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# REGULATION OF OOCYTE MATURATION IN VITRO IN PIGS

BY

JAINCHI DING

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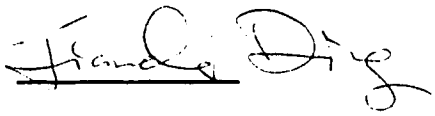

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# ABSTRACT

The main objectives of this thesis are 1) to investigate the importance of protein synthesis during maturation and fertilization and 2) to investigate effects of follicular cells on oocyte cytoplasmic maturation (male pronuclear development).

In a first series of experiments, the importance of protein synthesis during maturation and fertilization were examined by treatments with protein synthesis inhibitors at different stages of maturation or during fertilization; and protein synthesis in oocytes during maturation were analyzed using [$S^{35}$]methionine labelling and SDS-PAGE analysis. Results showed that, in the absence of protein synthesis during fertilization, sperm penetration, oocyte meiosis and female and male pronuclear development were not affected. By contrast, inhibiting protein synthesis during maturation severely impaired the completion of meiosis and pronuclear development. Protein synthesis between 24 and 48 h maturation culture were essential for oocytes to obtain the ability to form male pronuclei after sperm penetration. This period of maturation coincided with the dominant phase of protein reprogramming in the oocyte.

The following three series of experiments examined effects of follicular cells on oocyte maturation using co-culture of follicular shells with oocytes or culturing oocytes in follicular conditioned media. Co-culture of oocyte complexes with follicular shells or culture of oocyte complexes in appropriately prepared follicular conditioned media greatly enhanced male pronuclear formation rate. Male pronuclear formation in penetrated oocytes was significantly affected by sizes and ages of follicles used for co-cultured with oocytes or for production of conditioned media.

A final series of experiments demonstrated that EGF stimulated both nuclear and

cytoplasmic maturation of oocytes; while the former effect is independent, the latter is a result of an interaction with gonadotropins.

In conclusion, 1) during oocyte maturation, oocytes synthesize proteins which are essential for oocytes to complete meiosis and to gain the ability to decondense sperm nuclei; 2) follicular cells surrounding the oocyte play essential roles in regulating full oocyte maturation; 3) EGF, which was found in follicular fluid, may be directly involved in regulation of oocyte maturation; and 4) diversity of follicular development in pigs affects the quality of oocyte ovulated and these effects may have important consequences for the developmental potential of the early embryo.

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I would like to dedicate this manuscript to my wife, Mingxian Shen and to my daughter, Guangning (Helen) for their love and support.

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# CHAPTER I INTRODUCTION

Since Pincus and Enzmann (1935) observed the spontaneous resumption of meiosis in mammalian oocytes freed of follicular cells more than half a century ago, oocyte nuclear maturation in vitro has been intensively studied. Oocytes from all the mammalian species studied are able to resume meiosis from the dictyate stage to metaphase II when cultured in a suitable medium (see Thibault, 1977). The in vitro study of oocyte maturation enriches our understanding of the nature of oocyte maturation. In vitro maturation of follicular oocytes obtained from abattoir materials has become a routine laboratory method to produce embryos for embryological studies in many species, to produce embryos for the embryo transfer industry in cattle (see Betteridge, 1993), or to produce recipient oocytes for production of genetically identical animals by nuclear transplantation. Thus the in vitro study of oocyte maturation has contributed and will continue to contribute a great deal to animal science and the animal industry.

Oocyte maturation involves two aspects, nuclear maturation and cytoplasmic maturation. Cytoplasmic maturation must run parallel with the nuclear maturation (Chang, 1955) in order for the oocyte to achieve developmental ability. The regulation of nuclear and cytoplasmic maturation of the oocyte, especially in pig, will be reviewed in Chapter II to give background information.

One aspect of oocyte cytoplasmic maturation is reprogramming of protein synthesis. Protein synthesis is crucial for the resumption of oocyte meiosis. Patterns of protein synthesis underwent dramatic changes during maturation, especially during germinal vesicle breakdown (GVBD), in mouse, rabbit, sheep and pig oocytes (Schultz

& Wassarman, 1977 a, b; Van Blerkom & McGaughey, 1978; Warnes et al., 1977; Moor & Gandolfi, 1987; McGaughey et al., 1977). Inhibition of protein synthesis blocks GVBD in cow (Hunter and Moor, 1987), sheep (Moor and Crosby, 1986), pig (Fulka et al., 1986b; Kubelka et al., 1988), rat (Ekholm and Magnusson, 1979), and mouse (Downs, 1990) oocytes. However, details of the molecular basis for changes in pig oocytes during maturation and fertilization are not available. This prevents us from fully understanding the physiology of porcine oocyte maturation, such as when male pronuclear growth factors (MPGF) are synthesized (Iwamatsu & Chang, 1972; Thibault et al., 1975), and whether the completion of the second meiosis also depends on de novo protein synthesis. Therefore a series of studies were initiated to analyze de novo synthesis of protein in pig oocytes during maturation and fertilization when working as a visitor scientist at Dr. Moor's laboratory in Cambridge, England. We found that major changes in the protein pattern during fertilization were closely associated with the period of male pronuclear formation (Ding et al., 1992). A particular protein band at a $M_r$ 25 kD synthesized during maturation was dephosphorylated to yield a 22 kD protein, which was independent of de novo protein synthesis. These results suggested that fertilization could occur in the absence of de novo synthesis of proteins during fertilization and therefore that the proteins synthesized during oocyte maturation may be crucial for fertilization. In order to further understand the importance of protein synthesis for oocyte maturation and fertilization, further studies (Chapter III) on de novo protein synthesis in oocytes during maturation and fertilization were carried out as an early part of the experimental progam reported in this thesis.

Cytoplasmic maturation of the oocyte seems to be regulated by follicular cells. Nuclear maturation of porcine cumulus enclosed-oocytes in vitro without granulosa cells has been demonstrated by Motlik and Fulka (1974a) but these in vitro matured oocytes failed to undergo normal fertilization and subsequent embryonic development. My own previous studies (Ding et al., 1988; Nagai et al., 1993) and those of others (Mattioli et al., 1988 a, b; 1989; Naito et al., 1988; Yoshida et al., 1992a) showed that oocyte-complexes co-cultured with follicular shells, cultured in follicular fluid containing medium or cultured in follicular cell conditioned medium, greatly improved male pronuclear formation rate and subsequent embryonic development after in vitro fertilization, when compared with those oocyte complexes cultured alone in medium containing no follicular secretions. These results suggest that factors produced by follicular cells are involved in mediating the cytoplasmic maturation of oocytes.

It is therefore obvious that oocyte maturation is dependent on follicular maturation before the oocytes are dissociated from the follicles (ovulation). Follicular maturation in vivo is initiated by the gonadotropins, FSH (follicular stimulating hormone) and LH (luteinizing hormone) (Foxcroft et al., 1993). During the follicular phase, FSH induces early maturational changes and stimulates estradiol production by follicular cells, resulting in the release of the preovulatory surge of LH. LH (and probably also prolactin) induces luteinization of follicular cells and ovulation and stimulates progesterone production (see review by Gore-Langton and Armstrong, 1988). This series of maturational changes is also paralleled by a complex series of events determining follicle-oocyte interactions and hence oocyte maturation (Foxcroft et al., 1993). Therefore, it is logical to suggest that

follicular cells at different maturational stages may secrete a different quantity and/or quality of maturational factors that may have different effects on oocyte maturation.

Heterogeneity in the development of follicles has been demonstrated in both naturally cyclic (Foxcroft and Hunter, 1985; Grant et al., 1989; Hunter et al., 1989) and eCG primed immature gilts (Wiesak et al., 1990) and in weaned sows (Foxcroft et al., 1987). Follicles even in the selected preovulatory populations differ in size by up to 2 mm and showed marked variability in steroid content and gonadotropin binding. It has been suggested that follicular heterogeneity might influence the quality of oocytes ovulated (Foxcroft & Hunter, 1985; Hunter and Wiesak, 1990).

In order to better understand the effect of follicular heterogeneity on oocyte maturation, it is important to investigate the functional relationship between follicle maturity in the preovulatory period and oocyte maturation and subsequent embryonic development. This kind of research will also be important for us to understand the regulation of oocyte maturation by follicular cells in vivo and will hence help us to set up the best in vitro culture system for porcine oocyte maturation.

The objectives of the series of studies presented in Chapters IV to VII were therefore to study the effect of follicular secretions on oocyte maturation in pigs and especially the effects of follicular maturity and diversity on oocyte nuclear and cytoplasmic maturation. In the second series of experiments (Chapter IV), preliminary studies were carried out to set up the follicular shell co-culture system and to test the effect of follicular shell co-culture on oocyte nuclear and cytoplasmic maturation. The main experiment tested the effect of follicular shells from various sizes of follicles

collected after various times of eCG treatment of prepubertal gilts. These two experiments confirmed an important effect of follicular shells on oocyte maturation, and that cytoplasmic maturation of in vitro matured oocytes was significantly correlated to maturity of the follicle whose shell was used for co-culture. These results also confirmed the early suggestions that variability in production of follicular regulatory factors existed among preovulatory follicles.

However, it has been showed that follicular heterogeneity in eCG/hCG stimulated prepubertal gilts was much greater than in cyclic gilts (Wiesak et al., 1990). A further question asked here was whether this variability among follicles in the production of regulatory factors was also present in cyclic gilts. This led us to the fourth series of experiments (Chapter VI). In order to avoid effects of exogenous gonadotropins, synchronization of estrus with eCG/hCG could not be used. The logistics of experiments using follicles from naturally cyclic gilts for co-culture with oocytes was nevertheless difficult because the current IVM-IVF system was not as consistent as desired. However, it has been reported that medium used for culture of follicular shells (follicle conditioned medium) contained follicular cell secretions which supported oocyte cytoplasmic maturation (Mattioli et al., 1988 a and b). Use of conditioned media produced by follicles of different maturity would allow us to study oocyte maturation in a more controlled experiment. This idea led us to a third series of studies (Chapter V) to set up a conditioned medium culture system for oocyte maturation. Since FSH and LH have different stimulatory effects on follicular cells, conditioned media produced by follicular shells stimulated by different gonadotropin combinations were tested for their ability to

support oocyte maturation. The experiment demonstrated that FSH-stimulated follicular secretions supported the best cytoplasmic maturation. Thus, the fourth series of experiments (Chapter VI) were carried out, based on the results of the third series. The experiment again confirmed that maturational status of the follicles affected production of 'regulatory factors' for oocyte maturation and thus affected the quality of oocytes matured in the conditioned media produced.

Another question was what kind of factors were secreted by follicular cells to support oocyte maturation. It has been proposed that ovarian growth factors could be act as local regulators by paracrine or autocrine action (Racowsaky, 1991). The effect of growth factors on nuclear maturation has been examined by a number of investigators and epidermal growth factor (EGF) was particularly effective for stimulating nuclear maturation in all species tested so far. Recent studies also showed that EGF supplementation alone to the culture medium (Coskun et al., 1991) or in combination with low doses of gonadotropins (Harper and Brackett, 1993) improved developmental ability of oocytes matured in vitro. Therefore a fifth series of experiment (Chapter VII) were carried out to test the effect of EGF, alone or in combination with gonadotropins and follicular shell co-culture on nuclear and cytoplasmic maturation in pig oocytes.

These five series of experiments are presented as five sequential chapters and are extended forms of papers submitted for publication or already published. The data presented in Chapters III and IV are published as Ding et al., 1992 b and Ding and Foxcroft, 1992, respectively.

Finally, a general discussion is presented in Chapter VIII in order to integrate all

the results presented in this thesis and to gain further insight into the regulation of oocyte maturation.

Literatures cited in this chapter is listed in the following chapter **LITERATURE REVIEW**.

# CHAPTER II LITERATURE REVIEW

The mammalian oocyte is matured inside a particular ovarian structure, the follicle. The particular cellular relationship between the oocyte and somatic follicular cells is fundamental to oocyte maturation. In addition to providing nutrients to the oocyte, the follicular cells play a crucial role in regulating oocyte meiotic arrest and the resumption of meiosis. Various systems of communication have evolved between oocyte and follicular cells in regulating oocyte maturation.

Spontaneous resumption of meiosis of fully grown mammalian follicular oocytes in vitro was demonstrated by Pincus and Enzmann (1935) half a century ago when follicular oocytes were freed from their follicular environment. Since then, oocyte maturation in vitro has been studied in the human, in almost all domesticated species and in some wild animals. These in vitro studies of oocyte maturation enrich our understanding of the nature of oocyte maturation. It has been demonstrated in a number of species that follicular oocytes matured in vitro possesses full developmental potential to produce live young (mouse, Schroeder and Eppig, 1984; Downs et al., 1986; Eppig and Schroeder, 1989; rat, Fleming et al., 1985; Vanderhyden and Armstrong 1989; sheep, Staigmiller and Moor, 1984; Crozet et al., 1987; cattle, Critser et al., 1986; Lu et al., 1987, 1988, 1989; Xu et al., 1987; Goto et al., 1988; Sirard et al., 1988; Stubbings et al., 1988; Utsumi et al., 1988; Greve et al.,; 1989; Pavlok et al., 1989; Pollard et al., 1989; Gordon and Lu, 1990; pig, Mattioli et al., 1989; Jiang et al., 1991; Yoshida et al., 1993b) if oocytes are cultured in a suitable environment.

In order to render an oocyte with fertilization and subsequent developmental competence, two chains of maturational events must occur in the oocyte (Thibault et al., 1987). One is nuclear maturation, comprising the completion of the first meiosis. The second is cytoplasmic maturation, which includes the reprogramming of synthetic activity, and re-organization and utilization of products sequestered during oocyte growth (Moor et al., 1990). Cytoplasmic differentiation events are not dependent upon nuclear regulation but instead control both the progression of the meiotic cycle and the other intracellular events that confer developmental competence on the fully grown oocytes (Moor et al., 1990; Parrish and First, 1993).

The purpose of this chapter is to review relevant literature about oocyte maturation, especially in the pig, and give a background picture for the studies presented in the following chapters.

## 1. Cellular relationship between oocyte and somatic follicular cells

The fully grown immature oocyte arrested at the diplotene (dictyate) stage of the first meiotic cycle is covered by a transparent extracellular matrix called the zona pellucida. Surrounding the zona pellucida are multi-layers of follicular cells called the cumulus cells. The cumulus, with the oocyte sitting in its centre, projects into the follicular cavity which is filled with follicular fluid. One side of the cumulus is associated with the mural granulosa layer, which is the inner layer of the follicle wall. Outside the mural granulosa are two layers of theca tissue, the theca interna and theca externa. This follicular structure therefore establishes a micro-environment for the oocyte and an

intimate relationship between oocyte and follicular cells (for detailed descriptions, see Racowsky 1991). There are, furthermore, no direct contacts between oocyte and body fluid. Therefore, all the messages from the other parts of the body mediating oocyte maturation must either pass through the follicle wall and the zona pellucida as a result of the active transport processes of the follicular cells or first act on the follicular cells and thereby exert their effects indirectly on the oocyte.

The intimate relationship between the oocyte and the surrounding cumulus cells (corona radiata) is accomplished by gap junctions (Baker, 1982; Cran et al., 1979; Szöllösi et al., 1988; Thibault et al., 1987). The corona radiata cells indent their cellular processes deep into the zona pellucida and some of these processes make direct contact with the oocyte plasma membrane. The oocyte also projects its microvilli into the zona pellucida. Between the follicular cells, there are also gap junctions to facilitate intercellular communication. This intimate relationship between the oocyte and the surrounding follicular somatic cells established a functional basis of oocyte-follicular cell interaction during oocyte maturation.

## 2. Oocyte nuclear maturation

Germ cells in female embryos enter the germinal ridge, complete a series of mitotic cycles, and then enter meiosis. When the dictyate (germinal vesicle, GV) stage is reached, meiosis is arrested from about the time of birth until oocytes have nearly completed their growth phase (Moor et al., 1990). Oocytes are then competent to resume meiosis under appropriate stimulation of pituitary hormones in vivo or under certain

culture conditions in vitro. The resumption of meiosis and then development to metaphase of the second meiotic division (MII), which enables oocytes to be fertilized by the sperm of the same species, is usually referred to as oocyte (nuclear) maturation. How meiotic arrest is maintained and what constitutes the trigger for the resumption of meiosis have yet to be fully understood. However, a number of factors are known to be regulators of oocyte maturation.

### 2.1. General features of oocyte nuclear maturation

Immature ovarian oocytes are characterized by having a large nucleus referred to as the germinal vesicle (GV) (Fig.II-1). In vivo, in the preovulatory follicle after the LH surge, the GV nuclear envelop of immature oocyte ruptures, a process called germinal vesicle breakdown (GVBD). GVBD is the most obvious morphological change of the oocyte nucleus and is also important as an indicator of the resumption of meiotic division. Before GVBD, the nuclear chromatin undergoes condensation. After the breakdown of the germinal envelope, the chromosomes disperse and then arrange themselves on the microtubules at the equator of the meiotic spindle at metaphase of the first meiotic division (MI). Then under the influence of the microtubules, the chromosome bivalents move to the opposite ends of the spindle, which rotates gradually through 90°, so that the axis becomes radially oriented. The set of the chromosomes at the more peripheral end, together with very little cytoplasm, is enclosed by a plasma membrane and extruded into the perivitelline space (between the oocyte plasma membrane and zona pellucida) as a polar body; the other set of chromosomes occupies almost all the cytoplasm and forms

the secondary oocyte. Shortly after the completion of the first meiotic division, the oocyte enters the second meiotic division. In a very short time, it reaches the metaphase of the second meiosis (MII). At this point, the meiosis arrests again and ovulation occurs. The in vivo ovulated MII oocyte is considered to be a mature oocyte, since cytoplasmic maturation in these oocytes are almost always running parallel with meiotic maturation and hence have obtained the ability to be fertilized and subsequently to undergo embryonic development. (More detail description, see Baker, 1982; and Thibault et al., 1987). A series of nuclear maturational events are depicted in Fig.II-1 (Ding et al., unpublished observations).

## 2.2. Regulation of oocyte nuclear maturation

### 2.2.1. Hormonal regulation (gonadotropins and steroids)

The pituitary gonadotropins are the main extra-ovarian regulators. It is generally believed that the LH surge triggers the preovulatory oocyte to resume meiosis in vivo (Tsafriri, 1978; Moor et al., 1981).

When isolated intact follicles are cultured in vitro, oocytes resume meiotic division if gonadotropins are added to the culture medium (rat: Tsafriri et al., 1972; Hillensjo et al., 1976; hamster: Gwatkin and Andersen, 1976; Mandelbaum et al., 1977; cow: Thibault et al., 1975; rabbit: Thibault and Gérard, 1973; monkey: Thibault et al., 1976; sheep: Miller and Jagiello, 1973; Moor and Trounson, 1977). When pig oocyte-cumulus-mural granulosa complexes (G-C-Oocytes) are co-cultured either with or without follicle shell (FS) in vitro, the addition of the gonadotropins (2.5 μg/ml LH, 2.5 μg/ml FSH and 20

ng/ml prolaction) is crucial to the resumption of meiotic division (Fig.II-2, Ding et al., unpublished observations). In contrast, nuclear maturation in cumulus-enclosed oocytes (C-oocytes) cultured in the presence or absence of two follicle shells were not affected by gonadotropin supplementation. These results suggest that gonadotropins can act on mural granulosa cells, which directly connected with cumulus cells by intercellular junction, to remove their inhibitory effect on oocytes. Naito et al. (1988) showed that, gonadotropins (FSH and hCG) did not affect nuclear maturation when porcine oocytes were cultured in modified Krebs-Ringer bicarbonate solution, whilst gonadotropin greatly enhanced nuclear maturation in oocytes cultured in porcine follicular fluid. These observations demonstrate the apparent effects of gonadotropins on oocyte nuclear maturation, especially when follicular cells or follicular fluid are present.

While the effect of LH on oocyte meiotic maturation is clearly established, the effect of FSH remains controversial. Some studies indicate that FSH has an inhibitory effect on meiotic maturation, mainly by increasing the production of oocyte maturation inhibitor (OMI) and/or cAMP by granulosa cells (Anderson et al., 1985; Eppig et al., 1983; Moor et al., 1981). Other studies, however, show that it increases the rate of oocyte meiotic maturation (Fukui et al., 1982; Johnston et al., 1989, Naito et al., 1988; Mattioli et al., 1991a). These contradictory results may result from the different culture conditions, since the use of different culture media (Fukui et al., 1982) or culture system (Nagai et al., 1993) may produce different results.

Recent observations by Hoshino (1988) show that prolactin also influences oocyte meiotic maturation. Prolactin enhances the transition of mouse oocyte from MI to MII,

but does not affect GVBD.

The gonadotropins affect oocyte maturation indirectly (Thibault et al., 1975; Hillensjo et al., 1976) by changing follicular cell metabolic pathways towards pyruvate and lactate formation, by increasing the production of progesterone (Nicosia and Mikhail, 1975), by enhancing the synthesis of glycoproteins by the Golgi apparatus (Moricard, 1968) and probably by breaking gap junctions (Thibault, 1977, Szöllösi et al., 1988).

Changes in follicular steroid hormones during final stage of oocyte maturation in vivo are the main features of changes in follicular environment surrounding oocytes resulted from gonadotropin, especially preovulatory surge LH, stimulation (Ainsworth et al., 1980; Eppig, 1985; Grant et al., 1989; Gore-Langton and Armstrong, 1988). It has been well known that pituitary extract and progesterone stimulate nuclear maturation in amphibian oocytes (Burgers and Li, 1960; Masui, 1967; Schultz, 1977) and has been suggested that the pituitary extract which contains gonadotropic hormones (GTHs) stimulates the follicle cells to produce progesterone, the latter acts on the oocytes to induce the resumption of meiosis (Masui, 1967; and Maller and Krebs, 1980). Therefore, steroid hormones may also play an important role in regulation of oocyte meiotic maturation.

LH induces both the maturation of oocytes and production of progesterone in intact follicles in vitro (Tsafriri, 1978). However, cyanoketone or aminoglutethimide (AG), two inhibitors of progesterone production, did not inhibit rat follicle-enclosed oocyte maturation induced by LH (Tsafriri, 1978), although inhibition of steroidogenesis blocked LH-induced meiosis at the MI stage. Furthermore, incubation of follicles in media

containing progesterone did not stimulate maturation (Tsafriri, 1978). These results suggest that LH does not promote oocyte maturation via the synthesis of progesterone (Eppig, 1985). In contrast in the sheep, when follicles are cultured in the presence of AG, the ratio of oocyte GVBD is reduced significantly (Osborn et al., 1986). Moreover, use of a 17α-hydroxylase inhibitor (SU10603) to inhibit the conversion from progesterone to testosterone dramatically decreased meiotic resumption of oocytes , whilst use of an aromatase inhibitor (1,4,6-androstatrien- 3,17-dione (AST)) to inhibit the conversion from testosterone to estradiol did not affect oocyte nuclear maturation. Based on these results, the authors concluded that, though total abolition of steroid secretion by AG has negative effects on oocyte nuclear maturation, the greatly distorted (increased) ratio of progesterone to testosterone and estrogen has a more severe negative effect on oocyte nuclear maturation (Osborn et al., 1986, Osborn and Moor, 1983). Since addition of exogenous estrogen failed to alleviate the effect of SU, it is further suggested that high level of progesterone generated by SU during the early part of maturation, rather than the depression of estrogen, is most damaging to the oocyte (Osborn et al., 1986). The abolition of steroid production by AG reduced the rate of pig oocyte maturation in vitro (Szöllösi and Gérard, 1983). However, our recent study (Nagai et al., 1993) showed that neither AG, nor AST altered nuclear maturation (MII) stimulated by gonadotropins. Clearly, the effects of follicular steroids on nuclear maturation observed were not in agreement and species differences or variation of culture conditions may contribute to the controversial results.

Conflicting results were also seen on the effect of addition of progesterone to

culture medium: either there is a lower incidence of maturation in denuded mouse oocytes (Eppig and Koide, 1978), in corona enclosed rabbit oocytes (Smith et al., 1978) and in cumulus enclosed oocytes (Nagai et al., 1993), or an enhancement of nuclear maturation of cumulus enclosed rabbit oocytes (Bae and Foote, 1975). The negative effects on oocyte maturation of estradiol (pig: McGaughey, 1977; mouse: Eppig et al., 1983) and androgens (pig: Rice and McGaughey, 1981; Racowsky, 1983; mouse: Schultz et al., 1983b) have been described in in vitro studies. Anderson et al. (1985), however, demonstrated that androgen enhanced pig oocyte meiotic maturation in vitro by inhibition of OMI production by granulosa cells.

Analysis of in vivo data (De Sutter et al., 1991) showed that, in human, oocyte maturity was associated with an increase of follicular fluid progestin content and a significant decrease of androstenedione levels. Vanluchene et al. (1991) also reported that oocyte maturity was correlated to higher level of follicular fluid progesterone, 17-hydroxyprogesterone, 16α-hydroxyprogesterone and 20α-dihydroprogesterone. Higher fertility was associated with higher levels of 20α-dihydroprogesterone, and progesterone, and lower levels of androstendione (Vanluchene et al. 1991). Similarly, Itskovitz et al. (1991) found that follicles containing immature human oocytes have a lower follicular fluid progesterone and estradiol concentration than those containing mature oocytes. These in vivo studies suggest that the follicular steroid environment may affect oocyte nuclear maturation and fertilization. However, using leuprolide acetate to stimulate follicular development in human IVF procedure, follicular fluid progesterone and estradiol-17ß were lower than those stimulated with gonadotropins, but oocyte nuclear maturation was not

affected (Brzyski et al., 1990), suggesting no functional relationship between follicular fluid steroids and nuclear maturation of oocytes.

### 2.2.2. Role of cAMP

cAMP is considered as one of the possible inhibitors of oocyte meiotic resumption in mammals. The supporting facts are that culture of murine oocytes in the presence of cAMP derivatives such as dibutyryl cAMP (dbcAMP) and 8-bromo-cAMP, or activators of adenylate cyclase such as forskolin, or inhibitors of phosphodiesterase (PDE) such as isobutyl methyl xanthine (IBMX), inhibit the resumption of meiotic division (Cho et al., 1974; Wassarman et al., 1976; Dekel and Beers, 1978; Magnusson and Hillensjo, 1977; Schultz et al., 1983b; Bornslaeger and Schulz, 1985a; Dekel et al.,1984; Olsiewski and Beers, 1983; Racowsky, 1985a, b; Sato and Koide, 1984; Urner et al., 1983). Porcine oocyte nuclear maturation in vitro is also inhibited by dbcAMP (Rice and McGaughey, 1981; Ding et al., unpublished observation), though the effective dose used is higher than that used in mouse oocytes (Ding et al., unpublished observation). Most of the experiments on the effect of forskolin on oocyte meiotic resumption have shown that forskolin increases the cAMP level not only in cumulus free oocytes (mouse: Urner et al., 1983; Sato and Koide, 1984; hamster and pig: Racowsky, 1985a and 1985b), but also in zona free oocytes (Bornslaeger and Schultz, 1985a). Similar results are also found in sheep ( Moor and Heslop, 1981) and rat (Olsiewski and Beers, 1983) oocytes.

It is suggested that cAMP must be at least partly synthesized in cumulus cells, and then enters the oocyte via the cumulus-oocyte gap junctions to inhibit GVBD directly,

since the exogenous cAMP derivatives inhibit oocyte meiotic maturation. However, experiments to demonstrate transfer of labelled cAMP from cumulus cells to the oocyte have been unsuccessful, although labelled choline or labelled uridine migrate into the oocyte (mouse: Schultz et al., 1983a; b; Eppig and Downs, 1984; sheep: Moor et al., 1980; Crosby et al., 1985). Observations of the natural transfer of cAMP have been reported when cAMP was dramatically increased in cumulus cells by both IBMX and FSH or forskolin (mouse, Bornslaeger and Schultz, 1985b) but it has been suggested that this apparent transfer might have been due to cAMP present in cumulus processes which remained embedded in the zona pellucida after cumulus removed (Eppig and Downs, 1984; Thibault et al. 1987). The indirect evidence supporting this suggestion is from the recent study that the cumulus cell processes embedded in the zona pellucida of denuded oocyte are able to hold a substantial amount of cumulus originated actin (Canipari et al., 1988), which is also considered to be transferred to oocyte from cumulus cells (Moor and Osborn, 1983; Osborn and Moor, 1982). Furthermore, evidence to suggest that cAMP only acts on the oocyte via regulating cumulus cell function is not convincing, because oocytes freed of cumulus cells also respond to exogenous cAMP (Dekel and Beers, 1978; Beers and Dekel, 1981; Freter and Schultz, 1984). Therefore, the mechanisms by which non-oocyte originated cAMP exerts its inhibitory effect on the oocytes are still unclear.

Given the inhibitory effect of cAMP, it is tempting to speculate that the oocyte cAMP concentrations should decrease during final meiotic maturation. In fact, a significant reduction in cAMP level in the murine oocytes does occur just prior to GVBD, both in vivo and in vitro (Dekel and Beers, 1980; Schultz et al., 1983a; Vivarelli et al.,

1983). However, sheep oocytes show an increase of cAMP before GVBD (Moor and Heslop, 1981). Furthermore, those agents inducing the maturation of follicle enclosed oocytes in vitro, also stimulate production of cAMP (Tsafriri et al., 1972; Lindner et al., 1974). Even injection of cAMP derivative dbcAMP into the follicular antrum (Tsafriri et al., 1972) or short term exposure of follicles to 8-bromo-cAMP (Hillensjo et al., 1978), dbcAMP, or IBMX (Dekel and Beers, 1980) triggered the resumption of meiosis. Based on these and some other aforementioned results, Tsafriri (1988) has suggested that the enhanced production of cAMP in the somatic (follicular) compartment is involved in the mediation of the meiosis-inducing action of LH, while elevated cAMP in the oocyte (long term effect) inhibits the resumption of meiosis. The recent study by Yoshimura et al. (1992) further supports this suggestion. In their study, they found that in in vitro perfused rabbit ovaries, exposure to forskolin or hCG accelerated the meiotic maturation of follicle enclosed oocytes. The intraoocyte cAMP increased significantly within 30 min and reached its maximum 2 h following exposure to forskolin or hCG. Thereafter, cAMP in oocytes decreased abruptly and return to normal level by 6 h and followed by resumption of meiosis.

The inhibitory effect of cAMP inside the oocyte on meiotic maturation of the oocyte has been suggested to be via cAMP dependent protein kinase (PK) system because mouse oocytes injected with the catalytic subunit of PK fail to undergo spontaneous meiotic resumption during culture in vitro in the absence of dbcAMP and IBMX (see Wassarman, 1988). This protein kinase will probably further affect maturation promoting factor (MPF) activity, which has been proved to induce the transition from G2 to

metaphase of the meiotic, as well as mitotic cell cycle (see later section).

### 2.2.3 Role of oocyte maturation inhibitor(s) (OMI)

The follicular cells synthesize some low molecular weight substances, called oocyte maturation inhibitors (OMI), which are considered to have an inhibitory effect on oocyte meiotic maturation. OMI has been well studied. Use of pig follicular fluid in culture of pig, rat and hamster oocytes inhibits the spontaneous meiotic maturation (Tsafriri and Channing, 1975; Gwatkin and Andersen, 1976). These observations led to the search for OMI from follicular fluid. Channing et al (1982) have concluded that there are probably two and possibly three OMI polypeptides present in pig follicular fluid which are not species-specific (more detail see reviews by Tsafriri and Pomerantz, 1986; Tsafriri, 1988). OMI may exert its inhibitory effect via cumulus cells, since it prevents maturation of cumulus-enclosed oocytes, but not oocytes denuded of cumulus (Hillensjo et al., 1979; Tsafriri and Bar-Ami, 1982). It is suggested that the OMI might be small enough ($M_r$: 2,000d) to pass through the gap junction between the oocyte and cumulus cells (Tsafriri, 1978; Tsafriri, 1985; Tsafriri et al., 1983). However, in his latest review, Tsafriri (1988) also suggests that OMI purified from follicular fluid may not necessarily be the native molecule regulating meiosis by direct transfer from follicular cells to the ovum, through the gap junctions between cumulus cells and the oocyte.

Furthermore, recent evidence suggests that hypoxanthine and/or adenosine (inhibit cAMP phosphodiesterase) are, in fact, the other two low-molecular-weight components of follicular fluid which inhibit oocyte meiotic maturation in vitro (Downs et al.,1985;

Eppig et al., 1985). However, when rabbit preovulatory cumulus-free oocytes were cultured in the presence of hypoxanthine and adenosine, nuclear maturation is not prevented, although meiotic resumption in rabbit oocytes from small follicles was completely blocked (Thibault et al., 1987).

### 2.2.4. Role of cumulus oocyte uncoupling

As discussed in preceding sections, cAMP and OMI seem to exert their actions on the oocyte meiotic maturation via follicular cells. Therefore, the spontaneous resumption of meiosis by removal of the follicular cells from oocytes (Pincus and Enzmann, 1935) may be due to the oocytes escaping from an inhibitory follicular environment. Culture of oocytes with follicular cells results in a decrease of meiotic resumption of oocytes, which is reversed by the addition of gonadotropins to the culture media (Fig.II-2). Thus the positive hormonal effects on oocyte maturation may also be due to the removal of inhibitory factors from the follicular compartment.

Dramatic changes in the cumulus oophorus take place after the gonadotropin surge in vivo or during in vitro culture when gonadotropin is present. The cumulus expands and the cellular processes of cumulus cells lose their intimate contacts with the oocyte plasma membrane (Dekel and Kraicer, 1978; Dekel et al., 1978; Gilula et al., 1978; Larsen et al., 1986; Moor et al., 1980; Szöllösi et al., 1988). It is suggested that uncoupling of cumulus-oocyte gap junctions is essential to meiotic resumption; this is consistent with the observation of spontaneous resumption in artificially denuded oocytes. However, cumulus-oocyte uncoupling does not occur before GVBD (Eppig, 1982; Moor et al., 1980; Moor

and Gandolfi, 1987; Mattioli et al., 1988b) and these results argue against the above concept. A recent study in sheep by Szöllösi and his colleagues (1988), however, supports this concept. They demonstrated that at the time of meiotic resumption the heterocellular gap junctions between oocyte and cumulus cells underwent substantial changes, fully or partly losing contacts with the oocyte plasma membrane, although a cellular relationship was still present. Thus full or partial loss of contact between cumulus cell processes and oocyte plasma membrane may reduce the quantity of maturation inhibitors entering the cocyte.

The pituitary gonadotropins, FSH and LH, are responsible for the induction of cumulus expansion and uncoupling of the cumulus from the oocyte (Thibault, 1972; Moor et al., 1980; Hillensjo and Chinning, 1980). The changes in cumulus cell morphology induced by gonadotropins appear to be induced by a cAMP-mediated, energy-dependent process in which microfilaments play an important role (Lawrence et al., 1979).

### 2.2.5. Role of growth factors

The involvement of growth factors in ovarian physiology has been an active area of research in recent years. Several laboratories have demonstrated growth factor production by cultured granulosa, theca and interstitial cells (pig, Hammond et al., 1985; Hsu and Hammond, 1987; Mondschein et al., 1988; rat, Koos, 1986; Skinner et al., 1987; Bendell and Dorrington, 1988; cattle, Neufeld et al., 1987; Dreke et al., 1988; hamster, Roy and Greenwald, 1991), and follicular fluid contains epidermal growth factor (EGF) (Hsu et al., 1987), Insulin-like growth factors (IGF-I and II) Mondschein et al., 1988), and

transforming growth factor-alpha (TGF-α) (Lobb et al., 1989). There are also binding sites for EGF within the ovary (Vlodavsky et al., 1978, Jones et al., 1982; St-Arnaud et al., 1983; Feng et al., 1987) that flux in relation to the physiological state of the follicle (St-Arnaud et al., 1983; Feng et al., 1987). These observations suggest that the growth factors are involved in the regulation of ovarian functions. Therefore, it is reasonable to assume that the growth factors may have regulatory effects on oocyte meiotic maturation.

Recent studies from several groups indicate that some growth factors do affect oocyte meiotic resumption. EGF (Dekel and Sherizly, 1985; Ueno et al., 1988; Downs et al., 1988; Downs, 1989; Reed et al., 1991; Coskun and Lin, 1992; Sommer et al., 1992; Illera et al., 1992; Das et al., 1991), insulin and fibroblast growth factor (FGF) (Downs, 1989), TGF-α (Brucker et al., 1991) have a stimulatory effect on meiotic resumption in murine, porcine, bovine and human oocytes in vitro. Though Feng et al. (1988) have reported a stimulatory effect of TGF-ß on rat oocyte maturation in vitro, TGF-ß seems to have little effect or even negative effects on rat oocyte maturation (Downs, 1989). Placenta derived GF (PDGF), bombesin, nerve GF (NGF), IGF-I, IGF-II, and orthovan have little or no effect on meiotic maturation of mouse oocytes (Downs, 1989).

How growth factors affect oocyte meiotic maturation remains to be determined. However, it is reasonable to suggest that they act indirectly on oocytes by regulating follicular cell functions. The effects of growth factors on ovarian function were reviewed by Tsafriri (1988).

### 2.2.6. Follicular cell support of oocyte meiotic maturation

While follicular cells are considered to be the sources of oocyte maturation inhibitors, their positive effect on oocyte meiotic maturation should not be neglected. Somatic cells surrounding an oocyte facilitate production of nutrients and their transport into an oocyte. In addition, they generate signals which control and regulate oocyte metabolism, as well as nuclear and cytoplasmic maturation (Osborn and Moor, 1982, Moor and Seamark, 1986). Oocytes cultured with cumulus and/or the other follicular cells show a significantly higher MII rate than in the control cultures (cattle, Fukui and Sakuma, 1980; Faundez et al., 1988; rat, Vanderhyden and Amstrong, 1989; pig, Byun et al., 1993). Using a static culture system, we found that the number of cumulus cells ($>15x10^3$ per oocyte) directly connected with oocytes were essential to support high rate of nuclear maturation (Nagai et al., 1993). Moreover when a non-static culture system was used, pig cumulus-enclosed oocytes cultured both in gonadotropin-supplemented and in gonadotropin-free media, had a higher MII rate when two opened follicles were added to the culture medium than when cumulus-enclosed oocytes were cultured alone; however, the proportions of oocytes that underwent GVBD were not different (Fig.II-2, Ding et al., unpublished observations). These results suggest that additional follicular cells apart from cumulus cells may not affect GVBD but support nuclear progression from GVBD to MII. Furthermore, Tesarik et al.(1991) demonstrated that treatment of female mice with anti-cumulus antibody impaired eCG/hCG induced oocyte meiotic competence. Previous absorption of anti-cumulus antibody with isolated cumulus cells removed the inhibitory effect of this antiserum on oocyte meiotic maturation. But the disturbance of the antisera

was not on GVBD, but on the progression from MI to MII. These results are consistent with our findings. It is therefore suggested that cumulus and/or granulosa cells sustain the development of the oocyte from GVBD to MII. The nature of the regulatory factors produced by these follicular cells are not known, but growth factors synthesized by follicular cells during oocyte maturation may be involved.

### 2.3. Role of protein synthesis and maturation promoting factor (MPF)

Mammalian oocytes have an absolute requirement for protein synthesis in the period immediately before GVBD for the normal progression of the meiotic cycle. Inhibition of de novo protein synthesis during maturation blocks GVBD in cow (Hunter and Moor, 1987; Kastrop et al., 1991), sheep (Moor and Crosby, 1986), pig (Fulka et al., 1986b; Kubelka et al., 1988) and rat (Ekholm and Magnusson, 1979) oocytes. It has been reported that inhibition of protein synthesis in mouse oocytes does not inhibit GVBD (Stern et al., 1972; Wassarman et al., 1976; Golbus and Stein, 1976; Schultz and Wassarman, 1977a; Fulka et al., 1986b). However, a recent study by Downs (1990) showed that cycloheximide inhibited GVBD in mouse oocytes when maturation were initially prevented by IBMX, or delayed by FSH, suggesting that a protein with a rapid turnover rate is involved in the spontaneous maturation of mouse oocytes and that de novo protein synthesis is a requirement for hormonal induction of GVBD in vitro. Induction of GVBD in amphibian oocytes is also dependent on de novo protein synthesis. Thus it is certain that protein factor(s) which is responsible for the resumption of meiosis is synthesized during the early stage of maturation.

It is known now that the protein factor(s) which induces oocyte maturation is maturation promoting factor (MPF). MPF promotes the G2 to M phase transition in the cell cycle. MPF is first described as an activity in cytoplasm of unfertilized metaphase II frog oocytes (Masui and Markert, 1971; and Smith and Ecker, 1971). The transfer of matured frog oocyte cytoplasm into an immature oocyte , which is naturally arrested in G2 stage, induces the recipient to undergo meiosis. Cell fusion studies have been used to examine the MPF activities in mammalian oocytes (Motlik and Fulka, 1986; Fulka et al., 1986a; 1988; Kubelka et al., 1988; Mattioli et al., 1991b; Fulka et al., 1992). Crucial MPF-like activity reached threshold level at 8-16 h after the induction of maturation and peaked at MI and MII stage, but was low at anaphase I-telophase I stages (Mattioli et al., 1991b) in pig oocytes. These results are similar to those found in mouse oocytes (Fulka et al., 1992). The appearance of MPF activity in oocytes requires protein synthesis (Mattioli et al., 1991b).

Many studies have showed that MPF activity with identical properties appears during M phase not only in a variety of oocytes, but also in somatic cells and in fission yeast (for reference, see Gautier et al., 1988), suggesting that the MPF be a fundamental and universal regulator which induces the cell to enter metaphase. In this respect, the maturation-promoting factor should be precisely called a **METAPHASE-PROMOTING FACTOR.**

The nature of MPF was just recently unveiled. The abundance of MPF does not alter during the cell cycle although changes in its state of activation occur especially as the cell enters mitosis. The core polypeptide in MPF is a 34 kD phosphoprotein, probably a

histone kinase H1, with close homology to the yeast cdc2 mitotic regulator (see Lee and Nurse, 1987; Dunphy and Newport, 1988; Lohka, 1989). Conversion of the latent 34 kD phosphoprotein into active MPF appears to involve both dephosphorylation and the formation of complexes between it and a family of cell-cycle oscillatory proteins called cyclins ( 45kD protein in Xenopus) (Dunphy and Newport, 1988; Draetta et al., 1989). Cyclin proteolysis at the metaphase/anaphase transition probably inactivates MPF and allows the escape from M phase.

A recent review by Albertini (1992) provides an integrated view on regulation of meiotic maturation in the mammalian oocyte. Meiotic maturation involves two key aspects. The first is MPF and the role MPF plays in the control of the G2/M cell cycle transitions as aforementioned. The second involves the modulation of centrosomes and microtubule (MT) assembly in meiotic and mitotic cells. Mammalian oocytes exhibit a series of cell cycle transition that coordinate the events of meiosis with the onset of embryogenesis at fertilization. The execution of these cell cycle transitions, at G2/M of meiosis-I, and metaphase/anaphase of meiosis-I and II, invoive both biosynthetic and post-translational modifications that directly modulate centrosome and microtubule behaviour. Specifically, somatic cells alter the signal transduction pathways in the oocyte and influence the expression of MPF and cytostatic factor (CSF) activity through a microtubule-dependent mechanism. The regulation of the oocyte cell cycle machinery by hormone-mediated somatic cell signals, involving both positive and negative stimuli, ensures that meiotic cell cycle progression is synchronized with the earliest pivotal events of mammalian reproduction ( for details, see Albertini 1992).

### 3. Cytoplasmic maturation.

In preparation of the oocyte maturation, not only must meiotic maturation occur, but also the cytoplasm of the oocyte must undergo some critical changes in order to achieve competency to support sperm chromatin decondensation and subsequent male pronuclear (MPN) formation (Thibault et al., 1987). In in vivo matured oocytes, cytoplasmic maturation usually runs parallel with meiotic maturation, and ovulated MII oocytes thus usually possess the ability to be fertilized and to undergo subsequent embryonic development. However, in vitro meiotically matured oocytes frequently dissociate with cytoplasmic maturation, resulting in a low male pronuclear formation rate and low frequency of embryonic development (Motlik and Fulka, 1974 a, b; Thibault 1977; Moor and Trounson, 1977; Trounson et al., 1977). The ability of the ooplasm to promote the formation of a normal male pronucleus increases with time after the induction of oocyte maturation in vivo (Iwamatsu and Chang, 1972; Niwa and Chang, 1975). Sperm microinjection experiments (Thadani 1980; Balakier and Tarkowski, 1980) also showed that sperm decondensing activity is maximal in mature MII oocytes, but minimal or absent in immature GV oocytes. Furthermore this activity declines after fertilization and is either substantially reduced or absent in pronuclear stage oocytes (Usui and Yanagimachi, 1976; Komar, 1982). It has been proposed that mature ooplasm contains components that promote the formation of the male pronucleus and has been referred to as male pronuclear growth factor (MPGF) (Thibault, 1977). The nature of MPGF is not known and whether it depends on protein synthesis is not clear.

#### 3.1 Cytoplasmic differentiation

The re-organization and utilization of the products sequestered during oocyte growth constitute a major component of the maturation programme (Moor et al., 1990). The changes involve all the major structural and functional components in the oocyte and are facilitated or regulated by the follicular somatic cells.

The relocation of the organelles within the oocyte during maturation involves both mitochondria and cortical granules. Mitochondria, which are uniformly scattered throughout the cytoplasm in GV oocytes, become aggregated around the nucleus at the time of its breakdown during maturation. This mitochondrial clustering and subsequent dispersal at anaphase/telophase is both microtubule-dependent and necessary for the progression of meiotic maturation (for details, see Thibault et al., 1987).

While the mitochondria migrate to occupy a perinuclear position during maturation, the cortical granules, which are involved in the cortical granule reaction during fertilization and are probably responsible for prevention of polyspermic fertilization, migrate outward to form an irregular monolayer a few nanometers beneath the cell membrane by the time of maturation is complete (Cran and Cheng, 1986). The cortical granules are distributed throughout the entire inner surface of sphere of the oocyte. A cytoplasmic zone devoid of organelles often forms beneath the monolayer whilst a fine actin filament layer is interposed between the cortical granule layer and oolemma. It is obvious that the migration of cortical granules is an essential step in preventing the oocyte from being penetrated by multiple spermatozoa during fertilization.

The cytological remodelling is accompanied by a major reprogramming of protein synthesis, involving the utilization of approximately 30% of the polyadenylated RNA

stored during oocyte growth (Bachvarova et al., 1985). The pattern of de novo protein synthesis undergoes dramatic changes during oocyte maturation, especially during GVBD, in mouse (Schultz and Wassarman, 1977 a, b; Wassarman et al., 1978; Schultz et al., 1978), rabbit (Van Blerkom and McGaughey, 1978), sheep (Warnes et al., 1977; Moor and Gandolfi, 1987), goat (Le Gal et al., 1992), pig (McGaughey and Van Blerkom, 1977) and human (Schultz et al., 1988) oocytes. As mentioned before, resumption of meiosis depends on new protein synthesis. Thus the changes of protein synthetic pattern during oocyte maturation must have significant physiological importance for nuclear maturation and subsequent fertilization and embryonic development. Our studies (Ding et al., 1992) on protein synthesis during fertilization in porcine oocytes showed that whereas de novo protein synthesis during fertilization was not important for male pronuclear formation, proteins synthesized during maturation underwent post-translational modification in the absence of new protein synthesis. These results suggest that proteins synthesized during maturation may be utilized during fertilization and early embryonic development.

**3.2 Regulation of cytoplasmic maturation.**

Until now there is no suitable biochemical criterion for cytoplasmic maturation. Thus bioassay for fertilization and sperm decondensation, male pronuclear formation and embryonic development are used as criteria for oocyte cytoplasmic maturation. In pig, the most common criterion for physiologically measuring cytoplasmic maturation of oocytes is male pronuclear formation, since it is closely related to oocyte cytoplasmic maturation (Mattioli et al., 1988a). Incomplete or inadequate conditions in vitro result in partial and/or delayed sperm decondensation (Moor et al., 1990). Also male pronuclear

development could be a measurement of MPGF. In the normal sequence of pig fertilization, detachment of the sperm tail and early decondensation occur at about 4 h after insemination in vitro and is synchronized with the beginning of the second meiotic division (anaphase II) in the egg. At 6h after insemination, incipient nuclear membranes appear around the decondensed sperm nucleus and around the female chromosomes; by 7h well formed male and female pronuclei could be observed and by 10h most of oocytes contain both male and female pronuclei (for details, see Ding et al., 1992 a).

### 3.2.1 Effect of follicular cells on cytoplasmic maturation

Follicular cells not only control nuclear maturation, but also facilitate cytoplasmic maturation in mammalian oocytes (see Thibault et al., 1987; Buccione et al., 1990; Racowsky et al., 1991; Moor et al., 1990). In some species including the mouse and rat, cytoplasmic maturation could be achieved when cumulus-enclosed oocytes were cultured alone, since these in vitro matured oocytes are fertilizable and can proceed to full term upon transfer to recipients (mouse, Schroeder and Eppig, 1984; Downs et al., 1986; Eppig and Schroeder, 1989; rat, Fleming et al., 1985; Vanderhyden and Armstrong 1989). However, cumulus-enclosed oocytes cultured alone in vitro failed to form male pronuclei or had low developmental ability after sperm penetration in rabbits (Thibault and Gérard, 1970; 1973; Motlik and Fulka, 1974b), pigs (Motlik and Fulka, 1974a; Mattioli et al., 1988a; Ding et al., unpublished observations, see Fig.II-3), cattle (Thibault et al., 1975, 1976; Trounson et al., 1977; Liebfried-Rutledge et al., 1986) and sheep (Moor and Trounson, 1977), suggesting that the cumulus alone can not support full oocyte

cytoplasmic maturation in vitro in these species. Furthermore, culturing cumulus-enclosed oocytes in these species together with granulosa cells or follicle shells greatly improved cytoplasmic maturation and developmental potential (rabbit, Motlic and Fulka, 1982; ovine, Staigmiller and Moor, 1984; Crozet et al., 1987; Fukui et al., 1988; bovine, Critser et al., 1986; Lu et al., 1987, 1989; Lutterbach et al., 1987; Fukui and Ono, 1988; porcine, Mattioli et al., 1988 a, b; 1989; Zheng and Sirard, 1992; Ding et al., unpublished observations, see Fig.II-3). Therefore, additional follicular cells (mural granulosa cells or follicle shells) seems to play an important role in supporting cytoplasmic maturation in oocytes of these species. The development of successful co-culture systems resulted in the birth of live young after fertilization of oocytes matured in vitro (sheep, Staigmiller and Moor, 1984; Crozet et al., 1987; cattle, Critser et al., 1986; Lu et al., 1987, 1989; pig, Mattioli et al., 1989). However, recent reports showed that cumulus-enclosed oocytes matured in vitro without additional follicular cells also possess full developmental potential to term (bovine, Fukuda et al., 1990; porcine, Jiang et al., 1991; Yoshida et al., 1993b). These results suggest that additional follicular cells are not absolutely required for cumulus-enclosed oocytes to achieve full cytoplasmic maturation in vitro. The difference probably results from the use of different culture systems in different laboratories. Most of experiments that led to the conclusion that additional follicular cells are essential for supporting cytoplasmic maturation of cumulus-enclosed oocytes used a non-static culture system (Staigmiller and Moor, 1984; Crozet et al., 1987; Mattioli et al., 1988 a, b; 1989), whilst those leading to the suggestion of no additional follicular cell requirement used static culture system (Fukuda et al., 1990; Jiang et al., 1991; Yoshida

et al., 1993b). A comparison experiment carried out recently by Dr. Nagai and myself in Dr. Moor's laboratory clearly demonstrated the differences between these two culture systems (Nagai et al., 1993). Porcine cumulus-enclosed oocytes with $15x10^3$ somatic cells/egg (compact and biggest cumulus) matured in static culture had about 80% male pronuclear formation rate, whereas cumulus-enclosed oocytes connected with a small piece of mural granulosa cells ($150x10^3$ follicular cells/egg) had low male pronuclear formation rate (33%). These two groups both had high MII rate (>90%). When a non-static culture system was used, oocytes with $15x10^3$ follicular cells/egg cultured alone had a very low nuclear maturation rate (26%); however, co-culture of this group of oocytes with two everted follicle shells, not only increased nuclear maturation to 94%, but also a similar percentage of oocytes also contained male pronuclei as when oocytes were cultured alone in static culture. When oocytes with $150x10^3$ follicular cells/egg were cultured in a non-static system, addition of two everted follicle shells to the culture did not affect MII rates (over 90% in both groups), however, addition of two everted follicle shells increased male pronuclear formation rate from 36% to 81%. Therefore, results reported from different laboratories using various culture systems may not be comparable (Nagai et al., 1993).

Regardless of whether induction of cytoplasmic maturation is dependent on the presence of membrana granulosa cells, cytoplasmic maturation is enhanced in all species by association with adherent cumulus cells (for review, see Vanderhyden and Armstrong, 1989). Thus if the cumulus cells are removed before maturation culture, the percentage of such cumulus-free oocytes that support normal male pronuclear formation is

substantially reduced (mouse, Schroeder and Eppig, 1984; rat, Vanderhyden and Armstrong, 1989; cow, Fukui and Sakuma, 1980; Sirard et al., 1988; sheep, Staigmiller and Moor, 1984). The percentage of such oocytes that undergoes female pronuclear formation after parthenogenic activation by 10% ethanol is also greatly reduced (Byun et al., 1993). The number of cumulus cells also affects cytoplasmic maturation. Using a static culture, we (Nagai et al., 1993) tested male pronuclear formation after in vitro fertilization of in vitro matured oocytes with various number of cumulus cells. Removal of cumulus cells from $15x10^3$ cumulus cells to $2\text{-}4x10^3$ cumulus cells/egg reduced MII rate from 93 to 34% and male pronuclear formation rate from 83 to 30% after these oocytes were matured and fertilized in vitro. Even using a static culture, when additional follicular cells are not required for oocyte cytoplasmic maturation, the number of cumulus cells are crucial for oocytes to achieve cytoplasmic maturation.

Furthermore, recently Niwa (1993) showed that cumulus-enclosed oocytes matured in vitro and fertilized in vitro without removal of expanded cumulus had a high male pronuclear formation rate of 73%. In contrast, cumulus enclosed oocytes matured in vitro and fertilized with their expanded cumulus removed had a male pronuclear formation rate of 32%, which was significantly lower than that without removal of cumulus cell partner, but similar to that in denuded oocytes matured in vitro (39%). Kikuchi et al. (1993) also reported that the expanded cumulus cells surrounding oocytes play an important role in increasing male pronuclear formation during fertilization in vitro and that denuded oocytes with supplementation of cumulus cells during fertilization did not improve male pronuclear formation. The enhancement of male pronuclear formation by not removing

expanded cumulus cells surrounding oocytes is also observed in bovine oocytes (Ball et al., 1983). The underlying mechanism is not clear. Since the somatic cell-oocyte uncoupling is completed at MII stage (Motlik et al., 1986; Mattioli et al., 1988b), this effect is unlikely to be mediated by cumulus cells directly on the oocyte through the intercellular coupling between them. Alternatively, it has been suggested that in vitro matured oocytes may be damaged by the treatment used for removing their cumulus cells (treatment by hyaluronidase and/or mechanically pipetting), resulting in reduced male pronuclear formation rate (Ball et al., 1983; Kikuchi et al., 1993). Other possibilities, such as a sperm selection effect by penetrating expanded cumulus and/or effect of cumulus on sperm capacitation, should also be considered.

### 3.2.2 Effect of follicular secretions on cytoplasmic maturation

Not only follicular cells have positive effect on oocyte cytoplasmic maturation, but follicular secretions (follicular fluid and follicular cell conditioned medium) also have a supportive effect (see review by Niwa, 1993). Although pig cumulus-enclosed oocytes cultured in medium containing porcine follicular fluid without gonadotropin supplementation severely inhibited nuclear maturation, this inhibitory effect was removed by gonadotropin supplementation (Naito et al., 1988). Furthermore, male pronuclear formation rate in oocytes matured in the medium supplemented with follicular fluid in the presence of gonadotropins was greatly improved (Naito et al., 1988; Naito et al., 1992). These findings are supported by recent studies by Yoshida and colleagues (1990, 1992a, 1993b) and also by Funahashi and Day (1993a). The effective factors are also present in

bovine follicular fluid and the efficacy of the follicular fluid in supporting cytoplasmic maturation is heat(56°C)-treatment stable (Naito et al., 1990; Funahashi and Day, 1993a). Follicular cell conditioned medium is also capable of supporting cytoplasmic maturation of cumulus-enclosed, but not cumulus-free oocytes (Mattioli et al., 1988 a, b). Collectively, it is clear that follicular cell secretions contain factors supporting cytoplasmic maturation and this effect is mediated via cumulus cells.

### 3.2.3 Effect of hormones on cytoplasmic maturation.

Since nuclear maturation is clearly affected by gonadotropins, porcine oocyte maturation medium usually containing gonadotropins (FSH, LH, hCG, eCG, for review, see Niwa, 1993). When follicular cell co-culture (Ding et al., unpublished observations) or follicular fluid (Naito et al., 1988) were used, both nuclear and cytoplasmic maturation can be properly achieved only in the presence of gonadotropins. When cumulus-enclosed oocytes were co-cultured with follicle shells in the absence of gonadotropins (2.5 μg/ml FSH, 2.5μg/ml LH and 20 ng/ml prolactin) male pronuclear formation rate was below 10%, while culture in the presence of gonadotropins increased male pronuclear formation rate to more than 60% (Fig.II-3, Ding et al., unpublished observations). Mattioli et al. (1991a) further examined LH and FSH effects on cytoplasmic maturation separately or in combination and showed that LH but not FSH is effective in stimulating cytoplasmic maturation of pig oocytes co-cultured with everted follicle shells. The combination of LH and FSH was no better than LH alone. Funahashi and Day (1993b) reported that culturing pig cumulus-enclosed oocytes in medium supplemented with eCG, hCG and estradiol for

the first 20 h, then in gonadotropin-free medium for another 20 h, enhanced cytoplasmic maturation (67% MPN formation) when compared with those cumulus-enclosed oocytes continuously cultured in the presence of gonadotropins for 40 h (36% MPN formation). These data suggest that hormonal effects on cytoplasmic maturation are achieved in the first 20 h of culture and that over exposure of oocytes to gonadotropins, and probably also to steroid hormones produced by cumulus cells stimulated by gonadotropins, has adverse effects on cytoplasmic maturation.

An ether extract of follicular shell conditioned medium re-dissolved in fresh medium enhanced sperm decondensation of in vitro matured porcine cumulus-enclosed oocytes, and supplementation of progesterone, but not estradiol or testosterone, to culture medium could mimic the effect of the ether extract (Mattioli et al., 1988b), suggesting that steroids are the components that support cytoplasmic maturation. This suggestion is supported by our experiment (Nagai et al., 1993) in which total abolition of steroid production by cumulus cells during maturation culture using aminoglutethimide, a 20α-cholesterol oxidase inhibitor, dramatically reduced male pronuclear formation. Addition of progesterone (1mg/ml) to culture, however, not only suppressed nuclear maturation, but also reduced male pronuclear formation rate from 78% to 31% (Nagai et al., 1993), indicating exposure of cumulus-enclosed immature follicular oocytes to a high progesterone environment may be detrimental to both nuclear and cytoplasmic maturation. This result is contradictory to Mattioli and his colleagues' (1988b) finding. Whether this conflict is caused by using different culture systems (non-static vs static) is not known. In addition, suppression of estrogen production by cumulus cells using 1,4,6-androstatrien,

an aromatizing enzyme inhibitor, did not affect male pronuclear formation (Nagai et al., 1993), suggesting estrogens are not essential for supporting cytoplasmic maturation.

### 3.2.4 Effect of growth factors.

It is logical to suggested that growth factors may be involved in oocyte cytoplasmic maturation because of their effect on nuclear maturation (discussed before). Two recent reports by Coskun et al. (1991) and Harper and Brackett (1993) that EGF supplementation enhanced the developmental potential of bovine oocytes matured in vitro, support this suggestion. Growth factor effects on cytoplasmic maturation in species other than bovine have not been reported.

## 3.3 Mechanism of regulation of cytoplasmic maturation.

How gonadotropins, steroid hormones, growth factors and follicular cells and follicular secretions mediate cytoplasmic maturation is not well understood. One thing seems to be in common; all these factors are much less effective in mediating cytoplasmic maturation in cumulus-free oocytes or oocytes with a low number of cumulus cells when compared to effects in cumulus-enclosed oocytes (Mattioli et al., 1988b; Nagai et al., 1993; also see review by Thibault et al., 1987 and Buccione et al., 1990). Thus it is clear that factors regulating oocyte cytoplasmic maturation act via follicular somatic cells and the coupling between follicular somatic cells and the oocyte is essential (Mattioli et al., 1988b). It has been reported that when oocytes were cultured in follicular conditioned medium, uncoupling between cumulus cells and the oocyte occurred later than when

oocyte-complexes were cultured in control medium (Mattioli et al., 1988b). Follicle shell co-culture also delayed the uncoupling process (Ding et al., unpublished observation). Furthermore, addition of an ether extract of follicular conditioned medium or addition of progesterone, which promoted sperm decondensation of oocytes, to fresh culture medium, partially mimicked the effect of follicular conditioned medium on intercellular coupling between follicular cells and oocytes (Mattioli et al., 1988b). Therefore, the delay of the uncoupling process between follicular cells and the oocyte by follicular factors (follicle shell co-culture or using follicular conditioned medium) during in vitro culture may allow the oocyte to receive more regulatory control, and/or more substrates for cytoplasmic reprogramming from surrounding somatic cells via intercellular gap junction and hence facilitate cytoplasmic maturation.

Hormonal action on oocyte cytoplasmic maturation is also likely to be mediated via follicular somatic cells and receptors for these hormones exist on follicular cells. Gonadotropins stimulate follicular cell differentiation and hence steroidogenesis (see review by Gore-Langton and Armstrong, 1988) and the reprogramming of the production of regulatory factors for oocyte maturation (Racowsky, 1991). All these may act together to affect cytoplasmic maturation. In addition, Mattioli et al. (1991a) demonstrated that gonadotropins, especially LH, stimulate intercellular coupling between follicular cells and the oocytes and this may provide another mechanism for stimulating cytoplasmic maturation.

Effects of growth factors via follicular cells clearly exist, since there are binding sites for growth factors on follicular cells (for review, see Harper and Brackett, 1993).

EGF, for example, binds to its receptors and induces the activation of tyrosine-specific kinase, an essential primary event in the EGF pathway. In turn, tyrosine kinase activation initiates phosphorylation of several cellular proteins as well as phosphorylation of the receptor itself (for review see Harper and Brackett, 1993). These changes lead to new gene expression (see Mercola and Stiles, 1988) and hence changes in protein synthesis and protein phosphorylation. Also the EGF effect on oocyte maturation may involve important interactions with gonadotropins, steroids, other growth factors and/or other local mediators (Racowsky, 1991). EGF binding sites in granulosa cells were increased by FSH treatment (pig, Fujinaga et al., 1992; rat, St-Arnaud et al., 1983; Feng et al., 1987), but were decreased by LH/hCG treatment (Feng et al., 1987). In turn, EGF suppressed the FSH-induced increase in granulosa cell LH receptors in both rats and pigs (Knecht and Catt, 1983; May and Schomberg, 1989) and suppressed FSH-induced LH receptor mRNA synthesis in rats (Piquette et al., 1991). It has been suggested that such interactions between EGF and gonadotropins in the regulation of receptors may play an essential role during oocyte maturation (Harper and Brackett, 1993).

Although the metabolic changes that underlie acquisition of oocyte cytoplasmic maturation are ill defined, de novo protein synthesis within oocytes is involved (see Thibault et al., 1987). These newly synthesized proteins may be associated with modulating cytoplasmic glutathione levels, since such levels increase during meiotic maturation in hamster oocytes and are significantly higher in mature MII oocytes (8 mM) than in either GV oocytes or pronuclear stage oocytes (4 mM and 6 mM, respectively; Perrault et al., 1988). Glutathione, the major intracellular free thiol (Meister and

Anderson, 1983), has been implicated as playing a key role in protamine disulphide bond reduction (Perrault et al., 1984; Calvin et al., 1986), which in turn is a prerequisite for sperm chromatin decondensation, and thus for male pronuclear formation (Perrault et al., 1984). Indeed, when glutathione synthesis is blocked during the early stages of oocyte maturation in vitro, the cytoplasm of chromosomally normal MII oocytes is incapable of supporting sperm nuclear decondensation (Perrault et al., 1988). Recent studies in pig oocytes also confirm the effect of glutathione on sperm decondensation (for review, see Niwa, 1993). Oocytes matured in medium containing high concentrations of glutathione and cysteine, which is the precursor of glutathione, greatly improved male pronuclear formation (Yoshida et al., 1992b). Although permeability of the plasma membrane to glutathione is low (De Felici et al., 1987), the intracellular concentration of glutathione is dependent upon availability of cysteine (Meister, 1983). Supplementation of cysteine to the medium containing low concentrations of glutathione and cysteine greatly enhanced male pronuclear formation (Yoshida et al., 1992b) and this effect is cancelled by inhibition of glutathione synthesis during maturation (Yoshida, 1993a). Microinjection of glutathione to oocytes matured in glutathione- and cysteine-deficient medium also increased male pronuclear formation rate (Naito and Toyoda, 1992). Furthermore, Yoshida et al. (1993a) found that the rate of male pronuclear formation increased in parallel with increasing glutathione in oocytes. These findings strongly suggest that glutathione is responsible for sperm decondensation and male pronuclear formation and probably is the so-called male pronuclear growth factor (MPGF). Glutathione concentrations, therefore, may be used as a biochemical criterion for cytoplasmic maturation, and those factors

affecting male pronuclear formation rate of in vitro matured oocytes may mediate glutathione synthesis in oocytes.

**4. Follicular heterogeneity and oocyte maturation**

Follicular heterogeneity in porcine follicular development has been reviewed recently by Hunter and Wiesak (1990). Heterogeneity in the development of follicles has been demonstrated in both naturally cyclic (Foxcroft et al., 1985; Grant et al., 1989; Hunter et al., 1989) and eCG primed immature gilts (Wiesak et al., 1990) and in weaned sows (Foxcroft et al., 1987). Follicles even in the selected preovulatory populations differ in size by up to 2 mm and showed marked variability in steroid content and gonadotropin binding.

A major problem in swine reproduction is early embryonic loss which ranges from 25 to 35% (Hammond, 1921; Day et al., 1959; Spies et al., 1959; Perry and Rowlands, 1962). This early embryonic loss greatly reduces the productivity of sows. The causes of this loss are not certain, but it has been suggested that embryonic diversity during early development may be one of the possible causes (Pope et al., 1990). Furthermore, it is possible that diversity of early embryonic development is correlated to the diversity of oocyte quality caused by the heterogeneous development of follicles (Pope et al., 1990; Hunter and Wiesak, 1990).

Direct evidence for an effect of follicular heterogeneity on oocyte maturation comes from the observations of the oocyte nuclear stage in individual preovulatory follicles in vivo in cyclic gilts and eCG/hCG treated gilts (reviewed by Hunter and Wiesak, 1990). Nuclear maturation status of the oocyte inside the follicle was

significantly correlated with follicular fluid volume and significantly, or almost significantly, correlated with both follicular diameter and steroids (estradiol or progesterone) secreted during incubation of the collapsed follicle. These data suggest that follicular heterogeneity might influence the quality of oocytes ovulated (Foxcroft and Hunter, 1985; Hunter and Wiesak, 1990). However, of 1677 spontaneously ovulated oocytes, all but three were at the metaphase II stage (Hancock, 1961), suggesting that spontaneously ovulated oocytes reached the metaphase II stage. However, their specific ages (relative time to the completion of the first polar body extrusion) were probably different. Furthermore, the degree of cytoplasmic maturation is likely to be affected by the diversity of follicular development, since production of regulatory factors promoting normal oocyte cytoplasmic maturation in vivo may vary among preovulatory follicles (Hunter and Wiesak, 1990). Further experiments should be designed to examine the effect of follicular heterogeneity on cytoplasmic maturation of porcine oocytes.

## 5. Summary

To integrate these data, it is appropriate to suggest the possible mechanisms controlling oocyte maturation (Fig.II-4). The interaction between the oocyte and follicular cells is crucial to this model. During meiotic arrest, the follicular cells produce at least two or three inhibitors: OMI, cAMP, and possibly purine nucleotides. When the growing follicles are stimulated by the preovulatory gonadotropin surge, the follicular cells differentiate and their functions change. The production of inhibitors for meiotic and probably also cytoplasmic maturation reduces, and some stimulatory factors such as

growth factors may be synthesized in the follicular cells. The increased steroid production and the change of the steroid ratio may also be involved in the regulation of meiotic resumption. At the same time, intercellular communication is gradually terminated as a consequence of the oocyte-cumulus uncoupling. Therefore, the inhibitory effect of meiosis from the follicular compartment is finally eliminated. During this period, oocyte synthetic activity increases, and substances such as cyclin, a component of maturation promoting factor (MPF) which is crucial in triggering the resumption of meiosis, are synthesized. In the meantime, another component of MPF, a cdc2 gene product, is dephosphorylated and thereby forms a complex with cyclin. This complex, constituting the active MPF, promotes the resumption of oocyte meiotic maturation by driving the oocyte from the dictyate (GV) to the metaphase stage (for MPF, see Dunphy and Newport, 1988; and Lohka, 1989). This process is accompanied by modulation of centrosomes and the microtubule assembly (Albertini, 1992), which are machineries for the completion of meiotic division. In addition, the oocyte synthesizes or accumulates male pronuclear growth factor (MPGF, probably glutathione) and other molecules for fertilization and early embryonic development.

Fig.II-1. Phase contrast photomicrographs of porcine oocytes prepared as whole mounts and stained with 1% lacmoid showing nuclear events during meiotic maturation in vitro.

A. An immature oocyte with a germinal vesicle (GV) intact nucleus and a nucleolus at the beginning of culture.

B. An oocyte with a GV stage nucleus during the early stage of culture. Chromatin becomes condensed surrounding the nucleolus forming a ring.

C. An oocyte with GV stage nucleus after about 18 h of culture. Nucleolus disappears and chromatin further condenses. Nuclear membrane starts to breakdown.

D. An oocyte at pre-metaphase I (PMI or GVBD) stage after about 18-20 h of culture. Chromatin condenses and contracts to a ball-like chromatin mass, and nuclear membrane completely disappears.

E. An oocyte at metaphase I (MI) stage after about 20 to 26 h of culture. Individual chromosomes form and are arranged on the metaphase plate.

F. An oocyte at anaphase I stage after 26-30 h of culture. Chromosomes form two groups and start to separate.

G. An oocyte at telophase I stage after 28-34 h of culture. Two groups of chromosomes have been separated completely, but they are still associated by a spindle.

H. An oocyte at metaphase II (MII) stage after 32-40 h of culture. One group of chromosomes form a condensed chromatin mass which is extruded as the first polar body (Pb); the other group of chromosomes are again individually arranged on the metaphase plate.

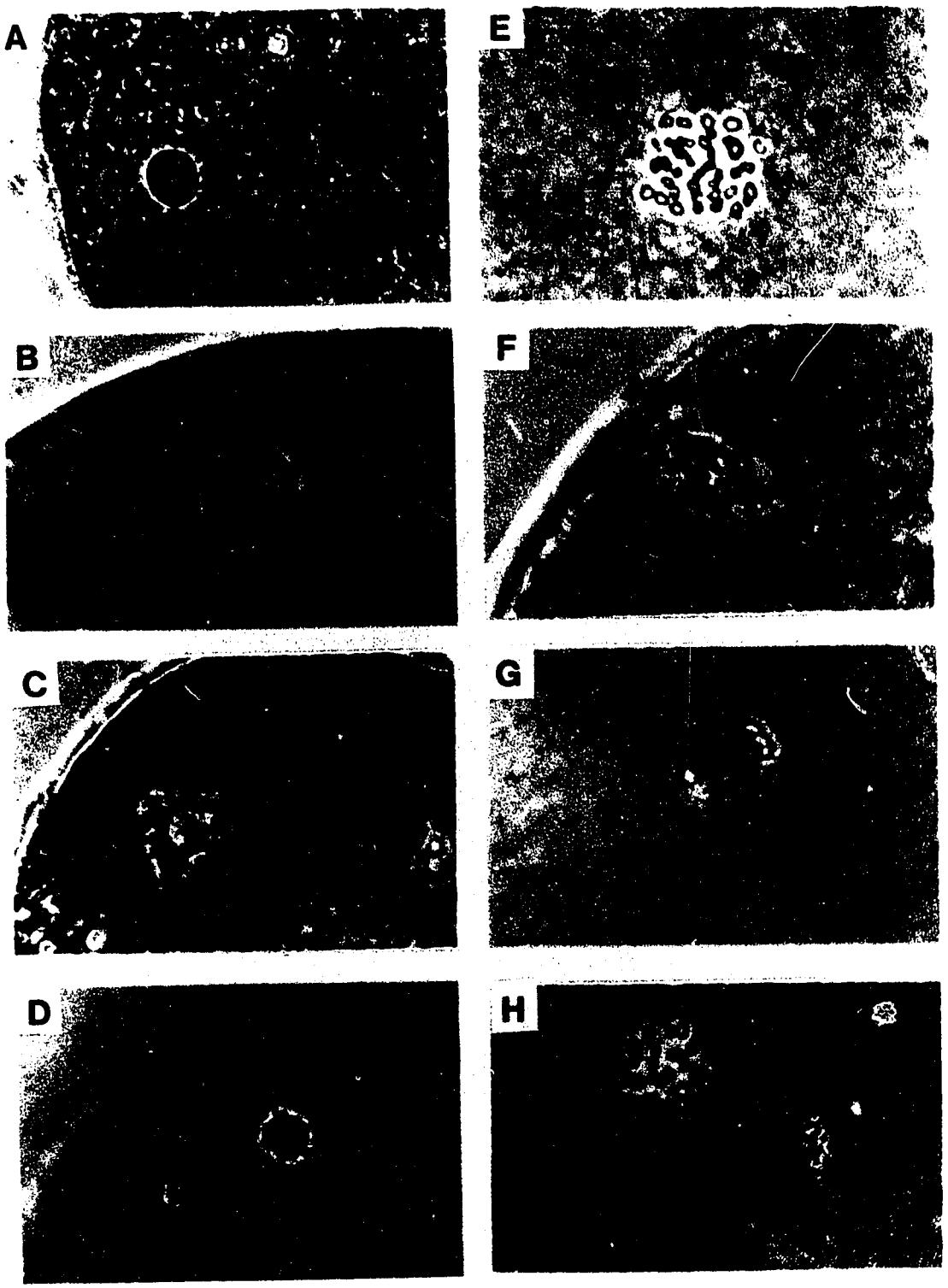

A
B
C
D
E
F
G
H

Fig.II-2. Effect of oocyte types, follicular shell co-culture and gonadotropin supplementation on nuclear maturation in porcine oocytes. Oocytes were cultured in TCM199 supplemented with 10% FCS in the absence (a) or presence (b) of gonadotropins (2.5μg/ml LH, 2.5μg/ml FSH and 20ng/ml prolactin) for 47 h on a rocking platform. Nuclear stages were examined under a phase contrast microscope after fixation and lacmoid staining. Type of oocytes: C-, cumulus-enclosed oocytes (approximately $15x10^3$ follicular cells per egg); G-C-, cumulus-enclosed oocytes directly associated with a small piece of mural granulosa tissue (approximately $150x10^3$ follicular cells per egg). GV: germinal vesicle; PMI: pre-metaphase I; MI: metaphase I; MII: metaphase II. FS: two follicle shells.

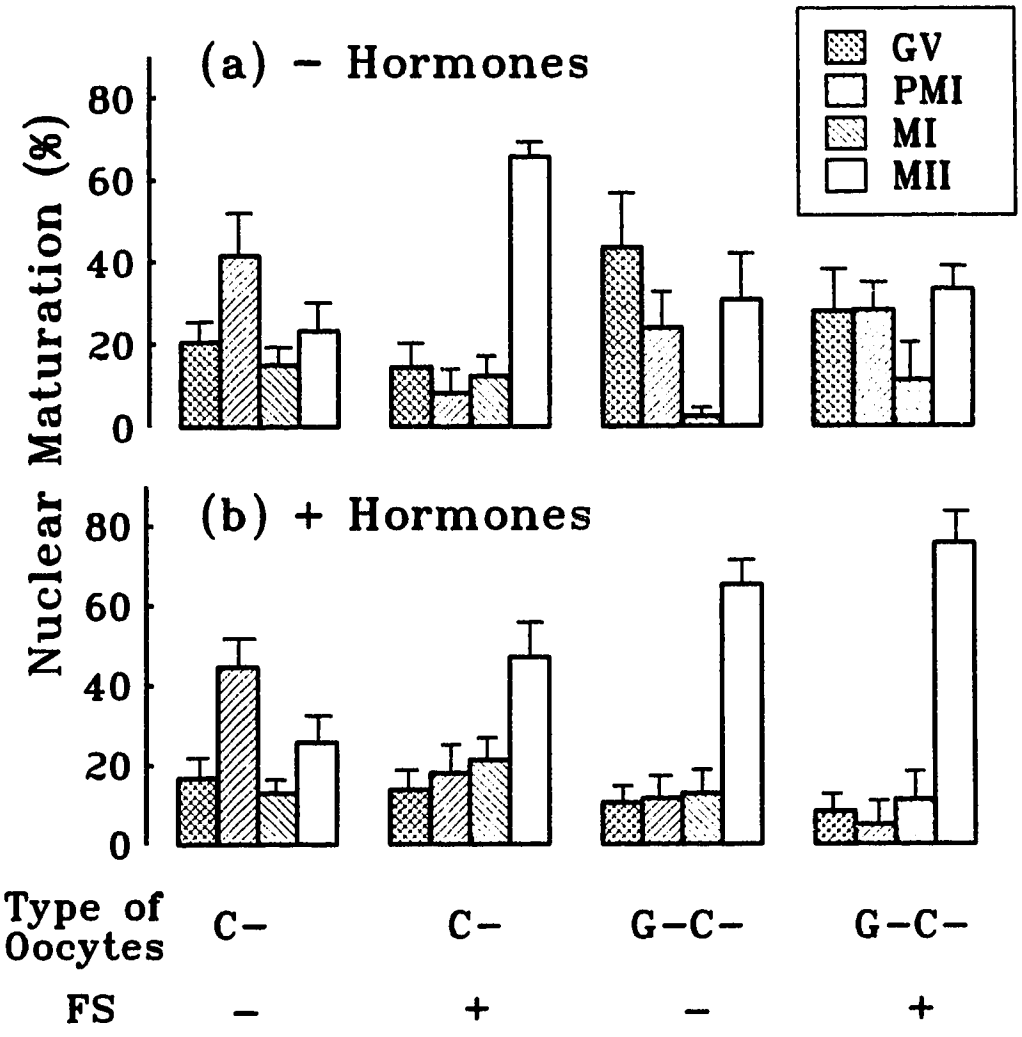

(a) – Hormones
(b) + Hormones
GV
PMI
MI
MII
Nuclear Maturation (%)
0
20
40
60
80
Type of Oocytes
C–
C–
G–C–
G–C–
FS
–
+
–
+

Fig.II-3. Effect of oocyte types, follicle shell co-culture and gonadotropin supplementation on male pronuclear formation (MPN) in penetrated oocytes. Oocytes were cultured in TCM199 supplemented with 10% FCS for 47 h on a rocking platform, then oocytes were inseminated and fixed 10 h post insemination for examination of sperm penetration and pronuclear development. Type of oocytes: C-, cumulus-enclosed oocytes (approximately $15x10^3$ follicular cells per egg); G-C-, cumulus-enclosed oocytes directly associated with a small piece of mural granulosa tissue (approximately $150x10^3$ follicular cells per egg). FS: two follicle shells. Hormones: 2.5μg/ml LH, 2.5μg/ml FSH and 20ng/ml prolactin.

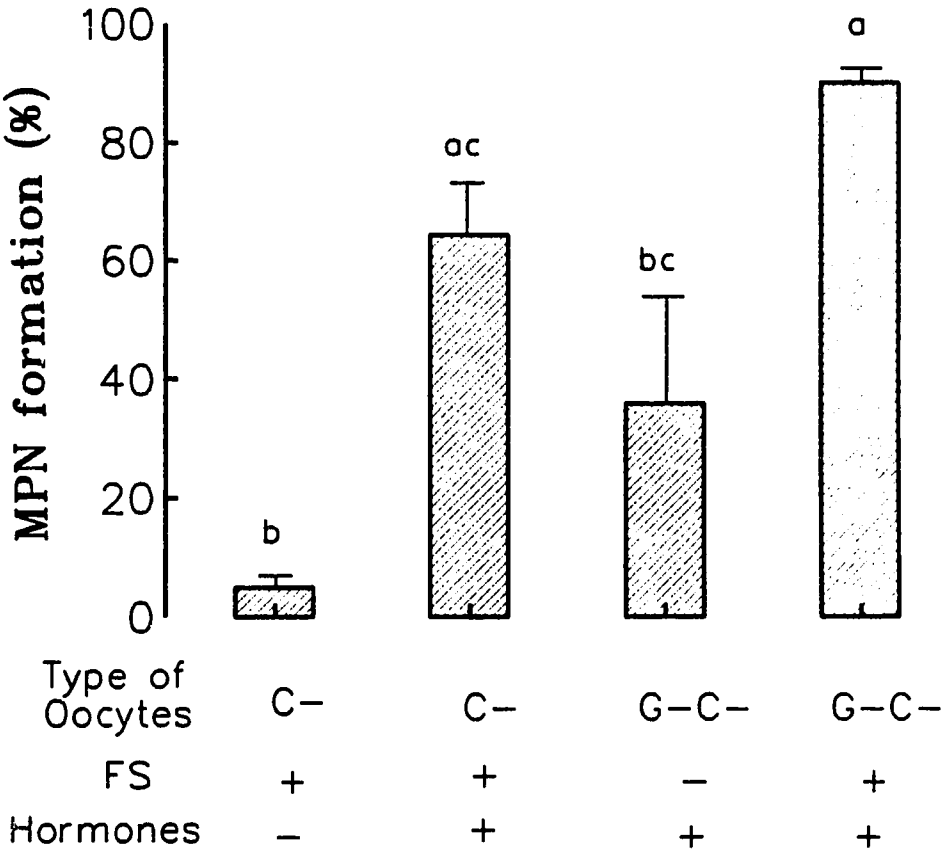
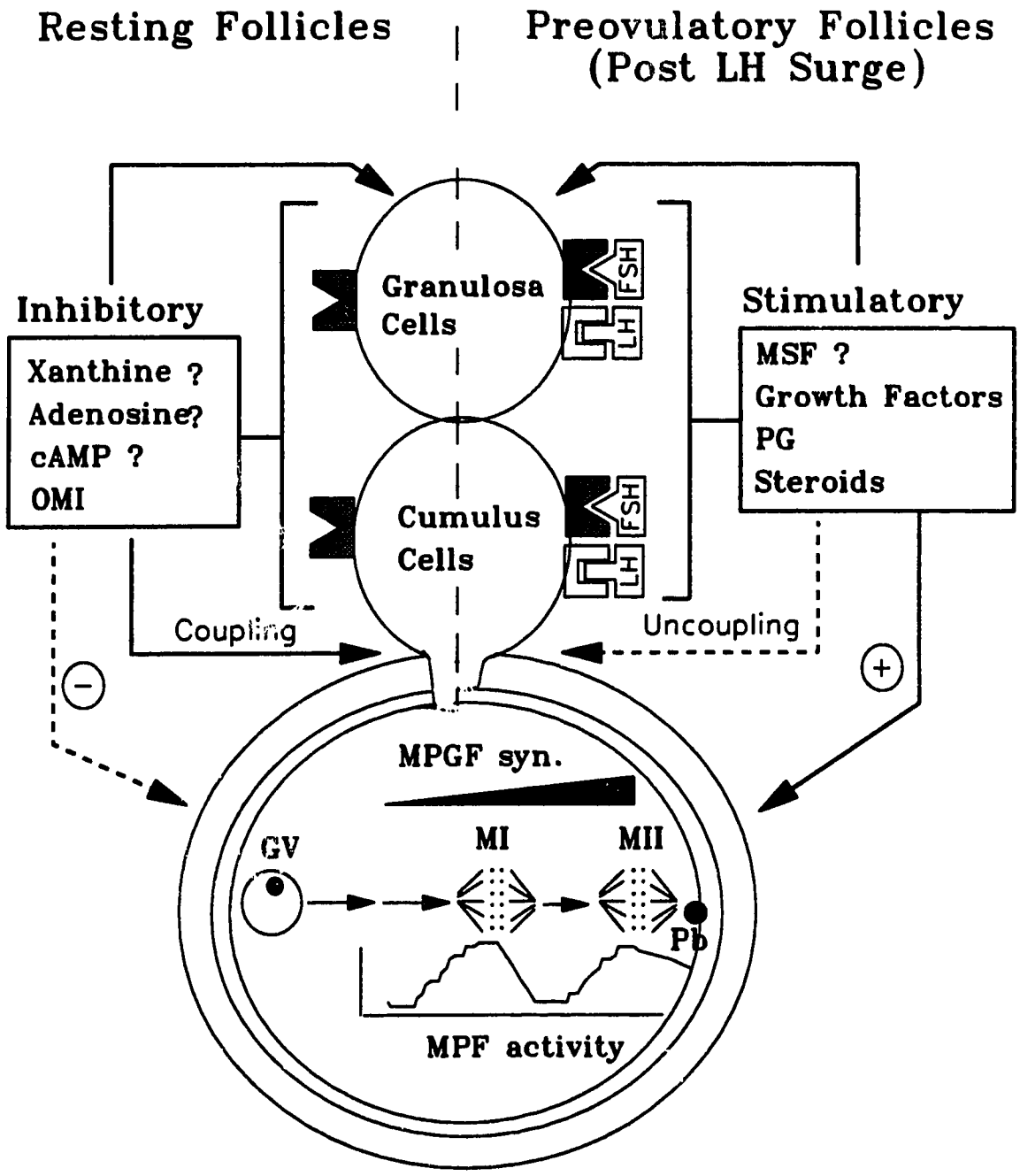

MPN formation (%)
100
80
60
40
20
0
b
ac
bc
a
Type of Oocytes
C−
C−
G−C−
G−C−
FS
+
+
−
+
Hormones
−
+
+
+

Fig.II-4. Diagrammatic representation of the mechanisms controlling oocyte maturation (see text for explanation). cAMP, cyclic adenosine monophosphate; OMI, oocyte maturation inhibitor(s); MSF, meiosis stimulating factor(s); PG, prostaglandins; GV, Germinal vesicle stage; MI, metaphase I; MII, metaphase II; Pb, polar body; MPGF, male pronuclear growth factor; MPF, maturation promoting factor; + and - indicate stimulatory and inhibitory effects, respectively.


Resting Follicles
Preovulatory Follicles
(Post LH Surge)
Granulosa
Cells
Cumulus
Cells
FSH
LH
Inhibitory
Xanthine ?
Adenosine?
cAMP ?
OMI
Stimulatory
MSF ?
Growth Factors
PG
Steroids
Coupling
Uncoupling
MPGF syn.
GV
MI
MII
Pb
MPF activity

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# CHAPTER III EFFECTS OF PROTEIN SYNTHESIS ON MATURATION, SPERM PENETRATION AND PRONUCLEAR DEVELOPMENT IN PORCINE OOCYTES[1]

## INTRODUCTION

*De novo* protein synthesis is essential for resumption of meiosis of ungulate oocytes (Hunter & Moor, 1987; Moor & Crosby, 1986; Fulka et al., 1986). Whether it is also essential for fertilization and pronuclear development has not been ascertained in domestic species. Biochemical results showed that proteins synthesized before fertilization undergo similar changes to those synthesized during fertilization (Ding et al., 1992). These findings suggest, therefore, that protein changes associated with fertilization occur primarily at the post-translational modification level using precursors accumulated during maturation. From this we hypothesize that *de novo* synthesis of proteins during fertilization may not be obligatory for sperm penetration, the second meiotic division or pronuclear development.

The present study was designed firstly to test this hypothesis by examining whether sperm penetration and subsequent pronuclear development occur in oocytes whose protein synthetic capacity is inhibited during fertilization. Having established that *de novo* synthesis of proteins during fertilization is not required, led to a second series

[1]The data in this chapter are published as **Ding J, Moor RM, Foxcroft GR (1992). Mol Reprod Dev 33:59-66.**

of experiments to identify the stage during oocyte maturation at which proteins required for activation, sperm decondensation and pronuclear formation are synthesized.

## MATERIALS AND METHODS

***Preparation of oocytes.***

Ovaries were obtained from slaughtered gilts at a local abattoir and transported to the laboratory within 40 min in a polystyrene box to prevent major changes in temperature. Follicles were dissected from the ovary and oocyte-cumulus-granulosa cell complexes (oocyte-complexes) were harvested from healthy follicles with a diameter of 3-6 mm. Dissection was carried out in medium 199 with 25 mM Hepes (Earle's salts, Gibco) containing 10% heat treated new born calf serum (Gibco) at a temperature of 24-26°C. The dissecting procedure was completed within 2.5 h. Groups of 10-15 randomly selected oocyte-complexes were incubated in 35 mm plastic petri dishes containing 2 ml medium 199 supplemented with 10% fetal calf serum (FCS, Gibco), 100µg/ml glutamine (Sigma, BDH), 70 µg/ml L-ascorbic acid (Sigma, BDH), 35 µg/ml insulin (Sigma,BDH) (see appendix) and gonadotrophins (2.5 µg/ml NIADDK-oLH-26,AFP-5551b; 2.5 µg/ml USDA-pFSH-B-1, AFP-5600) and prolactin (20 ng/ml USDA-pprl-B-1, AFP-5000) (see appendix). Two everted follicle shells (4 to 6 mm diam) were added to each dish and co-cultured with the oocytes. Culture was carried out with gentle agitation under an atmosphere of 5% $CO_2$ in air at 39°C for 47±1 h.

***Sperm preparation and in vitro fertilization (IVF) and nuclear examination***

Preparation of spermatozoa. Four mature Landrace boars were used for supplying semen. For each IVF study, sperm-rich fractions were obtained from two boars by a gloved-hand method. After removing the gel-fraction, the two semen samples were mixed (1:1) and held at 20°C for 16 h (Cheng et al., 1986). Spermatozoa were washed three times by centrifugation (1,200g for 4 min) in 4 ml of 0.9% w/v saline solution containing 1 mg/ml BSA (Fraction V) (see appedix). The sperm pellet was thereafter resuspended at $4x10^8$ cells/ml in preincubation medium at pH 7.8 (Cheng, 1985, see appendix) and incubated for 90 min before being co-cultured with oocytes as described by Ding et al. (1992).

In vitro fertilization. Cumulus-enclosed oocytes matured for 47±1 h in vitro were incubated with preincubated boar spermatozoa at a concentration of $5x10^5$ cells/ml in B.O. medium (Brackett & Oliphant, 1975, see appendix) containing 10 mg/ml BSA (fraction V) and 2 mM caffeine for 10 h. All the chemicals were from Sigma, BDH).

Examination of nuclear status. After 47±1 h maturation culture or 10 h after insemination, oocytes were denuded of cumulus cells, mounted on a slide using a whole mount technique and fixed for 48 h in ethanol/acetic acid (3:1). The nuclear status of oocytes was examined under a phase-contrast microscope after staining with 1% lacmoid in 45% acetic acid solution. The stage of nuclear maturation was identified basically according to Hunter and Polge (1966) and the nuclear status of oocytes after fertilization was identified as described previously (Ding et al.1992). Some unusual nuclear configurations are shown in Fig.III-1. Those penetrated oocytes with two or more polar bodies (Pb) and one full sized female pronucleus were considered as having completed the second meiosis

and normal female pronuclear development. Finally, those penetrated oocytes with full sized male pronucleus/pronuclei were considered to have undergone normal male pronuclear development.

***Measurement of protein synthesis***

Freshly prepared oocyte complexes (see above) were labelled with [$^{35}$S]-methionine at a final concentration of 500 μCi/ml (specific activity > 1000 Ci/mM, Amersham, U.K.) for 3 h at 39°C (see Moor et al., 1981) at various times during in vitro maturation (for details see Fig.III-4 legend). After incubation, oocytes were washed with [$^{35}$S]-methionine-free labelling medium and transiently cultured (15-20 min) in medium containing 10μg/ml DAPI (4,6-diamidino-2-phenylindole, Sigma), a DNA fluorescent stain material. Oocytes were thereafter denuded of cumulus cells and loaded, in a small amount of the same medium (2-3μl), into small individual compartments on a slide and covered gently with a cover slip to prevent quick evaporation of the medium. Both support of the cover slip and separation of individual oocytes were achieved using thin lines of Vaseline. Nuclear status was determined under a fluorescent microscope (see Fig.III-2 a-h), following which the cover slip was carefully removed and oocytes were individually recovered from the slide and placed in groups according to their nuclear configuration. A group of 1-3 radio-labelled oocytes with the same nuclear configuration was washed briefly in 10 mM tris-0.1mM EDTA solution, transferred in a small volume (<4μl) of this solution to a 0.5 ml micro-tube, lyophilized and stored at -70°C until required for gel electrophoresis. Oocytes

were prepared for protein analysis and polypeptides were separated using 8 - 15% linear gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) as described by Moor et al. (1981).

***Inhibition of protein synthesis***

Inhibition of protein synthesis by inhibitors of different components of the protein synthetic pathway was determined by measuring the levels of amino acid incorporation into oocyte proteins. After maturation, cumulus enclosed oocytes were labelled with [$^{35}$S]-methionine at a final concentration of 500 μCi/ml (specific activity > 1000 Ci/mM, Amersham, U.K.) at 39°C for 3 h, in the presence or absence (control) of a chain elongation inhibitor (cycloheximide at 35 μM or 350 μM) or a combination of inhibitors of chain elongation (35 μM cycloheximide), ribosomal subunit function (0.1 μM emetine) and an inducer of premature peptide chain release (20 μM puromycin).

Following labelling, the oocytes were denuded of cumulus cells and washed four times before transfer of single oocytes in 2 μl of 10 mM Tris-0.1 mM EDTA to 0.5 ml microtubes for lyophilization and subsequent storage at -70°C. Assays for non-specific binding were carried out by taking, before labelling of oocytes, 2 μl of [$^{35}$S]-methionine containing medium and thereafter treating these blank samples in an identical manner to those containing eggs. Lyophilized samples were prepared for the further analysis by adding 20 μl of gel SDS buffer (O'Farrell, 1975), followed by three cycles of freezing and heating. A 5 μl aliquot of each sample was used to measure the total radioactivity as a parameter of total uptake of [$^{35}$S]-methionine by the oocyte and another 5 μl aliquot was

used to determine incorporation of [$^{35}$S]-methionine into trichloroacetic acid (TCA) precipitable material as described by Van Blerkom (1978). In addition to the blank samples (radiolabelled medium), non-specific binding was further analyzed by loading SDS gels with the same number of TCA-precipitable counts from untreated controls and from inhibitor-treated groups. Pooling of a large number of samples of eggs subjected to the same inhibitor regimen, was necessary to obtain adequate counts for these experiments.

***Statistical analysis***

Data in Study 1 were subjected to an one way analysis of variance and percentage data from Study 2 and 3 were subjected to a two way variance analysis of a random block design after arcsine transformation; multiple comparisons of means were made by Student-Newman-Keul's tests.

# RESULTS

## *Study 1* *Effect of protein synthesis inhibitors on uptake and incorporation of [$^{35}$S]-methionine into proteins in porcine oocytes*

To determine the efficiency of blocking protein synthesis with a variety of inhibitors, a series of the three experiments and associated assays for non-specific binding was carried out. In vitro matured (cultured for 48 h) oocytes were used in this study.

Uptake studies. The total uptake of radiolabelled methionine was reduced by 25% in the presence of 35 μM cycloheximide [30190±1309 (mean±s.e.m.) cpm/oocyte, n=7] as compared to the control [40211±1908 cpm/oocyte, n=8; $P < 0.01$]. At the higher level of cycloheximide (350 μM), uptake was further inhibited to 63% of the control values, whilst the combination of inhibitors further reduced uptake to only 31% of controls.

Amino acid incorporation. Before correction for non-specific binding, TCA analysis indicated that incorporation of [$^{35}$S]-methionine was reduced by 75%, 81% and over 95% of controls in the presence of different concentrations of cycloheximide (35 or 350 μM) or a combination of inhibitors (cycloheximide, emetine and puromycin), respectively. Between 25-40% of the TCA precipitable counts in inhibitor-treated oocytes was accounted for by non-specific binding. Analysis of radiolabelled oocyte proteins by SDS-gel electrophoresis in the presence of protein synthesis inhibitors showed that the residual amount of protein synthesis, even with the single inhibitor used at the lowest concentrations (35 μM cycloheximide), was even lower than that suggested by the corrected TCA results. The apparent cycloheximide-resistant protein ran as a faint smear on the gel, represented by densitometry readings of less than 10% of the overall control synthesis, and probably consisted largely of peptide fragments. However, in order to adopt the most conservative interpretation of these data, the uncorrected TCA values were used as the maximum possible residual protein synthesis after insertion of different inhibitors; in reality synthesis of discrete, full length polypeptides is virtually abolished even by inhibition with 35 μM cycloheximide. Higher concentration of cycloheximide or inhibitor

combinations not only prevent synthesis but also act to suppress uptake and have therefore not been extensively used in our developmental studies.

**Study 2** ***Effect of inhibiting protein synthesis during fertilization on sperm penetration and pronuclear formation***

In total, 266 in vitro matured oocytes were used in five replicates to test the effect of protein synthesis inhibitors on fertilization. For each of the replicated experiments, oocytes were harvested, pooled and randomly divided into four groups of 10-15 oocytes fertilized in B.O. medium supplemented with (i) 35 μM cycloheximide, (ii) 350 μM cycloheximide, (iii) a combination of emetine (0.1 μM), cycloheximide (35 μM) and puromycin (20 μM), and (iv) a control group receiving no protein synthesis inhibitors. At 10 h post insemination (hpi) oocytes were fixed for examination of nuclear status. It is clear from Table III-1 that neither sperm penetration ($P > 0.05$) nor female and male pronuclear formation ($P > 0.05$) were significantly affected by suppressing protein synthesis with either the single or combined inhibitor treatments.

**Study 3** ***Effects of inhibiting protein synthesis at different stages of maturation on nuclear maturation, sperm penetration and pronuclear formation***

From the results presented in study 2 it is evident that protein synthesis was not required during fertilization for pronuclear formation. The aim of study 3 was to identify

the requirement for the synthesis of fertilization-dependent proteins during maturation, and particularly during the two critical meiotic transition phases at 20-24 h (GV to MI) and 34-36 h (entry to MII).

a. Protein inhibition and nuclear maturation. GV oocytes were randomly divided into four groups (two replicate dishes per group), treated respectively with 35 μM cycloheximide at 0, 24 or 36 h after explantation or left untreated (control). All groups of oocytes were fixed at 47 h after explantation. Inhibition of protein synthesis throughout maturation (0 h group) suppressed GVBD (TableIII-2). The addition of cycloheximide at 24 h resulted 24 h later in half the oocytes containing condensed female chromatin (Fig.III-1A) and the other half forming an incipient nucleus with one polar body (picture not shown but similar to Fig.III-1C). After inhibition of protein synthesis at 36 h of culture, over 90% of oocytes matured beyond the MII stage; of these 90% appeared to have a normal MII nuclear configuration with their chromosomes in a dispersed arrangement on the MII plate (same as Fig.III-1B). Only a very small proportion (<10%, see table III-2) formed incipient nuclei. In the control group, more than 90% of the oocytes had a normal MII nuclear configuration (Fig.III-1B).

b. Sperm penetration, meiosis progression and pronuclear development. The next series of replicated experiments examined the effects on pronuclear formation of inhibiting protein synthesis during maturation. The treatment groups were the same as those used above except that the 0 h inhibitor group was excluded since all oocytes in that group

were blocked at the GV stage (see study 3a). After 47 h of culture, matured oocytes were incubated with in vitro capacitated boar spermatozoa in B.O. medium containing 35 μM cycloheximide (including the control group) for a further 10 before being fixed for nuclear examination.

Mean penetration rates (% of eggs examined, ±sem) of 80±3.8, 96±1.5 and 95±2.6% for the groups in which protein synthesis were inhibited from 24 or 36 h on and for the control group, respectively, were not affected by treatments ($P > 0.05$). However, both the second meiotic division and female and male pronuclear formation were affected by inhibition of protein synthesis during maturation (Fig.III-3). After the inhibition of protein synthesis at 24 h of culture, 28% of penetrated oocytes were at the MI stage and 40% were at the MII, in both situations female chromatin was condensed (see Fig.III-1D) and about 23% of oocytes possessed female pronuclei, with either one Pb or multiple female pronuclei. Only about 5% of oocytes successfully completed second meiosis but subsequently failed to develop a female pronucleus. The penetrated oocytes (including MI oocytes) were able to decondense the sperm nucleus (Fig.III-1D and E) but none formed a male pronucleus (Fig.III-3 d). Following inhibition of protein synthesis at 36 h of culture, about 30% of penetrated eggs completed the second meiosis and formed female pronuclei (Fig.III-3 a), compared to 81% in the control group ($P < 0.01$), whereas 54% of penetrated oocytes developed putative female pronuclei without undergoing second meiosis (Fig.III-3 b). About half (52%) of the penetrated oocytes developed full sized male pronuclei, compared to 73% in the control group (Fig.III-3 d, $P < 0.05$).

c. Changes in protein synthesis during maturation. A brief re-examination of the polypeptide changes that take place in oocytes during maturation was undertaken both to complement that of McGaughey and Van Blerkom (1977) and to focus specifically on the correlation between protein synthesis and pronuclear formation. After labelling with [$^{35}$S]-methionine (see Materials and Methods), oocytes were grouped accurately according to nuclear status (by DAPI analysis) and sets of three similar staged oocytes were prepared for electrophoresis. The experiments focused primarily on the changes during the G2- to M-phase transition (at 24 h) and more particularly on the appearance of those polypeptides which change as a result of fertilization (see Ding et al., 1992). Apart from confirming the results of McGaughey and Van Blerkom (1977) our analysis highlighted the appearance of Mr 25K and 63K polypeptides during GVBD and the concomitant disappearance of Mr 70K, 59K, 42K and 26K species (Fig.III-4). The synthesis of the 25K polypeptide increases further between MI and MII and at fertilization this protein undergoes a major series of post-translational changes (see Ding et al., 1992). However, the functional relevance of the protein analysis will remain uncertain until a clear relationship has been established between individual polypeptide changes and the fertilization abberations that occur in eggs in which protein synthesis is inhibited.

## DISCUSSION

Protein synthesis is essential for the resumption of the first meiotic division in most mammalian oocytes. Inhibition of protein synthesis in oocytes by cycloheximide

effectively blocks germinal vesicle breakdown (GVBD) in bovine (Hunter & Moor, 1987), ovine (Moor & Closby, 1986) , porcine (Fulka et al., 1986; Kubelka et al, 1988) and rat (Ekholm & Magnusson, 1979) oocytes, consistent with our observations. Having completed first meiosis, the oocyte remains arrested until it is activated by sperm penetration or by other parthenogenetic factors. In this study, we examined the importance of protein synthesis on meiosis, sperm penetration, and pronuclear development in pig oocytes.

Cycloheximide, an inhibitor of peptide chain elongation, was used as a major protein synthesis inhibitor, since it specifically inhibited the incorporation of [$^{35}$S]-methionine into proteins without markedly affecting amino acid uptake or other cellular events. However at the concentration of 35 μM cycloheximide, some putative incorporation was still detectable and we are unable to be certain whether this residual radioactivity reflected non-specific binding, partial chain synthesis or a low level of normal synthesis. Gel electrophoresis and autoradiography showed that after loading the same amount of TCA precipitable radioactivity as controls onto 8-15% gradient SDS-polyacrylamide gels, no protein bands were found, indirectly suggesting that the residual radioactivity may not have been incorporated into proteins. Furthermore, we observed that 35μM cycloheximide successfully inhibited GVBD (Table III-2) even after 47 h of culture, suggesting that protein synthesis was severely suppressed. For caution's sake, a higher dose of cycloheximide (350 μM) or a combination of protein synthesis inhibitors (selected because of their different actions on the synthetic pathway) were used in part of the studies, since they further reduced or almost entirely eliminated residual protein

synthesis (study 1). However, a marked reduction in amino acid uptake also occurred in eggs treated with a high concentration of cycloheximide (350 μM) or the combination of puromycin, emetine and cycloheximide, suggesting that these two inhibitors may have had some adverse effect on membrane transport function. This prevented us from using them for prolonged protein synthesis inhibition in study 3.

The results showed that neither sperm penetration nor pronuclear development were affected by reduction of protein synthesis to 25% of its control value (using 35 μM cycloheximide) or by almost total elimination of protein synthesis (using a mixture of inhibitors) during fertilization. These results supported our earlier suggestion (Ding et al., 1992), that during maturation porcine oocytes have accumulated ample amount of these factors or their precursors which are important for reactivation of second oocyte meiosis and female and male pronuclear formation. Sperm penetration induced the post-translational modification of endogenous protein synthesized during maturation in the presence of cycloheximide (35 μM). Moreover the post-translational pattern of protein modification in the presence of cycloheximide (35 μM) was identical to that observed in newly labelled proteins during an 8 to 11 h co-incubation with spermatozoa (Ding et al., 1992). Therefore it seems that oocyte protein synthesis during fertilization is not obligatory for normal fertilization.

Inhibition of protein synthesis after 24 h of culture (at the early MI stage) totally inhibited male pronuclear development (Fig.III-3 d). However, almost all penetrated oocytes (even MI oocytes) possessed the ability to decondense the sperm nucleus/nuclei (Fig.III-1 D and E, and Fig.III-3 e and f). These results are similar to findings of Clarke

and Masui (1987) in mice; these authors suggested that the oocyte cytoplasmic factors required for the dispersion of sperm chromatin, which appeared to coincide with GVBD in many species (Longo, 1985), developed in the maturing oocytes independently of continuing protein synthesis, whereas the transformation to pronucleus required continuing protein synthesis during maturation. After 36 h of culture (at early MII stage) in the present studies, inhibition of protein synthesis did not totally block MPN formation, although the rate of MPN formation was reduced compared to the control group when inhibiting protein synthesis during fertilization only (Fig.III-3 d, $P < 0.05$). These results suggest that synthesis of male pronuclear growth factor(s) (MPGF) (Iwamatsu & Chang, 1972; Thibault et al., 1975) may commence immediately after oocytes reached MI; by the early MII stage, MPGF has accumulated sufficient amounts to provide oocytes with the ability to decondense sperm heads and subsequently form male pronuclei.

Inhibition of protein synthesis after both 24 and 36h of maturation impaired the completion of second meiosis. Inhibition of protein synthesis by puromycin at MI induced the extrusion of a polar body (Pb) and formation of an interphase nucleus in mouse oocytes (Clarke and Masui, 1983). If protein synthesis was resumed the decondensed chromosomes became condensed and returned to the metaphase stage. From their studies it is suggested that the maintenance of condensed chromosomes during the transition from MI to MII depends on protein synthesis; in the absence of protein synthesis the chromosomes decondense and form an interphase nucleus. Clarke and Masui (1983) also reported that inhibition of protein synthesis at MII caused the parthenogenetic activation of the oocytes. We found that about one third of the oocytes developed incipient nuclei

when protein synthesis was inhibited at early MI, suggesting that the above mechanism also exists in porcine oocytes. However, about 40% of the oocytes had a condensed chromatin mass (Table III-2) and even after sperm penetration this chromatin retained a similar configuration (Fig.III-3 c). Inhibiting protein synthesis either after 36 h of culture (at early MII) or after 48 h of culture (at late MII stage, unpublished observation) did not significantly stimulate parthenogenic activation of the oocyte. These results seem to differ from those in mice (Clarke and Masui, 1983). After sperm penetration of oocytes in which protein synthesis was inhibited at early MII, as in those oocytes in which protein synthesis was inhibited at early MI, the second meiosis was dramatically inhibited. Instead, the female chromatin in most of oocytes proceeded directly to form a female pronucleus (Fig.III-3 b).

Dramatic reprogramming of protein synthesis occurred during GVBD (about 24 h, see Fig.III-4). Before GVBD, there were no apparent qualitative, but obvious quantitative changes in protein synthesis (Fig.III-4 lanes 1-6), which might be essential to oocyte maturation since GVBD was totally blocked in the absence of protein synthesis. During the transition from MI to MII (about 36 h, Fig.III-4), there were quantitative but no obvious qualitative changes in protein synthesis. When oocytes reached full maturation the synthesis of 26 K polypeptides ceased. These results are similar to the early observations by McGaughey and Van Blerkom (1977). We conclude from our combined results that the reprogramming of protein synthesis after GVBD is absolutely essential for oocytes to complete meiotic maturation and pronuclear development.

In a previous study (Ding et al., 1992) we observed that a group of 25 K phospho-

polypeptides (25K at the present Fig.III-4) underwent post-translational modification to 22 K polypeptides as a result of dephosphorylation after activation of the oocytes. This was a slow process and took 6 h to complete; this time coincided with the period of male pronuclear development. Thus it was suggested that the 25/22 K polypeptides might be responsible for MPN formation. In the present study we further demonstrated that the synthesis of 25 K polypeptides occurred at about 24 h after initiation of maturation culture and that its synthesis had increased dramatically by 36 h (see Fig.III-4); together with the data from the experiments using protein synthesis inhibitors, these data confirm that the 25K/22K proteins are probably central to MPN development.

In conclusion, continued protein synthesis during maturation is absolutely essential for the completion of oocyte meiosis. While decondensation of the sperm nucleus may be independent of continued protein synthesis, the transformation of the sperm nucleus to a male pronucleus requires protein synthesis lasting at least until the early MII stage. During fertilization, protein synthesis is not essential for sperm penetration, the completion of the second meiosis, nor for pronuclear development.

**Table III-1. Effect of Inhibiting Protein Synthesis After In Vitro Maturation* on Subsequent Sperm Penetration And on Male (MPN) and Female (FPN) Pronuclear Formation.**

| Treatments | 35 µM cyclo. | 350 µM cyclo. | Inhib. mix. | Control |
|---|---|---|---|---|
| Nos. replicates | 5 | 5 | 5 | 5 |
| Nos. MII oocytes | 62 | 69 | 77 | 58 |
| Penetrated MII oocytes | | | | |
| - Nos. | 57 | 50 | 69 | 53 |
| - % (mean±s.e.) | 92.9±7.2 a | 74.3±14.7 a | 87.9±8.5 a | 92.5±4.8 a |
| Penetrated oocytes with 2Pb+1FPN | | | | |
| - Nos. | 47 | 41 | 56 | 40 |
| - % (mean±s.e.) | 81.8±5.9 a | 83.8±7.5 a | 83.4±5.9 a | 75.0±6.3 a |
| Penetrated oocytes with MPN | | | | |
| - Nos, | 42 | 37 | 45 | 34 |
| - % (mean±s.e.) | 73.1±2.8 a | 72.5±3.4 a | 68.5±7.0 a | 65.3±3.0 a |

a With different subscripts $P < 0.05$.

* In vitro matured oocytes were cocultured with in vitro capacitated boar spermatozoa for 10 h in B.O. medium containing 1) 35 µM cycloheximide (cyclo.), 2) 350 µM cyclo., and 3) a combined inhibitors (inhib. mix.) and 4) a control receiving no protein synthesis inhibitors. Fixed oocytes were stained with 1% lacmoid and examined under a phase contrast microscope.

**Table III-2. Effect of Inhibiting Protein Synthesis at Different Maturational Stages on Nuclear Maturation of Porcine Follicular Oocytes*.**

| | Time of inhibitor addition | | | |
|---|---|---|---|---|
| | 0 hour | 24 hour | 36 hour | Control |
| Nos. replicates | 2 | 4 | 4 | 4 |
| Nos. oocytes examined | 38 | 99 | 105 | 99 |
| GV - Nos. | 34 | 1 | | |
| - % | 89.5 | 1.0 | | |
| GVBD-MI | | | | |
| - Nos. | 4 | 14 | 7 | 4 |
| - % | 10.5 | 14.1 | 6.7 | 4.0 |
| MII, condensed chromatin (see Fig.III-1 A) | | | | |
| - Nos. | | 38 | 4 | |
| - % | | 38.4 | 3.8 | |
| MII, dispersed chromosomes (see Fig.III-1 B) | | | | |
| - Nos. | | 2 | 84 | 94 |
| - % | | 2.0 a | 80.0 b | 94.9 b |
| 1 Pb + 1 FPN | | | | |
| - Nos. | | 33 | 8 | |
| - % | | 33.3 | 7.6 | |
| Other | | | | |
| - Nos. | | 11 | 2 | 1 |
| - % | | 11.1 | 1.9 | 1.0 |

a,b Only MII with dispersed chromosomes was statistically analyzed. With different subscripts: $P < 0.01$.

*Oocytes were cultured for 47 h in 2 ml culture medium per dish using a non-static incubation system at an atmosphere of 5% $CO_2$ in air at 39°C. 35 μM (final concentration) cycloheximide was added into culture medium after 0, 24 and 36 h of culture and a control group was run parallel. Oocytes were then fixed and nuclear status were examined under a phase contrast microscope after 1% lacmoid staining.

Fig.III-1 Phase Contrast Photomicrographs of Oocytes Prepared as Whole Mounts And Stained With 1% Lacmoid. Immature oocyte-cummulus complexes were cultured in vitro with addition of 35 μM cycloheximide (final concentration) was added at either 24 or 36 h; control untreated cultures were run in parrallel. After 47 h of culture, oocytes were fixed immediately or after a further 10 h coincubation with in vitro capacitated boar spermatozoa in the presence of 35 μM cycloheximide.

A. An oocyte fixed after 47 h of culture in which protein synthesis was inhibited after 24 h of culture. The first meiotic division was completed with extrusion of the first polar body (Pb). However female chromosomes (arrow) were coalesced into a dot of pycnotic chromatin.

B. An oocyte recovered after 48 h of culture from the control group (no protein synthesis inhibition). The oocyte completed the first meiotic division (extrusion of the first Pb). Note that female chromosomes were dispersed on the metaphase plate.

C. An oocyte fixed 10 h post insemination (hpi) in which protein synthesis was inhibited after 24 h of maturation culture. The oocyte was penetrated and a swollen sperm head was found but out of focus. Although female chromosomes was decondensed and formed an incipient nucleus (arrow) it appeared different to a pronucleus. Furthermore, the oocyte had only one Pb.

D. An oocyte harvested 10 hpi in which protein synthesis was blocked after 24 h of maturation culture. Female chromosomes were coalesced into a dot (arrow) with only one Pb (on a different focal plane). The spermatozoa head was decondensed (SH1).

E. A polyspermic oocyte fixed 10 hpi in which protein synthesis was inhibited after initial 24 h maturation culture. Sperm heads were highly decondensed (SH2) and similar to male pronuclei, except their size was much smaller.

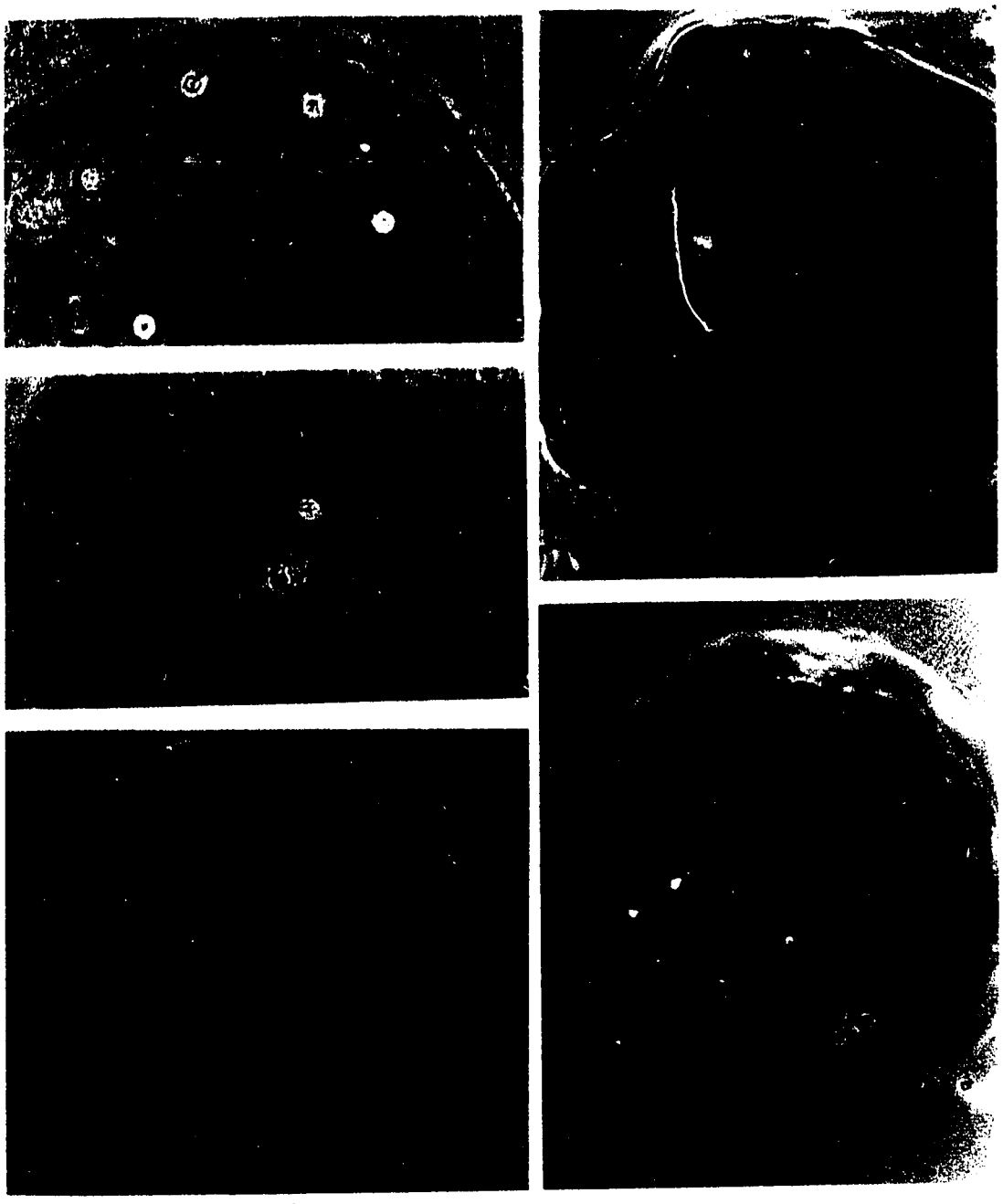


Fig.III-2 Changes of nuclear configurations during oocyte maturation in vitro stained with 4,6-diamidino-2-phenylindole (DAPI), a DNA fluorescent staining material.

a. An immature oocyte with a germinal vesicle (GV) intact nucleus. Chromatin was evenly dispersed inside the nucleus.

b. An oocyte with GV stage nucleus during the early stage of culture. Chromatin becomes condensed surrounding the nucleolus forming a ring.

c. An oocyte with GV stage nucleus after about 18 h of culture. Nucleolus disappears and chromatin further condenses.

d. An oocyte at pre-metaphase I stage (PMI or GVBD) stage after about 18-20 h of culture. Chromatin condenses and contracts to a ball-like chromatin mass.

e-f. Oocytes at metaphase I stage (MI) stage after about 20 to 26 h of culture. Individual chromosomes form.

g. An oocyte at anaphase I to telophase I stage after 26-34 h of culture. Chromosomes form two groups and separate.

h. An oocyte at metaphase II (MII) stage after 32-40 h of culture. One group of chromosomes form a condensed chromatin mass which is extruded as the first polar body (Pb); the other group of chromosomes are individually arranged on the metaphase plate.

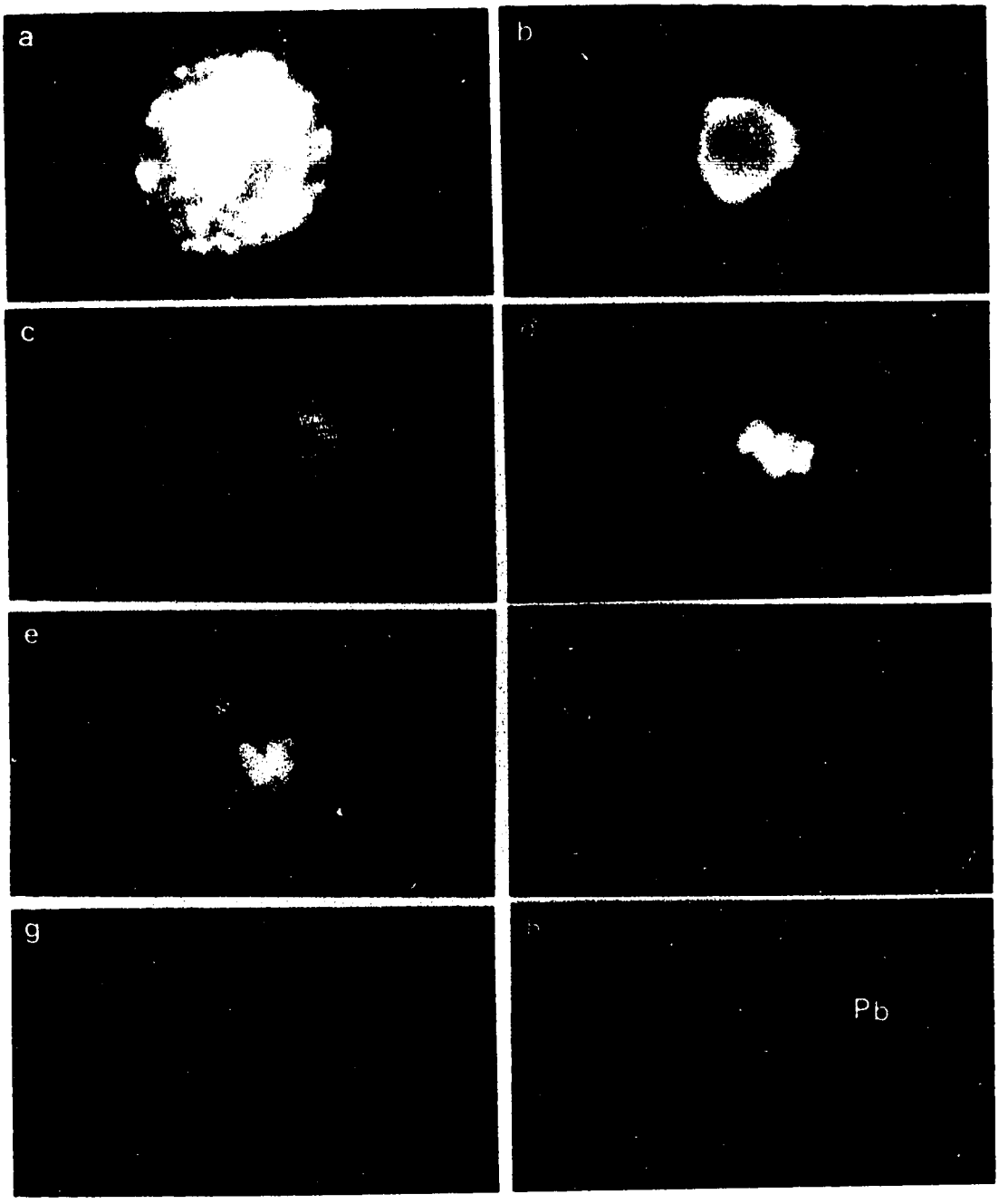

a
b
c
e
g
Pb

Fig.III-3 Effects of Inhibiting Protein Synthesis During Maturation on Female (a-c) And Male (d-f) Nuclear Transformation. Oocyte complexes were cultured in vitro with addition of 35 μM cycloheximide at either 24 (24 h) or 36 h (36 h) of culture; control (CTRL) cultures received no cycloheximide. After 47 h of culture oocytes were inseminated with boar spermatozoa in the presence of 35 μM cycloheximide. 10 h after insemination, oocytes were fixed to examine female and male nuclear transformation.

**2Pb+1F**: one full size female pronucleus with two or more polar bodies. **1Pb+1F**: one full size female pronucleus with polar body only. **1Pb+CC**: one polar body with condensed chromatin. **MPN**: full size male pronucleus. **SH1**: slightly decondensed sperm head. **SH2**: further decondensed sperm head.

* With different letter, $P < 0.05$.

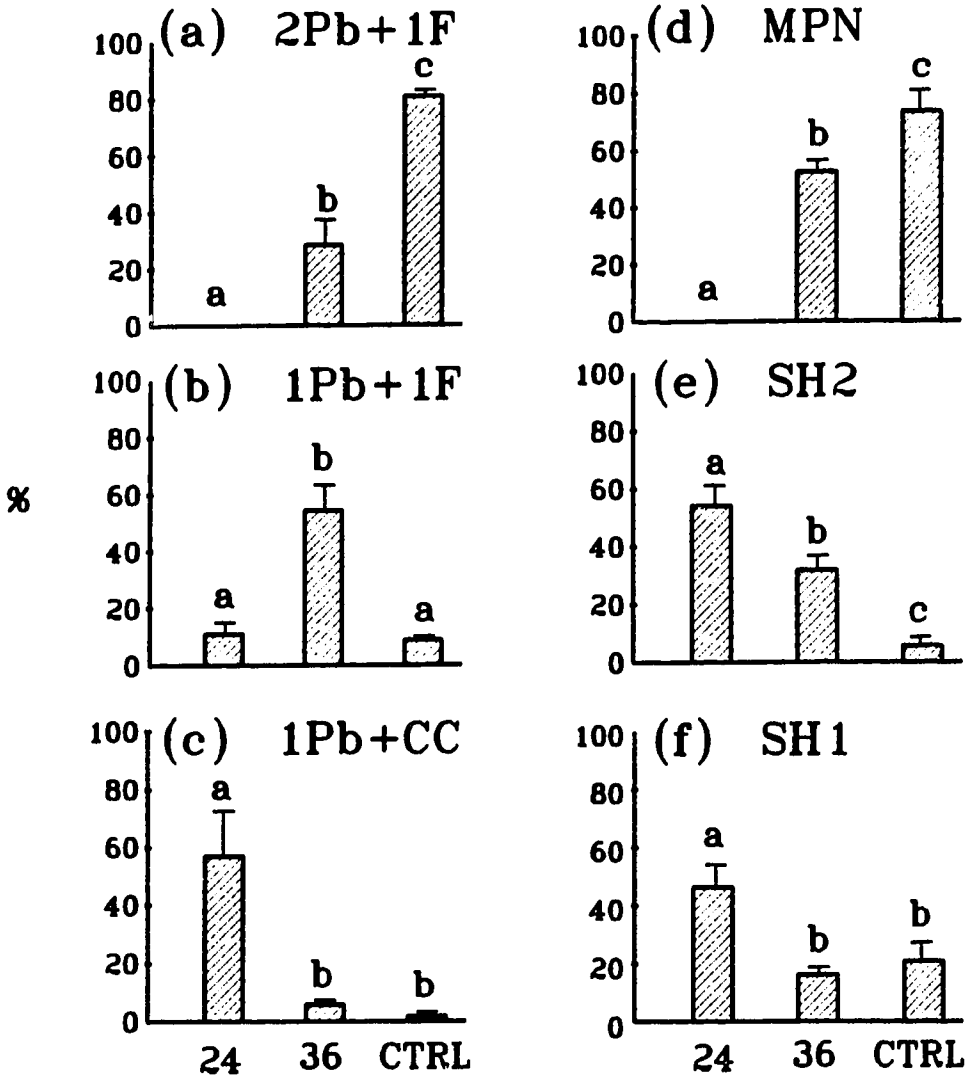
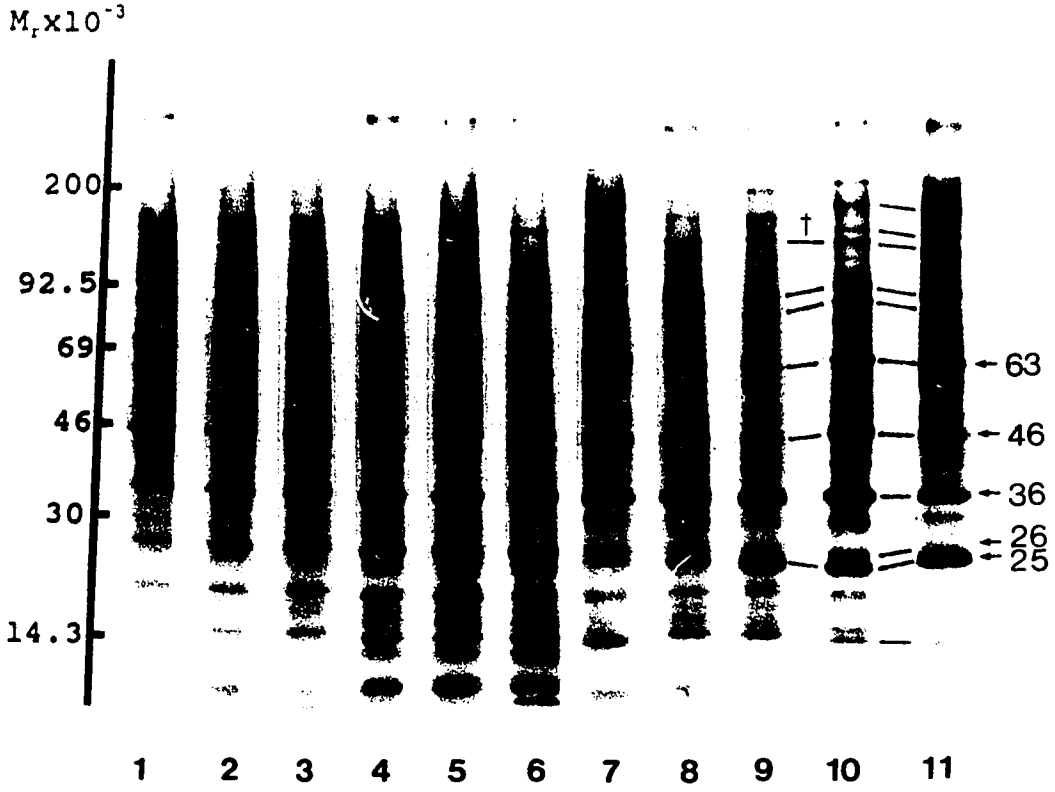

(a) 2Pb+1F
(b) 1Pb+1F
(c) 1Pb+CC
(d) MPN
(e) SH2
(f) SH1
%
100
80
60
40
20
0
a
b
c
24
36
CTRL

Fig.III-4 Changes In Patterns of Protein Synthesis During Maturation. A group of about 13 eggs were taken from culture every 4 h from 0 to 36 h and also at 47 h and then labelled with [$^{35}$S]-methionine for 3 h. After labelling, oocyte nuclear status were established by DAPI staining. Oocytes with the same nuclear configurations were grouped and prepared for SDS/PAGE. At least three replicates were made and the protein pattern was consistant corresponding to their nuclear configurations.

Lanes 1 to 6 show the GV patterns from 0 h to 20 h. Lanes 7 and 8 represent MI at 24 and 28 h, respectively. Lanes 9, 10 and 11 represent MII at 32, 36 and 47 h, respectively.

+: Black solid lines between lanes 9, 10 and 11 connect bands with same molecular weight.


$M_r x10^{-3}$
200
92.5
69
46
30
14.3
†
63
46
36
26
25
1
2
3
4
5
6
7
8
9
10
11

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# CHAPTER IV FOLLICULAR HETEROGENEITY AND OOCYTE MATURATION IN VITRO IN PIGS[1]

## INTRODUCTION

The mammalian oocyte is matured inside a particular ovarian structure, the follicle. The cellular relationship between the oocyte and somatic follicular cells is fundamental to oocyte maturation (Moor et al., 1990). In addition to providing nutrients to the oocyte, the follicular cells play a crucial role in regulating oocyte meiotic arrest and the resumption of meiosis. Various systems of communication have evolved between the oocyte and follicular cells which regulate oocyte maturation.

Since Pincus and Enzmann (1935) observed the spontaneous resumption of meiosis in mammalian oocytes freed of follicular cells more than half a century ago, oocyte nuclear maturation in vitro has been intensively studied. It is generally believed that follicular cells surrounding the oocyte exert an inhibitory effect on oocyte nuclear maturation.

Recent studies of pig oocyte maturation by either co-culturing oocytes with follicular cells (Mattioli et al., 1988a) or culturing oocytes in medium supplemented with porcine follicular fluid (Naito et al., 1988), however, indicate that the somatic follicular cells play a crucial role in oocyte cytoplasmic maturation. Further evidence for the

[1] Data in this chapter are published as **Ding J, Foxcroft GR (1992). Biol Reprod 47: 648-655.**

importance of maturational factors has come from experiments which have resulted in the birth of lambs (Staigmiller and Moor, 1984; Crozet et al., 1987), calves (Lu et al., 1987) and piglets (Mattioli et al., 1989) after co-culture of cumulus-oocyte complexes with follicular cells during in vitro oocyte maturation.

As the follicle matures, follicular cells undergo differentiation in function. Therefore, it is logical to suggest that follicular cells at different developmental stages may secrete different amounts of maturational factors and may have different effects on oocyte maturation. Follicular heterogeneity has been demonstrated in both naturally cyclic (Grant et al., 1989; Hunter et al., 1989) and eCG-stimulated immature gilts (Wiesak et al., 1990) and in weaned sows (Foxcroft et al., 1987). Follicles in the selected preovulatory population differ in size by up to 2 mm and show marked variability in steroid content and gonadotropin binding ability, and it has been suggested that this heterogeneity might influence the quality of oocytes ovulated (Foxcroft and Hunter, 1985; Hunter and Wiesak, 1990).

The present study comprised two experiments: 1) to confirm the effect of follicular shells on porcine oocyte maturation in vitro in our laboratory and 2) to test the effects of the maturational state of follicles on oocyte maturation.

## MATERIALS AND METHODS

***Experiment I:***

This experiment was designed to compare the quality of in vitro matured oocytes

in the presence and absence of follicular shells. Ovaries were obtained from late prepubertal gilts with live body weights of approximately 105 kg within 15 minutes of slaughter at a local abattoir and transported to the laboratory within 40 min in a polystyrene box to prevent major changes in temperature. Follicles were dissected from ovaries with no corpora lutea (prepubertal) and oocyte-cumulus-granulosa cell complexes (oocyte-complexes) were harvested from healthy follicles with a diameter of 3-6 mm. Dissection was carried out in medium 199 with 25 mM HEPES (N-1-hydroxyethylpiperazine N-2-ethanesulfonic acid; Earle's salts, Gibco) and 10% heat treated new born calf serum (Gibco) at a room temperature of 24-26°C. The dissecting procedure was completed within 2.5 h. Pooled oocyte-complexes were randomly divided into two groups. One group of 10-15 oocytes was cultured with two everted follicular shells selected from 5-6 mm follicles (FS) and the other was cultured without addition of follicular shells (control). Each plastic petri culture dish contained 2 ml medium 199 supplemented with 10% fetal calf serum (FCS, Gibco), 100μg/ml glutamine (Sigma, BDH), 70 μg/ml L-ascorbic acid (Sigma, BDH), 35 μg/ml insulin (Sigma,BDH) (see appendix) and gonadotropins (2.5 μg/ml NIADDK-oLH-26,AFP-5551b; 2.5 μg/ml USDA-pFSH-B-1, AFP-5630) and prolactin (20 ng/ml USDA-pprl-B-1, AFP-5000). Culture was carried out with gentle rocking agitation under an atmosphere of 5% $CO_2$ in air at 39°C for 47±1 h.

**Sperm preparation and in vitro fertilization (IVF).**

***Preparation of spermatozoa***: Four mature large white boars held at the Alberta Swine AI

Centre were used for supplying semen. For each IVF, sperm-rich fractions were obtained from two boars by a gloved-hand method. After removing the gel-fraction, the two semen samples were mixed in a 1:1 ratio and the pooled semen was held at 20°C for 16 h (Cheng et al., 1986). 1 ml of this pooled semen was treated as described previously (Ding et al., 1992) prior to use for IVF.

***IVF***: Cumulus-enclosed oocytes matured for 47±1 h in vitro were incubated with preincubated boar spermatozoa at a concentration of 5 x $10^5$ cells/ml in B.O. medium (Brackett and Oliphant, 1975, see appendix) (all the chemicals were from Sigma, BDH; BSA: fraction V.) for 10 h.

***Examination of nuclear status*:**

Ten hours post insemination (hpi) oocytes were denuded of cumulus cells, mounted on a slide with a whole mount technique and fixed for 48 h in ethanol/acetic acid (3:1). The nuclear status of oocytes was examined under a phase-contrast microscope after staining with 1% lacmoid in 45% acetic acid solution. Nuclear status (GV, MI and MII) was identified according to the criteria of Hunter and Polge (1966). Sperm penetration and male pronuclear development were determined as described by Ding et al. (1992). Those oocytes with sperm head(s) and/or swollen sperm head(s) and/or male pronuclei (MPN) with detached sperm tail(s) were classified as being penetrated, irrespective of the number of sperm heads present in the cytoplasm. Those penetrated oocytes with one or more full size male pronuclei were considered to have undergone normal male pronuclear development (MPN formation).

Maturation, penetration and male pronuclear formation were expressed as the percentage of MII to the number of oocytes examined, penetrated oocytes to MII oocytes and number of oocytes with one or more male pronuclei to number of oocytes penetrated, respectively, for each culture dish.

***Experiment II*:**

In Experiment I follicular shells played an important role in supporting oocyte maturation, as indicated by a higher rate of male pronuclear development. This experiment was therefore designed to further examine the effect of follicular cells at various stages of differentiation on oocyte maturation. This experiment was designed as a split-split-plot design, in which five replicate-experiments (plots) were carried out. In each replicate-experiment, follicular shells were obtained from the ovaries of prepubertal gilts at either 36 or 72 h after eCG injection; within each time of collection after eCG treatment, follicles were further split into small and large sizes. Follicles from each of these classes were then individually co-cultured with oocyte-complexes taken from a pool derived from the follicles obtained 36h after eCG treatment.

***Animal treatment:*** In total, 35 prepubertal gilts (7 animals in each of five replicates) weighing about 100kg were used. Gilts (PIC, Canada Ltd., Camborough x Canabrid) were raised at the Swine Research Centre of the University of Alberta under commercial conditions. In each sub-experiment, five animals were injected with 750 I.U. eCG (Ayerst Laboratory, Montreal, Canada) 36 h, and two animals 72 h, before slaughter.

***Follicular shell and oocyte preparation*:** Ovarian tissue, individually identified by

treatment, was obtained as described for the first experiment. All follicles with a diameter ≥ 3 mm were dissected from the ovaries of two gilts from each eCG treatment group and pooled within treatment. Two or three of the smallest and two or three of the largest follicles were randomly selected from each pool. Follicular diameter (mm) was measured and then follicular fluid was aspirated with a micro-syringe and follicular fluid volume was recorded; follicular fluid was then diluted with 1 ml of autoclaved PBS (Sigma) and stored at -70°C for determination of steroid concentrations. The follicular shells obtained were weighed and washed four times in Medium 199 with 25 mM HEPES supplemented with 10% new born calf serum (Sigma).

***Oocyte maturation:*** Oocyte-complexes isolated from 3-9 mm follicles of 36 h eCG treated gilts were pooled and used as a source of oocytes for the whole experiment. According to the number of oocyte-complexes available, they were randomly and equally (10 to 15 oocyte-complexes per dish) aliquoted into 8 or 12 culture dishes and subjected to four treatments; co-cultured with 1) 36 h (eCG treatment) small follicular shells (S36), 2) 36 h large follicular shells (L36), 3) 72 h small follicular shells (S72) and 4) 72 large follicular shells (L72). One follicular shell was added to each culture dish. At the completion of culture, cumulus-enclosed oocytes were harvested for IVF and the culture media were collected for steroid determination.

All the dissection procedures, in vitro maturation, IVF and the examination of nuclear status were the same as in Experiment I. The data for maturation, penetration and male pronuclear formation were also expressed as described in Experiment I.

***Radioimmunoassay of steroids*:**

Concentrations of progesterone ($P_4$), testosterone (T) and estradiol-17ß ($E_2$) in follicular fluid and culture medium were determined by direct (no extraction) radioimmunoassay (RIA).

Progesterone was assayed using a double antibody, $^{125}I$ RIA as described by Pharazyn et al (Pharazyn et al., 1991) with modification as a direct assay. Validity of the direct assay was established by demonstrating parallelism among a series of dilutions (1/50, 1/100, 1/200, 1/400, 1/800) of quality control follicular fluid and culture medium and the standard curve, and by quantitative recovery (101.9±2.8% and 97.8±0.4%) of standard progesterone to follicular fluid and culture medium prior to RIA, respectively.

Estradiol-17ß was determined by RIA using an antiserum (NCR Rabbit A11, obtained from Dr. N.C. Rawlings, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada) raised in rabbit against estradiol-17ß [1,3,5(10)-estratrien-3,17ß-diol-6-one-6-CMO:BSA; STERALOIDS E1361]. Cross-reactions for this antiserum reported by the supplier were: estrone 8%, estriol 0.4%, 17α-estradiol 0.4%, testosterone 0.08%, dihydrotestosterone 0.1%, 5α-androstan-3ß,17ß-diol 0.08% and cortisone 0.03%. Androstenedione, 5α-androstan-3α,17ß-diol, progesterone, pregnenolone, cholesterol, corticosterone and cortisol do not crossreact with this antiserum. 100 µl of sample diluted 1:500 and 1:100 for follicular fluid and culture medium, respectively in assay-buffer [$NaH_2PO_4 \cdot H_2O$ 2.77mM, $Na_2HPO_4$ 7.22mM, NaCl 139.66mM, $NaN_3$ 15.38mM and 0.1% (W/V) Gelatin, all chemicals from Sigma], 150 µl of assay-buffer (4°C) and 200 µl antiserum (final dilution 1:440,000) were added to a 12x75 mm glass

tube. After vortexing, the mixture was cultured at room temperature (20°C) for one hour, and followed by the addition of 100 µl (≈20,000 dpm) $^{3}$H-labelled estradiol-17ß (Du Pont NEN Products) to each assay tube. After incubation at 4°C for approximately 20 h, 200 µl of well mixed, dextran coated charcoal (Dextran 0.125 g, Carbon Decolouring Alkaline Norit-A 1.25 g, mixed thoroughly in 250 ml assay-buffer and cooled to 4°C; all chemicals were from Sigma Chemical Company) was added to each assay tube, incubated for 10 min at 4°C and then centrifuged at 1450xg at 4°C for 10 min. The supernatant from each tube was poured off into a 6 ml vial containing 5 ml liquid scintillation fluid (#8824757), mixed thoroughly and then counted in a beta-counter for 2 min. Triplicate tubes were assayed for each sample. A series of standards was prepared by diluting estradiol-17ß (Sigma) in assay buffer. Specificity was confirmed by showing parallelism between a series of diluted quality control follicular fluid and culture medium pools (1/50, 1/100, 1/200, 1/400, 1/800 and 1/1600) and standard inhibition curves. Accuracy (using direct assay) was determined from the recovery of added standard estradiol-17ß to follicular fluid and culture medium, which averaged 104±2.9 (sem) and 98±3.5%, respectively.

Testosterone was analyzed by a highly specific solid-phase $^{125}$I RIA using a 'Coat-A-Count' kit purchased from Diagnostic Products Corporation (Los Angeles, USA) according to the kit instructions. Direct assay was validated again by parallelism tests (1/10, 1/20, 1/40 and 1/80) and recoveries of 96.8±0.53% and 87±3.2% of added testosterone from follicular fluid and culture medium, respectively.

Standard curves for progesterone, estradiol-17ß and testosterone ranged from 0.00313 to 6.40, 0.0025 to 0.80, and 0.0025 to 0.80 ng per tube, respectively.

Sensitivities of the assays, defined as the dose at binding (%) = (CBmax - 2SDBmax) x 100% / CBmax (CBmax: mean cpm of maximal binding, SDBmax: standard deviation of CPM of maximal binding tubes), were 0.003, 0.0033, 0.006 ng per tube for progesterone, estradiol-17ß and testosterone, respectively. Before assay, samples were diluted more than 100 times in assay buffer, except for the testosterone assay for culture medium in which a 50 times dilution was used. All the samples were assayed in a single batch to remove any effects of inter-assay variability on treatment differences. The intra-assay coefficients of variation were 6.1, 8.96 and 8.94% for progesterone, estradiol-17ß and testosterone assays, respectively.

***Statistical analysis***

The data in Experiment I were subjected to ANOVA for a randomized complete block design (replicates were blocks) (Steel and Torrie, 1980), (Model Y = Block + Treatment + error). The data in Experiment II were subjected to ANOVA for a split-plot design (Steel and Torrie, 1980). Effects of the time after eCG treatment were tested against the interaction term between plot (replicate-experiment) and eCG treatment; the effect of follicular size, and the interaction between the time after eCG treatment and follicular size, were tested against the interaction terms between plot and size and among plot and eCG treatment and follicular size. Those data, expressed as percentages, were analyzed after arcsine transformation. Statistical analysis was carried out using the general linear model procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Multiple comparisons were made using the PDIFF (LSD) function of SAS. Since there

were variations in follicular diameters in each treatment group and also because of the known heterogeneity in follicular fluid steroid concentrations, correlation and regression analyses were conducted as a more appropriate method for revealing relationships between oocyte maturation and their corresponding maturation environment. In this study, simple correlations and multiple regressions were analyzed by SAS procedures in which the effect caused by plots (replicate-experiments) was removed by creating dummy variables for plots (four dummy variables of D1, D2, D3 and D4 for five replicate-experiments) and obtaining partial correlations or multiple regressions when dummy variables were forced into the analyses (Draper and Smith, 1981). Data shown in Tables are expressed as least squares means and standard errors of least squares means are given in each case.

## RESULTS

### Experiment I: Effects of follicular shells on maturation, sperm penetration and male pronuclear development

Rates of nuclear maturation, sperm penetration and male pronuclear development after 47±1 h maturation culture and 10 h co-incubation with in vitro preincubated boar spermatozoa are shown in Table IV-1. Co-culture of oocyte-complexes with follicular shells did not alter the rate of nuclear maturation (P=0.25), but sperm penetration rate was inhibited (P=0.04). There was also a marked elevation of MPN formation in penetrated oocytes when follicular shells were added to the culture (P=0.0004).

**Experiment II: Effects of follicular size and time after eCG on maturation, sperm penetration and MPN development**

**1. Morphological parameters of follicles used for co-culture and steroid concentrations in follicular fluid and in culture medium collected at the completion of the maturation culture**

Least squares means (LSM) of diameters, fluid volumes and shell weights of the follicles used in co-culture with oocyte-complexes, and steroid concentrations in corresponding follicular fluid and in culture medium, are presented in Table IV-2. Progesterone in follicular fluid was not affected by time after eCG treatment (P=0.08), but was very significantly affected by follicular size (P = 0.003). The interaction effect between time after eCG and size on progesterone concentrations was not significant (P = 0.24). Follicular fluid testosterone concentrations were not significantly affected by either time after eCG treatment (P=0.97), follicular size (P=0.45), or their interaction. Estradiol-17ß concentrations in follicular fluid were not significantly different among times after eCG treatment (P = 0.63), but were significantly different among follicular sizes (P = 0.01); there was no interaction among these treatments. The ratio of follicular fluid progesterone to follicular fluid testosterone was not affected by time after eCG treatment (P=0.84), was affected by follicular size (P= 0.03 ), but not by their interaction (P=0.77). The ratio of follicular fluid $E_2$:T was not affected by time after eCG treatment (P=0.6), but was probably affected by follicular size (P=0.051); there was no effect of their interaction (P=0.55). Progesterone concentrations in culture medium were not

affected by either time after eCG treatment (P=0.28) or by size (P=0.46), but their interaction was significant (P=0.03). Multiple comparisons showed that progesterone in culture media was higher after culture with L36 follicular shells than with S36 (P=0.04) or L72 (P=0.02) follicular shells. Medium concentrations of testosterone were not affected either by time after eCG treatment (P=0.1) or by follicular size (P=0.56) and there was no interaction between these two main factors (P=0.06). Culture medium concentrations of estradiol-17ß were significantly (P=0.04) affected by the interaction among follicular size and time after eCG treatment. Multiple comparisons showed that culture medium $E_2$ concentrations were higher after culture with L72 follicular shells than in the other groups ($P < 0.04$; see Table IV-2). The ratio of culture medium $P_4$:T was greater in the 36h group than in the 72h group (P=0.04), but was not affected by size (P=0.97) and there were no interactions among these two main factors (P=0.36). The ratio of culture medium $E_2$:T was not altered either by time after eCG or by follicular size and there was no interaction among these factors.

**2. Maturation, sperm penetration and male pronuclear development**

Co-culture of follicular shells obtained at different times after eCG and of different sizes, with oocyte complexes, did not significantly affect nuclear maturation, sperm penetration and the average number of sperm in penetrated oocytes (Table IV-3). MPN formation in penetrated oocytes, however, was significantly affected by follicular size (P=0.01), but not by time after eCG treatment (P=0.39). The interaction between time after eCG and follicle size was also not significant (P=0.97).

### 3. Correlation analysis

Correlation coefficients of male pronuclear formation rate to nuclear maturation rate (MII), penetrability, follicular diameter, follicular fluid volume, follicular shell weight, and steroid concentrations and their ratios in follicular fluid and culture medium are shown in Table IV-4. In the case of MPN formation, there were three observations (one in S72 and two in L72) of very low MPN formation rates which were considered as extreme outliers by student's t test ($P < 0.05$) of their residuals (Weisberg, 1985) in a regression analysis between MPN formation rate and shell weight or follicular diameter; they were therefore excluded from correlation analysis.

MPN formation rate was positively correlated with diameter, fluid volume and shell weight of follicular shells co-cultured with the oocytes and to follicular fluid progesterone, estradiol-17ß and to the ratios of $P_4$:T and $E_2$:T, but not to follicular fluid testosterone (see Table IV-4). There were no correlations among MPN formation rate and steroid concentrations ($P_4$, T and $E_2$) or their ratios in culture medium. The rate of oocyte maturation (MII), but not oocyte penetrability, was correlated with MPN formation rate.

A separate correlation analysis of 36 h and 72 h follicular shell data showed that morphorlogical parameters and hormonal environment of the follicular shells harvested 72 h after eCG treatment was more significantly correlated to the MPN formation rate than that of follicles harvested 36 h after eCG treatment (see Table IV-4).

### 4. Multiple regression analysis

Multiple stepwise regression of male pronuclear formation rate on follicular

diameter, follicular fluid volume, follicular shell weight, steroids and their ratios in follicular fluid and in culture medium after culture (see variables in Table IV-4) showed that, MPN rate was correlated to diameter (r=0.45, P=0.005), the ratio of $P_4$:T in medium (r=0.37, P=0.02 when follicular diameter was constant) and progesterone (r=-0.35, P=0.04 when follicular diameter and $P_4$:T in medium were constant).

## DISCUSSION

Follicular somatic cells play a vital role in reprogramming the maturation of the oocyte (Moor et al., 1983). In the pig, co-culture of follicular cells with cumulus enclosed oocytes increased the rate of MPN formation in penetrated oocytes after IVF (Mattioli et al., 1988a; Ding et al., 1988). Our present study (Experiment I) further confirmed this effect of follicular cells on oocyte cytoplasmic maturation. Naito et al. (1988) reported that addition of porcine follicular fluid to culture medium also greatly increased MPN formation rate after IVF. Furthermore, Mattioli et al. (1989) obtained piglets from oocytes matured in vitro in the presence of the follicular shell. From these results it is evident that follicular cells secrete factors that support cytoplasmic, if not nuclear, maturation.

Although the time per se at which follicular shells were obtained after eCG treatment in the present study did not affect oocyte nuclear or cytoplasmic maturation, follicular size had a significant impact on MPN formation rate. Correlation analysis showed that MPN formation rate was correlated to diameter and shell weight of co-cultured follicles. Large follicles were more supportive of cytoplasmic maturation, whilst smaller

follicles within the presumed ovulatory population were less supportive of oocyte cytoplasmic maturation in the pig. Such differences in the ability of follicles within the ovulatory population to support oocyte maturation would provide one mechanism by which follicle heterogeneity could affect early embryonic development.

The number of granulosa cells per follicle was strongly and positively correlated with the diameter of the follicle before the LH surge (Foxcroft and Hunter, 1985; Grant, 1989). The number of granulosa cells therefore might affect the amount of maturational factors secreted by follicle shells during co-culture with oocytes. However, MPN formation rate did not differ between 36 h and 72 h eCG treated groups, although the mean follicle diameter of 5.61±0.28 (s.e. of LSM) mm 36 h post eCG stimulation was smaller (P=0.018) than the mean follicle diameter of 7.18±0.28 mm 72 h post eCG stimulation. Hence, the amount of maturational factors secreted by the follicle shell is probably not determined by the number of granulosa cells alone.

Changes in steroid concentrations in follicular fluid have been reported in both cyclic gilts (Babalola et al., 1988, Grant 1989; Wiesak et al., 1990) and in prepubertal gilts stimulated with eCG (Ainsworth et al., 1980; Weisak et al., 1990). Generally, our data from large follicles showed a similar pattern, in that progesterone and estradiol increased during follicular development. Although the pattern of steroid changes was similar to that reported by Ainsworth et al. (1980) and Wiesak et al. (1990) in eCG stimulated prepubertal gilts, the absolute values of steroids were much higher in the follicles used in our co-cultures; this might be due to the differences in the body weights of the gilts used (>100Kg in our study, 70 Kg in the studies of Ainsworth et al., 1980 and

Wiesak et al., 1990). Follicular fluid of small follicles had a higher testosterone than progesterone or estradiol concentration and the ratios of $E_2$:T and $P_4$:T were lower in small follicles than in large follicles (P=0.0514 and 0.0265, respectively, Table IV-2). It has been reported that atretic follicles from sheep (Moor et al., 1978), pig (Meinecke et al., 1982) and human (Mori et al., 1982; McNatty and Baird, 1978) secreted increased amounts of androgen and very small amounts of estrogen. However, Moor et al. also reported a high androgen content in normal small ovine follicles. Therefore the low $E_2$:T ratio in the small follicles in this study may indicate that these follicles were either partially atretic or at an early developmental stage, which may be an alternative explanation for small follicles being less supportive of oocyte maturation.

In this study we used eCG treated gilts to supply follicles. Wiesak et al. (1990) have reported that the heterogeneity of preovulatory follicles in eCG primed gilts is greater than in cyclic gilts. One effect of eCG on follicular development is to 'rescue' atretic follicles (Hay and Moor, 1978; Hay et al., 1979; McNatty et al., 1982), but this 'rescuing' effect disappeared 24 h after eCG injection in sheep (McNatty et al., 1982). In pig this 'rescuing' effect may, however, extend to the time of ovulation, since the number of ovulations can be greatly increased by eCG treatment; however, the developmental ability of ovulated oocytes is markedly reduced (Holtz and Schlieper, 1991). Therefore in our model, small follicles at 72 h post eCG injection may represent those eCG rescued atretic follicles that may be less supportive of oocyte cytoplasmic maturation.

After 47 h co-culture, steroid conditions in culture medium were similar in all four groups and characterized by high progesterone and low testosterone and estradiol

concentrations, regardless of the differences in follicular fluid steroid concentrations. Because both LH and prolactin were added to culture medium, luteinization of follicular cells may have occurred. These results are similar to reports of an increase of progesterone and a decrease of testosterone and estrogen production by follicular tissues after LH/hCG stimulation in vivo (Grant, 1989) and in vitro (Ainsworth et al., 1990).

The involvement of steroids in the reprogramming of mammalian oocyte maturation is controversial (see review by Thibault et al., 1987). Most of the conclusions are drawn from steroid inhibitor experiments and addition of steroids to culture medium. The use of aminoglutethimidine (AG, blocks cholesterol to P5), or cyanoketone or trilostane (block P5 to P4), or SU10603 (blocks P4 to 17α-OH-P4; 4-OH-Δ4: T to $E_2$-17ß) did not affect the resumption of meiosis in rat follicle-enclosed oocytes; similarly trilostane does not alter the rate of meiotic resumption of either cumulus enclosed or denuded hamster oocytes (see Thibault et al. 1987 and the references therein). In contrast, AG reduced the incidence of pig oocyte maturation (Szollosi and Gerard, 1983) and the 17α-hydroxylase inhibitor prevents the transition of ovine oocytes from MI to MII, even if estradiol is present in the medium. Osborn et al. (1986) demonstrated that diminishing the entire steroidogenic pathway with AG reduced the incidence of GVBD in ovine oocytes; moreover in the ewe, inhibition of the conversion from P4 to T by SU10603 severely inhibited oocyte meiotic resumption, whilst inhibition of aromatase activity by 1,4,6-androstatrien-3,17-dione did not affect nuclear maturation. Therefore, though total abolition of steroid secretion has a negative effect on oocyte nuclear maturation, a greatly distorted (increased) ratio of P4 to T and E2 has a more severe negative effect on oocyte

nuclear maturation (Osborn et al., 1986; Osborn and Moor, 1983).

Addition of $P_4$ to the culture either inhibited nuclear maturation in naked mouse oocytes (Eppig and Koide, 1978) and of corona enclosed rabbit oocytes (Smith et al., 1978), or enhanced nuclear maturation of cumulus enclosed rabbit oocytes (Bae and Foote, 1975). Estrogen (McGaughey, 1977) and androgen (Rice and McGaughey, 1981) have been reported to inhibit pig oocyte maturation. Mattioli et al. (1988b) demonstrated an ether extract of follicle conditioned medium or progesterone, but not estradiol nor androgens, partially mimicked the effect of conditioned medium on cytoplasmic maturation of pig oocytes. These results suggest that progesterone may exert a positive effect on full oocyte maturation.

Recently, De Sutter et al. (1991) reported that, in human, oocyte maturity was associated with an increase of follicular fluid progestin content and a significant decrease of androstenedione levels. Vanluchene et al. (1991) also reported that oocyte maturity was correlated to higher level of follicular fluid P4, 17-hydroxyprogesterone, 16α-hydroxyprogesterone and 20α-dihydroprogesterone. Higher fertility was associated with higher levels of 20α-dihydroprogesterone, and progesterone, and lower levels of androstendione (Vanluchene et al. 1991). Similarly, Itskovitz et al. (1991) found that follicles containing immature human oocytes have a lower FF progesterone and estradiol concentration than those containing mature oocytes. Collectively these results suggest that the follicular steroid environment may affect oocyte nuclear maturation and fertilization. However, using leuprolide acetate to stimulate follicular development in human IVF procedure, FF $P_4$ and $E_2$ were lower than those stimulated with gonadotrophins, but oocyte

nuclear maturation was not affected (Brzyski et al., 1990), suggesting no functional relationship between FF steroids and nuclear maturation of oocytes.

In the present study, we found MPN formation was positively correlated to follicular fluid progesterone and estradiol content, and their ratios to testosterone, in follicles whose shells were used for co-culture with immature oocytes. The estrogen and progesterone contents and their ratios to testosterone in follicular fluid in this study, may only partially reflect the physiological status of follicles from which follicle shells were obtained for co-culture with oocyte-complexes. Thus the significant correlation of MPN formation to follicular fluid progesterone and estradiol may not necessarily reflect a cause and effect relationship between these variables; rather it may reflect the relationship between MPN formation rate and the physiological status of co-cultured follicles, similar to the correlation between MPN formation and follicle size (diameter).

There were no simple correlations between MPN formation and steroids in culture medium and their ratios. After the main effect of follicular diameter was accounted for, however, the ratio of progesterone to testosterone was positively correlated (P=0.003) with MPN formation rate. Furthermore, after the effects of both follicular diameter and progesterone to testosterone ratio in culture medium were removed, progesterone content in culture medium was negatively correlated (P=0.038) with MPN formation rate. Collectively, these results suggest that cytoplasmic maturation of porcine oocytes was mainly affected by non-steroid factors secreted by the follicular shell. Steroid factors could also be involved. However, whether these are true cause-effect relationships again awaits further clarification.

The correlation coefficients between MPN formation and morphological parameters and the steroid environment of the follicles used for co-culture were more significant in the 72h, than in the 36h, follicular data (see Table IV-4). This suggests that, the physiological diversity of follicles at the later stages of development has a greater impact on their ability to support oocyte maturation.

Although a significant effect of co-culture with follicle shells obtained at different times after eCG and from different sized follicles, on nuclear maturation was not detected (data not shown), correlation analysis showed a significant correlation between nuclear maturation rate and MPN formation rate, suggesting that factors that affect cytoplasmic maturation (MPN formation) would also affect nuclear maturation.

In conclusion, our data support the two hypotheses tested; 1) that follicular cells play an important role in supporting full oocyte maturation, 2) that the extent to which this supportive effect is expressed is affected by the confirmed heterogeneity in follicular development.

Table IV-1. Effects of follicle shells on oocyte maturation, sperm penetration and male pronuclear development.

| Treatment | Two follicle shells | No follicle shells |
|---|---|---|
| No. of replicates | 8 | 8 |
| No. eggs examined | 179 | 177 |
| MII oocytes | | |
| No. | 163 | 169 |
| % (LSM±se)[1] | 90.49±2.97 | 94.81±2.97 |
| Sperm penetration | | |
| No. | 122 | 140 |
| % (LSM±se)[2] | 73.53±2.22 | 81.65±2.22 * |
| MPN formation | | |
| No. | 99 | 69 |
| % (LSM±se)[3] | 78.15±4.37 | 38.56±4.37 ** |

[1] Least squares means (LSM) of the percentage of oocytes cultured reaching MII against the total number of eggs examined.
[2] Least squares means of the percentage of MII oocytes penetrated by sperm of all MII oocytes.
[3] Least squares means of the percentage of penetrated oocytes with MPN of all penetrated MII oocytes.

* Means within a row differ (P=0.04); ** Means within a row differ (P=0.0004)

Table IV-2. Morphological parameters of follicles whose shells were used for co-culture and steroid concentrations in follicular fluid and in culture medium collected at the completion of the maturation culture (LSM).

| Source of follicle shell | 36 h post eCG | | 72 h post-eCG | | Standard error of LSM |
|---|---|---|---|---|---|
| | small | large | small | large | |
| Nos of follicles | 11 | 11 | 11 | 11 | |
| Follicle diameter (mm) (range) | 4.13a (3.5-5.0) | 7.08b (6.0-9.0) | 5.60c (4.0-7.0) | 8.75d (7.5-11.0) | 0.30 |
| Follicle shell weight (mg) (range) | 10.79a (6.0-15.0) | 30.79b (20.0-58.0) | 17.54a (5.7-38.6) | 47.69c (31.4-73.8) | 2.759 |
| Follicular fluid volume (µl) (range) | 33.11a (14-50) | 140.27bc (71-317) | 70.46ab (10-166) | 278.2c (171-512) | 22.56 |
| **Steroids in follicular fluid** | | | | | |
| $P_4$ | 420.14a | 1219.94ab | 459.96a | 1975.40b | 282.22 |
| $E_2$ | 208.33a | 270.49ab | 169.08a | 391.91b | 42.76 |
| T | 787.20a | 1054.97a | 1358.26a | 440.12a | 413.86 |
| $P_4$:T | 0.6388a | 8.6863a | 0.9114a | 7.3527a | 2.693 |
| $E_2$:T | 0.296a | 1.957a | 0.340a | 1.293a | 0.576 |
| **Steroids in culture medium** (ng/ml) | | | | | |
| $P_4$* | 1104.47a | 1396.47b | 1198.00ab | 1040.04a | 86.71 |
| $E_2$* | 40.11a | 29.95a | 47.84a | 75.52b | 7.70 |
| T | 50.22ab | 12.52a | 46.12ab | 113.04b | 23.89 |
| $P_4$:T | 239.76ab | 305.78a | 119.08ab | 47.44b | 71.47 |
| $E_2$:T | 3.309a | 5.486a | 3.483a | 2.955a | 1.483 |

a-d: Multiple comparisons were made regardless of the interactions and means within a row without common subscripts were significantly different ($P < 0.05$).
*: There is an interaction between time after PMSG (36 and 72 h) and follicle size.

Table IV-3. Effect of maturational state of follicular shells on oocyte maturation (MII), sperm penetration of MII, male pronuclear formation rate and the average number of sperm in penetrated oocytes.

| Sources of follicular shells | 36 h post-eCG | | 72 h post-eCG | | Standard error of LSM |
|---|---|---|---|---|---|
| | small | large | small | large | |
| No. of dishes | 11 | 11 | 11 | 11 | |
| No. eggs examined | 143 | 145 | 136 | 133 | |
| Maturation | | | | | |
| No. | 129 | 127 | 123 | 118 | |
| % (LSM[1]) | 89.21a[4] | 86.83a | 89.78a | 85.91a | 2.31 |
| Sperm penetration | | | | | |
| No. | 107 | 113 | 98 | 103 | |
| % (LSM[2]) | 80.45a | 85.97a | 77.79a | 86.76a | 5.90 |
| Male pronuclear formation | | | | | |
| No. | 75 | 89 | 60 | 77 | |
| % (LSM[3]) | 67.15ab | 77.49a | 53.13b | 66.66ab | 4.05 |
| Average No. of sperm in penetrated oocytes (LSM) | 4.20a | 3.39a | 3.32a | 3.50a | 2.7 |

[1] Least squares means (LSM) of the percentage of oocytes cultured reaching MII of the total number of eggs examined.

[2] Least squares means of the percentage of MII oocytes penetrated by sperm of all MII oocytes.

[3] Least squares means of the percentage of penetrated oocytes with male pronucleus/pronuclei (MPN) of all penetrated MII oocytes penetrated.

[4] Multiple comparisons were made regardless of the interactions and means within a row without common subscripts were significantly different ($P < 0.05$).

Table IV-4. Correlation coefficients of male pronuclear formation rate (MPN) to other variables measured.

| Variables* | Overall (n=41) | | 36 h (n=22) | | 72 h (n=19) | |
|---|---|---|---|---|---|---|
| | r | P | r | P | r | P |
| Follicular morphology | | | | | | |
| FD | 0.45 | 0.005 | 0.54 | 0.020 | 0.73 | 0.002 |
| FFV | 0.40 | 0.015 | 0.48 | 0.043 | 0.64 | 0.009 |
| FSW | 0.43 | 0.008 | 0.50 | 0.034 | 0.68 | 0.005 |
| Follicular fluid | | | | | | |
| $P_4$ | 0.37 | 0.023 | 0.32 | 0.195 | 0.55 | 0.033 |
| $E_2$ | 0.40 | 0.015 | 0.34 | 0.156 | 0.70 | 0.004 |
| T | -0.04 | 0.813 | -0.01 | 0.961 | -0.15 | 0.588 |
| $P_4$/T | 0.39 | 0.018 | 0.40 | 0.098 | 0.47 | 0.073 |
| $E_2$/T | 0.43 | 0.007 | 0.58 | 0.011 | 0.42 | 0.114 |
| Culture medium after culture | | | | | | |
| $P_4$ | -0.12 | 0.471 | 0.14 | 0.568 | -0.37 | 0.172 |
| $E_2$ | 0.04 | 0.828 | -0.17 | 0.508 | 0.40 | 0.140 |
| T | 0.09 | 0.600 | -0.25 | 0.315 | 0.37 | 0.167 |
| $P_4$/T | 0.25 | 0.122 | 0.38 | 0.121 | -0.22 | 0.428 |
| $E_2$/T | 0.24 | 0.150 | 0.19 | 0.456 | 0.32 | 0.241 |
| Oocyte maturation | | | | | | |
| MII | 0.45 | 0.0032 | 0.38 | 0.086 | 0.53 | 0.019 |

* FD: follicular diameter; FFV: follicular fluid volume; FSW: follicular shell weight; $P_4$: progesterone; $E_2$:estradiol-17ß; T: testosterone; MII: metaphase II.

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# CHAPTER V FOLLICULAR STIMULATING HORMONE STIMULATED FOLLICULAR SECRETIONS SUPPORT BETTER OOCYTE MATURATION IN PIGS

## INTRODUCTION

Follicular cell co-culture supports both nuclear (Ding et al., unpublished observations) and cytoplasmic (Mattioli et al., 1988 a and b; Moor et al., 1990; Ding and Foxcroft, 1992; Zheng and Sirard, 1992; Nagai et al., 1993) maturation in porcine oocytes. Furthermore, it has been reported that supplementation of porcine follicular fluid to oocyte maturation medium (Naito et al., 1988, 1989; Yoshida et al., 1992; Funahashi and Day, 1993) or use of follicle conditioned medium (Mattioli et al., 1988 a and b) to culture pig immature oocytes in vitro increased the rate of male pronuclear formation and developmental capacity after in vitro fertilization. These results, therefore, suggest that factors secreted by follicular cells are involved in regulating oocyte maturation.

Gonadotropins are known to affect follicular cell differentiation. During the follicular phase in vivo, FSH induces early maturational changes and stimulates estradiol production by follicular cells, resulting in the release of the preovulatory surge of LH. LH (and probably also prolactin) induces luteinization of follicular cells and ovulation and stimulates progesterone production (see review by Gore-Langton and Armstrong, 1988).

This series of maturational changes is also paralleled by a complex series of events determining follicle-oocyte interactions and hence oocyte maturation (Foxcroft et al., 1993). Therefore, follicular cell conditioned media produced in the presence of different gonadotropin combinations may contain factors secreted by follicular cells which in both quantitative and qualitative terms may differentially affect oocyte maturation. Furthermore, since follicular cells may undergo maturational differentiation chronologically during culture, conditioned media collected over different periods may vary in their ability to support oocyte maturation. The objectives of present studies are, therefore, to test the above hypotheses.

## MATERIALS AND METHODS

**Preparation of oocytes and follicular shells:** Ovaries were collected from slaughtered prepubertal gilts of about 100 kg liveweight, at a local abattoir and transported to the laboratory in a polystyrene box to prevent major changes in temperature. Follicles with a diameter 3-7 mm were dissected from the ovaries and oocyte-cumulus-mural granulosa cell complexes (oocyte-complexes) were isolated from healthy follicles and were used as the source of immature oocytes. Follicular shells from 5-7 mm healthy follicles with oocytes and follicular fluid removed, were used to produce conditioned media. All dissection procedures have been described previously (Ding and Foxcroft, 1992).

**Production of follicular shell conditioned medium:**

Five or ten follicular shells were cultured in a 50 ml (25 $cm^2$) tissue culture flask (Falcon 3103) containing 10 ml fresh oocyte maturation medium (as described by Ding and Foxcroft, 1992, without supplementation of FSH, LH and prolactin, see appendix) on a rocking platform in 5% $CO_2$ in humidified air at 39°C. Gonadotropins were added to cultures according to the experimental protocols described below (see studies 1 and 2). After culture, conditioned media were centrifuged at 2083 x g for 10 min and supernatants were filtered using a 0.22μm micro-syringe filter and preserved at -30°C until used.

**Oocyte maturation and fertilization:**

Oocyte-complexes (10-15 oocytes per dish) were cultured in 2 ml culture medium according to the experimental protocols described below (see study 1 and 2). Cultures were carried out under the same gas and temperature conditions as described above. After 47 h in culture, cumulus enclosed oocytes from each culture dish were transferred to 1 ml fertilization medium (B.O. medium, see appendix) containing 5 x $10^5$ spermatozoa obtained by in vitro capacitation of ejaculated boar spermatozoa as described in detail previously (Ding et al, 1992 a; Ding and Foxcroft, 1992). After 6 h of co-culture with spermatozoa, oocytes were transferred into 1 ml sperm-free embryo culture medium (modified Krebs'-Ringer-bicarbonate medium, Petters et al., 1990, without $KH_2PO_4$; see appendix), cultured for another 5 h and then prepared for evaluation of nuclear status.

Oocytes were denuded of cumulus cells, mounted on a slide with a whole-mount technique, and fixed for at least 48 h in 25% acetic acid in ethanol. Oocytes were then

stained with 1% lacmoid in 45% acetic acid and their nuclear status were examined under a phase-contrast microscope. Oocytes were identified as GV, PMI, MI, MII and penetrated oocytes with detached unswollen or slightly swollen sperm head(s) (SH1), with further swollen sperm head(s), and/or with male pronucleus/pronuclei, as described previously (Ding et al, 1992 b, Ding and Foxcroft, 1992).

**Steroid measurements of conditioned medium:**

Progesterone and estradiol in conditioned medium were determined by direct (none extraction) RIA, as described previously (Ding and Foxcroft, 1992). Standard curves for progesterone and estradiol-17ß ranged from 0.00313 to 6.40 and 0.0025 to 0.80 ng per tube, respectively. Sensitivities of the assays, defined as the dose at binding (%) = $CB_{max}$ - $2SDB_{max}$) x 100% / $CB_{max}$ ($CB_{max}$: mean cpm of maximal binding, $SDB_{max}$: standard deviation of cpm of maximal binding tubes), were 6.25 and 6.25 pg per tube for progesterone and estradiol, respectively. All samples were assayed in a single assay with intraassay CVs of 5.31 and 6.48% for progesterone and estradiol-17ß, respectively, to remove any effects of interassay variability on treatment differences.

**Statistical Analysis**

Data were analyzed using ANOVA for a randomized complete block design (replicated experiments were blocks) (Model Y = Block + Treatment + Error). Percentage data were subjected to arcsine transformation before analysis. Statistical analysis was carried out using the general linear model procedures of the Statistical Analysis System

(Version 6.07, SAS Institute, Cary, NC). Multiple comparisons were made by the PDIFF (LSD) function of SAS. Orthogonal contrast analysis was also used to analyze main effects in study 1 (FSH-stimulated vs FSH-LH-Prl-stimulated conditioned media; further addition of FSH-LH-Prl during oocyte maturation vs no FSH-LH-Prl addition, etc) (Steel and Torrie, 1980).

## RESULTS

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batches of follicular conditioned media were produced. In each batch, five follicular shells were cultured in 10 ml oocyte culture medium (2 ml/shell) containing 2.5μg/ml FSH (USDA-pFSH-B-1,AFP-5600) only (FSH-stimulated conditioned medium): another five follicular shells were cultured in 10 ml of culture medium containing 2.5 μg/ml FSH, 2.5 μg/ml LH (NIADDK-oLH-26, AFP-5551b) and 20 ng/ml prolactin (USDA-pprl-B-1, AFP-5000) (a hormone combination previously used for oocyte maturation, see Ding and Foxcroft, 1992) (FSH-LH-Prl-stimulated conditioned medium). After 48 h, conditioned media were collected and preserved at -30°C until use.

A randomized complete block experiment was designed to examine the effect of follicular conditioned media on oocyte maturation (oocyte maturation experiment). The experiment was replicated five times using four batches of conditioned media, and immature oocyte-complexes (see Materials & Methods) used in each replicated experiment were randomly divided into six culture dishes (10-15 oocytes per dish) and subjected to different culture conditions (Table V-1).

Table V-2 shows the effect of follicular conditioned media on oocyte maturation. The proportion of oocytes developing beyond the second metaphase (>MII) and the penetrability of MII oocytes were not affected by supplementation treatment. Male pronuclear development in penetrated oocytes, however, was significantly affected by treatment (see Table V-2). Multiple comparisons showed that the high rate of male pronuclear formation in penetrated oocytes matured in FSH-stimulated follicular conditioned medium supplemented with the FSH-LH-Prl mixture during maturation culture was similar to that in oocytes matured in fresh maturation medium co-cultured with one follicular shell (group 6, P=0.82) but significantly higher than in oocytes matured in fresh maturation medium without co-culture with a follicular shell (group 5, P=0.05). Oocytes matured in FSH-LH-Prl stimulated follicular conditioned medium (groups 3 and 4) had lowest MPN formation rates. Orthogonal contrasts also showed that oocytes matured in FSH-stimulated follicular conditioned medium (groups 1 and 2) had a significantly higher MPN formation rate than those oocytes matured in FSH-LH-Prl-stimulated follicular conditioned medium (groups 3 and 4, P=0.0.001). Furthermore, further addition of the FSH-LH-Prl mixture during the oocyte maturation period may increase MPN formation rate (comparison of groups 2 and 4 with groups 1 and 3, P=0.06).

With respect to the steroid content of conditioned media neither estradiol concentrations [13.76±2.98 (n=4) and 18.75±2.47 (n=4) ng/ml, respectively] nor progesterone concentrations [537.46±136.3 (n=4) and 864.6±346.0 (n=4) ng/ml, respectively] in FSH- and FSH-LH-Prl-stimulated follicular conditioned media were

significantly different (P>0.19).

**Study 2:**

Study 1 showed that follicular secretions stimulated by FSH during the production of conditioned medium supported better cytoplasmic maturation of oocytes. In a second study we further tested the effect of follicular secretions produced during the different periods of conditioning under the stimulation of FSH. The 48 h condition period was divided into consecutive 24 h periods and because the conditioning period was halved, the number of follicular shells was doubled. To ensure an adequate supply of the conditioned media, seven batches of follicular conditioned media were produced by culturing ten follicular shells in 10 ml oocyte culture medium (1 ml/shell) supplemented with 2.5 µg/ml FSH. After 24 h of culture, medium was collected (1st 24h) and replaced with 10 ml fresh medium supplemented with 2.5µg/ml FSH followed by culture for another 24 h. Medium was then collected again (2nd 24h). Conditioned media were stored until use as described earlier.

A randomized complete block design was used again for the oocyte maturation experiment. The experiment consisted three treatment groups: 1) oocyte-complexes cultured in 1st 24h FSH-stimulated follicular conditioned medium, 2) cultured in 2nd 24h FSH-stimulated follicular conditioned medium and 3) cultured in fresh culture medium (Control). The FSH-LH-Prl mixture was added to each treatment during the oocyte maturation period (doses as in study 1). The experiment was replicated four times using four of the seven batches of conditioned media produced and duplicate oocyte cultures

for each treatment.

Table V-3 shows that treatments did not alter maturation rates ($P>0.05$) or penetrability ($P>0.05$) of in vitro matured oocytes. However, MPN formation rate was higher in oocytes matured in both conditioned media than in those matured in fresh culture medium ($P<0.05$). MPN formation rates were similar in both 1st 24h and 2nd 24h groups, which were similar to those matured in FSH-stimulated follicular conditioned medium supplemented with FSH-LH-Prl during oocyte maturation observed in Study 1.

The progesterone concentration of 179.05±21.3 ng/ml (n=6) in 1st 24h conditioned medium was significantly lower than that of 307.51±50.22 ng/ml (n=7) in 2nd 24h conditioned medium ($P=0.046$). Conversely, the estradiol concentration of 15.76±1.66 ng/ml (n=7) in 1st 24h conditioned medium was higher than that of 10.48±1.78 ng/ml (n=6) in 2nd 24h conditioned medium ($P=0.043$).

## DISCUSSION

Follicular secretions obtained from culture of follicular shells under the stimulation of FSH, LH and prolactin has been reported to support better cytoplasmic maturation in porcine oocytes than in the absence of hormones (Mattioli et al., 1988 a and b). Our results, however, did not confirm this effect. The present results showed that follicular secretions stimulated by FSH alone supported better cytoplasmic maturation, which was comparable to the results obtained from co-culture with follicular shells. If anything, follicular secretions stimulated by a mixture of FSH, LH and prolactin (similar to

treatment of Mattioli et al., 1988 a, b) exerted an inhibitory effect on cytoplasmic maturation when compared with the fresh culture medium. The precise reasons for this are not clear. However, several differences exist between these two culture system. 1) Oocytes were fixed 10-11 h after insemination in our system but at 14 h in Mattioli's study. 2) Cumulus enclosed oocytes associated a piece of mural granulosa were used as a source of immature oocytes in our culture, while cumulus-enclosed oocytes were used by Mattioli et al. 3) Follicular tissue and oocytes used in our cultures were obtained from pre-pubertal gilts, as compared to cyclic gilts. 4) Follicular conditioned media in our study were produced by culturing follicular shells alone; Mattioli et al. produced conditioned media in the presence of oocytes and factors resulting from interactions between follicular cells and oocytes were therefore probably involved.

The reason for FSH stimulated follicular secretion is supporting better cytoplasmic maturation is unknown. Physiologically, FSH effects are dominant before the time of the LH surge. Granulosa and theca cells stimulated by FSH at this stage are able to convert androgens of thecal origin to estrogen, resulting in an estrogen dominated environment in the follicle. After the LH surge, steroid production is substantially altered. Production of estrogen declines and production of progesterone increases (Gore-Langton and Armstrong, 1988; Ainsworth et al., 1980; Grant et al., 1989). Estradiol and progesterone production by follicular shells stimulated by FSH alone or a mixture of FSH, LH and Prl in the present study were not significantly different ($P > 0.05$), although there was a trend for follicular shells stimulated by FSH alone to be less steroidogenic than when stimulated by a mixture of FSH, LH and Prl (Estradiol: 13.76±2.98 to 18.75±2.47; Progesterone:

537.46±136.3 to 864.6±346.0 ng/ml). It seems that follicular cells stimulated by FSH alone also underwent luteinization during culture, which is not comparable to the in vivo situation. The overall change in the progesterone to estradiol ratio in both conditioned media were similar to those found in follicular fluid just before ovulation (100-108 h/30-36 h post eCG/hCG, Ainsworth et al., 1980). Mattioli et al. (1988 b) showed that steroid hormones (progesterone in particular) were mainly responsible for the improvement of cytoplasmic maturation of oocytes matured in conditioned medium. Our results do not imply this. Rather they further support earlier suggestions that non-steroidal follicular factors are also involved in regulation of oocyte cytoplasmic maturation (Ding and Foxcroft, 1992; Yoshida et al., 1992).

Further supplementation of a mixture of FSH, LH and Prl to both types of conditioned media during oocyte culture marginally improved the degree of cytoplasmic maturation of oocytes (P=0.06, see Table V-2). It has been reported by Mattioli et al. (1991) that oocytes matured in medium containing FSH only had low male pronuclear formation rate (44%), while those matured in medium containing LH had high male pronuclear formation rate (72%). Thus the effect of further supplementation of gonadotropins during oocyte maturation, in conditioned medium stimulated by FSH alone, may be explained by an LH effect on cytoplasmic maturation. However, it could not explain this effect in oocytes matured in conditioned medium stimulated by a mixture of FSH, LH and Prl. One possibility is that the hormones had been used up by follicular cells during follicle conditioning period.

Conditioned medium produced in the first or second 24 h period of culture in the

presence of FSH had a similar effect on oocyte nuclear and cytoplasmic maturation, suggesting that the concentrations of stimulating factors in these two conditioned medium are similar, although the degree of luteinization of follicular cells was lower in the first 24 h period than in the second 24 h period of culture. This suggests that steroidal factors may not be the most critical regulators of cytoplasmic maturation.

In summary, these results suggest that FSH stimulated follicular secretions are most suitable for supporting porcine oocyte maturation in vitro.

Table V-1. Oocyte Maturation Media

| Treatment Groups | Conditioned Medium (M.) | FSH-LH-Prl Supplementation During Oocyte Maturation | Follicular Shell Co-culture |
|---|---|---|---|
| 1 | FSH-stimulated Conditioned M. | - | - |
| 2 | FSH-stimulated Conditioned M. | + | - |
| 3 | FSH-LH-Prl stimulated Conditioned M. | - | - |
| 4 | FSH-LH-Prl stimulated Conditioned M. | + | - |
| 5 | Fresh M. (Control I) | + | - |
| 6 | Fresh M. (Control II) | + | + |

Table V-2. Effect of Follicular Shell Conditioned Medium on Oocyte Nuclear Maturation, Sperm Penetration and Male Pronuclear Development[1]

| Conditioned Medium | FSH-LH-Prl Addition during Oocyte Maturation | No. Eggs Examined | No. Eggs >MII (mean±se%)[2] | No. Eggs Penetrated (mean±se%)[3] | No. Eggs With MPN (mean±se%)[4] |
|---|---|---|---|---|---|
| FSH-stimulated Conditioned medium | - | 63 | 58 a[6] (93.1±5.2) | 53 a (90.9±9.1) | 27 ab (54.1±10.6) |
| | + | 62 | 60 a (96.9±3.1) | 58 a (96.0±4.0) | 40 a (71.1±9.7) |
| FSH-LH-Prl-stimulated Conditioned medium | - | 68 | 62 a (92.3±5.7) | 60 a (97.5±2.5) | 16 c (28.5±7.7) |
| | + | 62 | 58 a (94.1±2.5) | 54 a (93.3±6.7) | 21 bc (40.0±8.6) |
| Fresh medium | + | 65 | 64 a (98.8±1.2) | 61 a (95.3±4.5) | 24 bc (41.0±8.4) |
| Fresh medium + 1FS[5] | + | 64 | 60 a (92.3±5.0) | 59 a (98.5±1.5) | 36 a (71.4±15.0) |

[1] The experiment was replicated for five times.

[2] Mean of the percentage of oocytes developped to beyond MII of the total number of oocytes examined.

[3] Mean of the percentage of MII oocytes penetrated by sperm of all >MII oocytes.

[4] Mean of the percentage of penetrated oocytes with MPN of all penetrated MII oocytes.

[5] One follicular shell was co-cultured with oocytes.

[6] Means within a column without common superscripts were significantly different ($P < 0.05$).

TableV-3. Effects of Follicular Secretions Produced during the Different Culture Periods under the Stimulation of FSH on Oocyte Maturation, Sperm Penetration and Male Pronuclear Development.

| Treatment Groups | No. Replicates | No. Eggs Examined | No. Eggs >MII (mean±se%)[1] | No. Eggs Penetrated (mean±se%)[2] | No. Eggs With MPN (mean±se%)[3] |
|---|---|---|---|---|---|
| 1st 24h | 8 | 75 | 72 a[4] (95.2±2.6) | 56 a (79.9±8.5) | 36 a (61.5±5.8) |
| 2nd 24h | 8 | 86 | 73 a (85.3±3.9) | 61 a (83.1±5.7) | 40 a (66.0±9.0) |
| Fresh Medium | 8 | 84 | 80 a (95.0±3.8) | 70 a (88.8±4.8) | 27 b (37.9±9.3) |

[1] Mean of the percentage of oocytes developped to beyond MII of the total number of oocytes examined.

[2] Mean of the percentage of MII oocytes penetrated by sperm of all >MII oocytes.

[3] Mean of the percentage of penetrated oocytes with MPN of all penetrated MII oocytes.

[4] Means within a column without common superscripts were significantly different ($P < 0.05$).

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# CHAPTER VI CONDITIONED MEDIA PRODUCED BY FOLLICULAR SHELLS OF DIFFERENT MATURITY AFFECT MATURATION OF PIG OOCYTES

## INTRODUCTION

The quality of in vitro matured porcine oocytes is greatly improved by follicle shell co-culture or by supplementation with follicular fluid or by culture in follicle cell conditioned medium (Ding et al., 1988; Mattioli et al, 1988 a, b; 1989; Naito et al., 1988; 1989; Ding and Foxcroft 1992; Yoshida et al., 1992; Zheng and Sirard, 1992; Nagai et al., 1993; Funahashi and Day, 1993). These studies indicate that follicle cells secrete stimulatory factors which support cytoplasmic maturation of the oocyte.

Follicular maturation is paralleled by, and functionally related to, maturation of the oocyte inside the follicle. During follicular maturation, follicle cells (theca and granulosa cells) undergo functional changes in terms of their secretory activities. Thus it is reasonable to assume that follicle cells at different maturational stages may produce a different quantity and/or quality of oocyte maturation stimulatory factors, which in turn may affect oocyte quality.

Heterogeneity in follicular development has been observed in both naturally cyclic gilts (Grant et al., 1989; Hunter et al., 1989), in eCG-stimulated immature gilts (Wiesak et al., 1990) and in weaned sows (Foxcroft et al., 1987). As follicles in the selected

preovulatory population differ in size by up to 2 mm and show marked variability in steroid content and gonadotropin binding ability, it has been suggested that this heterogeneity might influence the quality of oocytes ovulated (Foxcroft and Hunter, 1985; Hunter and Wiesak, 1990). Our recent study (Ding and Foxcroft; 1992), using oocyte-cumulus-granulosa complexes co-cultured with individual follicle shells obtained from eCG stimulated gilts, showed that the extent of oocyte cytoplasmic maturation (male pronuclear formation rate) was affected by follicle size. Small follicle shells were less supportive than large follicle shells of oocyte cytoplasmic maturation, supporting the above hypothesis. However, it has been reported that follicular heterogeneity was greater in eCG treated gilts than that in naturally cyclic gilts (Wiesak et al., 1990; Hunter and Wiesak, 1990). Therefore the results obtained using eCG treated follicles may not represent the situation in naturally cyclic gilts. The present experiment was therefore designed to further test the hypothesis that follicle secretions produced by follicle shells of different maturational status would affect oocyte maturation in vitro when follicle shells from cyclic gilts were used.

## MATERIALS AND METHODS

To facilitate the development of oocyte maturation experiments using a split plot design mixed with a nest factor, conditioned media produced by follicles with different maturational status were used in this experiment. A previous experiment (Ding and Foxcroft, 1993) showed that follicle conditioned medium stimulated FSH supported

oocyte cytoplasmic maturation.

**Production of follicle conditioned media**

***Animal treatment.*** Twenty-five crossbred pubertal gilts (Camborough x Canabrid; Pig Improvement (Canada) Ltd., Acme, AB, Canada) weighing about 100 kg were raised at the Swine Research Center of the University of Alberta under commercial conditions. Estrus was checked daily using vasectomized boars. Acceptance of mounting by a vasectomized boar was defined as day 0 of the estrous cycle. Nineteen gilts showing a normal estrous cycle length of 20-22 days were selected for the experiment and assigned into one of two groups. One group of nine gilts was injected with 500 ng/head Estrumate (cloprostenol, a prostaglandin $F_{2\alpha}$ agonist, Coopers Agropharm Inc. Ontario) at 0900 h on day 15 and were slaughtered at 0900 h on day 17 of the cycle. Another group of ten gilts, also treated with Estrumate at 0900 h on day 15, was slaughtered at 0900 h on day 20 of the cycle. All gilts were slaughtered at a local abattoir.

***Preparation of follicle tissue.*** Immediately after slaughter, ovaries from individual animals were recovered and packed separately in plastic bags, clearly labelled and placed in a polystyrene box to prevent any rapid change of temperature and transported to the laboratory within 40 min of slaughter. All healthy-looking follicles (classified under a dissecting microscope) with a diameter ≥ 3 mm were dissected from each pair of ovaries. Detailed dissection procedures were described previously (Ding and Foxcroft, 1992). The 15 biggest follicles (assuming that all these follicles would be destined to ovulate) were selected, from which the five largest and five smallest were chosen as large and

follicles. Diameters were measured and then follicles were opened in a petri dish and the follicular fluid from each group of five follicles was pooled and stored at -30°C awaiting steroid measurement. The five follicle shells were weighed and then used to produce follicle conditioned medium.

***Production of conditioned media***. The five follicle shells in each category were washed four times using dissection medium (see Ding and Foxcroft, 1992 but without FSH, LH and prolactin) and then cultured in 10 ml of oocyte maturation medium containing 2.5 μg/ml FSH (USDA-pFSH-B-1, AFP-5600) (see Ding and Foxcroft, 1993) in a 50 ml (25 $cm^2$) tissue culture flask (Falcon 3103). Culture was carried out on a rocking platform in 5% $CO_2$ in humidified air at 39°C. After 48 h culture, culture media were centrifuged at 2083 g for 10 min and supernatants were filtered using a 0.22μm micro-syringe filter and preserved at -30°C until use.

**Oocyte maturation and fertilization in vitro**

***Oocyte maturation***. The experiment involved a split plot design including nested factors. Six batches of conditioned media produced by follicle shells of six gilts for each Day of treatment (Day 17 and Day 20) were randomly selected for oocyte maturation. Thes conditioned media were randomly assigned to three plots; thus, each plot contained conditioned media produced by follicle shells of two Day 17 and two Day 20 gilts (nested within Plot and Day). For each gilt, there were two conditioned media produced by small and large follicle shells, respectively, which was a split factor. The entire experiment was design was shown in Table VI-1. Therefore, on each experimental date, there were eight

dishes (2 Day x 2 Gilt x 2 Size = 8] (see Table VI-1). Each dish contained 2 ml conditioned medium and supplemented with 2.5 μg/ml FSH (USDA-pFSH-B-1,AFP-5600), 2.5 μg/ml LH (NIADDK-oLH-26, AFP-5551b) and 20ng/ml prolactin (USDA-pprl-B-1, AFP-5000)[1] (see Ding and Foxcroft, 1993). Immature cumulus-enclosed oocytes directly connected with a small piece of mural granulosa (oocyte-complexes) were obtained from ovaries of slaughtered prepubertal gilts of unknown origin from a local abattoir (see Ding et al., 1992 a). Oocyte-complexes in each experimental date were pooled and randomly divided into eight culture dishes (10-14 oocytes per dish) and cultured on a rocking platform under the same conditions as for the production of conditioned media.

***Fertilization.*** After 47 h culture, oocytes from each dish were transferred to 1 ml fertilization medium (B.O. medium, Brackett and Oliphant, 1975) and inseminated with 1 x $10^5$ in vitro capacitated boar spermatozoa. After 6 h culture, oocytes were transferred to 1 ml sperm free embryo culture medium (Modified Krebs'-Ringer-bicarbonate medium, see Petters et al., 1990, but without $KH_2PO_4$) and cultured for another 5 h. Oocytes were then denuded of cumulus cells and fixed for evaluation of nuclear status. These procedures were described in detail previously (Ding et al., 1992 b; Ding and Foxcroft, 1992).

**Evaluation of nuclear status**

After at least 48 h fixation in 25% acetic acid in absolute alcohol, nuclear status

[1] FSH, LH and prolactin were gifts from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

was examined under a phase contrast microscope after 1% lacmoid staining. Nuclear status was classified as described in detail previously (Ding and Clarke et al., 1992; Ding and Foxcroft, 1992).

**RIA of steroids**

Progesterone and estradiol-17ß in follicular fluid and follicle conditioned media were measured using direct (non extraction) immunoradioassay (RIA) established in our laboratory (see Ding and Foxcroft, 1992). Sensitivities of the assay, defined as the dose at binding (%) = $CB_{max}$ - $2SDB_{max}$) x 100% / $CB_{max}$ ($CB_{max}$: mean cpm of maximal binding, $SDB_{max}$: standard deviation of cpm of maximal binding tubes), were and 6.25 pg per tube for progesterone and estradiol, respectively. All the samples were assayed in a single assay to remove any effects of interassay variability on treatment differences. The intraassay coefficients of variation were 5.31 and 6.48% for progesterone and estradiol-17ß, respectively.

**Statistical analysis**

The data were subjected to ANOVA for a split plot design (Steel and Torrie, 1980). The general linear model for analysis of morphological parameters and steroids in follicular fluid and in conditioned media were Y = Day + Gilt(Day) + Size + Day*Size + error. Effect of Day was tested using Gilt(Day) as an error term. Effects of Size and Day*Size were tested using error as an error term. The general linear model for analysis of oocyte maturation and fertilization data was: Y = Plot + Day + Gilt(Plot*Day) + Size

+ Day*Size + Size*Gilt(Plot*Day) + error. Effect of Day was tested using Gilt(Plot*Day) as an error, and effects of Size and Day*Size were tested using Size*Gilt(Plot*Day) as an error. The percentage data were analyzed after arcsine transformation. Statistical analysis was carried out using the general linear model procedures of the Statistical Analysis System (Release 6.07, SAS Institute, Cary, NC 27512-8000). Data are presented in the Tables (Table VI-2 and -3) as least square's means (LSM) ±se of LSM.

## RESULTS

***Morphological parameters of follicles used for production of conditioned media and steroid concentrations in follicular fluid and in conditioned media.***

Least squares means for follicle diameter and fresh shell weight of the follicles used for production of conditioned media, and progesterone (P4), estradiol (E2) and ratios of P4 to E2 in corresponding follicular fluid and in conditioned media are presented in Table VI-2. Both P4 and E2 concentrations in follicular fluid were affected by Day and Size (Fig.VI-1 a and b), but were not affected by the interaction between Day and Size. Both P4 and E2 productions by follicles during the conditioning period were significantly affected by Day, but not by Size (Fig.VI-1 c and d), nor by Day*Size interaction. Ratios of progesterone to estradiol in follicular fluid and in conditioned media were affected neither by Day, nor by Size and nor by Day*Size interaction.

***Maturation, sperm penetration and male pronuclear formation.***

In total, conditioned medium produced by follicles from 12 gilts (6 for Day 17 and

6 for Day 20) were used for oocyte maturation. Each conditioned medium was used twice in two different replicated experiments. Data on oocytes that developed beyond the second metaphase (MII) after 58 h (47 h maturation culture plus 11 h culture post insemination), penetrability, oocytes with normal female pronuclear formation and male pronuclear formation are shown in Table VI-3. The percentage of oocytes developing beyond MII was not affected by Day, but was affected by Size. Oocytes cultured in conditioned media produced by large follicles had slightly higher nuclear maturation rates than those cultured in conditioned media produced by small follicles. There was no interaction effect between Day and Size on nuclear maturation. Neither penetration rates, nor normal female pronuclear formation, were affected by either Day or Size. After penetration, the proportion of penetrated oocytes that contained unswollen or slightly swollen sperm nuclei (SH1) was higher when cultured in conditioned media produced by Day 17 follicles than when cultured in conditioned media produced by Day 20 follicles (Fig.VI-2 a), but was not affected by either Size or Day*Size. The proportion of penetrated oocytes that contained further decondensed sperm nuclei (SH2) was not affected by either Day or Size or Day*Size (Fig.VI-2 b). The proportion of penetrated oocytes that developed male pronuclei was significantly higher when cultured in Day 20 than when cultured in Day17 follicle conditioned media (Fig.VI-2 c), while neither Size, nor Day*Size, had an effect on MPN formation.

## DISCUSSION

In a previous study (Ding and Foxcroft, 1992), it was demonstrated that the ability of oocytes to decondense sperm nuclei and to form male pronuclei (a criterion of

cytoplasmic maturation) after sperm penetration, was correlated with the size of the co-cultured follicle shells and with progesterone and estradiol concentrations and their ratios to testosterone concentration in corresponding follicular fluid. These results suggest that follicle cells with different maturity secrete a different quantity and/or quality of factors which stimulate cytoplasmic maturation of oocytes. These results were obtained using follicles from eCG primed gilts (36 and 72 h after eCG treatment of gilts). In the present study, we tested this hypothesis again using follicles from cyclic gilts, since follicular heterogeneity is reported to be greater in eCG primed prepubertal animals than in cyclic gilts (Wiesak et al., 1990).

Estrous synchronization could not be used to provide follicles because of the potential effects of exogenous hormones on the physiological condition of the follicles. Logistically, this prevented us from using our previously described follicle shell co-culture system in this experiment, since it was difficult to obtain follicles at defined ages (Day 17 and Day 20 of estrous cycle) on the days when the oocyte maturation experiments were to be carried out (this is essential to control the day to day variabilities in IVM-IVF system). As it has been reported that follicle conditioned medium supports porcine oocyte cytoplasmic maturation (Mattioli et al, 1988 a, b), we considered that using follicle shell conditioned medium would allow us to produce conditioned media at any time that gilts and hence follicles of defined age were available and thus to carry out the oocyte maturation experiment in a more controlled way. A preliminary experiment was used to test the effect of follicle conditioned media on oocyte maturation (Ding and Foxcroft, 1993). This study showed that follicle shells cultured using a medium containing 2.5

μg/ml FSH alone, supported optimal oocyte maturation, especially when a gonadotropin combination (2.5 μg/ml FSH, 2.5 μg/ml LH and 20 ng/ml prolactin) was used during oocyte maturation. Therefore, in the present study, the same conditions for the production of follicle conditioned media and oocyte maturation were used.

Morphological characteristics of the follicles used for production of conditioned media and steroid concentrations in follicular fluid showed significant maturational differences between Day 17 and Day 20, and between small and large follicles. Day 20 follicles and large follicles were more steroidogenic than Day 17 and small follicles, respectively. These results are similar to those reported by Grant et al. (1989) and indicate that Day 20 and large follicles were more mature than Day 17 and small follicles, respectively. Progesterone and estradiol secreted by collapsed follicles during conditioning period showed similar pattern to those in follicular fluid, suggesting that maturational status of follicles affected the secretory activities during in vitro culture. However, the amounts of progesterone and estradiol produced during a 48 period culture were substantially different from those in follicular fluid. Progesterone concentrations were much higher, and estradiol concentrations were much lower than in follicular fluid, thus causing a great increase in ratios of P4 to E2 in conditioned media (see Table VI-2). These results are similar to those reported previously when eCG primed follicles were used (Ding and Foxcroft, 1992) and suggest that follicle cells had undergone luteinization during in vitro culture.

The follicles obtained from cyclic gilts were substantially different from those obtained from eCG primed prepubertal gilts (Ding and Foxcroft, 1992) as shown in Table

VI-4. Follicle size was more variable in eCG treated gilts than in cyclic gilts and steroid concentrations in follicular fluid were also very different. Progesterone concentrations were much lower in cyclic gilts than that in eCG primed gilts (overall mean, cyclic: 185.5 ng/ml vs eCG primed: 1018.6 ng/ml). Estradiol-17ß was also lower in cyclic gilts than in eCG treated gilts (overall mean, cyclic: 143.6 ng/ml vs eCG primed: 260.0 ng/ml). Furthermore, the ratio of progesterone to estradiol was much higher in eCG treated follicles than in cyclic follicles. After only 36 h of eCG treatment, the follicular fluid steroid milieu was dominated by progesterone and after another 36 h (72 h after eCG), this trend was more apparent. Although in cyclic animals, Day 17 follicles contained similar amount of estradiol and progesterone, whilst Day 20 follicles contained more progesterone than estradiol, the change in steroid ratios was not as greater as in eCG treated animals. Also differences in the progesterone to estradiol ratio between small and large follicles were much greater in eCG primed than in cyclic follicles. These comparisons confirmed previous observations by Wiesak et al. (1990) that eCG treatment promotes steroidogenesis, induces accelerated luteinization of follicle cells, and induces greater variabilities among individual follicles.

Nuclear maturation of oocytes was affected by follicle conditioned media produced by the different sizes of follicle shell, but the effect was not great (small:93.5% vs large:96.9%) and all four treatment groups had nuclear maturation rates greater than 92%. Neither penetrability nor normal female pronuclear formation were affected by treatment and were similar to those observed previously (Ding and Foxcroft, 1992; Ding and Moor et al., 1992). However, cytoplasmic maturation as determined by MPN formation rates

in penetrated oocytes was significantly higher when oocyte complexes were cultured in conditioned media produced by Day 20 than Day 17 follicles, indicating that more mature follicles could support better oocyte maturation. However, follicle size did not affect MPN formation. These results were in contrast to those reported in a previous study using eCG primed follicles (Ding and Foxcroft, 1992) in which follicle size but not follicle age (36 and 72 h after eCG treatment) affected MPN formation rates. As discussed previously, the differences in steroid patterns in eCG treated follicles were wider between follicle size categories than between the times after eCG treatment. In contrast in cyclic follicles, differences were greater between follicle ages (Day 17 vs Day 20) than between follicle size categories (Fig.VI-1). Such differences in the pattern of follicular maturation and steroidogenesis may explain the different results between these two studies.

Male pronuclear formation rates seemed to be related to steroid concentrations in follicular fluid in the present experiment. Previously, we have also shown that male pronuclear formation rates were correlated to steroids in fluid of follicles whose shells were used for co-culture with oocytes. As suggested earlier, significant correlation between male pronuclear formation and steroid hormones in follicular fluid does not reflect a cause and effect relationship between these variables, rather it may reflect the relationship between MPN formation and the physiological status of the follicles used for co-culture or for production of conditioned medium. The present study also showed that MPN formation seemed to be correlated with steroid hormones in conditioned medium. In a previous study, however, we did not find any correlation between male pronuclear formation and steroids in culture medium collected at the completion of culture (Ding and

Foxcroft, 1992) and concluded that the extent of cytoplasmic maturation of porcine oocytes in our culture system was mainly affected by factors other than steroid hormones in the maturation environment.

Although the effect of follicle size on MPN formation was not statistically significant, oocyte complexes cultured in conditioned medium produced by large follicle shells tended to have higher MPN formation rate than those cultured in conditioned medium produced by small follicle shells within Day 20 (see Table VI-3). This again suggests that maturational variability of follicles within the preovulatory pool may affect oocyte quality.

Overall, MPN formation rates were lower in the present study when compared to the previous study of Ding and Foxcroft (1993). The cause for this is not clear, but probably related to the quality of spermatozoa used for IVF. In a parallel experiment carried out over the same period, oocyte complexes cultured in fresh oocyte maturation medium had no MPN formation at all, while we previously showed that oocytes matured in such conditions had about 40% MPN formation rate (Ding and Foxcroft, 1992; 1993).

Mean sperm number in penetrated oocytes was greater when oocytes were matured in conditioned media generated by large follicle than by small follicle shells, which again supports our overall hypothesis.

In conclusion, these results confirm that diversity of follicular development in pigs affects the quality of oocytes ovulated. These effects may have important consequences for the developmental potential of the early embryo.

Table VI-1. Experimental design for oocyte maturation, indicating the origin of the different conditioned media used for each in vitro maturation experimental replicate.

| Plot | Repeat (Expt. date) | Day of the cycle | Gilt 1 | | Gilt 2 | |
|---|---|---|---|---|---|---|
| | | | Small Follicles | Large follicles | Small follicles | Large follicles |
| 1 | Sept 8 | 17 | CM17I | CM17I | CM17II | CM17II |
| | | 20 | CM20I | CM20I | CM20II | CM20II |
| | Sept 1[illegible] | 17 | CM17I | CM17I | CM17II | CM17II |
| | | 20 | CM20I | CM20I | CM20II | CM20II |
| 2 | Sept 16 | 17 | CM17III | CM17III | CM17IV | CM17IV |
| | | 20 | CM20III | CM20III | CM20IV | CM20IV |
| | Sept 22 | 17 | CM17III | CM17III | CM17IV | CM17IV |
| | | 20 | CM20III | CM20III | CM20IV | CM20IV |
| 3 | Oct 13 | 17 | CM17V | CM17V | CM17VI | CM17VI |
| | | 20 | CM20V | CM20V | CM20VI | CM20VI |
| | Oct 14 | 17 | CM17V | CM17V | CM17VI | CM17VI |
| | | 20 | CM20V | CM20V | CM20VI | CM20VI |

Table VI-2. Morphological characteristics of follicles used for production of conditioned media and steroid concentrations in follicular fluid and conditioned media. All means presented in the Table are Least Squares Means (LSM) ± standard error (SE) of LSM.

| | Source of the follicular shells | | | | Statistics for Main Effects (P value)[a] | | |
|---|---|---|---|---|---|---|---|
| | Day 17 | | Day 20 | | | | |
| | Small | Large | Small | Large | Day | Size | D*S. |
| Number of Gilts | 9 | 9 | 10 | 10 | | | |
| Diameter (mm) | | | | | | | |
| Mean | 4.4±0.10 | 5.7±0.10 | 6.2±0.098 | 7.5±0.098 | 0.001 | 0.001 | 0.88 |
| Range | 3.5-5.0 | 4.4-7.2 | 5.7-6.9 | 6.8-8.2 | | | |
| Fresh Weight (mg)/5 Shells | | | | | | | |
| Mean | 84.2±0.004 | 117.6±0.004 | 141.7±0.003 | 185.9±0.003 | 0.0001 | 0.0001 | 0.132 |
| Steroid Concentration in Follicular Fluid (ng/ml)[b] | | | | | | | |
| P4 | 91.1±11.7 | 127.1±13.0 (n=8)[c] | 238.5±11.1 | 286.2±11.1 | 0.0001 | 0.003 | 0.625 |
| E2 | 89.0±13.2 | 134.5±13.2 | 167.4±12.6 | 183.5±15.4 (n=8)[c] | 0.032 | 0.039 | 0.299 |
| P4/E2 | 1.[illegible] | 1.20±0.2[illegible] (n=8) | 1.57±0.18 | 1.49±0.22 (n=8) | 0.95 | 0.11 | 0.23 |
| Steroid Concentration in Conditioned Media (ng/ml)[b] | | | | | | | |
| P4 | 469.8±209.9 | 469.1±209.9 | 828.6±199.1 | 1416±199.1 | 0.076 | 0.170 | 0.168 |
| E2 | 18.3±2.34 | 24.2±2.34 | 34.2±2.22 | 36.7±2.22 | 0.012 | 0.083 | 0.462 |
| P4/E2 | 32.6±6.36 | 24.6±6.36 | 31.2±6.04 | 44.0±6.03 | 0.58 | 0.70 | 0.11 |

[a] P values for Day were comparisons between Day 17 and Day 20, for Size were comparisons between small and large follicle shells and for D*S were interaction effects between Day and Size.

[b] P4: progesterone; E2: estradiol-17ß.

[c] Number of observations that were different from the number of gilts are indicated in the parentheses.

Table VI-3. Effect of maturational state of follicular shells on oocyte maturation (MII), sperm penetration of MII, normal female (2Pb+1F) and male (MPN) pronuclear formation rates, and the average number of sperm in penetrated oocytes.

| | Source of follicular shells | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 17 | | Day 20 | | SE of | Main Effect (P value)[a] | | |
| | Small | Large | Small | Large | LSM | Day | Size | D*S |
| No. batches of conditioned media used | 6 | 6 | 6 | 6 | | | | |
| No. of Dishes | 12 | 12 | 12 | 12 | | | | |
| No. eggs examined | 131 | 136 | 136 | 138 | | | | |
| No. egg beyond MII | 129 | 129 | 130 | 126 | | | | |
| % (LSM) | 95.0 | 98.7 | 92.0 | 94.9 | 1.33 | 0.10 | 0.046 | 0.45 |
| No. eggs penetrated | 115 | 108 | 110 | 104 | | | | |
| % (LSM) | 86.0 | 85.8 | 83.1 | 82.5 | 2.57 | 0.88 | 0.61 | 0.67 |
| No. of penetrated oocytes with 2Pb+ 1F | 84 | 74 | 81 | 81 | | | | |
| % (LSM) | 72.2 | 69.7 | 67.3 | 69.5 | 3.13 | 0.80 | 0.91 | 0.29 |
| No. penetrated eggs with SH1 | 71 | 57 | 48 | 30 | | | | |
| % (LSM) | 58.7 | 60.1 | 35.9 | 30.4 | 5.93 | 0.007 | 0.94 | 0.83 |
| No. penetrated eggs with SH2 | 24 | 28 | 34 | 26 | | | | |
| % (LSM) | 22.7 | 23.7 | 30.9 | 28.1 | 4.21 | 0.11 | 0.89 | 0.55 |
| No. penetrated eggs with MPN | 20 | 23 | 28 | 48 | | | | |
| % (LSM) | 18.6 | 16.2 | 33.1 | 41.5 | 4.72 | 0.011 | 0.61 | 0.22 |
| Average No sperm in penetrated oocytes (LSM) | 2.50 | 2.82 | 2.72 | 2.91 | 0.150 | 0.54 | 0.11 | 0.66 |

[a].P values for Day were comparisons between Day 17 and Day 20, for Size were comparisons between small and large follicle shells and for D*S were interaction effects between Day and Size.

Table VI-4. Comparisons of follicular size, progesterone (P4) and estradiol-17β (E2) levels in follicular fluid between follicles obtained from eCG treated gilts and cyclic gilts. Least Square's Means (LSM) are presented in the table.

| eCG treated follicles[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 36 h post eCG | | | 72 h post eCG | | | Difference between Size | Difference between Time |
| | Small | Large | Diff. | Small | Large | Diff.[2] | | |
| Diameter (mm) | 4.1 | 7.1 | 3 | 5.6 | 8.8 | 3.2 | 3.1 | 1.6 |
| P4(ng/ml) | 470.1 | 1219.9 | 799.8 | 460 | 1975.4 | 1515.4 | 1157.6 | 372.7 |
| E2(ng/ml) | 208.3 | 270.5 | 62.2 | 169.1 | 391.9 | 222.8 | 142.5 | 41.1 |
| P4:E2[3] | 2.01 | 4.51 | 2.50 | 2.72 | 5.04 | 2.32 | 2.41 | 0.62 |
| Cyclic follicles | | | | | | | | |
| | Day 17 of the cycle | | | Day 20 of the cycle | | | Difference between Size | Difference between Day |
| | Small | Large | Diff.[2] | Small | Large | Diff.[2] | | |
| Diameter(mm) | 4.4 | 5.7 | 1.3 | 6.2 | 7.5 | 1.3 | 1.3 | 1.8 |
| P4(ng/ml) | 91.1 | 127.1 | 36 | 238.5 | 286.2 | 47.7 | 41.9 | 153.25 |
| E2(ng/ml) | 89 | 134.5 | 45.5 | 167.4 | 183.5 | 16.1 | 30.8 | 63.7 |
| P4:E2[3] | 1.02 | 0.94 | -0.08 | 1.42 | 1.56 | 0.14 | 0.03 | 0.51 |

[1]. Data are from Ding and Foxcroft (1992).
[2]. Diff.: difference, values of small follicles minus values of large follicles.
[3]. The ratios of P4 to E2 are simply converted from the two Least Square's Means.

Fig.VI-1. Progesterone and estradiol-17ß (ng/ml) in follicular fluid (FF) and conditioned media (CM). Comparisons between two levels of main factors (LSM±SE of LSM) are presented. (a) Progesterone in FF; (b) Estradiol-17ß in FF; (c) Progesterone in CM; and (d) Estradiol-17ß in CM. P: P values for the comparisons of two (least squares) means adjacent.

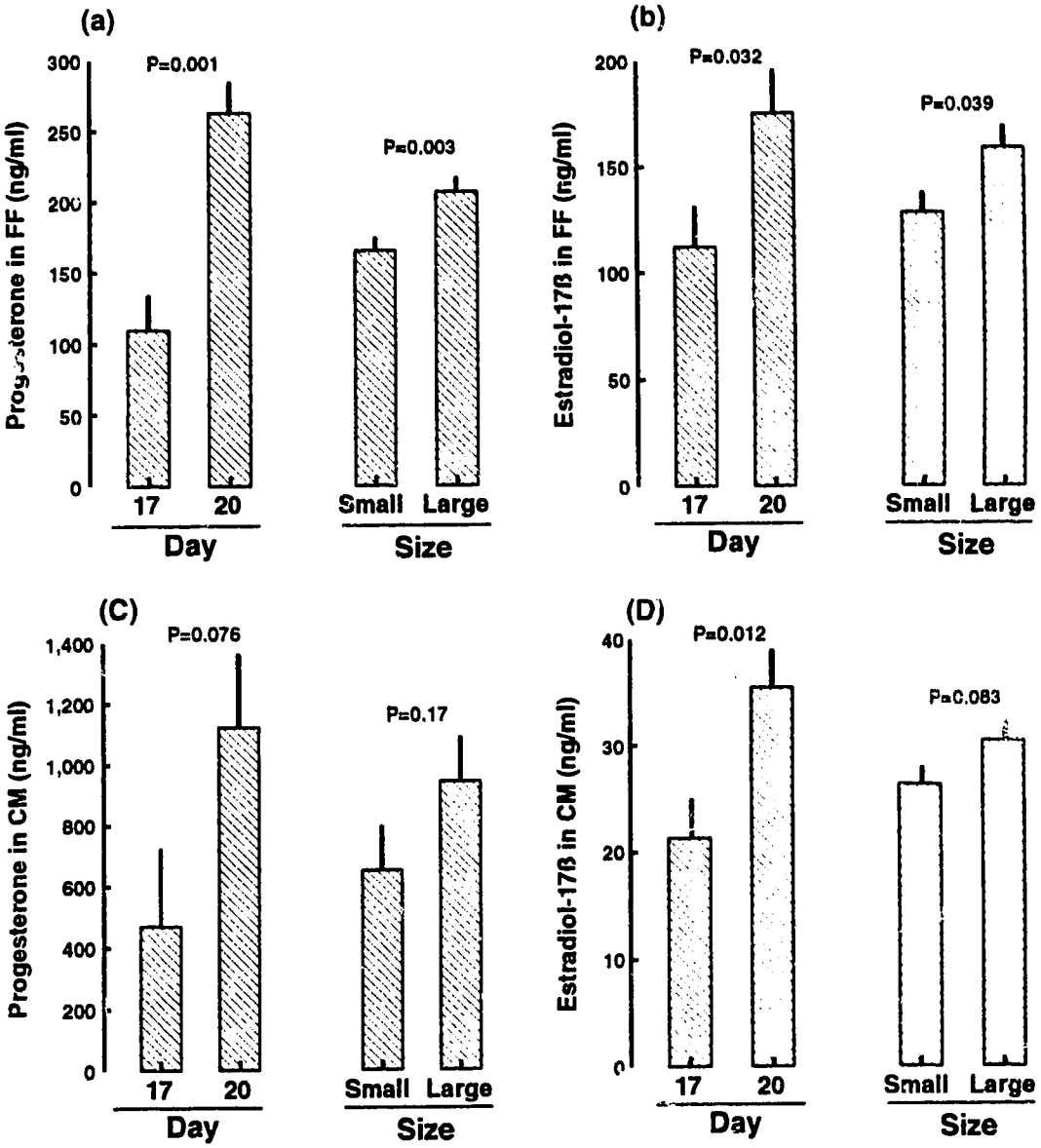
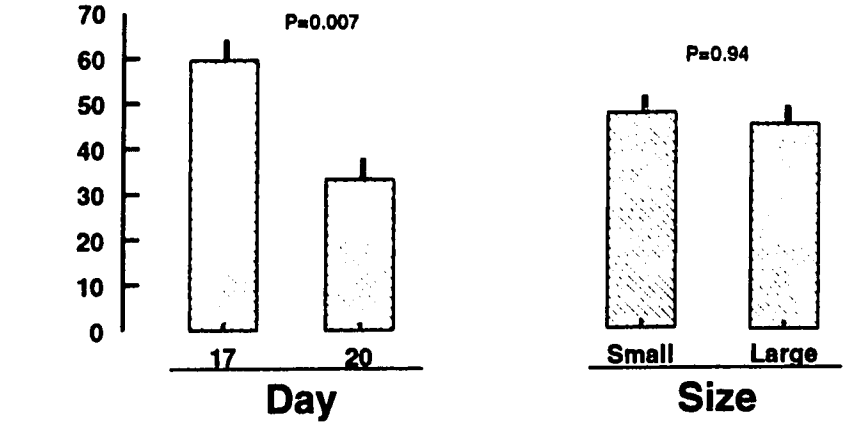
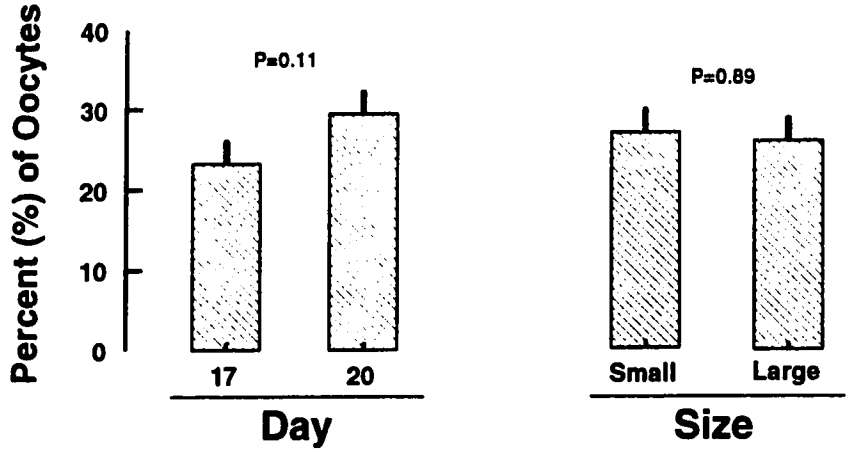

(a)
Progesterone in FF (ng/ml)
300
250
200
150
100
50
0
P=0.001
17
20
Day
P=0.003
Small
Large
Size
(b)
Estradiol-17β in FF (ng/ml)
200
150
100
50
0
P=0.032
17
20
Day
P=0.039
Small
Large
Size
(C)
Progesterone in CM (ng/ml)
1,400
1,200
1,000
800
600
400
200
0
P=0.076
17
20
Day
P=0.17
Small
Large
Size
(D)
Estradiol-17β in CM (ng/ml)
40
30
20
10
0
P=0.012
17
20
Day
P=0.083
Small
Large
Size

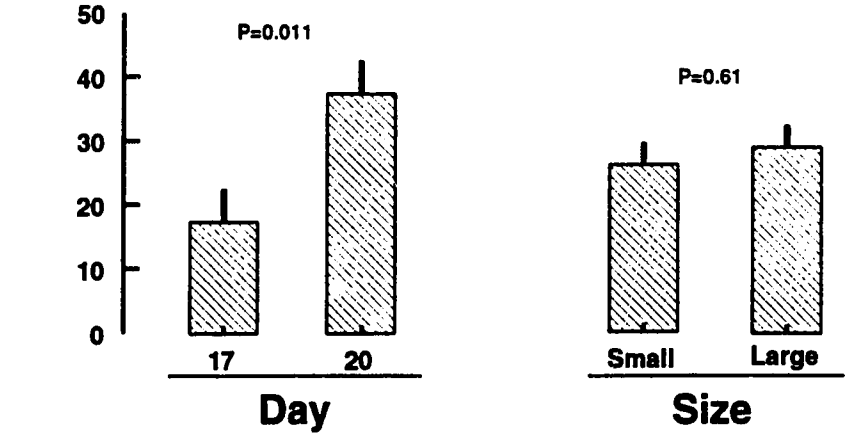
Fig.VI-2. Effects of conditioned media produced by follicular shells obtained from different day of the estrous cycle (Day 17 and Day 20) and from different follicle size categories (Small and Large), on the incidence of sperm decondensation in oocytes matured in vitro. Comparisons of means (LSM±SE of LSM) between two levels of main factors are presented. P: P values for the comparisons of two (least squares) means adjacent. SH1: undecondensed or slightly decondensed sperm nuclei. SH2: Further decondensed sperm nuclei. MPN: full-size male pronuclei.




(a) Penetrated Oocytes with SH1




(b) Penetrated Oocytes with SH2




(c) Penetrated Oocytes with MPN

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# CHAPTER VII EPIDERMAL GROWTH FACTOR STIMULATES OOCYTE MATURATION IN PIGS

## INTRODUCTION

Follicular secretions are believed to contain stimulating factors which mediate oocyte cytoplasmic maturation in the pig (Mattioli et al, 1988 a, b; Naito et al., 1988, Yoshida et al, 1992; Funahashi and Day, 1993). Our own studies (Ding and Foxcroft, 1992, Ding and Foxcroft, 1993 a, b) further confirmed the existence of stimulating factors in follicular secretions. The nature of these stimulating factors are not clear. Mattioli et al. (1988 b) showed that steroids (progesterone in particular) secreted by follicular cells were responsible for stimulating oocyte cytoplasmic maturation, while our own study (Ding and Foxcroft, 1992) suggested that other follicular factors were also involved since detailed analysis showed no correlations between steroid concentrations secreted by follicular cells during oocyte maturation and male pronuclear formation rates. Yoshida et al. (1992) reported that the stimulatory effect of follicular fluid on oocyte nuclear maturation was lost after heating at 56°C for 30 min, but there was no significant decrease after defatting. These results suggest that the nature of the stimulatory factors may be polypeptides.

Recent studies showed that growth factors stimulate oocyte nuclear maturation (see review by Racowsky, 1991). Epidermal growth factor (EGF) is a most potent growth factor in this regard. Cultured ovarian cells in a number of species have been shown to synthesize growth factors (cattle, Neufeld et al., 1987; pig, Hammond et al., 1985; rat,

Skinner et al., 1987). Porcine follicular fluid contains significant levels of EGF, and there are binding sites for EGF within the ovary (for review, see Feng et al., 1987) that fluctuate in relation to the maturational status of the follicle. In vitro studies showed that EGF could stimulate nuclear maturation in rats (Dekel and Sherizly, 1985; Feng et al., 1987; Ueno et al., 1988), mice (Downs, 1989; Das et al., 1991), cattle (Illera et al., 1992), pigs (Reed et al., 1991; Sommer et al., 1992) and human (Das et al, 1991). The developmental potential of in vitro matured bovine oocytes to eight-cell stage (Coskun et al., 1991) and to the blastocyst stage (Harper and Brackett, 1993) was also stimulated by EGF. These results suggest that EGF might be one of the follicular factors responsible for stimulating oocyte cytoplasmic maturation as well as nuclear maturation. However, an EGF effect on cytoplasmic maturation has not been tested in species other than the cow. The objectives of the present studies were, therefore, to examine the effects of epidermal growth factor, alone or in combination with gonadotropins and follicular shell co-culture, on cytoplasmic maturation in porcine oocytes.

## MATERIALS AND METHODS

### Preparation of follicular tissues

Ovaries from slaughtered prepubertal gilts (weighing about 100 kg) were collected from a local abattoir and transported to the laboratory in a polystyrene box to prevent major changes of temperature. Then ovaries were washed four times with phosphate-buffered saline (Sigma). Follicles with diameters ranging from 3 to 7 mm were dissected

and only healthy looking follicles were used. Oocytes with a compact cumulus directly associated with a piece of mural granulosa tissue (oocyte complexes) were isolated from dissected follicles. Follicular shells (follicular fluid and oocytes removed) from 5-7 mm follicles were used for co-culture with oocytes according to the experimental protocols described below. The whole dissection procedure was carried out in TCM 199 with 25 mM Hepes (Earle's salts; Gibco, Grand Island, NY) supplemented with 10% new born calf serum (Gibco, 56°C treated for 30 min) at a temperature of 24-26°C and completed within four hours of slaughter. Oocyte complexes and follicular shells were washed four times in the dissection medium (see above) before use.

**Oocyte maturation in vitro**

Oocyte complexes were cultured in 30 mm plastic petri culture dishes (Falcon) containing 2 ml TCM 199 (with Earle's salts, Gibco) per dish supplemented with 10% fetal calf serum (FCS, Gibco, 56°C treated for 30 min), 100µg/ml glutamine (Sigma, BDH), 70 µg/ml L-ascorbic acid (Sigma, BDH) and 35 µg/ml insulin (Sigma,BDH) (see appendix). Gonadotropins, EGF and follicular shells were added to culture according to the experimental designs described below (see Experiments I and II). Culture was carried out with gentle rocking agitation under 5% $CO_2$ in humidified air at 39°C for 47±1 h.

**Evaluation of cumulus expansion**

After 47 h maturation culture, oocyte-complexes were evaluated for cumulus expansion. Oocytes with an almost fully expanded cumulus (except corona radiata, see

Fig.VII-1 A) or those which had lost most of their cumulus cells (see Fig.VII-1 C right) and those which had lost almost all their cumulus cells including the corona radiata (see Fig.VII-1 E) were classified as cumulus expanded oocytes. Oocytes with dark looking intact cumulus (See Fig.VII-1 B and C left), were classified as having no cumulus expansion. A subjective assessment of the degree of mucification associated with the cumulus expansion was made during microscopic examination (see Fig.VII-1).

**Examination of nuclear status**

After 47 h maturation culture or 11 h after insemination, oocytes were denuded of cumulus cells, mounted on a slide with a whole mount technique and fixed for 48 h in ethanol/acetic acid (3:1). The nuclear status of oocytes was examined under a phase-contrast microscope after staining with 1% lacmoid in 45% acetic acid solution. Nuclear status (GV, premetaphase I, MI and MII) was identified according to the criteria of Hunter and Polge (1966). Sperm penetration and male pronuclear development were determined as described by Ding et al. (1992b). Those oocytes with sperm head(s) and/or swollen sperm head(s) and/or male pronuclei (MPN) with detached sperm tail(s) were classified as being penetrated. Those penetrated oocytes with full size male pronucleus/pronuclei were considered to have undergone normal male pronuclear development. Normal female pronuclear formation was defined as an oocyte containing one female pronucleus and two or more polar bodies.

## Experimental Design and Analysis

### Experiment I

A preliminary experiment was designed to determine an effective dose of EGF for oocyte nuclear maturation in our culture system. Oocyte complexes were randomly divided into five culture dishes (16-25 oocyte complexes per dish) and subjected to five different treatments: 1) control group, medium containing a gonadotropin mixture[1] (2.5 μg/ml NIADDK-oLH-26,AFP-5551b; 2.5 μg/ml USDA-pFSH-B-1, AFP-5600, and 20 ng/ml USDA-pProlactin-B-1, AFP-5000) as used for standard oocyte maturation culture in our previous studies (see Ding et al., 1992 b; Ding and Foxcroft, 1992); 2) no gonadotropins or EGF; 3) 0.1 ng/ml EGF (E-4127, isolated from mouse submaxillary glands, Sigma, Lot 101H8824-1); 4) 1.0 ng/ml EGF and 5) 10 ng/ml EGF. Oocyte complexes were cultured for 47 h for evaluation of cumulus expansion and nuclear maturation. The experiment was replicated three times.

The data were subjected to analysis of variance for a randomized complete block design (replicated experiments as blocks, see Steel and Torrie, 1980). Percentage data were analyzed after arcsine transformation. The analysis was accomplished using the general linear model procedures of the Statistical Analysis System (release 6.07, SAS Institute, Cary, NC 27512-8000). Multiple comparisons were made using the PDIFF (LSD) function of SAS. Data presented in the tables and figures were expressed as means±se of means.

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[1] Gonadotropins and prolactin were gifts from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

**Experiment II**

Experiment II was designed to test the effect of EGF, alone or in combination with gonadotropins and follicular shell co-culture on oocyte cytoplasmic maturation. The study was designed as a 2x2x2 factorial experiment in which oocyte complexes were randomly divided into eight culture dishes containing 10-15 oocyte complexes per dish cultured in oocyte maturation medium supplemented with different EGF, gonadotropins and follicular shell combinations (see Table VII-2). The EGF dose used was 1ng/ml as this dose effectively stimulated both cumulus expansion and nuclear maturation in Experiment I. The gonadotropin mixture was the same as that used in Experiment I (treatment 2). One follicular shell was added to the culture if needed.

After 47±1 h in culture, oocytes were transferred to fertilization medium and inseminated with in vitro capacitated boar spermatozoa. Freshly collected sperm-rich fraction semen was provided by the Alberta Swine AI Center. Spermatozoa were treated as described previously (Ding et al., 1992 b; Ding and Foxcroft, 1992). Fertilization medium was modified TCM 199, supplemented with 3.05 mM D-glucose (BDH), 2.92 mM calcium lactate (BDH), 0.91 mM sodium pyruvate (BDH), 12% (V/V) fetal bovine serum (Sigma) and 10mg/100ml Kanamycin (Sigma) (see appendix). pH was adjusted to 7.4 after equilibration under 5% $CO_2$ in air (see Cheng, 1985). Six hour after insemination oocytes were washed, transferred to 1 ml sperm-free embryo culture medium (modified Krebs'-Ringer-bicarbonate medium, see Petters et al., 1990, without $KH_2PO_4$, see appedix)), cultured for a further 5 h and then fixed for evaluation of nuclear status. The experiment was replicated five times.

Maturation media were collected after completion of oocyte cultures, centrifuged at 2083 x g for 10 min and supernatants stored at -30°C for measurement of progesterone and estradiol by direct radioimmunoassay (Ding and Foxcroft, 1992). Sensitivities of the assays, defined as the dose at binding (%) = $CB_{max}$ - $2SDB_{max}$) x 100% / $CB_{max}$ ($CB_{max}$: mean cpm of maximal binding, $SDB_{max}$: standard deviation of cpm of maximal binding tubes), were 6.25 and 6.25 pg per tube for progesterone and estradiol, respectively. All samples were assayed in a single assay with intra-assay CVs of 5.31 and 6.48% for progesterone and estradiol-17ß, respectively, to remove any effects of interassay variability on treatment differences.

Data obtained in the experiment were analyzed using variance analysis for a randomized complete block factorial design (replicated experiments were blocks, see Steel and Torrie, 1980). The statistical analysis was completed using the general linear model procedures of the SAS as described in Experiment I. Data presented in the tables and figures were expressed as means±se of means.

## RESULTS

**Experiment I:**

Oocyte-complexes in the control group stimulated with the gonadotropin combination had 100% cumulus expansion (see Table VII-1). EGF significantly stimulated cumulus expansion in a dose dependent manner. Oocyte-complexes cultured in medium containing 0 ng/ml EGF had no cumulus expansion, whereas addition of 0.1 ng/ml EGF to the culture increased cumulus expansion to 68.2%. Increases of the EGF dose to 1.0

and 10 ng/ml further increased cumulus expansion rates to 95.1 and 97.7, respectively, which were significantly higher than in those oocytes cultured in medium containing 0.1 ng/ml EGF ($P < 0.0004$), but not different from those oocytes cultured in medium containing the gonadotropin combination ($P > 0.07$). Although EGF effectively stimulated cumulus expansion, it did not stimulate mucification of cumulus cells as did treatment with gonadotropins (see Fig.VII-1, compare A with C, D and E).

Nuclear maturation was also stimulated by EGF (see Table VII-1). All three EGF treated groups had significantly higher rates of nuclear maturation than the EGF control group (no EGF and no gonadotropins) ($P < 0.05$) and although the response appeared to be dose-dependent, nuclear maturation rates were not significantly different among the 0.1, 1.0 and 10 ng/ml EGF dose groups ($P > 0.14$). Oocytes stimulated with gonadotropins had a significantly higher nuclear maturation (MII) rate than EGF control and the 0.1 ng/ml EGF treated groups ($P < 0.011$), but not different from 1.0 and 10 ng/ml EGF treated groups ($P > 0.124$).

**Experiment II**:

Effects of EGF, the gonadotropin combination, follicular shell co-culture and their interactions on nuclear maturation, sperm penetration and pronuclear development after sperm penetration were shown in Table VII-2. The proportion of oocytes maturing beyond MII after 47 h maturation culture and another 11 h fertilization culture was significantly affected by EGF ($P=0.0001$) and gonadotropin ($P=0.0001$) treatments, but not by follicular shell co-culture ($P=0.29$) (see Fig.VII-2). The only interaction effect on nuclear maturation

among three main factors was between EGF and gonadotropin treatments (P=0.0001), as shown in Fig.VII-3. Multiple comparisons showed that, without considering the follicular shell co-culture treatment, oocytes cultured in the presence of gonadotropins only had the highest (100%) nuclear maturation rate, significantly different from those oocytes cultured in the presence of EGF only (P=0.0058) or in the absence of both gonadotropins and EGF (P=0.0001), but not different from those cultured in the presence of both gonadotropins and EGF (P=0.145). Oocytes cultured in the absence of both EGF and gonadotropins again showed very low nuclear maturation rate (28.11%). Nuclear maturation rates were similar in those oocytes cultured in the presence of EGF with or without gonadotropins (P=0.1438).

EGF (P=0.022) and gonadotropin combination (P=0.05) treatments both significantly increased penetrability of MII oocytes, but follicular shell co-culture (P=0.598) did not have this effect (see Fig.VII-4). There were no significant interaction effects on oocyte penetrability among the three main factors (P > 0.05). Normal female pronuclear formation was not affected by any of three main factors (P > 0.11, see Fig.VII-5) and there were no interaction effects among treatments (P > 0.363).

Male pronuclear formation rate in penetrated oocytes was not altered by EGF treatment (P=0.475) during maturation culture (Fig.VII-6). However, gonadotropin addition (P=0.0001) and follicular shell co-culture (P=0.0001) during oocyte maturation significantly increased male pronuclear formation rates (see Fig.VII-6). In addition, the interaction effect between EGF and gonadotropins on male pronuclear formation was significant (P=0.05), although the other interactions among main factors were not

significant (P > 0.0147). Multiple comparison of interaction means (Fig.VII-7) showed that oocytes matured in media supplemented with EGF in combination with gonadotropins had the highest male pronuclear formation rate, which was significantly different from the other three interaction means (P < 0.0023). EGF alone had no effect on male pronuclear formation when compared to the group with no EGF and no gonadotropins (P=0.16).

Mean sperm numbers in penetrated oocytes were significantly affected by gonadotropin treatment during maturation culture (GTH-: 3.13 vs GTH+: 5.3 sperm/egg, P=0.032), but not affected by EGF (P=0.82) and follicular shell co-culture (P=0.43). There were no interaction effects among the three main factors (P>0.17).

Estradiol and progesterone concentrations in maturation media collected at the completion of maturation culture are shown in Table VII-3. Estradiol concentrations were significantly affected by follicular shell co-culture (P=0.0001), but not affected by EGF (P=0.17) or gonadotropins (P = 0.32) (Fig.VII-8) and there were no interactions among the three main factors (P> 0.255). Progesterone concentrations in culture media were significantly increased by gonadotropin addition during oocyte maturation, but not affected by EGF addition (P=0.82) or follicular shell co-culture (P=0.85) (Fig.VII-9). In addition, the interaction effect between gonadotropin addition and follicular shell co-culture on progesterone production was significant (P=0.07), although the other interactions among factors were not significant (P>0.355). Multiple comparison of progesterone interaction means (Fig.VII-10) showed that in the presence of gonadotropins, addition of one follicular shell to the culture reduced progesterone concentrations in culture media (P=0.036), while in the absence of gonadotropins, addition of one follicular

shell increased progesterone production (P=0.03).

## DISCUSSION

In preparation of the oocyte for fertilization, not only must meiotic maturation occur, but also the cytoplasm of the oocyte must undergo critical changes in order to achieve competency to support sperm chromatin decondensation and subsequent male pronuclear formation (for review, see Thibault et al, 1987). Although nuclear maturation (extrusion of first polar body) of oocytes could be achieved spontaneously in vitro, these in vitro (meiotically) matured oocytes may lack the ability to decondense sperm chromatin and subsequently to form male pronuclei. Granulosa cells play a very important role in the cytoplasmic maturation of rabbit oocytes (Thibault and Gérard, 1970; 1973) and further studies showed that co-culture of cumulus enclosed oocytes with granulosa cells (rabbit, Motlic and Fulka, 1982; ovine, Staigmiller and Moor, 1984; Crozet et al., 1987; bovine, Critser et al., 1986) or with follicle shells (pig, Mattioli et al., 1988 a, 1989; Ding et al., 1988; Ding and Foxcroft, 1992; Nagai et al.; 1993; Zheng and Sirard, 1993) promoted cytoplasmic maturation of in vitro matured oocytes and their developmental competence. In addition, cumulus enclosed oocytes cultured in media supplemented with follicular fluid (Naito et al., 1988, 1989; Yoshida et al., 1992) and in follicular cell conditioned media (Mattioli et al., 1988 a, b; 1989; Ding and Foxcroft, 1993 a, b) also increased the ability of oocytes to form male pronuclei after sperm penetration. These results strongly indicate that follicular cells secrete factors regulating cytoplasmic maturation of the oocyte via paracrine and/or autocrine mechanism.

The precise nature of such stimulating factors in follicular secretions (follicular fluid and follicle conditioned media) are not clear. Growth factors such as EGF (Dekel and Sherizly, 1985; Feng et al., 1987; Ueno et al., 1988; Downs, 1989) and the transforming growth factors (TGF-ß, Feng et al., 1988; TGF-α, Brucker et al., 1991) have been demonstrated to stimulate or enhance nuclear maturation in murine oocytes. Porcine (Reed et al., 1991; Coskun et al., 1992; Sommer et al., 1992), bovine (Illera et al., 1992) and human (Das et al, 1991) oocyte nuclear maturation are also stimulated by EGF. In addition, recent studies by Coskun et al. (1991) and Harper and Brackett (1993) showed that EGF could enhance cytoplasmic maturation of in vitro matured bovine oocytes. These results suggest that growth factors may be one group of follicular factors involved in mediating both oocyte nuclear and cytoplasmic maturation.

The present study confirmed that EGF could stimulate nuclear maturation of porcine oocytes in vitro in an appearently dose dependent manner with maximal effects at a dose of 1.0 ng/ml and above (Table VII-1). These results were similar to the previous report by Sommer et al. (1992). Although nuclear maturation rates in 1.0 and 10 ng/ml EGF groups were not statistically different from the control group stimulated by the gonadotropin combination, the usually high level of nuclear maturation (>90%) observed in several experiments was not achieved in response to EGF treatment alone. Nuclear maturation was also stimulated by EGF when oocyte-cumulus-granulosa complexes were co-cultured with the follicular shell (Experiment II).

EGF stimulated cumulus cell expansion (Table VII-1), as observed in mice (Downs, 1989) and cattle (Harper and Brackett, 1993). However, in contrast to the results

of Downs, cumulus mucification was not obvious in EGF stimulated groups under the light microscope as compared to the control groups (comparison of C-E with A, Fig.VII-1). Most of the cumulus cells were still clumped together in EGF treated groups although they had separated from, or only loosely maintained contact, with oocytes. In the control group, cumulus cells were evenly dispersed around the oocytes as a result of mucification. These results suggest that the mechanism for cumulus expansion stimulated by EGF alone differs from that stimulated by gonadotropins. When culture medium contained both the gonadotropin combination and EGF, cumulus cell mucification was similar to that observed when gonadotropin combination alone was used (data not shown).

Although the addition of EGF to the in vitro maturation media stimulated both nuclear maturation and the penetrability of in vitro matured oocytes, EGF alone did not stimulate cytoplasmic maturation. Consistent with our previous observations (Ding et al, 1988; Ding and Foxcroft, 1992), treatment with gonadotropins and follicular shell co-culture both significantly stimulated cytoplasmic maturation ($P<0.05$).

However, EGF significantly interacted with gonadotropins in stimulating cytoplasmic maturation (male pronuclear formation, see Fig.VII-7). Coskun et al. (1991) reported that EGF alone could improve the developmental potential of in vitro matured bovine oocytes. Harper and Brackett (1993) showed that whilst EGF alone or combined with a high dose of LH (50 μg/ml) or FSH (10 μg/ml) did not affect blastocyst formation rate, EGF combined with a low dose of LH (0.5 μg/ml) or FSH (0.5 μg/ml) promoted blastocyst formation. Collectively, these data indicate that EGF, alone or most probably in concert with gonadotropins and/or some other follicular factors, is a potential stimulator

for both nuclear and cytoplasmic maturation of oocytes.

Such EGF effects on oocytes seem to be mediated at least in part by follicular (cumulus) cells since denuded murine and porcine oocytes showed no significant increase in GVBD when treated with EGF (Downs et al., 1988; Coskun and Lin, 1992). Stimulatory effect of TGF-α on mouse oocyte maturation was also dependent on cumulus cells surrounding the oocytes (Brucker et al., 1991). However, Illera et al. (1992) reported a significant effect of EGF on GVBD in denuded bovine oocytes and Das et al. (1991) reported that EGF stimulated both GVBD and first polar body formation in both denuded and cumulus-enclosed murine and human oocytes. Mechanisms capable of mediating EGF effects via actions of follicular cells clearly exist. EGF receptors have been demonstrated in rat granulosa cells (Charbot et al., 1986) and specific binding of EGF to bovine cumulus and small antral granulosa cells (Rose et al., 1991) and to porcine granulosa cells (Fujinaga et al., 1992), have been observed. Binding of EGF to its receptors induces the activation of tyrosine-specific kinase, an essential primary event in the EGF pathway. In turn, tyrosine kinase activation initiates phosphorylation of several cellular proteins such as phospholipase C1 (which allows release of profilin and inositol triphosphate), PI-3 kinase, GTPase activation protein, membrane-associated protein kinase and *raf* kinase, as well as phosphorylation of the receptor itself (for review see Harper and Brackett, 1993). These changes lead to new gene expression (see Mercola and Stiles, 1988) and hence changes in protein synthesis and protein phosphorylation. Such changes in protein synthesis and protein phosphorylation may directly affect oocyte maturation (Ding et al., 1992 a, b). However, our own results support previous suggestion that EGF

effect on oocyte maturation may involve important interactions with gonadotropins, steroids, other growth factors and/or other local mediators. For example, EGF binding sites in granulosa cells were increased by FSH treatment (pig, Fujinaga et al., 1992; rat, St-Arnaud et al., 1983; Feng et al., 1987), but were decreased by LH/hCG treatment (Feng et al., 1987). In turn, EGF suppressed the FSH-induced increase in granulosa cell LH receptors in both rats and pigs (Knecht and Catt, 1983; May and Schomberg, 1989) and suppressed FSH-induced LH receptor mRNA synthesis in rats (Piquette et al., 1991). It has been suggested that such interactions between EGF and gonadotropins in the regulation of receptors may play an essential role during oocyte maturation (Harper and Brackett, 1993).

From a functional viewpoint, interaction between EGF and gonadotropins may result in changes in steroid production in follicular tissues and thus indirectly affect oocyte maturation. It has been reported that EGF inhibited FSH-stimulated estradiol production in granulosa cells (Hsueh et al., 1981; Jones et al., 1982) while enhancing FSH-stimulated progesterone synthesis (Jones et al., 1982). However, our results showed that EGF had no significant effect on estradiol and progesterone (Fig.VII-8 a and 9 a) production by follicular cells during oocyte maturation culture. We suggest, therefore, that EGF effects on oocyte nuclear and cytoplasmic maturation in vitro in the pig is unlikely to result from the modulation of steroid production by follicular cells during culture.

It is possible that EGF acts as an intraovarian regulator of oocyte nuclear and cytoplasmic maturation in response to gonadotropin stimulation. Recent studies using radioimmunoassay and immunohistochemical staining by Roy and Greenwald (1991 a)

showed that EGF expression in hamster ovarian cells was controlled by gonadotropins, especially FSH. Ovarian EGF content peaked on Day 1 (estrus as determined by copious vaginal discharge) and FSH treatment intensified EGF staining in granulosa cells. Hypophysectomy for 13 days resulted in almost complete absence of EGF specific staining in the remaining nonatretic follicles, which could be reversed by exogenous FSH treatment. Furthermore, Roy and Greenwald (1991 b) found that, when hamster preantral follicles were cultured in vitro, follicular DNA synthesis was significantly stimulated by FSH and EGF. Conversely, FSH- and EGF-induced DNA synthesis and Br-cAMP induced follicular DNA synthesis were drastically decreased by EGF antiserum treatment. These results suggest that FSH action on hamster preantral follicular DNA synthesis is mediated by EGF. If the assumption that EGF acts as an intraovarian regulator of oocyte nuclear and cytoplasmic maturation in response to gonadotropin stimulation is correct, EGF supplementation to the oocyte culture medium could replace the addition of FSH and follicular shell to the culture in terms of stimulating full oocyte maturation. However, our results and others (Dekel and Sherizly, 1985; Downs et al., 1988; Downs, 1989; Reed et al., 1991; Sommer et al., 1992) showed that, although EGF alone is equally effective as gonadotropins in promoting nuclear maturation, EGF alone did not stimulate cytoplasmic maturation. Overall therefore, we conclude that oocyte maturation is regulated by many factors including gonadotropins and EGF.

The present results also provide further insight into potential gonadotropin-dependent steroidal effects on oocyte maturation. Gonadotropin supplementation to the maturation medium did not affect estradiol but greatly elevated progesterone production

by follicular cells (Fig.VII-8 b and 9 b). Follicular shell addition to the culture dramatically increased estradiol secretion, but did not affect progesterone production (Fig.VII- 8 c and 9 c). Our results also showed that the interaction between follicular shell co-culture and gonadotropin supplementation affected the progesterone concentrations in culture media (Fig.VII-10) and the ratios of progesterone to estradiol concentrations in culture media (Fig.VII-11). Irrespective of the effects of EGF treatment, media derived from the culture of oocyte-cumulus-granulosa complexes in the presence of the gonadotropin combination (FS- & GTH+) had extremely high ratio of progesterone to estradiol (4073), while addition of one follicular shell (FS+ & GTH+) dramatically reduced this ratio (166.21). As we have shown that oocytes matured under the former environment had significant lower male pronuclear formation rate than that matured in the latter environment (Ding et al., 1988; Ding and Foxcroft, 1992), we suggest that a high progesterone to estradiol ratio in the absence of follicular shell may create an unsuitable environment for oocyte cytoplasmic maturation.

We therefore conclude: 1) EGF can stimulate both nuclear and cytoplasmic maturation in pig oocytes; 2) while EGF effects on nuclear maturation is independent of gonadotropin action, effects on cytoplasmic maturation result from interactions with gonadotropins or other follicular factors; 3) indirect effects of EGF on oocyte maturation are unlikely to be mediated by changes in follicular cell steroid production; whereas 4), actions of gonadotropins and the inclusion of follicular shells may affect oocyte maturation partially through regulation of the steroid milieu of the oocyte.

Table VII-1. Experiment I. Effect of EGF on Cumulus Expansion and Nuclear Maturation in Porcine Oocytes

| Treatment | Control (GTH*) | EGF (ng/ml) 0 | 0.1 | 1.0 | 10.0 |
|---|---|---|---|---|---|
| No. of Replicates | 3 | 3 | 3 | 3 | 3 |
| No. Eggs Examined | 60 | 54 | 65 | 65 | 65 |
| Cumulus Expansion | | | | | |
| No. | 60 a | 0 c | 45 b | 62 a | 63 a |
| (Mean±sem%) | (100±0.0) | (0.0±0.0) | (68.2±4.7) | (95.1±2.5) | (97.0±1.5) |
| Germinal Vesicle | | | | | |
| No. | 0 a | 33 b | 7 b | 1 b | 1 b |
| (Mean±sem%) | (0.0±0.0) | (62.9±7.3) | (11.4±3.1) | (1.3±1.3) | (1.3±1.3) |
| Pre-metaphase I | | | | | |
| No. | 0 | 8 | 5 | 5 | 6 |
| (Mean±sem%) | (0.0±0.0) | (14.7±2.3) | (8.3±4.2) | (8.2±2.4) | (8.7±4.7) |
| Metaphase I | | | | | |
| No. | 1 | 2 | 9 | 7 | 6 |
| (Mean±sem%) | (1.4±1.4) | (4.3±2.3) | (13.1±3.6) | (9.4±7.4) | (9.3±5.8) |
| Metaphase II | | | | | |
| No. | 57 a | 11 c | 44 b | 52 ab | 52 ab |
| (Mean±sem%) | (95.5±2.5) | (18.1±9.4) | (67.2±4.6) | (81.1±8.6) | (80.7±5.2) |
| Degenerated Oocytes | | | | | |
| No. | 2 | 0 | 0 | 0 | 0 |
| (Mean±sem%) | (3.0±1.5) | (0.0±0.0) | (0.0±0.0) | (0.0±0.0) | (0.0±0.0) |

*GTH: gonadotropins, FSH 2.5 μg/ml, LH 2.5 μg/ml and prolactin 20 ng/ml.

Table VII-2. Experiment II. The interactive effect of EGF, gonadotropins and follicular shell (FS) co-culture during maturation on sperm penetration and pronuclear development in porcine oocytes.

| | Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FS Co-culture | - | - | - | - | + | + | + | + |
| EGF | - | - | + | + | - | - | + | + |
| Gonadotropins | - | + | - | + | - | + | - | + |
| No. Replicates | 4 | 5 | 5 | 5 | 4 | 5 | 5 | 5 |
| No. Examined | 55 | 68 | 55 | 71 | 55 | 71 | 69 | 70 |
| GV No. | 37 a | 0 c | 4 b | 0 c | 33 a | 0 c | 5 b | 3 c |
| Mean±sem%[1] | 66.7±5.7 | 0.0±0.0 | 11.8±3.6 | 0.0±0.0 | 58.4±13.6 | 0.0±0.0 | 6.8±3.3 | 4.4±1.9 |
| PMI - MI No. | 4 | 0 | 1 | 0 | 5 | 0 | 1 | 0 |
| Mean±sem%[1] | 7.7±7.7 | 0.0±0.0 | 1.3±1.3 | 0.0±0.0 | 8.9±3.4 | 0.0±0.0 | 1.4±1.4 | 0.0±0.0 |
| >MII No. | 14 c | 68 a | 50 ab | 70 a | 16 c | 70 a | 59 b | 65 ab |
| Mean±sem%[1] | 25.6±5.0 | 100±0.0 | 90.7±3.8 | 98.6±1.4 | 30.8±12.9 | 100±0.0 | 86.6±5.9 | 93.0±2.1 |
| Penetration No. | 10 b | 63 a | 47 a | 67 a | 10 b | 65 a | 52 ab | 62 a |
| Mean±sem%[2] | 71.3±10.9 | 92.6±2.4 | 95.4±4.2 | 95.4±4.2 | 67.5±17.5 | 91.4±5.3 | 87.4±5.3 | 95.7±2.9 |
| 2Pb+1F No. | 8 a | 51 a | 41 a | 56 a | 7 a | 49 a | 41 a | 50 a |
| Mean±sem%[3] | 83.3±9.6 | 81.7±6.9 | 88.7±3.4 | 84.6±3.9 | 71.1±19.7 | 75.2±3.5 | 77.0±8.5 | 82.0±10.4 |
| Penetrated Oocytes With SH1 | | | | | | | | |
| No. | 2 bc | 22 ab | 32 a | 15 bc | 5 a | 11 bc | 30 a | 4 c |
| Mean±sem%[3] | 16.7±9.6 | 35.5±7.9 | 66.7±7.8 | 20.8±6.6 | 57.8±21.8 | 17.2±6.1 | 59.0±12.8 | 7.2±3.7 |
| Penetrated Oocytes With SH2 | | | | | | | | |
| No. | 8 a | 22 b | 15 b | 22 b | 2 b | 15 b | 15 b | 13 b |
| Mean±sem%[3] | 83.3±9.6 | 34.5±6.8 | 33.3±37.8 | 34.1±7.5 | 17.8±9.67 | 23.7±9.2 | 28.7±10.9 | 20.3±3.2 |
| Penetrated Oocytes With MPN | | | | | | | | |
| No. | 0 f | 19 cd | 0 f | 30 bc | 3 de | 39 ab | 7 e | 45 a |
| Mean±sem%[3] | 0.0±0.0 | 30.0±4.4 | 0.0±0.0 | 45.1±3.4 | 24.4±12.5 | 59.0±5.4 | 12.4±3.5 | 72.5±1.5 |
| Mean Sperm Number in Each Penetrated Oocytes | b | a | ab | a | ab | ab | ab | a |
| Mean±sem | 1.8±0.35 | 5.6±2.15 | 2.9±0.44 | 5.5±1.78 | 4.6±2.86 | 4.6±1.45 | 3.6±1.46 | 5.4±1.41 |

[1] means of percentage of nuclear maturational stages of the total oocytes examined.

[2] means of the percentage of MII oocytes penetrated by sperm of all MII oocytes.

[3] means of the percentage of penetrated oocytes with 2Pb+1F, SH1, SH2 and MPN of all penetrated MII oocytes.

a-f: Within a row without common letters were significantly different ($P<0.05$).

GV: germinal vesicle stage; PMI: pre-metaphase I stage; MI: metaphase I stage; >MII: beyond metaphase II stage; 2Pb+1F: oocytes containing one female pronucleus and two polar bodies; SH1: oocytes containing intact or slightly swollen sperm heads; SH2: oocytes containing swollen sperm heads; MPN: penetrated oocytes with male pronucleus/pronuclei.

Table VII-3. Experiment II. Estradiol and progesterone concentrations in culture media collected at the completion of maturation culture.

| | Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FS Co-culture | - | - | - | - | + | + | + | + |
| EGF | - | - | + | + | - | - | + | + |
| Gonadotropins | - | + | - | + | - | + | - | + |
| Estradiol-17ß concentrations in (ng/ml) culture media | | | | | | | | |
| No. replicates | 2* | 4 | 3* | 4 | 4 | 4 | 4 | 4 |
| | d | cd | d | cd | abc | bcd | ab | a |
| Mean±sem | 0.50±0.06 | 0.40±0.07 | 0.31±0.11 | 0.39±0.05 | 11.66±2.38 | 8.68±2.56 | 13.30±3.68 | 22.92±10.44 |
| Progesterone concentrations (ng/ml) in culture media | | | | | | | | |
| No. Replicates | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | e | a | e | ab | de | bcd | cde | abc |
| Mean±sem | 387.89±88.11 | 1552.23±135.9 | 343.98±21.57 | 1415.10±358.1 | 631.60±160.36 | 1026.93±72.83 | 763.52±157.38 | 1177.22±108.88 |

* Three samples were off the standard curve at the low dose end and were treated as missing samples.
a-e Within a row without common letters were significantly different (P<0.05).

Fig.VII-1 Photomicrographs of live oocyte-cumulus complexes cultured in medium containing a gonadotropin combination (FSH, LH and prolactin, control) or different doses of EGF for 48 h using a non-static culture system. **A**: oocyte complexes from the control group stimulated by gonadotropins (2.5μg/ml FSH, 2.5μg/ml LH and 20 ng/ml prolactin), showing cumulus expansion. Cumulus cells were evenly dispersed around the oocytes except the corona radiata. Cumulus was very sticky because of mucification of cumulus cells. **B**: oocytes complexes from 0 EGF group (with no EGF and no gonadotropins), showing no cumulus expansion. Cumulus was compact with and dark-looking with no mucification. **C**: oocyte complexes from 0.1 ng/ml EGF group. The left oocyte shows little cumulus expansion, and is compact and dark-looking and similar to those in B. The right oocyte has lost most of the cumulus cells and was classified as a cumulus expanded oocyte. However, the cumulus was not musified. **D**: oocytes complexes from the 1.0 ng/ml EGF group. The upper oocyte has a partially expanded cumulus, whereas the lower one has lost most of its cumulus cells and was partially denuded. The cumulus had no mucification. **E**: oocyte complexes from 10 ng/ml EGF group. Both oocytes were almost denuded.

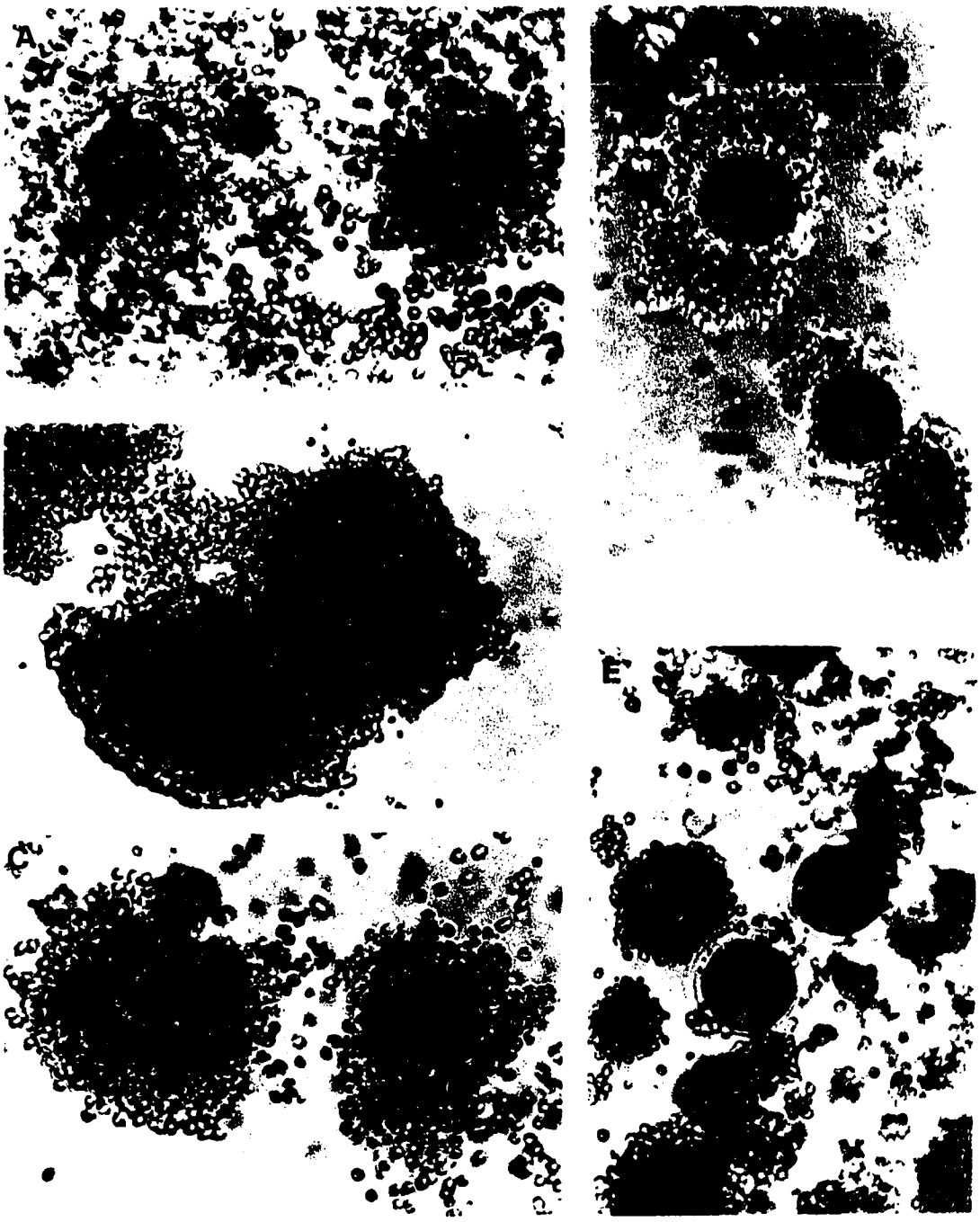

A
E

Fig.VII-2 Effects of EGF (a), gonadotropins (GTH, b) and follicular shell (FS, c) co-culture on nuclear maturation (MII) of oocytes. '*' indicates significant ($P<0.05$) difference between two means within a treatment.

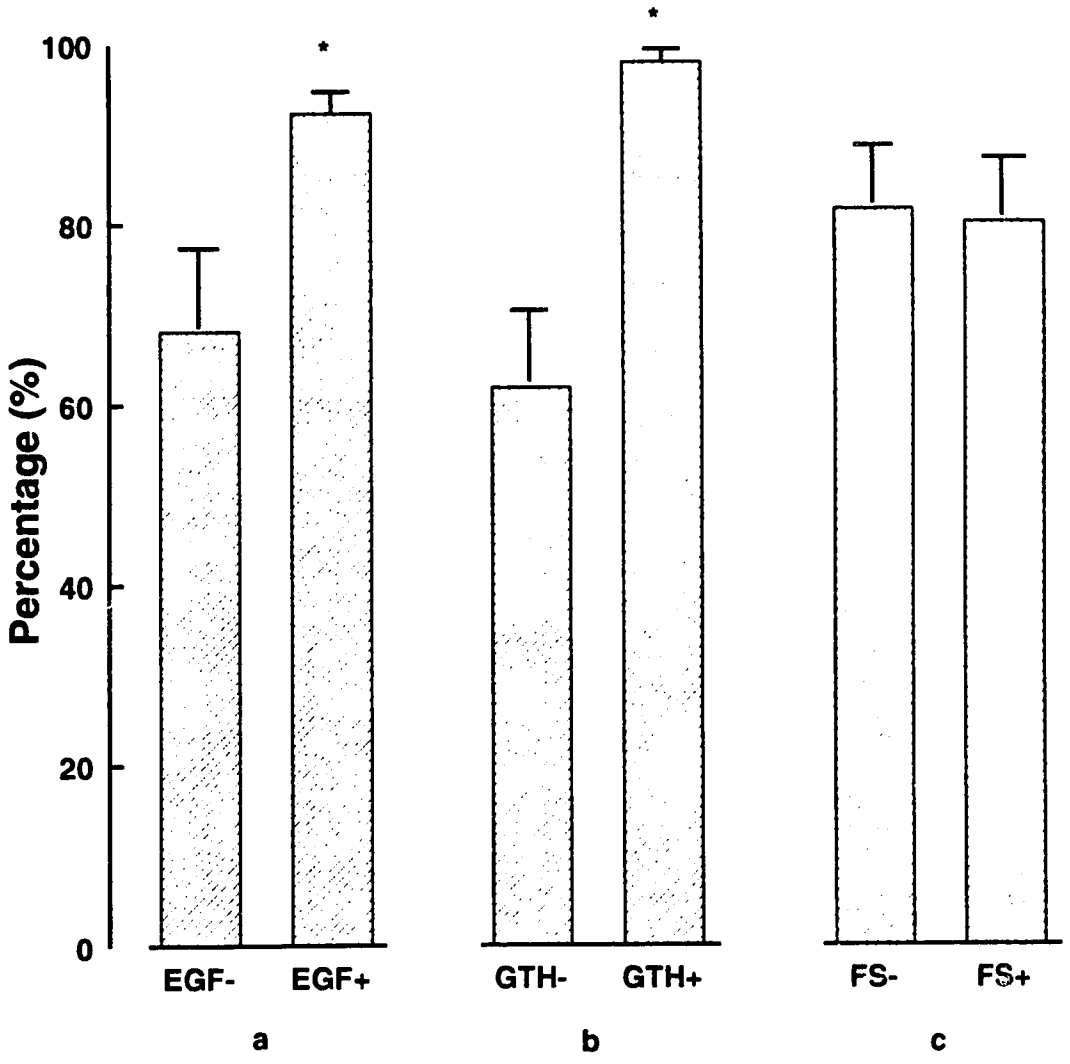

Metaphase II Rates---Main Factors
Percentage (%)
100
80
60
40
20
0
*
*
EGF-
EGF+
GTH-
GTH+
FS-
FS+
a
b
c

Fig.VII-3 Interaction effects between EGF and gonadotropins (GTH) on nuclear maturation of oocytes. Among the four interaction means, means with different subscripts differ significantly ($P < 0.05$).

## Metaphase II Rates---Interaction between EGF and GTH

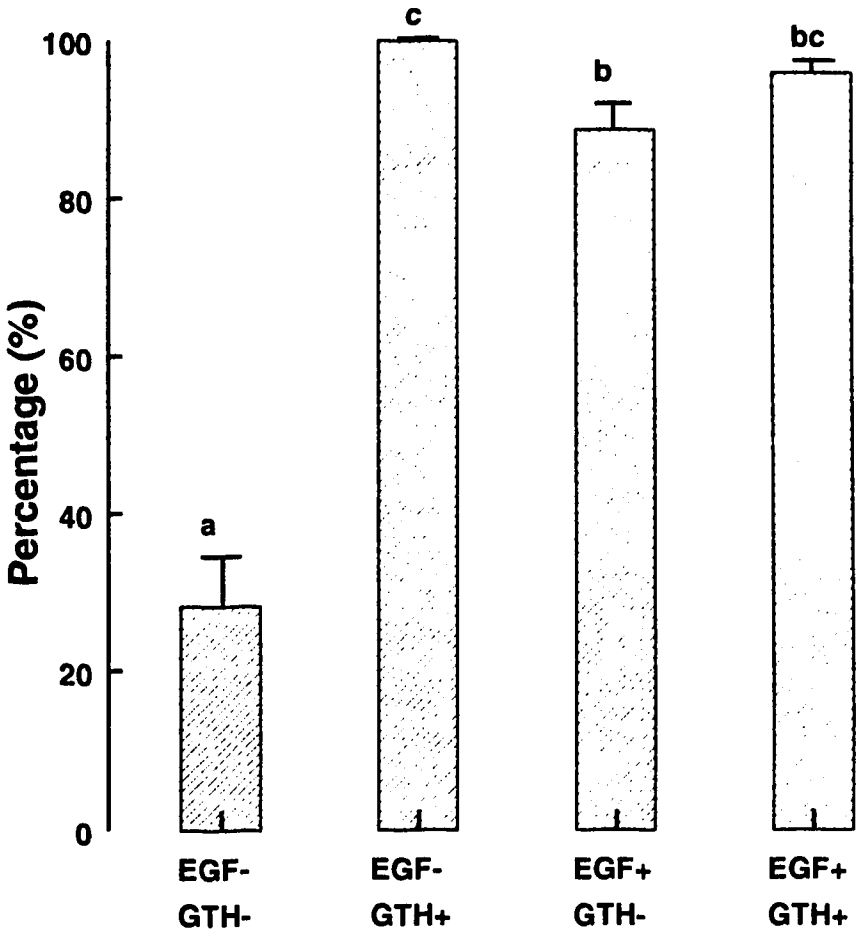


Fig.VII-4 Effects of EGF (a), gonadotropins (GTH, b) and follicular shell (FS, c) co-culture on sperm penetration rates of oocytes. '*' indicates significant ($P<0.05$) difference between two means within a treatment.

## Sperm Penetration Rates---Main Factors

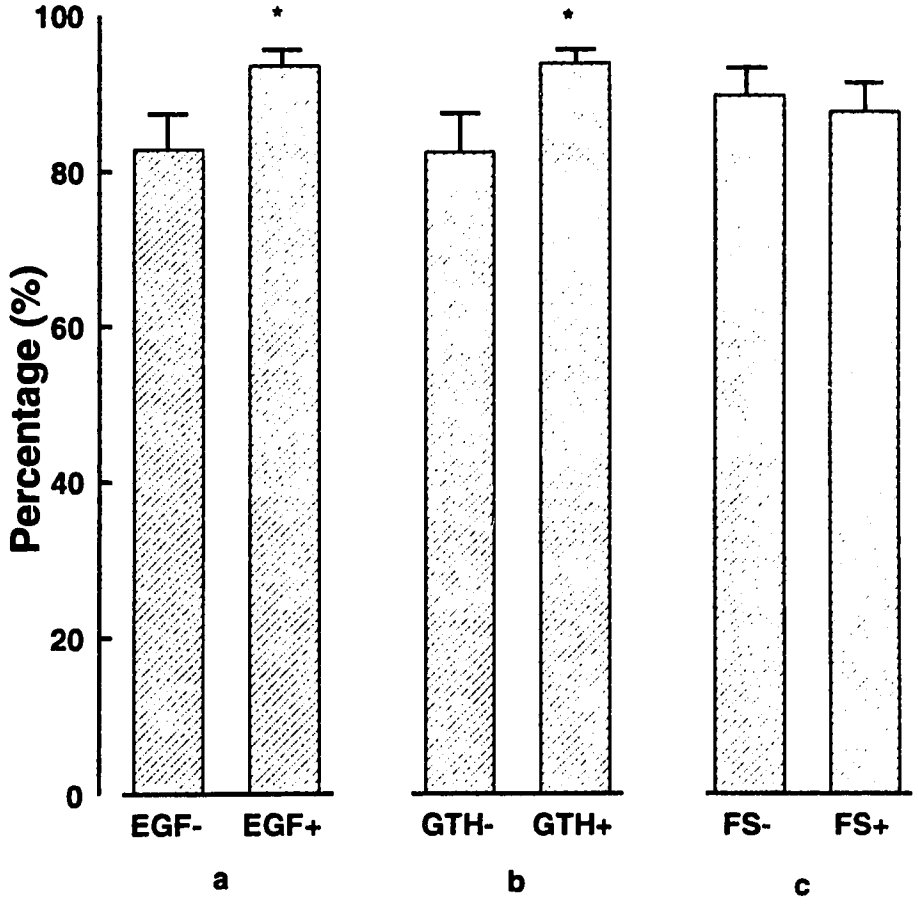


Fig.VII-5 Effects of EGF (a), gonadotropins (GTH, b) and follicular shell (FS, c) co-culture on normal female pronuclear formation in penetrated oocytes. '*' indicates significant ($P<0.05$) difference between two means within a treatment.

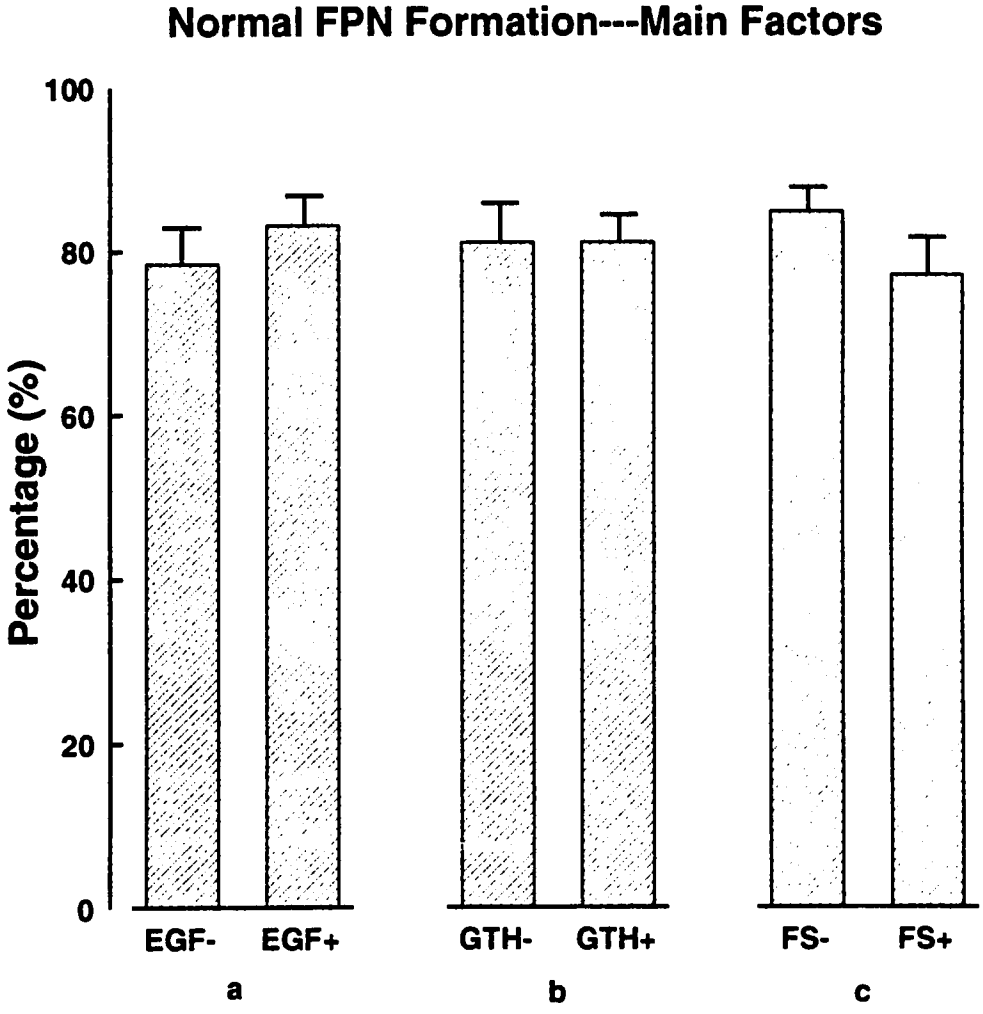

Normal FPN Formation---Main Factors
Percentage (%)
100
80
60
40
20
0
EGF-
EGF+
GTH-
GTH+
FS-
FS+
a
b
c

Fig.VII-6 Effects of EGF (a), gonadotropins (GTH, b) and follicular shell (FS, c) co-culture on male pronuclear formation in penetrated oocytes. '*' indicates significant ($P<0.05$) difference between two means within a treatment.

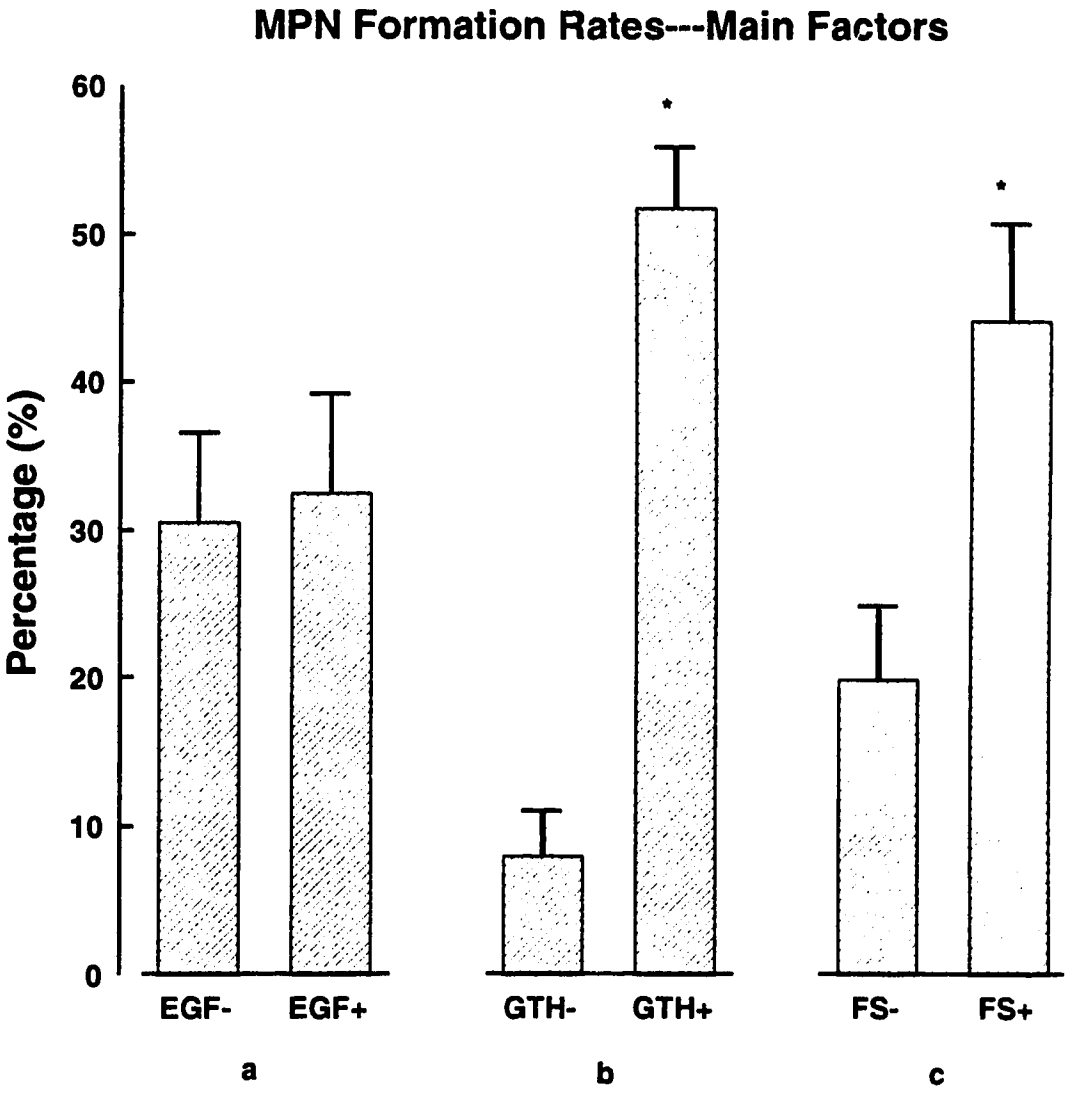

MPN Formation Rates---Main Factors
Percentage (%)
60
50
40
30
20
10
0
*
*
EGF-
EGF+
GTH-
GTH+
FS-
FS+
a
b
c

Fig.VII-7 Interaction effects between EGF and gonadotropins (GTH) on MPN formation in penetrated oocytes. Among the four interaction means, means with different subscripts differ significantly ($P < 0.05$).

**MPN Formation Rates--Interaction between EGF and GTH**

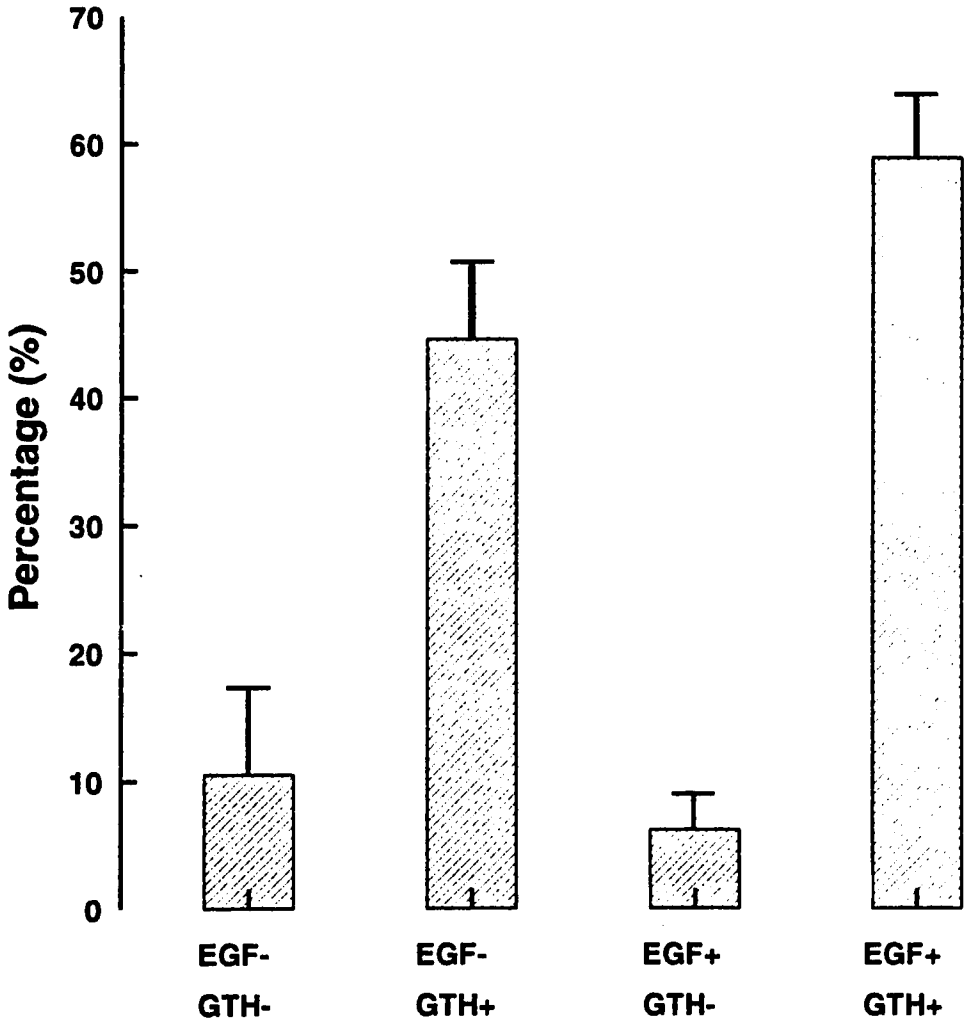


Fig.VII-8 Effects of EGF (a), gonadotropins (GTH, b) and follicular shell (FS, c) co-culture on estradiol-17ß concentrations in culture media which were harvested at the completion of oocyte maturation culture. '*' indicates significant ($P<0.05$) difference between two means within a treatment.

## Estradiol Concentrations in Culture Media---Main Factors

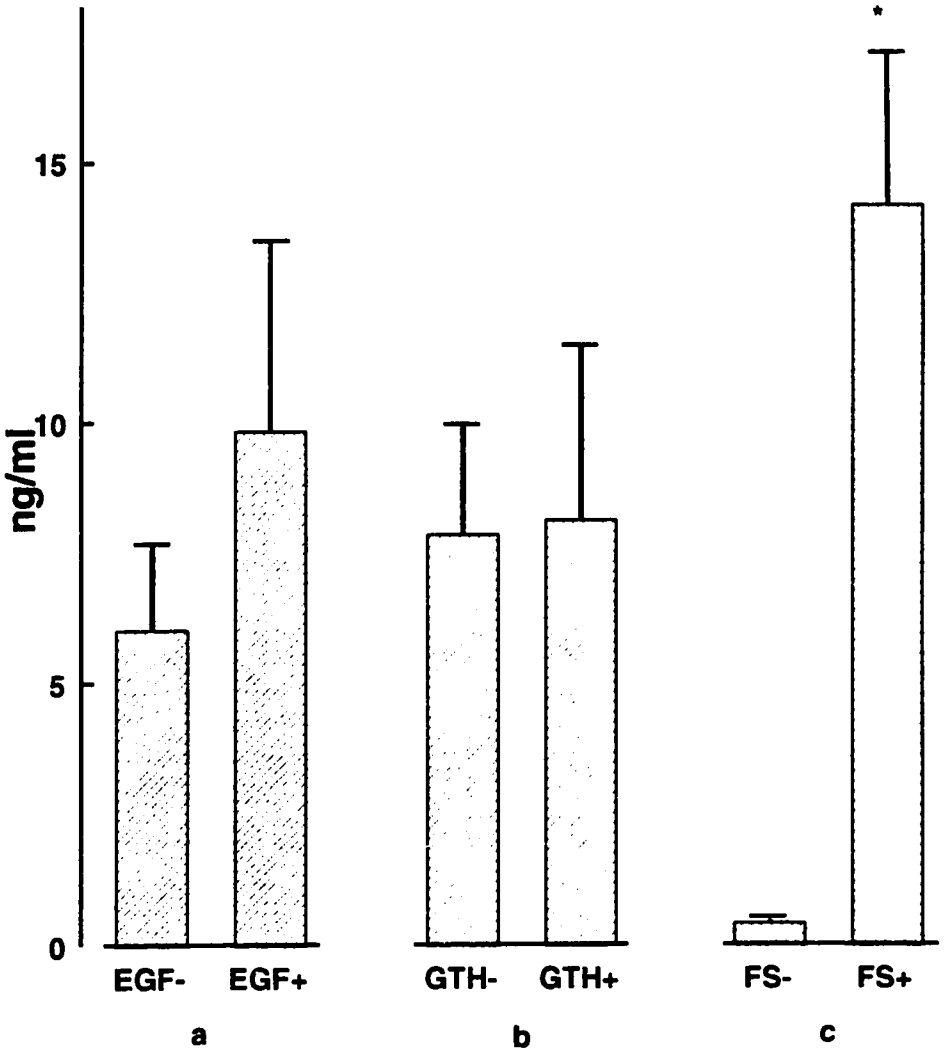


Fig.VII-9 Effects of EGF (a), gonadotropins (GTH, b) and follicular shell (FS, c) co-culture on progesterone concentrations in culture media which were harvested at the completion of oocyte maturation culture. '*' indicates significant ($P<0.05$) difference between two means within a treatment.

**Progesterone Concentartions in Culture Media---Main Factors**

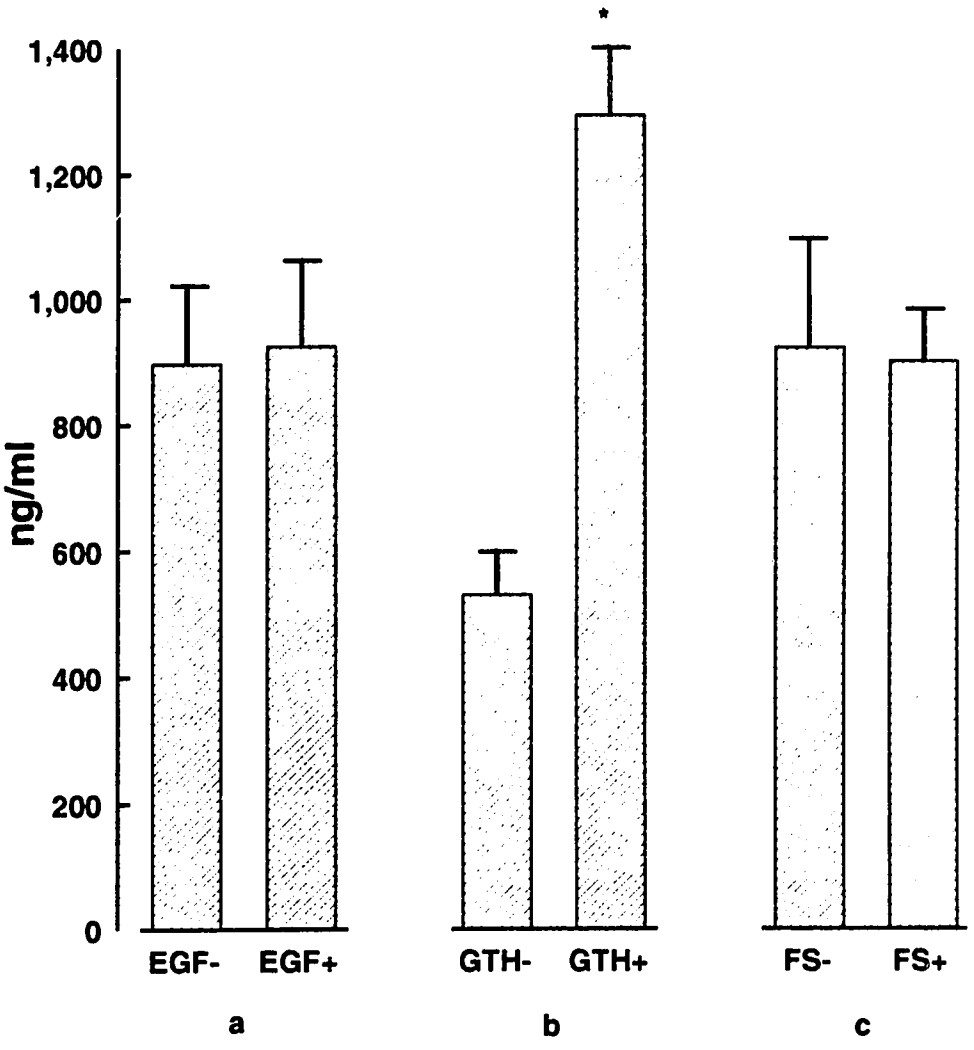


Fig.VII-10 Interaction effects between gonadotropins (GTH) and follicular shell co-culture on progesterone concentrations in culture media which were harvested at the completion of oocyte maturation culture. Among the four interaction means, means with different subscripts differ significantly ($P < 0.05$).

**Progesterone in Culture Media---Interaction Between GTH and FS**

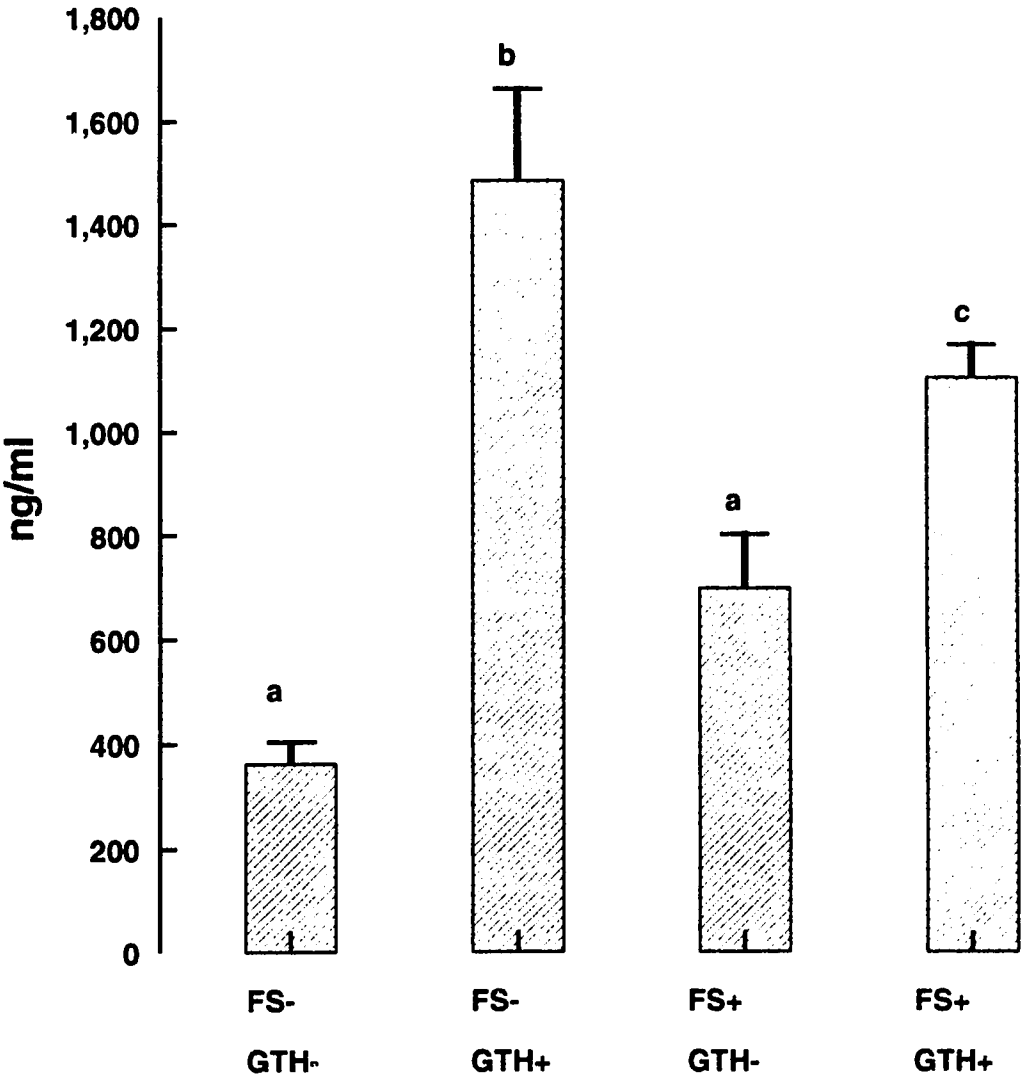
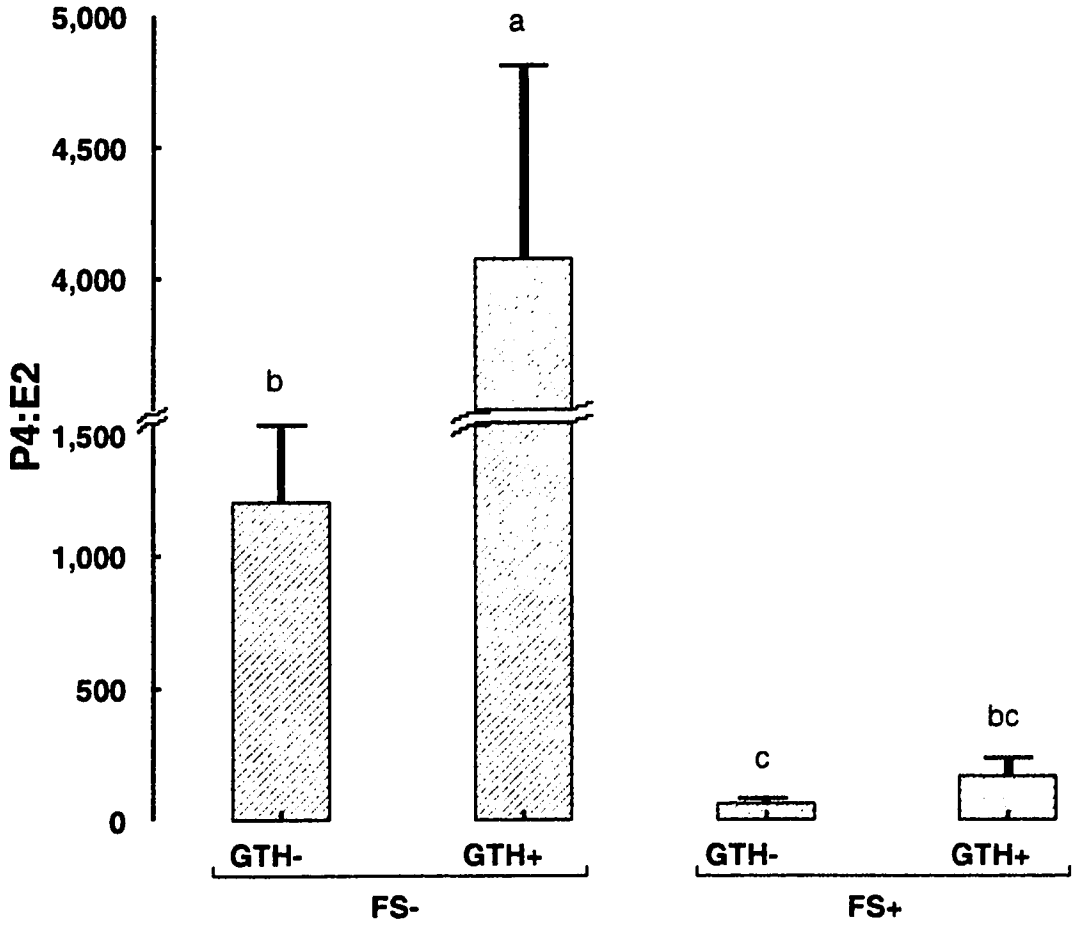


Fig.VII-11 Interaction effects between gonadotropins (GTH) and follicular shell co-culture on the ratios of progesterone (P4) to estradiol-17ß (E2) in culture media which were harvested at the completion of oocyte maturation culture. Among the four interaction means, means with different subscripts differ significantly ($P < 0.05$).

## Ratio of Progesterone to Estradiol

### ---Interaction between FS and GTH



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# CHAPTER VIII GENERAL DISCUSSION

Oocyte maturation involves both nuclear and cytoplasmic components (Chang, 1955; Thibault, 1977) and cytoplasmic maturation must run parallel with nuclear maturation in order for an oocyte to achieve full developmental potential if fertilization occurs (for review, see Thibault et al., 1987). During porcine oocyte maturation in vitro, cytoplasmic maturation is frequently impaired, resulting in low developmental potential after fertilization (see Mattioli et al., 1988a). Our understanding of the regulation of cytoplasmic maturation of oocytes is limited and further researches will facilitate our knowledge of oocyte maturation and allow the establishment of better maturation environments for porcine oocytes in vitro. In this thesis, a series of experiments were carried out and the results presented will help us to further understand the regulation of pig oocyte maturation in a number of respects. The purpose of this final chapter is to integrate all the results presented in this thesis and to gain further insight into the regulation of oocyte maturation from these general discussions.

## 1. Effect of protein synthesis on oocyte maturation.

Changes in protein synthesis during oocyte maturation and their functional importance in the cytoplasmic maturation of porcine oocytes are an active area of investigation. In a previous study we examined the changes in protein synthesis during fertilization (Ding and Clarke et al., 1992). Protein synthetic patterns underwent significant changes during fertilization in oocytes matured in vitro, which were the same

as those observed in oocytes matured in vivo. These changes in protein synthetic pattern were similar to the post-translational changes in presynthesized proteins in the absence of protein synthesis during fertilization. These results suggest that those proteins accumulated during maturation are important for fertilization and perhaps also for early embryonic development. Also we suggested that new protein synthesis during fertilization may not be crucial for sperm penetration and male pronuclear development. Our results (Chapter III) from protein inhibition experiment during fertilization confirmed our hypothesis that fertilization and male pronuclear formation could occur normally in the absence of new protein synthesis. Female pronuclear formation was not affected either. Thus protein synthesis during maturation is probably crucial for oocytes to achieve fertilization ability.

Mammalian oocytes have an absolute requirement for protein synthesis in the period immediately before GVBD for the normal progression of the meiotic cycle. Inhibition of de novo protein synthesis during maturation blocks GVBD in cow (Hunter and Moor, 1987; Kastrop et al., 1991), sheep (Moor and Crosby, 1986), pig (Fulka et al., 1986; Kubelka et al., 1988), rat (Ekholm and Magnusson, 1979) and mouse (Downs, 1990; Szöllösi et al., 1991) oocytes. Our results showed that after culture of porcine oocyte-cumulus-granulosa cell complexes (oocyte-complexes) with two follicular shells in medium containing protein synthesis inhibitor (cycloheximide) for the entire maturation period, almost 90% of oocytes remained at GV stage after 47 h of culture. It is known now that the protein factor(s) which induces oocyte maturation is maturation promoting factor (MPF). MPF initiates the transition from G2 to M phase in the meiotic and mitotic

cell cycle (see review by Masui, 1992). Crucial MPF-like activity reaches threshold level at 8-16 h after the induction of maturation and peaked at MI and MII stage (Mattioli et al., 1991) in pig oocytes. The appearance of MPF activity in oocytes requires protein synthesis (Mattioli et al., 1991). One of the MPF components called cyclin is synthesized continuously during interphase and degraded rapidly at the onset of cell division (see review by Masui, 1992). Inhibition of protein synthesis therefore blocks MPF activity and thus suppresses the resumption of meiosis.

After inhibition of protein synthesis starting at 24 h of culture and maintained for the rest of culture period (to 47h), about 72% of oocytes extruded their first polar body (Table III-2). However, a majority of them had either a condensed female chromatin mass (Fig.III-1, A; about 38%) or an incipient female nucleus (Fig.III-1 C; about 33%). A previous experiment showed that GVBD occurs after about 20 h in culture and by about 24 h, most of oocytes reached MI stage in our culture system (Ding, unpublished observations). These results suggest that protein synthesis after MI is crucial for maintaining normal MII nuclear configuration but is not important for the completion of the first meiotic division. When protein synthesis in pig oocytes was inhibited after 32 h of culture, nuclear formation was also observed (Mattioli et al., 1991). Inhibition of protein synthesis at MI induced the extrusion of a polar body (Pb) and formation of an interphase nucleus in mouse oocytes (Clarke and Masui, 1983; Kang et al., 1991; Szöllösi et al., 1991). If protein synthesis is resumed, the decondensed chromosomes become condensed and return to the metaphase stage (Clarke and Masui, 1983). From these studies it is suggested that the maintenance of condensed chromosomes during the

transition from MI to MII depends on protein synthesis; in the absence of protein synthesis the chromosomes decondense and form an interphase nucleus. Our observation that about one third of the oocytes developed incipient nuclei when protein synthesis was inhibited at early MI, suggests that the above mechanism also exists in porcine oocytes. However, about 40% of the oocytes had a condensed chromatin mass and even after sperm penetration this chromatin retained a similar configuration (Fig.III-3 c), suggesting differences between porcine and mouse oocytes. The maintenance of a metaphase configuration is dependent on MPF activity (see review by Masui, 1992). However, MPF activity decreases during anaphase I to telophase I (Mattioli et al., 1991, Naito et al., 1991, 1992), which is assumed to be caused by degradation of cyclin (see review by Moor et al., 1990). Thus re-entering metaphase after the completion of first meiosis depends on the synthesis of cyclin to achieve an increase in MPF activity. Inhibition of protein synthesis from early MI stage onwards prevents the oocytes from accumulating sufficient cyclin and hence MPF activity, resulting in prevention of oocytes from re-entering metaphase after the completion of first meiosis.

In contrast, after inhibition of protein synthesis from 36 h (at early MII stage) culture onwards, about 80% of oocytes had a normal MII nuclear configuration with dispersed individual chromosomes (Fig.III-1, B). This suggests that, although there is a reduction of MPF activity during anaphase and telophase transition between 26 to 33 h of culture (Mattioli et al., 1991; Naito et al., 1991), by 36 h oocytes have accumulated sufficient amounts of MPF and thus, immediately after the completion of first meiotic division, oocytes enter the second meiotic division and reaches MII stage with a normal

nuclear configuration. Inhibition of protein synthesis either after 36 h of culture (at early MII) or after 48 h of culture (at late MII stage, unpublished observation) did not significantly stimulate parthenogenetic activation of the oocyte. These results seem to be different to the findings in mice (Clarke and Masui, 1983). It seems that, in the mouse, maintenance of a normal MII configuration during MII arrest is dependent on continuous synthesis of cytostatic factor (CSF) which stabilizes MPF activity during MII arrest (for references, see review by Masui, 1992), while in porcine oocytes CSF activity is probably not dependent on continuous synthesis of this factor and thus inhibition of protein synthesis does not cause the de-stabilization of MPF, similar to results in *Rana* oocytes (Ziegler and Masui, 1976).

Although inhibition of protein synthesis at the early MII stage (36 h after onset of maturation culture) did not affect nuclear configuration at MII stage, normal completion of the second meiosis was impaired. After sperm activation, most of oocyte metaphase nuclei did not undergo normal nuclear division by extrusion of the second polar body. Instead, they formed female pronuclei directly. In contrast, inhibition of protein synthesis at the late MII stage (48 h after culture) did not affect the normal completion of second meiosis. These results suggest that, between 36 to 47 h of culture, oocytes synthesize some factors which are important for re-initiation of second meiosis after sperm activation. This may further indicate that if oocytes are activated by sperm immediately after reaching MII stage, the completion of the second meiosis may be affected.

Inhibition of protein synthesis after 24 h of culture (at early MI stage) totally

inhibited male pronuclear formation. However, almost all penetrated oocytes (even MI oocytes) possessed the ability to decondense the sperm nucleus/nuclei (Fig.III-1 d and e, and Fig.3). These results are similar to findings of Clarke and Masui (1987) in mice. These authors suggested that oocyte cytoplasmic factors required for the dispersion of sperm chromatin, which appeared to coincide with GVBD in many species (Longo, 1985), developed in the maturing oocytes independently of continuing protein synthesis, whereas the transformation to a pronucleus required continuing protein synthesis during maturation. After 36 h of culture (at early MII stage), inhibition of protein synthesis did not totally block MPN formation, although the rate of MPN formation was reduced compared to the control group when inhibiting protein synthesis during fertilization only (Fig.3, $P < 0.05$). These results suggest that synthesis of male pronuclear growth factor(s) (MPGF) (Iwamatsu and Chang, 1972; Thibault et al., 1975) may commence immediately after oocytes reach MI; by the early MII stage, MPGF has accumulated in sufficient amounts to provide oocytes with the ability to decondense sperm heads and subsequently to form male pronuclei. Recently it has been found that male pronuclear formation is correlated to the level of glutathione in oocytes (hamster, Perrault et al., 1984; 1988; mouse, Calvin et al., 1986; pig, Yoshida et al., 1993a). Glutathione concentration in porcine oocytes increased sharp between 24 and 36 h of culture when oocytes were cultured in the presence of cysteine, a precursor of glutathione (Yoshida et al., 1993a). Thus it seems that in our system, inhibition of protein synthesis after 24 h of culture may block the glutathione synthetic pathway, resulting in the prevention of male pronuclear formation. Another alternative explanation comes from the recent suggestion that a low

male pronuclear formation rate is related to the low MPF activity at the MII stage (mouse, Borsuk, 1991; pig, Naito et al., 1992), since high MPF/H1 kinase activity is a prerequisite for removal of the (sperm) nuclear membrane (Peter et al., 1990). Oocytes matured in control medium with lower H1 kinase activity at the MII stage had a lower male pronuclear formation rate when compared with oocytes matured in medium supplemented with follicular fluid. Therefore inhibition of protein synthesis after 24 h of culture, as discussed previously, would inhibit the second increase of MPF activity, and in turn causes inhibition of male pronuclear formation, as well as loss of a normal metaphase II configuration.

Dramatic reprogramming of protein synthesis were seen during GVBD. Before GVBD and during the transition from MI to MII, there were no apparent qualitative, but obvious quantitative, changes in protein synthesis. From 32 to 47 h of culture (early MII to late MII), the synthesis of 26 K polypeptides diminished and finally ceased, and a 25 kD band, which first appeared at the MI stage and was post-translationally modified during the pronuclear formation period by dephosphorylation (Ding and Clarke et al., 1992), became dominant in that region. We conclude from our combined results that the reprogramming of protein synthesis after GVBD is absolutely essential for oocytes to complete meiotic maturation and male and female pronuclear development.

In conclusion, cytoplasmic reprogramming in oocytes during porcine oocyte maturation is absolutely essential for completion of oocyte meiosis and formation of both pronuclei after sperm penetration. While decondesation of the sperm nucleus may be independent of continued protein synthesis, the transformation of a sperm nucleus to a

male pronucleus requires protein synthesis lasting at least until the early MII stage. During fertilization, protein synthesis is not essential for sperm penetration, the completion of the second meiosis, nor for pronuclear development.

**2. Effect of follicular shell co-culture and follicle shell conditioned medium on porcine oocyte maturation.**

Achievement of full oocyte maturation requires interaction between follicular somatic cells and oocytes during final maturation period (Moor et al., 1983). Follicle shell co-culture with porcine oocyte-complexes increased MPN formation (Mattioli et al., 1988a; Ding et al., 1988; Nagai et al., 1993). Furthermore, oocyte-complexes co-cultured with follicle shells in vitro have resulted in birth of piglets after in vitro fertilization (Mattioli et al., 1989). In the second series of experiments in this thesis (Chapter IV), we confirmed that male pronuclear formation was greatly enhanced (78.15%) by co-culture of oocyte-complexes with two follicle shells when compared with male pronuclear formation for those oocyte-complexes cultured in the absence of follicle shells (38.58%). This follicle shell effect on MPN formation was demonstrated again in a later series of experiment (Chapter V, Table V-2). These results suggest that follicle shells secrete stimulatory factors which are important for supporting cytoplasmic maturation of porcine oocytes. Further evidence supporting this hypothesis come from experiments in which the use of follicle conditioned medium (Mattioli et al., 1988 a, b) or medium supplemented with pig follicular fluid (Naito et al., 1988; Yoshida et al., 1992; Funahashi et al., 1993)

improved cytoplasmic maturation of pig oocytes. Indeed, oocytes matured in medium supplemented with a certain fraction of follicular fluid and cysteine have yielded live birth of piglets recently (Yoshida et al., 1993b).

In order to establish a follicle conditioned medium culture system for pig oocytes in our laboratory, we tested effects of both FSH- and FSH-LH-Prl-stimulated follicle conditioned medium on cytoplasmic maturation of pig oocytes (Chapter V). Results showed that FSH-stimulated follicle conditioned medium provided better maturation environment than FSH-LH-Prl-stimulated conditioned medium (Fig.V-2). In addition, further supplementation of gonadotropins (FSH, LH and Prl) to conditioned media during oocyte maturation period had beneficial effect on cytoplasmic maturation. It seems that high concentrations of gonadotropins are required when oocyte-complexes are cultured in follicular secretions, since oocytes cultured in follicular fluid also required gonadotropin supplementation in order to achieve high cytoplasmic maturation (Naito et al., 1988).

The mechanism by which follicle cells and/or follicular secretions exert their effect on oocyte cytoplasmic maturation is not well defined. On one hand, it seems that follicular factors secreted by follicle cells could insert their effect on the oocyte via cumulus cell absorption of those soluble factors, since conditioned medium did not support maturation of oocytes denuded of cumulus cells immediately before maturation culture (Mattioli et al., 1988b). On the other hand, we found that in the presence of two follicle shells, a small piece of mural granulosa tissue directly associated with cumulus-enclosed oocytes (G-C-oocytes) by intercellular gap junctions, more effectively stimulated

cytoplasmic maturation when compared with cumulus-enclosed oocytes (C-oocytes) without direct association with mural granulosa tissue (Ding et al., unpublished observations, see Fig.II-3). This suggests that the factors synthesized by mural granulosa cells may exert their effects on cumulus-enclosed oocytes more effectively by intercellular communication via the intercellular gap junctions between granulosa and cumulus cells. Another alternative explanation is the association of a small piece of granulosa tissue with cumulus increased the absorption area for follicular factors secreted by follicle shells and thus affected cytoplasmic maturation.

**3. Effect of follicular heterogeneity on oocyte maturation.**

Heterogeneity in pig follicular development has been established (for references see Chapter II) and it has been suggested that this diversity of follicular development may affect oocyte quality (Foxcroft and Hunter, 1985; Hunter and Wiesak, 1990) and hence embryo quality (Pope et al., 1990). In this thesis, two series of experiments were carried out to examine the possibility that follicles with different maturity will affect oocyte maturation, particularly cytoplasmic maturation. In one experiment (Chapter IV) in which follicles from eCG primed prepubertal gilts (36 h and 72 h after eCG) were used, oocyte cytoplasmic maturation was significantly affected by the size of co-cultured follicle shells, but was not affected by the time at which co-cultured follicles were harvested after eCG treatment. Cytoplasmic maturation was positively correlated to size, follicular fluid progesterone and estradiol and their ratios to testosterone of co-cultured follicles, but not with steroid hormones in culture medium. These data are interpreted as indicating that

cytoplasmic maturation is correlated with maturational status of the follicle shell used for co-culture and hence supporting the hypothesis that follicular heterogeneity may cause diversity of oocyte quality. In another experiment (Chapter VI) in which conditioned media produced by follicle shells from cyclic gilts were used, cytoplasmic maturation was improved in oocyte-complexes cultured in conditioned media produced by Day 20 follicles than by Day 17 follicles, again suggesting oocyte quality could be affected by maturity of follicular somatic cells.

Although both experiments indicate that maturity of follicle cells would affect oocyte quality, the results of the two experiments were substantially different. One showed that follicle size, not follicle age (36 and 72 h after eCG) affected cytoplasmic maturation, whilst the other showed that follicle age (Day 17 and Day 20), not follicle size, was important. However, if follicle size and follicular steroidogenesis are used as parameters of follicular maturity, then the difference in maturity was bigger between sizes than between times after eCG, when using eCG primed follicles. In contrast, the difference in maturity was greater between follicle ages (Day 17 and Day 20 of estrous cycle) than between size categories when follicles were obtained from naturally cyclic gilts (see Table VI-4). Indeed, the time difference in eCG primed follicles were only 36 h, while in cyclic follicles it was 72 h. These differences may be, at least partly, the cause of the conflicting results in the two experiments.

Size of follicles did not affect cytoplasmic maturation when using cyclic follicles, as it did when using eCG primed follicles, indicating that follicles in preovulatory pool in cyclic gilts were more uniform in their maturational status than those in eCG primed

gilts. Another alternative explanation for lack of a size effect on cytoplasmic maturation when using cyclic follicles, is that we pooled five follicles in each category to produce conditioned medium. Even if the mean level of maturity was lower for small follicles, if there were one or two follicles among them with average or equal maturity as compared to large follicles, then qualitatively the conditioned medium would contain similar atory factors for cytoplasmic maturation to those produced by large follicles and have similar ability to support cytoplasmic maturation.

It was shown that the size effect on cytoplasmic maturation was more significant in 72 h than in 36h eCG primed follicles (Table IV-3 and IV-4). A similar trend exists for cyclic follicles when comparing Day 17 and Day 20 follicles (see Table VI-3). Altogether, these results suggest that the maturational status of follicles in a preovulatory pool is more segregated at later stages than at early stages of follicular development.

The other supporting fact for the concept of follicular heterogeneity affecting oocyte quality comes from observations of nuclear maturation in individual preovulatory follicles in vivo in cyclic and eCG/hCG treated prepubertal gilts (Hunter and Wiesak, 1990; Wiesak et al., 1990). Oocytes collected at late follicular maturational stage (day 20-21, after LH ovulatory surge) from cyclic gilts or at 35 to 40 h after hCG from eCG/hCG treated prepubertal gilts both showed considerable variability in individual animals in the stage of nuclear maturation. This variability was greater in eCG/hCG treated prepubertal than cyclic gilts. The stage of nuclear maturation was correlated with follicular fluid volume in cyclic pigs and with follicle size in eCG/hCG treated prepubertal animals.

In summary, our results provide direct experimental support for the hypothesis that follicular heterogeneity in pig follicular development affects quality of oocytes.

**4. Possible identity of factors secreted by follicle cells ?**

How follicle cells exert their effects on oocyte maturation is not well understood. Mattioli et al. (1988b) examined the intercellular coupling between cumulus cells and the oocyte and found that follicle conditioned medium prolonged the coupling when compared with fresh control maturation medium. When oocyte-complexes were co-cultured with follicle shells, uncoupling between the oocyte and cumulus cells was also significantly delayed when compared with cultured without follicle shells (unpublished observations). The prolonged coupling between the oocyte and surrounding cumulus cells will allow maximal interaction between the oocyte and follicular somatic cells during the final maturation period. This interaction is essential for oocytes to obtain cytoplasmic maturation, since conditioned medium had no effect on denuded oocytes. Therefore, factors which can prolong the oocyte-cumulus coupling may stimulate cytoplasmic maturation.

An ether extract of follicle conditioned medium or progesterone, but not estradiol, nor androgens, partially mimicked the effect of conditioned medium on metabolic coupling between the oocyte and cumulus cells and on cytoplasmic maturation (Mattioli et al., 1988b). It was thus concluded that the effective components that stimulate cytoplasmic maturation in conditioned medium are steroid hormones, and in particular progesterone. Nagai et al. (1993) reported that total abolition of steroid production by

follicular cells during maturation impaired male pronuclear maturation; in contrast, inhibition of estrogen production did not affect male pronuclear formation. These results support Mattioli and his colleagues' (1988b) findings. However, supplementation of progesterone impaired not only nuclear, but also cytoplasmic maturation (Nagai et al., 1993), which is contradictory to Mattioli and his colleagues' results (1988b). But the dose (1 mg/ml) used by Nagai et al. (1993) is much higher than that (1μg/ml) used by Mattioli et al. (1988b). Probably this pharmacological dose of progesterone was toxic to the oocytes.

Yoshida et al. (1992) fractionated porcine follicular fluid and found that a partially purified fraction with molecular weight between 10,000 to 200,000 was effective in stimulating both cytoplasmic and nuclear maturation. However, Naito et al. (1990) suggested that glycosaminoglycans and inhibin might be strong candidates for the effective factors in porcine follicular fluid.

Our data in Chapter IV showed that there were no simple correlations between male pronuclear formation rate and the steroid hormones in their maturation environment (culture medium), although significant simple correlations between male pronuclear formation and steroid hormones in follicular fluid were found. Multiple regression analysis showed that male pronuclear formation was correlated with follicle diameter ($r=0.045$ $p=0.005$), the ratio of progesterone (P4):testosterone (T) in medium ($r=0.37$ $p=0.02$ when follicle diameter was constant), and P4 in medium ($r=-0.35$ $p=0.04$ when follicle diameter and P4:T in medium were constant). These correlation analyses suggest that steroid hormones in the maturation environment were not the main regulatory factors

for cytoplasmic maturation. On the strength of our data we therefore suggest that factors other than steroid hormones secreted by follicle cells are involved in mediating cytoplasmic maturation.

Other steroid data in this thesis also support the above conclusion. Chapter V showed that estradiol and progesterone in FSH-stimulated follicle conditioned medium were not different from those in FSH-LH-Prl-stimulated follicular conditioned medium, but the former supported better cytoplasmic maturation than the latter. Progesterone and estradiol concentrations in the first 24 h FSH-stimulated conditioned medium were significantly different from those in the second 24 h FSH-stimulated conditioned medium, but male pronuclear formation rates were similar. In addition, data in Chapter VII showed that the progesterone concentration in medium was higher when oocyte-complexes were cultured in gonadotropin supplemented medium in the absence rather than in the presence of the follicle shells (Fig.VII-10). Conversely, male pronuclear formation was lower when oocyte-complexes were cultured in the absence than in the presence of the follicle shell. Further, EGF, through an interaction with gonadotropins, stimulated cytoplasmic maturation (Fig.VII-7), but had no effects on steroid production. All these data suggest that cytoplasmic maturation of oocytes is independent of the oocyte's steroid environment in our culture system. However, there was one exception. Data in Chapter VI showed a clear trend for cytoplasmic maturation being correlated with both estradiol and progesterone concentration in the conditioned media (see Table VI-2 and VI-3).

However, effects of follicle shell co-culture on oocyte cytoplasmic maturation is related to changes in ratio of progesterone to estradiol in the maturation environment

(Chapter VII). Follicle shell co-culture greatly decreased this ratio when compared with no follicle shell co-culture (Fig.VII-11), suggesting that the severely altered steroid ratio in the absence of follicle shell may affect cytoplasmic maturation.

In summary, prolonged intercellular coupling between the oocyte and surrounding follicular cells, stimulated by follicular factors during maturation, allows the maximal interaction between the oocyte and follicular somatic cells. This may provide one mechanism through which the oocyte acquires sufficient amounts of substrate (for syntheses of active molecules) and regulatory signals from follicular somatic cells for the stimulation of cytoplasmic maturation. These effects may finally act via synthesis of glutathione (Yoshida et al., 1993 a) and/or achievement of high MPF/H1 kinase activity (Naito et al., 1992). Total abolition of steroid production or severely altered steroid ratios may affect oocyte maturation, suggesting that basic amounts of steroid hormones and their proper ratios in the maturation environment are important for oocyte maturation. However, factors other than steroid hormones play an important role in regulating cytoplasmic maturation in pig oocytes. The nature of non-steroid factors are not known and experiments for identifying these active stimulatory factors should be carried out in the future in order to improve maturation environment or to provide a defined maturation medium for porcine oocytes.

**5. Effect of EGF on oocyte maturation.**

As suggested above, factors other than steroid hormones play an important role in regulation of cytoplasmic maturation in pig oocytes. As reviewed earlier, growth

factors may be involved in regulating cytoplasmic, as well as nuclear, maturation and EGF is one of the most potent growth factors which affect oocyte nuclear maturation (see Racowsky, 1991). The data in Chapter VII showed that EGF stimulated both nuclear and cytoplasmic maturation. The effect on nuclear maturation was independent of other factors, while the effect on cytoplasmic maturation involved an interaction with gonadotropins. These results were similar to those reported in bovine oocyte maturation (Harper and Brackett, 1993). As discussed above, this effect did not appear to involve modulation of steroid production by follicular cells during culture. Mechanisms involving interactions with gonadotropins are clearly possible since EGF binding sites are modulated by both FSH and LH. Also EGF affects LH receptor number (see Chapter II). Whether EGF affects uncoupling between the oocyte and cumulus cells, or affects synthesis of glutathione and MPF activity in oocytes requires further clarification.

**6. The development of improved conditions for pig oocyte maturation in vitro.**

In order to mimic the in vivo oocyte maturation environment, follicular cell co-culture, or culture of oocytes in follicular fluid or in follicular cell conditioned medium, are used for oocyte maturation in vitro. Beneficial effects on oocyte nuclear and cytoplasmic maturation in the pig have been clearly demonstrated by a number of investigators (Mattioli et al., 1988 a, b; 1989; Ding et al., 1988; Nagai et al., 1993; Naito et al., 1988; 1989; 1990; Yoshida et al., 1990; 1992). Our data also demonstrated this effect and further suggested that the supportive effects of follicular secretions on oocyte

maturation vary with the different maturity of follicle cells. More mature follicles provide a better maturation environment for oocytes when they are used for co-culture or for production of conditioned medium. Thus in order to achieve an optimal effect, selection of the best developmental stage of follicles would be important for co-culture or production of conditioned medium, or for obtaining follicular fluid.

Although use of a co-culture system or of conditioned medium promoted cytoplasmic maturation, comparisons of steroid hormones in culture medium collected at the completion of culture or in conditioned medium with follicular fluid steroid hormones showed dramatic differences between them (Table IV-2 and VI-2). Generally speaking, progesterone concentrations in follicular conditioned medium (2ml per follicle shell) or culture medium (2ml per follicle shell) increased by about 3 to 5 fold for cyclic and 0.5 to 3 fold for eCG primed follicles when compared with corresponding follicular fluid. In contrast, estradiol concentrations decreased by 4 to 6 fold. These changes resulted in increases of the ratios of progesterone to estradiol by up to 40 fold. These changes in steroid concentration during culture suggest that follicle cells undergo dramatic differentiation, physiologically or non-physiologically, in vitro. Therefore, even with these "improved" culture system, oocytes are actually exposed to micro-environment dramatically different from that occurring in vivo during the final stage of maturation. In order to provide a maturation environment for oocytes in vitro very similar to that in vivo, current co-culture or conditioned medium systems should be improved to take account of these differences.

Furthermore, existing systems for the in vitro maturation and fertilization of

porcine oocytes are invariably associated with a high incidence of polyspermy and poor development of the fertilized egg. Therefore, some caution is appropriate in applying the results presented in this thesis to future studies. Only when in vitro culture systems have been developed which obviate the problems of polyspermic fertilization will it be possible to provide unequivocal evidence for the role of follicular factors on oocyte maturation. A major emphasis in future studies should therefore be an attempt to reduce the incidence of polyspermic fertilization, probably through improved techniques for the in vitro maturation (capacitation) of fresh ejaculated boar spermatozoa.

**7. General conclusion.**

In conclusion, 1) during oocyte maturation, oocytes synthesize proteins which are essential for the resumption and completion meiosis and as a consequence, oocytes gain the ability to decondense sperm nuclei and subsequently to form male pronuclei; 2) follicular cells surrounding the oocyte play essential roles in regulating full oocyte maturation; 3) although basic amounts of steroid hormones in the in vitro maturation environment are absolutely essential for both nuclear and cytoplasmic maturation, non-steroidal factors play a major role in regulation of cytoplasmic maturation in our culture system; 4) EGF, which was found in follicular fluid, may be directly involved in regulation of oocyte maturation; 5) maturity of follicles used for co-culture or for production of conditioned medium affects the quality of the oocyte; and 6) heterogeneity of follicular development may result in diversity in the quality of the oocyte ovulated and this effect may have important consequences for the developmental potential of the early

embryo.

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# APPENDIX

## 1. PREPARATION OF OOCYTE MATURATION MEDIUM

### A. Basic medium:

Medium 199 Gibco order # 320-1150
With NaHCO3 2.2gm/l, with l-glutamine

### B. Additive:

| | | | |
|---|---|---|---|
| M199 | 100 ml | Gibco 320-1150 | |
| Glutamine | 200 mg | BDH | Prod 37107 (S G5763) |
| L-Ascorbic Acid | 140 mg | BDH | ACS111 (S A4544) |
| Insulin | 70 mg | Sigma | I6634 |
| PVA | 100 mg | Sigma | P8136 |

Acidify with HCl until insulin dissolved (pH about 3.3). Then sterilized with millipore (0.20 μm, Nalgene, 190-2020) filtration and aliquot to 1.2 ml sterilized tubes (NS Foud Plat, T500-1, Simport LTD) and stored below -20°C.

### C. Fetal calf serum (FCS):

Sigma F4884
FCS is heat treated at 56°C in a water bath for 30 min. Then it is aliquoted to 2 ml autoclaved tubes (NS Foud Plat, T500-2, Simpot LTD), 2 ml FCS per tube.

**D. Oocyte maturation medium.** Three to four hours before oocyte culture, make maturation medium using medium 199 and stored additive and FCS as follows:

| | |
|---|---|
| Medium 199 | 17 ml |
| Additive | 1 ml |
| FCS | 2 ml |
| | |
| pH | 7.4 after equilibration in 5% $CO^2$ |
| Osmolarity | about 285 mOs. |

Each culture dish contains 2 ml oocyte maturation medium and is placed in a incubator under an atmosphere of 5% $CO_2$ in air at 39°C until use.

## 2. PREPARATION OF SPERM WASHING MEDIUM

| | mg/100ml | Source |
|---|---|---|
| NaCl | 900 | BDH ACS 783 |
| Bovine serum albumin | 100 | Fraction V, Sigma A 4503 |
| Kanamycin | 10 | Sigma K 1377 |

| | |
|---|---|
| pH | 7.2 after adjusting with a few drops of 0.10 N NaOH under pure air. |
| Osmolarity | about 308 (mOs) |

Medium is sterilized with millipore (0.20μm, Nalgene 190-2020) filtration, then kept at 4°C. Use within 2 weeks.

## 3. PREPARATION OF MODIFIED MEDIUM 199 FOR SPERM PRE-INCUBATION

| | mg/100ml | mM | Source |
|---|---|---|---|
| M199 | 88ml | | Gibco 320-1150 |
| D-glucose | 55 | 3.05 | BDH ACS 369 |
| Ca-lactate | 90 | 2.92 | BDH Prod 27604 |
| Na-pyruvate | 10 | 0.91 | BDH Prod 44094 |
| Kanamycin | 10 | | Sigma K 1377 |
| Fetal calf serum | 12 ml | (56°C treated for 30 min) | Sigma F4884 |

pH — Adjusted to 7.8 using 0.1 N NaOH after equilibration under 5% $CO_2$, 95% air for 10 mins.

Osmolarity — about 310 mOs.

Medium is sterilized with millipore (0.20μm, Nalgene 190-2020) filtration and kept at 4°C. Use within two weeks.

## 4. PREPARATION OF MODIFIED MEDIUM 199 FOR FERTILIZATION

| | mg/100ml | mM | Source |
|---|---|---|---|
| M199 | 88ml | | Gibco 320-1150 |
| D-glucose | 55 | 3.05 | BDH ACS 369 |
| Ca-lactate | 90 | 2.92 | BDH Prod 27604 |
| Na-pyruvate | 10 | 0.91 | BDH Prod 44094 |
| Kanamycin | 10 | | Sigma K 1377 |
| Fetal calf serum | 12 ml | (56°C treated for 30 min) | Sigma F4884 |

| | |
|---|---|
| pH | Adjusted to 7.4 using 0.1 N NaOH or 0.1 N HCL after equilibration under 5% $CO_2$, 95% air for 10 mins. |
| Osmolarity | about 310 mOs. |

Medium is sterilized with millipore (0.20μm, Nalgene 190-2020) filtration and kept at 4°C. Use within two weeks.

## 5. PREPARATION OF B.O. MEDIUM FOR FERTILIZATION

**A. Stock Solution 1:**

| | Weight | Source |
|---|---|---|
| NaCl | 4.3092 g | BDH ACS 783 |
| KCl | 0.1974 g | BDH ACS 645 |
| $CaCl_2.2H_2O$ | 0.2171 g | BDH ACS 186 |
| $NaH_2PO_4.2H_2O$ | 0.0840 g | BDH B 30132 |
| $MgCl_2.$ $5H_2O$ | 0.0697 g | BDH ACS 474 |
| Phenol Red | 1.25 mg | Sigma P3532 |
| Deionized water | add to 500 ml | |

Filtered with 0.20µm millipore filter and stored at 5°C.

**B. Stock Solution 2:**

| | Weight | Source |
|---|---|---|
| $NaHCO_3$ | 5.1746g | BDH B10247 |
| Phenol Red | 2 mg | Sigma P3532 |
| Deionized water | add to 400 ml | |

Filtered with 0.20µm millipore filter and stored at 5°C.

**C. Using stock solution 1 and 2 to make B.O. fertilization medium:**

| | make 100 ml | Source |
|---|---|---|
| Solution A: | 76 ml | |
| Solution B: | 24 ml | |
| Glucose | 250 mg | BDH ACS 369 |
| Na Pyruvate | 13.75 mg | BDH Prod 44094 |
| Kanamycin | 15 mg | Sigma K1377 |
| Caffeine | 38.84 mg | Sigma C4144 |
| BSA | 1000 mg | Sigma A4503 |

pH 7.4 after equilibration in 5% $CO^2$ in air.

Osmolarity 310 mOs.

Medium is sterilized by filtration through millipore filter (0.20µm, Nalgene 190-2020) and stored at 4°C. Use within two weeks.

## 6. PREPARATION OF KREBS'-RINGER-BICARBONATE MEDIUM

| | mg/100 ml | mM | Source |
|---|---|---|---|
| NaCl | 670.5 | 114.7 | BDH ACS 783 |
| KCl | 35.6 | 4.78 | BDH ACS 645 |
| $CaCl_2.2H_2O$ | 24.5 | 1.70 | BDH ACS 186 |
| $MgSO_4.7H_2O$ | 29.4 | 1.19 | BDH ACS 496 |
| $NaHCO_3$ | 210.6 | 25.07 | BDH B 10247 |
| D-Glucose | 100 | 5.55 | BDH ACS 369 |
| Glutamine | 14.6 | 1.00 | BDH Prod 37107 |
| BSA (fraction V) | 400 | | Sigma A 4503 |
| Kanamycin | 10 | | Sigma K 1377 |
| Deionized water | add to 100 ml | | |
| Osmolarity | 293 mOs | | |

Medium is sterilized by filtration through millipore filter (0.20μm, Nalgene 190-2020) and stored at 4°C. Use within two weeks.