "Germ Cells and Fertilization", Cambridge University Press, 1972, p. 29.

continue growth is determined by the available quantity of follicle stimulating hormone (FSH), a product of the anterior pituitary gland. FSH causes further multiplication of the granulosa cells and also increases the amount of fluid ("liquor folliculi"), that passes through the spaces between them. The follicular fluid contains increasing concentrations of estrogen, which is one product of the granulosa cells. Estradiol increases the responsiveness of granulosa cells to luteinizing hormone (LH), also a product of the anterior pituitary (Tepperman and Tepperman 1986). As the volume of fluid increases,the spaces between the cells become confluent,forming an antrum. The follicle is now said to be of the Graafian type.

In order for ovulation to occur, the Graafian follicle, and the oocyte within it, must undergo further changes which require precisely controlled hormonal conditions. Circulating FSH levels remain elevated for only a short time at the beginning of the growth phase, after which the levels of circulating gonadotrophins (FSH and LH), remain relatively constant until a short time before ovulation. The last phase of maturation,

preovulatory maturation, is marked by a sudden increase in the release of gonadotrophins from the pituitary, especially LH (the LH-surge). The release of FSH also increases and it has been suggested that the FSH/LH ratio is just as important in controlling preovulatory maturation as that of LH alone (Austin and Short. 1972). This ratio may also regulate to some extent the number of oocytes that ovulate, which is fairly constant for each species. Following the LH surge, granulosa cells increase in numbers and the quantity of follicular fluid increases dramatically. The oocyte becomes loosely attached to the surrounding granulosa cells and may become free-floating in the follicular fluid shortly before ovulation.

In addition to the changes which occur in the Graafian follicle, there is a resumption of meiosis within the oocyte. The oocyte still contains lampbrush chromosomes, but the synthesis of RNA that was characteristic of early stages of growth has now terminated. The germinal vesicle subsequently breaks down (nuclear membrane ruptures), and the first meiotic prophase is completed. During Metaphase I, the bivalents (paired

chromosomes), arrange themselves on, and become attached to, microtubules at the equator of the meiotic spindle (see Fig. 2). During Anaphase I bivalents move to opposite ends of the spindle, which rotates gradually through 90^0. By early Telophase I, rotation is nearly complete, and the surface of the egg near the chromosomes may show a slight elevation. Unequal cytoplasmic division begins as a furrow develops around the outermost set of chromosomes (Austin and Short. 1972). One daughter cell, the secondary oocyte, recieves most of the cytoplasm while the other recieves the minimum of ooplasm and is the abortive polar body (see figure 2).

After extrusion of the first polar body the secondary oocyte begins the second meiotic division. Prophase II is very short and results in the positioning of the condensed chromosomes at the periphery of the oocyte (chromatin mass stage). Metaphase II begins as spindle fibers appear adjacent to the chromatin and the chromosomes align themselves on the metaphase plate. In most species Metaphase II is an arrested stage of development like the dictyate stage of Metaphase I and usually occurs before ovulation.

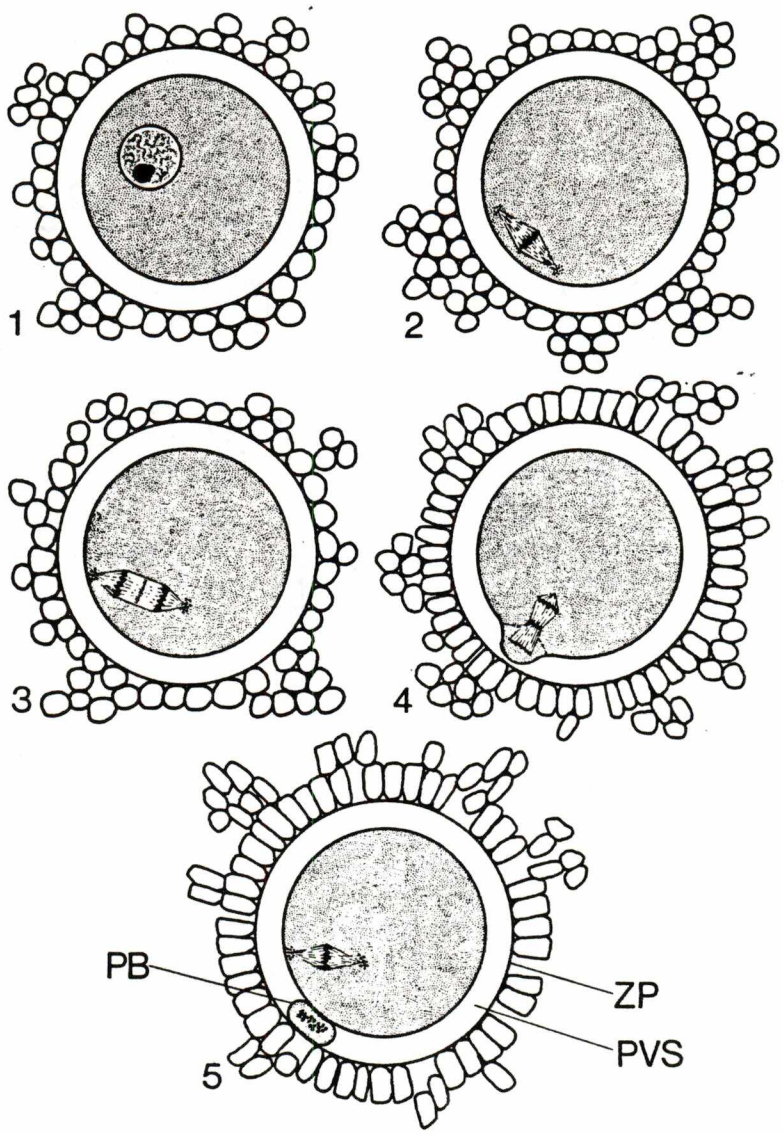


Figure 2. Pre-ovulatory maturation of the oocyte. (1.) germinal vesicle; (2.) metaphase I; (3.) anaphase I and rotation of the spindle; (4.) polar body extrusion; (5.) secondary oocyte at metaphase II, surrouded by a perivitelline space (PVS) between oocyte and zona pellucida (ZP). PB is the first polar body.

Source: Austin and Short, "Germ Cells and Fertilization", Cambridge University Press, 1972, p. 35

The ruptured follicle becomes the corpus luteum within the ovarian cortex following ovulation. The remaining granulosa cells rapidly divide and become transformed into luteal cells. These luteal cells contain secretory granules and a yellow pigment from which the name corpus luteum is derived ("yellow body"). The corpus luteum secretes progesterone, a steroid hormone which is important in controlling the length of the reproductive cycle and maintaining pregnancy in many species. Regression of the corpus luteum, which occurs if pregnancy does not ensue, involves a halt in progesterone secretion, degeneration of the luteal cells, and infiltration of fibroblasts which convert the gland into a mass of scar tissue (corpus albicans). Fig. 3 is an idealized drawing of the structure of the mammalian ovary showing follicles at various stages in their development and the formation and regression of corpora lutea.

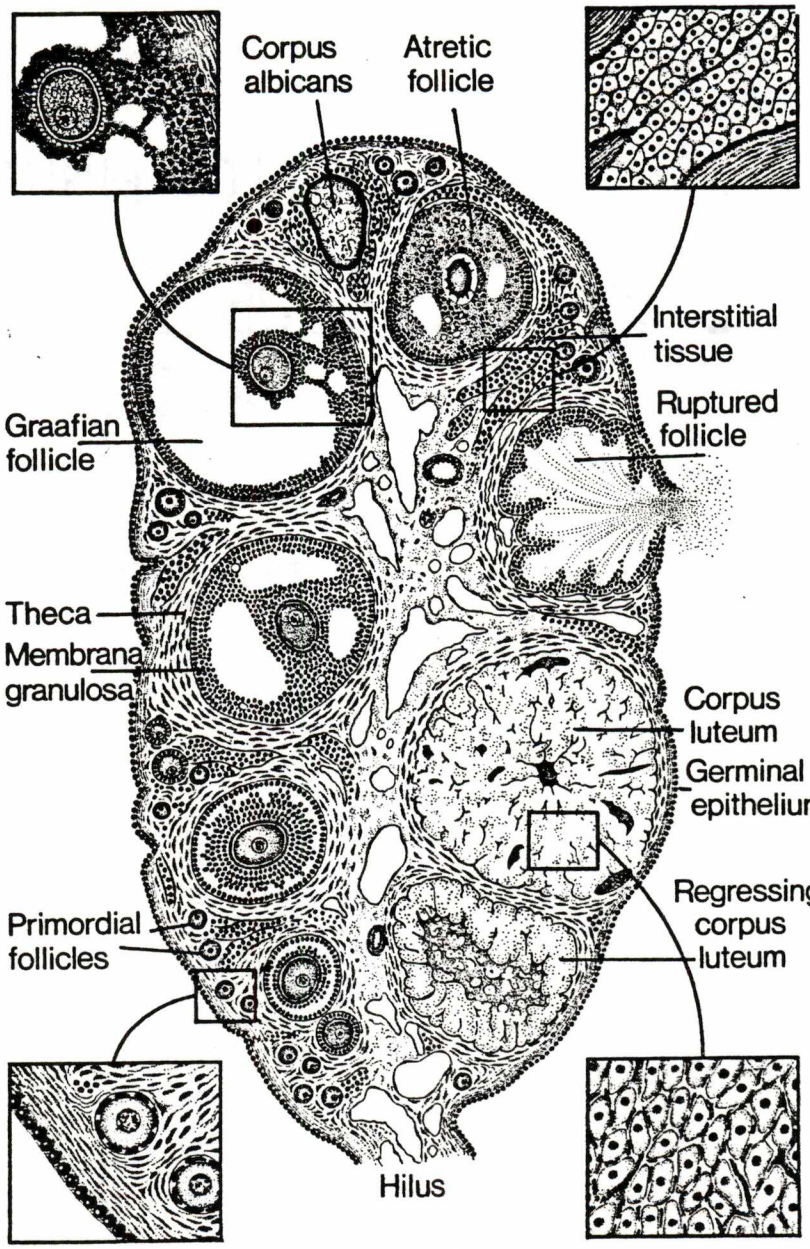


Figure 3. Cross section of the mammalian ovary showing follicles at various stages of development.

Source: C. D. Turner, "General Endocrinology", W. B. Saunders Co., 1966, Fig. 12-7.

2. MATERIALS AND METHODS

Oocyte Isolation

Bovine ovaries were collected at a local slaughterhouse and immediately transported to the laboratory. The ovaries were placed in Ham's F-10 media and macerated in a vegetable chopper which was modified to use razor blades for chopping. The mixture was then poured into a 250 ml Nalgene flask and shaken vigorously for 25 seconds to remove the attatched cumulus and corona cells. Following shaking, the mixture was poured through two layers of nylon filters (Fig. 4). The pore size of the upper filter was 220 um, and the bottom filter had a pore size of 100um. The 100 um filter was then carefully rinsed with Ham's F-10 media into a 90 mm petri dish. The dish was then placed under a dissecting microsope for selection of oocytes. When murine ovaries were used, the ovary was macerated in a 90mm petri dish with a no. 10 scalpel. Any remaining cumulus or corona cells were removed with a pipette. Shaking was not necessary in the case of murine oocytes.

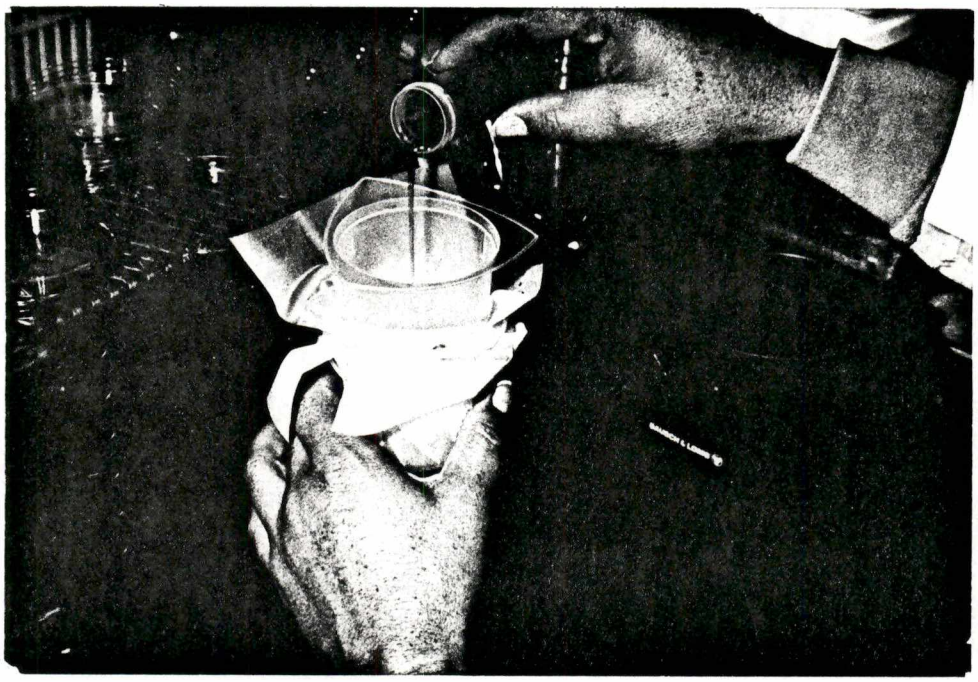


Figure 4. Double-filtering process. Oocytes will pass through the top (220um) filter and are found on the bottom filter (100um).

Although oocytes at many stages of maturation were released from the ovary during the maceration procedure, only primary oocytes were chosen to be put into culture. Primary oocytes were identified by the presence of the germinal vesicle. The germinal vesicle was clearly visible under a dissecting microscope or in some cases a nuclear stain was used. DAPI, 4',6-diamidino-2-phenylindole (*van der Kooy and Kuypers*.1979) or Hoechst dye 33342, (*Greggoire*. 1984) are nuclear stains which were used for visualization of the germinal vesicle. Following selection of primary oocytes, (using dissecting scope), a random sample of the oocytes was exposed to one of the nuclear stains and viewed under a fluorescent microscope (Zeiss) for confirmation of the maturational stage.

Culture Conditons

Ham's F-10 powdered culture medium (Sigma), used for oocyte maturation, was supplemented with 2.2 g/L sodium bicarbonate (Sigma), 242.2 mg/L calcium lactate (Sigma), 75.0 mg/L

penicillin-G , K salt (Sigma), and 50.0 mg/L streptomysin sulfate (Sigma). The supplements were dissolved in sterile distilled water (Flow Laboratories). The osmolarity of the medium was adjusted to 280 mOsM and pH was 7.35 after equilibration with 5% CO_2 in air.

In some cases, the medium was further supplemented with various concentrations of one or more of the following: (1) pregnant mare's serum gonadotrophin (PMSG), (2) B-estradiol-3 benzoate (E_2), (3) human chorionic gonadotrophin (HCG),or (4) ovine prolactin (PRL). Following isolation, primary oocytes were placed in 1 ml center-well tissue culture dishes with 1 ml of culture medium and incubated at 37.5^0 C under air with 5% CO_2 and 100% humidity. The media and supplements were changed at 12 hour intervals. Following the culture period the oocytes were examined, as described above, for signs of maturation. Sterile techniques were observed throughout the entire procedure to avoid the risk of contamination.

Experimental Group I

Isolated bovine oocytes were placed in modified Ham's F-10 culture medium supplemented with 1.0 ug/ml E_2 and 1.0 IU/ml HCG (Sigma; Lot 35F0479), and incubated for 48 hrs. (Newcomb; 1978).

Experimental Group II

Isolated bovine primary oocytes were placed in modified Ham's F-10 medium supplemented with 10 ug/ml PMSG (Sigma; 31 IU/mg; Lot 67F-0041), for 24 hrs., then 1.0 ug/ml E_2 and 1.0 IU/ml HCG for the next 24 hrs.

Experimental Group III

Isolated murine oocytes were placed in modified Ham's F-10 culture medium supplemented with 1 ug/ml E_2 and PRL (Sigma; 1940 IU/MG; Lot 66F-04851), at one of the following concentrations: (1) .32 ng/ml, (2) 3.2 ng/ml, (3) 32 ng/ml, (4) 320 ng/ml, (5) 3.2 ug/ml, or (6) 32 ug/ml and incubated for 23-26 hrs.

Experimental Group IV

Isolated murine oocytes were placed in modified Ham's F-10 culture medium supplemented with PRL at one of the following concentrations: (1) .32ng/ml, (2) 3.2 ng/ml, (3) 32 ng/ml, (4) 320 ng/ml, (5) 3.2 ug/ml, or (6) 32 ug/ml,and incubated for 23-26 hrs.

Experimental Group V

Isolated bovine primary oocytes were placed in modified Ham's F-10 culture medium supplemented with PRL at concentrations of either 30 ng/ml, 300 ng/ml, 3.2 ug/ml, or 32 ug/ml and incubated for 23-26 hrs.

Control Groups

All experimental groups were tested along with appropriate control groups. The controls consisted of isolated oocytes (either bovine or murine), which were placed in modified Ham's F-10 culture medium. No additional hormones were added to the

medium. All control groups were subjected to the same environmental conditions and remained in culture for the same amount of time as their corresponding experimental group before their maturational status was determined.

3. EXPERIMENTAL RESULTS

Oocyte Isolation

Following isolation of the oocytes from the ovary, as described above, oocytes at various stages of maturation were examined under a dissecting microscope (Fig. 5). Primary oocytes were identified by the presence of a germinal vesicle. Figure 6 shows a murine primary oocyte as seen with light microscopy under higher magnification. The germinal vesicle is visible. Nuclear staining,as mentioned earlier, was also used for confirmation of the maturational status of the oocyte. Figure 7 shows a bovine oocyte following treatment with DAPI. The germinal vesicle was visible as a large fluorescing body, when viewed with the fluorescent microscope (Zeiss).

Indicators of Maturation

Germinal vesicle breakdown must occur in order for the oocyte to resume meiosis. That this event has occured is easily detected

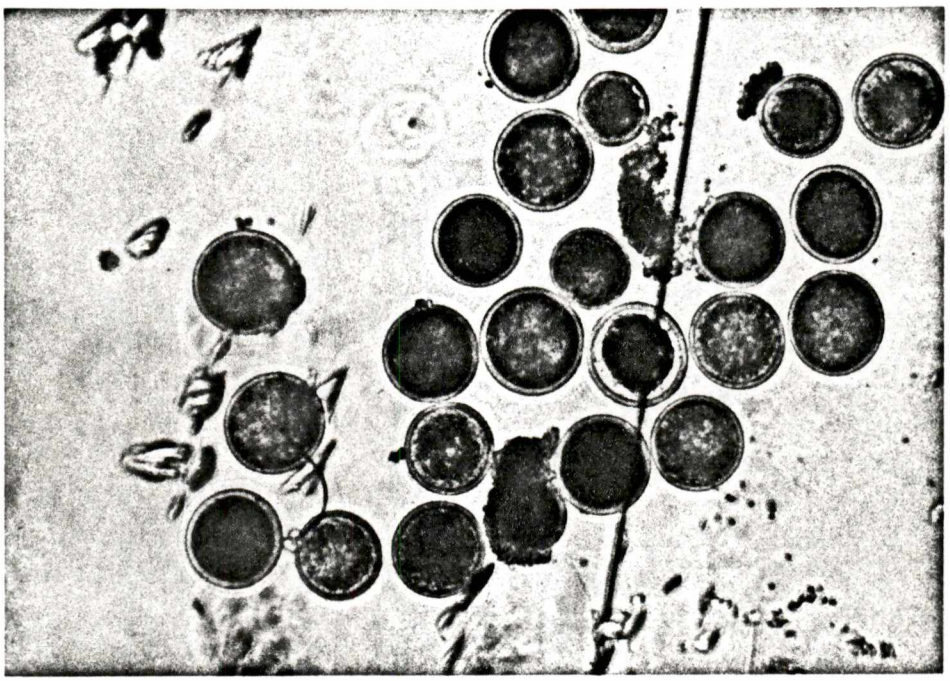


Figure 5. Bovine oocytes at various stages of development which were isolated from the ovary by maceration (60X).

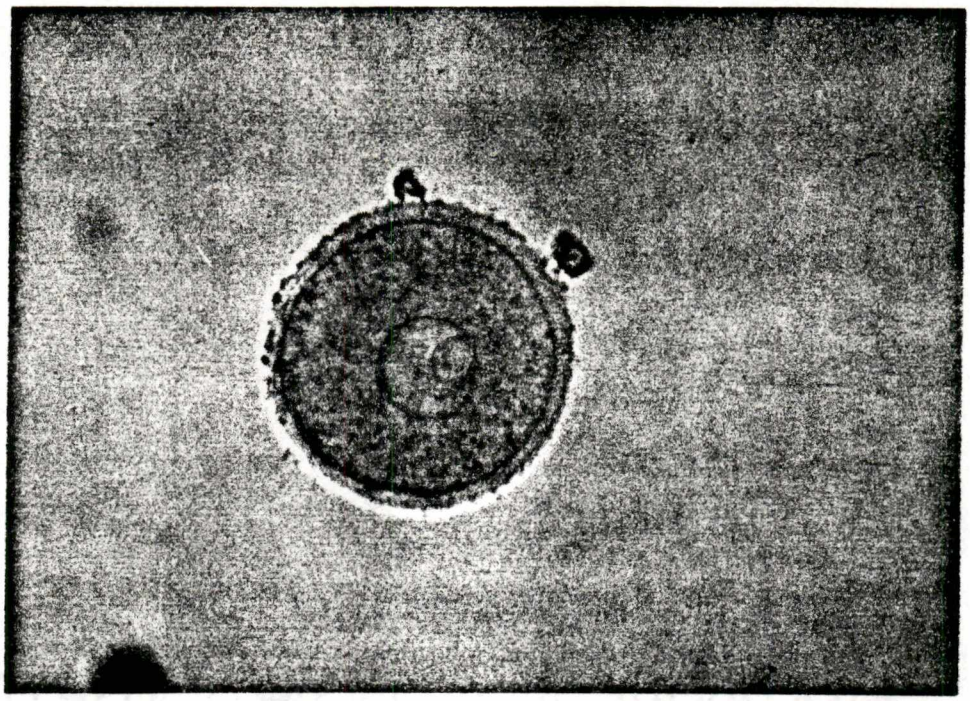


Figure 6. Germinal vesicle stage murine oocyte. Germinal vesicle and nucleolus are visible (160X).

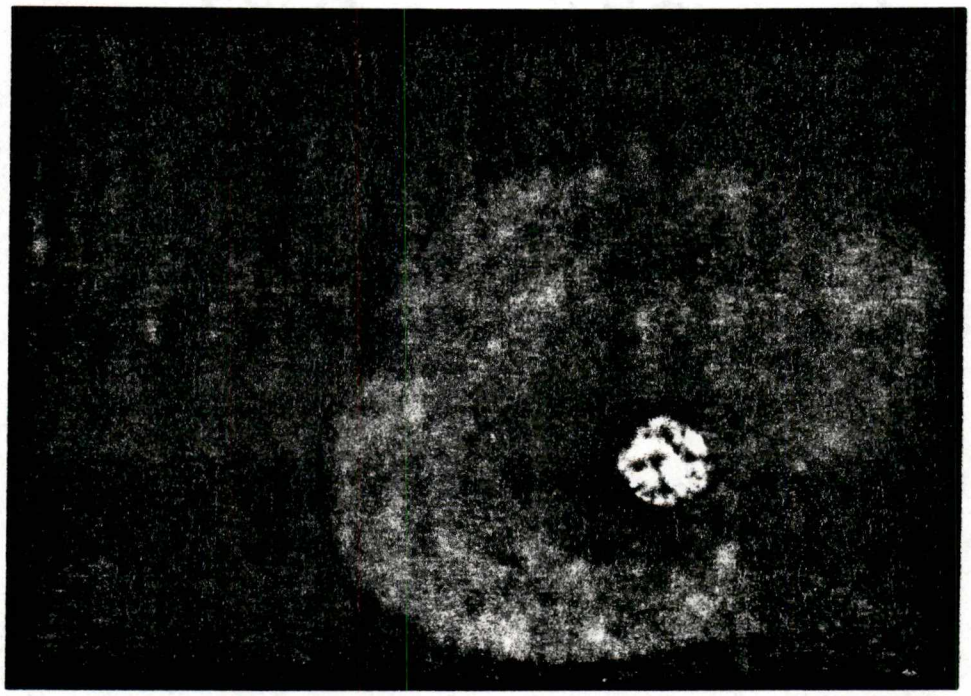


Figure 7. Germinal vesicle stage bovine oocyte following DAPI staining (128X, Zeiss Optics).

through the use of either light or fluorescent microscopy. Germinal vesicle breakdown will usually occur approximately 12-14 hours following placement of the oocytes into culture.

Extrusion of the first polar body occured as the oocyte concluded the first meiosis and was easily seen with a dissecting microscope. Maturation continued to the point of Metaphase II where the metaphase plate was visualized with the use of one of the previously mentioned nuclear stains. Figures 8 and 9 show *in vitro* matured oocytes visualized using light and fluorescent microscopy, respectively. These three events: (1) germinal vesicle breakdown, (2) extrusion of the first polar body, and (3) presence of the Metaphase II plate, were used to judge the maturational status of the oocytes following incubation.

Control Groups

The mean percentage of bovine oocytes in control groups (Groups I, II, and V), which matured to Metaphase II following culture was 9.3%. Murine control groups (Groups III and IV), had a

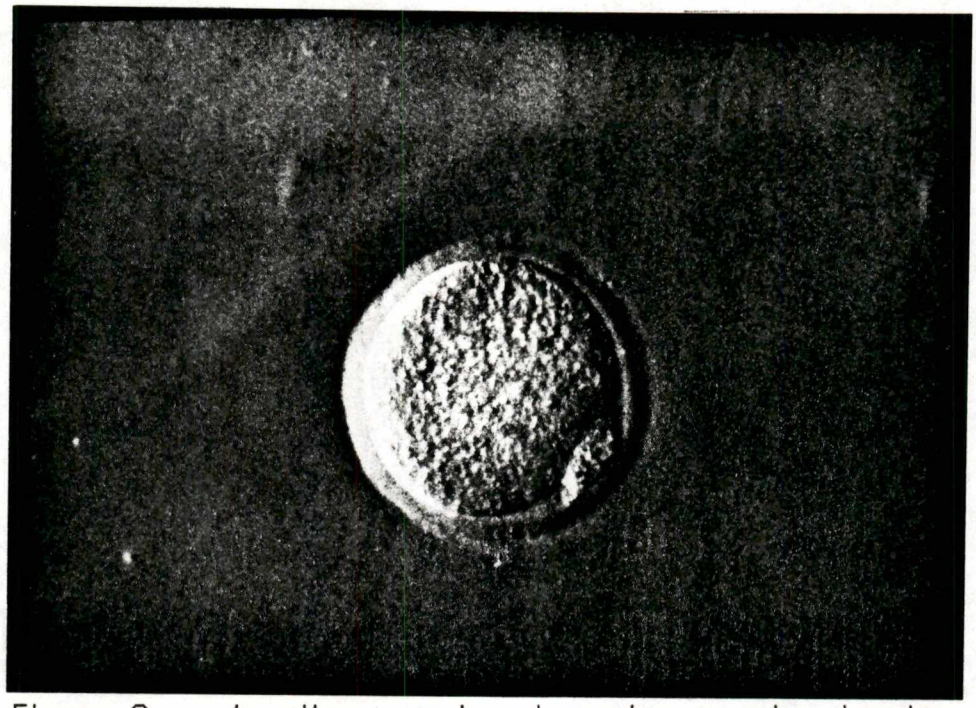


Figure 8. *In vitro* matured murine oocyte showing first polar body (Hoffmann Optics; 120X)

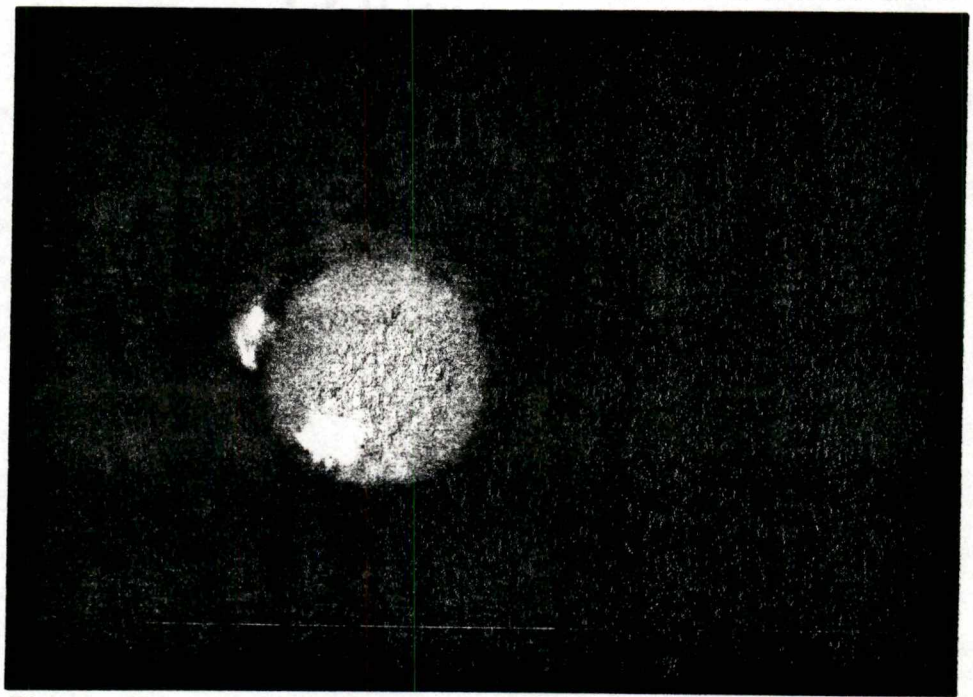


Figure 9. *In vitro* matured bovine oocyte following DAPI staining. Metaphase plate and first polar body are visible (Zeiss fluorescent Optics 128X).

mean maturation rate (No. of mature oocytes/ No. of total oocytes), of 25.3% following the culture period.

Experimental Group I

Culture of bovine oocytes in modified Ham's F-10 medium containing 1.0 ug/ml E_2 and 1.0 IU/ml HCG had a maturation rate of 19.4% compared with 10.8% for controls (Table 1).

Experimental Group II

Bovine oocytes cultured in modified Ham's F-10 medium supplemented with 10 ug/ml PMSG for 24 hrs. followed by 1.0 IU/ml HCG and 1.0 ug/ml E_2 for an additional 24 hrs. had a maturation rate of 22.9% (Table 2). The control groups had a maturation rate of 7.6%

TABLE 1

EFFECT OF HCG AND E_2 ON MATURATION
OF BOVINE OOCYTES

[HCG and E2]	N[a]	No. of Mature Oocytes[b]	(%)[c]
Controls	16/16/14	2/2/1	(10.8)
1.0 ug/mlE_2 + 1.0 IU/ml HCG	26/26/25	6/5/4	(19.4)

a Numbers represent three different experiments.

b Absence of GV; Oocytes at Metaphase II; First polar body present.

c Mean of three experiments.

TABLE 2

EFFECT OF PMSG[a], HCG[b], AND E_2[b] ON
MATURATION OF BOVINE OOCYTES

[Hormone]	N[c]	No. of Mature Oocytes[d]	(%)[e]
Controls	21/23/22	2/1/2	(7.6)
1.0 IU/ml PMSG +	30/30/30/30	6/7/7/7	(22.9)
1.0 IU/ml HCG +	30/30/20/22	6/7/5/6	
1.0 ug/ml E_2			

[a] In media for the first 24 hrs. of culture only.

[b] In media for second 24 hrs. of culture only.

[c] Numbers represent individual experiments.

[d] Absence of GV; Oocytes at Metaphase II; First polar body present.

[e] Mean of all experiments.

Experimental Group III

Isolated murine oocytes cultured in modified Ham's F-10 culture medium supplemented with 1.0 ug/ml E_2 and Prl at concentrations of 0.32 ng/ml, 3.2 ng/ml, 32 ng/ml, 320 ng/ml, 3.2 ug/ml, or 32.0 ug/ml had maturation rates of 36.6%, 43.3%, 33.3%, 39.4%, 46.7%, 50.0%, and 50.0%, respectively (Table 3). The control groups had a maturation rate of 27.7%.

Experimental Group IV

Isolated murine oocytes cultured in modified Ham's F-10 medium supplemented with increasing concentrations of PRL (0.32 ng/ml, 3.20 ng/ml, 32.0 ng/ml, 320 ng/ml, 3.20 ug/ml, 32.0 ug/ml), had maturation rates of 26.7%, 36.7%, 43.3%, 40.0%, 53.3%, and 46.7% respectively (Table 4). The control groups had a maturation rate of 23.3 %.

TABLE 3

EFFECT OF INCREASING PROLACTIN CONCENTRATIONS ON THE MATURATION OF MURINE OOCYTES IN PRESENCE OF 1.0 ug/ml E_2

PRL Concentration	(N)[a]	No. of Matured Oocytes[b]	(%)[c]
Control	10/10/10	4/2/2	(27.7)
0.32 ng/ml PRL	10/10/10	3/6/4	(43.3)
3.20 ng/ml PRL	10/10/10	3/3/4	(33.3)
32.0 ng/ml PRL	10/10/10	3/5/5	(39.4)
320 ng/ml PRL	10/10/10	4/6/4	(46.7)
3.20 ug/ml PRL	10/10/10	3/7/5	(50.0)
32.0 ug/ml PRL	10/10/10	5/5/5	(50.0)

[a] Numbers represent three different experimental groups.

[b] Absence of GV; Oocytes at Metaphase II; First polar body present.

[c] Mean of three experiments.

TABLE 4

EFFECT OF INCREASING PROLACTIN CONCENTRATIONS ON MATURATION OF MURINE OOCYTES

[PRL]	N [a]	No. of Matured Oocytes[b]	(%)[c]
Controls	10/10/10	3/2/2	(23.3)
0.32 ng/ml PRL	10/10/10	4/1/3	(26.7)
3.20 ng/ml PRL	10/10/10	4/4/3	(36.7)
32.0 ng/ml PRL	10/10/10	5/4/4	(43.3)
0.32 ug/ml PRL	10/10/10	4/3/5	(40.0)
3.20 ug/ml PRL	10/10/10	5/6/5	(53.3)
32.0 ug/ml PRL	10/10/10	4/5/5	(46.7)

[a] Numbers represent three different experimental groups.

[b] Absence of GV; Oocytes at Metaphase II; First polar body present.

[c] Mean of three experiments.

Experimental Group V

Isolated bovine oocytes cultured in modified Ham's F-10 medium supplemented with 30.0 ng/ml, 300 ng/ml, 3.00 ug/ml, or 30.0 ug/ml PRL had maturation rates of 17.8%, 22.2%, 33.3%, and 35.5%, respectively. The maturation rate for controls was 11.1% (Table 5).

TABLE 5

EFFECT OF INCREASING PROLACTIN CONCENTRATIONS ON MATURATION ON BOVINE OOCYTES

Prolactin Concentration	(N)[a]	No. of Matured Oocytes[b]	(%)[c]
Controls	15/15/15	2/1/2	(11.1)
30.0 ng/ml PRL	15/15/15	3/3/2	(17.8)
300 ng/ml PRL	15/15/15	3/4/3	(22.2)
3.00 ug/ml PRL	15/15/15	4/6/5	(33.3)
30.0 ug/ml PRL	15/15/15	5/6/5	(35.5)

a Numbers represent three different experimental groups.

b Absence of GV; Oocytes at Metaphase II; First polar body present.

c Mean of three experiments.

4. DISCUSSION

Much research has been done which relates to *in vitro* maturation of oocytes of many different species. However, much of the work involved cumulus-enclosed oocytes which were aspirated from follicles of various sizes. (*Rutledge, et al.* 1986; *Liehman, et al.* 1986.) The oocytes used in this project were cumulus-free, non-follicular, germinal vesicle stage oocytes, and therefore were at a much earlier developmental stage.

Various culture media supplements have also been used to increase the maturational rate of immature oocytes. Since LH is believed to overcome the inhibitory action of follicular fluid on meiosis (*Tsafriiri, et al.* 1976.), many workers have added LH to culture media (*Bae and Foote,* 1975). HCG is similar to LH in its biological activity (*Rajaniemi and Vanha-Pettula,*1972), and is also utilized in culture media for the induction of meiosis (*Newcomb, et al.* 1978). The effects of steroids on maturation have also been investigated, but with controversial results. It has been reported that estradiol-17B reduced significantly the

incidence of maturation of cumulus enclosed rabbit, bovine (*Robertson and Baker,* 1969), and mouse oocytes (*Smith and Tenney,* 1980). Other researchers have indicated that estrogens constitute one essential component in cytoplasmic maturation (*Thibault*, 1977; *Dahlhausen et al.* 1981). It has also been reported that ovine (Moor and Trounson, 1977), and bovine (*Newcomb et al.* 1978) oocytes, cultured in medium containing both gonadotrophin (LH or HCG) and E_2 17-B, resumed meiosis and developed to blastocyst stages. The effects of steroids on oocyte maturation have been shown to be concentration dependent (*Smith and Tenney,* 1980), but their effect also depends on the characteristics of the oocytes cultured (cumulus-enclosed or denuded oocytes) and the species of animal from which the oocytes were obtained (*Tyler, et al.* 1980).

In the present study, estradiol 17-B, in conjunction with HCG (Group1), HCG and PMSG (Group II), and PRL (Group III), increased the numbers of oocytes which resumed meiosis and matured to Metaphase II, compared to control groups. The effect of PRL alone on the maturation of murine oocytes (Group IV), was very

similar to its effect in the presence of 1.0 ug/ml E_2 17-B (Group III). The mean maturation rate for all oocytes in Group III was 42.8% compared to 41.1% for Group IV. This suggests that the response seen in Group III is due to PRL alone. The results of Group IV also demonstrate that the effects of PRL are concentration related. Figure 10 shows the dose-response effect exhibited by murine oocytes treated with increasing concentrations of PRL (From Table 4). PRL also increased the numbers of maturing bovine oocytes (Table 5), compared to controls. Although the response of bovine oocytes to PRL (Table 5) was not as marked as that of murine oocytes (Table 4), the mean maturation rate was 26.6% for all oocytes in Group V. This work indicates that PRL alone may sufficiently increase the maturation of bovine and murine oocytes when added to culture media. That is, other steroids or hormones may not be necessary.

The response of the oocytes to PRL may also suggest the presence of PRL receptors on the oocyte surface. A "leveling-off effect", in terms of response, was seen at the highest concentrations of PRL, in both murine and bovine experiments.

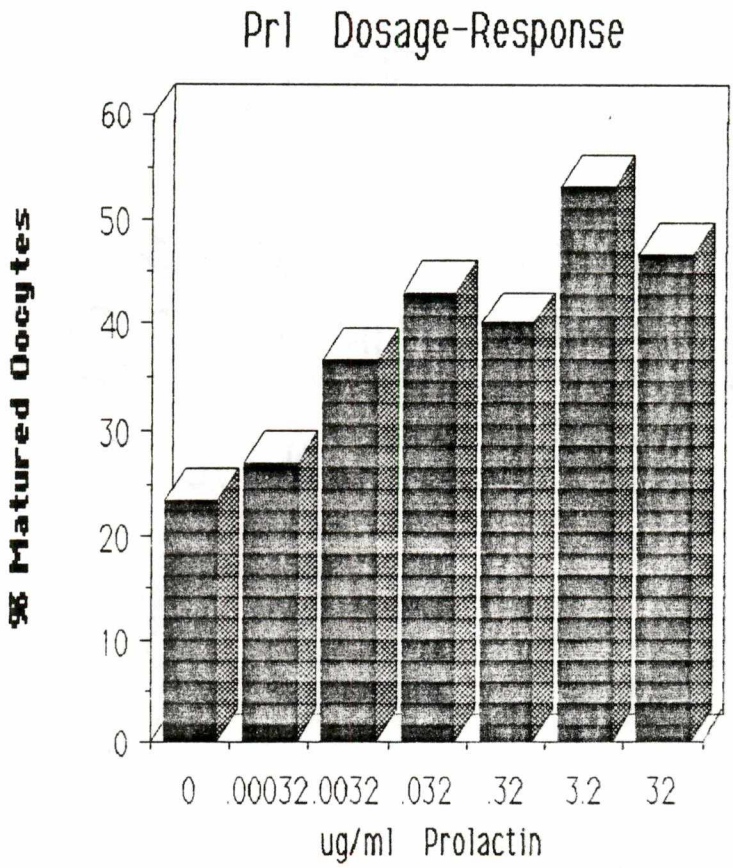


Figure 10. The effect of increasing prolactin concentrations on the maturation of murine oocytes.

This effect is indicative of hormone-receptor interactions, and would occur due do saturability of receptors (*Tepperman and Tepperman*. 1986).

The prolactin molecule is a single polypeptide containing 198 amino acids residues with a molecular weight of 22,000 (*Shome and Parlow*. 1977). Most of the experimental data on the function of PRL are derived from the result of *in vivo* and *in vitro* use of ovine PRL (*Nicoll*, 1980); the use of this PRL in different species limits the conclusions to be drawn regarding the physiology of this hormone (*Yen et al*. 1986). Although ovine Prl increased the maturation of both murine and bovine oocytes in the present work, more conclusive data could be drawn from experiments using murine and bovine PRL.

Tremendous progress has been made in *in vitro* maturation, fertilization and early embryo development. The potential that these procedures will have on the animal industry is one reason these areas have recieved so much research attention. There is much interest in increasing the variety of genetic material for use in artificial insemination. While males produce billions of

gametes throughout their lifetimes, females release fewer than 1% of their oocytes during their reproductive lifespan (*Ball et al.*, 1984). Superovulation followed by embryo transfer is the most common method used to increase the number of female gametes available. The oocyte isolation technique described above, in conjunction with in vitro maturation, would greatly increase the number of oocytes per animal available for embryo production.

The field of genetic engineering could also make use of in vitro maturation. Currently, if nuclear genes are to be inserted into mammals, they must be microinjected into the male pronucleus of single-cell embryos (*Hensleigh and Hunter*. 1984). However, only a small percentage of the injected genes become incorporated by chromosomes and expressed. Therefore, a large, inexpensive, readily available source of embryos is required. The large numbers of immature oocytes present in the ovary could be a valuable resource for genetic engineering research. Following isolation of several hundred oocytes from the ovary, in vitro maturation and fertilization would provide numerous embryos for microinjection of foreign DNA, or other genetic manipulations.

In vitro maturation of oocytes could also be a useful procedure for quality control testing of media in human *in vitro* fertilization procedures (*Quinn and Whittingham*. 1982). The mouse embryo system is widely used for culture media testing in IVF laboratories. Development of hatched mouse blastocysts from two-cell embryos at high frequency is indicative of suitable culture media. The maturation of germinal vesicle-stage oocytes could also be a test for media quality. Due to the mechanisms involved, germinal vesicle breakdown followed by extrusion of the first polar body may be a more sensitive test of culture media quality than development of the mouse embryo.

Fertility evaluations, such as the sperm-penetration assay (SPA), require a large supply of mature oocytes (*Yanagimachi et al.* 1976). Currently, this procedure requires superovulation and subsequent oviductal flushing of hamsters for oocyte collection. In view of the large numbers of oocytes in the ovary, *in vitro* maturation could increase the oocyte yield per hamster and would also reduce the number of hamsters necessary.

5. CONCLUSION

The oocyte isolation and *in vitro* maturation procedures presented here are applicable to many areas of the reproductive sciences. Large numbers of oocytes, which are easily isolated from the ovary by maceration, can be matured *in vitro* with PRL and fertilized or used in many other reproductive techniques. Of all hormonal supplements tested, PRL treatment alone increased the maturation of both bovine and murine oocytes at higher rates, when compared to controls.

Further work will include *in vitro* fertilization of the *in vitro* matured oocytes. The role of PRL in maturation should also be investigated further. Receptor-binding assays would be useful in determining whether PRL receptors are actually present on the surface of the oocyte.

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