1988

Studies On The Regulation Of Oocyte Maturation In The Rat With Assessment By In Vitro Fertilization And Fetal Development

Barbara Catharine Vanderhyden

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, tests publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

STUDIES ON THE REGULATION OF OOCYTE MATURATION IN THE RAT WITH ASSESSMENT BY IN VITRO FERTILIZATION AND FETAL DEVELOPMENT

by

Barbara Catharine Vanderhyden

Department of Physiology

Submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario

September, 1988

ISBN 0-315-43308-6

ABSTRACT

The purposes of this study were: 1) to develop techniques for *in vitro* maturation of rat oocytes which would permit fertilization and normal embryonic development, and 2) to investigate the role of somatic cell-germ cell interactions, and hormonal influences, in the physiological regulation of oocyte maturation as assessed by these techniques.

Procedures for *in vitro* fertilization (IVF) of rat oocytes were developed and validated by transferring the resulting embryos to recipient females. Although embryos resulting from IVF were less successful in establishing pregnancy than appropriate *in vivo* fertilized controls, optimization of culture and transfer techniques enabled minimization of embryonic losses and permitted valid use of these techniques.

Oocytes matured in the presence of their cumulus cells and serum were as capable of IVF, embryonic and fetal development as ovulated oocytes. Oocytes matured in the absence of cumulus cells showed a high incidence of abnormal pronuclear formation during fertilization, indicating that the cumulus cells played a role in ensuring normal cytoplasmic maturation.

Oocytes matured in the absence of cumulus cells, serum or follicular fluid had an increased resistance to sperm penetration. When this penetration problem was overcome by drilling a hole in the zona pellucida, cumulus-free oocytes

continued to show a high incidence of abnormal fertilization, verifying a role for cumulus cells in cytoplasmic maturation.

Immature oocytes obtained from prepubertal rats were capable of spontaneous nuclear maturation, and a small proportion was capable of being fertilized. Administration of pregnant mares' serum gonadotropin prior to oocyte collection increased the proportion of oocytes which subsequently underwent fertilization. Follicle-stimulating hormone (FSH) stimulation of *in vitro* maturing oocytes delayed nuclear maturation but did not affect the proportion of oocytes capable of undergoing fertilization. Conditioned media from cultures of FSH- or LH-stimulated granulosa cells could substitute for serum or follicular fluid in preventing zona hardening.

The results of this research indicate that a granulosa cell product(s), present in follicular fluid and serum, helps to maintain the penetrability of *in vitro* matured oocytes. Cumulus cells play an essential role in ensuring normal cytoplasmic maturation and, therefore, normal pronuclear formation in oocytes during fertilization.

ACKNOWLEDGEMENTS

During my sojourn as a graduate student, I have had the good fortune to work and interact with a group of extremely talented individuals. Their willingness to share their skills and their friendship made this experience a most enjoyable one. Although numerous people have been helpful during various parts of this project, I wish to acknowledge particularly the following people:

I would like to thank Dave Armstrong for his encouragement and guidance and, most of all, for his faith in my judgement. His trust in my abilities fostered my independence and confidence, both scientifically and personally.

I am most grateful to Tom Kennedy for his many helpful suggestions and especially for his guidance in the realm of statistics. Thanks for keeping tabs on me.

Thanks to Anne Rouleau for suggesting the bursa-peeling technique and for making long afternoons of tedious tasks an often enjoyable experience.

Thanks also to Jean Rutledge for her skilful assistance, particularly in the zona drilling experiments, and for her willingness to repeatedly stick her hand into cages holding very large male rats.

I owe many thanks to Gerry Barbe for his help in dealing

with computers and labortory equipment, and to Joan Barber for providing the necessary animals, laboratory supplies and entertainment.

I thank Dr. Evelyn Shaver and Dr. Barry Tepperman, members of my Advisory Committee, for their helpful criticisms and suggestions throughout the development of this project.

I am grateful to John McLaughlin who taught us the zona drilling technique.

I am indebted to Nancy Wilson and Barb McDougall for transforming my scribble into respectable literature and for being so very flexible to accommodate my deadlines.

I thank Paul Morley for teaching me how to culture granulosa cells and for performing most of the steroid assays for the granulosa cell studies. A special thanks for his moral support and his friendship.

And, finally, many thanks to my family whose continuous support and encouragement throughout this work are most gratefully appreciated.

TABLE OF CONTENTS

List of Photographic Plates

LIST OF TABLES

List of Figures

LIST OF ABBREVIATIONS

AGP	aminoglutethimide phosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CF	cumulus-free
CI	cumulus-intact
dbcAMP	dibutyryl cyclic adenosine monophosphate
DPBS	Dulbecco's phosphate buffered saline
DPBS-S	Dulbecco's phosphate buffered saline with 5% heat-inactivated, charcoal-treated rat serum
estradiol	estradiol-17β
FSH	follicle-stimulating hormone
g	gram
GAGs	glycosaminoglycans
GnRH	gonadotropin releasing hormone
GVBD	germinal vesicle breakdown
h	hour
hCG	human chorionic gonadotropin
IBMX	isobutylmethylxanthine
IU	international units
l	liter
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
MEM	minimal essential medium
MPF	maturation-promoting factor
1-MA	1-methyladenine

ug	microgram
uM	micromolar
mg	milligram
ul	microliter
um	micrometer
ml	milliliter
ng	nanogram
P_4	progesterone
PMSG	pregnant mares' serum gonadotropin
PB	polar body
saline	0.9% sodium chloride
S.E.M.	standard error of the mean
wt	weight
~	approximately
>	greater than
<	less than

CHAPTER 1

INTRODUCTION

The central role played by the oocyte in all mammalian reproduction is exemplified by the statement "Omne vivum ex ovo" ("All living things come from eggs"), which is attributed to William Harvey. The mammalian oocyte achieves the capacity for fertilization and subsequent development after a lengthy period of growth and differentiation. In rodents and humans, oogenesis begins with primordial germ cell formation in early fetal development and ends, weeks to years later, in the sexually mature adult. Around the time of birth, the ovary is populated with nongrowing primary oocytes that have reached the diplotene stage of prophase I of meiosis. At this stage meiosis is arrested, the chromosomes decondense and a prominent nucleus or germinal vesicle is formed. This diffuse diplotene or dictyate stage of meiosis persists until puberty. In response to the surge of luteinizing hormone (LH) in each estrous or menstrual cycle, some of the oocytes resume the meiotic process, by a mechanism that is not yet understood, and progress to the metaphase II stage, at which time the oocytes enter a second meiotic arrest. The resumption of meiosis after the first period of arrest is characterized by the dissolution of the nuclear membrane, condensation of the diffuse chromatin, separation of homologous chromosomes and emission of the

first polar body. The secondary oocyte, ovulated while in the second meiotic arrest, completes meiosis and extrudes the second polar body following fusion with the spermatozoan at fertilization.

Primary oocytes liberated from their follicles before the LH surge are capable of spontaneous resumption of meicsis (Pincus & Enzmann, 1935). This phenomenon has been used extensively in studies that attempt to determine the mechanisms involved in meiotic arrest and oocyte maturation. The resumption of meiosis is morphologically seen as the disappearance of the germinal vesicle and the extrusion of the first polar body and these changes are widely used as criteria for the resumption of meiosis. However, there is no assurance that these oocytes mature in vitro by a mechanism similar to that occurring in the follicular environment and, indeed, the developmental competence of these oocytes is often limited (Cross & Brinster, 1970; Thibault & Gerard, 1973; Leibfried & Bavister, 1983). Until it can be demonstrated that spontaneously maturing oocytes can attain the same level of fertilizability and developmental competence as ovulated oocytes, the results of experiments that use germinal vesicle breakdown as the criterion of success will be clouded with some degree of doubt.

Although the rat is one of the most commonly used animals in laboratory studies of reproductive biology and endocrinology, the fertilization of rat oocytes in vitro has been difficult to achieve, being carried out

successfully only after similar success with several other species. There are numerous reports of inconsistent or irreproducible results (Shalgi et al., 1979; Blandau, 1980; Quigley, 1980, 1982), but successful in vitro fertilization has been reported by three groups (Toyoda & Chang, 1974a; Kaplan & Kraicer, 1978; Evans & Armstrong, 1984).

The purpose of the present research was twofold: 1) to develop a technique for in vitro fertilization of rat oocytes that would allow developmental success of these oocytes following transfer to a recipient uterine environment, and 2) to use this technique of in vitro fertilization with in vitro matured rat oocytes to assess their developmental capabilities following various manipulations in vitro. In doing so, possible factors regulating oocyte maturation in vitro were studied.

CHAPTER 2

LITERATURE REVIEW

2.1 OOCYTE DEVELOPMENT

2.1.1 Oocyte Growth and Acquisition of Meiotic Competence

At the time of birth, the rodent ovary is populated with small, nongrowing oocytes arrested in meiotic prophase and surrounded by a layer of follicular cells. During the first 2 weeks after birth, some of these oocytes and their surrounding follicles begin to grow, with the growth being apparently regulated within the ovary (Krarup et al., 1969). In rodents, oocytes complete growth in 2-3 weeks, before the formation of the follicular antrum. During this period of time, oocytes grow from a diameter of ~10 µm to a size of ~80 µm and they acquire a proteinaceous extracellular coat called the zona pellucida. Throughout its growth phase, the oocyte remains arrested in the dictyate stage of the first meiotic prophase.

Although the oocyte has completed its growth relatively quickly, the follicle continues to grow. The stromal cells differentiate into an external theca cell layer separated by a basement membrane from the internal granulosa cells. The follicular cells undergo rapid proliferation and a fluid-filled antrum appears, resulting in a mature, Graafian follicle with a diameter greater than

500 μm. The follicular fluid is comprised of serum transudate and of glycosaminoglycans and other substances secreted by the granulosa cells. At this stage the oocyte is enclosed by a dense mass of granulosa cells called the cumulus oophorus or cumulus cells and is suspended in the follicular fluid connected only by a stalk of cells to the mural granulosa cells. The innermost layer of cumulus cells, the corona radiata, forms specialized intercellular gap junctions with the oolemma. Oocytes appear to be dependent on the cumulus cells for their growth (Eppig, 1977, 1979a), since the cumulus cells may be a primary source of nutrients (Herlands & Schultz, 1984). In one study, however, the authors reported the growth of naked oocytes, provided that ovarian cells were included in the culture (Bachvarova et al., 1980). The discrepancy in these reports may be due to differences in the culture conditions.

During oocyte growth, the dictyate-stage oocytes acquire meiotic competence, i.e. the ability to undergo spontaneous meiotic maturation following isolation from their follicles (Pincus & Enzmann, 1935; Szybek, 1972; Sorensen & Wassarman, 1976). As it occurs within the follicle, this maturation involves the breakdown of the germinal vesicle and progression to metaphase II of meiosis, with extrusion of the first polar body. The acquisition of meiotic competence appears to be correlated with the stage of oocyte and follicular development (Erickson & Sorensen, 1974, Iwamatsu & Yanagimachi, 1975;

Tsafriri & Channing, 1975a). Meiotic competence is acquired between 15-21 days of age in mice (Szybek, 1972; Sorensen & Wassarman, 1976), at 23 days in hamsters (Iwamatsu & Yanagimachi, 1975) and between days 20-26 in rats (Bar-Ami & Tsafriri, 1981). The mean diameter of competent mouse oocytes is larger than that of incompetent ones (Sorensen & Wassarman, 1976) and there is a high correlation between rat oocyte diameter and the ability to undergo spontaneous maturation (Bar-Ami & Tsafriri, 1981). However, oocyte diameter does not seem to be the only determinant of meiotic competence in the rat, since the average diameter of the few competent oocytes on day 20 is less than that of incompetent oocytes on day 26. This is in agreement with other evidence that indicates that the acquisition of meiotic competence is not only time-dependent, but is also independent of the presence of follicle cells, heterologous cell contacts and cell growth (Canipari et al., 1984). There is some evidence to suggest that the time-dependence is related to the time of antrum formation (Erickson & Sorensen, 1974; Bar-Ami & Tsafriri, 1981).

When mouse oocytes are explanted on day 7 (Bachvarova et al., 1980) or day 8 (Eppig, 1977, 1979a) and grown in culture, only 10-20% undergo meiosis in culture. It is clear that growth in vitro deprives these oocytes of some essential factor or condition necessary for them to acquire meiotic competence. In attempting to identify the

correlation between antrum formation and the acquisition of meiotic competence, the observation that the development of the antrum is dependent on gonadotropin stimulation (Richards, 1980) naturally led to investigations of the role of these hormones on the acquisition of meiotic competence. Hypophysectomy of immature rats on day 15 (prior to the acquisition of meiotic competence) reduces the percentage of competent oocytes on day 26 by 58% (Bar-Ami & Tsafriri, 1981). This response can be reversed by treatment of the hypophysectomized rats with pregnant mares' serum gonadotropin (PMSG) or follicle-stimulating hormone (FSH). Further study revealed that the action of the gonadotropins is, at least partly, mediated by ovarian estrogen production (Bar-Ami et al., 1983). It remains to be determined whether the proportion of oocytes acquiring meiotic competence after growth in vitro can be increased by addition of these hormones.

Numerous studies have investigated the details of RNA synthesis and accumulation during oogenesis and the changes in the qualitative pattern of protein synthesis during oocyte growth (reviewed by Bachvarova, 1985; Schultz, 1987). These changes precede acquisition of meiotic competence and become more pronounced with increasing oocyte diameter (Schultz et al., 1979), which suggests that the synthesis of specific proteins may be responsible, at least in part, for acquisition of meiotic competence.

2.1.2 Oocyte Maturation

One of the major problems in developmental biology and reproductive physiology today is the elucidation of the mechanisms regulating mammalian oocyte maturation. Several factors have been implicated in the maturation processes; however, there is no generally accepted model for the coordination of these factors in the regulation of oocyte maturation. Oocyte maturation *in vivo* is the result of the preovulatory surge of gonadotropins, leading one to suspect that oocyte maturation is under the positive control of gonadotropins. However, when fully grown dictyate stage oocytes are isolated from their follicles and cultured in the absence of any hormones, they undergo spontaneous meiotic maturation (Pincus & Enzmann, 1935; Edwards, 1965a,b). This observation led to the conclusion that some component(s) of antral follicles inhibits oocyte maturation. Consequently the action of the gonadotropins could be to reverse this inhibition through modification or inactivation of the inhibitory factor. Subsequent sections of this chapter will present evidence to suggest that steroid hormones, oocyte-cumulus cell coupling, cyclic adenosine monophosphate (cAMP) and a follicular oocyte maturation inhibitor may all play roles in maintaining the follicular oocyte in meiotic arrest. The possible existence of a maturation-promoting factor will also be discussed.

A Phases of Maturation

Investigators of mammalian oogenesis usually refer to maturation as the processes occurring during the final stages of preovulatory meiosis in oocytes. A more restrictive, yet more accurate, definition of maturation may be those developmental processes by which the immature mammalian oocyte at the germinal vesicle stage becomes fertilizable (McGaughey, 1983). Complete maturation of the cumulus-oocyte complex thus involves three separate processes: 1) nuclear or meiotic maturation, 2) cytoplasmic maturation, and 3) cumulus cell maturation, all of which are under regulation by the intrafollicular environment. These three phases of maturation will be considered here briefly, with greater detail provided where appropriate in other sections.

Nuclear maturation

Throughout the growth phase, the oocyte remains at the dictyate stage of prophase I. The nucleus, or germinal vesicle, of the oocyte is prominent and the chromosomes are highly diffuse and transcriptionally active. Following the acquisition of meiotic competence, resumption of meiosis can be mediated by a hormonal stimulus *in vivo* or simply by the removal of the oocyte from its ovarian follicle into a suitable culture medium *in vitro*. Meiotic maturation of the fully grown dictyate oocyte is characterized by the dissolution of the nuclear membrane, condensation of the diffuse chromatin into distinct bivalents, separation of

homologous chromosomes and emission of the first polar body, with arrest at metaphase II. This sequence of events is morphologically seen as the disappearance of the germinal vesicle or germinal vesicle breakdown (GVBD) followed several hours later by polar body extrusion. Since GVBD is the first prominent change observed, it is widely used as a criterion for the resumption of meiosis.

Cytoplasmic maturation

Earlier studies have shown that the spontaneous maturation of liberated oocytes results in morphologically normal secondary oocytes, but the developmental competence of these oocytes is limited. Spontaneously matured oocytes from mice (Cross & Brinster, 1970; Mukherjee, 1972), rabbits (Thibault & Gerard, 1973), rats (Niwa & Chang, 1975a), pigs (Motlik & Fulka, 1974) and hamsters (Leibfried & Bavister, 1983) exhibit very low fertilization rates. Thibault & Gerard (1970, 1973) noted that follicle-free rabbit oocytes undergoing spontaneous maturation are incapable of transforming sperm nuclei into pronuclei in the cytoplasm of the penetrated oocyte. Similar deficiencies in pronuclear formation have been found in rats (Niwa et al., 1976), pigs (Motlik & Fulka, 1974) and hamsters (Leibfried & Bavister, 1983). When pronuclear development does occur, the rate of cleavage and the frequency of preimplantation development to blastocyst stage are low, with development to live offspring even lower when compared to oocytes matured in vivo (Cross &

Brinster, 1970; Niwa et al., 1976, Moor & Trounson, 1977; Shalgi et al., 1979). It has been suggested, therefore, that deficient cytoplasmic maturation occurs in spontaneously maturing oocytes even though nuclear maturation appears normal (Thibault, 1972, 1977).

Changes in the cytoplasmic activity of mammalian oocytes during the course of maturation have been studied by the insemination of oocytes at various stages of maturation. Using hamster oocytes, Usui & Yanagimachi (1976) observed that, although sperm penetration of the zona-free oocytes can occur at any stage of maturation, decondensation of the sperm chromatin will occur only after GVBD. Similar results were reported in studies using mice (Iwamatsu & Chang, 1972a) and rats (Niwa & Chang, 1975a). Thus the process of GVBD may cause the release of some nuclear factor(s) or induction of some cytoplasmic changes that are necessary for male pronucleus development following penetration of the oocyte. Support for this theory is provided by a study with starfish oocytes, in which the mechanical breakdown of the germinal vesicle, in the absence of any hormone treatment, was able to induce complete cytoplasmic maturity (Guerrier et al., 1983).

Cytoplasmic maturation has been found to be somewhat dependent on the conditions under which the oocytes are induced to mature. Further details will be provided in subsequent sections (see Section 2.1.2 B).

Cumulus maturation

Cumulus cells surround the oocyte and send out processes that traverse the zona pellucida and terminate on the oolemma. Fully grown oocytes are coupled with the surrounding cumulus cells by gap junctions between these projections and the oocyte (Albertini & Anderson, 1974; Amsterdam et al., 1976; Gilula et al., 1978). During oocyte growth, gap junctions mediate intercellular communication between the oocyte and the cumulus cells. The cells are both metabolically and ionically coupled (Gilula et al., 1978; Moor et al., 1980a; Racowsky & Satterlie, 1985) and experimental evidence indicates that the cumulus cells furnish nutrients to the oocyte via these gap junctions, with the conclusion that this heterologous intercellular communication is essential for oocyte growth (Cross & Brinster, 1974; Eppig, 1979a; Bachvarova et al., 1980; Heller et al., 1981; Herlands & Schultz, 1984).

Following the preovulatory surge of gonadotropins, morphological transformation of the cumulus oophorus occurs. There is a significant decrease in the number of gap junctions associated with a decrease in the extent of metabolic and ionic coupling (Gilula et al., 1978; Heller & Schultz, 1980; Eppig, 1982a). Cumulus cell cytoplasmic processes retract from the oolemma (Gilula et al., 1978; Dekel et al., 1978) and the cumulus oophorus undergoes expansion or mucification. Mucification refers to the mechanical separation of the cells due to the intercellular deposition of hyaluronic acid. This separation is

necessary to allow sperm penetration to occur after ovulation. Complete cumulus maturation therefore involves the 2 processes: uncoupling of the oocyte-cumulus cell complex and mucification. In vitro studies have determined that FSH is a very effective uncoupling agent (Moor et al., 1981; Eppig, 1982b) as well as a stimulus for cumulus expansion (Dekel & Kraicer, 1978; Eppig, 1979b). Although both processes occur simultaneously in vivo, FSH-stimulated cumulus expansion is not required for cumulus cell-oocyte uncoupling in vitro (Eppig & Ward-Bailey, 1982; Salustri & Siracusa, 1983).

In addition to their nutritional role, cumulus cells are involved in regulating several functions of the oocyte. Their influence on nuclear and cytoplasmic maturation, on quality of fertilization and on subsequent developmental potential are discussed in detail in other sections.

It should be stressed at this point that spontaneous oocyte maturation refers only to the meiotic maturation (i.e. GVBD) that occurs when an oocyte is removed from its follicle. As such, this phenomenon can be used to study the ultrastructural and biochemical changes that occur during meiotic maturation. However, there is evidence to indicate that cytoplasmic and cumulus maturation do not occur spontaneously. The cumulus oophorus remains tightly packed around spontaneously maturing oocytes (Dekel & Kraicer, 1978; Eppig, 1979b) and the oocytes may have difficulty in forming the male pronucleus after sperm

penetration (see Section 2.1.2) Thus the spontaneously maturing oocyte has potential problems if it is used to determine the mechanisms of oocyte maturation, particularly if the criterion of successful maturation is simply GVBD. It is clear that the fertilizability and the developmental competence of the *in vitro* matured oocyte are criteria of equal and possibly greater importance.

B Oocyte Maturation *in vitro*

The investigations of oocyte maturation in mammals have been pursued through the use of several different approaches, using: 1) oocytes matured *in vivo*, 2) ovaries or ovarian fragments in organ culture, 3) isolated ovarian follicles in culture, or 4) the culture of isolated oocytes. The advantages and limitations of each approach will be presented here briefly.

In most mammals, meiotic maturation occurs in the oocytes of a select group of antral follicles as a result of the endogenous preovulatory gonadotropin surge, or the administration of exogenous gonadotropin to a suitably prepared female. Blocking the preovulatory surge of gonadotropin in the rat by Nembutal prevents the resumption of meiosis (Ayalon *et al.*, 1972), while administration of exogenous gonadotropins to the Nembutal-treated rats results in GVBD within 2-3 hours (Vermeiden & Zeilmaker, 1974; Magnusson *et al.*, 1977). The basic method for examining the maturation of oocytes *in vivo* has been to

administer exogenous gonadotropins to females and to recover ovarian or oviductal oocytes at various times following the hormone administration. The oocytes analyzed from intact animals have been matured under physiological conditions and, therefore, accurate observations can be made concerning the processes that occur in vivo. Using this method, gross observations have been made regarding the relationships between meiotic maturation, the dose and time of gonadotropin administration and follicular growth and steroidogenesis (reviewed by Donahue, 1972; Baker, 1979). In addition, oocytes matured in vivo are fertilizable and competent to undergo embryonic and fetal development. Unfortunately, the complexity of the in vivo system makes it less effective for the investigation of mechanisms involved in oocyte maturation.

The culture of whole ovaries or ovarian fragments has proved to be of limited value in the detailed investigations into the regulation of oocyte maturation. Gonadotropins induced complete meiotic maturation of oocytes in large (but not smaller) follicles of these cultures (Baker & Neal, 1972; Neal & Baker, 1973, 1974) and this maturation was reported to be similar to maturation of oocytes in vivo (Baker & Neal, 1972, Baker, 1979). Although this approach can be used for describing maturation in vitro, it resembles the in vivo model in that it is a multicellular system in which complex, intercellular interactions are difficult to interpret.

Like the organ culture, the culture of preovulatory

follicles provides an *in vitro* model in which the normal association between various follicular cell types and compartments are maintained. Follicle-enclosed oocytes are dependent upon hormonal stimulation to induce the resumption of meiosis. Meiotic maturation can be induced when follicles are cultured in the presence of gonadotropins (Tsafriri *et al.*, 1972; Neal & Baker, 1973; Lindner *et al.*, 1974; Hillensjo, 1976; Meinecke & Meinecke-Tillman, 1981), prostaglandins (Tsafriri *et al.*, 1972; Lindner *et al.*, 1974), dbcAMP (Tsafriri *et al.*, 1972; Lindner *et al.*, 1974), forskolin (Dekel & Sherizly, 1983), gonadotropin releasing hormone (GnRH) (Hillensjo & LeMaire, 1980, Ekholm *et al.*, 1981; Dekel *et al.*, 1983a), epidermal growth factor (Dekel & Sherizly, 1985) or tranforming growth factor-B (Feng *et al.*, 1988). Follicle-enclosed oocytes matured *in vitro* appear to resemble those maturing *in vivo* since they are fertilizable and developmentally competent. This system is ideal for studying the mechanisms underlying the hormonal induction of meiosis, i.e. the role of gonadotropins, steroids, cyclic AMP, prostaglandins, growth factors, protein synthesis and energy metabolism. Like the previous two systems, however, this model suffers the disadvantage of being multicellular and therefore has limitations for studies examining the site of gonadotropic action or localization of the response.

In 1935, Pincus & Enzmann demonstrated that germinal

vesicle stage rabbit oocytes will resume meiosis spontaneously when removed from antral follicles and cultured in hormone-free media. This study, with the support of a subsequent one by Chang (1955), led the way into one of the largest areas of study in the field of oocyte maturation: the elucidation of the mechanism by which follicular components maintain the intrafollicular oocyte in meiotic arrest (see Section 2.1.2 C). Most of the investigations using isolated mammalian oocytes attempt to determine the chronology of maturational stages in vitro or to identify the culture conditions optimal for successful maturation in various species. This latter goal has led to a tremendous diversity in the culture media and culture conditions employed, thus making interpretation and comparison of studies a difficult task.

The major advantage of culturing isolated oocytes is its relative simplicity in comparison with the other approaches. Only one or two cell types are involved, thereby alleviating much of the difficulty in interpretation of results posed by the more complex, multicellular systems. From ovaries of immature mice and rats, it is possible to obtain a large number of oocytes at the same developmental stage (Mangia & Canipari, 1977), facilitating large-scale, reliable studies. The most important drawback of this model is that it may not represent physiological events. Oocyte maturation in vivo is dependent on gonadotropic stimulation and therefore, unless special precautions are taken, it cannot be assumed

that hormone-independent spontaneous maturation accurately reflects the physiological processes of oocyte maturation.

Published accounts of studies in which isolated oocytes were matured *in vitro* vary greatly in their descriptions of optimal culture media and culture conditions. Most studies employ complex media containing amino acids, nucleic acid precursors, vitamins, biological fluids and various hormones (mouse: Cross & Brinster, 1970; hamster: Gwatkin & Haidri, 1973; rabbit: Thibault *et al.*, 1975; sheep: Moor & Trounson, 1977; cow: Newcomb *et al.*, 1978; rat: Shalgi *et al.*, 1979). The purpose of these experiments was to determine *in vitro* conditions capable of allowing normal fertilization and development of the oocytes after maturation. In contrast, some investigations sought to describe the minimal requirements for normal cytogenetic maturation leading to the secondary oocyte (pig: McGaughey, 1977a; rat: Fleming *et al.*, 1986) with moderate success. In all studies, the oocytes were able to undergo GVBD and polar body formation; however, the fertilizability and developmental competence appeared to be dependent on the presence of two key features in the culture systems: cumulus cells and serum.

As most of the experimental methods that will be presented in this thesis involves the culture of isolated oocytes, the remainder of this chapter predominantly will consider previous literature concerning the selection of oocytes, regulation of maturation, fertilizability and

developmental competence of isolated oocytes matured *in vitro*.

C Inhibitors Of Meiotic Maturation *in vitro*

The finding that mammalian oocytes removed from their follicles mature spontaneouly in culture led to the suggestion that within the follicle meiosis is prevented through follicular inhibitory actions (Pincus & Enzmann, 1935; Chang, 1955). Several factors have been implicated in the mechanism by which intrafollicular oocytes are maintained in meiotic arrest: steroid hormones, granulosa cells, follicular fluid, cAMP, purines, cumulus cells and a specific maturation-inhibiting factor. The following discussion will present the evidence that supports the involvement of these factors in meiotic inhibition.

Follicular fluid, granulosa cells, OMI and purines

The existence of a factor in follicular fluid which prevents oocytes from spontaneously maturing was first demonstrated with isolated rabbit oocytes cultured in media containing follicular fluid (Chang, 1955). Maturation-inhibiting activity has also been demonstrated by porcine (Tsafriri & Channing, 1975b; Jagiello *et al.*, 1977) and bovine follicular fluid (Gwatkin & Andersen, 1976). The effects of the follicular fluid inhibitor of oocyte maturation do not seem to be species-specific (Gwatkin & Andersen, 1976; Tsafriri *et al.*, 1977; Hillensjo *et al.*, 1978a; Chari *et al.*, 1983; Downs & Eppig, 1984). As

support for the existence of a physiologically relevant factor in follicular fluid, Channing et al. (1983) and Hillensjo et al. (1985) have reported that follicular fluid from preovulatory human follicles contains significantly less inhibitory activity than do follicles that contain immature oocytes. In addition rat oocytes show a decrease in sensitivity to the inhibitor during the course of follicular development (Tsafriri et al., 1977). Despite the variety of evidence available to support the existence of an inhibitory factor in follicular fluid, several other investigators have failed to confirm the maturation-inhibiting effect of porcine follicular fluid on bovine (Leibfried & First, 1980a), porcine (Leibfried & First, 1980b; Racowsky & McGaughey, 1982a) or rat oocytes (Fleming et al., 1983).

There is considerable evidence to suggest that the inhibitory activity in follicular fluid is a product of granulosa cells. Cocultures of porcine granulosa cells with oocytes result in a density-dependent inhibition of oocyte maturation (Tsafriri & Channing, 1975b) which is reversible by the addition of LH (Tsafriri et al., 1977). Since follicular fluid from large follicles contains less inhibitory activity (see above), it is not surprising that granulosa cells collected from small follicles inhibit meiosis more effectively than cells from medium or large follicles (Tsafriri & Channing, 1975b; Centola et al., 1981). Further studies also determined that cell contact

between the oocytes and granulosa cells is not necessary, as extracts from granulosa cells or granulosa cell-conditioned medium elicited similar inhibitory effects (Gwatkin & Andersen, 1976; Centola et al., 1981; Sato & Koide, 1984; Andersen et al., 1985). The addition of FSH or prolactin to cultures of porcine granulosa cells facilitates the accumulation of the inhibitory factor in the medium, whereas androgens reduce it (Channing et al., 1982; Andersen et al., 1985). Although there is much evidence to indicate that the inhibitory factor is a granulosa cell product, numerous other studies have demonstrated that granulosa cells were ineffective in inhibiting the maturation of oocytes from the pig (Jagiello et al., 1977; Leibfried & First, 1980a; Rice & McGaughey, 1980), cow (Jagiello et al., 1977; Leibfried & First, 1980a), sheep (Jagiello et al., 1977) or mouse (Nekola & Smith, 1974).

The results of coculture of oocytes with various follicular components corroborated the concept of follicular inhibition of meiotic maturation and led to the partial characterization and purification of a polypeptide, the oocyte maturation inhibitor (OMI) (Tsafriri & Channing, 1975b; Tsafriri et al., 1976; Stone et al., 1978; reviewed by Tsafriri, 1988). OMI apparently exerts its inhibitory action via cumulus cells since it prevents the maturation of cumulus cell-enclosed oocytes, but has no effect on the maturation of denuded oocytes (Hillensjo et al., 1979) and it has been suggested that OMI passes through the gap

junctions between the cumulus cells and the oocyte to prevent the maturation of the oocyte (Tsafriri, 1985). A physiological role for OMI *in vivo* has not been demonstrated and this factor requires further purification and identification before the necessary studies can be pursued.

OMI has not been the only inhibitory factor identified in follicular fluid. Recent evidence suggests that hypoxanthine, adenosine and/or guanosine are components of porcine follicular fluid that prevent the spontaneous maturation of murine oocytes *in vitro* (Down *et al.*, 1985; Eppig *et al.*, 1985).

Oocyte-cumulus cell communication

The presence of gap junctions between the cumulus cell projections and the oocytes results in bidirectional metabolic and ionic coupling. During maturation the cumulus cells surrounding the oocyte become more loosely organized and the cytoplasmic projections withdraw from the oocyte, thereby decreasing the number of gap junctions and reducing the degree of intercellular coupling (see Section 2.1.2 A). These observations led to the hypothesis that, in response to LH, the release of the oocyte from follicular suppression of meiosis is the result of the uncoupling of the cumulus cells from the oocyte, thereby preventing the transfer of an inhibitory factor from the cumulus cells to the oocyte (Lindner *et al.*, 1974; Dekel & Beers, 1978, 1980; Gilula *et al.*, 1978; Moor *et al.*,

1980a). Several studies have produced results that argue against this hypothesis. The timing of the disruption of the cumulus-oocyte coupling has been found to be inconsistent with this model of gap junctional regulation of oocyte maturation. Studies in which the transport of choline or uridine (Moor et al., 1980a; Eppig, 1982a) or ionic coupling (Dekel & Beers, 1980) was measured indicate that the disruption of oocyte-cumulus cell communication temporally follows rather than precedes GVBD. Another study demonstrated that low concentrations of FSH suppress intercellular coupling but do not induce resumption of meiosis in follicle-enclosed ovine oocytes, whereas LH induces resumption of meiosis but not disruption of coupling (Moor et al., 1981). This dissociation between GVBD and oocyte-cumulus uncoupling casts doubt on the hypothesis that the transfer of an inhibitory factor from the cumulus cells to the oocyte is reduced by the uncoupling mechanism, allowing meiosis to resume. It is possible, however, that oocyte maturation occurs because of a quantitative or qualitative change in the inhibitory signal communicated from the cumulus cells to the oocyte (Moor et al., 1981).

Since the transfer of amino acids (Colonna & Mangia, 1983) and of uridine (Salustri & Siracusa, 1983) to the oocyte increases in direct proportion to their uptake by cumulus cells, more recent studies have expressed oocyte uptake as a fraction of total cumulus uptake (as opposed to

the absolute values of label incorporated into treated versus control oocytes reported in earlier studies). When measuring relative uptake, Salustri & Siracusa (1983) noted a temporal correlation between the uncoupling in mouse oocyte-cumulus complexes and the resumption of meiosis. In addition to this correlation, Dekel et al. (1984), Dekel & Sherizly (1985) and Racowsky & Satterlie (1985) reported that metabolic uncoupling preceded GVBD in rat and hamster oocytes both in vivo and in vitro, thereby adding support for the original hypothesis. The evidence provided by these most recent studies suggests that the reduction in coupling between the cumulus cells and the oocyte may serve to signal the resumption of meiosis in the oocyte; however, it has yet to be demonstrated that the temporal relationship between these two events is not merely a result of a common trigger.

Cyclic AMP

Although the mechanisms that maintain meiotic arrest in vivo remain to be elucidated, it is generally accepted that cAMP plays a central role in this process (reviewed by Masui & Clarke, 1979; Eppig & Downs, 1984; Schultz, 1987). Neither the origin of the cAMP nor its mode of action has been clearly established. The first evidence for a possible regulatory role for cAMP came from experiments showing that dbcAMP injected into cultured rat follicles induces oocyte maturation (Tsafriri et al., 1972). Similarly, many of the agents capable of inducing

maturation of follicle-enclosed oocytes *in vitro* also stimulate the production of cAMP (Tsafriri *et al.*, 1972; Lindner *et al.*, 1974). In contrast, the LH-stimulated resumption of meiosis in follicle-enclosed oocytes and the spontaneous maturation of isolated oocytes are prevented by the continuous presence of cAMP derivatives or inhibitors of phosphodiesterase (Cho *et al.*, 1974; Lindner *et al.*, 1974; Dekel & Beers, 1978; Hillensjo *et al.*, 1978b, Rice & McGaughey, 1981). The addition of the adenylate cyclase activators cholera enterotoxin (Dekel & Beers, 1980) or forskolin (Dekel *et al.*, 1983b) inhibits the spontaneous maturation of rat oocytes cultured within their cumulus but not in denuded oocytes. A similar result has been reported by Eppig *et al.* (1983) who showed that suboptimal concentrations of dbcAMP produce a greater inhibitory effect on cumulus-intact oocytes than on denuded oocytes. Gonadotropins reverse the inhibitory action of dbcAMP on the maturation of cumulus-intact rat oocytes but have no effect on the dbcAMP inhibition of denuded oocytes (Dekel & Beers, 1978, 1980). These results, together with the knowledge that there are no LH receptors on the oocyte (Amsterdam *et al.*, 1975), suggest that the cAMP analogs stimulate the cumulus cells to suppress oocyte maturation by either the transfer of cAMP to the oocyte to maintain high levels there or perhaps by the generation and/or activation of a maturation-inhibiting factor that is transferred to the oocyte. To support a direct role for cAMP, recent studies have shown that exposure of oocyte-

cumulus complexes to agents that elevate the cumulus cell cAMP levels also increase the intraoocyte cAMP levels relative to denuded oocytes similarly exposed (Racowsky, 1984, 1985a; Bornslaeger & Schultz, 1985).

These above studies clearly establish a central role of oocyte cAMP in the maintenance of meiotic arrest within the follicle. This led to the hypothesis that maturation is initiated by a fall in intra-oocyte cAMP and, indeed, a decrease in cAMP levels has been found to occur prior to GVBD in mouse oocytes (Schultz et al., 1983a). This observation lends support to the idea that the LH surge induces the disruption of intercellular communication between the cumulus cells and the oocyte, thereby leading to oocyte maturation (Dekel et al., 1981). Such a reduction in coupling could result in the maturation-associated decrease in oocyte cAMP while overall follicle cell cAMP levels are increasing in response to the LH surge. It remains to be determined whether the intra-oocyte cAMP has direct effects in the oocyte or whether the levels of this compound are merely a reflection of the influx of another substance of cumulus cell origin, the decreased levels of which allow GVBD to proceed.

Steroids

Steroids have also been implicated in the intrafollicular maintenance of meiotic arrest. As described above, it is generally accepted that cAMP plays

a central role in the mechanisms that maintain meiotic arrest and steroids have been tested as participants in these mechanisms. Androgens have been reported to augment the maturation-inhibiting action of dibutyryl cyclic AMP (dbcAMP) on porcine oocytes (Rice & McGaughey, 1981, Racowsky, 1983; Daniel et al., 1986). Estradiol can directly arrest the maturation of pig (McGaughey, 1977b, Racowsky & McGaughey, 1982b) and hamster oocytes (Racowsky, 1985b); however, the inhibitory effect of estradiol was expressed only in the absence of exogenous protein, an unphysiological state (Racowsky & McGaughey, 1982c). In the presence of forskolin and estradiol, the maturation of denuded and cumulus intact oocytes is inhibited (Racowsky, 1985b). The maturation-inhibiting action of FSH, forskolin, cholera toxin or suboptimal concentrations of dbcAMP on denuded or cumulus intact mouse oocytes is augmented by progesterone or testosterone (Eppig et al., 1983; Schultz et al., 1983b). It has been postulated that the synergistic interaction between the steroids and cAMP may be due to a steroid hormone-induced increase in the activity of the regulatory subunit of cAMP-dependent protein kinase (Liu et al., 1981). However, the role of steroids in the maintenance of meiotic arrest requires further study, particularly to determine how the preovulatory surge of gonadotropins, while causing a brief rise in follicular steroidogenesis, can at the same time induce the resumption of meiosis (reviewed by Tsafriri, 1985).

D Stimulation of Maturation

While there are clear differences between the control of maturation in mammals and lower vertebrates, there are indications that these groups may share some fundamental mechanisms. A cytoplasmic factor known as maturation-promoting factor (MPF) appears in starfish (Kishimoto & Kanatani, 1976) and amphibian oocytes (Masui & Markert, 1971) undergoing GVBD and a similar factor present in maturing mouse oocytes is capable of inducing meiosis in immature oocytes (Balakier, 1978; Sorensen et al.,1985).

In the starfish, follicle cells produce a maturation-inducing substance, identified as 1-methyladenine (1-MA) (Kanatani et al, 1969). 1-MA then acts on the plasma membrane of the oocyte (Kanatani & Hiramoto, 1970; Doree & Guerrier, 1975) to induce the appearance of the cytoplasmic MPF which is the direct trigger for GVBD (Kishimoto & Kanatani, 1976). In amphibians, progesterone is the stimulus for MPF production (Masui & Markert, 1971). Enucleated oocytes produce much less MPF than intact oocytes; some germinal vesicle material is thus required for MPF production (Kishimoto et al., 1981). In most species the germinal vesicle must break down and release its contents into the cytoplasm before the oocyte acquires the capacity to induce sperm pronuclear formation. It has recently been shown that mechanical breakdown of the germinal vesicle, in the absence of any hormone treatment, induces complete cytoplasmic maturity in starfish (Guerrier

et al., 1983). The role of MPF in this species, then appears to be simply the induction of GVBD.

Since the cytoplasmic control of nuclear behavior is well documented for both mitotic and meiotic mammalian cells (Gurdon & Woodland, 1968; Johnson & Rao, 1971; Gerhart et al., 1984), it seems likely that the control of nuclear progression during maturation of the mammalian oocyte is, at least in part, regulated by cytoplasmic factors. Evidence to support this suggestion comes from a variety of studies. Fusion of fully grown, meiotically mature mouse oocytes with meiotically incompetent oocytes results in GVBD of the nucleus of the incompetent oocyte (Balakier, 1978). In another study, cytoplasmic extracts of meiotically mature mouse oocytes injected into immature X. laevis (Sorensen et al., 1985) or Asterina pectinifera oocytes (Kishimoto et al., 1984) causes GVBD within 2 hours, in the absence of progesterone or 1-MA, respectively. In addition, cytoplasm obtained from oocytes inhibited from resuming maturation does not elicit GVBD in these cases (Sorensen et al., 1985). In contrast to non-mammalian species, however, the mammalian MPF appears to be generated in the cytoplasm in a manner independent of nuclear control (Balakier & Czolowska, 1977). Enucleate fragments of fully grown but immature oocytes gain the ability to cause chromosome condensation in inactive cells (after fusion) during the same period of time required for the nucleate fragments to undergo GVBD. The results of all these experiments suggest that mammalian oocytes generate

cytoplasmic MPF activity during the course of maturation.

Immature mammalian oocytes have the capability to undergo GVBD spontaneously when removed from follicular influences; however, it appears that GVBD induced in this manner is insufficient for cytoplasmic maturity as indicated by the low incidence of sperm pronuclear formation during fertilization (See section 2.1.2 A). The putative MPF may therefore play a more complex role in the maturation of the mammalian oocytes, one that involves the regulation of cytoplasmic maturation. In this respect, the timing of acquisition of cytoplasmic maturity may be relevant. In starfish, the oocyte cytoplasm develops this capacity soon after GVBD, with maximal levels of MPF 20-40 min. after exposure to 1-MA (Meijer & Guerrier, 1984). In mammals, cytoplasmic maturity (as measured by the ability to form the male pronucleus after fertilization) develops gradually during maturation, requiring 8-10 hours to be complete (Niwa & Chang, 1975a). These results indicate that the action of MPF may involve more than the induction of GVBD in mammalian oocytes. Alternatively, another factor or combination of factors may be required to attain complete cytoplasmic maturation.

Maturation of the oocyte is stimulated physiologically by LH but can be induced effectively in follicle-enclosed oocytes *in vitro* by GnRH (Hillensjo & LeMaire, 1980; Ekholm *et al.*, 1981; Dekel *et al.*, 1983a), forskolin (Dekel & Sherizly, 1983), epidermal growth factor (Dekel &

Sherizly, 1985), transforming growth factor-B (Feng et al., 1988) as well as gonadotropins (Lindner et al., 1974). Beyond the investigations into the various inducers of oocyte maturation, however, the mechanism for this induction has yet to be fully determined. A recent study by Downs et al (1988) demonstrated that FSH and EGF induce GVBD in isolated oocytes arrested with dbcAMP, isobutylmethylxanthine (a phosphodiesterase inhibitor), guanosine or hypoxanthine plus adenosine. Brief exposure of the oocytes to a high concentration of dbcAMP also induces GVBD in oocytes maintained in meiotic arrest. Since both FSH and EGF stimulate cAMP production in oocyte-cumulus complexes, the authors suggested that, in response to hormone treatment, the cumulus cells generate a positive signal that acts upon the oocytes to stimulate GVBD. GVBD is stimulated by acute, but not chronic, exposure of cultured follicles to dbcAMP (Hillensjo et al., 1978; Dekel et al., 1981), which suggests that oocyte maturation is modulated according to the duration and magnitude of elevated cAMP levels.

From other studies, evidence has been provided for a mechanism of oocyte maturation with a different role for cAMP. It has recently been shown that phospholipase C and direct activation of protein kinase C can mimic the effects of GnRH on rat oocytes (Aberdam & Dekel, 1985). The results suggest that the GnRH-induced meiotic maturation of rat oocytes is mediated by the phospholipid-dependent protein kinase C. Protein kinase C is not independent of

cAMP but rather can be inhibited by the presence of this cyclic nucleotide (Nishizuka et al., 1984). Since many studies have established that cAMP inhibits the resumption of meiosis in mammalian oocytes (Cho et al., 1974; Dekel & Beers, 1978; Ball et al., 1983), it is possible that activation of phospholipid turnover and protein kinase C by GnRH may reverse that negative control exerted by cAMP on the oocyte leading to resumption of meiosis. This process would be particularly effective if the cellular levels of cAMP were to decrease at the same time, as demonstrated by Schultz et al. (1983a). It has yet to be determined if LH-stimulated meiotic maturation is mediated by the same mechanism.

A study by Powers & Paleos (1982) has shown the effect of a calcium ionophore (A23187) and 2 inhibitors of transmembrane calcium transport (verapamil and tetracaine) on denuded mouse oocytes incubated in dbcAMP-supplemented media. An increase in the external calcium concentration or addition of the calcium ionophore decrease the inhibitory effect of dbcAMP on GVBD. Furthermore, it was found that verapamil or tetracaine (which would reduce calcium influx) can potentiate the inhibitory effect of suboptimal concentrations of dbcAMP on GVBD. These results suggest that there may be an interaction between cAMP levels and intracellular calcium levels such that raising the intracellular concentration of free calcium may cause oocyte maturation. This work supports the mechanism

described above. Clearly, a spatial and temporal analysis of intracellular free calcium concentrations during oocyte maturation is necessary to elucidate further the role of this ion in the maturation process.

The ability of gonadotropins to alter the physiological environment of the follicle is sufficent to allow the oocyte to resume meiosis and there is much evidence to support the view that follicular steroids play a significant role in the maturation of oocytes. The role of steroids in the mediation of the gonadotropic stimulus for the resumption of meiosis in lower vertebrates (amphibians and fish) is well established. As in mammals, gonadotropins act on ovarian follicles in Rana pipiens to induce maturation of the enclosed oocytes (Heilbrunn et al., 1939); however, progesterone added to incubated follicles will also induce oocyte maturation (Schuetz, 1967a). Follicle-free oocytes undergo maturation in response to progesterone (Masui, 1967; Schuetz, 1967b; Smith et al., 1968) or to gonadotropins, when they are incubated with follicle cells (Masui, 1967). In addition, gonadotropin treatment has been shown to induce follicles to convert pregnenolone to progesterone (Fortune et al., 1975; Thibier-Fouchet et al., 1976). Although progesterone is the most potent inducer of maturation in R. pipiens and Xenopus laevis, other steroids such as testosterone and deoxycorticosterone can be effective (Schuetz, 1967b; Smith et al., 1968; Schorderet-Slatkine, 1972; Jacobelli et al., 1974).

Evidence for a physiological role for steroid hormones in the regulation of mammalian oocyte maturation is much less consistent. Resumption of meiosis can be triggered in rat follicles explanted on the day of proestrus by LH, human chorionic gonadotropin (hCG), immunochemically pure FSH, or prostaglandin E_2. All of these substances induce an immediate rise in cAMP accumulation in granulosa cells and follicular fluid and a somewhat later increase in progesterone synthesis (Tsafriri et al., 1972, 197[illegible]; Lindner et al., 1974, 1977). Similar results were obtained by the culture of preovulatory follicles from immature PMSG-treated rats (Hillensjo, 1976). In the rat, cumulus cells isolated from preovulatory follicles after the LH surge have enhanced production of progesterone in culture compared to those isolated before the surge (Hillensjo et al., 1981). The post-LH rise in progesterone could indicate a possible role for progesterone in mediating the action of LH on oocyte maturation. Progesterone has been shown to facilitate the maturation of rabbit oocytes (Bae & Foote, 1975); however, there was no effect observed on the maturation of human (Shea et al., 1975), porcine (McGaughey, 1977b) or mouse (Eppig & Koide, 1978) isolated oocytes.

Addition of steroids to the culture medium did not induce the maturation of bovine, porcine (Foote & Thibault, 1969) or rat (Tsafriri et al., 1972) follicle-enclosed oocytes. Suppression of steroidogenesis by the inhibitors

cyanoketone or aminoglutethimide did not affect meiotic maturation of follicle-enclosed oocytes induced to mature with LH (Lieberman et al., 1976). In contrast, the normal maturation of follicle-enclosed sheep oocytes appears to be dependent upon the presence of estradiol in the culture medium (Moor & Trounson, 1977; Moor et al., 1980b). In studies using isolated oocytes, exogenous estradiol and progesterone were unable to alter the maturation of mouse oocytes in vitro (Eppig & Koide, 1978, Richter & McGaughey, 1979; Smith & Tenney, 1980). However, porcine oocytes cultured in media containing both progesterone and estradiol showed a higher incidence of normal haploidy than did control oocytes (McGaughey, 1977b). In the human, higher follicular fluid estradiol levels correlated well with successful fertilization and an enhanced cleavage rate of oocytes associated with pregnancy following in vitro fertilization (Botero-Ruiz et al., 1984). Thus, although steroids may affect the maturation of oocytes in some species, their specific action(s) during this process is still unclear.

E Fertilization and Developmental Capabilities

The fertilizability and developmental capabilities of oocytes matured in vitro are largely dependent on the conditions of culture. Under certain conditions, isolated maturing oocytes may exhibit chromosomal abnormalities (McGaughey & Polge, 1971) or ultrastructural abnormalities

(Thompson et al., 1971; Zamboni, 1971) that may influence further development. In addition numerous investigators have observed abnormal pronuclear formation possibly resulting from inadequate cytoplasmic maturation in oocytes matured in vitro (see Section 2.1.2 A). These studies suggest that the spontaneous maturation of isolated oocytes in vitro may be an anomalous, non-physiological process. In contrast, however, a review of all studies in which in vitro matured oocytes have been normally fertilized and found capable of normal embryonic and fetal development leads one to the inescapable conclusion that this success is dependent upon the addition of serum to the culture medium.

Serum appears to improve the fertilizability of the oocytes compared with those matured in media supplemented with bovine serum albumin (BSA). Although Niwa & Chang (1975a) and Fleming et al., 1986) reported fertilization of rat oocytes following maturation in BSA-supplemented media, only 18-32% of the oocytes were penetrated by spermatozoa, few cleaved to normal 2-cell embryos and only 2.3% developed to viable fetuses following transfer into recipient uteri. A possible beneficial effect of serum has not been examined in this species; however., Schroeder & Eppig (1984), Leibfried-Rutledge et al. (1986) and Choi et al. (1987) have shown the importance of serum in the maturation media in maintaining the fertilizability and developmental competence of mouse, cow and hamster oocytes. These studies report no abnormalities in pronuclear

development and the developmental capability of a proportion of the fertilized oocytes, thereby suggesting that cytoplasmic maturation was not deficient in all oocytes. The mechanism by which serum has a positive effect on cytoplasmic maturation requires further study.

Few studies have examined the factors that may influence the fertilizability and developmental competence of *in vitro* matured rat oocytes, primarily because attempts at *in vitro* fertilization of rat oocytes has led to somewhat inconsistent results by various investigators. However, the effect of LH on the fertilizability of *in vitro* matured rat oocytes has been studied using *in vivo* (Shalgi *et al.*, 1979) and *in vitro* fertilization (Fleming *et al.*, 1986). Unfortunately the results of these two studies conflict. Oocytes matured *in vitro* in the presence of LH and fertilized *in vivo* showed a 3-fold increase in fertilizability compared to non-stimulated oocytes. In contrast, there was no detectable difference in the *in vitro* fertilization rate as a result of exposure of isolated oocytes to hCG. Clearly the influence of other factors in these studies confound any attempt at comparison.

2.2 TECHNIQUES FOR FERTILIZATION *IN VITRO*

The maturation, capacitation and activation of the male gamete and the union of the male and female gametes for fertilization is a complex series of events whose details are beyond the scope of this discussion. However, a brief summary of the process of fertilization as it occurs *in vivo* is necessary to understand the description of *in vitro* fertilization techniques in later sections.

Following ejaculation, spermatozoa require a period of time in the female genital tract to undergo some physiological/functional changes (capacitation) which render them capable of fertilization. There has been much speculation about the conditions or factors directly controlling capacitation within the female genital tract, and *in vitro* studies have identified changes in adenylate cyclase/protein kinase activity, metabolism, intracellular ions and the plasma membrane (reviewed by Yanagimachi, 1988). However, it is not certain which of these represents changes necessary for capacitation.

The acrosome is a membrane-bound, cap-like structure covering the anterior portion of the sperm nucleus. Although it contains numerous enzymes, hyaluronidase and acrosin appear to be particularly important for sperm function in fertilization. Hyaluronidase and acrosin are believed to assist in the digestion of the extracellular cumulus matrix of hyaluronic acid which surrounds the oocyte and in the passage of the spermatozoa through the

zona pellucida of the oocyte. In addition the acrosome reaction renders the spermatozoa capable of fusing with the plasma membrane of the oocyte. There is some evidence to suggest that components of the cumulus may promote the acrosome reaction of mammalian spermatozoa (Bavister, 1982; Tesarik, 1985; Westrick et al., 1985), while the ability of the zona pellucida to induce the reaction is clear (reviewed by Meizel, 1985).

Acrosome-reacted spermatozoa probably use both mechanical and enzymatic means in passing through the zona pellucida (reviewed by Yanagimachi, 1988). Acrosomal enzymes appear to alter the zona structure such that vigorous tail movement can force the spermatozoon through this layer. Once through the zona pellucida, the equatorial membrane of the sperm head fuses with the oolemma. The sperm-egg fusion is completed by the gradual incorporation of first the sperm head and then the entire tail (reviewed by Gaddum-Rosse, 1985).

In response to the fusion with the spermatozoon, the oocyte undergoes activation, a process characterized primarily by the exocytosis of cortical granules and the resumption of meiosis (for the second time). Cortical granules are small, membrane-bound vesicles containing hydrolytic enzymes and saccharide components (Gulyas, 1980). The contents of these granules, when released by exocytosis during oocyte activation, alter the physical and chemical characteristics of the zona pellucida such that the zona becomes impenetrable by other spermatozoa. This

process, called the zona reaction, is largely responsible for the block to polyspermy in such species as hamsters, dogs, sheep and humans (Austin & Braden, 1956; Barros & Yanagimachi, 1972; Wolf, 1981; Sathananthan & Trounson, 1985). An alternative mechanism to block polyspermy is that presented by the oocyte plasma membrane (Austin & Braden, 1956; Wolf, 1981). Although this block is an effective means of rejecting excess spermatozoa in the rabbit and mole, the nature and mechanism of its action are poorly understood. Numerous species, including the rat, mouse, cat and guinea pig, appear to benefit from the presence of a block at both the zona pellucida and the plasma membrane of the oocyte.

During the process of meiotic maturation within the follicle, the nucleus of the oocyte undergoes GVBD and extrusion of the first polar body. At ovulation the secondary oocyte, arrested at metaphase of the second meiotic division, is released. At the time of sperm-egg fusion, meiosis is resumed again, with the resulting haploid nucleus being transformed into the female pronucleus. Meanwhile the nuclear envelope of the sperm head disintegrates and the sperm nucleus decondenses and is transformed into the male pronucleus. It is interesting to note that the sperm nuclei decondense only very slightly without the presence of germinal vesicle material in the oocyte cytoplasm (Balakier & Tarkowski, 1980; Usui & Yanagimachi, 1976). Thus GVBD apparently prepares the

oocyte for fertilization by providing a cytoplasmic factor needed for decondensation of the sperm nucleus. In comparison, an additional cytoplasmic factor (male pronucleus growth factor) appears to be essential for the formation of the male pronucleus (Thibault & Gerard, 1973; Yanagimachi, 1981), but may appear in the ooplasm independently of the breakdown of the germinal vesicle (Iwamatsu & Ohta, 1980). Both the sperm nucleus decondensing factor and the male pronucleus growth factor apparently fail to appear within the oocyte cytoplasm when oocytes are matured in vitro under suboptimal conditions (Usui & Yanagimachi, 1976; Masui & Clarke, 1979).

In the final stages of fertilization, the fully developed male and female pronuclei migrate towards the center of the zygote, their nuclear envelopes disintegrate and their chromosomes intermix. Thus, the resumption of meiosis that occurs during oocyte activation has initiated a series of nuclear events that culminates in the combination of the male and female chromosomes for the first mitotic division, signalling the end of fertilization and the beginning of embryonic development.

2.2.1 In the Human

The birth of Louise Brown in July, 1978 was the first case in which a human oocyte was recovered from a woman's ovary, fertilized in vitro, developed in culture and implanted into the uterine lumen where it developed to term

(Steptoe & Edwards, 1978). Although human oocytes had been fertilized *in vitro* a decade earlier (Edwards *et al.*, 1969), it took the birth of a "test tube" baby to bring attention to the technique. The procedure immediately became recognized as one of the most advanced, and ethically complicated, techniques for the treatment of infertility.

Human oocytes have been collected by various aspiration techniques during laparoscopy, laparotomy or with the aid of ultrasonic methods. Since the number of oocytes recovered in cycling women is fairly low, administration of gonadotropins has been used to stimulate follicular growth and oocyte maturation (reviewed by Soupart, 1981). Up to 14 oocytes per patient were obtained by stimulation with FSH (Jagiello *et al.*, 1968). Similar techniques were introduced later using other superovulatory agents: human menopausal gonadotropin (Steptoe & Edwards, 1970), human pituitary gonadotropin (Talbot *et al.*, 1976) and clomiphene citrate (Lopata *et al.*, 1978). As efforts are made to recover all the oocytes from follicles of various sizes, the stage of development of these oocytes is uncertain, as are their normality and capabilities for future development. The success of *in vitro* fertilization thus depends on the collection of oocytes at the right stage and/or the improvement in the culture methods for the proper maturation of immature oocytes.

Reports on the success of *in vitro* fertilization and embryo transfer are often difficult to evaluate and even

more difficult to compare as the end-points vary greatly. Criteria for the success of *in vitro* fertilization have included the presence of a sperm tail in the vitellus, the presence of male and female pronuclei, the presence of two polar bodies or cleavage of the zygote. None of the criteria alone is sufficient to assess fertilization accurately since rapid sperm tail digestion, spontaneous or parthenogenetic activation, abnormal fertilization or fragmentation can confound the evaluation. Fertilization and cleavage normally occur within the oviduct where they cannot be visualized directly. Therefore identification of the normality of *in vitro* fertilized and cleaving human embryos becomes a critical problem.

The evaluation of the proportions of oocytes/embryos giving pregnancies following *in vitro* fertilization can be as complicated as the assessment of fertilization. Investigators publish pregnancy rates in a variety of ways, i.e. per treatment cycle, per laparoscopy/ultrasound or per embryo transfer. Results reported from two well-established *in vitro* fertiliztion-embryo transfer programs indicate that both have a pregnancy rate of less than 20% per treatment cycle (Jones *et al.*, 1984; Wood *et al.*, 1985). A review summarizing the pregnancy rates of several programs in the United States, Europe and Australia found an average pregnancy rate per laparoscopy of 8% (Grobstein *et al.*, 1983). A major problem for the successful development of *in vitro* fertilized oocytes following

placement in the uterus may be the procedure used to collect the oocytes. The stimulation of a large number of follicles alters the maternal endocrine environment which may affect embryo development in a number of ways: 1) by causing degeneration of the embryo due to asynchronous development of the embryo and the uterus (Chang, 1950; Dickmann & Noyes, 1960; El-Badrawi & Hafez, 1982), 2) by causing expulsion of embryos from a non-receptive uterus (Adams, 1980), 3) by failure of the transferred embryos to develop sufficiently to stimulate the maternal system for recognition and maintenance of pregnancy, or 4) by improper formation and development of the corpus luteum after aspiration from the follicles. Most of these problems might possibly be overcome by freezing the embryos and delaying transfer to a non-stimulated cycle. Testart (1987) has shown a high pregnancy rate when transfers were performed with frozen-thawed embryos coming from in vitro fertilization cycles in which pregnancy did not occur after transfer of fresh embryos. Although this study has yet to be confirmed, it is a promising means of dealing with the tentative problems associated with stimulated cycles.

It is clear that there is much room for improvement at all stages of the in vitro fertilization-embryo transfer process. In view of its importance for practical animal breeding and the high demand for clinical application, considerable research effort has been given to the various aspects of the techniques in other species. As knowledge of the natural processes accumulates, an increase in the

pregnancy rate following transfer of *in vitro* fertilized mammalian oocytes can be expected.

2.2.2 In other mammals

The clinical application of the *in vitro* fertilization-embryo transfer techniques is a culmination of two decades of research on similar techniques in other species. Chang (1959) was the first to obtain live offspring following the transfer of rabbit oocytes fertilized *in vitro* by capacitated sperm. Successful *in vitro* fertilization has also been achieved in the hamster (Yanagimachi & Chang, 1963), mouse (Iwamatsu & Chang, 1969), cat (Hamner *et al.*, 1970), guinea pig (Yanagimachi, 1972), squirrel monkey (Gould *et al.*, 1973), rat (Miyamoto & Chang, 1973a), dog (Mahi and Yanagimachi, 1976), cow (Iritani & Niwa, 1977) and pig (Iritani *et al.*, 1978). As *in vitro* fertilization of mouse and hamster oocytes is relatively easy to achieve, a variety of culture systems have been developed by different investigators (reviewed by Rogers, 1978) and the most extensive studies involving *in vitro* fertilization have been done with these species.

Although the rat is one of the most commonly used animals in laboratory studies in reproductive biology and endocrinology, the fertilization of rat oocytes *in vitro* was apparently more difficult to achieve, being carried out successfully only after similar success with several other species, including humans. Early studies in rats required

that the zona pellucida be dissolved by chymotrypsin before *in vitro* fertilization (15 to 53%) could be achieved (Toyoda & Chang, 1968). Successful capacitation of rat spermatozoa *in vitro* followed by *in vitro* fertilization was first reported by Toyoda & Chang (1974a). Until 1984, all published reports of successful *in vitro* fertilization in the rat had come from this same group of investigators with the exception of one abstract published in 1980. Pfeifer *et al.* (1980) claimed success at *in vitro* fertilization in the rat without assessing fertilization directly. Their success was based on cleavage of oocytes that had been exposed 2 days earlier to sperm for 6h. Because unfertilized oocytes will often undergo segmentation in a manner similar to that of normal, fertilized oocytes *in vivo* (Pincus, 1931; Blandau, 1980), cleavage is probably the least reliable evidence of fertilization.

Blandau (1980) has noted the frequent lack of reproducibility of results in experiments using in vitro fertilization. Quigley (1980) attempted duplication of the procedures of Toyoda & Chang (1974a), but did not achieve fertilization. Even after extensive studies involving variations of many components of the media and several changes in methodology, none of the rat oocytes underwent fertilization. Later Quigley (1982) published a critical analysis of 16 papers published by the only group achieving successful rat *in vitro* fertilization between 1973 and

1978. A comparison of the sperm penetration rates in the control groups of the various studies revealed a range of 15 to 100%. Presuming that the media, techniques and culture conditions of the investigators in this group are similar, it is clear that the optimal culture conditions for *in vitro* fertilization of rat oocytes had not been fully identified.

Following the reports of inconsistent or irreproducible results (Shalgi *et al.*, 1979; Quigley, 1980, 1982), successful *in vitro* fertilization in the rat was finally described by two other groups (Kaplan & Kraicer, 1978; Evans & Armstrong, 1984). Both groups used modifications of the methodology described by Toyoda and Chang (1974a) and have achieved similar fertilization rates (77% - Evans & Armstrong, 1984; 81% - Shalgi, 1984).

One of the most important considerations in the development of successful *in vitro* fertilization procedures has been the identification of the media components essential for sperm capacitation and fertilization. Early experiments used follicular fluid (Gwatkin & Andersen, 1969; Yanagimachi, 1969a,b), blood sera (Barros & Garavagno, 1970; Yanagimachi, 1970) or tubal fluid (Barros & Austin, 1967; Iwamatsu & Chang, 1972) to supplement the media. More chemically defined media were described by Bavister (1969), Toyoda *et al.* (1971), Miyamoto & Chang (1973b) and Davis (1976) in which the importance of serum albumin and metabolic intermediates was emphasized. They concluded that serum albumin, lactate and pyruvate could

replace tissue fluids in the induction of sperm capacitation and the acrosome reaction for *in vitro* fertilization. A later study (Tsunoda & Chang, 1975a) revealed that cumulus-intact oocytes could be fertilized in the absence of pyruvate, substantiating an earlier observation that the cumulus cells produced pyruvate (Donahue & Stern, 1968). Investigations of various sugars by Niwa & Iritani (1978) indicated that D-glucose was most effective in permitting fertilization.

Further investigations into the conditions that permit fertilization included the determination of optimal media concentration of calcium (Miyamoto & Ishibashi, 1975), sodium (Niwa & Chang, 1975b) and the potassium/sodium ratio (Toyoda & Chang, 1974b). Maximal rates of fertilization in the rat could be obtained with a pH range of 7.5-7.8, a more restricted range of pH than for the hamster (6.8-8.2) (Miyamoto *et al.*, 1974). However, the pH in these experiments was not determined or controlled during the incubation period so that the optimal pH during continued culture has not been determined. In the mouse and hamster systems (Miyamoto & Chang, 1973c), the ideal range of osmolarity for fertilization was between 310 and 390 mOsmoles (mOsm)/l with low fertiliztaion rates at osmolarities greater than 410 mOsm/l or less than 250 mOsm/l. Niwa & Chang (1975b) demonstrated that the proportion of rat oocytes fertilized was the same when osmolarity was increased from 309 to 357 or 397 mOsm/l,

indicating that rat oocytes are less sensitive to osmolarity than to pH.

Additional variables affecting in vitro fertilization of rat oocytes were reported by Niwa & Chang (1974a). Supplemental CO_2 (5% CO_2 in air) gave a higher proportion of oocytes penetrated (77%) than air alone (24%). When the size of the incubation drops was altered, the percentage of oocytes undergoing fertilization in volumes of 5, 10, 50, 100 and 400 ul was 6, 13, 47, 86 and 78% respectively. These authors also performed studies in which oocytes, in the presence or absence of their cumulus cells, were exposed to capacitated sperm. There was no change in the fertilization rate of oocytes if their surrounding cumulus cells were removed.

It is clear that various factors play important roles in achieving in vitro fertilization in the rat. The inconsistent and often irreproducible results, however, indicate that the optimal conditions need to be defined more completely. In addition, there are significant intangible factors (described by Quigley, 1982) that seem to be overcome by experience and technical expertise. Comments such as these have come from the authors themselves:

> According to our experience, the rat sperm are very sensitive to environmental changes such as temperature, dilution and other unknown factors (Niwa & Chang, 1974b).
>
> Since, however, the rates of fertilization in the early trials were always lower than in later trials the

experience of the researcher to conduct such an experiment must also be of importance (Niwa & Chang, 1973).

The improved fertilization rate for oocytes of rats in the present study is unlikely to be due to strain difference and is most probably related to technical improvement (Niwa et al., 1976).

2.2.3 Sperm preparation

Mammalian spermatozoa undergo morphological and biochemical maturation in the epididymis, but they require further maturation in the female reproductive tract before they attain the ability to penetrate oocytes. The changes that the spermatozoa undergo in the female tract are not well-defined, but there are two particular events that have relevance to their use in in vitro fertilization procedures: capacitation and the acrosome reaction. Capacitation involves changes in the motility of the spermatozoa and also changes in the character of the sperm membrane. This latter aspect of capacitation appears to be an essential pre-requisite for the acrosome reaction to occur (see Section 2.2). The need for spermatozoa to undergo capacitation to achieve fertilizability was first described by Austin (1951) and Chang (1951). Because the mechanisms of capacitation were not understood at the time, the earliest successful attempts at in vitro fertilization of rat oocytes were achieved using in vivo capacitated spermatozoa (Miyamoto & Chang, 1973a,d). Spermatozoa were recovered from the uterus of mated females and it was reported that the proportions of penetrated oocytes were

higher following insemination with spermatozoa which had been removed 4-5 h after mating than with spermatozoa obtained 0.5-1 h or 10-11 h after mating.

Although *in vitro* capacitation and fertilization were described for the hamster in 1963 (Yanagimachi & Chang, 1963), the first report of *in vitro* fertilization in the rat using epididymal spermatozoa capacitated *in vitro* did not appear until 11 years later (Toyoda & Chang, 1974a). The vast majority of reports on *in vitro* fertilization have used preincubated epididymal spermatozoa. Although two other groups have reported successful *in vitro* fertilization using *in vitro* capacitated spermatozoa (Kaplan & Kraicer, 1978; Evans & Armstrong, 1984), the former group has since gone back to using spermatozoa capacitated *in vivo* (Shalgi *et al.*, 1985). The two most important factors controlling fertilizability of preincubated spermatozoa are the concentration and the length of the preincubation period. Components of the incubation media that are optimal for capacitation *in vitro* (See Section 2.2.2) have been identified in the same studies that determined optimal conditions for *in vitro* fertilization, as the latter process is dependent upon successful capacitation.

Several studies (Niwa & Chang, 1973, 1974b,c, 1975c) have shown maximal rates of fertilization of rat oocytes when spermatozoa are preincubated in a dilute (0.5-1.5 x 10^6 spermatozoa/ml) rather than a concentrated (5-15 x 10^6 spermatozoa/ml) suspension. The minimal number of

spermatozoa/oocyte required for fertilization was 3000-6000 spermatozoa, compared with 100-800 spermatozoa/oocyte for the mouse (Tsunoda & Chang, 1975b). There was little apparent difference in spermatozoa motility regardless of whether the spermatozoa were incubated in low or high dilutions. It was also found that increasing the number of oocytes in each drop increased the proportion of oocytes penetrated, indicating that the presence of a certain number of oocytes may facilitate capacitation of sperm or penetration of oocytes.

Attempts to determine the optimal length of the preincubation period has led to some contradictory results. In the first report of successful in vitro capacitation of rat spermatozoa (Toyoda & Chang, 1974a), the spermatozoa were preincubated for 1 h before adding the oocytes. Subsequently two papers were published whose results were directly contradictory. The first (Toyoda & Chang, 1974b) reported a progressive decline in the percentage of oocytes penetrated as the preincubation time was increased from 0.5 h to 5.5 h, whereas the second paper (Niwa & Chang, 1974b) noted a progressive increase in fertilization rate over the same period of preincubation. Clearly factors other than the length of preincubation may be affecting the fertilization capabilities of these spermatozoa.

Although ejaculated spermatozoa are used routinely for the in vitro fertilization of oocytes from humans and large animals, they have limited use in the in vitro

fertilization of rodents because of difficulties in obtaining ejaculates. The use of ejaculated rat spermatozoa has been reported (Tsunoda & Chang, 1975a) but with a fertilization rate of 0-8%, compared with 80% using epididymal spermatozoa. Conditions for preincubation of both sources of spermatozoa were similar; however, it is apparent that those conditions optimal for epididymal spermatozoa are not adequate for ejaculated spermatozoa.

The penetration of more than one sperm into the vitellus of an oocyte, or polyspermy, is rare (<1%) in in vivo fertilized oocytes when mating occurs at the normal time (Braden et al., 1954; Austin, 1956). In contrast, polyspermy is frequently observed in in vitro fertilized oocytes at rates of 10 to 30% of penetrated hamster (Yanagimachi & Chang, 1964), mouse (Iwamatsu & Chang, 1969) and rat oocytes (Miyamoto & Chang, 1973a; Toyoda & Chang, 1974a). This high incidence of polyspermy may be due to the larger number of spermatozoa around the oocytes during in vitro fertilization (3000-6000; Niwa & Chang, 1974d) as opposed to during in vivo fertilization (20-60; Blandau & Odor, 1949). In addition, polyspermy in vivo is apparently prevented by the formation of a block to sperm penetration at the level of the zona pellucida and the oocyte plasma membrane following penetration by the first sperm (Fukuda & Chang, 1978). Under the conditions of in vitro fertilization, this block to polyspermy may develop more slowly, at a rate not fast enough to prevent the penetration of more than one spermatozoon.

2.2.4 Developmental capability

The ability to fertilize mammalian oocytes *in vitro* does not necessarily bestow on them the normal capacity for further development. Numerous factors affect the probability of successful development of *in vitro* fertilized oocytes. As described earlier (Chapter 2.1.2 D), rates of *in vitro* fertilization are highest when the oocytes are collected just before or soon after ovulation. Generally oocytes that are matured *in vitro* have lower fertilization rates and survive to viable fetuses in proportions lower than those of *in vivo* fertilized oocytes (Chang, 1955; Cross & Brinster, 1970; Niwa *et al.*, 1976; Moor & Trounson, 1977; Shalgi *et al.*, 1979; Leibfried-Rutledge *et al.*, 1987). Thus improvement in the culture methods for the proper maturation of oocytes is of great importance for the successful transfer of oocytes fertilized *in vitro*.

In contrast to humans, the probability of pregnancy ensuing in the recipient animals after transfer of *in vitro* fertilized oocytes is relatively high (>60%) (reviewed by Chang, 1982). This may be due to the fact that the recipient animals are better prepared, not necessarily exposed to the hormonal stimulation required for oocyte recovery, whereas in human studies, the donor and the recipient normally are the same patient. The ability to use different individuals as donor and recipient in animals studies lends itself to an even greater advantage. Embryos

can be transferred to recipient animals that are at a stage of pregnancy that will be optimal for continued development and implantation of the embryo (See section 2.3.3).

Despite all the advantages available with the use of laboratory animals, the potential for development of *in vitro* fertilized oocytes still appears to be considerably lower than that of *in vivo* fertilized oocytes. The proportions of *in vitro* fertilized oocytes that develop into viable fetuses or normal offspring is relatively low, about 20-30% (Cross & Brinster, 1970; Mills *et al.*, 1973; Toyoda & Chang, 1974a; Shalgi, 1984) whereas 75% of *in vivo* fertilized oocytes are expected to develop into young in these species. Several studies have reported that the majority of embryonic losses occurs before implantation; however, significant postimplantation losses have also been noted. In one study, 31% of *in vitro* fertilized rabbit oocytes implanted, but only 19% developed to term (Mills *et al.*, 1973). Similarly in the rat, 34-42% of transferred *in vitro* fertilized oocytes had undergone implantation, but only 24-26% developed into viable fetuses (Toyoda & Chang, 1974a; Shalgi, 1984). The cause(s) of the pre- and postimplantation losses are as yet unknown; however, 31-55% of transferred *in vivo* fertilized rat or rabbit oocytes in these studies were capable of development to full-term fetuses. This suggests that the cause of the developmental incompetence arises during the procedure for *in vitro* fertilization rather than as a consequence of the embryo transfer technique. Defects may be due to unfavorable

conditions *in vitro* (culture media, exposure to environmental stresses, handling) or possibly due to chromosomal aberrations (Wood *et al.*, 1982). Since some embryos derived from *in vitro* fertilization are able to develop to term under these conditions, it is apparent that improvement of the culture conditions will probably lead to an increase in the number of viable embryos.

2.3 EMBRYO TRANSFER

2.3.1 Embryo development *in vitro*

The most commonly used criterion for the viability of oocytes that have been manipulated *in vitro* is fertilization. More rigid criteria of developmental competence would be the demonstration of normal embryonic and fetal development. The development of techniques for *in vitro* fertilization in many species naturally led to efforts to determine the appropriate culture conditions for preimplantation embryo development *in vitro*. Culturing embryos fertilized *in vitro* to the blastocyst stage would be a feasible criterion of successful fertilization and is more convincing as an indicator of normalcy than is penetration, pronuclear formation or cleavage. Embryo development *in vitro* would also be less costly, less difficult and less time-consuming than embryo transfers. In early studies investigating the developmental capacity

of in vitro fertilized hamster (Yanagimachi & Chang, 1964; Whittingham & Bavister, 1974) and rat (Toyoda & Chang, 1974a) oocytes, more than 90% cleaved in culture to produce 2-cell embryos but no further cleavage occurred. This "2-cell block" has been demonstrated with in vivo fertilized oocytes as well (Whittingham & Bavister, 1974), so abnormalities resulting from in vitro fertilization procedures can be discounted as causative factors. In addition, the viability of in vivo fertilized oocytes, as determined by oviductal embryo transfer, is compromised within minutes of exposure to various culture media (Sato & Yanagimachi, 1972; Farrell & Bavister, 1984). The rapidity of these detrimental effects suggests that the 2-cell block is caused by some physico-chemical imbalance and/or the deficiency of some critically important factor in the culture environment. It was initially thought that some special oviductal factor was necessary for the embryos to proceed from the 2- to 4-cell stage (Whittingham & Biggers, 1967). It soon became apparent, however, that fertilized mouse oocytes were able to develop to the blastocyst stage in BSA-supplemented medium. Optimal culture conditions were determined by altering osmotic pressure (Brinster, 1965a; Whitten, 1971), pH (Brinster, 1965a) and energy sources (Brinster, 1965b; Whitten & Biggers, 1968; Whitten, 1971). The first successful transfers of embryos that had been fertilized in vitro and cultured to ~~the~~ blastocyst stage were reported in the mouse (Mukherjee & Cohen, 1970; Hoppe & Pitts, 1973). With the ability to develop mouse

embryos *in vitro*, this species was (and still is) used extensively in studies of early embryonic development. Unfortunately, embryos of most other mammalian species including the hamster (reviewed by Bavister, 1987), other laboratory animals (Whittingham, 1975), domestic animal species (Wright & Bondioli, 1981) and even outbred strains of mice (Goddard & Pratt, 1983) appear to be much more difficult to maintain in culture. In fact, there are no reports of culture of hamster or rat 2-cell embryos that supported further development.

The inability to support the development of embryos of many species *in vitro* suggests that these embryos may require specific environmental factors that have yet to be identified. Reliable information on the composition of oviductal secretions is scarce (Borland *et al.*, 1977; Shalgi *et al.*, 1977; Bavister, 1981) making it difficult to mimic that environment *in vitro*. As an alternative, Biggers *et al.*, 1962) devised a technique in which embryos are cultured within an oviduct which is itself maintained in culture. The oviduct thus provides the environment that directly nourishes the embryos. Difficulty with the culture of the oviducts of some species has led to cross-species culture techniques; i.e. culture of the oviduct of one species containing the embryos of another (Bavister & Minami, 1986). There are also numerous reports of successful cross-species preimplantation embryo development *in vivo*, where embryos are surgically transferred into the

oviduct of another species and recovered several days later for transfer into an appropriate recipient for implantation and fetal development (Boland, 1984; Eyestone et al., 1985; Sirard et al., 1985). Although these techniques currently have variable degrees of success, their improvement should serve to increase the proportion of embryos able to develop to morulae or blastocysts and therefore perhaps improve the success of these embryos following transfer.

2.3.2 Embryo transfer - The technique

The first experiments employing transplantation of mammalian fertilized oocytes were described by Heape in 1890. In these experiments, rabbit embryos were transferred to the uterus of recipient animals for embryonic and fetal development. Similar experiments were performed in the rat more than 40 years later (Nicholas, 1933) to test for embryo viability. In this particular study, albino and pigmented rats were used so that pigmentation served as the decisive criterion of the origin of the developing embryos. Of 18 transferred embryos, 11 implanted in the uterus and developed to viable fetuses. The success of embryo transfers in small animals signalled the beginning of many studies in which this technique was used to investigate the developmental capabilities of oocytes or embryos undergoing in vitro manipulations or to determine the importance of various aspects involved in implantation. These studies became the groundwork leading to the development of the

human *in vitro* fertilization-embryo transfer programs that are now used for the treatment of infertility. The treatment attempts to circumvent many causes of infertility including anovulation, blocked Fallopian tubes and oligospermia.

Numerous factors may affect the success of embryo transfers in mammals but they can be divided into 3 main categories: 1) the source of embryos, involving embryo number, age and quality, 2) *in vitro* manipulation, including method of collection, exposure to environmental factors, treatments such as freezing or micromanipulation, and method of transfer, and 3) the recipient, in which the site of transfer, the maternal endocrine environment, genetic disparity and donor-recipient synchrony are important factors. Fortunately, most of these factors can be controlled to allow maximal success rates.

Embryo transfer in farm animals has become a technique of growing commercial value. High pregnancy rates have been achieved after embryo transfers using surgical techniques in cows (Rowson *et al.*, 1969; Betteridge & Mitchell, 1974; Sreenan & Beehan, 1974), sheep (Hunter *et al.*, 1955, Averill, 1958; Moore, 1968), goats (Nishikawa *et al.*, 1963a,b; Moore, 1974) and pigs (Pope *et al.*, 1972). Surgical techniques performed via laparotomy under local or general anaesthesia involve transfer of the embryos into the infundibulum or ampulla of the oviduct or into the lumen of the uterus using a capillary pipette (reviewed by

Sugie et al., 1980). Non-surgical transfers of cattle embryos is now practised widely. Embryos are deposited directly into the uterus through the cervix (Sugie, 1965), similar to the technique used with humans. While pregnancy rates may be 10 to 30% lower and less consistent following non-surgical (reviewed by Greve & Del Campo, 1986) compared with surgical transfers (reviewed by Betteridge, 1977), non-surgical transfers involve less cost, less risk, are faster and are therefore becoming the method of choice.

In small animals, embryo transfers are largely performed surgically, although fairly successful techniques for non-surgical embryo transfer have been described for the rat (Vickery et al., 1969) and the mouse (Marsk & Larsson, 1974). As mentioned earlier, the first transfers performed in the rat involved the transfer of embryos to the uteri of genetically distinct recipient rats that were mated at the same time as the donors (Nicholas, 1933). Embryo transfers into the uterus have been used commonly to investigate the relationship between the age of the embryos and endometrial development in rabbits (Chang, 1950), mice (Fekete & Little, 1942; McLaren & Michie, 1956) and rats (Dickmann & Noyes, 1960; Noyes & Dickmann, 1960). During embryo transfers into the uterus, the recipients are usually anaesthetized and a surgical needle is passed through the antimesometrial wall of the uterus in the area close to the uterotubal junction. A micropipette containing the embryos is then inserted through the puncture and the embryos are gently injected into the

uterine cavity. Although the technique itself is relatively simple, this method suffers from the restrictions imposed by the critical developmental requirements of the embryo and the endometrium (See Section 2.3.3).

Initial attempts to transfer rat oocytes into the oviducts of recipient rats failed because of inaccessibility of the tubal ostium and because the infundibulum was too small to receive the pipette carrying the oocyte-cumulus cell complexes (Noyes, 1952). Special consideration was necessary in these animals due to the presence of the bursa, a membrane enclosing the ovary. However, no successful results were obtained when the bursal membrane was pierced by a pipette and the oocytes were transferred under the ovarian bursa (Noyes, 1952). Alden (1942) had described a foramen in the bursal membrane which became critical for the success of oocyte transfers. Noyes (1952) passed the transfer pipette through the foramen and expelled the oocytes into the bursal sac. With this technique, 41% of mature follicular oocytes developed to term following fertilization and transfer into mated recipients. After consideration of several factors that may limit the development of a greater proportion of oocytes, Noyes suggested that the success rate may be limited by a relative progesterone deficiency, since with this technique there is always an unphysiological excess of oocytes compared with corpora lutea. A similar experiment

performed 30 years later (Walton & Armstrong, 1983) resulted in a success rate of 56.5%; however, in this experiment only 2-4 oocytes were transferred to each recipient as compared with 4-10 oocytes in the early experiments. If success is dependent on progesterone levels, then the transfer of fewer oocytes/recipient would ensure an overall greater proportion of oocytes developing to fetuses. A more likely possibility is that the transfer of a fewer number of embryos reduces the incidence of crowding in the uterus, an occurrence related to greater mortality (Bowman & Roberts, 1958; Harper, 1964).

When cumulus-enclosed oocytes are transferred into the ovarian bursae of rats, ciliary activity of the tubal mucosa transports them rapidly (within 1 h) into the oviduct; however, denuded oocytes are not picked up as efficiently by the oviduct (Noyes & Dickmann, 1961). Since the time that elapses between fertilization and denudation is only a few hours, the method of intrabursal transfer suffers from even more stringent timing restrictions than does uterine transfer (Noyes & Dickman, 1961). To attain the highest proportion of viable fetuses from transferred oocytes/embryos, it was necessary to develop a method to transfer the oocytes/embryos directly into the oviduct.

Although Bittner & Little (1937) reported the transfer of denuded oocytes to the oviducts of mice, they did not report the technical details. Tarkowski (1959) successfully transferred 2-cell mouse embryos into the oviducts of the recipients but, unfortunately, he too did

not report on his method of dealing with the presence of the bursa. The first rat oviductal transfers were described in detail by Noyes & Dickmann (1961). In these studies, an incision was made in the bursal membrane with a high frequency current electroscalpel to prevent bleeding. The pipette was then passed through the incision into the infundibulum of the oviduct and the zygotes were expelled. 51% of the zygotes developed to near-term fetuses following synchronous transfer to recipients. The authors concluded that, although oviductal transfers are more difficult, they offer a wider margin of safety in cases when the exact stage of development of the embryos and the recipient endometrium is not known. A modification of this technique was described by Toyoda & Chang (1974a) in which a small cut was made at the antimesometrial side of the bursal membrane and the bursa was removed from around the ovary by means of a small forceps. The fimbriated end of the oviduct was then recognized as an extrusion between the ovary and the oviduct with the ostium clearly exposed. Unfortunately other variables of these two experiments prevent the comparison of their success rates, but it is clear that both methods of oviductal transfer can be used effectively to determine the viability of transferred oocytes or embryos.

2.3.3 Asynchronous vs synchronous transfers

Early studies in rabbits (Chang, 1952) and mice

(Bowman & Roberts, 1958) demonstrated the ability of nearly all ovulated oocytes to be fertilized and develop normally in the oviduct. However, a significant proportion of reproductive loss in these species results from functional defects occurring around the time of implantation. Extensive studies have been performed in the mouse (Fekete & Little, 1942; McLaren & Michie, 1956), rabbit (Chang, 1950) and rat (Dickmann & Noyes, 1960; Noyes & Dickmann, 1960,1961) to determine the relationship between the embryo and the endometrium at the time of implantation. In all these studies embryos at various stages of maturity were transferred to the oviducts or uteri of recipient animals that were at various preimplantation stages of pregnancy; thus, transfers were either synchronous (eg. Day 3 embyros into Day 3 pregnant animals) or asynchronous (eg. Day 3 embryos into Day 4 pregnant animals). Generally these experiments indicated that transferred embryos that were the same developmental stage or 1 day more advanced than the uterus survived to viable fetuses in the highest proportions. Transferred embryos that were 1 day younger than the recipient uterus developed at the usual rate until the fifth day of pregnancy (normal day of implantation) and then degenerated rapidly and failed to implant. The factor detrimental to younger embryos was not uniformly distributed throughout the early days of pregnancy, but was limited to a comparatively brief period around the time of implantation (Dickmann & Noyes, 1960). It was concluded

that the uterine environment undergoes changes in the afternoon of Day 5, becoming detrimental to younger embryos, but stimulating Day 5 blastocysts in such a way that they become attached to the endometrial epithelium and elicit the decidual reaction. The suggestion that the uterus can exert a negative influence on the development of the embryos has been demonstrated in numerous hormonal and metabolic studies (Gulyas & Daniel, 1969; Psychoyos, 1973; Weitlauf, 1971, 1973). In summary, normal implantation can take place only when the embryo and the endometrium have simultaneously reached specific stages in their development.

CHAPTER 3

RATIONALE AND OBJECTIVES

A current treatment for some causes of human infertility employs the techniques of *in vitro* fertilization and embryo transfer. The success of these techniques relies partly on the collection of mature oocytes from their follicles prior to ovulation. The precise timing of oocyte collection is necessary because the mechanisms regulating oocyte maturation have not been fully elucidated. Numerous investigations of the regulation of oocyte maturation have concentrated on the resumption of meiosis in response to the LH surge. Most of these studies have used spontaneously maturing oocytes, i.e. oocytes removed from their follicles prior to the LH surge. However, there is evidence to suggest that spontaneously matured oocytes may not have complete developmental competence. Thus, the observation of nuclear maturation as seen by germinal vesicle breakdown and polar body formation may not be an accurate evaluation of complete oocyte maturation. A better criterion of successful maturation and developmental competence of spontaneously maturing oocytes would be the ability of these oocytes to be fertilized. If fertilization was used to assess the developmental competence of spontaneously maturing oocytes, the regulation of both nuclear and cytoplasmic maturation could be investigated by maturing oocytes under various conditions *in vitro*.

The overall objective of this research project was to study the regulation of oocyte maturation. To achieve this objective, it was first necessary to develop a method for assessing developmental competence. Thus, the first part of the project concentrated on establishing a method for *in vitro* fertilization and transfer of rat oocytes to recipient animals for further development. The development of a technique for producing unilateral pregnancy in rats has been described in Chapter 5. The non-pregnant uterine horn was a natural environment in which *in vitro* fertilized oocytes could undergo embryonic and fetal development. The ability of *in vitro* fertilized oocytes to develop to various stages (Chapter 6) led to a study which attempted to improve the proportion of *in vitro* fertilized oocytes that would undergo normal development to near-term fetuses (Chapter 7).

As oocytes fertilized *in vitro* were shown to be capable of embryonic and fetal development, the second part of the project included the use of *in vitro* fertilization to assess complete oocyte maturation. Initial studies determined the appropriate culture conditions to achieve nuclear maturation and fertilizability of spontaneously maturing oocytes, and the developmental competence of some of these oocytes was tested (Chapter 8). As oocytes matured under sub-optimal conditions showed a low incidence of fertilization, the cause of this inability was investigated further by testing the penetrability of these *in vitro* matured oocytes (Chapter 9). Oocytes maturing *in*

vivo do so in response to the LH surge; therefore, the last group of studies in this project examined the influence of various hormonal factors on the maturation-fertilization process.

To summarize, the specific aims of this research project were:

1) To develop a system in which fertilized rat oocytes could undergo embryonic and fetal development.
2) To determine the ability of in vitro fertilized oocytes to develop to various embryonic and fetal stages.
3) To determine the cause of embryonic losses following the transfer of in vitro fertilized oocytes to pregnant recipients.
4) To increase the proportion of in vitro fertilized oocytes capable of development to near-term fetuses.
5) To determine the appropriate culture conditions to achieve fertilizability of in vitro matured oocytes.
6) To determine the developmental competence of rat oocytes matured in vitro.
7) To increase the penetrability of in vitro matured oocytes by drilling a hole in the zona pellucida of each oocyte.
8) To establish the role of cumulus cells in the cytoplasmic maturation of oocytes matured in vitro.
9) To determine the possible roles for hormonal factors (PMSG, FSH and granulosa cell products) in regulating nuclear and cytoplasmic maturation as well as fertilizability of in vitro matured rat oocytes.

CHAPTER 4

GENERAL MATERIALS AND METHODS

4.1 Animals

Three strains of rats were used in these experiments: Sprague-Dawley (Crl: CD ® (SD)BR), Long-Evans (Crl: (LE)BR) and Wistar (Crl: (WI)BR). All rats were purchased from Charles River Canada Inc., St. Constant, Quebec. Unweaned Wistar female pups were caged with their mothers until the time of sacrifice. Prepubertal Sprague-Dawley female rats were housed until they weighed 60-65 g (approximately 26 days old) before treatment began. Adult Sprague-Dawley and Long-Evans rats were obtained at 110-120 g and were allowed a minimum period of one week to adjust to their environment before experiments were initiated. The animals were housed in air-conditioned quarters with free access to food and water. Lighting was provided for 14 h daily with the lights on from 0500 to 1900. In some experiments the timing of the period of illumination was altered to manipulate the time of ovulation. Ovulation in the low-dose PMSG-treated immature rat occurs between 0200 and 0400 of Day 1 (Day 1 = day of estrus) during a normal light cycle (Walton et al., 1983). Therefore, animals used for the collection and/or transfer of ovulated oocytes were exposed to light from 1700 to 0700, causing ovulation to occur during mid-afternoon. To avoid confusion, all times cited in the remaining text refer to the diurnal cycle of

the animals, i.e. 0000 refers to the midpoint of the dark period and 1200 is the midpoint of the light period.

4.2 Experimental Methods

The methods used in this study vary considerably among experiments; therefore details of each are given in subsequent sections. There are, however, two procedures that are used in many of the experiments: in vitro fertilization and the maturation of oocytes in vitro. The details of the in vitro fertilization technique are described in Section 4.2.1. The general methodology for maturing oocytes in vitro is given in Section 4.2.2.

4.2.1 In vitro Fertilization

The technique of in vitro fertilization has been used in most experiments of this research project. The method used for fertilization was basically that described by Toyoda & Chang (1974a) with some minor modifications. The fertilization medium was prepared as two stock solutions using triple distilled water. Solution A comprised 6.636 g NaCl/l, 427.7 mg KCl/l, 301.7 mg $CaCl_2$/l, 194.4 mg KH_2PO_4/l, 171.9 mg $MgSO_4$/l and 2 mg phenol red/l. Solution B comprised 12.940 g $NaHCO_3$/l and 2 mg phenol red/l. With the exception of KH_2PO_4, all components were obtained from Fisher Scientific Company, Fair Lawn, New Jersey; KH_2PO_4 was obtained from the McArthur Chemical Co. Ltd., Montreal.

Solutions A and B were stored at 4°C for a maximum period of three months. The final solution, prepared 12-24 h before use, comprised a mixture of 41.7 ml Solution A, 8.1 ml Solution B and 0.2 ml sodium lactate (60% solution: Fisher Scientific). To this were added 2.75 mg sodium pyruvate (Gibco Laboratories, Grand Island, New York), 50 mg dextrose (Fisher Scientific), 1250 IU penicillin and 1.25 mg streptomycin (Gibco Laboratories). The solution was gassed for 10 minutes with 5% CO_2 in air and then 200 mg bovine serum albumin (BSA, Fraction V; Sigma Chemical Co., St. Louis, Missouri) was added. The fertilization medium was sterilized using a 0.20 µm positive pressure filtration system (Nalgene: Sybron/Nalge, Rochester, New York). The medium was allowed to equilibrate for a minimum of 6 h in a humidified incubator at 37°C with 5% CO_2 in air as the gas phase. The final pH was 7.4-7.6 and the osmolarity was 290-300 mOsm/l.

In most experiments ovulated oocytes were used as a control for the technique of in vitro fertilization. At body weight 60-65 g (~26 days of age), immature rats were injected subcutaneously with 4 or 6 IU PMSG (Equinex: Ayerst, Montreal) at 0800 to 1000 on Day -2. Although ovulation would occur in response to the endogenous LH surge in these animals, an intraperitoneal injection of 15 IU hCG at 1000 on Day 0 was used, when it was necessary, to ensure a more accurate synchronization of ovulation. The rats were killed by cervical dislocation on Day 1 between 0200 and 0500. The oviducts were dissected out in

Dulbecco's phosphate-buffered saline (DPBS; Gibco Laboratories) with 5% heat-inactivated, charcoal-treated rat serum (DPBS-S). The swollen ampullae were torn open with fine forceps to allow the oocytes to escape. The ovulated oocytes were then transferred to the fertilization medium.

Whether the oocytes were ovulated or matured in vitro, they were washed in DPBS or DPBS-S before transfer to 50 µl droplets of fertilization medium. The droplets were incubated under mineral oil (U.S.P. Heavy, Drug Trading Co., Toronto or U.S.P., Ingram & Bell Ltd., Don Mills) in 10 x 35 mm plastic tissue culture dishes (Nunc: Roskilde, Denmark). Each droplet contained 1-8 oocytes.

To prepare the sperm suspension, adult males of proven fertility were anaesthetized with ether and killed by cervical dislocation. The cauda epididymis and vas deferens of each testis were removed and kept moist with fertilization medium. Approximately 4 cm of the epididymal duct proximal to the vas deferens and 1 cm of the vas deferens were dissected free of fat and blood vessels. Using fine forceps, the sperm suspension was expressed from the duct into a drop of fertilization medium. This drop was added to 0.6 ml of fertilization medium under oil and allowed to disperse for 2-3 minutes in the incubator. Only sperm suspensions showing vigorous, progressive motility were used. Using a hemocytometer, the concentration of sperm was determined and the sperm suspension was then

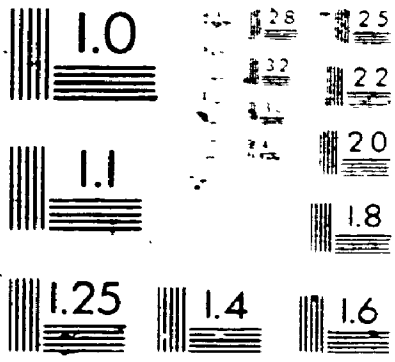
diluted to a concentration of 1.25 x 10^6 spermatozoa/ml. Droplets of fertilization medium already containing oocytes were inseminated with 40 µl of the diluted sperm suspension to give a final concentration of 5 x 10^5 spermatozoa/ml. In early experiments the diluted sperm preparation was incubated for 1-2 h before insemination. This period was soon reduced to 15-30 minutes, an alteration that resulted in a slight increase in the proportion of oocytes undergoing fertilization.

Fertilization of the oocytes was assessed by phase-contrast microscopy 14-18 h after insemination. Oocytes were considered normally fertilized if they had 2 pronuclei and 1 sperm tail within the vitellus. Oocytes with >1 sperm tail were classed as polyspermic; those with only 1 pronucleus were classed as abnormally fertilized. The remaining oocytes were assessed as unfertilized, or degenerating if they showed fragmentation or abnormal structure. In most experiments the oocytes were examined again after a further 24 h to assess development to the 2-cell stage.

4.2.2 Oocyte Maturation *in vitro*

COLLECTION OF IMMATURE OOCYTES

At body weight 60-65 g, immature Sprague-Dawley rats were treated with 6 IU PMSG at 1400 h on Day -2. Between 0100 and 0600 on Day 0 (unless otherwise stated), the animals were killed by cervical dislocation and their



5.2.3), 12% showed complete re-encapsulation and 8% showed partial re-encapsulation of the ovary, but only 8% of the animals had fetuses in the uterine horn ipsilateral to the peeled ovary.

In the first experiment, the reasons for the increase in the number of corpora lutea between Weeks 1 and 5 both in the control and peeled ovaries are not yet known. There is evidence to suggest that the number of ovulations increases to a maximum during the early reproductive life of the female (Biggers et al., 1962; Kennedy & Kennedy, 1972).

Oocytes in the oviduct ipsilateral to the bursa-peeled ovary were not always found in rats with complete resacculation. On four occasions oocytes were found in the oviduct next to an ovary that was still completely exposed. These results agree with those of Neugebauer (1935) who concluded that the ovarian bursa is practically indispensable for oocyte pick-up and that bilateral pregnancy rarely occurs after unilateral bursa peeling.

The results of these experiments indicate that this preparation could be used to produce unilateral pregnancy with only a small margin of error (<8%). This error may be further reduced by excluding animals in which the peeled bursa has completely re-encapsulated the ovary. The surgical technique appears to have no effect on the ability of that ovary to ovulate or on the ability of zygotes to develop into viable fetuses in the ipsilateral uterine horn.

volume of serum had been collected, the pooled serum was thawed and heat-inactivated for 30 minutes in a 56°C shaking waterbath. Charcoal (50 mg/ml, Norit A: J.T. Baker Chemical Co., Phillipsburg, New Jersey) was added to remove steroids and other low molecular weight substances and the solution was stirred overnight at 4°C. The following morning the charcoal was removed by centrifugation at 1800 x g for 30 minutes. The supernatant was then centrifuged for 60 minutes at 27,000 x g in an ultracentrifuge. The serum was sterilized by positive pressure filtration (pore size = 0.20 um) and stored at -20°C until use.

REMOVAL OF CUMULUS CELLS

In studying the role of cumulus cells in the maturation process, oocytes were matured either with their cumulus cell layer intact (CI) or the cumulus cells were removed and the oocytes were matured cumulus-free (CF). To remove the cumulus cells, oocyte-cumulus complexes were placed in DPBS with 0.1% hyaluronidase (bovine testes: Sigma Chemical Co.). The cumulus cells were stripped away from the oocyte by repeated pipetting with a fine-bore, heat-polished pipette. In most experiments the cumulus cells were removed from those oocytes that had been cultured with their cumulus cell layer intact such that all oocytes were cumulus-free prior to insemination. The oocytes were washed in DPBS and transferred to fertilization medium for *in vitro* fertilization.

Although in all experiments the GVBD and the GVBD + polar body (PB) oocytes were separated and placed into different droplets for fertilization, the proportions of GVBD and GVBD + PB oocytes fertilized in each group were never significantly different. This observation could be expected as polar bodies are transient structures that degenerate quite rapidly and, therefore, the separation of GVBD and GVBD + PB was rather artificial, depending entirely on the time of analysis. Only when the length of culture was strictly controlled (see Section 8.2.2, 10.2.2 B and 10.2.3 C) could the event of PB extrusion be considered important.

By combining 2 or more replicates of each experiment, a minimum of 100 oocytes/treatment was used. The fertilization of ovulated oocytes (see Section 4.2.1) was normally used as a control.

4.3 Statistical Analyses

The results of the studies presented here have been largely reported as the proportion of oocytes achieving a certain stage of development following one of several treatments. For experiments with 2 treatments, the proportions were compared using X^2 analyses with Yates' correction. For more complex designs involving a factorial arrangement of treatments, proportions were compared using the R X C test for independence and X^2 analyses (Steel & Torrie, 1980). Most experiments were performed at least twice and inter-replicate and treatment differences were

identified by analysis of variance using arcsin transformation of the proportions (Steel & Torrie, 1980). If the analysis revealed no inter-replicate differences, the data were pooled and have been presented as such. Presumably due to the variability in the penetrating abilities of each sperm preparation, some studies showed inter-replicate differences and, therefore, only the results of one experiment have been presented. Although some studies showed inter-replicate differences, the effect of similar treatments was always the same between experiments.

For experiments in which the effects of a treatment resulted in a measurable response in each individual of that treatment, the mean $\pm$ the standard error of the mean (S.E.M.) were used for comparison. When the experimental design involved 2 treatments, the means of the 2 groups were compared using Student's two-tailed t-test. For experiments with more than 2 treatments, comparisons of the means were made using one-way analysis of variance with Bartlett's test of homogeneity of variance and Duncan's new multiple range test (Steel & Torrie, 1980).

CHAPTER 5

REMOVAL OF THE OVARIAN BURSA

5.1 Introduction

Currently rat embryos cannot develop in culture and, therefore, most investigations into the viability of *in vitro* fertilized rat oocytes have involved transfer into pseudopregnant recipients (Toyoda & Chang, 1974a; Shalgi, 1984). Pseudopregnancy is commonly induced by electrical stimulation of the cervix on the morning of estrus or by mating with a vasectomized male. With these techniques, however, ovulated oocytes will be present in the oviduct and may interfere with investigations of early embryonic development of transferred oocytes. Using mated recipients, some investigators have used the genetic marker of eye colour to distinguish between endogenous and transferred embryos (Noyes, 1952; Walton & Armstrong, 1983), but this technique is useful only when examining development to the later stages of gestation, when pigmentation is at detectable levels.

To study embryonic development during early gestation, a system was needed in which endogenous embryos could be distinguished from transferred embryos, or in which transferred embryos could remain isolated from endogenous embryos. As endogenous embryos cannot be distinguished morphologically from transferred embryos, identification could be difficult, requiring specific genetic or

biochemical markers. On the other hand, physical separation of the embryos may be possible by taking advantage of the fact that the rat has two uterine horns with separate cervices (Hamilton, 1947). Using methods to produce unilateral pregnancy, it would be possible to use the "empty" horn as a recipient for transferred embryos while the contralateral side would bear the endogenous embryos. Two methods have been reported for the production of unilateral pregnancy: 1) ovariectomy, and 2) opening of the ovarian bursa.

To develop a method for in vivo fertilization of transferred oocytes, Shalgi et al (1979) removed the ovary at the time of transfer into the ipsilateral oviduct. When this technique was performed before ovulation had occurred in the recipient rat, the oviduct could contain only transferred oocytes. Although potentially a useful method to produce unilateral pregnancy, removal of the ovary has uncertain effects on the subsequent pregnancy and can cause bleeding that is sometimes difficult to control.

In the rat and the mouse, encapsulation of the ovary by the bursa ovarica is almost complete (Robinson, 1887; Alden, 1942; Wimsatt & Waldo, 1945), unlike in women, in which the ovarian bursa is absent or nearly so. In the rat, destruction or opening of the bursa before mating has been shown to be of moderate success in limiting the number of fetuses found in the ipsilateral uterine horn (Neugebauer, 1935; Kelly, 1939). Perhaps more complete

removal of the bursa would have resulted in a higher rate of successful unilateral pregnancy. In the present study this procedure was modified and its usefulness for the study of transferred oocytes or embryos was examined.

5.2 Methods and Results

5.2.1 Effects on Ovulation

Adult Long-Evans rats were anaesthetized by intraperitoneal injection of tribromoethanol solution (2%, 0.01 mg/g body weight) and the bursa was peeled back from around the left ovary of each rat (Plate 1). The bursa around the right ovary was left intact and this side was used as a control. The left ovary was exposed through a flank incision and was drawn out by pulling on the fat pad. After lifting the bursa away from the ovary with a pair of fine forceps, a small hole was made in the bursa using a pair of iridectomy scissors, care being taken to avoid bleeding. It was presumed that, with a small hole, the bursa would be less likely to slip back on the ovary. The ovary was manipulated gently through the hole with fine forceps until the entire ovary was exposed. The ovary was then replaced in the abdominal cavity.

At 2 days after the operation (Day -4), the cycles of about one-fifth of the rats were synchronized with an LHRH agonist (des-Gly^{10}, D-Ala^{6}, L-ProNHEt^{9} LHRH; Sigma Chemical Co.) using a method described by Vickery & McRae (1980) and modified by Walton & Armstrong (1983). Briefly, the rats

were injected subcutaneously with 40 µg of the LHRH agonist at 0600 on Day -4. With this treatment, ovulation occurs in the early morning of Day 1. On the morning of Day 1 (1 week after the operation), vaginal smears were taken and only rats in estrus were subsequently killed. The bursa-free ovary was examined for adhesions or possible re-encapsulation of the ovary. Ovaries and oviducts from both sides were removed. Corpora lutea associated with ruptured follicles were counted. The oocytes were collected by inserting a 30-gauge needle into the infundibulum of the oviduct and flushing with 0.2 ml DPBS. The synchronization of rat cycles and subsequent killing during estrus were repeated so that 9-16 rats were killed each week for 5 weeks from the day of bursa peeling.

The average number of corpora lutea and ovulated oocytes on the control and the peeled sides of the rats have been summarized in Table 1. On the control side the average number of corpora lutea was not significantly different from the average number of ovulated oocytes ($P>0.1$). In contrast, although all the rats ovulated from the peeled ovary, as indicated by the presence of corpora lutea, only 18% contained oocytes in the ipsilateral oviduct. In the rats in which ovulated oocytes were found on the operated side, the average number of oocytes (2.0 ± 0.4) was significantly less than the average number of corpora lutea (6.5 ± 1.1) on that side ($P<0.01$). There was a statistically significant increase in the number of

PLATE 1

A series of photographs showing the technique for peeling the bursa from around the rat ovary. (a) The bursa is lifted away from the ovary with a pair of fine forceps. (b) Using iridectomy scissors, a small hole is made between blood vessels of the bursal membrane. (c) Fine forceps are used to indicate the edges of the small hole that has been made in the bursa. (d) The ovary is gently manipulated through the small hole. In this photograph the upper portion of the ovary is already free of the bursal covering. (e,f) Due to its elastic properties, the bursa remains as an intact covering around the oviduct and, in addition, now acts as barrier between the infundibulum of the oviduct and the ovary.

a

b

c

d

e

f

TABLE 1 Average number of corpora lutea (CL) and oviductal oocytes (± S.E.M.) in rats 1-5 weeks after unilateral peeling of the bursa

Time after bursa peeling (weeks)	No. of rats	Control side		Peeled side		
		Average no. of CL	Average no. of oocytes	Average no. of CL	No. of rats with oocytes	Average no. of oocytes/ rat with oocytes
1	10	6.1 ± 0.6	5.6 ± 0.6	5.5 ± 0.7	0	0
2	15	6.2 ± 0.6	6.6 ± 0.8	4.2 ± 0.8	3	2 ± 1.0
3	10	6.4 ± 0.6	6.7 ± 0.6	4.6 ± 0.4	3	1.7 ± 0.7
4	16	9.5 ± 0.5	8.4 ± 0.8	8.1 ± 0.7	4	1.8 ± 0.5
5	9	8.0 ± 0.5	7.4 ± 0.9	8.2 ± 0.9	1	4

corpora lutea between Weeks 1 and 5 in both the control and peeled ovaries ($P<0.05$).

The degree of re-encapsulation of the peeled ovary is indicated in Table 2). In most cases presence of oocytes on the operated side was associated with partial or complete re-encapsulation of the ovary. In each of 4 rats (out of a total of 60 operated animals) one oocyte was found in the oviduct when the ipsilateral ovary appeared to be completely free of the bursa (Table 3). Complete re-encapsulation of the ovary was possible within 2 weeks of the removal of the bursa from around the ovary. The percentage of rats with the ovary still completely exposed did not change significantly over the 5-week period (80-89%) ($P>0.1$).

5.2.2 Effects on Fetal Development

The bursa was peeled from around the left ovary of 35 mature Sprague-Dawley rats and 19 Long-Evans rats, as described in Section 5.2.1. After 3-4 days for recovery, the animals were caged with males for 5 days and allowed to mate. The female rats were killed 13 days later and the number of fetuses in each uterine horn was counted. The bursa-free ovary was examined for adhesions or re-encapsulation, and the number of corpora lutea was counted. Only rats with fetuses in the control horn were used in the analysis.

The degree of re-encapsulation of the ovary on the

TABLE 2 Degree of re-encapsulation of ovary 1-5 weeks after unilateral peeling of the bursa

Time after bursa peeling (weeks)	No. of rats	Re-encapsulation of ovary		
		None	Partial	Complete
1	10	8	2	0
2	15	12	2	1
3	10	8	1	1
4	16	14	0	2
5	9	8	0	1

TABLE 3 Degree of re-encapsulation of the ovary and the presence of oocytes in oviducts ipsilateral to the peeled ovary

Degree of ovary re-encapsulation	No. of rats	No. of rats with oocytes	No. of oocytes	No. of oocytes per rat
None	10	4	4	1
Partial	5	2	2	1
Complete	5	5	16	3.2

operated side and the presence of fetuses in the ipsilateral uterine horn are summarized in Table 4. The presence of fetuses in the uterine horn of the peeled ovary was normally associated with complete re-encapsulation of the ovary. In only one case, fetuses (3) were present in the uterine horn of a rat in which the peeled ovary still appeared to be completely exposed. In the Sprague-Dawley rats (n=32), ovulation on the operated side was verified by the presence of corpora lutea, and the average number of corpora lutea on that side (5.5 $\pm$ 0.4) was not significantly different from the number on the control side (5.2 $\pm$ 0.4) ($P>0.1$).

5.2.3 Embryo Transfers

Immature female Sprague-Dawley (albino) rats were treated on Day -2 with 4 IU PMSG to induce follicular growth. On or within 2 weeks before Day -3, the bursa was peeled from around the left ovary of each Long-Evans (pigmented) rat. The Sprague-Dawley and Long-Evans r[redacted] were caged with Sprague-Dawley males of proven fertility from 2000 on Day 0 to 0300 on Day 1, with copulatory plugs or spermatozoa in the vaginal smears taken as evidence of insemination. The immature rats were killed at 2000 on Day 1 and the oviducts were flushed with DPBS. Oocytes with 2 pronuclei and 1 sperm tail were transferred immediately into the left oviduct of the mated recipient (Long-Evans) rats. To perform the transfers, the recipient rats were

TABLE 4 Numbers of fetuses (± s.e.m.) surviving in each uterine horn after unilateral peeling of the ovarian bursa

Strain of rat	No. of rats	Control side		Peeled side		Re-encapsulation of ovary		
		No. of rats with fetuses	Average no. of fetuses	No. of rats with fetuses	Average no. of fetuses/ rat with fetuses	None	Partial	Complete
Sprague-Dawley	32	32	5.3 ± 0.3	3	2 ± 0.6	27	1	4
Long-Evans	19	17	6.8 ± 0.3	1	3	14	3	2

anaesthetized with tribromoethanol solution and the bursa-free (transfer) ovary was drawn out through a lateral incision. Using a finely drawn (150-250 µm, internal diameter) heat-polished glass pipette, 4-5 zygotes were transferred to the infundibulum of the left oviduct of each recipient. The recipient rats were killed on Day 20 and the fetuses in each uterine horn were counted and weighed. In the transfer horn (i.e. the uterine horn associated with the peeled ovary) the fetuses were identified as pigmented (endogenous) or albino (transferred).

The recovery of viable fetuses after transfer of 85 in vivo fertilized oocytes was 60% (Table 5). In all cases but one, the transfer horn contained only fetuses with non-pigmented eyes, indicating the albino donor as the source. In one case a single black-eyed fetus was found in the transfer horn, along with the non-pigmented fetuses. In all rats the control horn contained only fetuses with pigmented eyes.

There was no significant difference between the control and transfer sides with respect to fetal or placental weights ($P>0.1$).

5.3 Discussion

Early experiments by Neugebauer (1935) and Kelly (1939) indicated that opening the ovarian bursa in the albino rat distinctly lessens fecundity on the operated side: 44% of the rats studied by Kelly and 65% of those

TABLE 5 Percentage of transferred zygotes recovered as viable fetuses and mean fetal and placental weights 20 days after zygote transfer

Uterine horn	Embryos recovered/ transferred	No. of rats with fetuses	Source of fetuses	Mean ± s.e.m. fetal wt (g)	Mean ± s.e.m. placental wt (mg)
Control	---	15	Endogenous	1.86 ± [illegible]08	466 ± 21
Transfer	51/85 (60%)	15	Transferred	1.79 ± 0.09	451 ± 23
		1	Endogenous	1.98	463

studied by Neugebauer were unilaterally pregnant. This figure has been increased up to 93% using the modifications of the procedure described here. The use of this preparation as a recipient for transferred oocytes of various origins should be considered.

Our technique for peeling the bursa has met with some success with regards to maintaining a bursa-free ovary. In his experiments Kelly (1939) "pulled open (the bursa) with forceps" and 2 weeks later found complete resacculation of the ovary in 57% of his animals, with partial resacculation in 16%. It is not surprising then that he should find fetuses on the bursa-peeled side in 56% of the animals. A possibly more refined technique of bursa peeling was used in the present experiments with minimal, if any, bleeding.

By passing the ovary through a small opening, this method had two major advantages. First, the elastic properties of the bursa made it difficult for the ovary to slip back into the bursa after peeling. Secondly, by peeling the bursa from around the ovary, the bursa was consequently in a position to cover the infundibulum of the oviduct, further inhibiting any ovulated oocytes from entering the oviduct.

During the 5 weeks of experiments described in Section 5.2.1, complete re-encapsulation of the ovary occurred in 8% of the rats and partial re-encapsulation in another 8% of the animals. Oocytes were found in oviducts next to the peeled ovary in 18% of the animals. When the animals were used within 2 weeks of the bursa peeling (Sections 5.2.2 &

5.2.3), 12% showed complete re-encapsulation and 8% showed partial re-encapsulation of the ovary, but only 8% of the animals had fetuses in the uterine horn ipsilateral to the peeled ovary.

In the first experiment, the reasons for the increase in the number of corpora lutea between Weeks 1 and 5 both in the control and peeled ovaries are not yet known. There is evidence to suggest that the number of ovulations increases to a maximum during the early reproductive life of the female (Biggers et al., 1962; Kennedy & Kennedy, 1972).

Oocytes in the oviduct ipsilateral to the bursa-peeled ovary were not always found in rats with complete resacculation. On four occasions oocytes were found in the oviduct next to an ovary that was still completely exposed. These results agree with those of Neugebauer (1935) who concluded that the ovarian bursa is practically indispensable for oocyte pick-up and that bilateral pregnancy rarely occurs after unilateral bursa peeling.

The results of these experiments indicate that this preparation could be used to produce unilateral pregnancy with only a small margin of error (<8%). This error may be further reduced by excluding animals in which the peeled bursa has completely re-encapsulated the ovary. The surgical technique appears to have no effect on the ability of that ovary to ovulate or on the ability of zygotes to develop into viable fetuses in the ipsilateral uterine horn.

CHAPTER 6

EMBRYONIC DEVELOPMENT FOLLOWING IN VITRO FERTILIZATION

6.1 Introduction

Techniques for in vitro fertilization of rat oocytes have been established (Toyoda & Chang, 1974a; Kaplan & Kraicer, 1978; Evans & Armstrong, 1984) and it has been shown that rat oocytes fertilized in vitro are able to develop to fetuses (Toyoda & Chang, 1974a; Shalgi, 1983; Fleming, Evans, Walton & Armstrong, 1986), although there was a trend for the proportion of oocytes or embryos that developed to Day 20 fetuses to decrease with increasing divergence from the natural situation. Oocytes fertilized in the donor and transferred to a recipient within the next 24 h exhibited the highest rates of development, while oocytes fertilized in vitro exhibited the lowest rate. Oocytes transferred to a mated recipient immediately after ovulation, for fertilization and development in the recipient, showed an intermediate rate of development (Fleming et al., 1986). It was therefore suggested that in vitro fertilization or fertilization in the recipient resulted in lower rates of normal embryo development because of greater susceptibility of unfertilized oocytes to damage incurred during handling or to other environmental stresses.

There are several differences between conditions of

fertilization *in vivo* and *in vitro*. Although it has been established that greater embryonic loss occurs after *in vitro* than after *in vivo* fertilization, the factor or combination of factors that is responsible for the embryonic loss has yet to be determined. In addition little is known as to when during development these losses are occurring. Shalgi (1984) has demonstrated that the proportion of *in vivo* or *in vitro* fertilized oocytes that develop to Day 20 fetuses is only 10% less than the proportion of viable Day 13 fetuses, suggesting that the embryonic losses due to *in vitro* fertilization are occurring before Day 13. As the most critical event before Day 13 is probably implantation, this study will pay particular attention to the development of the embryo before implantation and the process of implantation itself.

The present set of experiments was designed to compare the development of fetuses to Day 20 after fertilization at different sites, and to determine the time of embryonic loss during the development of oocytes fertilized *in vitro*.

6.2 Methods and Results

The methodologies of the three experiments in this study are similar, differing only in the final stages of the experiment. The general protocol is, therefore, described first and presented in Figure 1. The distinctive features of each experiment are described in the subsequent

FIGURE 1

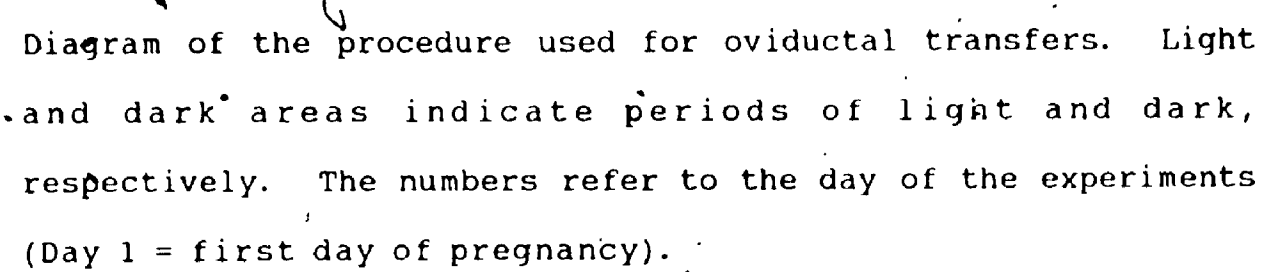
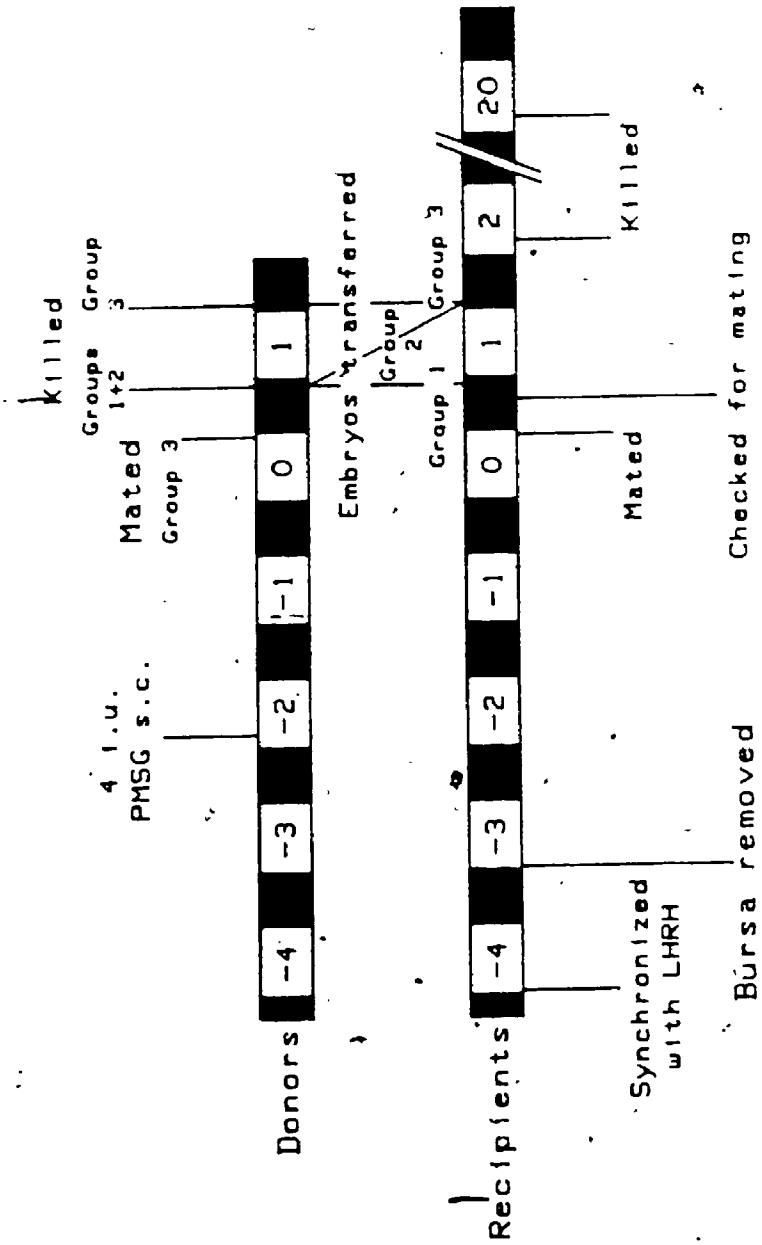


Diagram of the procedure used for oviductal transfers. Light and dark areas indicate periods of light and dark, respectively. The numbers refer to the day of the experiments (Day 1 = first day of pregnancy).


Donors
Recipients
-4
-3
-2
-1
0
1
2
20
4 I.u. PMSG s.c.
Mated Group 3
Killed Groups 1+2
Group 3
Embryos transferred
Group 1
Group 2
Group 3
Synchronized with LHRH
Bursa removed
Mated
Checked for mating
Killed

sections.

DONORS

At body weight 60-65 g, immature Sprague-Dawley rats were treated with 4 IU PMSG at 0800 h on Day -2. On Day 0 the rats were allocated randomly to 3 groups. Rats in the groups for transfer of unfertilized oocytes (Group 1) and of oocytes fertilized *in vitro* (Group 2) were killed on Day 1 between 0230 and 0500. The oviducts were dissected out in DPBS-S and the swollen ampullae were torn open with fine forceps to allow the oocytes to escape. The oocytes were either fertilized *in vitro* or transferred immediately to mated recipients (see below). Fertilization of all oocytes in Group 2 was assessed by phase-contrast microscopy 14-16 h after insemination. Zygotes with 2 pronuclei and 1 sperm tail within the vitellus were then transferred. Rats in Group 3 were caged with Sprague-Dawley males of proven fertility from 2000 on Day 0 to 0200 on Day 1, when they were assessed for mating. The presence of a copulatory plug and/or spermatozoa in the vaginal smear was taken as evidence of insemination. Mated donors were killed at 1900 to 2200 on Day 1 and the ova were flushed from the oviducts with DPBS-S, using a 30-gauge needle. Fertilized oocytes were collected and transferred immediately. *In vitro* and *in vivo* fertilized oocytes not transferred were cultured for a further 24 h period and development to the 2-cell stage was assessed.

RECIPIENTS

The recipients for the transfers were naturally cycling female Long-Evans rats, but when synchronization was necessary to ensure an adequate number of mated female rats, the rats were treated with an LHRH agonist as described in Section 5.2.1. At 3-5 days before the day of transfer, one ovary of each rat was exposed by bursa-peeling. At 2000 on Day 0, the recipient rats were caged with males of proven fertility. The rats were assessed for mating at 0200 on Day 1 and only mated rats were subsequently used. Transfers of unfertilized oocytes (Group 1) were performed at 0230 to 0500 on Day 1. Oocytes fertilized in the donor (Group 3) or *in vitro* (Group 2) were transferred at 1900 to 2200 on the same day. Oviductal transfers were performed as described in Section 5.2.3. Animals with evidence of large adhesions, significant blood clots, or whose peeled bursae had slipped back and enclosed the ovary were not used as recipients. Volume of transfer material varied between 0.5 and 1 µl for cumulus-enclosed oocytes (Group 1) to <0.4 µl for zygotes (Groups 2 & 3).

6.2.1 Effect of Site of Fertilization

One-third of rats in Group 1 were killed 16 h after transfer (1800, Day 1) to assess efficacy of fertilization and transfer. Both oviducts were removed and flushed with 0.2 ml DPBS-S (0.1 ml was passed through the oviduct from

the infundibulum; 0.1 ml was flushed in a retrograde manner, from the uterotubular junction). The recovered oocytes and zygotes were counted and examined under a phase-contrast microscope to assess the stage of development and the occurrence of fragmentation or other degenerative changes.

Recipients from all three groups were killed 40 h after insemination to assess efficacy of transfer and development to the 2-cell stage, or at 0800 on Day 20 to permit estimation of embryonic losses after the first cleavage. For animals killed on Day 20, the number of fetuses and the total fetal and placental weights were recorded for the control and transfer horns. The number and size of resorption sites in each horn were also noted.

At 16 h after the transfer of unfertilized oocytes into mated recipients (Group 1), 84.2% (n=38) of the oocytes recovered were fertilized. A similar proportion (85.7%, n=84) of endogenous oocytes in the control horn were fertilized. At 40 h, 77.1% of recovered oocytes were 2-cell embryos, which was significantly lower ($P<0.05$) than the percentage of 2-cell embryos recovered after transfer of zygotes following fertilization *in vivo* (93.9%, n=49) or *in vitro* (98.4%, n=61) (Groups 3 & 2). However, when adjusted for the 84% fertilization rate, the percentage of 2-cell embryos recovered after transfer of unfertilized oocytes increased to 91.5% (n=35), which was not significantly different from the values for the other two groups.

Pronuclear zygotes resulting from *in vitro* and *in vivo* fertilization and not transferred, but cultured for a further 24 h period, showed statistically similar *in vitro* cleavage rates of 89.9% (n=109) and 98.2% (n=56), respectively.

The proportion of transferred oocytes or zygotes recovered 40 h after insemination was significantly different between all three groups ($P<0.01$). (Table 6). The percentage of transferred oocytes or zygotes recovered on Day 20 reflected, in part, losses that occurred due to the transfer technique or to failure of fertilization. To indicate more accurately the developmental abilities of the oocytes, these numbers were corrected for the losses occurring before Day 2. After this correction, less than 20% of zygotes produced by *in vitro* fertilization were recovered as live fetuses. This low percentage of recovery on Day 20 was associated with a significantly higher ($P<0.005$) percentage of resorption sites, compared to Groups 1 & 3. About 90% of all resorption sites were <6 mm in diameter.

6.2.2 Embryonic Mortality after *in vitro* Fertilization

In this experiment, zygotes were derived from fertilization *in vitro* (Group 2) or *in vivo* in the donors (Group 3) and transferred as described. Recipients were killed at 1800 on Day 2 (40 h), 0800 on Day 5, 2000 on Day 8, or 0800 on Day 20, to identify more accurately the

significantly higher ($P<0.001$) percentage of resorption sites (32.7%); only 10% of Group 3 zygotes were recovered as resorption sites. All resorption sites were 6 mm or less in diameter.

6.2.3 Effect of Environmental Conditions on Oocyte Development

To determine possible effects of incubation and handling on the ability of oocytes fertilized *in vivo* to develop to Day 20 fetuses, donors (Group 3) were mated as described previously. At 6 h after mating, one-half of the mated rats were killed and the oocytes were collected and incubated for 10-12 h before transfer, under conditions similar to those used for *in vitro* fertilization, but without the addition of spermatozoa. The remaining mated rats were killed 16-18 h after mating and the oocytes and zygotes were collected, assessed for fertilization and the zygotes were transferred immediately. All recipients were killed at 0800 on Day 20.

The recovery of viable fetuses after transfer of *in vivo* fertilized oocytes with or without *in vitro* incubation was not significantly different (68.5%, n=92, and 62.8%, n=43, respectively). There was also a statistically similar proportion of resorbing fetuses in both groups (18.5%, n=92, and 11.6%, n=43, respectively).

The mean fetal and placental weights combined for all experiments are summarized in Table 10. There was no

timing of embryonic mortality following transfer of *in vitro* fertilized oocytes. For animals that were killed on Days 2 or 5, both oviducts were removed and flushed with DPBS-S, as described in Section 6.2.1. On Day 5, the uterine horns were flushed as well, in a manner similar to that described for flushing oviducts. The recovered oocytes, zygotes and embryos were counted and examined microscopically to assess the stage of development. On Day 8, implantation sites were dissected out, counted and weighed. They were fixed in Bouins's solution, dehydrated and embedded in paraffin wax. Serial sections of 10 um were stained with haematoxylin and eosin and examined for evidence of normal implantation. Animals killed on Day 20 were analyzed as described in the previous experiment.

There were notable differences between *in vitro* and *in vivo* fertilized oocytes (Groups 2 & 3, respectively) with respect to the stage of development at which the transferred zygotes were lost (Table 7). Zygotes transferred after *in vivo* fertilization were lost primarily during the 24 h after transfer (26%) with no significant loss between Days 2 and 20. In contrast, 24% of the *in vitro* fertilized oocytes were lost during the development from 2-cell zygotes to morulae or blastocysts, with further losses occurring after the blastocyst stage (40%).

In each group the percentage of recovered zygotes that had cleaved to 2 cells was comparable to that in the first experiment, with 89.7% of *in vitro* fertilized oocytes and

TABLE 7 Recovery of embryos at 2, 5, 8 and 20 days after the transfer of *in vitro* (Group 2) or *in vivo* (Group 3) fertilized oocytes

Time killed	Stage of development	% (of total no.) of zygotes recovered	
		Group 2	Group 3
Day 2	2-cell zygotes	86.7 (45)[a]	74.0 (50)[a]
Day 5	Blastocysts/morulae	62.4 (85)[a]	67.7 (65)[a]
Day 8	Implantation/ decidualization sites	57.5 (80)[a]	62.9 (70)[a]
Day 20	Fetuses	22.7 (110)[a]	73.3 (90)[b]

a,b Within each time, values with different superscripts are significantly different ($P<0.001$).

100% of *in vivo* fertilized oocytes developing to the 2-cell stage ($P>0.05$).

Embryos produced by *in vivo* fertilization and recovered on Day 5 were primarily at the blastocyst stage of development, although a small percentage were still at the morula stage (Table 8). These results were not statistically different from the development of embryos in the control horns of both groups. In contrast, more than half of the embryos produced in Group 2 were developing at a slower rate than those in the control horn, as indicated by the significantly higher proportion of Group 2 embryos that were at the morula stage ($P<0.001$). In both groups, <3% of the recovered embryos were degenerate.

On Day 8 there was a significant difference ($P<0.05$) between the mean weights (g) of the implantation sites in the transfer horns of rats in Groups 2 & 3 (Table 9). Histological examination of the implantation sites from the transfer horn in Group 2 rats revealed that 60% of the 20 sites examined showed no evidence of an embryo or trophoblastic invasion, suggesting that the embryos elicited a decidual response, but developed no further. The remaining 40% of sites and those from the transfer horn in Group 3 ($n=16$) and the control horns in both groups ($n=27$) all contained embryos with evidence of implantation.

The percentage of transferred zygotes that developed to Day 20 fetuses in this experiment (Table 7) was comparable to the recoveries in the first experiment. Associated with the great embryonic losses in Group 2 was a

TABLE 8 Development of transferred *in vitro* (Group 2) and *in vivo* (Group 3) fertilized oocytes to Day 5 embryos

	Stage of development	% (of total no.) of zygotes recovered	
		Group 2	Group 3
Control horn	Blastocyst	89.2 (93)[a]	86.3 (73)[a]
	Morula	8.6 (93)[c]	13.7 (73)[c]
Transfer horn	Blastocyst	45.3 (53)[b]	88.6 (44)[a]
	Morula	52.8 (53)[d]	9.1 (44)[c]

a,b,c,d Values with different superscripts for each developmental stage are significantly different ($P<0.001$).

TABLE 9 Mean weights of implantation sites 8 days after zygote transfer

	Mean weight of implantation sites (g)	
	Group 2	Group 3
Control horn	35.8 ± 1.2 (15)[a]	33.9 ± 2.2 (13)[a]
Transfer horn	29.4 ± 1.8 (15)[b]	35.2 ± 1.4 (13)[a]

Values are mean ± s.e.m. for the number of rats in parentheses.

[a,b] Values with different superscripts are significantly different ($P<0.05$).

significantly higher ($P<0.001$) percentage of resorption sites (32.7%); only 10% of Group 3 zygotes were recovered as resorption sites. All resorption sites were 6 mm or less in diameter.

6.2.3 Effect of Environmental Conditions on Oocyte Development

To determine possible effects of incubation and handling on the ability of oocytes fertilized *in vivo* to develop to Day 20 fetuses, donors (Group 3) were mated as described previously. At 6 h after mating, one-half of the mated rats were killed and the oocytes were collected and incubated for 10-12 h before transfer, under conditions similar to those used for *in vitro* fertilization, but without the addition of spermatozoa. The remaining mated rats were killed 16-18 h after mating and the oocytes and zygotes were collected, assessed for fertilization and the zygotes were transferred immediately. All recipients were killed at 0800 on Day 20.

The recovery of viable fetuses after transfer of *in vivo* fertilized oocytes with or without *in vitro* incubation was not significantly different (68.5%, n=92, and 62.8%, n=43, respectively). There was also a statistically similar proportion of resorbing fetuses in both groups (18.5%, n=92, and 11.6%, n=43, respectively).

The mean fetal and placental weights combined for all experiments are summarized in Table 10. There was no

TABLE 10 Mean fetal and placental weights 20 days after transfer of unfertilized oocytes (Group 1) or zygotes derived from *in vitro* (Group 2) or *in vivo* (Group 3) fertilization

Group	No. of rats	Fetal weight (g)		Placental weight (mg)	
		Control side	Transfer side	Control side	Transfer side
1	10	2.22 ± 0.05[a]	2.00 ± 0.07[a]	489 ± 12[a]	592 ± 50[b]
2	11	2.07 ± 0.05[a]	1.79 ± 0.11[a]	485 ± 16[a]	510 ± 44[a]
3					
No incub.	31	2.02 ± 0.05[a]	1.90 ± 0.05[a]	465 ± 12[a]	456 ± 13[a]
Incub.*	19	2.05 ± 0.03[a]	1.98 ± 0.04[a]	454 ± 11[a]	451 ± 15[a]

* Incubation for 10-12 h.

Values are mean ± s.e.m. for the number of rats in parentheses.

Values with different superscripts are significantly different ($P<0.005$), within columns and between relevant columns.

significant difference between experiments in any of the values measured. There were no significant differences in the mean fetal and placental weights in the control horn, although Bartlett's test indicated heterogeneity of variance amongst the fetal weights. In the transfer horn there was no significant difference between the mean fetal weights of the four groups. However, mean placental weights of the fetuses in Group 1 was significantly greater than those in Group 2 and Group 3 ($P<0.005$).

6.3 Discussion

The experiments reported here have examined a number of the potential factors that may be responsible for the reduced developmental abilities of embryos produced by _in vitro_ fertilization. It is clear that handling of the unfertilized oocyte with subsequent fertilization _in vivo_ had no detrimental effect on the development of the oocyte. Following fertilization in the recipient (Group 1), the transferred oocytes were able to develop into 2-cell embryos or Day 20 fetuses in proportions comparable to oocytes that were fertilized in the donor and then transferred at the pronuclear stage (Group 3).

Transfer of unfertilized oocytes to mated recipients for _in vivo_ fertilization gave a significantly lower rate of recovery of fetuses than did transfer of oocytes fertilized in the donor. When corrected for losses within 40 h of transfer, the rate of recovery indicated that only

a small portion of the loss of embryos occurred after the 2-cell stage of development, i.e. the proportion of 2-cell embryos that developed to Day 20 fetuses was similar in both groups after in vivo fertilization. The low recovery of 2-cell embryos at 40h was not significantly different from the percentage of oocytes recovered as zygotes 16 h after transfer, suggesting that embryo loss following transfer of unfertilized oocytes may be at least partly due to losses of the oocytes shortly after transfer. When oocytes were collected immediately after ovulation and transferred, the presence of cumulus cells necessitated the use of a larger bore pipette and a larger volume of medium, which has been suggested to increase losses due to regurgitation (Shalgi et al., 1979).

The results of the experiments presented here show that, compared to oocytes fertilized in vivo, a lower proportion of oocytes fertilized in vitro develop to viable fetuses. A comparable decline in development was observed when in vitro fertilized oocytes were transferred at the 2-cell stage of development (Shalgi, 1983). By recovering embryos on Day 2, the present study determined that there was no deficiency in the ability of in vitro fertilized oocytes to undergo the first cleavage division. With correction for losses before Day 2, it is evident that the majority of the embryonic losses occurred after the 2-cell stage of development, which is in contrast to the time of loss of oocytes transferred before fertilization. This finding is supported by the number of resorption sites that

are seen on Day 20. The increased number of resorption sites found in Group 2 suggests that some of the losses occurred at or after implantation.

Shalgi (1984) indicated that 34% of *in vitro* fertilized rat zygotes developed into Day 13 fetuses, when examined by laparotomy. The present study demonstrated that a fairly high percentage (58%) of zygotes derived from *in vitro* fertilization will develop to embryos capable of eliciting decidualization, but a considerable proportion of these undergo no further development. The slower rate of development of such zygotes to the blastocyst stage suggests a possible reason for the high rate of unsuccessful implantation in Group 2. When the embryo is delayed in development, as it was in 53% of the embryos from *in vitro* fertilization, implantation may or may not occur, depending on the degree of asynchrony in development of the embryo and the uterine environment (Dickmann & Noyes, 1960; Mantalenakis & Danezis, 1968). Since a variety of artificial physical stimuli can increase endometrial vascular permeability and bring about decidualization (Psychoyos, 1973), it is possible that, during the period of optimal endometrial sensitivity, the physical presence of the embryo, even at an earlier stage of development, may be sufficient to initiate the decidual cell reaction, without subsequent implantation. This postulate is supported by the results of the histological examination of the uterine swellings. For example, some of

what were recorded as implantation sites on Day 8 were simply sites of decidualization in the absence of implantated embryos. It is possible that these sites represented early implantation of an embryo in which the inner cell mass was incapable of further development. The lower mean weight of the implantation sites in Group 2, as compared to that in Group 3, is indicative of the higher proportion of decidualization sites as opposed to implantation sites with developing embryos. The majority of the resorption sites seen on Day 20 (i.e. those <6 mm) were probably necrotic masses that resulted from the regression of these deciduomata (Lobel, Tic & Shelesnyak, 1965).

In the present study the majority of the embryonic losses after *in vitro* fertilization occurred after the 2-cell stage of development. As there was no evidence for developmental inability after *in vivo* fertilization, it may be concluded that embryo losses beyond the 2-cell stage of development are due to defects in the embryos produced by *in vitro* fertilization. Losses might be due to the absence, in culture, of some factor provided *in vivo* during the 16 h after fertilization, or culture conditions may have had a detrimental effect which was not manifested in the zygote until later stages of development. The results of the third experiment indicate that, after fertilization *in vivo*, handling of zygotes, incubation or exposure to environmental conditions such as light or changes in temperature do not have any detrimental effects on the

development to Day 20 fetuses.

During in vitro fertilization, the conditions for gamete interaction vary considerably from conditions in vivo. Exposure of the oocytes to a large number of spermatozoa leads to relatively high rates of polyspermic fertilization (Niwa & Chang, 1975a). In this experiment oocytes fertilized in vitro and intended for transfer were examined and only those with two pronuclei and one sperm tail within the vitellus were transferred. These criteria were insufficient, however, to assess the developmental abilities of the zygotes. Shalgi & Phillips (1982) have demonstrated a different morphological pattern of sperm attachment and penetration into oocytes in vitro compared to in vivo. It is clear that further investigation is needed into the relevance of this difference, and others resulting from in vitro conditions, in the development of the zygotes.

Development of fetuses after the transfer of unfertilized oocytes into mated recipients was associated with placental weights that were greater than those of the other groups. Although it was not significant, placentas associated with fetuses developing from oocytes fertilized in vitro were also heavier. The reason for these elevated weights is not known, but it may be associated with the fact that generally there are fewer fetuses developing in the transfer uterine horn of rats in these two groups.

The similarity in the percentage survival of fetuses

developing from rat oocytes fertilized *in vitro* in our studies and the overall pregnancy rates generally reported in the most successful human *in vitro* fertilization programs (Grobstein *et al.*, 1983) suggests that similar mechanisms may be operating to cause the high rate of post-fertilization embryonic losses in both species.

CHAPTER 7

MODIFYING PREIMPLANTATION EMBRYONIC DEVELOPMENT FOLLOWING *IN VITRO* FERTILIZATION

7.1 Introduction

Most human oocytes retrieved can be successfully fertilized *in vitro* but, even though several embryos are returned to the women, usually less than 20% of embryo transfers result in full-term pregnancy (Grobstein *et al.*, 1983; Jones *et al.*, 1984; Wood *et al.*, 1985). Animal models have been developed to facilitate the study of embryonic and fetal development of transferred embryos resulting from fertilization *in vitro* (Noyes, 1952; Toyoda & Chang, 1974a; Walton & Armstrong, 1983). In these models early investigations indicated that, after transfer, oocytes fertilized *in vitro* were capable of development to full-term fetuses in the low proportions similar to those seen in humans. In addition, experiments in the previous chapter demonstrated that many embryos from *in vitro* fertilized oocytes were slower to advance to the blastocyst stage than were those from oocytes fertilized *in vivo*. When an embryo is delayed in development, implantation may or may not occur, depending on the degree of asynchrony in development of the embryo and the uterine environment (Dickmann & Noyes, 1960). Therefore, the slower rate of development of the *in vitro* fertilized embryos may be the

cause of unsuccessful implantation. The present studies were undertaken to determine whether the ability of *in vitro* fertilized oocytes to produce full-term fetuses could be increased by providing them with additional time for preimplantation development.

7.2 Methods

As the experimental protocol is rather complex, the sequence of events has been summarized in Figure 2.

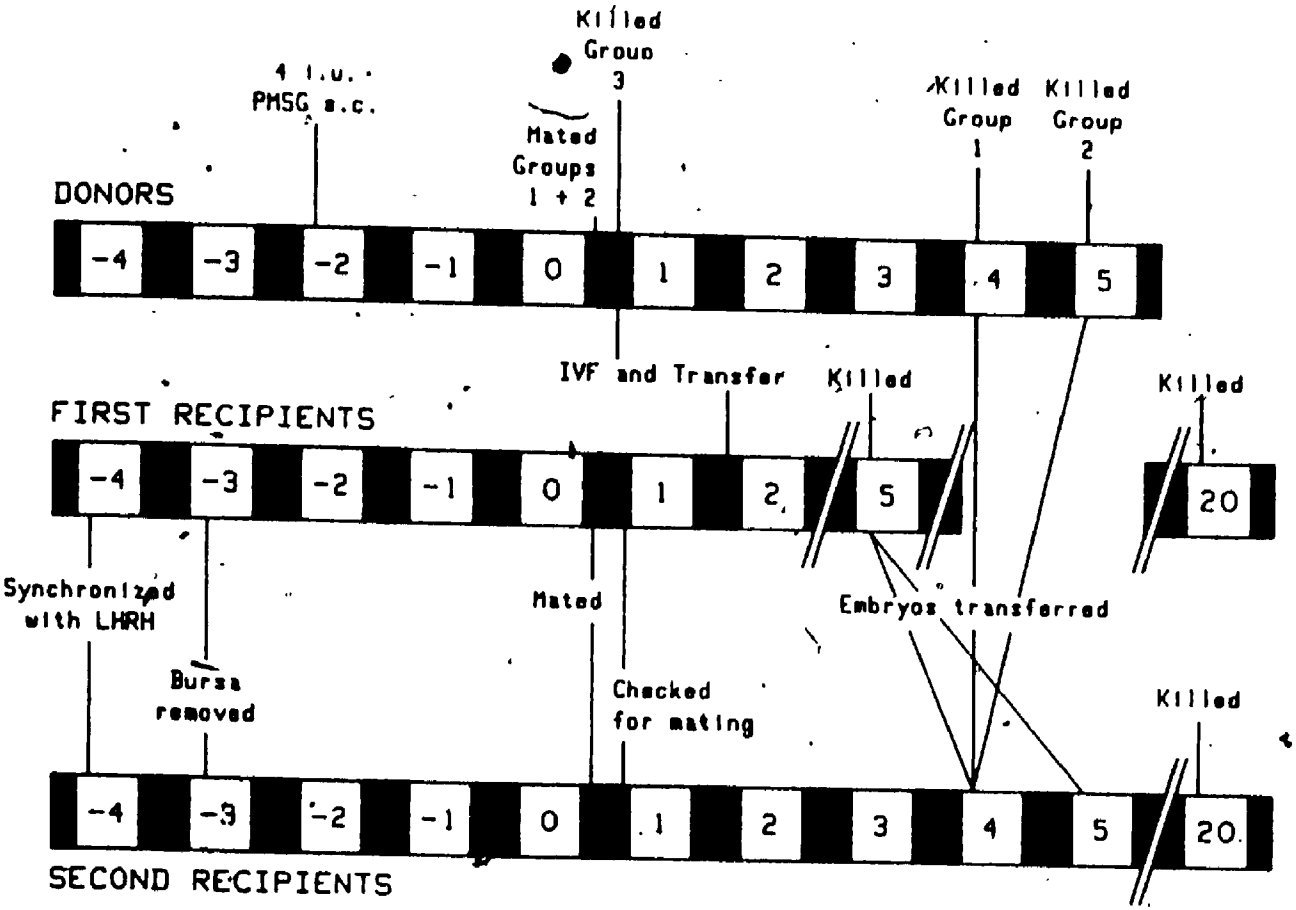
DONORS

At a body weight of 60-65 g, immature Sprague-Dawley rats were treated with 4 IU PMSG at 0800 on Day -2. On Day 0 the rats were allocated randomly to 3 groups. Rats in the group for transfer of Day 4 control embryos (Group 1) and of Day 5 control embryos (Group 2) were caged with males of proven fertility from 2000 on Day 0 to 0400 on Day 1, when they were assessed for mating. The presence of a copulatory plug and/or spermatozoa in the vaginal smear was taken as evidence of insemination. Mated donors were killed at 2000 to 2200 on Day 4 (Group 1) or at 0800 to 1000 on Day 5 (Group 2). Using a 30-gauge needle, the embryos were flushed from the oviducts and/or uterine horns with DPBS-S, as described in Section 6.2.1. Embryos were collected and transferred immediately to mated recipients (see below).

Rats in the group for transfer of oocytes fertilized

FIGURE 2

Diagram of the procedure used for embryo transfers. Light and dark areas indicate periods of light and dark, respectively. The numbers refer to the day of the experiment (Day 1 = first day of pregnancy).


4 I.U. PMSG s.c.
Killed Group 3
Mated Groups 1 + 2
Killed Group 1
Killed Group 2
DONORS
-4
-3
-2
-1
0
1
2
3
4
5
IVF and Transfer
Killed
Killed
FIRST RECIPIENTS
-4
-3
-2
-1
0
1
2
5
20
Synchronized with LHRH
Bursa removed
Mated
Checked for mating
Embryos transferred
Killed
-4
-3
-2
-1
0
1
2
3
4
5
20
SECOND RECIPIENTS

in vitro (Group 3) were killed on Day 1 between 0100 and 0300. The oviducts were dissected out in DPBS-S and the swollen ampullae were torn open with fine forceps to allow the oocytes to escape. The oocytes were then fertilized *in vitro* as described previously with fertilization rates ranging from 85-100%. Fertilization was assessed by phase-contrast microscopy 14-16 h after insemination and only zygotes with 2 pronuclei and 1 sperm tail within the vitellus were transferred to mated recipients.

RECIPIENTS

The recipients for the transfers were prepared as in previous experiments. The cycles of female Long-Evans rats were synchronized with 40 µg LHRH agonist and at 3-14 days before the day of mating, the ovarian bursa was peeled from around the left ovary of each recipient. At 2000 on Day 0, the recipient rats were caged with males of proven fertility. They were assessed for mating at 0400 on Day 1 and only mated rats were subsequently used. Some of the mated rats were used at 2000 to 2300 as Day 1 recipients for oocytes that had been fertilized *in vitro*. The remaining rats were left until Day 4 of pregnancy at which time they became recipients of Day 4 or Day 5 embryos (see below).

COLLECTION AND TRANSFER OF EMBRYOS

Some of the Day 1 recipients of *in vitro* fertilized oocytes were allowed to reach Day 20 of gestation, at which

time the transferred zygotes were assessed for their ability to develop to near-term fetuses. At 0600 to 0800 on Day 5 the remaining recipients were killed and both the transfer and the control oviducts and uterine horns were removed and flushed with DPBS. The previous study indicated that half of the embryos developing from in vitro fertilized oocytes and collected at this time were morulae, while the remaining embryos were at the blastocyst stage. Therefore, when the embryos were collected in the current experiment, they were divided into 2 groups, depending on their stage of development (Group 3m = morulae; Group 3b = blastocysts). Groups of four or 5 embryos were transferred into the left oviduct or uterine horn of each of the Day 4 pregnant recipients. Control embryos from the Day 4 (Group 1) and Day 5 (Group 2) donor rats were collected and transferred in a similar manner. As an additional control, some of the Day 5 Group 3 embryos (morulae and blastocysts) were transferred into the uterine horn of Day 5 pregnant recipients. Oviductal transfers were performed as described earlier. For uterine transfers a small portion of the uterine horn near the uterotubular junction was drawn through an incision by pulling the fat pad. A small hole was made near the top of the horn with the point of an 18-gauge needle. The transfer pipette was inserted into this hole and the embryos were gently expelled.

To determine the stage of embryo development more accurately, some of the embryos from each group were used

for cell counting. Using a method described by Fujimoto et al. (1975), an embryo was fixed in a solution of methanol:glacial acetic acid:deionized water (4:1:3, by vol.) which caused dissolution of the zona pellucida. The embryo was quickly transferred to a glass slide. The cells were separated by allowing drops of a methanol:glacial acetic acid (3:1, v/v) solution to fall on the embryo from a height of 10-12 cm. The embryo was then stained with haemotoxylin and the number of cells was counted.

The recipient rats were killed at 0800 on Day 20. The number of fetuses and the total fetal weights and placental weights were recorded for the control and transfer horns of each rat. The number and size of resorption sites in each horn were also noted.

7.3 Results

As in the previous study, the control embryos produced by in vivo fertilization and recovered on Day 5 were primarily at the blastocyst stage of development (Table 11). In contrast, half of the embryos developing from in vitro fertilized oocytes were still at the morula stage, indicating that the development of these embryos was retarded compared with those in the control horn.

Embryos from each group were collected and the number of cells per embryo was determined (Table 12). As could be expected, Day 5 control embryos had a significantly greater mean number of cells per embryo than did the Day 4 control

TABLE 11 Stage of development of *in vitro* fertilized rat oocytes on Day 5 after transfer to recipients

	Stage of development	% (of total no.) of zygotes recovered
Control horn	Blastocyst	80.9 (444)[a]
	Morula	19.1 (444)[b]
Transfer horn	Blastocyst	51.1 (377)[c]
	Morula	48.8 (377)[c]

a,b,c Values with different superscripts are significantly different ($P<0.001$).

TABLE 12 Mean number of cells per embryo at time of transfer into Day 4 pregnant recipient rats

Group	Embryo	Source	Mean (±s.e.m.) no. of cells/embryo (n)
1	Day 4 morula	control	22.4 ± 0.6 (57)[a]
2	Day 5 blastocyst	control	26.1 ± 0.8 (39)[b]
3m	Day 5 morula	IVF	17.9 ± 1.1 (21)[c]
3b	Day 5 blastocyst	IVF	24.5 ± 0.8 (25)[ab]

[a,b,c] Values with different superscripts are significantly different ($P<0.01$).

embryos ($P<0.01$). Blastocysts derived from *in vitro* fertilized oocytes contained a mean number of cells per embryo that was not significantly different from that of the control blastocysts or morulae. In agreement with the morphologically evident retardation in development of the morulae from *in vitro* fertilized oocytes, these embryos showed a significantly lower mean number of cells per embryo compared to the other three groups ($P<0.01$).

The proportion of embryos recovered as near-term fetuses after transfer into Day 4 recipients is summarized in Table 13. Embryos developing from *in vitro* fertilized and control oocytes were equally capable of implantation and fetal development when transferred into the oviducts of the recipients. However, the survival of the Group 3 morulae after transfer into the recipient uterine horn was significantly less than that of Group 3 blastocysts or the control morulae or blastocysts ($P<0.01$), with no significant differences between the last three groups. Within Groups 1, 2 and 3b, the embryos were noticeably more capable of implantation and fetal development when transferred into the uterine horn rather than into the oviduct of the Day 4 recipient, although this improvement was statistically significant ($P<0.001$) only in Group 1. There was no significant difference in the ability of Group 3 morulae to develop to fetuses after transfer into the oviduct or uterus.

The proportion of transferred embryos recovered as resorption sites in the transfer uterine horn on Day 20 is

TABLE 13 Number of embryos recovered as fetuses and resorption sites (R.S.) on Day 20 after transfer into Day 4 pregnant recipient rats

			% (of total no.) of transferred embryos recovered			
			Into oviduct		Into uterus	
Group	Embryo	Source	Fetuses	R.S.	Fetuses	R.S.
1	Day 4 morula	control	$27.4^{a,x}$	3.2 (62)	$66.1^{a,y}$	13.8 (65)
2	Day 5 blastocyst	control	$40.7^{a,x}$	8.6 (81)	$54.6^{a,x}$	15.6 (77)
3a	Day 5 morula	IVF	$26.7^{a,x}$	12.9 (60)	$18.4^{b,x}$	18.4 (49)
3b	Day 5 blastocyst	IVF	$34.1^{a,x}$	13.4 (82)	$45.4^{a,x}$	13.6 (44)

For fetuses within columns, values with different alphabetical superscripts (a,b) are significantly different ($P<0.01$); within rows values with different superscripts (x,y) are significantly different ($P<0.001$).

also summarized in Table 13. There were no significant differences among the four groups in the incidence of resorption sites following embryo transfer into the oviducts or uterine horns. In addition, the proportion of embryos recovered as resorption sites after oviductal transfer was not significantly different from that of embryos recovered after uterine transfer within each group. Most resorption sites (92%) were <1 cm in diameter.

The results of transfers of zygotes/embryos derived from *in vitro* fertilization are summarized in Table 14. Some of the *in vitro* fertilized zygotes transferred on Day 1 were assessed on Day 20 of gestation for their ability to develop to near-term fetuses without an intermediate transfer. A large proportion of the transferred zygotes became viable fetuses with 17.6% of the zygotes being recovered as resorption sites. This success rate is comparable to the proportions of Day 5 blastocysts derived from *in vitro* fertilized oocytes (Group 3) that were recovered as fetuses after synchronous transfer into Day 5 pregnant recipients or asynchronous transfer into Day 4 recipients. The transfer of Group 3 morulae into Day 4 or 5 recipients showed a significantly lower success rate ($P<0.01$).

The mean fetal and placental weights for each group with transfers of embryos on Day 4 are summarized in Table 15. Within each treatment there was no significant difference between the mean fetal or placental weights of

TABLE 14 Number of *in vitro* fertilized zygotes/embryos recovered as fetuses and resorption sites (R.S.) on Day 20 after transfer into pregnant recipient rats

Stage of development	Stage of recipient	Transferred into	No. embryos	% Fetuses	% R.S.
Day 1 zygote	Day 1	oviduct	119	47.1[a]	17.6
Day 5 morula	Day 4	uterus	49	18.4[b]	18.4[c]
Day 5 morula	Day 5	uterus	43	9.3[b]	23.5[c]
Day 5 blastocyst	Day 4	uterus	44	45.4[a]	13.6[c]
Day 5 blastocyst	Day 5	uterus	42	42.9[a]	14.3[c]

a,b,c Values with different superscripts are significantly different ($P<0.05$).

TABLE 15 . Mean fetal and placental weights on Day 20 of gestation in rats after transfer of embryos on Day 4

Group	No. of rats	Fetal weight (g)		Placental weight (mg)	
		Control side	Transfer side	Control side	Transfer side
1 Cont. morula					
Oviduct	5	2.08 ± 0.06	1.97 ± 0.07	532 ± 26	541 ± 45
Uterus	11	2.03 ± 0.05	1.99 ± 0.05	468 ± 16	465 ± 16
2 Cont. blastocyst					
Oviduct	11	1.97 ± 0.04	1.95 ± 0.05	476 ± 16	497 ± 24
Uterus	15	1.98 ± 0.04	2.14 ± 0.08	478 ± 11	486 ± 17
3m IVF morula					
Oviduct	9	2.01 ± 0.06	1.91 ± 0.07	473 ± 17	441 ± 15
Uterus	7	2.07 ± 0.08	2.15 ± 0.10	500 ± 15	499 ± 30
3b IVF blastocyst					
Oviduct	10	2.01 ± 0.06	2.02 ± 0.07	465 ± 8	526 ± 29
Uterus	7	2.06 ± 0.08	2.15 ± 0.10	501 ± 35	492 ± 19

Values are mean ± s.e.m. for the number of rats in parentheses.

the transfer side compared with those of the control side.

7.4 Discussion

These experiments have examined the possibility that low embryonic survival of *in vitro* fertilized oocytes is due to asynchronous development of the embryo and the uterine environment at the time of implantation. In the previous study it was determined that *in vitro* fertilized oocytes showed a delay in development such that, at 0800 on Day 5, half of the embryos were at the morula stage whereas most control embryos were blastocysts. This observation has been confirmed in the current experiments. It has been demonstrated that *in vitro* development of mouse 1-cell embryos to the blastocyst stage is delayed compared to development within the reproductive tract of pregnant (Bowman & McLaren, 1970; Harlow & Quinn, 1982), pseudopregnant or immature (Papaioannou & Ebert, 1986) females. Although the oocytes in the present experiments were in culture for only 16-18 h, it possible that this period *in vitro* was sufficient to cause the delay in the development of these oocytes. The previous study indicated, however, that a 10-12 h period of in vitro culture before transfer had no detrimental effect on the ability of *in vivo* fertilized oocytes to develop to Day 20 fetuses. The delay in embryonic development in this experiment was quantified by counting the number of cells in embryos from each group. The mean number of cells in

the Day 5 control blastocysts (26.1 ± 0.8) agrees well with that found in a study by Surani (1975) with a value of 27.5 ± 4.0 (n=23). The lower mean number of cells in the Group 3 morulae supports the suggestion that fertilization *in vitro* and/or the time *in vitro* associated with it caused a delay in the development of the embryos that ultimately became detrimental at the time of implantation.

The experiments reported here demonstrate the increased ability of some Group 3 embryos (IVF morulae) to develop to fetuses if given additional time for preimplantation growth. The transfer of Group 3 morulae into the oviduct resulted in a proportion of embryos developing to fetuses that is similar to the other three groups. Transfer of these embryos into the uterus, however, is not conducive to implantation and subsequent fetal growth. Furthermore, transfers of Group 3 morulae into Day 5 uterine horns, i.e. not given additional time for preimplantation growth, result in a rate of survival significantly less than that for transfers into Day 4 recipients. These results lead to a number of possible explanations: (1) that the oviduct is a more favorable environment for embryos retarded in development; (2) that the younger embryos are too delayed in development to catch up with the state of the uterus; or (3) that the uterus is actually a hostile environment, less tolerant of embryos that are not at the appropriate stage of development.

Early studies in rabbits (Chang, 1952) and mice (Bowman & Roberts, 1958) demonstrated the ability of nearly

all ovulated oocytes to be fertilized and develop normally in the oviduct. A significant proportion of the reproductive losses in these species resulted from functional defects occurring around the time of implantation. Implantation takes place only when the embryo and the endometrium have simultaneously reached specific stages in their development (Noyes & Dickmann, 1960, 1961; Dickmann & Noyes, 1960; Noyes et al., 1963). Transferred embryos that were 1 day younger than the recipient uterus developed at the usual rate until the fifth day of pregnancy and then degenerated rapidly and failed to implant. It was concluded that the uterine environment undergoes changes in the afternoon of Day 5, becoming detrimental to younger embryos, but stimulating Day 5 blastocysts in such a way that they elicit the decidual reaction. These results possibly explain the poor survival of the Group 3 morulae when transferred directly into the uterus in the current experiments.

In contrast to transfers into the uterus, oviductal transfers may offer some advantages to the embryo, particularly if it is delayed in development. With asynchronous development of the embryo and the recipient uterine environment, there appears to be a wider margin of safety for oviductal than for uterine transfers. Noyes & Dickmann (1961) determined that embryos 1 to 5 days of age were capable of surviving transfer to the oviduct while only 3- to 5-day-old embryos survived transfer to the

uterus. While the oviduct may offer advantages when the exact stage of development of the donor embryos is uncertain (as it was with the Group 3 embryos), the uterus obviously has its own benefits when receiving embryos of more appropriate stages: slightly larger percentages of embryos survived transfer to the uterus than to the oviduct. This is in agreement with the work done by Noyes & Dickmann (1961).

It is tempting to extrapolate the results of these experiments to the human programs for in vitro fertilization. In natural cycles, the blastocyst normally enters the uterus 3-4 days after ovulation (Jones, 1984). In these programs, however, 4-cell embryos are commonly transferred into the uterus 2 days after follicular aspiration. In addition, endometrial maturation may be more advanced as a consequence of the endocrine manipulations used for follicular stimulation (Martel et al., 1987). Therefore, an embryo is being transferred into a uterine environment which may be 1-3 days advanced in relation to the development of the embryo; this difference may be sufficient to prevent implantation. Testart (1987) has shown a high pregnancy rate when transfers were performed with frozen-thawed embryos coming from in vitro fertilization cycles in which pregnancy did not occur after transfer of fresh embryos. He suggested that the unsuccessful in vitro fertilization cycles were the consequence of an unfavorable uterine environment resulting from the ovarian stimulation procedures and that an

increase in the success rate of *in vitro* fertilization can be predicted if embryos are frozen and stored to be transferred at a later cycle into a more normal uterine environment not influenced by exogenous hormonal stimulation. In contrast, there is some evidence to suggest that the advanced endometrium may be more conducive to implantation, regardless of the state of development of the embryo (Jones, 1984). Clearly more information about the mechanism of implantation in humans is necessary before comparisons can be made.

In conclusion, some embryos derived from in vitro fertilized oocytes have a slower rate of development that appears to be detrimental at the time of implantation. If given additional time for preimplantation development, many of these embryos are capable of implantation and normal fetal growth. The developmental potential of transferred *in vitro* fertilized rat oocytes therefore appears to be limited, at least partly, by the asynchrony between the embryo and the uterine environment, rather than by a defect in the oocyte caused by the fertilization procedure.

CHAPTER 8

OOCYTE MATURATION IN VITRO

8.1 Introduction

It is accepted widely that the endogenous LH surge triggers the resumption of meiosis in oocytes in vivo. As a result the chronological relationship between these two events can be established accurately by the administration of exogenous gonadotropin (usually hCG) and observation of subsequent maturational events. In this manner it has been determined that, following gonadotropin injection, GVBD occurs after 2 h in the mouse (Edwards & Gates, 1959) and rabbit (Moricard & Henry, 1967), 2-3 h in the rat (Vermeiden & Zeilmaker, 1974) and hamster (Iwamatsu & Yanagimachi, 1975), 18 h in the pig (Hunter & Polge, 1966) and an estimated 25 h in the human (Edwards, 1965b). If large preovulatory follicles from adult cycling rats are explanted before the preovulatory LH surge, the resumption of meiosis must be triggered by hormonal stimulation (see Section 2.1.2 D). A 4 h culture period is needed for GVBD to occur in follicle-enclosed oocytes exposed to LH (Lindner et al., 1974). In most studies, however, a large number of synchronously growing follicles and oocytes are obtained from immature animals treated with PMSG (Cole & Hart, 1930). In vivo the majority of mammalian oocytes degenerate through follicular atresia and never reach the

stage of normal maturation or ovulation (Peters et al., 1975; Peluso et al., 1977). Thus, a major benefit of collecting follicles or oocytes from PMSG-treated immature animals is that this procedure ensures the selection of non-atretic follicles for culture.

Administration of PMSG to immature animals induces the development of a number of follicles, but the oocytes are maintained in the dictyate stage of meiosis. Since the oocytes are usually meiotically competent at the time of PMSG injection, it is clear that the LH content of the PMSG preparation is not sufficient to initiate the resumption of meiosis.

This system is ideal, then, for ensuring that the oocyte maintains a physiological relationship with the other components of the follicle until just prior to the preovulatory LH surge. At this time the oocyte can be removed from the follicle and maintained in meiotic arrest for a further period of time in vitro (see Section 2.1.2 C) to facilitate our understanding of the inhibition imposed by the follicular environment. Alternatively, the isolated oocytes can be allowed to undergo spontaneous maturation under various conditions, in attempts to understand the requirements of the oocyte in achieving fertilizability and developmental competence.

The purpose of this study was to isolate oocytes from PMSG-treated rats and to identify the essential features of a culture system for maturation in vitro. In doing so the

optimal conditions for in vitro maturation were determined such that maximum fertilizability of the oocytes and normal fetal development could be achieved.

8.2 Methods and Results

8.2.1 Follicle vs Oocyte Cultures

The specific aim of this study was to compare the fertilizability of oocytes following maturation within the follicle or as isolated oocyte-cumulus cell complexes, in the presence or absence of LH. Immature rats were injected subcutaneously with 6 IU PMSG at 0800 on Day -2. They were given an intraperitoneal injection of 15 IU hCG 24 h later and were divided into 2 groups. At 4 h after the administration of hCG, all rats were killed. From one ovary of each rat, oocytes were collected as described in Section 4.2.2. The CI oocytes were washed and transferred to wells containing 0.5 ml MEM supplemented with 0.5% BSA. For half the oocytes, the medium also contained 100 ng/ml LH (USDA-bLH-B5). From the other ovary of each rat, the largest 4 to 7 follicles were dissected out using 25-gauge needles. The follicles were placed on metal grids in the wells of 24-well plates (Falcon: Becton Dickinson and Company, Rutherford, New Jersey). MEM + BSA $\pm$ LH was added until the follicles were just submerged beneath the surface. The oocytes and follicles were cultured for 8 h at which time the oocytes were retrieved from the follicles. All the oocytes were washed in DPBS-S and transferred to droplets of fertilization medium for

insemination. The oocytes were assessed 14-16 h later for fertilization.

This experiment was performed in 3 replicates. Although the effects of the treatments were the same in all replicates, the proportion of ovulated oocytes that were fertilized differed by 15% among the replicates and therefore the data could not be pooled. Table 16 summarizes the results of one of the replicates. Oocytes matured within their follicles were fertilized in proportions significantly greater than isolated oocytes ($P<0.05$). The addition of LH to the culture medium had no effect on the fertilizability of isolated oocytes or of oocytes matured within their follicles.

8.2.2 Length of Culture Period

Since oocytes can mature spontaneously when placed in a culture system, it was of interest to note the effect of certain protein supplements in the maturation medium on the maturation and fertilization of *in vitro* matured oocytes. Immature oocytes were collected as described in Section 4.2.2 and half were transferred to the maturation medium. The remaining oocytes were stripped of their cumulus cells using a fine-bore, heat-polished pipette. The oocytes were then washed and transferred to the culture medium. The CI and CF oocytes were cultured for up to 24 h in MEM + 0.5% BSA or MEM + 10% heat-inactivated, charcoal-treated rat serum. Samples of oocytes were collected at 1-2 h

TABLE 16 Effect of LH on the fertilization of oocytes matured either intrafollicularly or as isolated oocyte-cumulus cell complexes

Group	LH	No. Oocytes	% Fertilized
Follicles	+	66	45.4[a]
	-	60	43.3[a]
Oocytes	+	58	13.8[b]
	-	62	9.7[b]
Ovulated		58	93.1[c]

a,b,c Values with different superscripts are significantly different ($P<0.05$).

intervals and examined for GVBD and PB formation. The experiment was organized such that all CI oocytes were stripped and assessed within a 2.5 h period and a common sperm preparation was used to inseminate all the oocytes. The oocytes were assessed for fertilization 14-16 h later.

The percentages of oocytes having undergone GVBD and PB formation at various times during a 24 h culture period are illustrated in Figures 3 (MEM + BSA) and 4 (MEM + serum). In both BSA- and serum-supplemented media, CI oocytes required a longer period of time to undergo GVBD, i.e. an additional 2-4 h compared to CF oocytes. In contrast the production of the first PB began slightly earlier in CI oocytes, approximately 1 h earlier for oocytes in MEM + BSA, 1.5 h for oocytes in MEM + serum. In addition the PB produced by CI oocytes were formed and disintegrated quite rapidly relative to PB formed by CF oocytes. Comparing CI oocytes matured in BSA- and serum-supplemented media, oocytes matured in the presence of serum showed more rapid GVBD + PB formation (by 2 h) than oocytes matured in MEM + BSA.

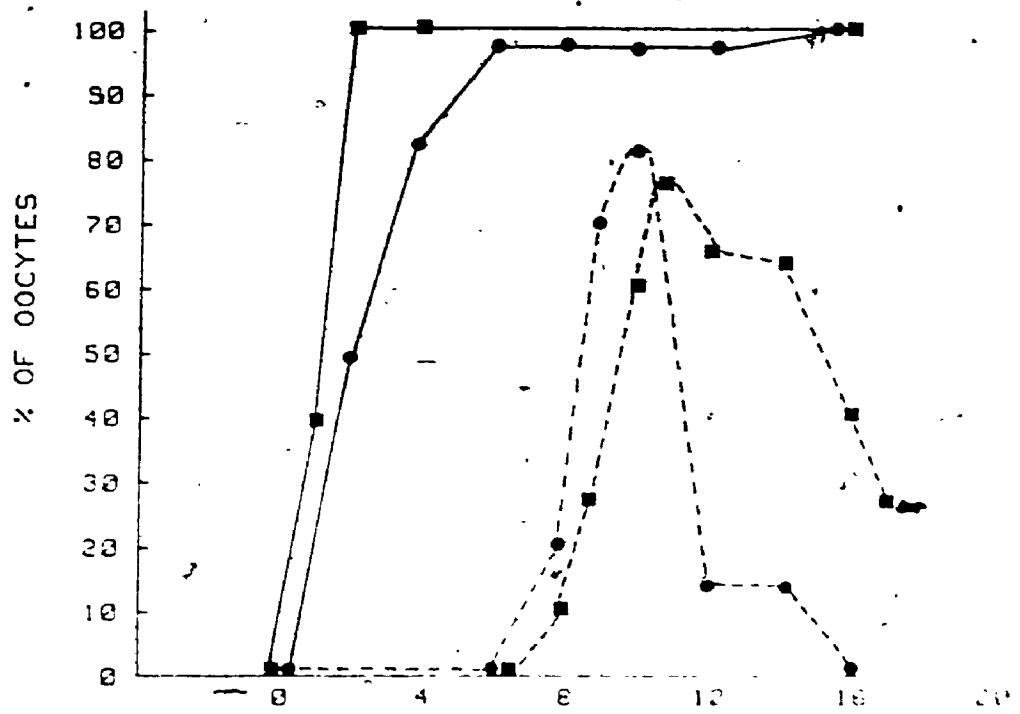
The fertilization rate of the CI oocytes matured in MEM + BSA or serum for various lengths of time is shown in Figure 5. Oocytes matured in the presence of serum showed an increase in fertilizability as GVBD occurred. The proportion of fertilized oocytes that cleaved to 2-cell embryos was 80%. A second increase occurred immediately following the production of the first PB. The maximum percent fertilization (96.8%, n=126) occurred with oocytes

FIGURE 3

Nuclear maturation of oocytes matured CI (●) or CF (■) in MEM + 0.5% BSA. The solid line indicates GVBD; the dashed line shows presence of PB. A minimum of 150 oocytes/group was assessed at each time.

FIGURE 4

Nuclear maturation of oocytes matured CI (●) or CF (■) in MEM + 10% serum. The solid line indicates GVBD; the dashed line shows presence of PB. A minimum of 150 oocytes/group was assessed at each time.


% OF OOCYTES
100
90
80
70
60
50
40
30
20
10
0
8
4
8
12
16

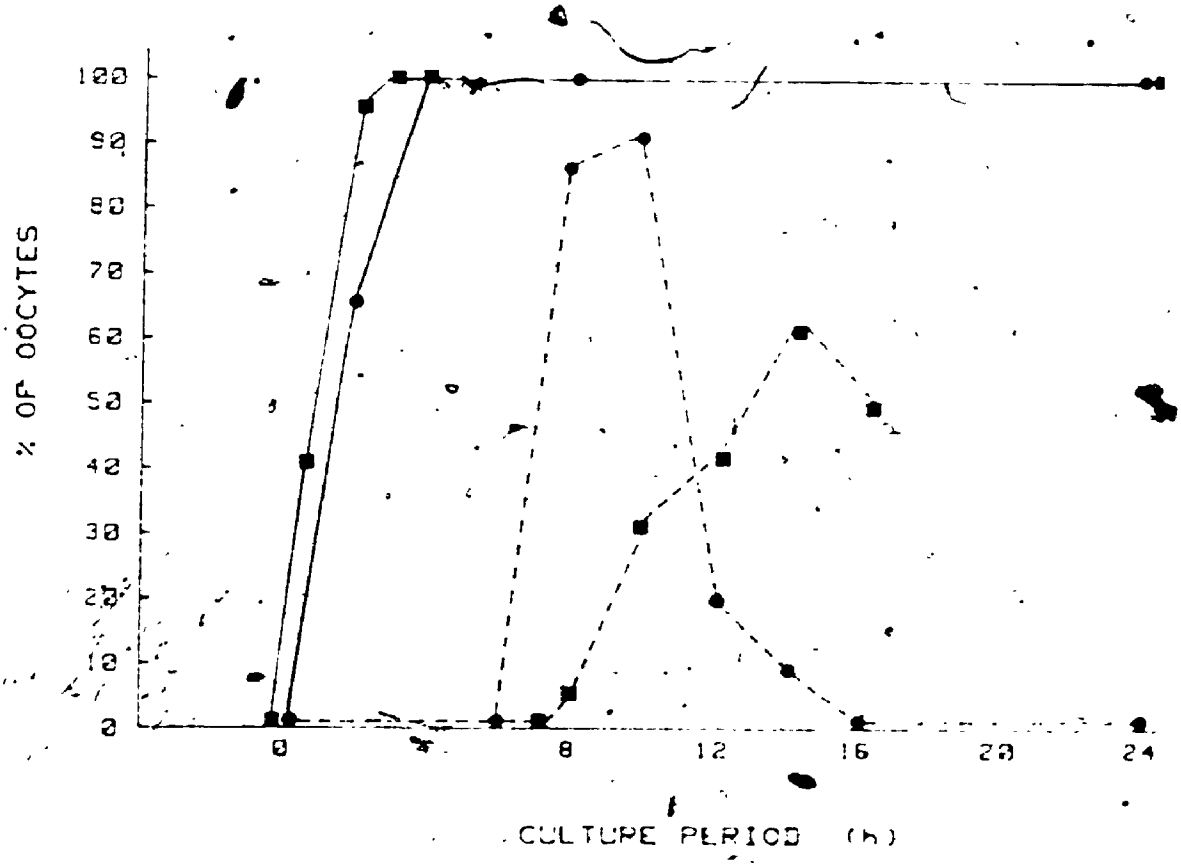
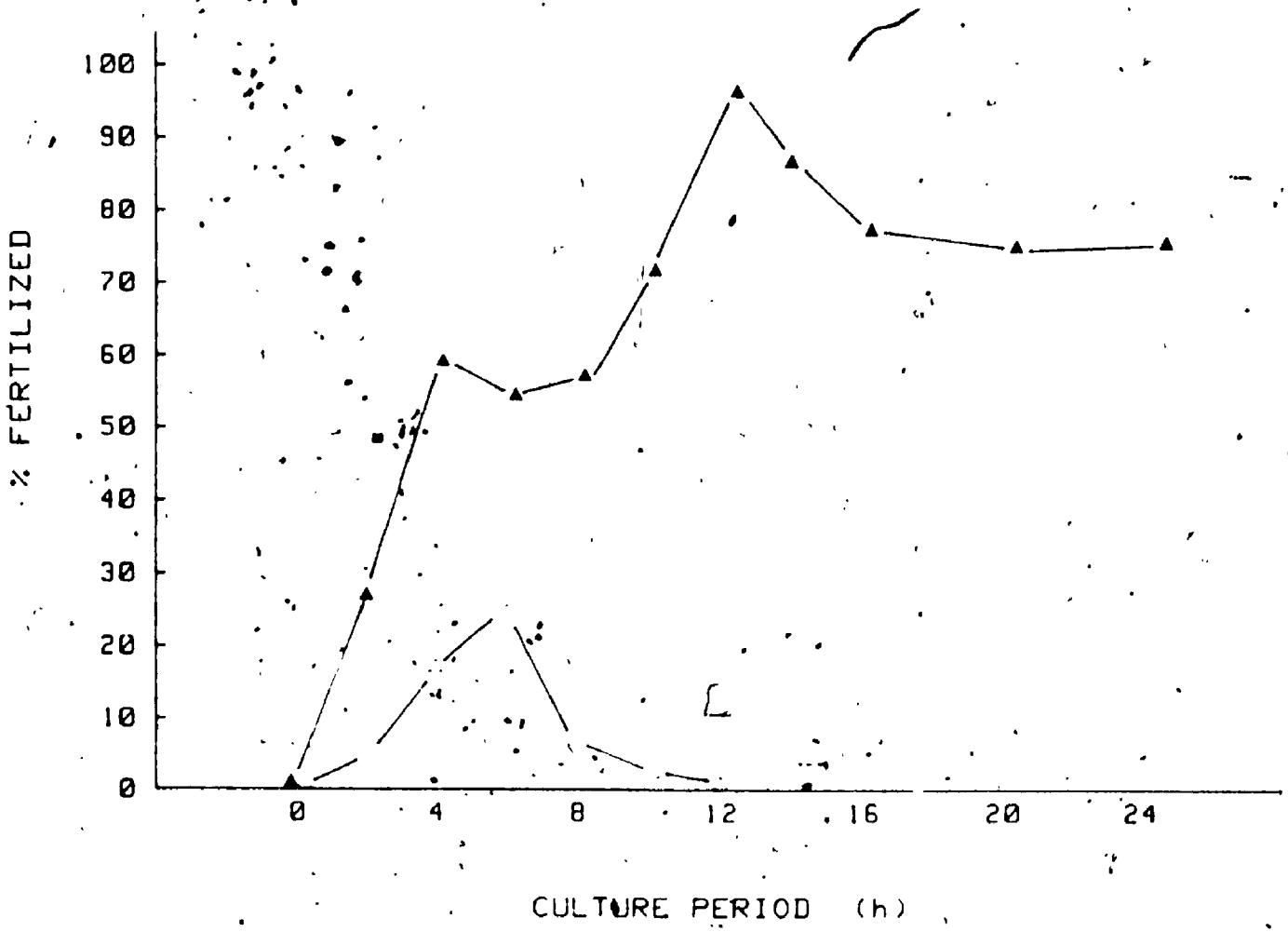

% OF OOCYTES
100
90
80
70
60
50
40
30
20
10
0
8
8
12
16
20
24
CULTURE PERIOD (h)

FIGURE 5

Fertilization of oocytes matured CI in MEM + 0.5% BSA (Δ) or MEM + 10% serum (▲) for varying lengths of time.


% FERTILIZED
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
CULTURE PERIOD (h)

matured for 12 h and is comparable to that of ovulated (control) oocytes (92.3%, n=104). Most (91.1-100.0%) of the oocytes that were fertilized following maturation from 6-16 h cleaved to 2-cell embryos in proportions comparable to that of ovulated oocytes (97.9%, n=96). Following a slight decline, the frequency of fertilization then plateaued at ~75% for oocytes cultured between 16-24 h. The cleavage rate of these oocytes dropped to 62.1% (n=87) for oocytes cultured for 24 h before insemination. In contrast to oocytes matured in MEM + serum, those matured in MEM + BSA exhibited a rapid increase in fertilizability when matured for 0-6 h; however, the proportion of oocytes fertilized fell quickly to 0 for oocytes matured longer than 10 h. The rate of cleavage of these oocytes reached 57.1% (n=28) for oocytes matured for 6 h.

8.2.3 Serum Concentration and Source

The result of the second experiment indicated that the presence of serum in the maturation medium particularly enhanced the fertilizability of the oocytes. To determine the optimal concentration of serum, immature oocytes, CI and CF, were matured in MEM or MEM + 5, 10, 15 or 20% rat serum for 10-12 h. After their stage of maturation was determined, the oocytes were fertilized <u>in vitro</u> and assessed for fertilization and cleavage to 2-cells.

The sera of other species were tested for their ability to maintain the fertilizability of oocytes during

their maturation *in vitro*. Immature CI oocytes were matured for 10 h in MEM + 10% untreated goat, pig or fetal calf serum, heat-inactivated human serum or heat-inactivated, charcoal-treated rat serum. As before the oocytes were examined for GVBD and PB formation and were then fertilized *in vitro*.

The proportion of oocytes fertilized following maturation in MEM + various concentrations of rat serum is shown in Figure 6. Oocytes matured CI were fertilized in significantly higher proportions than their CF counterparts ($P<0.01$). The proportions undergoing fertilization increased with increasing serum concentration and reached its maximum at a minimum concentration of 15%. The proportion of oocytes fertilized following maturation in MEM + 15% serum was not significantly different from the proportion of ovulated oocytes fertilized. The cleavage rate of the CI oocytes of all groups was >92%.

Table 17 summarizes the maturation, fertilization and cleavage of oocytes matured CI in MEM + serum from various sources. GVBD occurred in oocytes in all sera; however, the percentage GVBD was significantly less in oocytes matured in MEM + porcine or fetal calf serum ($P<0.05$) than in oocytes matured in rat serum. Of the oocytes in all groups having undergone GVBD, >88% also extruded the first PB. Goat, pig and fetal calf serum enhanced the fertilizability of the oocytes to a degree similar to that of rat serum. Although some oocytes that matured in MEM +

FIGURE 6

Fertilization of oocytes matured CI or CF in MEM + various concentrations of rat serum. At each concentration of serum, oocytes matured CI were fertilized in significantly higher proportions than oocytes matured CF ($P<0.01$). The number of oocytes matured in each group ranged from 64 to 224.

OVUL = ovulated

● = % of fertilized oocytes undergoing cleavage to 2-cell embryos

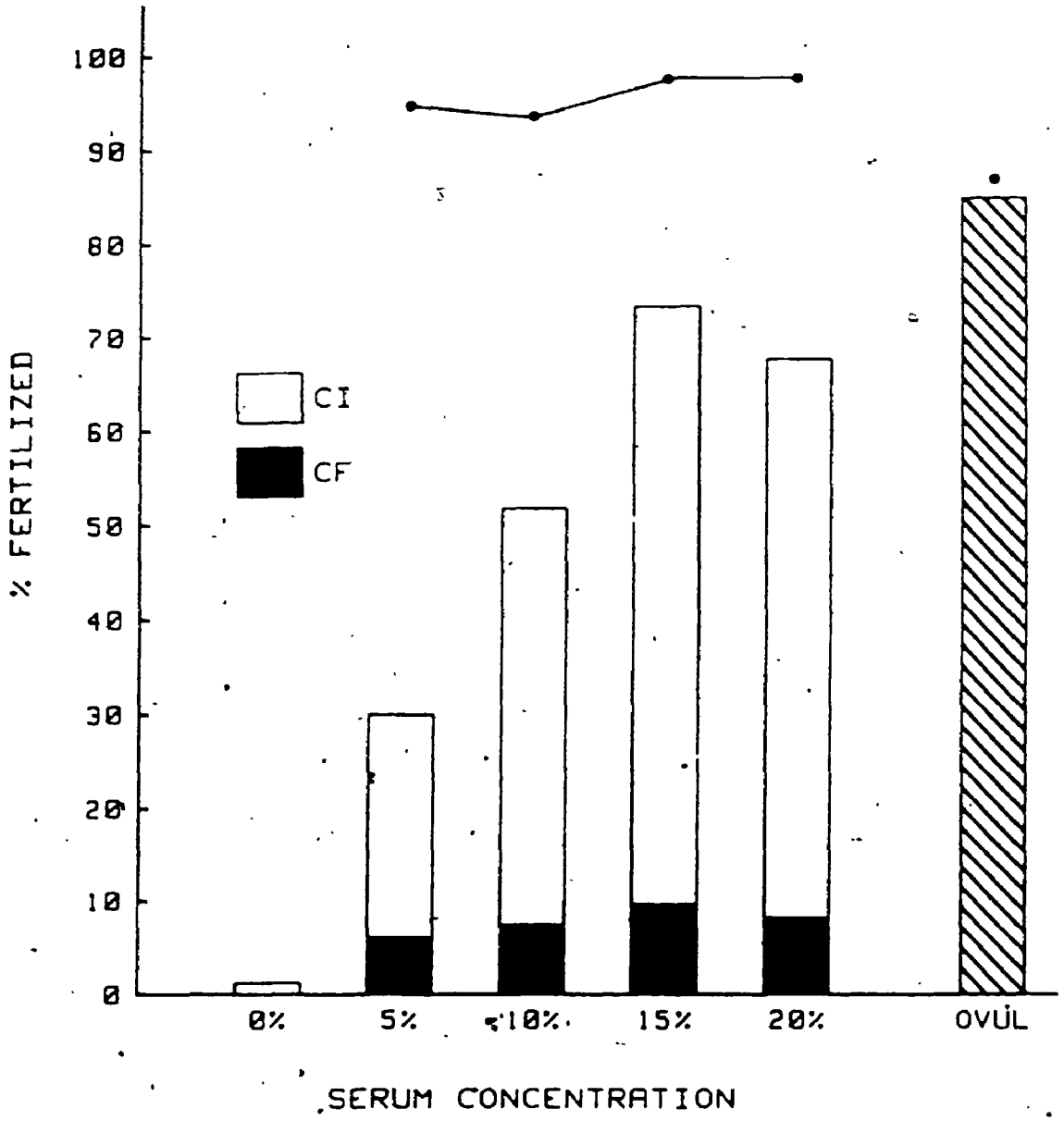

100
90
80
70
60
50
40
30
20
10
0
% FERTILIZED
CI
CF
0%
5%
10%
15%
20%
OVÜL
SERUM CONCENTRATION

TABLE 17 Nuclear maturation and fertilization of oocytes matured CI in MEM + 10% serum from various sources

Type of serum	% GVBD (n)	% (of GVBD) + PB	% Fertilized (n)	% (of Fert.) 2-cell
Rat	100.0 (62)[a]	100.0 (62)[a]	50.0 (62)[a]	83.9 (31)[a]
Goat	97.6 (85)[a]	94.0 (83)[ab]	50.6 (81)[a]	87.8 (41)[a]
Pig	83.8 (80)[bc]	92.5 (67)[ab]	58.5 (65)[a]	81.6 (38)[a]
Fetal calf	70.3 (74)[b]	88.5 (52)[b]	51.4 (72)[a]	75.7 (37)[a]
Human	92.8 (83)[ac]	98.7 (77)[a]	30.7 (75)[b]	69.6 (23)[a]

[a,b,c] Within each column, values with different superscripts are significantly different ($P<0.05$).

human serum could be fertilized, the proportion was significantly less than that of the other 4 groups ($P<0.05$).

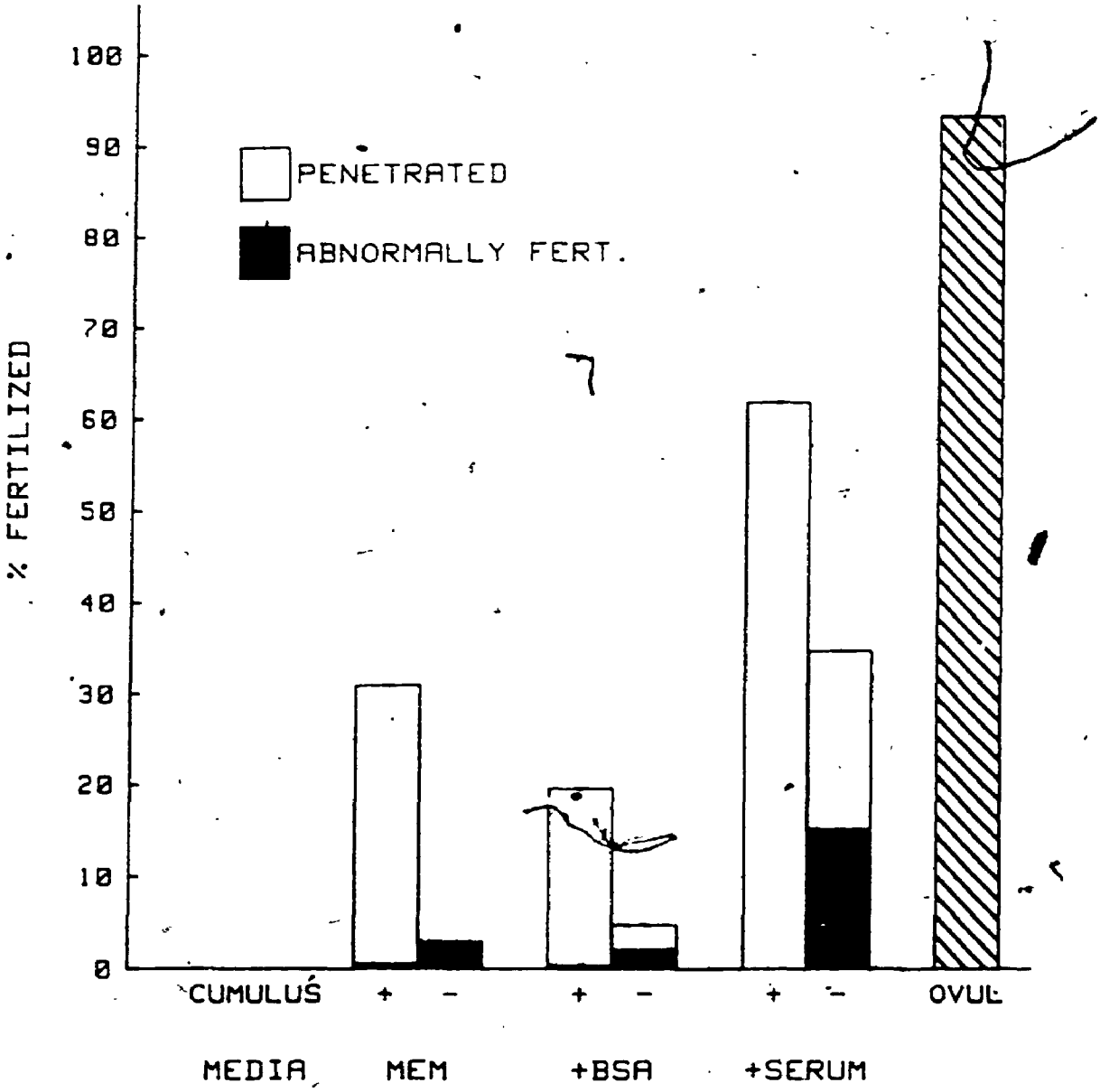
8.2.4 Normality of Fertilization and Cleavage

An experiment with methodology similar to that described in Section 8.2.2 was performed. Immature oocytes were cultured CI or CF for 8-10 h in MEM, MEM + 0.5% BSA or MEM + 10% serum. Again, the CI oocytes were stripped of their cumulus cells at the end of the period of maturation and all oocytes were exposed to epididymal sperm for *in vitro* fertilization. At 14-16 h after insemination, the oocytes were assessed for sperm penetration and the presence of pronuclei. Cleavage rate of the fertilized oocytes was assessed after an additional 24 h.

Figure 7 indicates the proportion of oocytes fertilized following maturation CI or CF for 8-10 h in various media. Careful analysis of the *in vitro* matured oocytes revealed that a proportion of the oocytes cultured CF and/or in the absence of serum showed evidence of abnormal fertilization. While normally fertilized oocytes show 2 pronuclei and 1 sperm tail within the vitellus, the majority of these abnormally fertilized oocytes showed 1 sperm tail and no pronuclei in the vitellus, or 1 sperm tail and only 1 pronucleus in the cytoplasm. This latter situation was particularly prominent in the MEM + serum, CF group. A small percentage of the fertilized oocytes (<10%)

FIGURE 7

Normal and abnormal fertilization of oocytes matured CI or CF in MEM, MEM + 0.5% BSA or MEM + 10% serum. The number of oocytes matured in each group ranged from 67 to 148. Within each medium group, the fertilization rate of oocytes matured CI is significantly higher than that of CF oocytes ($P<0.01$). The addition of serum significantly increased the proportion of oocytes undergoing fertilization compared with those in serum-free medium.


100
90
80
70
60
50
40
30
20
10
0
% FERTILIZED
PENETRATED
ABNORMALLY FERT.
CUMULUS
+
−
+
−
+
−
OVUL
MEDIA
MEM
+BSA
+SERUM

were recorded as abnormally fertilized due to polyspermy.

Within each medium group, the fertilization rate of the oocytes matured CI was significantly higher than that of the CF oocytes ($P<0.01$). The fertilization rate of the CI oocytes was also significantly different among the 3 types of media and the ovulated oocytes ($P<0.05$). The presence of serum in the medium enhanced the fertilizability of the *in vitro* matured oocytes to a 2- to 3-fold increase in fertilization rate over the other 2 groups.

Of the fertilized oocytes in the MEM and MEM + BSA groups, 75-80% cleaved to 2-cells. This was not significantly different from the cleavage rate of oocytes matured CF in MEM + serum (65.6%); however, these cleavage rates were all significantly less than the rate observed for CI oocytes matured in media supplemented with serum (92.9%) and that of ovulated oocytes (100.0%; $P<0.05$).

8.2.5 Developmental Capabilities

Having demonstrated that immature oocytes can be matured and fertilized *in vitro*, it was necessary to show that these oocytes were capable of complete embryonic and fetal development. Immature oocytes were collected 36 h after PMSG and matured CI in MEM + 10% serum for 10-12 h. The oocytes were fertilized *in vitro* with ovulated oocytes being used as controls. Oocytes assessed as being normally fertilized 14-16 h later were transferred immediately into

Day 1 pregnant recipient rats. Recipient rats were mature Long-Evans rats prepared as described in Section 5.2.3. Briefly the cycles of the recipients were synchronized with an LHRH agonist. At 3-5 days before the day of transfer, the ovarian bursa was peeled back from around the left ovary of each recipient such that the ovary was completely exposed. In vitro matured and ovulated oocytes with 2 pronuclei and 1 sperm tail were considered normally fertilized and were transferred into the left, non-pregnant (transfer) oviduct of the recipients. The recipients were killed on Day 20 of gestation and the number of fetuses and the total fetal and placental weights were recorded for the control and transfer horns. The number and size of the resorption sites in each horn were also noted.

Pronuclear zygotes resulting from in vitro fertilization of in vitro matured or ovulated oocytes showed statistically similar proportions of zygotes developing to fetuses following transfer (57.8%, n=45 and 55.0%, n=40, respectively). Within each group, the mean fetal weight and placental weight in the transfer horn were not significantly different from those in the control horn (Table 18).

8.3 Discussion

The meiotic maturation of rat oocytes under various conditions and their subsequent ability to be fertilized have been studied in these experiments. In the first

TABLE 18 Mean fetal and placental weights 20 days after zygote transfer

Group	Uterine horn	No. of rats	Mean ± s.e.m. fetal weight (g)	Mean ± s.e.m. placental wt (mg)
In vitro matured	control	11	2.15 ± 0.06[a]	514 ± 7[c]
	transfer	11	2.01 ± 0.06[a]	574 ± 34[c]
Ovulated	control	10	2.08 ± 0.07[b]	462 ± 15[d]
	transfer	10	1.89 ± 0.07[b]	487 ± 29[d]

a,b,c,d Within each group, values with the same superscripts are not significantly different ($P<0.05$).

experiment it was demonstrated that oocytes matured within their follicles had greater fertilizability than isolated oocytes. The later experiments revealed that the poor fertilization of isolated oocytes was primarily due to suboptimal culture media. LH had no apparent effect on the fertilizability of isolated oocytes or of oocytes matured within their follicles which suggests that continued exposure to LH was not necessary for maturation to occur. This observation indicates that the LH surge acted only as a trigger for the initiation of oocyte maturation. The effect of LH on the fertilizability and developmental capacity of *in vitro* matured rat oocytes has been studied previously (Shalgi *et al.*, 1979). Although that study reported that the addition of LH increased the fertilizability of isolated oocytes 3-fold, the oocytes had been collected prior to the gonadotropin surge and matured with or without LH. The advantage of the experiment described here is that all oocytes had been exposed to a gonadotropin surge by administration of an ovulatory dose of hCG. In this way the role of the LH surge could be differentiated from a possible role for LH during the maturation process.

There is much evidence to suggest that elevated cAMP levels in the oocyte are responsible for maintaining the oocyte in meiotic arrest (Cho *et al.*, 1974; Schultz & Wasserman, 1977; Dekel & Beers, 1978). In the present experiments, meiotic maturation, as seen by GVBD, occurs within 2 h for isolated CF oocytes and is completed by 4-6

h for CI oocytes. If high cAMP levels maintain meiotic arrest, then it appears that the cumulus cells may contribute to the levels of cAMP in the oocyte, thus delaying GVBD for 2-4 h. This is in agreement with results described by Dekel & Beers (1980) who showed that both LH and denudation accelerate the rate of GVBD compared to cumulus-oocyte complexes alone. Once maturation has been initiated, however, the presence of cumulus cells accelerates the process, particularly in serum-supplemented media. At 8 h of culture, polar body extrusion had occurred in >80% of the CI oocytes, whereas this phase of maturation was slower to begin and more prolonged for CF oocytes. Thus the presence of cumulus cells and serum appear to enhance completion of maturation, including polar body extrusion, through some unknown mechanism.

The beneficial effect of cumulus cells on maturation has been reported for the human (Kennedy & Donahue, 1969), the rabbit and the cow (Robertson & Baker, 1969) and the mouse (Cross & Brinster, 1970). In these studies, serum did not appear to contribute any additional maturation-enhancing factors to those provided by the cumulus cells. In contrast the present study indicated that serum and cumulus cells have beneficial effects on maturation that are separate and possibly additive. Although the nature of the beneficial effect of cumulus cells has yet to be determined, they may provide an energy source for oocyte maturation or may produce factors or hormones capable of

regulating maturation. Serum may maintain or enhance coupling of the cumulus cells to the oocytes and thus improve the transport of nutrients, hormones or factors involved in controlling the rate of maturation. To support this suggestion, a recent study has shown that serum improved cumulus cell viability and completion of the first meiotic division in bovine and hamster oocyte-cumulus complexes compared with BSA (Leibfried-Rutledge et al., 1986).

In addition to its influence on maturation, serum also appears to improve the fertilizability of the oocytes compared with those matured with BSA. Although Niwa & Chang (1975a) and Fleming et al. (1986) reported fertilization of rat oocytes following maturation in BSA-supplemented media, only 18-32% of the oocytes were penetrated by spermatozoa, few cleaved to normal 2-cell embryos and <3% developed to viable fetuses following transfer. Schroeder & Eppig (1984), Leibfried-Rutledge et al. (1986) and Choi et al. (1987) have shown the importance of serum in the maturation media in maintaining the fertilizability and developmental competence of mouse, cow and hamster oocytes.

Rat serum was initially used in these experiments to present in vitro conditions to the oocytes that resembled their in vivo environment as closely as possible. The caution, however, was unnecessary as sera from goat, pig and fetal calf were all capable of promoting maturation, fertilization and cleavage in vitro in proportions similar

to rat serum.

In the present study, oocytes matured without cumulus cells and/or without serum frequently showed deficiencies in pronuclear formation that led to poor cleavage rates. Similar deficiencies have been reported in the rabbit (Thibault, 1977), hamsters (Leibfried & Bavister, 1983) and cows (Leibfried-Rutledge et al., 1987). These authors suggested that the developmental problems of the oocytes were due to deficient cytoplasmic maturation. From our experiments, it became clear that, although serum was able to enhance the penetrability of CF oocytes, the presence of cumulus cells (even in the absence of serum) promoted normal fertilization with proper pronuclear formation. Cumulus cells then not only controlled the rate of nuclear maturation, but their presence also ensured normal cytoplasmic maturation as well.

Much evidence has been brought forth suggesting that meiosis is inhibited in vivo by a follicular factor (Chang, 1955; Tsafriri & Channing, 1975b) with cAMP playing a major role in this inhibition (Schultz et al., 1983b; Downs & Eppig, 1984; Eppig & Downs, 1984). In addition purines have been found to be effective inhibitors of meiotic maturation (Downs et al., 1985; Eppig et al., 1985). The regulation of these factors and the mechanism(s) by which they are removed or modified to allow maturation to occur is still unclear. The role of the cumulus cells in this process is equally unclear. It has been postulated that

the inhibitory factor is transported from the cumulus cells to the oocyte via the gap junctions that join them. The surge of gonadotropins then must either modify the inhibitory activity or cause the production of a maturation-promoting factor (MPF) that, when delivered to the oocyte, can act to over-ride the inhibition. The inclusion of a MPF in this mechanism would make it comparable to mechanisms which are already elucidated in lower vertebrates. In the starfish, follicle cells produce a factor (1-methyladenine) that acts on the plasma membrane of the oocyte (Kanatani & Hiramoto, 1970; Doree & Guerrier, 1975) to induce the appearance of a cytoplasmic MPF which is the direct trigger for GVBD (Kishimoto & Kanatani, 1976). In amphibians progesterone is the stimulus for MPF production (Masui & Markert, 1971). A similar mechanism can be hypothesized for mammalian oocytes. In response to the gonadotropin surge, cumulus cells produce a factor, possibly progesterone, that acts on the oolemma and/or ooplasm to induce the production of a cytoplasmic MPF. This MPF could be a trigger for GVBD and may also play a role in facilitating cytoplasmic maturation. Thus, as in the present experiments, oocytes matured in the absence of cumulus would undergo meiotic maturation, due to the removal of the inhibitory factor, but cytoplasmic maturation would not occur due to the absence of the MPF.

These experiments have demonstrated that immature, cumulus cell-enclosed oocytes can be matured <u>in vitro</u> in serum-supplemented media, can be fertilized and can develop

to viable fetuses in proportions similar to oocytes matured in vivo. The fertilizability of the oocytes is dependent on the presence of both serum and cumulus cells. It appears that these two factors may play separate roles in enabling the oocytes to acquire their developmental competence. The beneficial effect of serum appears to lie in its ability to maintain penetrability of the oocytes. Cumulus cells also help to maintain the penetrability of the oocytes, and appear to have an additional role in ensuring normal cytoplasmic maturation, as indicated by the reduced frequency of abnormal fertilization of oocytes matured in the presence of their cumulus cells. The experimental model developed in the present studies should prove useful in elucidation of the mechanisms by which cumulus cells exert their beneficial effect on cytoplasmic maturation of oocytes.

CHAPTER 9

HARDENING OF THE ZONA PELLUCIDA

9.1 Introduction

When establishing the optimal conditions to achieve maximal fertilization rates for *in vitro* fertilization systems, the ratio of sperm to oocyte is usually increased to several thousand to one. Fertilization is clearly far less efficient *in vitro* than in the oviduct where only a small number of sperm is needed. Possible reasons for the necessity of the supernumerary sperm include decreased proportions of sperm undergoing capacitation or the acrosome reaction *in vitro* and/or reduced sperm motility or viability under the culture conditions. In addition to alterations in the fertilizing capability of the sperm, conditions *in vitro* may also affect the penetrability of the oocyte.

As a consequence of fertilization *in vivo*, the zona pellucida shows an increased resistance to further sperm penetration, a modification that is attributed to the secretory products of the cortical granules of the oocytes and is thought to prevent polyspermy. It has been demonstrated, however, that the zona pellucida of mouse oocytes cultured *in vitro* in serum-free medium also become progressively more resistant to solubilization by chymotrypsin, independent of cortical granule release (DeFelici & Siracusa, 1982). This "hardening" of the zona

pellucida in culture has been correlated with a decrease in the frequency of fertilization (Gianfortoni & Gulyas, 1985; Downs et al., 1986). Other studies have indicated that spontaneous zona hardening is reduced in the presence of cumulus cells (DeFelici & Siracusa, 1982; Gianfortoni & Gulyas, 1985) or serum (Downs et al., 1986) or glycosaminoglycans (GAGs; DeFelici et al., 1985). GAGs are synthesized actively by granulosa and cumulus cells in vitro (Gebauer et al., 1978; Ax & Ryan, 1979; Yanagishita & Haskell, 1979; Bellin et al., 1983) and are found in high concentrations in follicular fluid (Yanagishita et al, 1979; Grimek & Ax, 1980). It is therefore probable that GAGs play a role in maintaining the zona pellucida of the oocytes during their maturation in vivo in a state capable of penetration.

The development of significant zona hardening during 4 h of culture (DeFelici et al., 1985) makes this phenomenon a major factor for consideration in developing procedures for in vitro fertilization. Furthermore, investigators must differentiate between a loss of fertilizability due to the unsuccessful maturation of the oocyte and that due to the increased resistance of the zona pellucida to sperm penetration. For this reason, almost all studies maturing oocytes in vitro use media supplemented with serum.

The zona pellucida may act as a potential barrier to sperm penetration that can be somewhat overcome by an appropriate culture medium or an increase in the sperm

concentration; however, oligospermia is a common cause of male infertility and *in vitro* fertilization is still an imperfect technique where the male gamete is concerned. Any advance that could improve penetrability of oocytes without requiring increased sperm concentrations would increase the success of *in vitro* fertilization in all species.

Previous work with the mouse suggests that removal of the zona pellucida can allow marked reduction of the sperm:oocyte ratio for fertilization (Fukuda & Chang, 1978; Thadani, 1982). However, the zona pellucida is believed to contribute to the block to polyspermy (Braden *et al.*, 1954; Barros & Yanagimachi, 1972) and may play as yet undefined roles in normal tubal transport or protection of the embryo prior to implantation. An alternative to zona removal has been provided recently by Gordon & Talansky (1986) who devised a method to weaken the zona pellucida such that fertilization was achieved with a lower sperm:oocyte ratio, without compromising the biological function of the zona or the viability of the oocyte. Mouse oocytes were placed in a micromanipulation device and acid Tyrode's solution was expelled from a micropipette, creating a small hole in the zona. These oocytes were not inferior in terms of fertilizability, susceptibility to polyspermy or developmental potential, indicating that this technique may be useful in both humans and animals to enhance the efficiency of fertilization.

Numerous investigators have noted that isolated oocytes undergoing spontaneous maturation were incapable of transforming sperm nuclei into pronuclei in the cytoplasm of the oocyte (Thibault, 1977; Niwa & Chang, 1976; Leibfried & Bavister, 1983; Leibfried-Rutledge et al., 1987). In experiments presented earlier, this phenomenon was closely related to the absence of cumulus cells during maturation. Therefore, it was suggested that the cumulus cells may play a role in facilitating cytoplasmic maturation as well as helping to maintain the penetrability of oocytes.

The purpose of this study was to investigate the role of cumulus cells in cytoplasmic maturation by alleviating the penetrability problems that result following in vitro maturation of cumulus-free oocytes.

9.2 Methods and Results

The first two experiments of this study attempted to reduce the incidence of spontaneous zona hardening by supplementing the maturation media with agents known to have this ability, namely serum, follicular fluid and GAGs. In the third experiment, the technique of zona drilling was used to by-pass the zona barrier to penetration.

9.2.1 Follicular Fluid

To investigate the importance of follicular fluid

components in the maturation process, immature CI oocytes were matured for 11-12 h in MEM, MEM + 10% or 20% serum, and MEM + 25% or 50% rat follicular fluid (rFF). For the collection of rFF, immature rats were treated at 0800 on Day -2 with 15 IU PMSG to induce the development of a large number of follicles. At 0600 on Day 0, the rats were injected with 15 IU hCG. The ovaries of the rats were collected in DPBS 7-9 h later. The largest follicles were punctured with a 25-gauge needle and the rFF was immediately collected using capillary tubes. The capillary tubes were centrifuged for 3 minutes to clear the rFF of granulosa cells. The rFF was refrigerated until its use in the maturation media 8 h later. Following the period of _in vitro_ maturation of the oocytes, they were stripped of their cumulus cells, rinsed in DPBS and transferred to the fertilization medium for insemination.

Of 51 immature oocytes cultured for 11-12 h in MEM, 86% underwent GVBD. When rFF or serum was added to the culture media, >97% showed GVBD. The percentage of these oocytes that were fertilized has been summarized in Table 19. The results indicated that both serum and rFF contain a factor(s) that enhanced the fertilizability of the oocytes, with rFF being slightly better than serum. Oocytes that had matured in serum or 25% rFF and had been fertilized showed cleavage rates that were not significantly different. Oocytes matured in 50% rFF and fertilized, however, cleaved in proportions significantly less ($p<0.05$).

TABLE 19 Fertilization and cleavage of oocytes matured in media containing serum or follicular fluid

Culture media	% Fertilized (n)	% (of Fert.) 2-cell
MEM	2.3 (44)[a]	0 (1)[a]
MEM + 10% serum	55.4 (74)[b]	82.9 (41)[b]
MEM + 20% serum	69.8 (63)[bc]	81.8 (44)[b]
MEM + 25% rFF	87.3 (71)[d]	71.0 (62)[b]
MEM + 50% rFF	76.5 (34)[cd]	42.3 (26)[c]

a,b,c,d Within each column, values with different superscripts are significantly different ($P<0.05$).

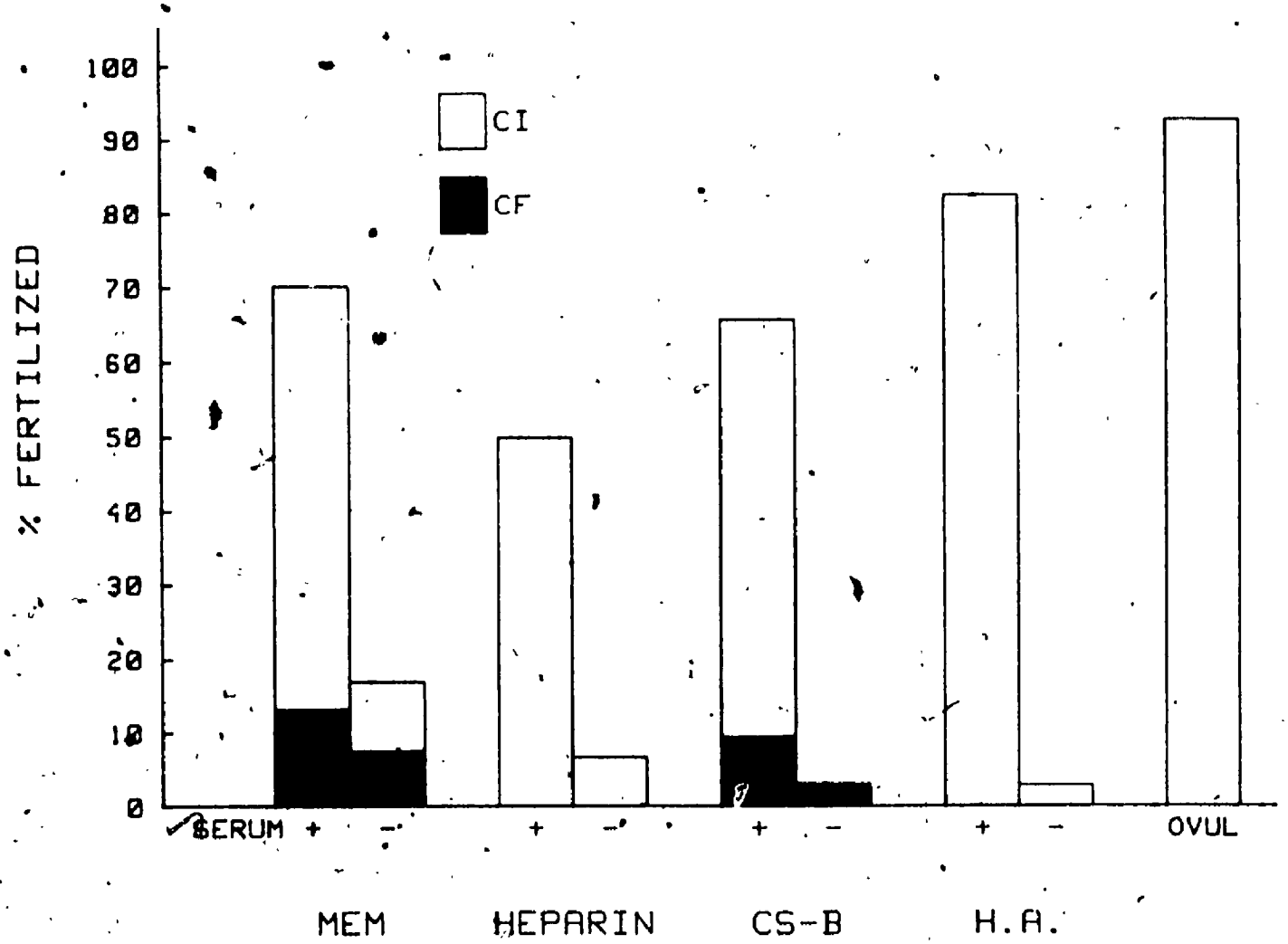
9.2.2 Glycosaminoglycans

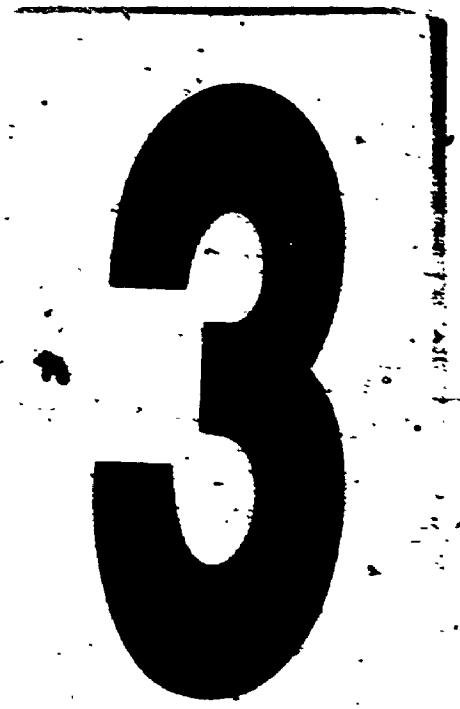
Since GAGs occur in fairly high concentrations in follicular fluid and since they have been implicated in maintaining the penetrability of the zona pellucida, several GAGs were tested for their effects on oocytes during maturation in the presence or absence of serum. Immature oocytes were cultured for 12 h with or without cumulus cells in MEM ± 20% serum and 2 mg/ml heparin, chondroitin sulfate-B or hyaluronic acid (Sigma Chemical Co.). Once again, the oocytes were then stripped, if necessary, washed and transferred to the fertilization media for *in vitro* fertilization.

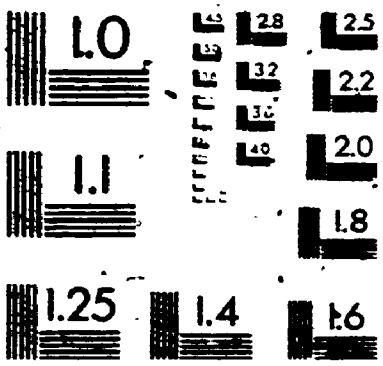
Figure 8 illustrates the percentage of oocytes fertilized following maturation CI or CF in MEM ± serum ± GAGs. The results show that, in all groups, the percentage of CI oocytes that were fertilized was significantly higher than that of CF oocytes in serum-supplemented media ($P<0.01$). The percentage of CI oocytes that underwent fertilization following maturation in MEM ± serum was not significantly different from the percentage fertilized following maturation in similar media with any of the GAG supplements. In GAG-supplemented media, oocytes matured CF or in serum-free media or both showed fertilization rates of <10.0%. Oocytes matured CI in MEM + serum ± GAGs and fertilized, had proportions that cleaved to 2-cells (90-100%) not significantly different from ovulated controls (88.9%).

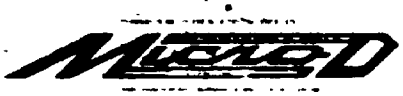
FIGURE 8

Fertilization of oocytes matured CI or CF in MEM ± serum + heparin, chondroitin-sulfate B (CS-B) or hyaluronic acid (H.A.). The addition of GAGs to the maturation medium had no effect on the penetrability of the oocytes ($P>0.1$). A minimum of 55 oocytes was used for each treatment. The histogram indicates the results of 1 of 2 replicates.


% FERTILIZED
100
90
80
70
60
50
40
30
20
10
0
CI
CF
SERUM + − + − + − + − OVUL
MEM
HEPARIN
CS-B
H.A.




1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8



9.2.3 "Drilling" of the Zona Pellucida

As follicular fluid was difficult to obtain in sufficient quantities to do an extensive study and since GAGs were found to be ineffective in our culture system, an alternate method was sought to increase the penetrability of *in vitro* matured oocytes. We used a method for "drilling" a small hole in the zona pellucida using an acidic medium, essentially as described by Gordon & Talansky (1986). The zonae pellucidae of oocytes were drilled using a Nikon phase contrast microscope with Leitz micromanipulators. Holding pipettes (1 mm, outer diameter; Clark Electromedical Instruments, England) were pulled with a Flaming Brown Micropipette Puller (Sutter Instrument Co., U.S.A.) and heat-polished on a microforge (Narishige Scientific Inst. Lab., Tokyo) to a tip diameter of 75 µm. Drilling pipettes were pulled to a tip diameter of 10 µm. Acid Tyrode's solution was used to "drill" the holes in the zonae. The solution was comprised of 8.0 g NaCl/l, 0.2 g KCl/l, 0.24 g $CaCl_2$/l, 0.1 g $MgCl_2$/l and 1 g glucose/l. The pH of the solution was adjusted to 2.5. The final solution was sterilized by positive pressure filtration through a Millex-GV 0.22 µm filter unit (Millipore Corporation, Bedford, MA) and stored at 4°C. On the day of the experiment, the solution was warmed to room temperature and loaded into the drilling pipette.

To drill the oocytes, groups of 25-75 oocytes were placed in 500 µl droplets of DPBS + 0.1% BSA under oil. An

oocyte was picked up with the holding pipette and the zona was held against the drilling pipette in a tangential position. Acid Tyrode's solution was slowly expelled from the drilling pipette until a dimple in the oolemma appeared, thereby signalling a rupture in the zona pellucida. At this point the oocyte was immediately withdrawn. For most oocytes, the hole was not visible with the phase contrast microscope. If the hole was too large, the zona would loosen its enclosure of the oocyte and/or occasionally fall off with handling; these oocytes were not used in the experiment.

This study included four experiments, to determine: A) an appropriate sperm concentration, B) the effect of drilling on the fertilizability of CI and CF in vitro matured oocytes, C) the developmental capability of drilled oocytes, and D) the effect of drilling and progesterone on the maturation and fertilization of in vitro matured oocytes.

A. Sperm Concentration

Immature oocytes were collected as described in Section 4.2.2 and were matured CI for 8-10 h in MEM + 15% rat serum. At the end of the culture period, all the oocytes were stripped of their cumulus cells and half had a holes drilled in their zonae pellucidae, one hole per oocyte. The oocytes were rinsed with fresh DPBS and divided into 50 µl droplets of fertilization medium. The droplets of medium were randomly divided into 5 groups,

each inseminated with one of 5 concentrations of spermatozoa: 10,000, 25,000, 50,000, 100,000 or 250,000 sperm/ml. Fertilization and incidence of polyspermy were assessed 14-16 h later and cleavage of fertilized oocytes was assessed after an additional 24 h.

The proportions of drilled and non-drilled oocytes that were fertilized are summarized in Figure 9. At all sperm concentrations, the proportions of drilled oocytes that were fertilized was significantly greater than that of non-drilled oocytes ($P<0.01$). There was no incidence of polyspermy in fertilized, non-drilled oocytes exposed to lower sperm concentrations; however, 17.4% of non-drilled oocytes in droplets with the highest sperm concentration showed incidence of polyspermy, although in all cases the polyspermy was limited to 2 sperm tails in the vitellus. The incidence of polyspermy in drilled oocytes is indicated in Figure 10. The percentage of polyspermic oocytes increased with increasing sperm concentrations, such that the incidence of polyspermy was significantly higher at concentrations greater than 50,000 sperm/ml than at concentrations less than 50,000 sperm/ml ($P<0.01$). At the higher concentrations, polyspermic oocytes were often characterized by 2 or more sperm tails within the vitellus.

In all groups the proportions of fertilized oocytes cleaving to 2-cell embryos was >60%. Of all the non-drilled, fertilized oocytes, 80.0% ($n=55$) cleaved to 2-cells, while 78.5% ($n=158$) of fertilized oocytes in the

FIGURE 9

Penetration of drilled and intact oocytes at various sperm concentrations. At each sperm concentration the proportion of drilled oocytes that was fertilized was significantly greater than that of non-drilled oocytes ($P<0.05$). The number of oocytes used for each treatment ranged from 98 to 132.

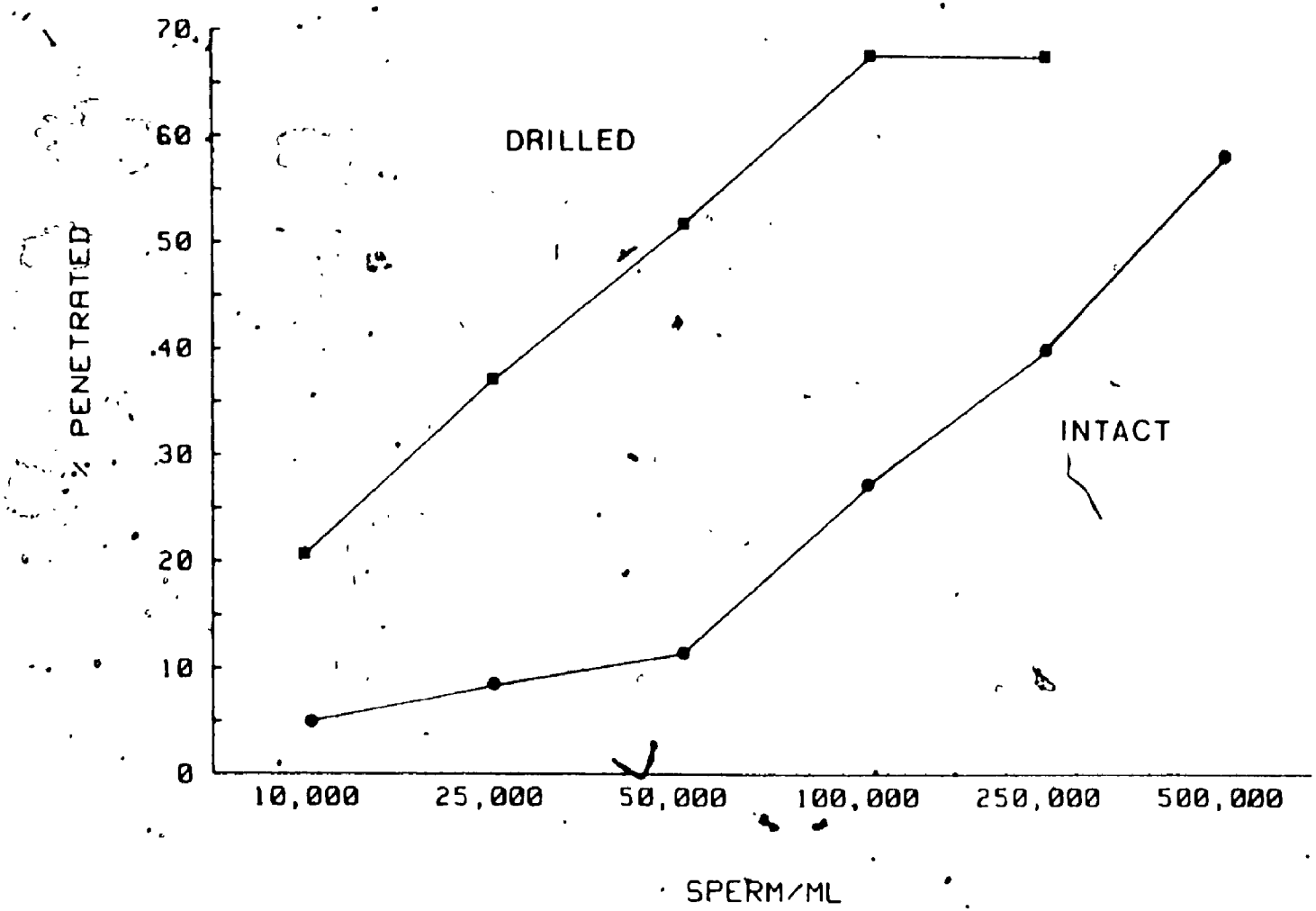
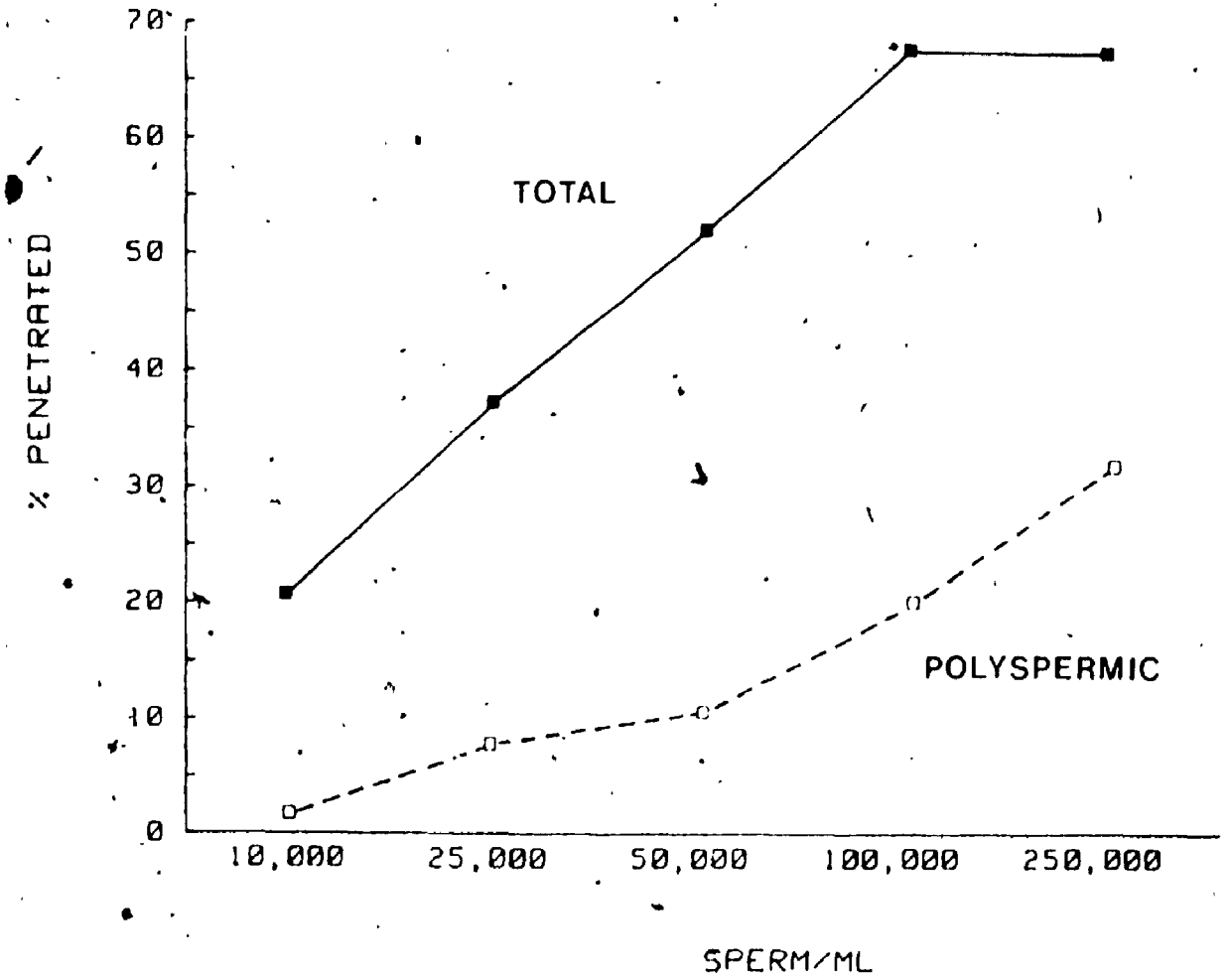

70
60
50
40
30
20
10
0
% PENETRATED
DRILLED
INTACT
10,000
25,000
50,000
100,000
250,000
500,000
SPERM/ML

FIGURE 10

Incidence of polyspermy in drilled oocytes exposed to various concentrations of sperm. At concentrations greater than 100,000 sperm/ml, penetrated oocytes showed a significant increase in polyspermy compared with those at low sperm concentrations ($P<0.05$). The number of oocytes used for each treatment ranged from 124 to 132.


70
60
50
40
30
20
10
0
% PENETRATED
TOTAL
POLYSPERMIC
10,000
25,000
50,000
100,000
250,000
SPERM/ML

drilled group cleaved to 2-cell embryos. At each sperm concentration, the proportion of fertilized oocytes in the non-drilled group that cleaved was not significantly different from that in the drilled group ($P>0.1$).

B. Fertilizability of Oocytes Matured under Various Conditions before Drilling

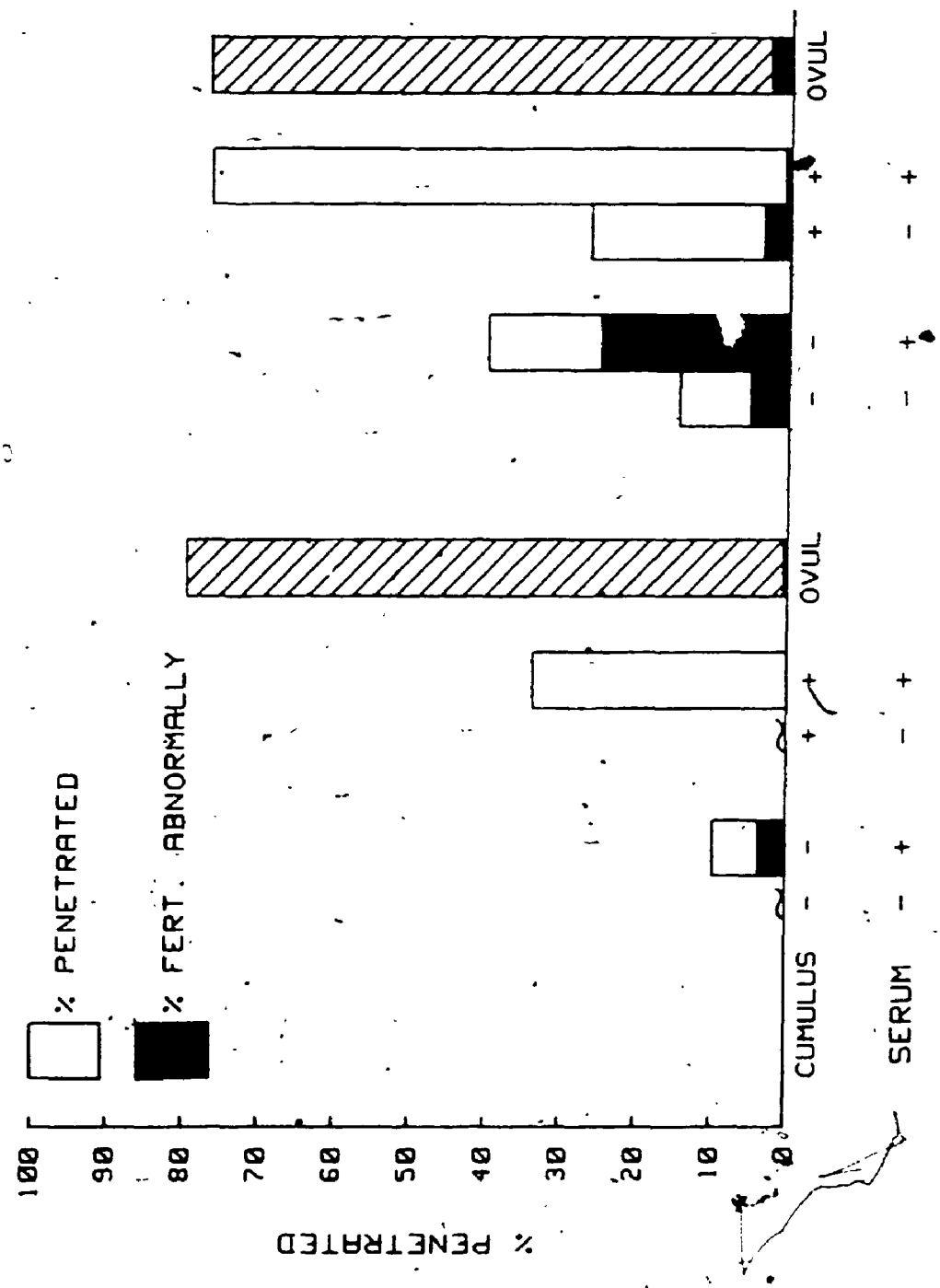
Immature oocytes were matured CI or CF for 8-10 h in MEM + 15% rat serum or 0.5% BSA. At the end of the culture period, the CI oocytes were stripped of their cumulus cells and half the oocytes of each group had a hole drilled in their zonae. Ovulated oocytes were collected as described in Section 4.2.1 and were treated similarly. All the oocytes were washed in DPBS and transferred to fertilization medium. The droplets were inseminated with 50,000 sperm/ml and the oocytes were assessed 14-16 h later for evidence of fertilization.

The results of the three replicates of this experiment have been summarized in Figure 11. As in the previous experiment, drilling significantly increased the proportions of *in vitro* matured oocytes undergoing fertilization ($P<0.01$); however, drilling had no effect on the fertilizability of ovulated oocytes. In agreement with experiments presented in Section 8.2.4, both serum and cumulus cells had beneficial effects on the fertilizability of *in vitro* matured oocytes.

All oocytes matured CF, regardless of the culture medium, showed a high incidence of abnormal fertilization,

FIGURE 11

Penetration of drilled and intact oocytes following maturation CI or CF in MEM + 0.5% BSA or MEM + 15% serum. Drilling significantly increased the proportions of oocytes undergoing fertilization in each treatment group ($P<0.01$). The number of oocytes used for each treatment ranged from 68 to 212.


% PENETRATED
% FERT. ABNORMALLY
100
90
80
70
60
50
40
30
20
10
0
% PENETRATED
CUMULUS - - + +
SERUM - + - +
OVUL
OVUL
INTACT
DRILLED

i.e. only one pronucleus instead of the normal two in the cytoplasm of the oocyte. Even when drilling overcame the barrier to penetration, the proportion of penetrated oocytes that were fertilized abnormally was not significantly different from that of non-drilled oocytes ($P>0.1$). In contrast to the high incidence of abnormal fertilization with CF oocytes, the incidence of abnormal fertilization in ovulated oocytes or oocytes matured CI was <5% with the exception of CI oocytes matured in MEM + BSA and then drilled; 14.3% of penetrated oocytes in this group were abnormally fertilized.

The incidence of polyspermy in oocytes undergoing these various treatments is summarized in Table 20. For most treatments the proportions of penetrated oocytes having at least two sperm tails within the vitellus were significantly greater for drilled oocytes than for non-drilled oocytes ($P<0.01$); for oocytes matured CF in MEM + serum, this increase was not significant.

C. Development Capability of Drilled Oocytes

Immature oocytes were collected and matured CI in MEM + 15% serum. Following an 8-10 h period of maturation, the oocytes were stripped of their cumulus cells and half the oocytes were treated with the zona drilling technique. The oocytes were washed and placed in droplets of fertilization medium. The droplets of non-drilled oocytes were inseminated with a concentration of 100,000 sperm/ml;

TABLE 20 Incidence of polyspermy in intact and drilled oocytes following maturation in vitro

Maturation conditions	Drilled	Total no. oocytes	No. oocytes penetrated	% (of Penetrated) polyspermic
MEM + BSA				
CF	-	68	0	0 [a]
	+	82	12	33.3 [b]
CI	-	79	0	0 [a]
	+	79	21	57.1 [b]
MEM + serum				
CF	-	174	17	0 [a]
	+	172	69	7.2 [a]
CI	-	148	50	10.0 [a]
	+	178	137	43.8 [b]
Ovulated	-	212	169	3.0 [a]
	+	142	110	38.2 [b]

[a,b] Within each type of media, values with different superscripts are significantly different ($P<0.01$).

drilled oocytes were exposed to 50,000 sperm/ml. Fertilization of the oocytes was assessed 14-16 h later. Drilled and non-drilled zygotes with 2 pronuclei and 1 sperm tail were transferred into mated recipients. As in previous experiments, the recipients were Long-Evans rats that had been unilaterally bursa-peeled (see Section 5.2.1). The recipient animals were killed on Day 20 and the number of fetuses and resorption sites and the total fetal and placental weights in each uterine horn were recorded.

The proportion of transferred zygotes recovered as fetuses or resorption sites on Day 20 have been summarized in Table 2. The proportion of fetuses recovered following the transfer of drilled zygotes was significantly less than that of non-drilled zygotes ($P<0.05$). In both drilled and non-drilled groups, the mean fetal weight in the transfer horn was significantly less than that in the control horn ($P<0.01$); however, the mean fetal weights in the transfer horns were not significantly different between groups ($P>0.1$). There was no significant differences among the placental weights within or between groups ($P>0.1$). The proportion of drilled oocytes recovered as resorption sites following transfer was not significantly different from that of non-drilled oocytes.

D. Effect of Progesterone on the Maturation of Oocytes

In previous experiments, the presence of cumulus cells during maturation <u>in vitro</u> was essential to ensure normal

TABLE 21 Percentage of transferred zygotes recovered as viable fetuses and resorption sites (R.S.) and mean fetal and placental weights 20 days after zygote transfer

Group	Uterine horn	No. Embryos transferred	% Fetuses	% R.S.	No. rats	Mean ± s.e.m. fetal wt. (g)	Mean ± s.e.m. placental wt. (mg)
Drilled	control				10	2.25 ± 0.04[a]	539 ± 30[c]
	transfer	68	30.9[a]	25.0[a]	10	2.13 ± 0.04[b]	537 ± 24[c]
Intact	control				8	2.33 ± 0.06[a]	517 ± 23[c]
	transfer	53	50.9[b]	17.0[a]	8	2.06 ± 0.04[b]	556 ± 37[c]

[a,b,c] Within each column, values with different superscripts are significantly different ($P<0.05$).

cytoplasmic maturation. As mentioned in the discussion of the previous study, progesterone plays a major role in the mechanism of maturation in non-mammalian species. The increased accumulation and release of progesterone by rat ovarian follicles in response to LH (Stoklosowa & Malbandov, 1972; Tsafriri et al., 1973; Hillensjo et al., 1981) suggests that progesterone may play a role in the maturation of mammalian oocytes. The present experiment examined the possible involvement of progesterone in the cytoplasmic maturation of rat oocytes. Immature oocytes were collected and divided into 4 groups: 1) non-drilled, 2) drilled after culture, 3) drilled before culture, and 4) CI + aminoglutethimide phosphate (AGP), a steroidogenesis inhibitor. The cumulus cells were removed from the oocytes of the first 3 groups. CF oocytes in groups 1 and 2 were immediately subdivided and transferred to one of 4 culture media: a) MEM + 0.5% BSA, b) MEM + BSA + 2 µg progesterone/ml, c) MEM + 15% serum, or d) MEM + serum + 2 µg progesterone/ml. CF oocytes in group 3 had holes drilled in their zonae as described earlier before being placed in one of the 4 types of culture media listed. Group 4 oocytes were matured CI in MEM + 15% serum, but half the oocytes were matured in media containing 10^{-5} M AGP as an additional supplement. Oocytes from all groups were matured for 8-10 h. At the end of the culture period, oocytes in groups 1 and 3 were washed and transferred to droplets of fertilization medium. Group 2 oocytes had

holes drilled in their zonae before being transferred to fertilization medium. CI oocytes in group 4 were stripped of their cumulus cells before being placed in fertilization droplets. The droplets were inseminated with 50,000 sperm/ml and fertilization was assessed 14-16 h later. The proportion of fertilized oocytes cleaving to 2-cell embryos was determined after an additional 24 h culture period.

The proportion of oocytes penetrated in each group and the incidence of abnormal fertilization are summarized in Figure 12 and Table 22. In most groups of media, CF oocytes drilled before maturation had a greater incidence of penetration than those drilled at the end of the culture period; however, this increase was only significant with oocytes matured in MEM + serum + progesterone ($P<0.05$). In all cases the proportion of drilled oocytes that underwent fertilization was significantly greater than that of non-drilled CF oocytes ($P<0.05$). The addition of progesterone to either serum-free or serum-supplemented media did not significantly alter the proportion of CF oocytes (drilled or non-drilled) achieving fertilization ($P>0.1$). For most treatments, the presence of progesterone did not affect the incidence of abnormal fertilization ($P>0.1$) (Table 22). In one treatment (oocytes matured CF in MEM + serum and drilled before the culture period), the addition of progesterone to the culture media significantly increased the incidence of abnormal fertilization ($P<0.05$).

The addition of AGP to the maturation media for CI oocytes significantly reduced the fertilizability of these

FIGURE 12

Penetration of intact oocytes and oocytes drilled (DR) before or after the period of maturation. In most groups oocytes drilled before maturation had a greater penetration rate than intact oocytes or those drilled at the end of the culture period ($P<0.05$). The number of oocytes used for each treatment ranged from 42 to 98.

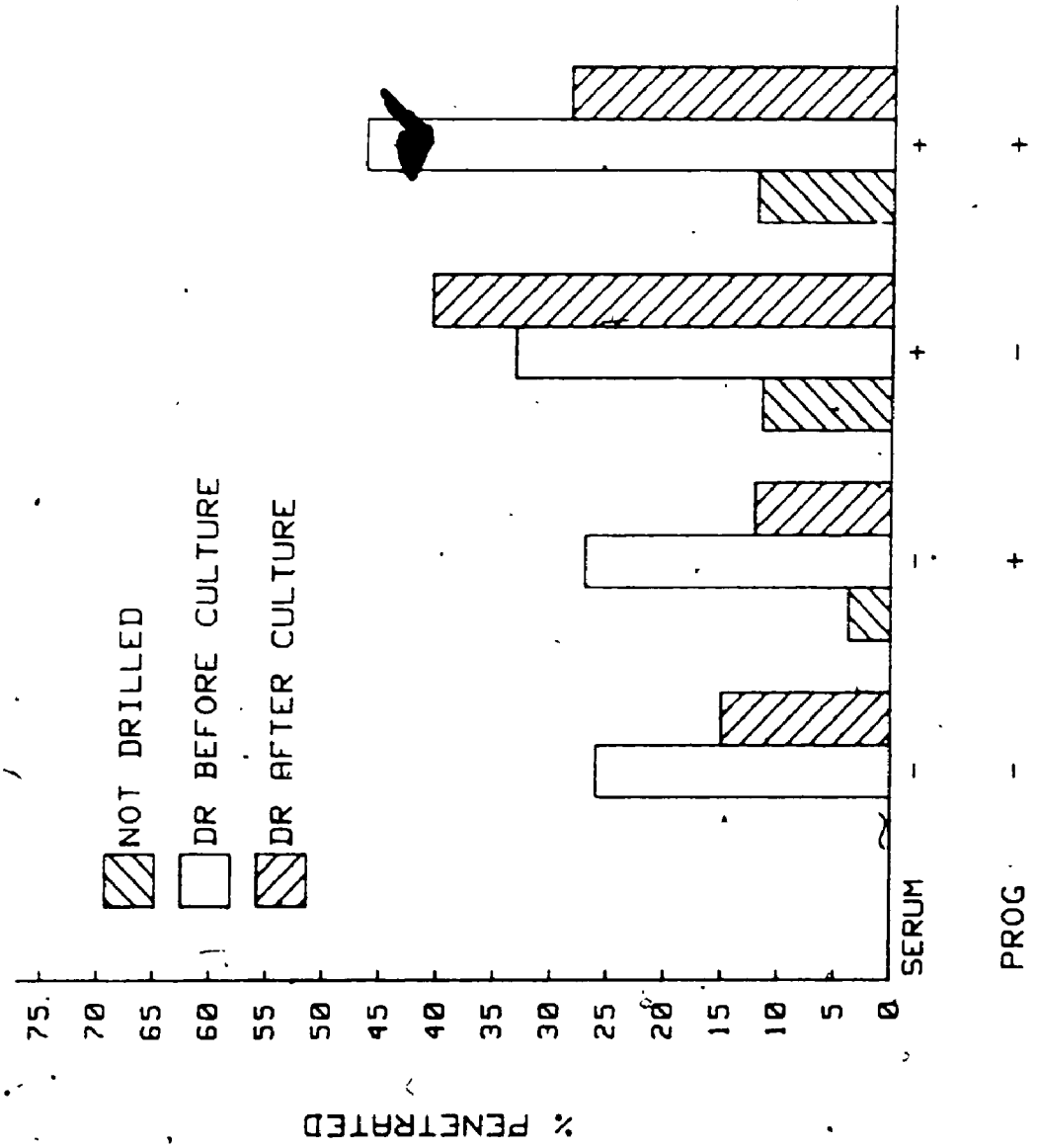

NOT DRILLED
DR BEFORE CULTURE
DR AFTER CULTURE
% PENETRATED
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
SERUM
PROG
+
-
+
-
+
-
+
-

TABLE 22 Incidence of abnormal fertilization in intact and drilled oocytes matured with or without progesterone (P_4)

Group	Maturation conditions Serum	P_4	No. oocytes	% Penetrated	% (of Pen) fertilized abnormally	% (of Pen) 2-cell
Intact	-	-	42	0	0	0
	-	+	52	3.8	100.0 (2)	0
	+	-	61	11.5	71.4 (7)	71.4
	+	+	58	12.1	71.4 (7)	57.1
Drilled before culture	-	-	50	26.0	92.3 (13)	30.8
	-	+	63	27.0	82.4 (17)	35.3
	+	-	57	33.3	47.4 (19)[a]	26.3
	+	-	75	46.7	94.3 (35)[b]	20.0
Drilled after culture	-	-	60	15.0	44.4 (9)	44.4
	-	+	58	12.1	57.1 (7)	42.9
	+	-	54	40.7	72.7 (22)	63.6
	+	+	98	28.6	82.1 (28)	64.3
CI	+	-	58	70.7[a]	2.4 (41)	92.7
CI + AGP	+	-	68	47.1[b]	0 (32)	100.0

a,b Within each column, values with different superscripts are significantly different ($P<0.05$).

oocytes ($P<0.05$); however, the incidence of abnormal fertilization in CI oocytes was not significantly altered by the presence of AGP during maturation (Table 22). In all oocytes matured CI, the proportion of oocytes undergoing abnormal fertilization was <3%.

The proportions of fertilized oocytes undergoing cleavage to 2-cell embryos are summarized in Table 22. Oocytes drilled before the period of maturation showed less ability to cleave to 2-cells after fertilization than oocytes drilled at the end of the culture period. For all treatments the addition of progesterone to the maturation media did not significantly affect the ability of fertilized oocytes to cleave to 2-cell embryos.

9.3 Discussion

DeFelici & Siracusa (1982) reported that immature, cumulus-free mouse oocytes cultured *in vitro* undergo spontaneous hardening of the zona pellucida, as indicated by an increased resistance to chymotrypsin digestion. In addition, hardening of the zona pellucida in culture has been correlated with a decrease in the frequency of fertilization (Gianfortoni & Gulyas, 1985; Downs *et al.*, 1986). In our experiments a decrease in the frequency of fertilization was also observed with oocytes matured CF. While this observation suggests that zona hardening had occurred, these oocytes were not exposed to chymotrypsin digestion and, thus, the possibility exists that other

changes in the zona pellucida had occurred; i.e. loss or damage to sperm receptors on the zona pellucida. For simplicity, however, the decrease in penetrability of oocytes matured CF will be referred to as zona hardening.

In our experiments serum and follicular fluid were both able to prevent or limit zona hardening such that fertilizability was maintained. Previous studies have indicated that spontaneous zona hardening is reduced in the presence of cumulus cells (DeFelici & Siracusa, 1982) or serum (Downs et al., 1986) or GAGs (DeFelici et al., 1985). Although GAGs were able to prevent zona hardening almost completely during a 5 h culture (DeFelici et al., 1985), their protective effect decreased to varying degrees during longer culture periods. In the present study, GAGs were unable to maintain fertilizability of oocytes matured in media containing the same concentrations of GAGs as reported previously. Since GAGs cannot completely prevent zona hardening during longer culture periods, it is possible that additional factors present in follicular fluid cooperate with GAGs to serve this function; this is supported by the fact that follicular fluid maintained fertilizability whereas GAGs alone did not.

As detailed in previous chapters, there is some evidence to suggest that meiosis is inhibited in vivo by a follicular factor; maturation-inhibiting activity has been demonstrated in rabbit (Chang, 1955), porcine (Tsafriri & Channing, 1975b), bovine (Gwatkin & Andersen, 1976) and

human follicular fluid (Chari et al., 1983). For meiotic maturation to occur in vivo, the inhibitory factor must be inactivated or its levels must decrease in response to the LH surge. Channing et al. (1983) and Hillensjo et al. (1985) have reported that follicular fluid from preovulatory human follicles contains significantly less activity of the putative maturation-inhibiting factor than do follicles that contain immature oocytes. Indeed, preovulatory rat follicular fluid in the present experiment supported maturation and enhanced fertilizability of in vitro matured oocytes, clearly indicating that levels of any maturation-inhibiting factor are low enough to be ineffective. This does not eliminate the possibility that a maturation-promoting factor may be involved in the modification or reduction of inhibiting activity.

The technique of drilling a hole in the zona pellucida was shown to be an effective method of increasing the penetrability of oocytes. At low sperm concentrations (i.e. 50,000 sperm/ml) the fertilization rate of non-drilled oocytes was 5-fold less than that of drilled oocytes, and yet the non-drilled oocytes showed only an ~50% greater capability for development to term fetuses. Thus, drilling was the more advantageous method to produce fetuses in cases of low sperm numbers.

The proportion of drilled oocytes that developed to fetuses following transfer was significantly less than that of non-drilled oocytes. Although the reasons for this difference are not known, it can be speculated that the

presence of a hole reduced the ability of the zona pellucida to protect the oocyte from possible adverse conditions in the oviductal or uterine environment.

Although originally designed to be helpful in cases of oligospermia, the zona drilling technique was especially useful in this study for by-passing the barrier to penetration that is normally presented by the hardening zona pellucida of *in vitro* matured CF oocytes. Oocytes matured CF in serum-free media had low fertilizability and a high incidence of abnormal pronuclear formation. The high incidence of abnormal fertilization in CF oocytes matured in MEM + serum suggested that the abnormal fertilization was related to the lack of cumulus cells rather than an inadequate culture medium. To provide evidence to support this suggestion, the drilling technique was used to increase the penetrability of CF oocytes matured in serum-free media. In doing so, the incidence of abnormal fertilization in these oocytes could be directly related to the absence of cumulus cells during maturation. Thus the lower penetration rate of these oocytes (which was overcome by drilling) was probably due to zona hardening during the period of maturation; whereas the high incidence of abnormal pronuclear formation was caused by the absence of cumulus cells.

In Chapter 8, a hypothesis was proposed for a mechanism by which the gonadotropin surge triggers the maturation of oocytes. Since progesterone plays a major

role in this mechanism in non-mammalian species, it was of interest to determine if progesterone was involved in the maturation of mammalian oocytes. In the rat, cumulus cells isolated from preovulatory follicles after the LH surge have enhanced production of progesterone in culture compared to those isolated before the surge (Hillensjo et al., 1981). It was a likely possibility then that, if progesterone was involved in the maturation of the oocytes, the cumulus cells would be the source of progesterone for the oocytes, probably delivering it to the oocytes by means of projections through the zona pellucida which communicate directly with the ooplasm via gap junctions. As it is not known if progesterone can enter the oocyte directly in the absence of these cumulus cell projections through the zona, the present study attempted to allow the progesterone access to the oolemma via a hole made by zona drilling. As the results indicated, progesterone had no effect on the penetrability of the oocytes nor on the incidence of abnormal fertilization. Thus, progesterone may not play a direct role in cytoplasmic maturation of mammalian oocytes.

The addition of an inhibitor of steroidogenesis, AGP, to the maturation media resulted in a significant decrease in fertilization. Although only a preliminary study, the concentration of AGP used was one known to inhibit fully granulosa cell steroidogenesis under similar culture conditions. The results suggest that some aspect of steroidogenesis is involved in the fertilization of rat oocytes, but the possibility that this concentration of AGP

had a toxic effect on the oocytes cannot be ignored.

In conclusion, this study provided additional evidence that cumulus cells play a dual role in the maturation of rat oocytes. Cumulus cells help to maintain the penetrability of the oocytes while, at the same time, ensuring that normal cytoplasmic maturation occurs. The factor(s) involved in the maturation-promoting action of the cumulus cells has yet to be identified.

CHAPTER 10

HORMONAL FACTORS AFFECTING THE IN VITRO MATURATION-FERTILIZATION PROCESS

10.1 Introduction

The previous two chapters have described experiments which sought to determine the optimal conditions for maturation of oocytes *in vitro*. Both serum and cumulus cells were apparently necessary to help maintain the penetrability of the oocytes during the culture period. In addition, cumulus cells were involved in regulating normal cytoplasmic maturation through some unknown mechanism. As maturation *in vivo* occurs in an environment abundant in hormones, it was of interest to investigate the possible roles of some of these hormones in the maturation of oocytes *in vitro*.

The increased accumulation and release of progesterone by rat ovarian follicles in response to LH (Stoklosowa & Nalbandov, 1972; Tsafriri *et al.*, 1973; Hillensjo *et al.*, 1981) suggests that progesterone may play a role in the maturation of oocytes. However, previous studies have failed to provide a link between steroidogenesis and oocyte maturation in the rat, when GVBD has been used as the end-point. Tsafriri *et al.* (1972) could not mimic the meiosis-inducting action of LH on cultured follicle-enclosed rat oocytes by addition to the medium of progesterone, 20α-

dihydroprogesterone or 17β-estradiol. These same authors found that the meiosis-inducing action of LH was not prevented by the addition of cyanoketone, an inhibitor of steroid hormone synthesis. The experiment performed in the previous chapter suggested that, while progesterone did not affect maturation directly, inhibition of steroidogenesis with AGP reduced the proportions of oocytes undergoing fertilization. Thus it appears that the steroidogenic response of the ovarian follicle to LH may not be involved in the ability of this hormone to induce meiotic maturation of oocytes, but may be important for providing the maturing oocyte with the ability to be fertilized.

Many of the studies that have investigated the hormonal control of oocyte maturation-fertilization have used follicle-enclosed oocytes. Although this system is more closely related to the *in vivo* environment than isolated oocytes, cultured follicles suffer the disadvantage of complexity. Due to interactions among the various cell types, the response to a stimulus to the entire follicle is difficult to interpret. For example, consider the following possibility. Since the gonadotropin surge induces complete maturation of the oocyte, LH may have a dual role in the process; i.e. removal or modification of the inhibitory factor and formation of a maturation-inducing substance. While removal of the inhibitory factor would allow GVBD to occur, the MPF (possibly a steroid) would elicit normal cytoplasmic

maturation. Thus, studies which examine the response of follicle-enclosed oocytes to changes in steroid hormone levels may indicate a lack of response where, in fact, the ooyctes cannot respond while the inhibitory factor is still present. Conversely, numerous investigators are pursuing the identity of the meiosis-inhibiting factor. In many studies the occurrence of GVBD has been made synonymous with oocyte maturation, without the appropriate attempts at fertilization or subsequent development. Clearly more information about both nuclear and cytoplasmic maturation must be obtained before a possible mechanism for complete oocyte maturation can be outlined.

The maturation of isolated oocytes, being a more simple system, may be useful in determining the importance of hormonal factors on nuclear and cytoplasmic maturation. Three groups of experiments were performed with the following objectives: 1) to determine the need for PMSG stimulation prior to the collection and maturation of immature oocytes; 2) to determine the effect of FSH on the in vitro maturation and subsequent fertilization of oocytes, and 3) to determine if substances released by FSH-stimulated granulosa cells can influence oocyte maturation and fertilizability.

10.2 Methods and Results

10.2.1 Varying Time after PMSG

The results of the previous experiments indicated that

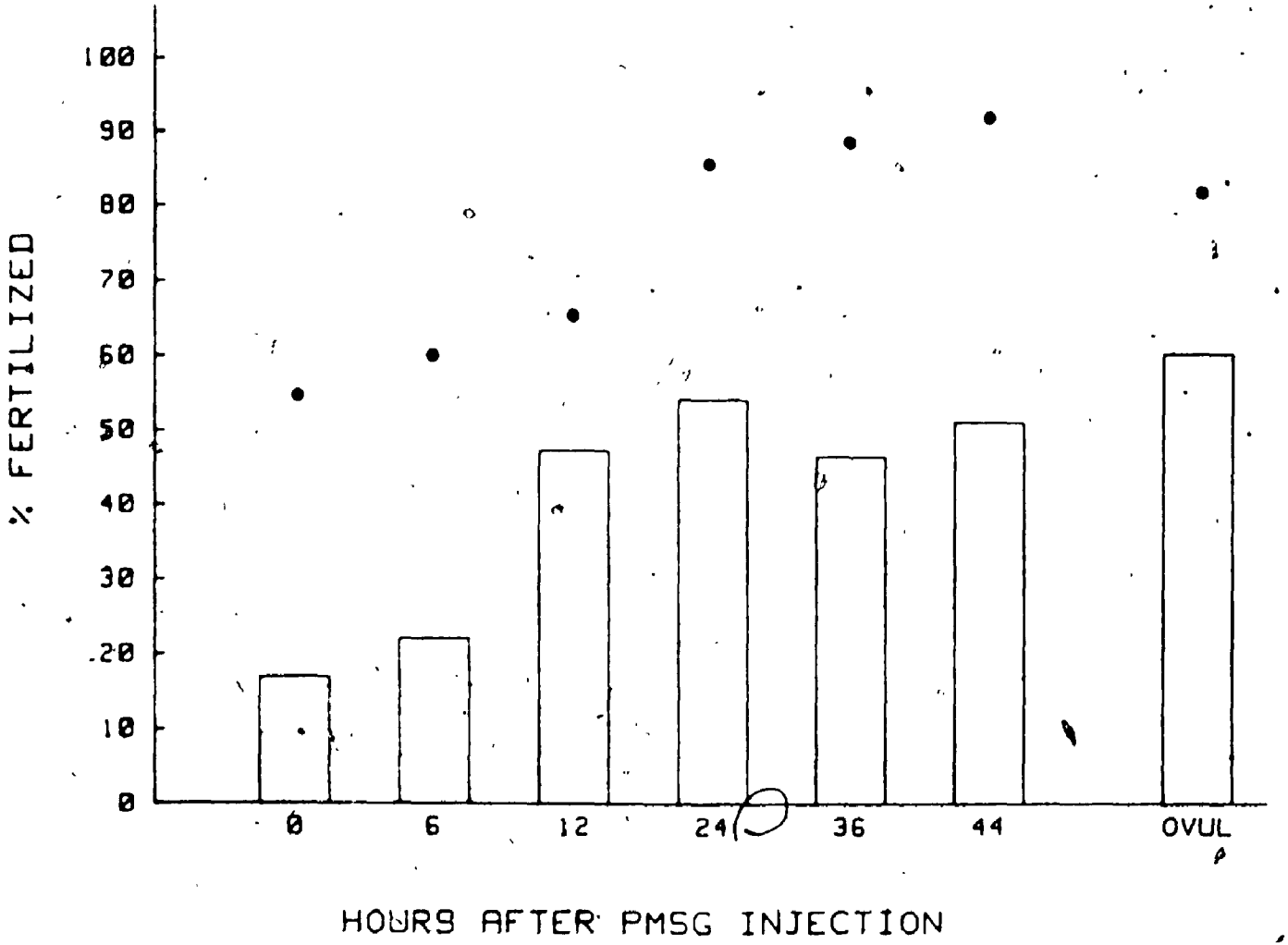
immature oocytes collected 35-40 h after the PMSG injection were capable of maturation and fertilization *in vitro*. To determine the minimal length of time post-PMSG that was necessary to allow this capability, immature oocytes were collected at 0, 6, 12, 24, 36 and 44 h after the PMSG injection and were matured in the presence of their cumulus cells in MEM + 20% rat serum for 10-11.5 h. The oocytes were assessed for GVBD and PB formation and then inseminated *in vitro*. The PMSG injections were staggered such that the period of maturation *in vitro* and the sperm preparation were common to all treatments. After 14-16 h, the oocytes were examined for evidence of fertilization. Cleavage of fertilized oocytes was assessed 24 h later.

More than 98% of the oocytes in each group had undergone GVBD by the end of the culture period, with 71-100% of these forming the first PB. The fertilization rate of these oocytes is shown in Figure 13. Oocytes collected from rats that did not receive any PMSG were capable of *in vitro* maturation and were fertilized, although at a significantly lower rate than oocytes collected at least 12 h after the PMSG injection ($P<0.01$). Maximum percentage of fertilization was obtained with oocytes collected as soon as 12 h after PMSG; however, maximum cleavage of fertilized oocytes occurred in oocytes collected at least 24 h post-PMSG.

FIGURE 13

Fertilization of *in vitro* matured oocytes collected at various times after PMSG injection. Fertilization of oocytes collected at least 24 h post-PMSG was significantly greater than those collected at 0 or 6 h post-PMSG. In each group the number of oocytes that were cultured ranged from 65 to 150.

● = % of fertilized oocytes undergoing cleavage to 2-cell embryos


% FERTILIZED
100
90
80
70
60
50
40
30
20
10
0
0
6
12
24
36
44
OVUL
HOURS AFTER PMSG INJECTION

10.2.2 Effect of FSH

In vitro studies have determined that FSH is a potent stimulus for cumulus expansion during the maturation of oocyte-cumulus cell complexes (Dekel & Kraicer, 1978; Eppig, 1979b). Since cumulus expansion and oocyte maturation occur during the same period of time, it was of interest to determine if, in addition to its effect on cumulus expansion, FSH also had an influence on nuclear and cytoplasmic maturation. The role of FSH was investigated in three experiments: A) FSH dose response; B) time course of maturation in FSH-supplemented media, and C) effects of FSH on oocytes maintained in meiotic arrest *in vitro*.

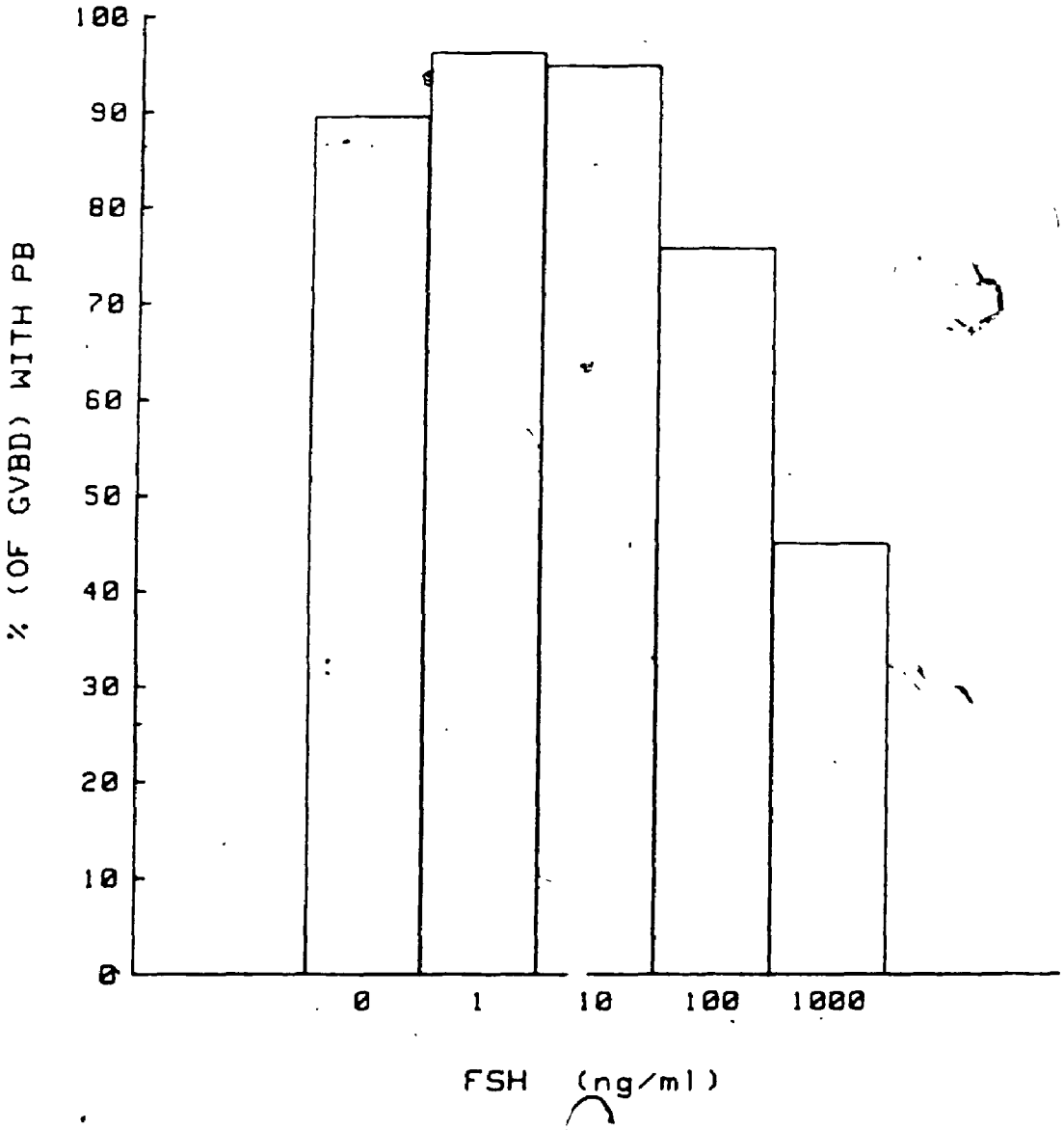
A FSH Dose Response

Immature oocytes were collected from PMSG-treated rats as described in Section 4.2.2. The oocytes were matured CI in wells containing MEM + 10% serum and 0, 1, 10, 100 or 1000 ng FSH (NIH-oFSH-S16)/ml. At the end of the 9-10.5 h culture period, the cumulus cells were stripped away from the oocytes and the oocytes were assessed for GVBD and PB formation. They were washed in fresh DPBS and transferred to droplets of fertilization medium for insemination with epididymal sperm.

The proportions of oocytes undergoing GVBD and PB extrusion during the 9-10.5 h culture period are shown in Figure 14. Higher concentrations (100 or 1000 ng/ml) of FSH in the maturation media significantly reduced the proportions of oocytes with PB at the end of the culture

FIGURE 14

Proportion of oocytes undergoing GVBD + PB extrusion during a 9-10.5 h culture period in MEM supplemented with various concentrations of FSH. Higher concentrations (100 or 1000 ng/ml) significantly reduced the proportion of oocytes with PB at the end of the culture period ($P<0.01$). The results for each concentration of FSH were obtained from the maturation of 124 - 436 oocytes.


100
90
80
70
60
50
40
30
20
10
0
% (OF GVBD) WITH PB
0
1
10
100
1000
FSH (ng/ml)

period ($P<0.01$).

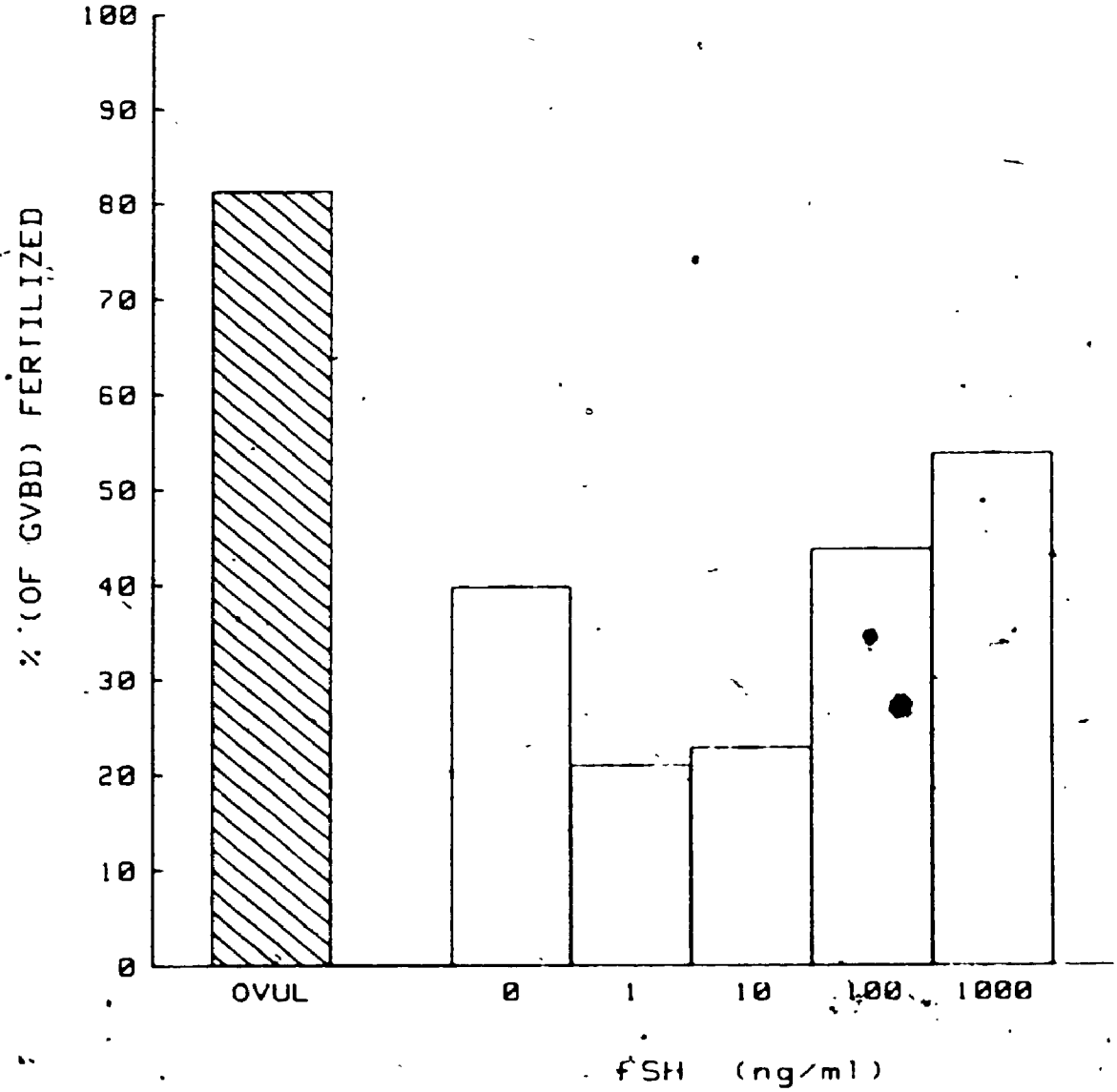
Oocytes matured in the presence of varying concentrations of FSH showed a biphasic change in fertilizability (Figure 15). Low concentrations of FSH (1 or 10 ng/ml) in the maturation media resulted in a significant decrease in fertilizability compared to non-stimulated oocytes, whereas high concentrations of FSH led to similar (at 100 ng/ml) or significantly higher (1000 ng/ml) proportions of oocytes undergoing fertilization ($P<0.01$).

B Time Course of Maturation

In the previous experiment, the higher concentrations of FSH reduced the proportions of oocytes with PB after 9-10.5 h of culture. This result suggested that FSH either decreased or increased the rate of nuclear maturation. If the rate of maturation was decreased, PB would not yet be formed; an increase in the rate of maturation would lead to earlier disintegration of PB. To determine which of these two possibilities was correct, immature oocytes were collected and matured CI in wells containing MEM + 10% serum. For half the oocytes, 500 ng FSH/ml was added to the culture medium. Oocytes were removed from the culture media at 2 h intervals after 6 to 16 h of culture. Cumulus cells were stripped from the oocytes and the oocytes were assessed for nuclear maturation. Following transfer to droplets of fertilization media, they were exposed to epididymal sperm for *in vitro* fertilization. The

FIGURE 15

Fertilization of oocytes matured CI in MEM supplemented with various concentrations of FSH (n = 124 - 225 oocytes/group). Low concentrations of FSH (1 or 10 ng/ml) caused a significant decrease in fertilization compared to non-stimulated oocytes. High concentrations of FSH led to similar (at 100 ng/ml) or significantly higher (1000 ng/ml) prpportions of oocytes undergoing fertilization ($P<0.01$). The fertilization of ovulated (OVUL) oocytes were used as a control.


100
90
80
70
60
50
40
30
20
10
0
% (OF GVBD) FERTILIZED
OVUL
0
1
10
100
1000
FSH (ng/ml)

experiment was organized such that all the oocytes were collected within a 2.5 h period and a common sperm preparation could be used to inseminate all the oocytes. The oocytes were assessed for fertilization 14-16 h later and were examined again after an additional 24 h for cleavage to 2-cell embryos.

The proportion of oocytes undergoing GVBD and PB formation after various lengths of time in culture are illustrated in Figure 16. All oocytes had undergone GVBD by 6 h of culture. FSH clearly decreased the rate of nuclear maturation such that maximum incidence of PB was seen at 12 h as compared with 8 h for unstimulated oocytes. In addition, PB were apparently more stable in FSH-supplemented media. In MEM + serum, PB were formed and disintegrated within a 6 h period. In contrast, 40% of oocytes in FSH-supplemented media still had PB 8 h after the time when PB were first evident.

It appears that individual oocytes in the FSH-supplemented media may have responded differently to the presence of FSH since the formation of PB by the oocytes occurred between 6-12 h of culture, whereas all the unstimulated oocytes formed PB within a 2 h period (6-8 h of culture). This extended period of PB formation may have contributed to the apparent PB stability described in the previous paragraph.

Figure 17 shows the proportion of oocytes undergoing fertilization after various lengths of time in culture.

FIGURE 16

Proportion of oocytes undergoing GVBD + PB extrusion after various lengths of time in MBM or MEM supplemented with 500 ng/ml FSH. The lines indicate the percentage of GVBD oocytes that have formed a PB. (n = 58 - 205 oocytes/group)

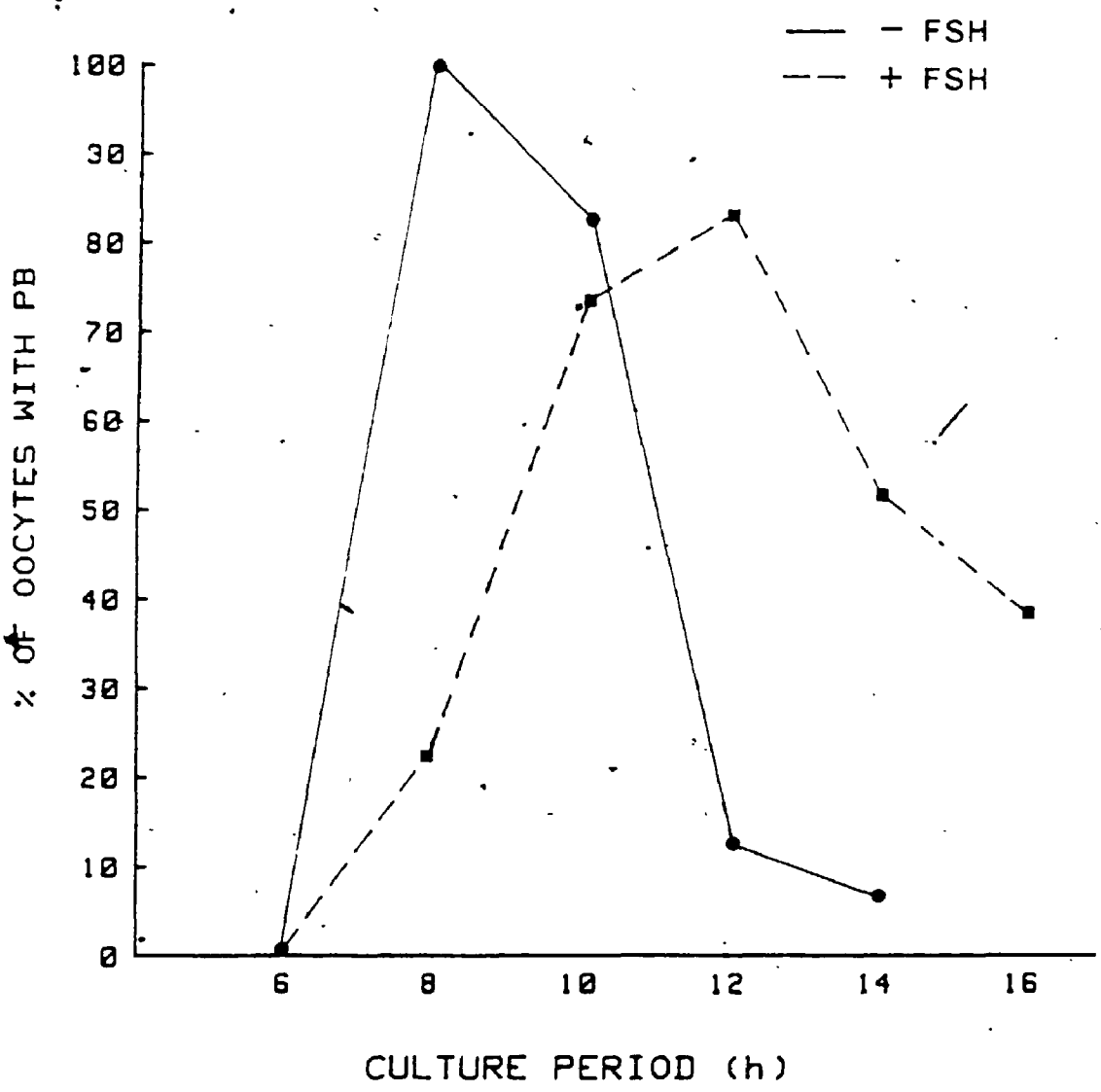
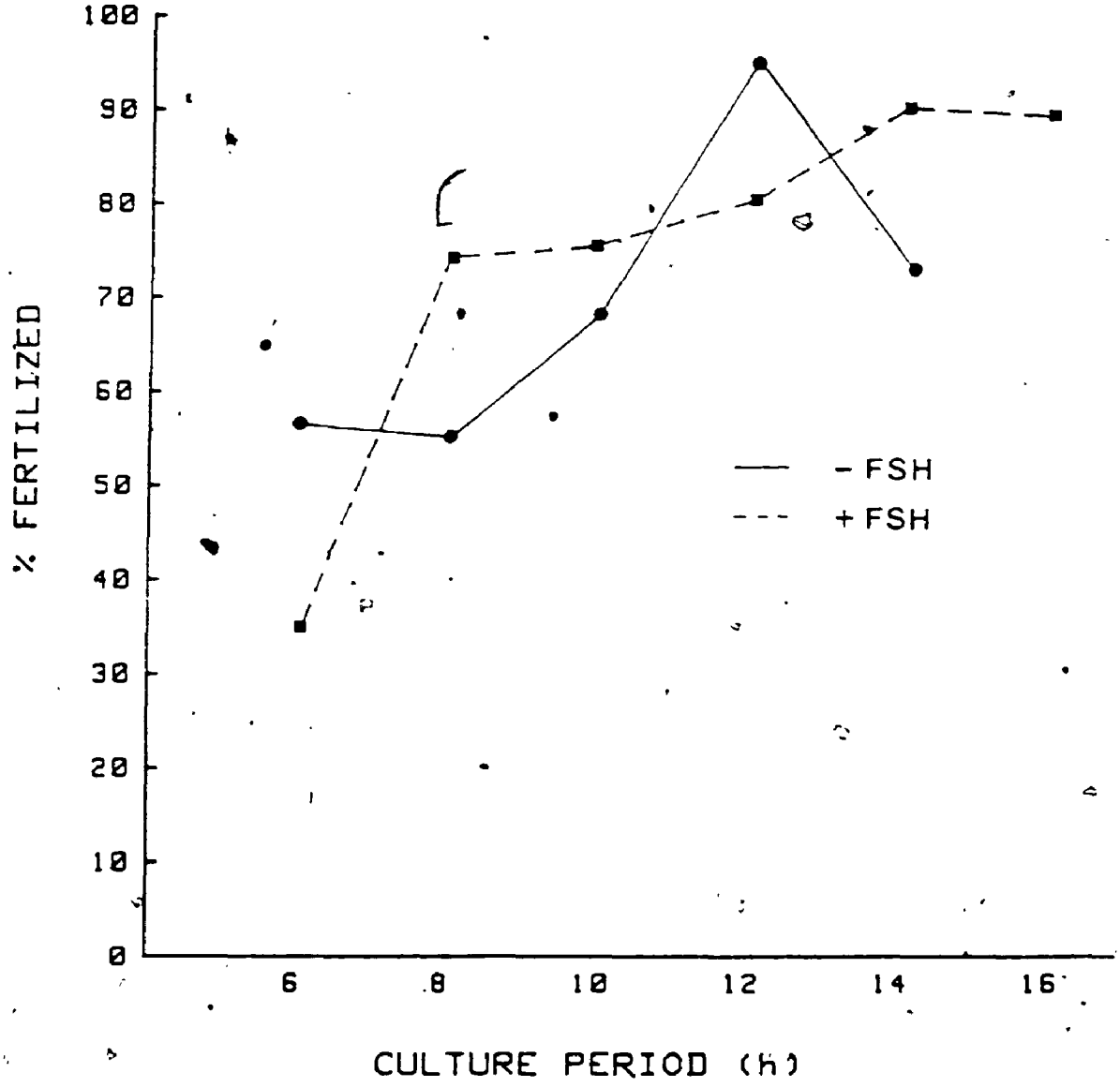

— FSH
– – + FSH
% OF OOCYTES WITH PB
100
30
80
70
60
50
40
30
20
10
0
6
8
10
12
14
16
CULTURE PERIOD (h)

FIGURE 17

Proportion of oocytes undergoing fertilization after various lengths of time in MEM or MEM supplemented with 500 ng/ml FSH. The maximum proportion of oocytes undergoing fertilization following maturation in the presence of FSH did not differ significantly from that of unstimulated oocytes ($P>0.1$). The number of oocytes used in each group ranged from 54 to 114.


100
90
80
70
60
50
40
30
20
10
0
% FERTILIZED
6
8
10
12
14
16
CULTURE PERIOD (h)
- FSH
+ FSH

The fertilizability of oocytes matured in MEM + serum was similar to that seen in Section 8.2.2, reaching peak percentage fertilization after 12 h of culture. The fertilizability of oocytes matured in the presence of FSH increased significantly between 6 and 8 h of culture, then slowly climbed to a maximum of 88.9% (n=81) at 14 h. Although delayed by 2 h, the maximum proportion of oocytes undergoing fertilization following maturation in the presence of FSH did not differ significantly from that of unstimulated oocytes (95.0%, n=100).

C FSH - An Inducer of Nuclear Maturation?

The results of the previous experiment indicated that FSH had an effect on the rate of nuclear maturation in spontaneously maturing oocytes. The purpose of the present experiment was to determine if FSH played a role in initiating nuclear maturation by observing its effects on oocytes maintained in meiotic arrest in vitro. Oocytes were maintained in meiotic arrest using isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor; i.e. IBMX increases cAMP levels by preventing its degradation. As described earlier, elevated levels of cAMP help to maintain the oocytes in meiotic arrest. A preliminary study indicated that IBMX at a concentration of 200 μM was effective in inhibiting GVBD in 86.7% (n=105) of oocytes matured for 10 h.

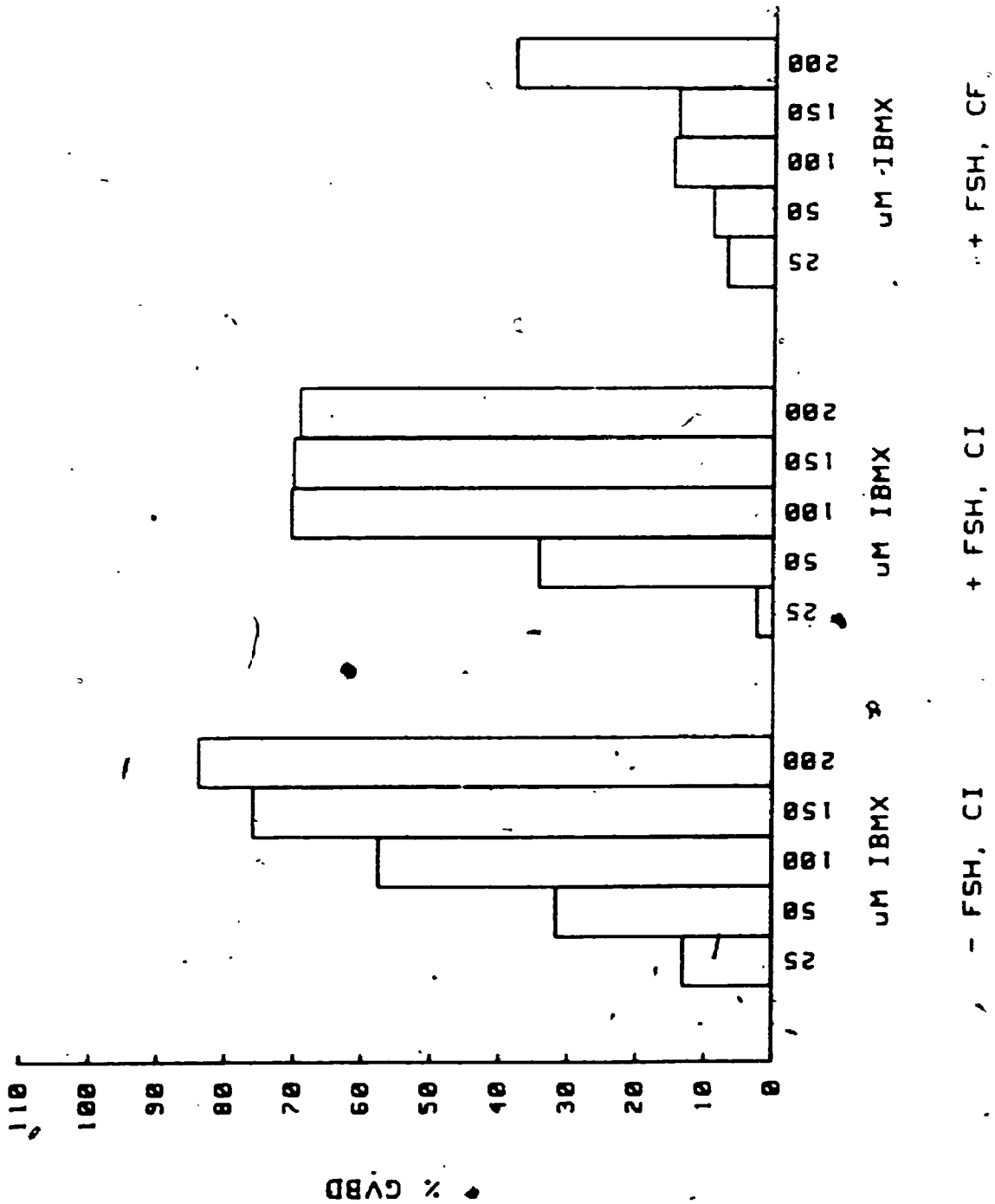
Immature oocytes were obtained 30 h after PMSG injection into the immature donor rats. They were

collected and rinsed in DPBS + 200 µM IBMX before transfer to wells containing MEM + 5% serum + 25, 50, 100, 150 or 200 µM IBMX. The oocytes were cultured for 6 h and then washed twice in DPBS + 200 µM IBMX. Approximately 25% of the oocytes from each treatment were stripped of their cumulus cells and assessed for GVBD and PB formation. All oocytes were then returned to media similar to that of their first period of culture with the exception that all wells contained 1000 ng FSH/ml as an additional supplement. Freshly collected immature oocytes were matured in MEM + 5% serum and used as a control. At the end of the 10 h culture period, the cumulus cells were removed from CI oocytes and the oocytes were examined for signs of nuclear maturation. Mature (GVBD) oocytes were washed in DPBS before transfer to droplets of fertilization media for insemination. Fertilization was assessed 14-16 h later.

During the first culture period, increasing concentrations of IBMX caused an increase in the proportion of oocytes maintained in meiotic arrest, as seen by the persistence of the germinal vesicle (Figure 18). The addition of FSH to the culture media of these oocytes for the second culture period did not significantly affect the proportions of CI oocytes with an intact germinal vesicle. Oocytes matured in FSH during the second period of culture, however, were not maintained in meiotic arrest as effectively as CI oocytes such that, at each concentration of IBMX (except 25 µM), the proportion of CI oocytes with germinal vesicle intact (GVI) at the end of the culture

FIGURE 18

Proportion of oocytes undergoing GVBD following culture in MEM + IBMX for 6 h (- FSH, CI), followed by a second culture period with (CI) or without cumulus cells (CF) in MEM + IBMX + 1000 ng/ml FSH. The addition of FSH to the culture media for the second culture period did not significantly affect the proportions of CI oocytes with an intact germinal vesicle. At each concentration of IBMX (except 25 uM), the proportion of CI oocytes with germinal vesicles was significantly greater than the proportion of oocytes with intact germinal vesicles following CF maturation ($P<0.01$). The number of oocytes used in each group ranged from 50 to 102. The data represent the results of 1 of 2 replicates.


% CYBD
0
10
20
30
40
50
60
70
80
90
100
110
- FSH, CI
uM IBMX
25
50
100
150
200
+ FSH, CI
uM IBMX
25
50
100
150
200
+ FSH, CF
uM IBMX
25
50
100
150
200

period was significantly greater than the proportion of GVI oocytes following CF maturation ($P<0.01$) (Figure 18). 100% of oocytes matured in MEM + serum underwent GVBD.

Figure 19 indicates the proportion of mature oocytes fertilized following maturation CI or CF in MEM + serum + IBMX + FSH. The incidence of fertilization was higher with oocytes matured in lower concentrations of IBMX, with a plateau in fertilization occurring at IBMX concentrations less than 100 µM IBMX. The maximum percentage of oocytes fertilized (60.0%; IBMX at 100 µM) was not significantly different from the percentage of oocytes fertilized in the MEM + serum group.

10.2.3 Granulosa Cell Preconditioned Media

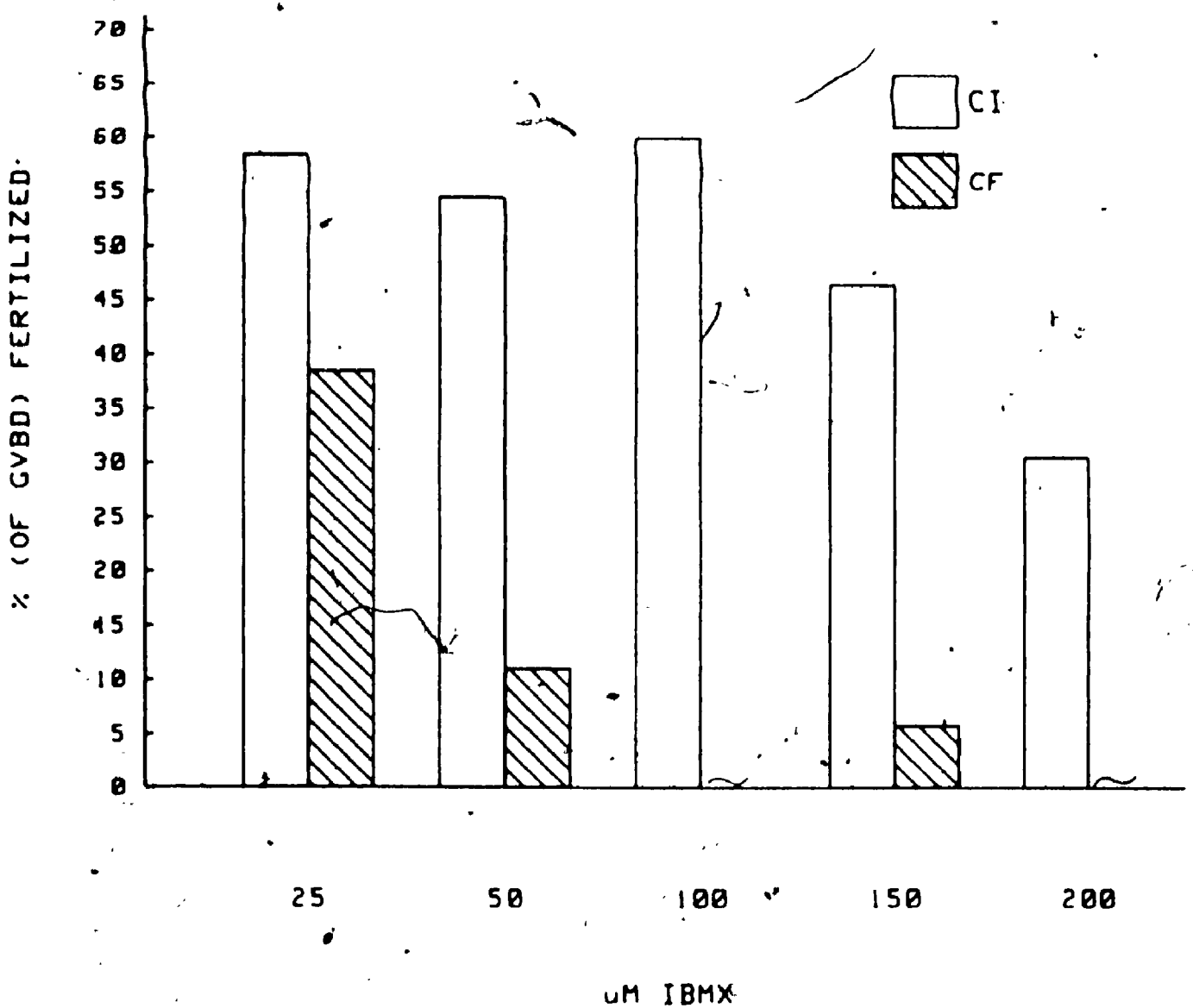
In response to the gonadotropin surge, intrafollicular oocytes undergo complete maturation in preparation for ovulation. As the oocytes themselves do not have receptors for gonadotropins (Amsterdam et al., 1975), it is reasonable to assume that the granulosa and cumulus cells respond to the gonadotropins in such a way as to provide an environment suitable for oocyte maturation. The purpose of this experiment was to observe the effects of granulosa cell products on the maturation and fertilization of oocytes.

A Pat and Porcine Granulosa Cells from Preovulatory Follicles

As the intrafollicular changes that induce oocyte

FIGURE 19

Proportion of mature oocytes undergoing fertilization following maturation CI or CF in MEM + serum + IBMX + 1000 ng/ml FSH. The incidence of fertilization was higher with oocytes matured in lower concentrations of IBMX. At each concentration of IBMX, oocytes matured CI fertilized in higher proportions than their CF counterparts ($P<0.01$). The number of mature oocytes inseminated in each treatment ranged from 9 to 77. The data represent the results of 1 of 2 replicates.


% (OF GVBD) FERTILIZED
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
CI
CF
25
50
100
150
200
uM IBMX

maturation occur during the few hours prior to ovulation, the aim of this first experiment was to obtain granulosa cells from preovulatory follicles. Immature rats (60-65 g) were treated with 40 I.U. PMSG at 2000 to induce the development of a large number of follicles. The rats were killed by cervical dislocation 60 h later and the follicles were punctured with a 25-gauge needle. Using gentle pressure, the granulosa cells were expressed into Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 (1:1) (DMEM:F12; Gibco Laboratories) containing 1.2 g $NaHCO_3$/l, 15 mM HEPES (pH 7.2) and antibiotics (50 U penicillin/ml, 50 µg streptomycin/ml and 0.625 µg fungizone/ml). The suspension of granulosa cells was centrifuged at 270 x g for 3 minutes. The supernatant was discarded and the cells were resuspended in 5 ml Hank's balanced salt solution (Gibco Laboratories) containing 2.5 mg collagenase/ml, 0.5 mg protease/ml and 0.5 mg hyaluronidase/ml, The cells were allowed to disperse in this solution for 15 minutes in a shaking water bath at 37°C. The suspension was centrifuged again at 270 x g for 3 minutes and the cells were resuspended in MEM. Aliquots of the cell suspension containing 125,000, 250,000, 500,000 or 1,000,000 cells were cultured in 24-well tissue culture plates (Falcon). FSH (NIH-oFSH-S14) was added to each well at a final concentration of 500 ng/ml. Cells were cultured in 1 ml of medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The preconditioned media were collected

after 40 h and, if necessary, was stored at -20°C.

Porcine ovaries were obtained from a local abattoir (Thorndale, Ontario) and transported to the laboratory on ice. Medium to large follicles (3-6 mm in diameter) were bisected and the granulosa cells gently scraped off into DMEM:F12. The suspension was centrifuged at 270 x g for 3 minutes, the supernatant was removed and the cells were resuspended in MEM. They were mechanically dispersed by repeated pipetting and cultured under the same conditions as for rat granulosa cells. The preconditioned medium was collected and stored as described above.

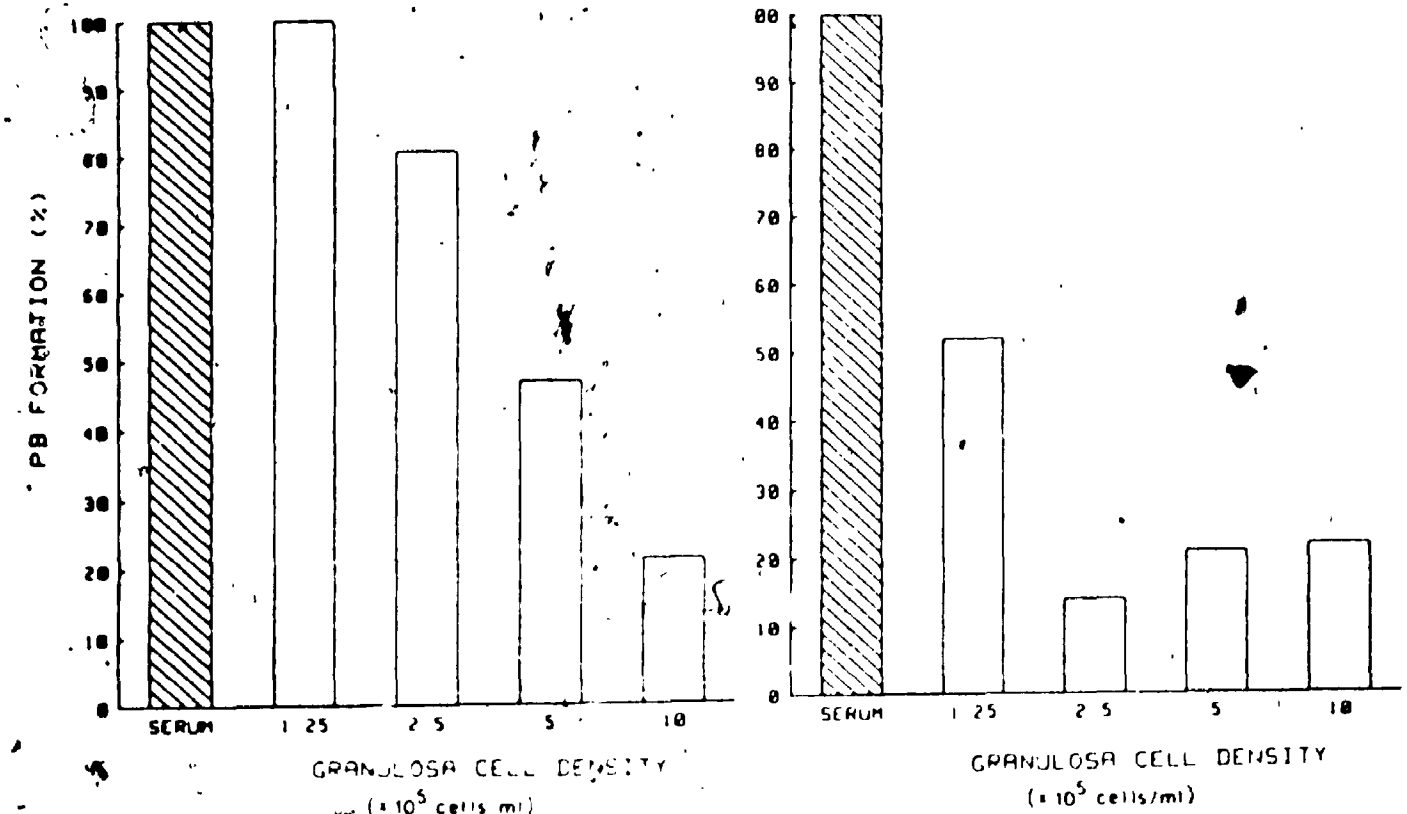
Immature oocytes were collected from PMSG-treated rats and matured CI for 9-10 h in the preconditioned media. At the end of the culture period the oocytes were stripped of their cumulus cells and assessed for GVBD and PB formation. They were washed in fresh DPBS and placed in droplets of fertilization medium for <u>in vitro</u> fertilization.

Preconditioned medium from porcine granulosa cells decreased the proportions of oocytes with PB in a dose-dependent manner (Figure 20). Preconditioned medium from rat granulosa cells at concentrations greater than 250,000 cells/ml also significantly reduced the proportion of oocytes with PB, compared to that from the lowest concentration ($P<0.05$). In both types of media, 100% of the oocytes had undergone GVBD.

Although the preconditioned medium from porcine granulosa cells at the higher concentrations reduced the incidence of PB formation at this time, this medium

FIGURE 20

Proportions of oocytes with PB following maturation in preconditioned media from porcine (left) or rat (right) FSH-stimulated granulosa cells (n = 42 - 68 oocytes/group). Preconditioned media from porcine granulosa cells decreased the proportion of oocytes with PB in a dose-dependent manner. Preconditioned media from rat granulosa cells at concentrations greater than 250,000 cells/ml also significantly reduced the proportion of oocytes with PB, compared to that from the lowest concentration ($P<0.05$). The data represent the results of 1 of 2 replicates.


PB FORMATION (%)
100
90
80
70
60
50
40
30
20
10
0
SERUM
1 25
2 5
5
10
GRANULOSA CELL DENSITY
(x 10^5 cells/ml)

significantly enhanced the fertilizability of the *in vitro* matured oocytes ($P<0.05$) compared with oocytes matured in preconditioned medium from lower density granulosa cell cultures (Figure 21). In contrast, there were no significant differences among the proportions of oocytes fertilized following maturation in preconditioned medium from rat granulosa cells.

B Granulosa Cells from DES-primed Rats

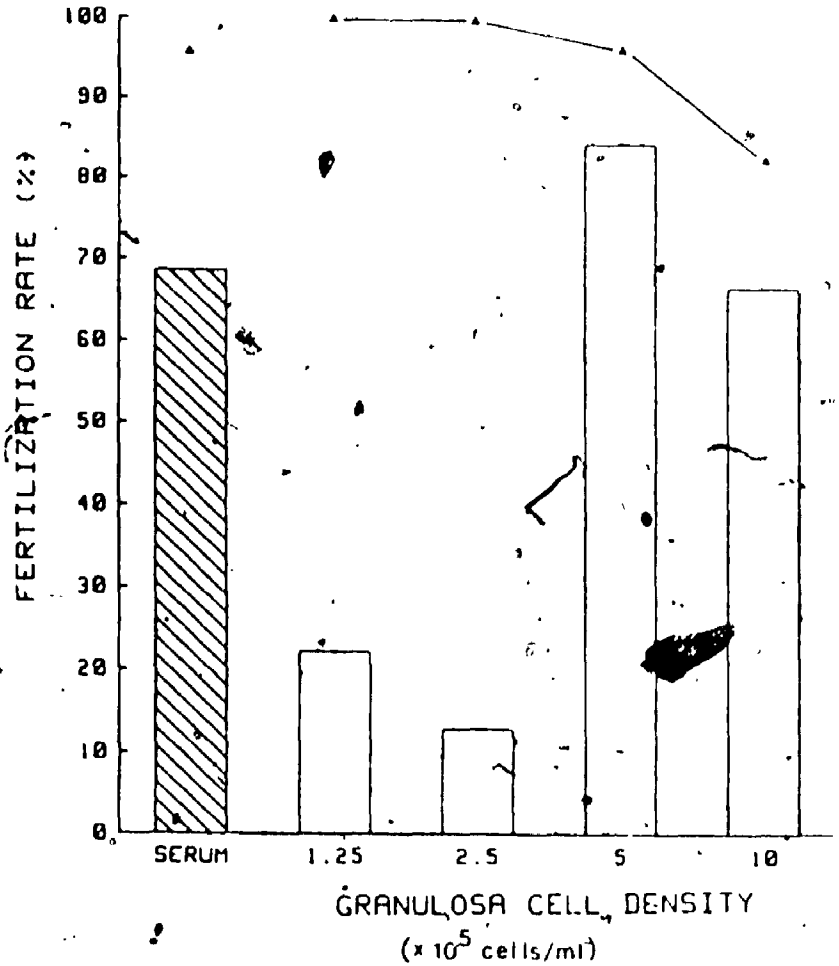
One of the major problems in the previous experiment was the dispersion of rat granulosa cells from preovulatory follicles. The granulosa cell suspension was extremely viscous and the cells were difficult to separate, even with enzymatic dispersion. For this reason, granulosa cells were collected from stimulated, but non-preovulatory, follicles for the remainder of the experiments.

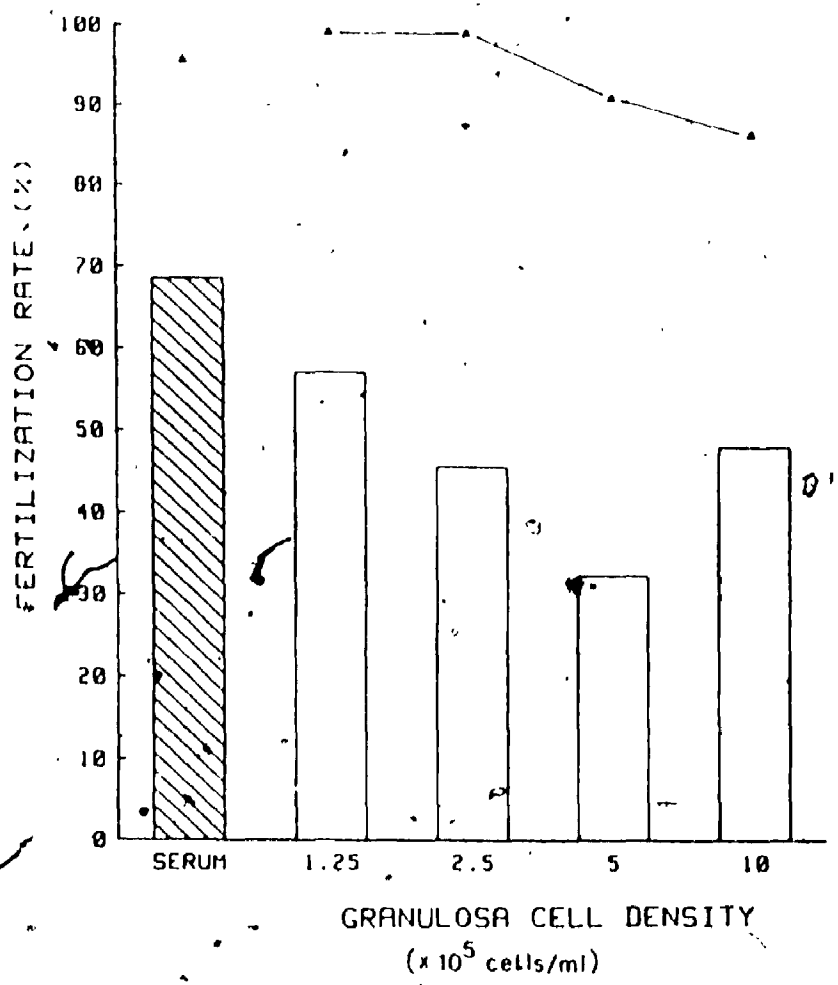
Immature female Wistar rats were injected subcutaneously at 0800 at 18, 19 and 20 days of age with 1 mg diethylstilbestrol (DES; Sigma Chemical Co.) in 0.1 ml sesame oil to increase cell yield (Goldenberg *et al.*, 1972). The animals were killed at 0800 at 21 days of age and the ovaries were collected. The ovaries were punctured with a 25-gauge needle and the granulosa cells were gently expressed to DMEM:F12. The suspension was centrifuged and resuspended in MEM as described earlier; however, no enzymatic dispersion was necessary. Wells containing 0, 500,000 or 1,000,000 cells/ml with or without 500 ng FSH/ml were incubated for 40 h. Immature oocytes were collected

FIGURE 21

Fertilization of oocytes matured in preconditioned media from porcine (left) or rat (right) FSH-stimulated granulosa cells (n = 42 - 68 oocytes/group). Preconditioned media from porcine granulosa cells at the higher concentrations increased the fertilizability of in vitro matured oocytes ($P<0.05$) compared with oocytes matured in preconditioned medium from the lower densities. There were no significant differences among the proportions of oocytes fertilized following maturation in preconditioned media from rat granulosa cells. The data represent the results of 1 of 2 replicates.

▲ = proportion of fertilized oocytes cleaving to 2-cell embryos


FERTILIZATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
SERUM
1.25
2.5
5
10
GRANULOSA CELL DENSITY
(x 10^5 cells/ml)


FERTILIZATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
SERUM
1.25
2.5
5
10
GRANULOSA CELL DENSITY
(x 10^5 cells/ml)

and matured CI in the preconditioned media for 10-11 h. The mature oocytes were then stripped of their cumulus cells, washed and transferred to fertilization medium for insemination. Fertilization was assessed 14-16 h later and cleavage rates were determined after an additional 24 h.

The results of one of the replicates of this experiment are summarized in Table 23. There were no significant differences among the treatments in the occurrence of GVBD. The proportion of oocytes extruding a PB was significantly reduced when oocytes were matured in medium from FSH-stimulated cell cultures as compared to unstimulated cultures or cultures without granulosa cells ($P<0.05$). Only oocytes matured in media from FSH-stimulated cell cultures were capable of fertilization; none of the oocytes in the remaining groups showed any evidence of fertilization. The proportion of fertilized oocytes that cleaved to 2-cell embryos was not significantly different between the 2 groups that had fertilized oocytes.

C. Time Course for Maturation in Preconditioned Media

It is clear from the previous experiment that FSH-stimulated granulosa cell cultures produced a substance that affected the incidence of PB formation and, therefore, possibly the rate of nuclear maturation. This possibility was investigated by maturing oocytes in preconditioned medium for varying lengths of time.

Granulosa cells were obtained from DES-primed rats as

TABLE 23 Effect of preconditioned media from FSH-stimulated or unstimulated granulosa cells on the maturation and fertilization of rat oocytes

Granulosa cell density (cells/ml)	FSH	No. Oocytes	% GVBD	% (of GVBD) with PB	% (of GVBD) fertilized	% (of Fert) 2-cell
1,000,000	-	63	96.8	82.0[a]	0	0
	+	79	97.5	48.1[b]	24.7	79.0
500,000	-	71	88.7	79.4[a]	0	0
	+	81	97.5	45.6[b]	19.4	100.0
0	-	56	100.0	69.6[a]	0	0
	+	20	100.0	75.0[a]	0	0

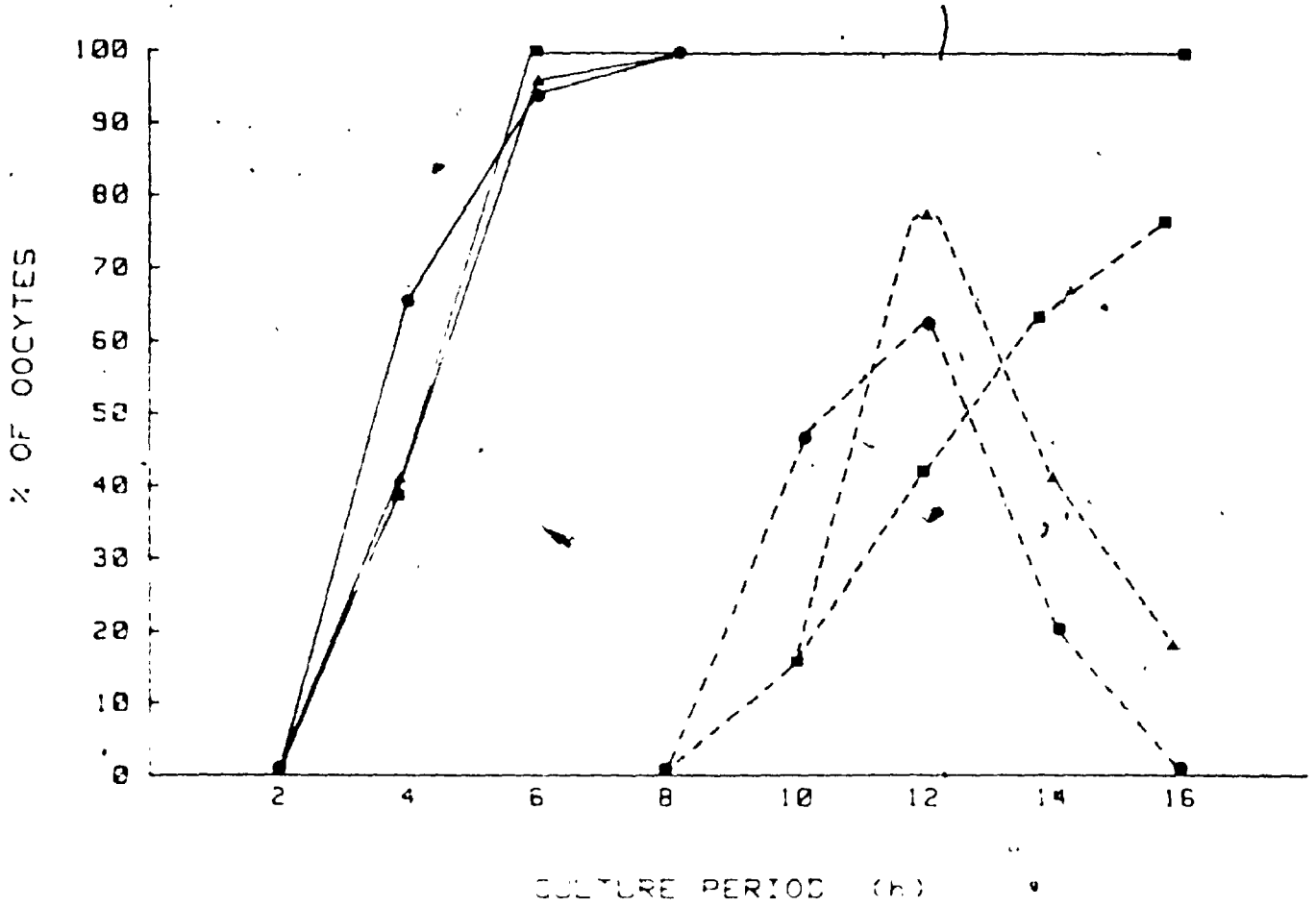
[a,b] Values with different superscripts are significantly different ($P<0.05$).

described in the previous experiment. They were cultured for 40 h unstimulated or supplemented with 500 ng FSH/ml or FSH (500 ng/ml) + LH (250 ng/ml). FSH was added to some wells without cells as an additional control. The preconditioned media were collected and a sample of each was stored at -20°C for later analyses of progesterone, 17β -estradiol and 20α -OH-progesterone levels. Radioimmunoassays for progesterone (Leung & Armstrong, 1979), 17β -estradiol (Daniel & Armstrong, 1984) and 20α -OH-progesterone (Morley et al., 1987) have been previously described and validated for direct measurements. The remaining volume of preconditioned media was used for maturing oocytes. Immature oocytes were collected and matured CI in the 3 types of preconditioned media from cell cultures. Oocytes were collected at 2 h intervals after 2 to 16 h of culture. They were stripped of their cumulus cells and assessed for nuclear maturation.

Spontaneous nuclear maturation of immature oocytes in preconditioned medium (Figure 22) occurred at a slower rate than in MEM + serum (cf Figure 4). GVBD was completed in most oocytes after 6 h of culture. Oocytes matured in medium from unstimulated granulosa cells showed the highest proportion with PB at 12 h of culture. FSH-stimulated granulosa cells produced medium in which oocytes matured much more slowly, with the incidence of PB formation still increasing at the end of the experiment. The addition of LH to FSH-stimulated cultures increased the rate of

FIGURE 22

Nuclear maturation of oocytes matured in preconditioned media from unstimulated granulosa cells (▲) or from those stimulated with FSH (■) or FSH + LH (●). The solid line indicates the proportion of oocytes with GVBD. The dashed line shows the proportion of GVBD oocytes with PB. The number of oocytes assessed in each group ranged from 21 to 88.


100
90
80
70
60
50
40
30
20
10
0
% OF OOCYTES
2
4
6
8
10
12
14
16
CULTURE PERIOD (h)

maturation until it was comparable to that of unstimulated cultures.

Table 24 summarizes the results obtained from radioimmunoassay of the samples of preconditioned media. 17β-estradiol concentrations were close to 0 in all types of media. The addition of FSH to the cultures significantly increased the levels of both progesterone and 20α-OH-progesterone ($P<0.05$). The concentrations of 20α-OH-progesterone in cultures supplemented with both FSH and LH was significantly greater than that of FSH alone ($P<0.05$).

D FSH- and LH-stimulated Granulosa Cell Cultures

In an earlier experiment (Section 10.2.3 B), it was demonstrated that oocytes matured in preconditioned media from FSH-stimulated granulosa cell cultures were capable of fertilization. The purpose of the present experiment was to determine a possible role for LH in this system.

Granulosa cells were obtained from DES-primed rats and cultured for 40 h unstimulated or with 500 ng FSH/ml, 250 ng LH/ml or both FSH + LH at these concentrations. The preconditioned media were collected and used for the maturation of oocytes. Immature oocytes were obtained from PMSG-treated rats and some were matured CI for 11-12 h in the preconditioned medium. As controls, the remaining oocytes were matured in MEM + 5% serum, or the same medium supplemented with 500 ng FSH/ml, 250 ng LH/ml or both FSH + LH at these concentrations. At the end of the culture

TABLE 24 Concentrations (± S.E.M.) of steroid hormones in preconditioned media from granulosa cells cultured for 40 h

Culture medium	17β-Estradiol ng/ml (n=2)	Progesterone ng/ml (n=2)	20α-OH-Progesterone ng/ml (n=1)
No cells + FSH	0 ± 0	0.96 ± 0.49	0.113
Cells alone	0 ± 0	0.25 ± 0.25	0.207
Cells + FSH	0.09	77.57 ± 17.15	133.17
Cells + FSH + LH	0.33	98.62 ± 8.02	193.32

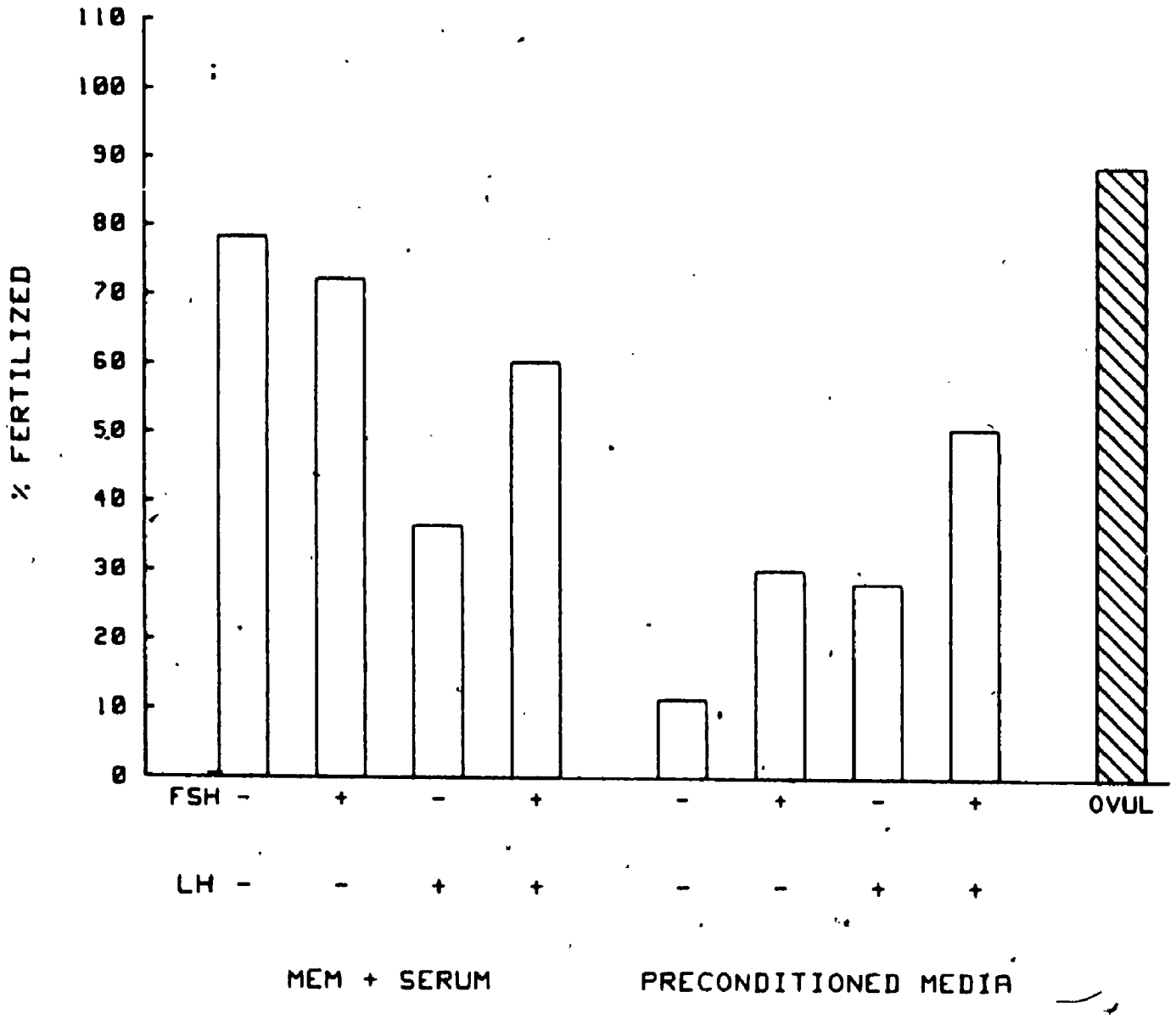
period the oocytes were stripped of their cumulus cells and washed in DPBS. Following transfer to droplets of fertilization medium, they were inseminated with epididymal sperm. Fertilization was assessed 14-16 h later and the proportions of fertilized oocytes undergoing cleavage to 2-cell embryos were determined after an additional 24 h.

The proportions of oocytes fertilized in each group are illustrated in Figure 23. Oocytes matured in MEM + serum + LH were fertilized in proportions significantly lower than oocytes matured in the other 3 serum-supplemented media ($P<0.01$). The addition of FSH or FSH + LH to serum-supplemented medium did not change significantly the proportion of oocytes undergoing fertilization following maturation in this medium ($P>0.1$). Oocytes were capable of being fertilized after maturation in all types of preconditioned media in the absence of serum. The addition of FSH or LH to the granulosa cell culture medium significantly increased the fertilizability of oocytes matured in that medium ($P<0.01$) compared with oocytes matured in medium from unstimulated granulosa cells. When both FSH + LH were present in the granulosa cell cultures, the fertilizability of oocytes matured in that medium was significantly higher than that of oocytes matured in only FSH- or LH-stimulated cultures ($P<0.01$).

For all treatments, the proportion of fertilized oocytes undergoing cleavage to 2-cell embryos was greater than 75%.

FIGURE 23

Proportions of oocytes undergoing fertilization following maturation in MEM + 5% serum supplemented with FSH, LH or both or in preconditioned media from rat granulosa cells stimulated with FSH, LH or both. Oocytes matured in MEM + serum + LH were fertilized in proportions significantly lower than oocytes matured in the other 3 serum-supplemented media ($P<0.05$). The addition of FSH or LH to the granulosa cell culture medium significantly increased the fertilizability of oocytes, with FSH + LH together resulting in a significantly higher proportion of oocytes fertilized than FSH or LH alone ($P<0.01$). The number of oocytes cultured in each type of medium ranged from 41 to 106. The data represent the results obtained from 1 of 2 replicates.


% FERTILIZED
110
100
90
80
70
60
50
40
30
20
10
0
FSH - + - + - + - + OVUL
LH - - + + - - + +
MEM + SERUM
PRECONDITIONED MEDIA

10.3 Discussion

The results of these experiments indicate that both maturation and fertilization of rat oocytes are strongly influenced by gonadotropins and the intrafollicular environment produced by gonadotropic stimulation. The first experiment demonstrated that oocytes obtained from immature rats (at approximately 26 days of age) were capable of spontaneous meiotic maturation. The ability of oocytes to resume meiosis spontaneously in culture is not acquired until the age of 15 days in mice (Szybec, 1972; Sorensen & Wassarman, 1976) and at 23 days in hamsters (Iwamatsu & Yanagimachi, 1975). Bar-Ami & Tsafriri (1981) demonstrated that rat oocytes acquire meiotic competence between 20 and 26 days of age, although their proportion of oocytes from 26-day-old rats undergoing GVBD (67.6%) was significantly less than that found in the present experiment (100.0%; $P<0.001$).

Short term (12 h) exposure to PMSG was sufficient to allow maximum fertilizability of *in vitro* matured oocytes. Although this exposure was beneficial, it was not necessary as 17% of oocytes obtained from rats without PMSG injection were capable of maturation and fertilization in vitro. The mechanism by which PMSG treatment improved the fertilizability and cleavage of *in vitro* matured oocytes is not known. It can be speculated that PMSG stimulated the follicles of the immature ovaries to produce an environment more suitable for oocyte development, perhaps by altering

the production and/or secretion of steroid hormones or growth factors.

The results of the time course of oocyte maturation in medium supplemented with FSH clearly indicated that FSH had an influence on oocyte nuclear maturation. Since it is understood that FSH increases intracellular cAMP levels (Eppig et al., 1983; Schultz et al., 1983) and cAMP in vitro inhibits oocyte maturation (Dekel & Beers, 1978), it is probable that FSH acted on the cumulus cells to initially sustain a level of cAMP sufficient to prolong meiotic arrest in the oocyte. Phosphodiesterase activity in the cumulus-oocyte complex may have then reduced the cAMP concentrations and nuclear maturation proceded. This scheme is supported by evidence that mouse cumulus-oocyte complexes incubated in media containing cAMP analogs or phosphodiesterase inhibitors were maintained in meiotic arrest for at least 24 h (Cho et al., 1974; Magnusson & Hillensjo, 1977; Dekel & Beers, 1978). It was also shown that the maturation of denuded oocytes was not affected by the presence of FSH and that cAMP analogs were more effective in inhibiting maturation in cumulus cell-enclosed oocytes than in denuded oocytes (Eppig et al., 1983). Therefore, the cumulus cells appear to be necessary for the cAMP-mediated inhibition of maturation. These studies support the hypothesis that oocyte maturation is inhibited by a cAMP-induced factor transmitted from cumulus cells to the oocytes via the gap junctions that join them.

The inhibition of nuclear maturation caused by the preconditioned medium from granulosa cells in this study is in agreement with results from a similar study reported previously. Cocultures of porcine granulosa cells with oocytes resulted in a dose-dependent inhibition of oocyte maturation (Tsafriri & Channing, 1975b) which was reversible by the addition of LH (Tsafriri et al., 1977). Further studies also determined that cell contact between oocytes and granulosa cells was not necessary, as extracts from granulosa cells or granulosa cell preconditioned medium elicited similar inhibitory effects (Gwatkin & Andersen, 1976; Centola et al., 1981; Sato & Koide, 1984; Andersen et al., 1985). In this study FSH stimulation of in vitro maturing oocytes reduced the rate of nuclear maturation. FSH-stimulated granulosa cells released a substance(s) into the medium that further reduced the rate of nuclear maturation in a density-dependent manner, which suggests that the action of FSH in influencing the rate of nuclear maturation is mediated by a cAMP-stimulated granulosa cell product.

Although FSH delayed nuclear maturation in this study, the maximum fertilizability of the mature oocytes was not altered, as long as serum was present in the medium. The results of the experiment using varying concentrations of FSH in the maturation medium were difficult to interpret until the time course of maturation in FSH-supplemented medium was performed. Then it became clear that oocyte fertilizability was a function of both the length of the

culture period and the presence of FSH. Thus the fertilizability of oocytes exposed to varying concentrations of FSH was influenced by the length of the culture (which varied by 1.5 h) and by the presence of different concentrations of FSH, which presumably might have delayed oocyte maturation to varying degrees.

FSH is known to be a potent stimulus for cumulus expansion in cumulus-oocyte complexes in vitro. Since cumulus expansion is initiated during the same period of time as nuclear maturation in vivo, it was of interest to determine if these two processes responded to a common stimulus, namely FSH. When meiotic maturation was inhibited by the presence of IBMX, FSH had no effect on the maturation or fertilizability of the oocytes. Thus, FSH did not act as a trigger for the induction of maturation of isolated rat oocytes in vitro, in contrast with a recent study which demonstrated that FSH induced GVBD in mouse oocytes meiotically arrested with 200 μM IBMX (Downs et al., 1988). Previous investigators have induced the resumption of meiosis in follicle-enclosed rat oocytes by the addition of FSH (Lindner et al., 1974). This observation suggests that FSH-stimulation of cultured follicles initiates a mechanism by which the enclosed oocytes will resume meiosis, but the machinery for the mechanism may be lacking or may be unable to perform at a level sufficient to induce the resumption of meiosis in isolated rat oocytes, particularly in the presence of IBMX.

It appears, however, that this observation may be species-specific.

The ability of oocytes matured in granulosa cell-conditioned media to be fertilized leads to an important observation. Granulosa cell cultures were clearly able to release a substance into the medium that was able to help maintain the penetrability of the oocytes during *in vitro* maturation. Although this substance did not act as effectively as serum in this study, it is possible that the amount released into the large volume of medium resulted in a concentration less than optimal to support penetrability of *in vitro* matured oocytes.

Medium from granulosa cell cultures stimulated by both FSH and LH supported oocyte fertilizability to a degree greater than cultures stimulated by either gonadotropin alone. This suggests that FSH and LH, additively or collaboratively, increased the concentration of some factor in the medium and thus increased the penetrability of the zona of *in vitro* matured oocytes. Alternatively, the medium produced when LH was added to the granulosa cell cultures was seen to prevent the FSH-induced delay in nuclear maturation in spontaneously maturing oocytes. In doing so, a high number of oocytes were completely mature at the time of insemination, possibly leading to an increased proportion of oocytes undergoing fertilization.

In all these studies, immature oocytes undergoing spontaneous maturation required cumulus cells and serum or follicular fluid to ensure normal cytoplasmic maturation

and fertilizability. Although associated with the role of maintaining penetrability of the oocytes, serum may have other less obvious forms of support to the oocytes maturing in vitro. The observation that oocytes can mature in medium from granulosa cell cultures and can be fertilized is an important step towards the identification of the component(s) that are essential for the normal maturation and fertilization of oocytes. Since progesterone and 20α-OH-progesterone are produced in significant amounts by FSH-stimulated granulosa cells, these substances may play a role in the maturation-fertilization process. Further analysis of granulosa cell-conditioned medium and its effects on maturing oocytes may help to identify the components involved in regulation of maturation-fertilization of oocytes.

SUMMARY AND CONCLUSIONS

A technique for the *in vitro* fertilization of rat oocytes was established such that normal embryonic and fetal development were possible. This technique was then applied to oocytes undergoing *in vitro* maturation under various conditions. In this way some of the factors regulating maturation and fertilization of rat oocytes were studied.

To be able to determine the developmental capability of the oocytes fertilized *in vitro*, it was necessary to establish a technique whereby these oocytes could undergo embryonic development. The technique for peeling the bursa was an effective means of producing unilateral pregnancy without having a detrimental effect on subsequent fetal development.

Using the unilaterally pregnant animal as a recipient for transferred oocytes, it was determined that, compared to oocytes fertilized *in vivo*, a lower proportion of oocytes fertilized *in vitro* developed to normal fetuses (Table 6). There was no deficiency in the ability of *in vitro* fertilized oocytes to undergo the first cleavage division. Furthermore, a fairly high percentage (58%) of zygotes derived from *in vitro* fertilization developed to embryos capable of eliciting decidualization; however,

these embryos were unable to undergo complete implantation or subsequent fetal development (Table 7). The slower rate of development of embryos derived from in vitro fertilization, compared with those from in vivo fertilization, may have been responsible for the incidence of unsuccessful implantation (Table 8). This is supported by the observation that these embryos, given additional time for preimplantation development in the oviduct, were as capable of development to Day 20 fetuses as morulae or blastocysts derived from in vitro fertilization (Table 12).

The technique of in vitro fertilization was used with in vitro matured oocytes as a criterion to indicate developmental competence. Oocytes matured cumulus-intact in medium supplemented with serum were capable of fertilization in proportions similar to ovulated oocytes (Figure 6). In addition these in vitro matured and ovulated oocytes developed to viable fetuses in similar proportions following transfer to unilaterally pregnant recipients. Maturation of oocytes in the absence of either or both cumulus cells and serum not only decreased the rate of nuclear maturation, but also reduced the proportion of oocytes capable of fertilization (Figure 7). Oocytes matured in the absence of cumulus cells showed a high incidence of abnormal pronuclear formation, which was indicative of abnormal cytoplasmic maturation. Progesterone stimulation of cumulus-free oocytes did not alter the incidence of abnormal fertilization (Table 21).

Oocytes matured cumulus-intact in serum-free medium

were fertilized in lower proportions than those matured in the presence of serum. The incidence of abnormal fertilization was low (Figure 7). The addition of follicular fluid to the maturation medium help to maintain the penetrability of *in vitro* matured oocytes (Table 19). An alternate method to increase penetrability, by drilling a hole in the zona pellucida, was also effective in increasing the proportion of oocytes penetrated (Figure 9). By increasing the number of oocytes penetrated following maturation cumulus-free, this method helped verify that the incidence of abnormal pronuclear formation was associated with the absence of cumulus cells (Figure 11).

Oocytes obtained from unstimulated rats (i.e. no PMSG injection) were capable of spontaneous meiotic maturation and a small proportion underwent fertilization. PMSG-stimulation of the ovary prior to oocyte collection increased the proportion of oocytes undergoing fertilization (Figure 13). Maturation of oocytes in the presence of AGP, a steroidogenesis inhibitor, significantly reduced the proportion of oocytes being fertilized, indicating that some aspect of the steroidogenic response to PMSG plays a role in maintaining the fertilizability of the oocytes.

FSH stimulation of *in vitro* maturing oocytes reduced the rate of nuclear maturation (Figure 16) but did not affect the proportion of oocytes capable of undergoing fertilization (Figure 17). FSH-stimulated granulosa cells

released a substance(s) into the medium that further reduced the rate of nuclear maturation in a density-dependent manner, which suggests that the action of FSH in influencing the rate of nuclear maturation is mediated by a granulosa cell product. Granulosa cell preconditioned medium was also capable of maintaining the penetrability of oocytes during *in vitro* maturation, provided the granulosa cells were stimulated with FSH, LH or both. Since progesterone and 20 -OH-progesterone are produced in significant amounts by FSH-stimulated granulosa cells, these substances may play a role in the maturation-fertilization process *in vivo*.

The results presented in this thesis can therefore be summarized as follows:

1) The removal of the bursa from around one ovary is an effective means of producing unilateral pregnancy, without affecting the number of ovulations or fetal development.

2) Following transfer, *in vitro* fertilized rat oocytes develop to viable fetuses in lower proportions than *in vivo* fertilized oocytes.

3) Following transfer to a recipient for embryonic development, *in vitro* fertilized rat oocytes show a slower development to the blastocyst stage that may be detrimental at the time of implantation. If given additional time for preimplantation development in the

oviduct, *in vitro* fertilized oocytes can become viable fetuses in proportions similar to *in vivo* fertilized oocytes.

4) Following the LH surge, continued exposure to LH is not necessary for normal maturation of oocyte-cumulus complexes.

5) The presence of cumulus cells and/or serum increases the rate of nuclear maturation in oocytes matured *in vitro*.

6) Oocytes matured *in vitro* in the absence of cumulus cells show a high incidence of abnormal pronuclear formation; the presence of cumulus cells ensures normal cytoplasmic maturation.

7) Oocytes matured *in vitro* in the presence of cumulus cells and serum are as capable of *in vitro* fertilization, embryonic and fetal development as ovulated oocytes. The presence of follicular fluid instead of serum in the maturation medium supports the normal maturation, fertilization and cleavage of *in vitro* matured oocytes.

8) Oocytes matured *in vitro* in the absence of cumulus cells, serum or follicular fluid have an increased resistance to sperm penetration, that can be overcome by drilling a hole in the zona pellucida.

9) Progesterone does not appear to directly influence cytoplasmic maturation of rat oocytes.

10) In vitro matured oocytes exposed to the zona drilling technique are capable of normal embryonic and fetal development.

11) Oocytes obtained from hormonally unstimulated rats were capable of spontaneous nuclear maturation, and a small proportion was capable of being fertilized normally. Exposure of the rats to gonadotropic stimulation increased the proportion of oocytes capable of in vitro fertilization.

12) The addition of FSH to the maturation medium decreases the rate of nuclear maturation in oocytes matured in vitro, but does not affect the proportion of oocytes undergoing fertilization. Oocytes matured in preconditioned medium from FSH-stimulated granulosa cells undergo a slower rate of nuclear maturation than oocytes matured in MEM supplemented with FSH.

13) Oocytes are capable of normal maturation, fertilization and cleavage when matured cumulus-intact (and serum-free) in preconditioned medium from FSH- or LH-stimulated granulosa cells.

The use of in vitro fertilization as the criterion to determine normal maturation of rat oocytes in these studies has led to the following general conclusions:

1) Cumulus cells play an essential role in ensuring normal cytoplasmic maturation and, therefore, normal pronuclear formation in oocytes matured in vitro.

2) A substance(s) produced by the gonadotropin-stimulated granulosa cells, and present in both follicular fluid and serum, is capable of maintaining the penetrability of oocytes matured in vitro.

3) Fully grown rat oocytes can spontaneously undergo meiotic maturation independent of hormonal stimulation. Gonadotropic stimulation of the follicle, probably through changes in follicular steroidogenesis, renders the oocytes fully capable of maturation and fertilization.

REFERENCES

Aberdam, E. and Dekel, N. (1985) Activators of protein kinase C stimulate meiotic maturation of rat oocytes. Biochem. Biophys. Res. Comm. 132: 570-574.

Adams, C.E. (1980) Retention and development of eggs transferred to the uterus at various times after ovulation in the rabbit. J. Reprod. Fert. 60: 309-315.

Albertini, D.F. and Anderson, E. (1974) The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. J. Cell Biol. 63: 234-250.

Alden, R.H. (1942) The periovarial sac in the albino rat. Anat Rec. 83: 421-435.

Amsterdam, A., Josephs, R., Lieberman, M.E. and Lindner, H.R. (1976) Organization of intramembrane particles in freeze-cleaved gap junction of rat Graafian follicles: Optical-diffraction analysis. J. Cell Sci. 21: 93-105.

Amsterdam, A., Koch, Y., Lieberman, M.E. and Lindner, H.R. (1975) Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat. J. Cell Biol. 67: 894-900.

Anderson, L.D., Stone, S.L. and Channing, C.P. (1985) Influence of hormones on the inhibitory activity of oocyte maturation present in conditioned media of porcine granulosa cells. Gamete Res. 12: 119-130.

Austin, C.R. (1951) Observations on the penetration of the sperm into the mammalian egg. Aust. J. Scient. Res. Ser. B. 4: 581-596.

Austin, C.R. (1956) Ovulation, fertilization and early cleavage in the hamster. J. R. Microsc. Soc. 75: 141-154.

Austin, C.R. and Braden, A.W.H. (1956) Early reaction of the rodent egg to spermatozoa penetration. J. Exp. Biol. 33: 358-365.

Averill, R.L.W. (1958) The production of living sheep eggs. J. Agric. Sci. 50: 17-33.

Ayalon, D., Tsafriri, A., Lindner, H.R., Cordova, T. and Harell, A. (1972) Serum gonadotrophin levels in pro-estrous rats in relation to the resumption of meiosis by the oocytes. J. Reprod. Fert. 31: 51-58.

Ax, R.L. and Ryan, R.J. (1979) FSH stimulation of ^{3}H-glucosamine incorporation into proteoglycans by porcine granulosa cells in vitro. J. Clin. Endocrinol. Metab. 49: 646-648.

Bachvarova, R. (1985) Gene expression during oogenesis and oocyte development in mammals. In: Developmental Biology. A Comprehensive Synthesis, Vol. 1 - Oogenesis. L.W. Browden, Ed. Plenum Press, New York. pp. 453-524.

Bachvarova, R., Baran, M.M. and Tejblum, A. (1980) Development of naked growing mouse oocytes in vitro. J. Exp. Zool. 211: 159-169.

Bae, I.H. and Foote, R.H. (1975) Effect of hormones on the maturation of rabbit oocytes recovered from follicles of various sizes. J. Reprod. Fert. 42: 357-360.

Baker, T.G. (1979) The control of oogenesis in mammals. In: Ovarian follicular development and function. A.R. Midgley, Jr. and W.A. Sadler, Eds. Raven Press, New York. pp. 353-364.

Baker, T.G. and Neal, P. (1972) Gonadotrophin-induced maturation of mouse Graafian follicles in organ culture. In: Oogenesis. J.D. Biggers and A.W. Schuetz, Eds. University Park Press, Baltimore. pp. 377-396.

Balakier, H. (1978) Induction of maturation in small oocytes from sexually immature mice by fusion with meiotic or mitotic cells. Exp. Cell Res. 112: 137-141.

Balakier, H. and Czolowska, R. (1977) Cytoplasmic control of nuclear maturation in mouse oocytes. Exp. Cell Res. 110: 466-469.

Balakier, H. and Tarkowski, A.K. (1980) The role of germinal vesicle karyoplasm in the development of male pronucleus in the mouse. Exp. Cell Res. 128: 79-86.

Ball, G.D., Lenz, R.W., Ax, R.L. and First, N.L. (1983) Effects of cAMP on bovine oocyte maturation and cumulus expansion. In: Factors regulating ovarian function. G.S. Greenwald & P.F. Terranova, Eds. Raven Press, New York. pp. 61-64.

Bar-Ami, S., Nimrod, A., Brodie, A.M.H. and Tsafriri, A. (1983) Role of FSH and oestradiol-17B in the development of meiotic competence in rat oocytes. J. Steroid Biochem. 19: 965-971.

Bar-Ami, S. and Tsafriri, A. (1981) Acquisition of meitoic competence in the rat: Role of gonadotropin and estrogen. Gamete Res. 4: 463-472.

Barros, C. and Austin, C.R. (1967) In vitro fertilization and the sperm acrosome reaction in the hamster. J. Exp. Zool. 166: 317-323.

Barros, C. and Garavagno, A. (1970) Capacitation of hamster spermatozoa with blood sera. J. Reprod. Fert. 22: 381-384.

Barros, C. and Yanagimachi, R. (1972) Polyspermy-preventing mechanisms in golden hamster eggs. J. Exp. Zool. 180: 251-266.

Bavister, B.D. (1969) Environmental factors important for in vitro fertilization in the hamster. J. Reprod. Fert. 18: 544-545.

Bavister, B.D. (1981) Analysis of culture media for in vitro fertilization and criteria for success. In: Fertilization and embryonic development in vitro. L. Mastroianni, Jr. and J.D. Biggers, Eds. Plenum Press, New York. pp. 41-60.

Bavister, B.D. (1982) Evidence for a role of post-ovulatory cumulus components in supporting fertilizing ability of hamster spermatozoa. J. Androl. 3: 365-372.

Bavister, B.D. (1987) Studies on the developmental blocks in cultured hamster embryos. In: The mammalian preimplantation embryo. B.D. Bavister, Ed. Plenum Publishing Corp., New York. pp. 219-249.

Bavister, B.D. and Minami, N. (1986) Use of cultured mouse oviducts to bypass in vitro development block in cleavage stage hamster embryos. Biol. Reprod. 34 (Suppl. 1): 191.

Bellin, M.M., Lenz, R.W., Steadman, L.E. and Ax, R.L. (1983) Proteoglycan production by bovine granulosa cells in vitro occurs in response to FSH. Mol. Cell Endocrinol. 29: 51-65.

Betteridge, K.J. (1977) Summary of factors affecting success rates in surgical embryo transfer. In: Embryo transfer in farm animals: A review of techniques and applications. K.J. Betteridge, Ed. Agriculture Canada, Monograph 16. pp. 29-31.

Betteridge, K.J. and Mitchell, D. (1974) Embryo transfer in cattle: experience of twenty-four completed cases. Theriogenology: 1: 69-82.

Biggers, J.D., Finn, C.A. and McLaren, A. (1962) Long-term reproductive performance of female mice. II. Variation of litter size with parity. J. Reprod. Fert. 3: 313-330.

Biggers, J.D., Gwatkin, R.B.L. and Brinster, R.L. (1962) Development of mouse embryos in organ cultures of fallopian tubes on a chemically defined medium. Nature, London 194: 747-749.

Bittner, J.J. and Little, C.C. (1937) The transmission of breast and lung cancer in mice. J. Hered. 28: 117-121.

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

Blandau, R.J. and Odor, D.L. (1949) The total number of spermatozoa reaching to various segments of the reproductive tract in the female albino rat at intervals after insemination. Anat. Rec. 103: 93-110.

Boland, M.P. (1984) Use of the rabbit oviduct as a screening tool for the viability of mammalian eggs. Theriogenology 21: 126-137.

Borland, R.M., Hazra, S., Biggers, J.D. and Lechene, C.P. (1977) The elemental composition of the environments of the gametes and preimplantation embryo during the initiation of pregnancy. Biol. Reprod. 16: 147-157.

Bornslaeger, E.A. and Schultz, R.M. (1985) Regulation of mouse oocyte maturation: Effect of elevating cumulus cell cAMP on oocyte cAMP levels. Biol. Reprod. 33: 698-704.

Botero-Ruiz, W., Laufer, N., DeCherney, A.H., Lake Polan, M. Haseltine, F.P. and Behrman, H.R. (1984) The relationship between follicular fluid steroid concentration and successful fertilization of human oocytes in vitro. Fert. Steril. 41: 820-826.

Bowman, J.C. and Roberts, R.C. (1958) Embryonic mortality in relation to ovulation rate in the house mouse. J. Exp. Biol. 35: 138-143.

Bowman, P. and McLaren, A. (1970) Cleavage rate of mouse embryos in vivo and in vitro. J. Embryol. Exp. Morph. 24: 203-207.

Braden, A.W.H., Austin, C.R. and David, H.A. (1954) The reaction of the zona pellucida to sperm penetration. Aust. J. Biol. Sci. Ser. B. 7: 391-409.

Brinster, R.L. (1965a) Studies on the development of mouse embryos in vitro. I. The effect of osmolarity and hydrogen ion concentration. J. Exp. Zool. 158: 49-58.

Brinster, R.L. (1965b) Studies on the development of mouse embryos *in vitro*. IV. Interaction of energy sources. J. Reprod. Fert. 10: 227-240.

Canipari, R., Palombi, F., Riminucci, M. and Mangia, F. (1984) Early programming of maturation competence in mouse oogenesis. Dev. Biol. 102: 519-524.

Centola, G.M., Anderson, L.D. and Channing, C.P. (1981) Oocyte maturation inhibitor (OMI) activity in porcine granulosa cells. Gamete Res. 4: 451-461.

Chang, M.C. (1950) Development and fate of the transferred rabbit ova or blastocyst in relation to the ovulation time of recipients. J. Exp. Zool. 114: 197-216.

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

Chang, M.C. (1952) An experimental analysis of female sterility in the rabbit. Fert. Steril. 3: 251-262.

Chang, M.C. (1955) The maturation of rabbit oocytes in culture and their maturation, activation, fertilization and subsequent development in fallopian tube. J. Exp. Zool. 28: 378-399.

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

Chang, M.C. (1982) The development of *in vitro* fertilization from laboratory animals to human. In: Oxford Reviews of Reproductive Biology, Volume 4. C.A. Finn, Ed. Clarendon Press, Oxford. pp. 85-100.

Channing, C.P., Anderson, L.D., Hoover, D.J., Kolena, J., Osteen, K.G., Pomerantz, S.H. and Tanabe, K. (1982) The role of non-steroidal regulators in control of oocyte and follicular maturation. Rec. Prog. Horm. Res. 38: 331-408.

Channing, C.P., Liu, C.O., Jones, G.S. and Jones, H. (1983) Decline in follicular oocyte maturation inhibitor coincident with maturation and achievement of fertilizability of oocytes recovered at midcycle of gonadotropin-treated women. Proc. Natl. Acad. Sci. U.S.A. 80: 4184-4188.

Chari, S., Hillensjo, T., Magnusson, C., Sturm, G. and Daume, E. (1983) *In vitro* inhibition of rat oocyte meiosis by human follicular fluid fractions. Arch. Gynecol. 233: 155-164.

Cho, W.K., Stern, S. and Biggers, J.D. (1974) Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. J. Exp. Zool. 187: 383-386.

Choi, T.S., Mori, M., Kohmoto, K. and Shoda, Y. (1987) Beneficial effect of serum on the fertilizability of mouse oocytes matured in vitro. J. Reprod. Fert. 79: 565-568.

Cole, H.H. and Hart, G.H. (1930) The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. Am. J. Physiol. 93: 57-68.

Colonna, R. and Mangia, F. (1983) Mechanisms of amino acid uptake in cumulus-enclosed mouse oocytes. Biol. Reprod. 28: 797-803.

Cross, P.C. and Brinster, R.L. (1970) In vitro development of mouse oocytes. Biol. Reprod. 3: 298-307.

Cross, P.C. and Brinster, R.L. (1974) Leucine uptake and incorporation at three stages of mouse oocyte maturation. Exp. Cell Res. 86: 43-46.

Daniel, S.A.J. and Armstrong, D.T. (1984) Site of action of androgens on follicle-stimulating hormone-induced aromatase activity in cultured rat granulosa cells. Endocrinology 114: 1975-1982.

Daniel, S.A.J., Khalil, M.W. and Armstrong, D.T. (1986) 19-Norandrostenedione (4-estrene-3,17-dione) inhibits porcine oocyte maturation in vitro. Gamete Res. 13: 173-184.

Davis, B.K. (1976) Influence of serum albumin on the fertilizing ability in vitro of rat spermatozoa. Proc. Soc. Exp. Biol. Med. 151: 240-243.

DeFelici, M., Salustri, A. and Siracusa, G. (1985) "Spontaneous" hardening of the zona pellucida of mouse oocytes during in vitro culture. II. The effect of follicular fluid and glycosaminoglycans. Gamete Res. 12: 227-235.

DeFelici, M. and Siracusa, G. (1982) "Spontaneous" hardening of the zona pellucida of mouse oocytes during in vitro culture. Gamete Res. 6: 107-113.

Dekel, N., Aberdam, E. and Sherizly, I. (1983b) Spontaneous maturation of cumulus-enclosed rat oocytes is inhibited by forskolin. Biol. Reprod. 28 (Suppl. 1): 86.

Dekel, N. and Beers, W.H. (1978) Rat oocyte maturation *in vitro*: Relief of cyclic AMP inhibition by gonadotropins. Proc. Natl. Acad. Sci. USA 75: 4369-4373.

Dekel, N. and Beers, W.H. (1980) Development of rat oocytes *in vitro*: Inhibition and induction of maturation in the presence or absence of the cumulus oophorus. Dev. Biol. 75: 247-252.

Dekel, N. and Kraicer, P.F. (1978) Induction *in vitro* of mucification of rat cumulus oophorus by gonadotropins and adenosine 3',5'-monophosphate. Endocrinology 102: 1797-1802.

Dekel, N., Kraicer, P.F., Phillips, D.M., Sanchez, R.S. and Segal, S.J. (1978) Cellular associations in the rat oocyte-cumulus cell complex: Morphology and ovulatory changes. Gamete Res. 1: 47-57.

Dekel, N., Lawrence, T.S., Gilulu, N.B. and Beers, W.H. (1981) Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. Dev. Biol. 86: 356-362.

Dekel, N. and Sherizly, I. (1983) Induction of maturation in rat follicle-enclosed oocytes by forskolin. FEBS Lett. 151: 153-155.

Dekel, N. and Sherizly, I. (1985) Epidermal growth factor induces maturation of rat follicle-enclosed oocytes. Endocrinology 116: 406-409.

Dekel, N., Sherizly, I. and Galiani, D. (1984) Maturation of rat oocytes follows uncoupling in the cumulus-oocyte complex. Biol. Reprod. 30 (Suppl. 1): 144.

Dekel, N., Sherizly, I., Tsafriri, A. and Naor, Z. (1983a) A comparative study of the mechanism of action of luteinizing hormone and a gonadotropin releasing hormone analog on the ovary. Biol. Reprod. 28: 161-166.

Dickmann, Z. and Noyes, R.W. (1960) The fate of ova transferred into the uterus of the rat. J. Reprod. Fert. 1: 197-212.

Donahue, R.P. (1972) The relation of oocyte maturation to ovulation in mammals. In: Oogenesis. J.D. Biggers and A.W. Schuetz, Eds. University Park Press, Baltimore. pp. 413-438.

Donahue, R.P. and Stern, S. (1968) Follicular cell support of oocyte maturation: Production of pyruvate *in vitro*. J. Reprod. Fert. 17: 395-398.

Doree, M. and Guerrier, P. (1975) Site of action of 1-methyladenine in inducing oocyte maturation in starfish. Exp. Cell. Res. 91: 296-300.

Downs, S.M., Coleman, D.L., Ward-Bailey, P.F. and Eppig, J.J. (1985) Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of porcine follicular fluid. Proc. Natl. Acad. Sci. U.S.A. 82: 454-458.

Downs, S.M., Daniel, S.A.J. and Eppig, J.J. (1988) Induction of maturation in cumulus cell-enclosed mouse oocytes by follicle-stimulating hormone and epidermal growth factor: Evidence for a positive stimulus of somatic cell origin. J. Exp. Zool. 245: 86-96.

Downs, S.M. and Eppig, J.J. (1984) Cyclic adenosine monophosphate and ovarian follicular fluid act synergistically to inhibit mouse oocyte maturation. Endocrinology 114: 418-427.

Downs, S.M., Schroeder, A.C. and Eppig, J.J. (1986) Serum maintains the fertilizability of mouse oocytes matured in vitro by preventing hardening of the zona pellucida. Gamete Res. 15: 115-122.

Edwards, R.G. (1965a) Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature 208: 349-351.

Edwards, R.G. (1965b) Maturation in vitro of human ovarian oocytes. Lancet 2: 926-929.

Edwards, R.G., Bavister, B.D. and Steptoe, P.C. (1969) Early stages of fertilization in vitro of human oocytes. Nature 221: 632-635.

Edwards, R.G. and Gates, A.H. (1959) Timing of the stages of the maturation, division, ovulation, fertilization the first cleavage of eggs of adult mice treated with gonadotrophins. J. Endocrinol. 18: 292-304.

Ekholm, C., Hillensjo, T. and Isaksson, O. (1981) Gonadotropin releasing hormone agonists stimulate oocyte meiosis and ovulation in hypophysectomized rats. Endocrinology 108: 2022-2024.

El-Badrawi, H.H. and Hafez, E.S.E. (1982) Factors and mechanisms affecting success of in vitro fertilization and embryo transfer. In: In vitro fertilization and embryo transfer, E.S.E. Hafez and K. Semm, Eds., MTP Press Ltd., Lancaster, England. pp. 361-379.

Eppig, J.J. (1977) Mouse oocyte development in vitro with various culture systems. Dev. Biol. 60: 371-388.

Eppig, J.J. (1979a) A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained in vitro. J. Exp. Zool. 209: 345-353.

Eppig, J.J. (1979b) Gonadotropin stimulation of the expansion of cumulus oophori isolated from mice.: General conditions for expansion in vitro. J. Exp. Zool. 208: 111-120.

Eppig, J.J. (1982a) The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev. Biol. 39: 268-272.

Eppig, J.J. (1982b) The relationship between parthenogenetic embryonic development and cumulus cell-oocyte intercellular coupling during oocyte meiotic maturation. Gamete Res. 5: 229-237.

Eppig, J.J. and Downs, S.M. (1984) Chemical signals that regulate mammalian oocyte maturation. Biol. Reprod. 30: 1-11.

Eppig, J.J., Freter, R.R., Ward-Bailey, P.F. and Schultz, R.M. (1983) Inhibition of oocyte maturation in the mouse: Participation of cAMP, steroid hormones, and a putative maturation-inhibitory factor. Dev. Biol. 100: 39-49.

Eppig, J.J. and Koide, S.L. (1978) Effects of progesterone and oestradiol-17B on the spontaneous meiotic maturation of mouse oocytes. J. Reprod. Fert. 53: 99-101.

Eppig, J.J. and Ward-Bailey, P.F. (1982) The mechanism of cumulus cell-oocyte uncoupling: Evidence for the participation of both cumulus cells and oocytes. Gamete Res. 6: 145-154.

Eppig, J.J., Ward-Bailey, P.F. and Coleman, D.L. (1985) Hypoxanthine and adenosine in murine ovarian follicular fluid: Concentrations and activity in maintaining oocyte meiotic arrest. Biol. Reprod. 33: 1041-1049.

Erickson, G.F. and Sorensen, R.A. (1974) In vitro maturation of mouse oocytes isolated from late, middle and preantral Graafian follicles. J. Exp. Zool. 190: 123-127.

Evans, G. and Armstrong, D.T. (1984) Reduction in fertilization rate in vitro of oocytes from immature rats induced to superovulate. J. Reprod. Fert. 70: 131-135.

Eyestone, W.H., Northey, D.L. and Leibfried-Rutledge, M.L. (1985) Culture of 1-cell bovine embryos in the sheep oviduct. Biol. Reprod. 32 (Suppl. 1): 100a.

Farrell, P.S. and Bavister, B.D. (1984) Short-term exposure of two-cell hamster embryos to collection media is detrimental to viability. Biol. Reprod. 31: 109-114.

Fekete, E. and Little, C.C. (1942) Observations on the mammary tumor incidence of mice born from transferred ova. Cancer Res. 2: 525-530.

Feng, P., Catt, K.J. and Knecht, M. (1988) Transforming growth factor-B stimulates meiotic maturation of the rat oocyte. Endocrinology 122: 181-186.

Fleming, A.D., Evans, G., Walton, E.A. and Armstrong, D.T. (1986) Developmental capability of rat oocytes matured in vitro in defined medium. Gamete Res. 12: 255-263.

Fleming, A.D., Khalil, W. and Armstrong, D.T. (1983) Porcine follicular fluid does not inhibit maturation of rat oocytes in vitro. J. Reprod. Fert. 69: 665-670.

Foote, W.D. and Thibault, C. (1969) Recherches experimentales sur la maturation in vitro des ovocytes de triue et de veau. Ann. Biol. Anim. Biochim. Biophys. 9: 329-349.

Fortune, J.E., Concannon, P.W. and Hansel, W. (1975) Ovarian progesterone levels during in vitro oocyte maturation and ovulation in Xenopus laevis. Biol. Reprod. 13: 561-567.

Fujimoto, S., Passantino, T.J., Koenczoel, I. and Segal, S.J. (1975) A simplified method for chromosome preparations of the rat preimplantation embryos. Cytologia 40: 469-475.

Fukuda, Y. and Chang, M.C. (1978) Relationship between sperm concentration and polyspermy in intact and zona free mouse eggs inseminated in vitro. Arch. Androl. 1: 267-273.

Gaddum-Rosse, P. (1985) Mammalian gamete interactions: What can be gained from observatons on living eggs? Am. J. Anat. 173: 347-356.

Gebauer, H., Lindner, H.R. and Amsterdam, A. (1978) Synthesis of heparin-like glycosaminoglycans in rat ovarian slices. Biol. Reprod. 18: 350-358.

Gerhart, J., Wu, M. and Kirschner, M. (1984) Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs. J. Cell Biol. 98: 1247-1255.

Gianfortoni, J.G. and Gulyas, B.J. (1985) The effects of short-term incubation (aging) of mouse oocytes on in vitro fertilization, zona solubility, and embryonic development. Gamete Res. 11: 59-68.

Gilula, N.B., Epstein, M.L. and Beers, W.H. (1978) Cell-to-cell communication and ovulation: A study of the cumulus-oocyte complex. J. Cell Biol. 78: 58-75.

Goddard, M.J. and Pratt, H.P.M. (1983) Control of events during early cleavage of the mouse embryo: An analysis of the "2-cell block". J. Embryol. Exp. Morphol. 73: 111-133.

Gordon, J.W. and Talansky, B.E. (1986) Assisted fertilization by zona drilling: A mouse model for correction of oligospermia. J. Exp. Zool. 239: 347-354.

Gould, K.G., Cline, E.M. and Williams, W.L. (1973) Observations on the induction of ovulation and fertilization in vitro in the squirrel monkey (Saimiri sciureus). Fert. Steril. 24: 260-280.

Greve, T. and Del Campo, M. (1986) Embryo loss following embryo transfer in cattle and swine. In: Embryonic mortality in farm animals. J.M. Sreenan and M.G. Diskin, Eds. Martinus Mijhoff for EEC. pp. 179-194.

Grimek, H.J. and Ax, R.L. (1982) Chromatographic comparison of chondroitin containing proteoglycans from small and large bovine ovarian follicles. Biochem. Biophys, Res. Commun. 104: 1401-1406.

Grobstein, C., Flower, M. and Mendeloff, J. (1983) External human fertilization: An evaluation of policy. Science 222: 127-133.

Guerrier, P., Meijer, L., Moreau, M. and Longo, F.J. (1983) Hormone-independent GVBD induces cytoplasmic maturity in the starfish oocyte. J. Exp. Zool. 226: 303-309.

Gulyas, B.J. (1980) Cortical granules of mammalian eggs. Int. Rev. Cytol. 63: 357-392.

Gulyas, B.J. and Daniel, J.C., Jr. (1969) Incorporation of labelled nucleic acid and protein precursors by diapausing and non-diapausing blastocysts. Biol. Reprod. 1: 11-20.

Gurdon, J.B. and Woodland, H.R. (1968) The cytoplasmic control of nuclear activity in animal development. Biol. Rev. 43: 233-267.

Gwatkin, R.B.L. and Andersen, O.F. (1969) Capacitation of hamster spermatozoa by bovine follicular fluid. Nature, 224: 1111-1112.

Gwatkin, R.B.L. and Andersen, O.F. (1976) Hamster oocyte maturation in vitro: Inhibition by follicular components. Life Sci. 19: 527-536.

Gwatkin, R.B.L. and Haidri, A.A. (1973) Requirements for the maturation of hamster oocytes in vitro. Exp. Cell Res. 76: 1-7.

Hamilton, C.E. (1947) The cervix uteri of the rat. Anat. Rec. 97: 47-62.

Hamner, C.E., Jennings, L.L. and Sojka, N.J. (1970) Cat (Felis catus, L.) spermatozoa require capacitation. J. Reprod. Fert. 23: 477-480.

Harlow, G.M. and Quinn, P. (1982) Development of preimplantation mouse embryos in vivo and in vitro. Aust. J. Biol. Sci. 35: 187-193.

Harper, M.J.K. (1964) Observations on amount and distribution of prenatal mortality in a strain of albino rats. J. Reprod. Fert. 7: 185-209.

Heape, W. (1890) Preliminary note on the transplantation and growth of mammalian ova within a uterine foster mother. Proc. R. Soc. Lond. 48: 457-458.

Heilbrunn, L.V., Daugherty, K. and Wilbur, K.M. (1939) Initiation of maturation in the frog egg. Physiol. Zool. 12: 97-100.

Heller, D.H., Cahill, D.M. and Schultz, R.M. (1981) Biochemical studies of mammalian oogenesis: Metabolic cooperativity between granulosa cells and growing mouse oocytes. Dev. Biol. 84: 455-464.

Heller, D.T. and Schultz, R.M. (1980) Ribonucleoside metabolism by mouse oocytes: Metabolic cooperativity between fully grown oocytes and cumulus cells. J. Exp. Zool. 214: 355-364.

Herlands, R.L. and Schultz, R.M. (1984) Regulation of mouse oocyte growth: Probable nutritional role for intercellular communication between follicle cells and oocytes in oocyte growth. J. Exp. Zool. 229: 317-325.

Hillensjo, T. (1976) Oocyte maturation and glycolysis in isolated pre-ovulatory follicles of PMS-injected immature rats. Acta Endocrinol. (Copenhagen) 82: 809-830.

Hillensjo, T., Batta, S.K., Schwartz-Kripner, A., Wentz, A.C., Sulewski, J. and Channing, C.P. (1978a) Inhibitory effect of human follicular fluid upon the maturation of porcine oocytes in culture. J. Clin. Endocrin. Metab. 47: 1332-1335.

Hillensjo, T., Brannstrom, M., Chari, S., Daume, E., Magnusson, C., Nilsson, L., Sjogren, A. and Tornel, J. (1985) Oocyte maturation as regulated by follicular factors. Ann. N.Y. Acad. Sci. 442: 73-79.

Hillensjo, T., Ekholm, C. and Ahren, K. (1978b) Role of cyclic AMP in oocyte maturation and glycolysis in the pre-ovulatory rat follicle. Acta Endocrinol. (Copenhagen) 87: 377-388.

Hillensjo, T., Kripner, A.S., Pomerantz, S.H. and Channing, C.P. (1979) Action of porcine follicular fluid oocyte maturation inhibitor in vitro: Possible role of cumulus cells. Adv. Exp. Med. Biol. 112: 283-290.

Hillensjo, T. and LeMaire, W.J. (1980) Gonadotropin releasing hormone agonists stimulate meiotic maturation of follicle-enclosed rat oocytes in vitro. Nature 287: 145-146.

Hillensjo, T., Magnusson, C., Svensson, U. and Thelander, H. (1981) Effect of luteinizing hormone and follicle-stimulating hormone on progesterone synthesis by cultured rat cumulus cells. Endocrinology 108: 1920-1924.

Hoppe, P.C. and Pitts, S. (1973) Fertilization in vitro and development of mouse ova. Biol. Reprod. 8: 420-426.

Hunter, G.L., Adams, C.E. and Rowson, L.E.A. (1955) Interbreed ovum transfer in sheep. J. Agric. Sci. 46: 143-149.

Hunter, R.H.F. and Polge, C. (1966) Maturation of follicular oocytes in the pig after injection of human chorionic gonadotrophin. J. Reprod. Fert. 12: 525-531.

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

Iritani, A., Niwa, K. and Imai, H. (1978) Sperm penetration in vitro of pig follicular oocytes matured in culture. J. Reprod. Fert. 54: 379-383.

Iwamatsu, T. and Chang, M.C, (1969) In vitro fertilization of mouse eggs in the presence of bovine follicular fluid. Nature 224: 919-920.

Iwamatsu, T. and Chang, M.C. (1972a) Sperm penetration in vitro of mouse oocytes at various times during maturation. J. Reprod. Fert. 31: 237-247.

Iwamatsu, T. and Chang, M.C. (1972b) Capacitation of hamster spermatozoa treated with rabbit tubal fluid or rabbit and bovine tubal cystic fluids. J. Exp. Zool. 182: 211-216.

Iwamatsu, T. and Ohta, T. (1980) The changes in sperm nuclei after penetrating fish oocytes matured without germinal vesicle material in their cytoplasm. Gamete Res. 3: 121-132.

Iwamatsu, T. and Yanagimachi, R. (1975) Maturation in vitro of ovarian oocytes of prepubertal and adult hamsters. J. Reprod. Fert. 45: 83-90.

Jacobelli, S., Nanocq, J., Baltus, E. and Brachet, J. (1974) Hormone-induced maturation of Xenopus laevis oocytes: Effects of different steroids and study of the properties of a progesterone receptor. Differentiation: 2: 129-135.

Jagiello, G., Graffeo, J., Ducayen, M. and Prosser, R. (1977) Further studies of inhibitors of in vitro mammalian oocyte maturation. Fert. Steril. 28: 476-481.

Jagiello, G., Karnicki, J. and Ryan, R.J. (1968) Superovulation with pituitary gonadotrophins: Method for obtaining meiotic metaphase figures in human ova. Lancet 1: 178-180.

Johnson, R.T. and Rao, P.N. (1971) Nucleo-cytoplasmic interactions in the achievement of nuclear synchrony in DNA synthesis and mitosis in multinucleate cells. Biol. Rev. 46: 97-155.

Jones, G.S. (1984) Update on in vitro fertilization. Endocrine Reviews 5: 62-75.

Jones, H.W., Acosta, A.A., Andrews, M.C., Garcia, J.E., Jones, G.S., Mayer, J., McDowell, J.S., Rosenwaks, Z., Sandow, B.A., Veeck, L.L. and Wilkes, C.A. (1984) Three years of in vitro fertilization at Norfolk. Fert. Steril. 42: 826-834.

Kanatani, H., Hirai, S., Nakanishi, K. and Kurokawa, T. (1969) Isolation and identification of meiosis inducing substance in starfish Asterias amurensis. Nature 221: 273-274.

Kanatani, H. and Hiramoto, Y. (1970) Site of action of 1-methyladenine in inducing oocyte maturation in starfish. Exp. Cell Res. 61: 280-284.

Kaplan, R. and Kraicer, P.F. (1978) Effect of elevated calcium concentration on fertilization of rat oocytes in vitro. Gamete Res. 1: 281-285.

Kelly, G.L. (1939) Effect of opening the ovarian bursa on fecundity in the albino rat. Anat. Rec. 73: 401-405.

Kennedy, J.F. and Donahue, R.P. (1969) Human oocytes: Maturation in chemically defined media. Science 164: 1292-1293.

Kennedy, T.G. and Kennedy, J.P. (1972) Effects of age and parity on reproduction in young female mice. J. Reprod. Fert. 28: 77-84.

Kishimoto, T., Hirai, S. and Kanatani, H. (1981) Role of germinal vesicle material in producing maturation-promoting factor in starfish oocyte. Dev. Biol. 81: 177-181.

Kishimoto, T. and Kanatani, H. (1976) Cytoplasmic factor responsible for germinal vesicle breakdown and meiotic maturation in starfish oocyte. Nature 260: 321-322.

Kishimoto, T., Yamazaki, K., Kato, Y., Koide, S.S. and Kanatani, H. (1984) Induction of starfish oocyte maturation by maturation-promoting factor of mouse and surf clam oocytes. J. Exp. Zool: 231: 293-295.

Krarup, T., Pedersen, T. and Faber, M: (1969) Regulation of oocyte growth in the mouse ovary. Nature 224: 187-188.

Leibfried, M.L. and Bavister, B.D. (1983) Fertilizability of in vitro matured oocytes from golden hamsters. J. Exp. Zool. 226: 481-485.

Leibfried, L. and First, N.L. (1980a) Effect of bovine and porcine follicular fluid and granulosa cells on maturation of oocytes in vitro. Biol. Reprod. 23: 699-704.

Leibfried, L. and First, N.L. (1980b) Follicular control of meiosis in the porcine oocyte. Biol. Reprod. 23: 705-709.

Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H., Northey, D.L. and First, N.L. (1987) Development potential of bovine oocytes matured in vitro or in vivo. Biol. Reprod. 36: 376-383.

Leibfried-Rutledge, M.L., Critser, E.S. and First, N.L. (1986) Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. Biol. Reprod. 35: 850-857.

Leung, P.C.K. and Armstrong, D.T. (1979) Estrogen treatment of immature rats inhibits ovarian androgen production in vitro. Endocrinology 104: 1411-1417.

Lieberman, M.E., Tsafriri, A., Bauminger, S., Collins, W.P., Ahren, K. and Lindner, H.R. (1976) Acta Endocrinol. (Copenhagen) 83: 151-157.

Lindner, H.R., Amsterdam, A., Salomon, Y., Tsafriri, A., Nimrod, A., Lamprecht, S.A., Zor, U. and Koch, Y. (1977) Intraovarian factors in ovulation: Determinants of follicular response to gonadotrophins. J. Reprod. Fert. 51: 215-235.

Lindner, H.R., Tsafriri, A., Lieberman, M.E., Zor, U., Koch, Y., Bauminger, S. and Barnea, A. (1974) Gonadotropin action on cultured Graafian follicles: Induction of maturation division of the mammalian oocyte and differentiation of the luteal cell. Rec. Prog. Horm. Res. 30: 79-138.

Liu, A.Y.-C., Walter, U. and Greengard, P. (1981) Steroid hormones may regulate autophosphorylation of adenosine-3',5' monophosphate-dependent protein kinase in target tissues. Eur. J. Biochem. 114: 539-548.

Lobel, B.L., Tic, L. and Shelesnyak, M.C. (1965) Studies on the mechanism of nidation. XVII. Histochemical analysis of decidualization in the rat. Part 4: Regression of deciduomata: Acta Endocr., Copenhagen 50: 537-559.

Lopata, A., Brown, J.B., Leeton, J.F., Talbot, J.M. and Wood, C. (1978) In vitro fertilization of preovulatory oocytes and embryo transfer in infertilie patients treated with clomiphene and human chorionic gonadotropin. Fert. Steril. 30: 27-35.

Magnusson, C., Hillensjo, T., Tsafriri, A., Hultborn, R. and Ahren, K. (1977) Oxygen consumption of maturing rat oocytes. Biol. Reprod. 17: 9-15.

Mahi, C.A. and Yanagimachi, R. (1976) Maturation and sperm penetration of canine ovarian oocytes in vitro. J. Exp. Zool. 196: 189-196.

Mangia, F. and Canipari, R. (1977) Biochemistry of growth and maturation in mammalian oocytes. In: Development in Mammals, Vol. 2. M.H. Johnson, Eds. North-Holland Publishing Co., New York. pp. 1-29.

Mantalenakis, S.J. and Danezis, J.M. (1968) Effect of blastocyst transfer of gestation period in rats. Fert. Steril. 19: 910-913.

Marsk, L. and Larsson, K.S. (1974) A simple method for non-surgical blastocyst transfer in mice. J. Reprod. Fert. 37: 393-398.

Martel, D., Frydman, R., Glissant, M., Maggioni, C. Roche, D. and Psychoyos, A. (1987) Scanning electron microscopy of postovulatory human endometrium in spontaneous cycles and cycles stimulated by hormone treatment. J. Endocrinol. 114: 319-324.

Masui, Y. (1967) Relative roles of the pituitary, follicle cells, and progesterone in the induction of oocyte maturation in Rana pipiens. J. Exp. Zool. 166: 365-376.

Masui, Y. and Clarke, H.J. (1979) Oocyte maturation. Int. Rev. Cytol. 57: 185-282.

Masui, Y. and Markert, C.L. (1971) Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes. J. Exp. Zool. 177: 129-146.

McGaughey, R.W. (1977a) The maturation of porcine oocytes in minimal defined culture media with varied macromolecular supplements and varied osmolarity. Exp. Cell Res. 109: 25-30.

McGaughey, R.W. (1977b) The culture of pig oocytes in minimal medium and the influence of progesterone and estradiol-17B on meiotic maturation. Endocrinology 100: 39-45.

McGaughey, R.W. (1983) Regulation of oocyte maturation. In: Oxford Reviews of Reproductive Biology, Volume 5. C.A. Finn, Ed. Clarendon Press, Oxford. pp. 106-130.

McGaughey, R.W. and Polge, C. (1971) Cytogenetic analysis of pig oocytes matured in vitro. J. Exp. Zool. 176: 383-396.

McLaren, A. and Michie, D. (1956) Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. J. Exp. Biol. 33: 394-416.

Meijer, L. and Guerrier, P. (1984) Maturation and fertilization in starfish oocytes. Int. Rev. Cytology 86: 129-196.

Meinecke, B. and Meinecke-Tillman, S. (1981) Induction and inhibition of meiotic maturation of follicle-enclosed porcine oocytes in vitro. Theriogenology 15: 581-589.

Meizel, S. (1985) Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. Am. J. Anat. 174: 285-302.

Mills, J.A., Jeitles, G.G. and Brackett, B.G. (1973) Embryo transfer following in vitro and in vivo fertilization of rabbit ova. Fert. Steril. 24: 602-608.

Miyamoto, H. and Chang, M.C. (1973a) Fertilization of rat eggs in vitro. Biol. Reprod. 9: 384-393.

Miyamoto, H. and Chang, M.C. (1973b) The importance of serum albumin and metabolic intermediates for capacitation of spermatozoa and fertilization of mouse eggs in vitro. J. Reprod. Fert. 32: 193-205.

Miyamoto, H. and Chang, M.C. (1973c) Effect of osmolality on fertilization of mouse and golden hamster eggs in vitro. J. Reprod. Fert. 33: 481-487.

Miyamoto, H. and Chang, M.C. (1973d) In vitro fertilization of rat eggs. Nature 241: 50-51.

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

Miyamoto, H., Toyoda, Y. and Chang, M.C. (1974) Effect of hydrogen-ion concentration on in vitro feritlization of mouse, golden hamster, and rat eggs. Biol. Reprod. 10: 487-493.

Moor, R.M., Osborn, J.C., Cran, D.G. and Walters, D.E. (1981) Selective effect of gonadotropins on cell coupling, nuclear maturation and protein synthesis in mammalian oocytes. J. Embryol. Exp. Morphol. 61: 347-365.

oocyte was picked up with the holding pipette and the zona was held against the drilling pipette in a tangential position. Acid Tyrode's solution was slowly expelled from the drilling pipette until a dimple in the oolemma appeared, thereby signalling a rupture in the zona pellucida. At this point the oocyte was immediately withdrawn. For most oocytes, the hole was not visible with the phase contrast microscope. If the hole was too large, the zona would loosen its enclosure of the oocyte and/or occasionally fall off with handling; these oocytes were not used in the experiment.

This study included four experiments, to determine: A) an appropriate sperm concentration, B) the effect of drilling on the fertilizability of CI and CF in vitro matured oocytes, C) the developmental capability of drilled oocytes, and D) the effect of drilling and progesterone on the maturation and fertilization of in vitro matured oocytes.

A. Sperm Concentration

Immature oocytes were collected as described in Section 4.2.2 and were matured CI for 8-10 h in MEM + 15% rat serum. At the end of the culture period, all the oocytes were stripped of their cumulus cells and half had a holes drilled in their zonae pellucidae, one hole per oocyte. The oocytes were rinsed with fresh DPBS and divided into 50 µl droplets of fertilization medium. The droplets of medium were randomly divided into 5 groups,

Moor, R.M., Smith, M.W., and Dawson, R.M.C. (1980a) Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. Exp. Cell Res. 126: 15-30.

Moor, R.M., Polge, C. and Willadsen, S.M. (1980b) Effect of follicular steroids on the maturation and fertilization of mammalian oocytes. J. Embryol. Exp. Morph. 56: 319-335.

Moor, R.M. and Trounson, A.O. (1977) Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J. Reprod. Fert. 49: 101-109.

Moore, N.W. (1968) The survival and development of fertilized eggs transferred between Border Leicester and Merino ewes. Aust. J. Agric. Res. 19: 295-302.

Moore, N.W. (1974) Multiple ovulation and ovum transfer in the goat. Proc. Aust. Soc. Anim. Prod. 10: 246-249.

Moricard, R. and Henry, R. (1967) The pharmacodynamics of gonadotropins. In: Proceedings of the Fifth World Congress on Fertility and Sterility. B. Westin and N. Wuqvist, Eds. Excerpta Medica, New York. pp. 15-20.

Morley, P., Armstrong, D.T. and Gore-Langton, R.E. (1987) Fibronectin stimulates growth but not follicle-stimulating hormone-dependent differentiation of rat granulosa cells in vitro. J. Cell. Physiol. 132: 226-236.

Motlik, J. and Fulka, J. (1974) Fertilization of pig follicular oocytes cultivated in vitro. J. Reprod. Fert. 36: 235-237.

Mukherjee, A.B. (1972) Normal progeny from fertilization in vitro of mouse oocytes matured in culture and spermatozoa capacitated in vitro. Nature 237: 397-398.

Mukherjee, A.B. and Cohen, M.M. (1970) Development of normal mice by in vitro fertilization. Nature 228: 472-473.

Neal, P. and Baker, T.G. (1973) Response of mouse ovaries in vivo and in organ culture to pregnant mare's serum gonadotrophin and human chorionic gonadotrophin. I. Examination of critical time intervals. J. Reprod. Fert. 33: 399-410.

Neal, P. and Baker, T.G. (1974) Response of mouse ovaries in vivo and in organ culture to pregnant mare's serum gonadotrophin and human chorionic gonadotrophin. II. Effect of different doses of hormones. J. Reprod. Fert. 37: 399-404.

Nekola, M.V. and Smith, D.M. (1974) Oocyte maturation and follicle cell viability in vitro. Eur. J. Obstet. Gynec. Reprod. Biol. Suppl. 4: S125-131.

Neugebauer, C. (1935) L'influence de la capsule ovarienne sur la fecondite de la rate blanche. C. r. Seanc. Ass. Anat. 30: 400-407.

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

Nicholas, J.S. (1933) Development of transplanted rat eggs. Proc. Soc. Exp. Biol. Med. 30: 1111-1113.

Nishikawa, Y., Horie, T., Sugie, T., Onuma, H. and Niwa, T. (1963a) Studies on the transplantation of ova (artificial pregnancy) in goats. III. Experiment on the speed of ova descending the genital tract, progress of cleavage of ova, and collection of ova from the donor. Proc. Jpn. Acad. 39: 671-676.

Nishikawa, Y., Horie, T., Sugie, T., Onuma, H. and Niwa, T. (1963b) Studies on the transplantation of ova (artificial pregnancy) in goats. IV. Experiments on synchronization of estrus and transplantation of ova into the recipient. Proc. Jpn. Acad. 39: 758-763.

Nishizuka, Y., Takai, Y., Kishimoto, A., Kikkawa, U. and Kaibuchi, K. (1984) Phospholipid turnover in hormone action. Rec. Prog. Horm. Res. 40: 301-345.

Niwa, K. and Chang, M.C. (1973) Feritilization in vitro of rat eggs as affected by the maturity of the female and the sperm concentration. J. Reprod. Fert. 35: 577-580.

Niwa, K. and Chang, M.C. (1974a) Various conditions for the fertilization of rat eggs in vitro. Biol. Reprod. 11: 463-469.

Niwa, K. and Chang, M.C. (1974b) Effects of sperm concentration on the capacitation of rat spermatozoa. J. Exp. Zool. 189: 353-356.

Niwa, K. and Chang, M.C. (1974c) Optimal sperm concentration and minimal number of spermatozoa for fertilization in vitro of rat eggs. J. Reprod. Fert. 40: 471-474.

Niwa, K. and Chang, M.C. (1975a) Fertilization of rat eggs in vitro at various times before and after ovulation with special reference to fertilization of ovarian oocytes matured in culture. J. Reprod. Fert. 43: 435-451.

Niwa, K. and Chang, M.C. (1975b) Effect of pretreatment of rat spermatozoa with high concentrations of NaCl on sperm capacitation and fertilization of eggs in vitro. Biol. Reprod. 13: 187-189.

Niwa, K. and Chang, M.C. (1975c) Requirement of capacitation for sperm penetration of zone-free rat eggs. J. Reprod. Fert. 44: 305-308.

Niwa, K. and Iritani, A. (1978) Effect of various hexoses on sperm capacitation and penetration of rat eggs in vitro. J. Reprod. Fert. 53: 267-271.

Niwa, K., Miyake, M., Iritani, A. and Nishikawa, Y. (1976) Fertilization of rat oocytes cultured in vitro from various stages of maturation. J. Reprod. Fert. 47: 105-106.

Noyes, R.W. (1952) Fertilization of follicular ova. Fert. Steril. 3: 1-12.

Noyes, R.W. and Dickmann, Z. (1960) Relationship of ovular age to endometrial development. J. Reprod. Fert. 1: 186-196.

Noyes, R.W. and Dickmann, Z. (1961) Survival of ova transferred into the oviduct of the rat. Fert. Steril. 12: 67-79.

Noyes, R.W., Dickmann, Z., Doyle, L.L. and Gates, A.H. (1963) Ovum transfers, synchronous and asynchronous, in the study of implantation. In: Delayed Implantation. A.C. Enders, Ed. University of Chicago Press, Chicago. p. 197-211.

Papaioannou, V.E. and Ebert, K.M. (1986) Development of fertilized embryos transferred to oviducts of immature mice. J. Reprod. Fert. 76: 603-608.

Peluso, J.J., Steger, R.W. and Hafez, E.S.E. (1977) Sequential changes associated with the degeneration of preovulatory rat follicles. J. Reprod. Fert. 49: 215-218.

Peters, H., Byskov, A.G., Himelstein-Braw, R. and Faber, M. (1975) Follicular growth: The basic event in the mouse and human ovary. J. Reprod. Fert. 45: 559-566.

Pfeifer, H., Reiche, W. and Mettler, L. (1980) In vitro early cleavage stages in the rat. Arch. Androl. 5: 84.

Pincus, G. (1931) Observations on the living eggs of the rabbit. Proc. R. Soc. Lond (Biol) 107: 132-157.

Pincus, G. and Enzmann, E.V. (1935) The comparative behaviour of mammalian eggs in vivo and in vitro. J. Exp. Med. 62: 655-675.

Pope, C.E., Christenson, R.K., Zimmerman-Pope, V.A. and Day, B.N. (1972) Effect of number of embryos on embryonic survival in recipient gilts. J. Anim. Sci. 35: 805-808.

Powers, R.D. and Paleos, G.A. (1982) Combined effects of calcium and dibutyryl cAMP on germinal vesicle breakdown in the mouse oocyte. J. Reprod. Fert. 66: 1-8.

Psychoyos, A. (1973) Endocrine control of egg implantation. In: Handbook of Physiology, Section 7, Vol. 2, Part 2. R.O. Greep, E.B. Astwood and S.R. Geiger, Eds. Physiological Society, Washington, D.C. pp. 187-215.

Quigley, M.M. (1980) In vitro fertilization in the rat: Art or science? Arch. Androl. 5: 61-62.

Quigley, M.M. (1982) In vitro fertilization in the rat. In: In vitro fertilization and embryo transfer, E.S.E. Hafez and K. Semm, Eds., MTP Press Ltd., Lancaster, England. pp. 77-88.

Racowsky, C. (1983) Androgenic modulation of cyclic adenosine monophosphate (cAMP)-dependent meiotic arrest. Biol. Reprod. 28: 774-787.

Racowsky, C. (1984) Effect of forskolin on the spontaneous maturation and cyclic AMP content of rat oocyte-comulus complexes. J. Reprod. Fert. 72: 107-116.

Racowsky, C. (1985a) Effect of forskolin on the spontaneous maturation and cyclic AMP content of hamster oocyte-cumulus complexes. J. Exp. Zool. 234: 87-96.

Racowsky, C. (1985b) Antagonistic actions of estradiol and tamoxifen upon forskolin-dependent meiotic arrest, intercellular coupling, and the cAMP content of hamster oocyte-cumulus complexes. J. Exp. Zool. 234: 251-260.

Racowsky, C. and McGaughey, R.W. (1982a) Further studies of the effects of follicular fluid and membrana granulosa cells on the spontaneous maturation of pig oocytes. J. Reprod. Fert. 66: 505-512.

Racowsky, C. and McGaughey, R.W. (1982b) Further studies concerning the effect of estradiol on the meiotic maturation of porcine oocytes in vitro. J. Exp. Zool. 221: 103-110.

Racowsky, C. and McGaughey, R.W. (1982c) In the absence of protein, estradiol suppresses meiosis of porcine oocytes in vitro. J. Exp. Zool. 224: 103-110.

Racowsky, C. and Satterlie, R.A. (1985) Metabolic, fluorescent dye and electrical coupling between hamster oocytes and cumulus cells during meiotic maturation in vivo and in vitro. Dev. Biol. 108: 191-202.

Rice, C. and McGaughey, R.W. (1980) Interactions between the granulosa cell and the oocyte in vitro. J. Anim. Sci. 51 (Suppl.): Abstract 521.

Rice, C. and McGaughey, R.W. (1981) Effect of testosterone and dibutyryl cAMP on the spontaneous maturation of pig oocytes. J. Reprod. Fert. 62: 245-256.

Richards, J.S. (1980) Maturation of ovarian follicles: Actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol. Rev. 60: 51-89.

Richter, J.D. and McGaughey, R.W. (1979) Specificity of inhibition by steroids of porcine oocyte maturation in vitro. J. Exp. Zool. 209: 81-90.

Robertson, J.E. and Baker, R.D. (1969) Role of female sex steroids as possible regulators of oocyte maturation. Second Annual Meeting of SSR. University of California, Davis. Abstract 57.

Robinson, A. (1887) On the position and peritoneal relations of the mammalian ovary. J. Anat. Physiol. 21: 169-179.

Rogers, B.J. (1978) Mammalian sperm capacitation and fertilization in vitro: A critique of methodology. Gamete Res. 1: 165-223.

Rowson, L.E.A., Moor, R.M. and Lawson, R.A.S. (1969) Fertility following egg transfer in the cow; effect of method and synchronization of oestrus. J. Reprod. Fert. 18: 517-523.

Salustri, A. and Siracusa, G. (1983) Metabolic coupling, cumulus expansion and meiotic resumption in mouse cumuli oophori cultured in vitro in the presence of FSH or dcAMP, or stimulated in vivo by hCG. J. Reprod. Fert. 68: 335-341.

Sathananthan, A.H. and Trounson, A.O. (1985) The human pronuclear ovum: Fine structure of monospermic and polyspermic fertilization in vitro. Gamete Res. 12: 385-398.

Sato, E. and Koide, S.S. (1984) A factor from bovine granulosa cells preventing oocyte maturation. Differentiation 26: 59-62.

Sato, A. and Yanagimachi, R. (1972) Transplantation of preimplantation hamster embryos. J. Reprod. Fert. 30: 329-332.

Schorderet-Slatkine, S. (1972) Action of progesterone and related steroids on oocyte maturation in Xenopus laevis. An in vitro study. Cell Differentiation 1: 179-189.

Schroeder, A.C. and Eppig, J.J. (1984) The developmental capacity of mouse oocytes that matured spontaneously in vitro is normal. Dev. Biol. 102: 493-497.

Schuetz, A.W. (1967a) Effect of steroids on germinal vesicle of oocytes of the frog (Rana pipiens) in vitro. Proc. Soc. Exp. Biol. Med. 124: 1307-1310.

Schuetz, A.W. (1967b) Action of hormones on germinal vesicle breakdown in frog (Rana pipiens) oocytes. J. Exp. Zool. 166: 347-354.

Schultz, R.M. (1987) Molecular aspects of mammalian oocyte growth and maturation. In: Experimental approaches to mammalian embryonic development. J. Rossant & R. Pedersen, Eds. Cambridge University Press. pp. 195-237.

Schultz, R.M., Letourneau, G.E. and Wassarman, P.M. (1979) Program of early development in the mammal: Changes in the patterns and absolute rates of tubulin and total protein synthesis during oocyte growth in the mouse. Dev. Biol. 73: 120-133.

Schultz, R.M., Montgomery, R.R. and Belanoff, J.R. (1983a) Regulation of mouse oocyte meiotic maturation: Implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. Dev. Biol. 97: 264-273.

Schultz, R.M., Montgomery, R., Ward-Bailey, P.F. and Eppig, J.J. (1983b) Regulation of oocyte maturation in the mouse: Possible role of intercellular communication, cAMP, and testosterone. Dev. Biol. 95: 294-304.

Schultz, R.M. and Wassarman, P.M. (1977) Biochemical studies of mammalian oogenesis: Protein synthesis during oocyte growth and meiotic maturation in the mouse. J. Cell. Sci. 24: 167-194.

Shalgi, R. (1983) Success of rat embryos derived from *in vitro* as compared to *in vivo* fertilization. Biol. Reprod. 28 (Suppl. 1): 40.

Shalgi, R. (1984) Developmental capacity of rat embryos produced by *in vivo* or *in vitro* fertilization. Gamete Res. 10: 77-82.

Shalgi, R., Dekel, N. and Kraicer, P.F. (1979) The effect of LH on the fertilizability and developmental capacity of rat oocytes matured *in vitro*. J. Reprod. Fert. 55: 429-435.

Shalgi, R., Kaplan, R. and Kraicer, P.F. (1977) Proteins of follicular, bursal and ampullar fluids of rats. Biol. Reprod. 17: 333-338.

Shalgi, R., Kaplan, R. and Kraicer, P.F. (1985) The influence of postovulatory age on the rate of cleavage of *in vitro* fertilized rat oocytes. Gamete Res. 11: 99-106.

Shalgi, R. and Phillips, D.M. (1982) Sperm penetration into rat ova fertilized *in vitro*. J. Androl. 3: 382-387.

Shea, B.J., Baker, R.D. and Latour, J.P.A. (1975) Human follicular oocytes and their maturation *in vitro*. Fert. Steril. 26: 1075-1082.

Sirard, M.A., Lambert, R.D., Menard, D.P. and Bedoya, M. (1985) Pregnancies after *in vitro* fertilization of cow follicular oocytes, their incubation in rabbit oviduct and their transfer to the cow uterus. J. Reprod. Fert. 75: 551-556.

Smith, D.M. and Tenney, D.Y. (1980) Effects of steroids on mouse oocyte maturation *in vitro*. J. Reprod. Fert. 60: 331-338.

Smith, L.D., Ecker, R.E. and Subtelny, S. (1968) *In vitro* induction of physiological maturation in Rana pipiens oocytes removed from their ovarian follicles. Dev. Biol. 17: 627-643.

Sorensen, R.A., Cyert, M.S. and Pedersen, R.A. (1985) Active maturation-promoting factor is present in mature mouse oocytes. J. Cell Biol. 100: 1637-1640.

Sorensen, R.A. and Wassarman, P.M. (1976) Relationship between growth and meiotic maturation of the mouse oocyte. Dev. Biol. 50: 531-536.

Soupart, P. (1981) Current problems in *in vitro* fertilization and embryo transfer. In: Bioregulators of Reproduction, G. Jagiello and H.J. Vogel, Eds, Academic Press Inc., New York. pp. 427-457.

Sreenan, J.M. and Beehan, D. (1974) Egg transfer in the cow: pregnancy rat and egg survival. J. Reprod. Fert. 41: 497-499.

Steel, R.G.D. and Torrie, J.H. (1980) Principles and Procedures of Statistics. McGraw-Hill, Inc., New York.

Steptoe, P.C. and Edwards, R.G. (1970) Laparoscopic recovery of preovulatory human oocytes after priming of ovaries with gonadotrophins. Lancet 1: 683-689.

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

Stoklosowa, S. and Nalbandov, A.V. (1972) Luteinization and steroidogenic activity of rat ovarian follicles cultured *in vitro*. Endocrinology 91: 25-32.

Stone, S.L., Pomerantz, S.H., Shwartz-Kripner, A. and Channing, C.P. (1978) Inhibitor of oocyte maturation from porcine follicular fluid: Further purification and evidence for reversible action. Biol. Reprod. 19: 585-592.

Sugie, T. (1965) Successful transfer of a fertilized bovine egg by non-surgical techniques. J. Reprod. Fert. 10: 197-201.

Sugie, T., Seidel, G.E., Jr. and Hafez, E.S.E. (1980) Embryo transfer. In: Reproduction in farm animals. E.S.E. Hafez, Ed. Lea & Febiger, Philadelphia. pp. 569-594.

Surani, M.A.H. (1975) Zona pellucida denudation, blastocyst proliferation and attachment in the rat. J. Embryol. Exp. Morph. 33: 343-353.

Szybek, K. (1972) *In vitro* maturation in oocytes from sexually immature mice. J. Endocrinol. 54: 527-528.

Talbot, J.M., Dooley, M., Leeton, J., Lopata, A., McMaster, R., Wood, C., Brown, J.B. and Evans, J.H. (1976) Gonadotrophin stimulation for ooycte recovery and *in vitro* fertilization in infertile women. Aust. N.Z.J. Obstet. Gynaecol. 16: 111-118.

Tarkowski, A.K. (1959) Experiments on the transplantation of ova in mice. Acta Theriol. 2: 251-257.

Tesarik, J. (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability in vitro. J. Reprod. Fert. 74: 383-388.

Testart, J. (1987) Evidence of uterine inadequacy to egg implantation in stimulated in vitro fertilization cycles. Fert. Steril. 47: 855-857.

Thadani, V. (1982) Mice produced from eggs fertilized in vitro at a very low sperm:egg ratio. J. Exp. Zool. 219: 277-283.

Thibault, C. (1972) Final stages of mammalian oocyte maturation. In: Oogenesis. J.D. Biggers and A.W. Schuetz, Eds. University Park Press, Baltimore. pp. 397-411.

Thibault, C. (1977) Are follicular maturation and oocyte maturation independent processes? J. Reprod. Fert. 51: 1-15.

Thibault, C. and Gerard, M. (1970) Facteur cytoplasmique necessaire a la formation du pronucleus male dans l'ovocyte de lapine. C.R. Acad. Sci. Paris 270: 2025-2026.

Thibault, C. and Gerard, M. (1973) Cytoplasmic and nuclear maturation of rabbit oocytes in vitro. Ann. Biol. Anim. Biochim. Biophys. 13: 145-155.

Thibault, C., Gerard, M. and Menezo, Y. (1975) Preovulatory and ovulatory mechanisms in oocyte maturation. J. Reprod. Fert. 45: 605-610.

Thibier-Fouchet, C., Mulner, O. and Ozon, R. (1976) Progesterone biosynthesis and metabolism by ovarian follicles and isolated oocytes of Xenopus laevis. Biol. Reprod. 14: 317-326.

Thompson, R.S., Chakraborty, J. and Van Pelt, L. (1971) An ultrastructural study of monkey follicular oocytes maturing in vitro and in vivo. Fourth Annual Meeting of SSR, Boston University. Abstract 25.

Toyoda, Y. and Chang, M.C. (1968) Sperm penetration of rat eggs in vitro after dissolution of zona pellucida by chymotrypsin. Nature 220: 589-591.

Toyoda, Y. and Chang, M.C. (1974a) Fertilization of rat eggs in vitro by epididymal spermatozoa and the development of eggs following transfer. J. Reprod. Fert. 36: 9-22.

Toyoda, Y. and Chang, M.C. (1974b) Capacitation of epididymal spermatozoa in a medium with high K/Na ratio and cyclic AMP for the fertilization of rat eggs in vitro. J. Reprod. Fert. 36: 125-134.

Toyoda, Y., Yokoyama, M. and Hosi, T. (1971) Studies on fertilization of mouse eggs in vitro. I. In vitro fertilization by fresh epididymal sperm. J. Anim. Reprod. 16: 147-151.

Tsafriri, A. (1985) The control of meiotic maturation in mammals. In: Biology of fertilization, Vol. 1. C. Metz and A. Monroy, Eds. Academic Press, New York. pp. 221-252.

Tsafriri, A. (1988) Local nonsteroidal regulators of ovarian function. In: The physiology of reproduction. E. Knobil and J. Neill, Eds. Raven Press, New York. pp. 527-565.

Tsafriri, A. and Channing, C.P. (1975a) Influence of follicular maturation and culture conditions on the meiosis of pig oocytes in vitro. J. Reprod. Fert. 43: 149-152.

Tsafriri, A. and Channing, C.P. (1975b) An inhibitory influence of granulosa cells and follicular fluid upon porcine oocyte meiosis in vitro. Endocrinology 96: 922-927.

Tsafriri, A., Channing, C.P., Pomerantz, S.H. and Lindner, H.R. (1977) Inhibition of maturation of isolated rat oocytes by porcine follicular fluid. J. Endocrinol. 75: 285-291.

Tsafriri, A., Lieberman, M.E., Barnea, A., Bauminger, S. and Lindner, H.R. (1973) Induction of ovum maturation and of steroidogenesis in the isolated Graafian follicle by luteinizing hormone; role of RNA and protein synthesis. Endocrinology 93: 1378-1386.

Tsafriri, A., Lindner, H.R., Zor, U. and Lamprecht, S.A. (1972) In vitro induction of meiotic division in follicle-enclosed rat oocytes by LH, cyclic AMP and prostaglandin E_2. J. Reprod. Fert. 31: 39-50.

Tsafriri, A., Pomerantz, S.H. and Channing, C.P. (1976) Inhibition of oocyte maturation by porcine follicular fluid: Parial characterization of the inhibitor. Biol. Reprod. 14: 511-516.

Tsunoda, Y. and Chang, M.C. (1975a) In vitro fertilization of rat and mouse eggs by ejaculated sperm and the effect of energy sources on in vitro fertilization of rat eggs. J. Exp. Zool. 193: 79-86.

Tsunoda, Y. and Chang, M.C. (1975b) Penetration of mouse eggs *in vitro*: Optimal sperm concentration and minimal number of spermatozoa. J. Reprod. Fert. 44: 139-142.

Usui, N. and Yanagimachi, R. (1976) Behaviour of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization and early development. J. Ultrastruct. Res. 37: 276-288.

Vermeiden, J.P.W. and Zeilmaker, G.H. (1974) Relationship between maturation division, ovulation and luteinization in the female rat. Endocrinology 95: 341-351.

Vickery, B.H., Erickson, G.I. and Bennett, J.P. (1969) Nonsurgical transfer of eggs through the cervix in rats. Endocrinology 85: 1202-1203.

Vickery, B.H. and McRae, G.I. (1980) Synchronization of oestrus in adult female rats by utilizing the paradoxical effects of an Lh-RH agonist. J. Reprod. Fert. 60: 399-402.

Walton, E.A. and Armstrong, D.T. (1983) Oocyte normality after superovulation in immature rats. J. Reprod. Fert. 67: 309-314.

Walton, E.A., Evans, G. and Armstrong, D.T. (1983) Ovulation response and fertilization failure in immature rats induced to superovulate. J. Reprod. Fert. 67: 91-96.

Weitlauf, H.M. (1971) Protein synthesis by blastocysts in the uteri and oviducts of intact and hypophysectomized mice. J. Exp. Zool. 176: 35-40.

Weitlauf, H.M. (1973) *In vitro* uptake and incorporation of amino acids by blastocysts from intact and ovariectomized mice. J. Exp. Zool. 183: 303-308.

Westrick, J.C., Boatman, D.E. and Bavister, B.D. (1985) Characteristics of acrosome reaction-inducing factor from hamster cumulus and follicular fluid. Biol. Reprod. 32 (Suppl. 1): 213.

Whitten, W.K. (1971) Nutrient requirements for the culture of preimplantation embryos *in vitro*. Adv. Biosci. 6: 129-141.

Whitten, W.K. and Biggers, J.D. (1968) Complete development *in vitro* of the preimplantation stages of the mouse in a simple chemically defined medium. J. Reprod. Fert. 17: 399-401.

Whittingham, D.G. (1975) Fertilisation, early development and storage of mammalian ova in vitro. In: The early development of mammals. M. Balls & A.D. Wild, Eds. Cambridge University Press, Cambridge, U.K. pp. 1-24.

Whittingham, D.G. and Bavister, B.D. (1974) Development of hamster eggs fertilized in vitro or in vivo. J. Reprod. Fert. 38: 489-492.

Whittingham, D.G. and Biggers, J.D. (1967) Fallopian tube and early cleavage in the mouse. Nature 213: 942-943.

Wimsatt, W.A. and Waldo, C.M. (1945) The normal occurrence of a peritoneal opening in the bursa ovarii of the mouse. Anat. Rec. 93: 47-53.

Wolf, D.P. (1981) The mammalian egg's block to polyspermy. In: Fertilization and embryonic development in vitro. L. Mastroianni and B.J. Biggers, Eds. Plenum Press, New York. pp. 183-197.

Wood, C., McMaster, R., Rennie, G., Trounson, A. and Leeton, J. (1985) Factors influencing pregnancy rates following in vitro fertilization and embryo transfer. Fert. Steril. 43: 245-250.

Wood, C., Trounson, A., Leeton, J.F., Renou, P.M., Walters, W.A.W., Buttery, B.W., Grimwade, J.C., Spensley, J.C. and Yu, V.Y.H. (1982) Clinical features of eight pregnancies resulting from in vitro fertilization and embryo transfer. Fert. Steril. 38: 22-29.

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

Yanagimachi, R. (1969a) In vitro acrosome reaction and capacitation of golden hamster sperm by bovine follicular fluid and its fractions. J. Exp. Zool. 170: 269-280.

Yanagimachi, R. (1969b) In vitro capacitation of hamster spermatozoa by follicular fluid. J. Reprod. Fert. 18: 275-286.

Yanagimachi, R. (1970) In vitro capacitation of golden hamster spermatozoa by homologous and heterologous blood sera. Biol. Reprod. 3: 147-153.

Yanagimachi, R. (1972) Fertilization of guinea pig eggs in vitro. Anat. Rec. 174: 9-20.

Yanagimachi, R. (1981) Mechanisms of fertilization in mammals. In: Fertilization and embryonic development in vitro. L. Mastroianni and J.D. Biggers, Eds. Plenum Press, New York. pp. 81-182.

Yanagimachi, R. (1988) Mammalian fertilization. In: The physiology of reproduction. E. Knobil and J. Neill, Eds. Raven Press, New York. pp. 135-185.

Yanagimachi, R. and Chang, M.C. (1963) Fertilization of hamster eggs in vitro. Nature 200: 281-282.

Yanagimachi, R. and Chang, M.C. (1964) In vitro fertilization of golden hamster ova. J. Exp. Zool. 156: 361-375.

Yanagishita, M. and Haskell, V.C. (1979) Biosynthesis of proteoglycans by rat granulosa cells cultured in vitro. J. Biol. Chem. 254: 12355-12364.

Yanagishita, M., Rodbard, D. and Haskell, V.C. (1979) Isolation and characterization of proteoglycans from porcine follicular fluid. J. Biol. Chem. 254: 911-920.

Zamboni, L. (1971) Fine structure of human follicular oocytes maturing in vitro. Fourth Annual Meeting of SSR, Boston University. Abstract 24.