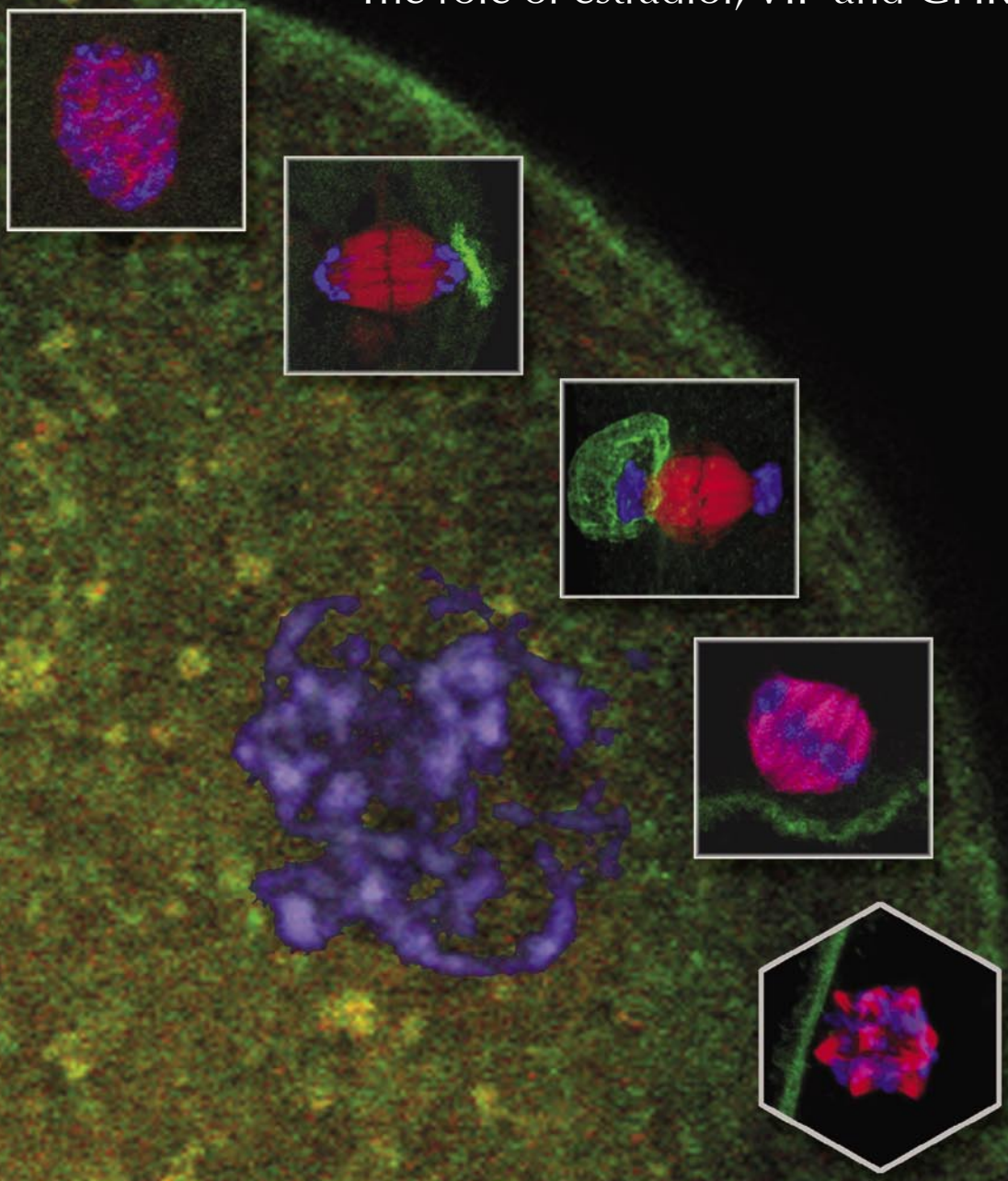
Fundamental aspects of bovine oocyte maturation

The role of estradiol, VIP and GHRH



Anna Rita Costa Lage Beker van Woudenberg

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Utrecht, 2004

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Fundamental aspects of bovine oocyte maturation
The role of estradiol, VIP and GHRH

Fundamentele aspecten van de eicelmaturatie bij het rund
De rol van oestradiol, VIP and GHRH
(met een samenvatting in het Nederlands)

Aspectos fundamentais da maturação oocitária em bovinos
Função do estrógeno, VIP e GHRH
(com resumo em Português)

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“The worst thing that can happen in someone’s life
is not when a project does not work,
is not when a plan does not work,
is not when a journey finished in the wrong place.
The worst is not to begin.
That is the biggest failure”

Amyr Klink- Mar sem fim

"Há quem diga que todas as noites são de sonhos. Mas há também quem garanta que nem todas, só as de verão. Mas no fundo isso não tem importância. O que interessa mesmo não são as noites em sí, são os sonhos. Sonhos que o homem sonha sempre. Em todos os lugares, em todas as épocas do ano, dormindo ou acordado."

William Shakespeare

To my family and friends

To Hans and Lucas

"A saudade é a dor da ausência"
Silva Lobo

"Good men might die, but death cannot kill their names"
Spanish proverb

To my dedicated father Franciscus Beker

To my great friend Eugênio Pacceli

To my mentor Mart Bevers

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

HISTORICAL ASPECTS

In the ancient world, the possibility that living creatures were generated from the inanimate was an accepted concept. Aristotle (384-322 B.C.) postulated that the male was the giver of "seed" and the female played a passive role by providing the "soil" in which the "seed" could grow. Galen (131-210 A.C.) on the contrary, imagined that the two seeds, masculine and feminine, participated equally in the constitution of the conceptus (embryo). These two hypotheses were taken throughout disputes and contrapositions, and for more than 1000 years no relevant discoveries to reproduction were made. The sixteenth century was the period where the ovaries first received recognition as structures, but it was only in the next century that Niels Stensen (1638-1686) proposed the name "ovaries" for what was previously named "female testicle". William Harvey (1578-1657) was one of the first scientists to contribute to our knowledge of the ovaries and he recognized that "*Ex Ovo Omnia*" i.e. "all living things come from eggs". Another decisive contribution was made by Reinier de Graaf (1641-1673) in his *De mulierum organis generationis inservientibus* (1672). De Graaf, who lived in Delft, in the Netherlands, has been credited for the discovery of the ovarian follicles, which in honor of his achievements were named after him. While the eggs of the viviparous animals were still imperceptible, the spermatozoa were not. Only a few years after the discoveries of de Graaf, another Dutchman, Antoni van Leeuwenhoeck (1632-1723), used his newly invented microscope to describe the human sperm, in which he falsely recognized minuscule creatures inside, the *animalculi* (later called *Homunculi*). Around 1770, Lazzaro Spallazani (1729-1799) made an important contribution with a precise and audacious experiment. He collected semen from a poodle and transported it into the vagina of a bitch, which littered pups afterwards. This was the first scientific description of artificial insemination (AI), although there is evidence that Arabs already around 700 B.C. were using

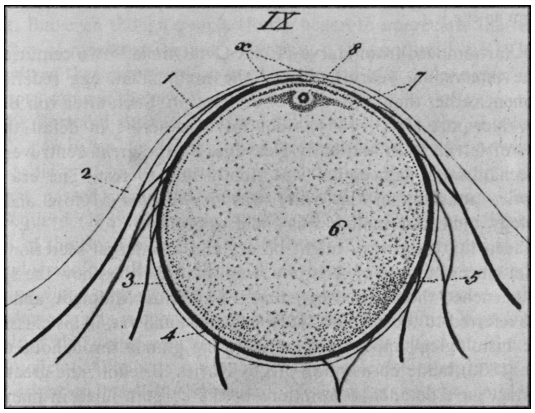

Fig.1-Von Baer's classical drawing of the mammalian egg within the Graafian follicle (*" De Ovi Mammalium et Hominis Genesis"*, 1827) (Short,1977)

AI empirically for horse breeding. Oocytes were finally visualized in 1827 by Karl Ernst von Baer (1792–1876) (Fig. 1), who also introduced the term "spermatozoa" for the male gametes. Using an optical instrument certainly more adequate than that of Spallanzani, Oscar Hertwig (1849-1922) in 1875 demonstrated penetration of the oocyte by spermatozoa.

Following the discovery of sperm, oocytes and their direct involvement in fertilization and embryogenesis, the investigations of the late nineteenth century centered on the physiological and endocrinological aspects of mammalian reproduction, setting then the Golden Age for gametology (Austin,1961; Short,1977; Capanna,1999; Houtzager,2000).

OOGENESIS AND FOLLICULOGENESIS IN MAMMALS

The formation of the oocyte (oogenesis) and the follicle (folliculogenesis) are elegantly orchestrated processes, which result in a unique female cell that, following fertilization, is able to give rise to a new individual that expresses and maintains the characteristics of the species. Oogenesis begins relatively early in fetal development and the oocyte population is defined shortly after birth, with no additional oocytes being formed after this period (for reviews see Driancourt,1991; 2001; Picton et al.,1998; Fair,2003). However, recently Johnson et al. (2004) demonstrated the existence of germline stem cells that sustain oocyte and follicle production in ovaries of postnatal mice.

Oogenesis initiates with **primordial germ cell** formation during gastrulation, followed by proliferation through mitosis (Wassarman,1988). These precursors of the oocyte migrate to the genital ridges (for review see Freeman,2003). It is noteworthy that the origin and migration of primordial germ cells to the genital ridges is similar in males and females (Wassarman,1988). Sex cell differentiation, to either spermatozoa or oocytes, will occur later in development and is regulated by factors from the gonadal somatic tissue (Matsui,1998; Tsang et al.,2001). Once established in the developing ovary, primordial germ cells proliferate to form cells that are connected with each other via intercellular bridges and then are called **oogonia** (Picton et al.,1998), which multiply and eventually undergo one last round of DNA replication, before they enter meiosis (see also section: oocyte meiosis) and are classified as **primary oocytes** (Gosden and Bownes,1995) (Fig. 2).

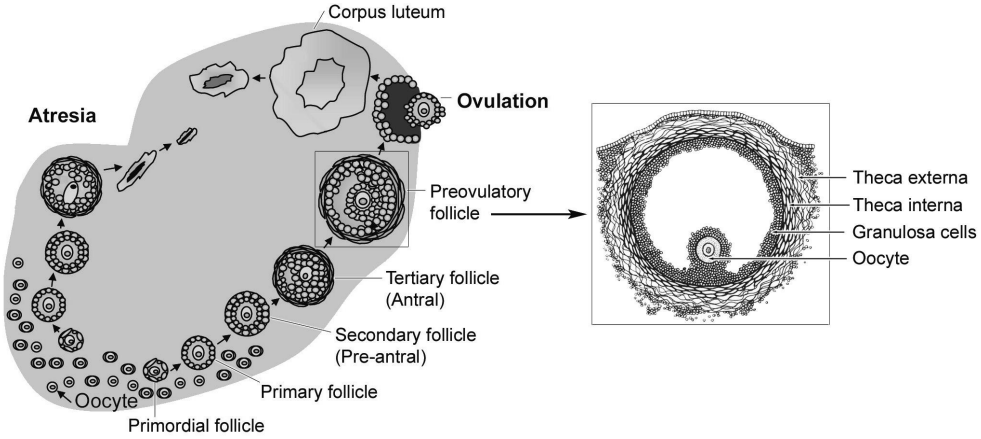

Fig. 2- Schematic representation of the ovary indicating relevant follicular structures which arise during development and atresia. On the right an illustration of an antral follicle with the respective cell layers. Adapted from Drummond et al. (2002).

The initiation of meiosis in the oocyte coincides with the onset of folliculogenesis. Oocytes then become enclosed in a single layer of (pre)granulosa cells thus forming the **primordial follicles** (Gosden and Bownes,1995). Those (pre)granulosa cells, which are from mesothelial and/or mesonephric origin (van den Hurk et al.,1995), are already able to synthesize steroids at this stage (for review see Juengel et al.,2002). Follicles will remain in the primordial state until they are recruited into the growing population. The primordial stage can take quite a long time, in humans for instance up to 4-5 decades. Primordial follicle activation is characterized by the change of the surrounding granulosa cells from a flattened to a cuboidal shape, by proliferation of the granulosa cell, and by the start of oocyte growth. The follicles are now called **primary follicles** (Fair et al.,1997). Progression to the **secondary (preantral) follicle** stage is characterized by the appearance of a second layer of granulosa cells (Driancourt,1999) and in non-rodent species by the initial deposition of zona pellucida glycoproteins around the oocyte. At this stage follicles appear to become responsive to gonadrotropins. In bovine, primordial, primary and secondary follicles appear in the fetal ovary at days 90, 140 and 210 post conception respectively (Russe,1983). The transition to the **tertiary (antral) follicle** is characterized by the continued proliferation and differentiation of the cells surrounding the oocyte, i.e. cumulus cells, theca interna and theca externa, as well as the formation of a fluid-filled antral cavity

(see Fig.2) (Driancourt,1991). After reaching the size of approximately 3 mm, those follicles might enter the process of recruitment, selection and dominance, which occurs in waves during the estrous cycle (cow 2-3 waves/cycle) (Garcia et al.,1999; Bo et al.,2003).

If there is a LH surge the dominant follicle continues to grow, becomes **pre-ovulatory,** and the oocyte within undergoes final maturation, culminating in follicle rupture and the ovulation of matured oocyte (Fair,2003).

OOCYTE MEIOSIS

Meiosis is a prerequisite for sexual reproduction in most animals. This process is unique to germ cells and its purpose is to form haploid, genetically balanced gametes. In mammals, meiosis can be divided in the following phases (Klug and Cummings,1986; Kleinsmith and Kish,1988) (Fig. 3):

Prophase I: The prophase of meiosis I is the longest phase and in most mammals starts during fetal life. It can be divided into five subphases designated *leptotene, zygotene, pachytene, diplotene and diakinesis*. At leptotene (Fig. 3.1) the chromosomes start to condense and become visible as long "threads". At zygotene (Fig. 3.2) the synaptonemal complex, which is a "zipper-like" protein structure that serves to align and join the homologous chromosomes, begins to develop (Heyting,1996). At this phase also chromosome pairing (synapsis) is initiated. During pachytene (Fig. 3.3), the chromosomes are fully paired and form a bivalent, allowing genetic recombination between homologous chromosomes (crossing-over) (Roeder,1990). In diplotene (Fig. 3.4) the synaptonemal complex disappears but the chromosomes remain connected to each other at the sites of recombination, the chiasmata. The meiotic process is arrested at this phase (first meiotic arrest) and the oocyte enters the dictyate stage (Fig. 3.5) which is generally a very long period and is characterized by the presence of a germinal vesicle (GV) which persists up to the preovulatory stage until activation by the LH surge (Fig. 3.6). Although the meiotic process is suspended at the dictyate stage, oocyte growth and synthetic activity take place during this time interval (Tsafriri,1978). The long prophase ends with diakinesis (Fig. 3.7) when the chromosomes condense progressively. The nuclear envelope is then dissolved, a process called germinal vesicle break down (GVBD) and metaphase I sets in.

Metaphase I: The spindle apparatus forms and the bivalents align on the equatorial plate. The centromeres of homologous chromosomes attach to spindle fibers arising from opposite poles of the cell (Fig. 3.8).

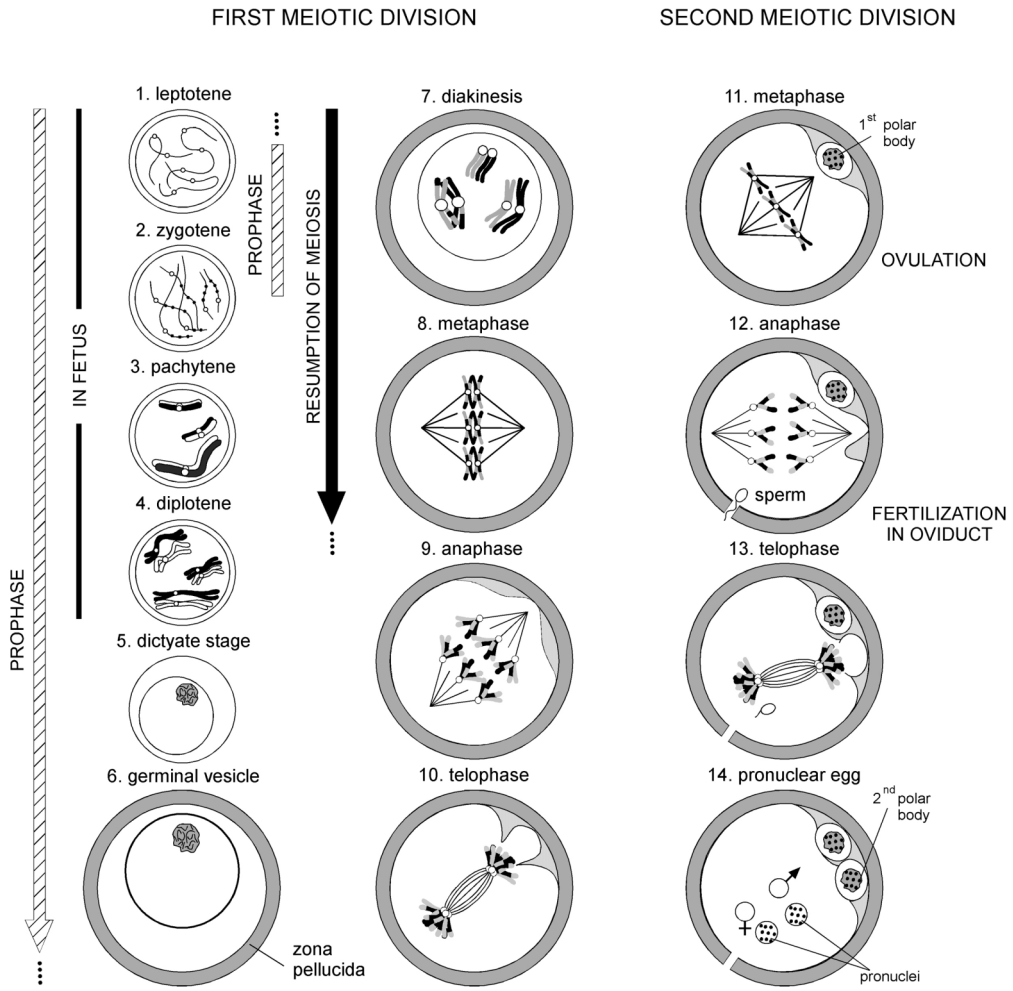

Fig. 3. Schematic illustration of the stages of meiosis in the mammalian oocyte. Prophase I, which starts in the fetal ovary, is divided in leptotene (1), zygotene (2), pachytene (3), diplotene (4) and diakinesis (7). Meiosis is arrested before the end of prophase I (at the dictyate stage; 5), generally for a long period. At puberty the process of follicle growth starts and the oocyte of the pre-ovulatory follicle will resume meiosis passing through metaphase I (8), anaphase I (9), telophase I (with extrusion of the first polar body; 9). In most mammals, the oocyte will arrest again at metaphase II (11) and ovulation occurs at this stage. After fertilization, the oocyte enters the anaphase II (12). Subsequently telophase II (13) sets in, culminating with the extrusion of the second polar body. Adapted from Tsafriri (1978).

Anaphase I: Homologous chromosomes (but not sister chromatids) of each tetrad separate from each other and move to opposite poles of the spindle (Fig. 3.9).

Telophase I: This stage brings the first meiotic division to a conclusion as the separated homologues aggregate, which in the oocyte culminate with the extrusion of the first polar body (Fig. 3.10).

Metaphase II: During metaphase II the paired chromatids become associated with spindle microtubules and migrate to the equatorial region of the spindle. In most mammals, the oocyte enters in the second meiotic arrest and ovulation usually occurs at this stage (Fig. 3.11).

Anaphase II: The second meiotic division is completed in the oviduct following sperm penetration. Sister chromatids separate from one another and are drawn to opposite poles of the spindle (Fig. 3.12).

Telophase II: The last phase of meiosis ending in the oocyte with the extrusion of second polar body (Fig. 3.13).

OOCYTE MATURATION

The whole mechanism of oocyte maturation is a harmonic interaction between cumulus cells, the nucleus and the cytoplasm of the oocyte. There are two key steps in oocyte maturation: nuclear and cytoplasmic maturation. Nuclear maturation is characterized by the resumption of meiosis from GV to the formation of the second metaphase plate (MII stage), while cytoplasmic maturation includes the molecular and structural changes that occur in the cytoplasm of the oocyte that enable it to support fertilization and early embryonic development (Eppig et al.,1996). Although nuclear and cytoplasmic maturation can take place at least partially independent from each other, the synchronization of those processes within the oocyte is of great importance for further development. Initially, the oocyte acquires the ability to fully condense its chromatin. This requires active Maturation Promoting Factor (MPF) (Masui and Markert,1971) which is predetermined by a sufficient amount of its subunits, cyclin B1 and P34cdc2, in an activated form (Levesque and Sirard,1996). In bovine, the average follicular size at which the oocyte acquires the ability to undergo nuclear maturation is 2-3 mm, corresponding to an oocyte diameter of 110 μm (Fair et al.,1995). After MPF activation, the oocyte forms a metaphase I (MI) plate and a functional spindle. Subsequently, progression to MII takes place simultaneously with extrusion of the polar body. In most mammals, the last meiotic event before ovulation is arrest at MII, a

process that requires functional cytostatic factor (CSF) (Masui,1990). To release the oocyte from this second meiotic arrest an activating sperm is necessary. Once the spermatozoon fertilizes the MII oocyte, the cell cycle resumes and interphase follows with pronuclei formation, fusion and DNA synthesis (Sirard,2001).

Although bovine oocytes from follicles >3mm are able to reach the MII stage, they are not competent to develop to blastocysts (Lonergan et al.,1994; Hendriksen et al.,2000). Additional but essential cytoplasmic modifications occur during subsequent follicular growth and dominance until ovulation (Fair,2003). The cytoplasmic reorganization that occurs from the time that a follicle is selected to become dominant (in bovine around 8 mm) until the LH surge is termed prematuration or capacitation. Some of those changes include: increase in number and size of the lipid droplets; decrease in the number of Golgi complexes; enlargement of the perivitelline space; movement of the cortical granules towards the subplasmalemal area (Hyttel et al.,1997). The final maturation is triggered in vivo by the LH surge (Driancourt,2001) or in vitro by removal of the oocyte from the follicle (Pincus and Enzmann,1935). The coupling between the oocyte and the cumulus cells then changes and the loss of gap-junctions between those cells culminate with cumulus expansion. At this stage, the interaction between cumulus cells and the oocyte becomes restricted to the corona radiata (layer of cumulus cells directly surrounding the oocyte) while the outer cumulus cells are uncoupled (Mattioli and Barboni,2000). In the oocyte cytoplasm there is a further increase in lipid content and reduction in the Golgi compartment. The cortical granules are aligned underneath the oocyte membrane, meiosis is resumed and the oocyte nuclear membrane breaks down releasing the oocyte nuclear contents into the cytoplasm. The oocyte will then progress through the final stages of meiosis I and arrest at metaphase of meiosis II. With these modifications the oocyte is considered mature and has developed and modulated the cell biological machinery for sustaining fertilization and initial embryonic development (Hyttel et al.,1997).

Innumerous factors, such as kinases, hormones, peptides and growth factors have been identified that influence (in vitro) maturation of oocytes (for review see Bevers et al. 1997). However, further research is essential to improve the knowledge on the mechanisms and factors involved in the process of oocyte nuclear and cytoplasmic maturation.

GHRH and VIP

Gonadotropins (FSH and LH) are the most important hormones for the two major functions of the ovary: the production of fertilizable oocytes and the secretion of sex steroids. In addition to the effects of the gonadotropins, the ovarian function is also modulated by locally produced factors such as growth hormone (GH) (Izadyar et al.,1999), insulin growth factors (IGFs) (for review see Adashi,1998), epidermal growth factor (EGF) and growth hormone releasing hormone (GHRH) (Bagnato et al.,1992).

GHRH and vasoactive intestinal peptide (VIP) are members of a family of regulatory peptides that includes secretin and glucagon. Based on the remarkable amino-acid sequence homology of the members of the secretin-glucagon family it has been suggested that receptors for these peptides are shared. Nowadays, other peptides have been added to the VIP-secretin-glucagon family: pituitary adenylate cyclase activating polypeptide (PACAP), gastric inhibitory peptide, parathyroid hormone and extendins. The receptors for GHRH/VIP/PACAP are G-protein-coupled transmembrane proteins, whose signaling mechanisms involves the activation of adenylate cyclase, protein kinase A and phospholipase C (for review see Nussdorfer and Malendowicz,1998).

The finding that VIP-containing nerve fibers innervate the ovary, showing a delicate network of these fibers in close relation to the theca cell layers (Ahmed et al.,1986) has prompted investigations on the role for this peptide in ovarian function. Indeed, there is evidence that VIP acts as a regulator and/or modulator of folliculogenesis and steroidogenesis. For instance, VIP directly stimulates progesterone, 20a-hydroxyprogesterone and estradiol production by isolated granulosa cells (Davoren and Hsueh,1985). Similarly, the identification of GHRH in the gonads, both at the level of gene transcription and protein production (Bagnato et al.,1992), also suggests a role for this hormone in paracrine or autocrine regulation of gonadal function. In males, GHRH is produced by Leydig cells in culture and acts as a positive regulator of Leydig cell function by itself and/or can facilitate LH-stimulated steroidogenesis and cAMP production, probably via a VIP receptor (Ciampani et al.,1992). GHRH significantly accelerated oocyte maturation in both follicle and cumulus-enclosed rat oocytes. However, no effect was observed when the cumulus cells were removed from the oocytes prior to culture, suggesting that GHRH influences oocyte maturation via the cumulus cells (Apa et al.,1995). Thus, GHRH and VIP may add to the array of paracrine modulators involved in the regulation of ovarian steroidogenesis.

GHRH is the major physiological stimulus of GH secretion from the anterior pituitary gland (Connor et al.,2002). In bovine, Izadyar et al. (1996) demonstrated that maturation of COCs in the presence of GH accelerates progression of meiosis and induces cumulus expansion. The expression of mRNA for a GH receptor in the oocyte supports the involvement of GH in the maturation of bovine oocytes (Izadyar et al.,1999), which could also be regulated by GHRH. Despite the evidence of GHRH and VIP as paracrine or autocrine regulators in the ovary, little is known about the effects of GHRH and VIP on bovine oocyte in vitro maturation.

ESTRADIOL

One of the major physiological roles of estrogenic steroids is the control of reproduction in the female. This covers a broad range of biological activities which in mammals includes: the development of the reproductive tract and secondary sex organs; the regulation of the estrus cycle including complex feedback loops with hypothalamic regulatory peptides and pituitary hormones; the development of the mammary gland and control of lactation; and the control of female reproductive behaviour (Sutherland et al.,1988). Estrogens also exert local effects within the ovary (for review see Rosenfeld et al.,2001) such as: stimulation of folliculogenesis; increase of expression of gonadotropin receptors by granulosa cells; increased formation of gap junctions between granulosa cells, permitting the transport of nutrients and other biochemical factors in the follicle; inhibition of granulosa cell apoptosis; formation and maintenance of the corpus luteum.

a) Estrogens promote folliculogenesis

As the ovarian follicles grow and differentiate, increasing amounts of estrogen are produced (Fortune,1994), which in turn upregulate the synthesis and release of the pituitary gonadotropins (FSH and LH) thereby promoting ovarian follicular growth. It has been reported that estrogen directly increases the number and size of ovarian follicles in vivo (Nakano et al.,1982) and the size of pre-antral follicles in culture (Gore Langton and Daniel,1990; Hulshof et al.,1995).

In mammals, two types of estrogen receptors are identified: estrogen receptor α (ERα) and β (ERβ). Mice lacking genes (knockout) for estrogen receptor α (αERKO), estrogen receptor β (βERKO), estrogen

receptor αβ (αβERKO) and for aromatase (ArKO) offer an opportunity to help defining the role of estrogen in early folliculogenesis.

Studies with αERKO and βERKO mice showed that folliculogenesis can occur in the absence of one of these estrogen receptors, although sub-normally. This indicates that in mice ERα and ERβ can compensate, at least partially, for the absence of the other to provide mature ovulatory follicles (for review see Couse and Korach,1999; Hewitt and Korach,2003). However, ovaries from αβERKO show a different phenotype from those observed in αERKO and βERKO mice. Young αβERKO mice exhibit normal follicles with a developing oocyte surrounded by granulosa cells. However when age progresses, follicles exhibit a lack of oocytes but instead contain what appear to be Sertoli cells. Additionally, intermediate structures with characteristics of both type of follicles, containing a degenerating oocyte and both granulosa and Sertoli-like cells are present. Thus, follicles that appear normal, begin to "trans-differentiate" as oocytes degenerate, into seminiferous tubule-like follicles. This suggests that the combined functions of ERα and ERβ are essential to maintain oocyte integrity and prevent follicular degeneration (Couse et al.,1999).

In ArKO mice, which lack the capacity to synthesize estradiol, follicle development is arrested at the antral stage rendering these mice infertile due to an inability to ovulate. As the animal ages, secondary and antral follicles become less common in the ovary. Additionally, Sertoli-like cells can be observed in ArKO mice similar to the αβERKO phenotype (Britt et al.,2001).

b) Estrogens are involved in the process of follicular selection to dominance.

The process of follicle selection results from an interplay between gonadotropins, steroids and peptides. An important stage during this process is follicle deviation, which is characterized by continued growth of the developing dominant (largest) follicle and reduced growth of the subordinate (smaller) follicles (Beg et al.,2003). Estradiol, together with other factors, such as inhibin, insulin-like growth factor (IGF-I) and its binding proteins (IGFBPs), plays a crucial role in this process (Ginther et al., 2003). By the time of morphological selection, the dominant follicle has much higher concentrations of estradiol in the follicular fluid, and in vitro its granulosa cells produce more estradiol than cells from subordinate follicles (Fortune et al.,2001). Moreover, estradiol is

involved in the continuing depression of FSH concentrations to less than the requirements of the smaller cohort follicles. Only the more-developed largest follicle is able to utilize the low FSH concentration (Ginther et al.,2001). Estradiol enhances the actions of FSH in granulosa cells, permitting rapid activation of genes such as those for LH receptor and aromatase (Richards et al.,1995). Concomitantly, serum concentrations of LH start to increase (Rosenfeld et al.,2001) and a shift occurs from FSH- to LH-dependence in the future dominant follicle. Therefore, estradiol plays an important role in determining the ability of a given follicle to respond to FSH and LH (Ginther et al.,2003). Thus, whether or not a particular follicle expresses estrogen receptors may control its destiny, to become a dominant follicle or a subordinated one.

THE ROLE OF ESTRADIOL DURING IVM

Follicular fluid of mammals contains steroid hormones. At the time of the LH surge, preovulatory follicles have a high concentration of estradiol (bovine: about 1 μg/ml) (Dieleman et al.,1983). However, 6h after the LH peak a sharp decline of estradiol occurs, which coincides with the GVBD. Although most in vivo effects of estradiol are well identified, the role of estradiol during IVM remains contradictory. Some studies describe that the presence of estradiol during IVM reduces the maturation rate of oocytes in mice (Eppig and Koide,1978; Smith and Tenney,1980; Kaji et al.,1987) and pigs (Richter and McGaughey,1979; Eroglu,1993; Bing et al.,2001), but others have not observed any effect in pigs (Singh et al.,1993). For human oocytes, the presence of estradiol in the culture medium had no effect on the progression of meiosis, but improved the fertilization and cleavage rates, suggesting that estradiol supports the cytoplasmic changes during maturation necessary for fertilization and early embryonic development (Tesarik and Mendoza,1995). In bovine, estradiol during IVM was described to increase the maturation rate in some studies (Fukui et al.,1982; Younis et al.,1989). But in another study the effect of estradiol was concentration-dependent. When oocytes were cultured in the presence of low concentrations (10-100 ng/ml) of estradiol a decrease in the developmental progression to morula and blastocyst stages was observed. However, when estradiol was present at high concentrations (1000 ng/ml) the developmental potential of matured bovine oocytes to morula and blastocyst stages was improved (Ali and Sirard,2002). In contrast, presence of high concentrations (>10 μg/ml) of estradiol during IVM had a negative effect on spindle formation

and polar body extrusion (Kruip et al.,1988). Regarding developmental competence of oocytes, estradiol during IVM had no effect on the percentages of cleaved and 4-8 cell stage embryos (Younis et al.,1989). Conversely, Gliedt et al. (1996) reported a negative effect of estradiol on cumulus expansion of bovine oocytes, and a decrease in blastocyst formation rate following in vitro fertilization.

The divergence between the reported effects of estradiol on in vitro maturation, besides species differences, is mainly due to the fact that in most of those studies culture media supplemented either with fetal calf serum (FCS), bovine serum albumin (BSA) or estrus cow serum were used. These compounds contain gonadotropins, growth factors, steroids and peptides (Keskintepe and Brackett,1996; Wang et al.,1997), that could influence the effect of the addition of estradiol during IVM. Therefore, for a proper examination on the possible influence of added components to the medium on oocytes and granulosa cells, the use of a clearly defined culture medium is of great importance.

STEROID MECHANISMS OF ACTION

To better understand how steroids could exert their effects in vitro, it is of value to review some aspects of steroid mechanisms of action in vivo.The complex physiological responses to steroids are generally presumed to involve transcriptional regulation of many genes, mediated via the so-called classic, or genomic, steroid pathway. This mechanism is summarized in the following steps: 1) free steroid enters the target cell by passive diffusion through the plasma membrane or assisted by a transporter, and binds with high affinity to a receptor; 2) the steroid-receptor complex undergoes activation and enables the receptor to bind to selective sites on the DNA; 3) the activated steroid-receptor complex interacts with specific DNA sequences referred to as steroid response elements, usually located in the promoter region of the steroid-responsive genes; 4) the activated steroid-receptor complex initiates transcription of its target genes (Couse and Korach,1999).

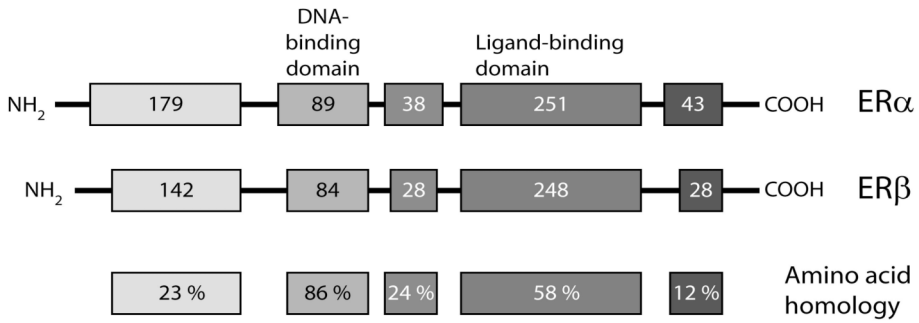

Fig. 4- Structure of the estrogen receptor α (ERα) and β (ERβ). Numbers in boxes indicate number of amino acids or percentage of homology between domains (as indicated). Adapted from McDonnell (1999).

The steroid hormone receptor superfamily includes glucocorticoid, mineralocorticoid, progestin, estrogen, androgen and 1α,25-dihydroxyvitamin D3 receptors. All members of this superfamily share similar characteristics and have highly conserved structural domains involved in DNA and ligand binding (Falkenstein et al.,2000). Currently two estrogens receptors, denominated ERα and ERβ, have been molecularly characterized (Mosselman et al.,1996; Pace et al.,1997). These receptors have considerable sequence identity in the DNA-binding domains, which permits both receptor types to interact with estrogen response elements (EREs) of various genes (Fig.4) (Tremblay et al.,1997).

Different tissue distribution is described for ERα and ERβ mRNA. ERα mRNA is predominantly expressed in the uterus, mammary gland, testis, pituitary, liver, kidney, heart, and skeletal muscle, whereas ERβ transcripts are significantly expressed in the ovary and prostate. Furthermore, even in those tissues expressing both ERs, there is often a distinct expression pattern within the heterogeneous cell types composing the tissues. In the ovary, for instance, ERβ is localized to the granulosa cells of maturing follicles, whereas ERα is detectable in the surrounding thecal cells (for review see Couse and Korach,1999).

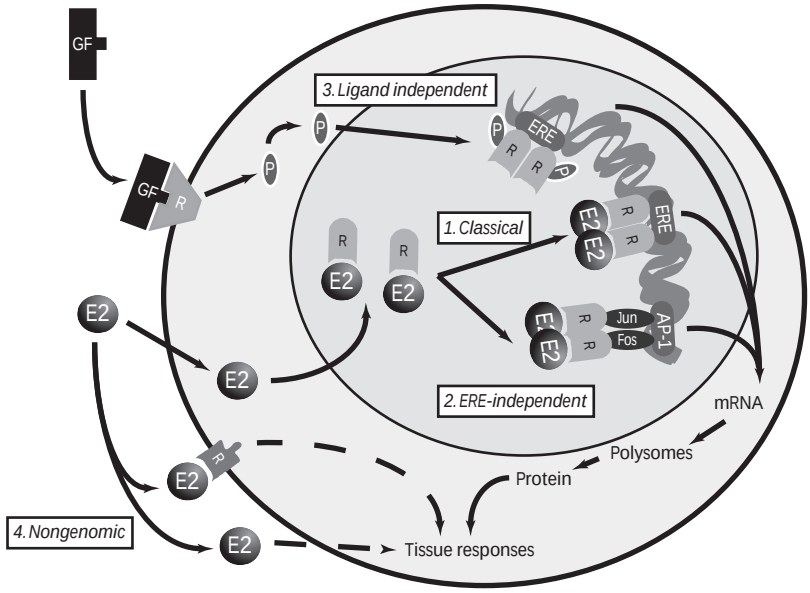

Fig. 5-Schematic illustration of the mechanisms of estradiol and estradiol receptor signaling:1) classical, ligand and estradiol response element (ERE) dependent; 2) ERE-independent; 3) ligand-independent and 4) non-genomic. See further explanation in the text. Adapted from Hall et al.(2001).

In recent years, several mechanisms of gene activation by the steroid receptors that deviate from the classical genomic model (Fig. 5-1) have been described. These include; a) activation by a ligand-bound steroid receptor without the evidence of direct DNA binding, but rather via interaction with other DNA-bound transcription factors such as the AP-1 complex (Fig. 5-2); b) ligand-independent activation of the receptor through pathways that alter the activity of cellular kinases and phosphatases leading to phosphorylation and activation of ER at ERE-containing promoters (Fig. 5-3). Additionally, some steroid effects are clearly incompatible with the involvement of genomic mechanisms. These effects are called nonclassic, nongenomic steroid effects (Fig. 5-4) (for review see Hall et al.,2001). The nongenomic pathway involves, possibly but not necessarily, a putative plasma membrane receptor (Fig. 5-4).

Evidence of non-genomic effects exerted by steroids has been described in a wide variety of cell types, such as pancreatic cells, neurons, sperm, endometrial cells, granulosa cells and oocytes (for reviews see Revelli et

al.,1998; Falkenstein et al.,2000; Levin,2002). In general, nongenomic steroid effects have the following characteristics (see also Fig. 6): 1) are too rapid (from seconds to few minutes) to be compatible with the involvement of changes in mRNA and protein synthesis; 2) can be elicited even by steroids coupled with high-molecular weight substances that cannot cross the plasma membrane and do not enter the cell (e.g. BSA-conjugates); 3) are not blocked by inhibitors of mRNA or protein synthesis; 4) can be observed even in highly specialized cells that do not accomplish mRNA and protein synthesis (e.g. spermatozoa) or by cells lacking the nuclear steroid receptors (Revelli et al.,1998; Moore and Evans,1999). Such nongenomic effects are postulated to be initiated at the plasma membrane, mediated by a membrane-bound receptor that seems to be similar to the classical intracellular estrogen receptors and involve conventional second messenger cascades (for review see Falkenstein et al.,2000; Losel et al.,2003). For instance, a rapid transient increase in the intracellular free calcium concentration occurs in human oocytes (Tesarik and Mendoza,1995) as well as in sperm (Luconi et al.,1999) shortly after exposure to estradiol.

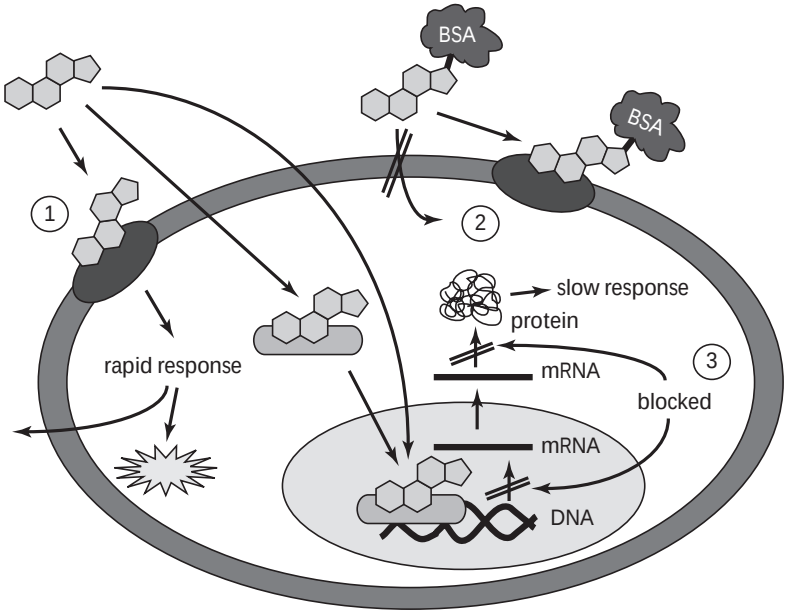

Fig. 6- Schematic representation of some of the characteristics of steroids non-genomic mechanism of action. 1) too rapid; 2) elicit even when conjugated with high molecular weight molecules; 3) not blocked by inhibitors of mRNA or protein synthesis. See text for further explanation. Adapted from Moore and Evans (1999).

Although several nongenomic effects of estradiol have been described, the nature and characteristics of the mediating plasma membrane receptor remains controversial. Moreover, although the involvement of nongenomic steroid pathways in oocytes is well described in lower vertebrates (*Xenopus*, fish), little is known about the putative plasma membrane-mediated effects of E2 during in vitro maturation of bovine oocytes.

ARTIFICIAL REPRODUCTIVE TECHNIQUES

At birth, the ovaries contain a large store of primordial follicle oocytes. After puberty ovarian follicular development is characterized by waves of growth during estrous cycles (cow 2-3 waves/cycle). Each wave of follicular growth is characterized by recruitment, selection and dominance. In monovular species (like equine, bovine and human), during this process a large number of follicles will be recruited, but in general only one follicle will be selected to continue its growth. This follicle will become dominant and will (eventually) ovulate (Garcia et al.,1999). The majority of the recruited follicles becomes atretic and degenerates, leading to a substantial waste of oocytes. In domestic animals, especially in bovine, superovulation and embryo transfer have been used to obtain more progeny from genetically valuable females for commercial purposes. The development of the technique of transvaginal ultrasound guided ovum pick up (OPU) enabled retrievement of oocytes in vivo. This technique allows the utilization of antral follicles that are potentially able to ovulate but because of the physiological process of selection would naturally undergo atresia after the rise of a dominant follicle. A step further in the attempt to maximize the utilization of the available oocytes is IVM. Unfortunately, this process is not completely understood and is still far from an extensive routine commercial application. For example, in 2002 only 15% of the bovine embryos transferred worldwide were produced in vitro (Thibier,2003). The main reason is the low efficiency of the process. Although a significant improvement in the IVM and in vitro fertilization (IVF) protocols has been achieved in the last decades, the efficiency of the process is still not optimal. For instance, in bovine only 30-40% of blastocyst are obtained after oocyte IVM/IVF/IVC. Therefore, research should be focused on understanding the physiology and pathophysiology of oocyte maturation, including the involvement of hormones, growth factors and cytokines in this process. Additionally, strategies should be developed to improve the quality of oocyte IVM that is vital to increase the number of usable oocytes per estrous cycle and finally a higher number of offspring per female.

SCOPE OF THE THESIS

The present study aims to increase our understanding of the processes of in vitro maturation of oocytes and subsequent embryo development in the bovine.

In chapter 2, the effects of VIP and GHRH during IVM of bovine oocytes were investigated. Nuclear stage was assessed at different time points. Evaluation of cortical granule distribution, by confocal laser scanning microscopy, was used as indicator of cytoplasmic maturation and developmental competence of oocytes. It was also evaluated whether these peptides affect cumulus expansion.

In chapter 3, we investigated the role of estradiol on the in vitro nuclear maturation of oocytes using a defined medium. Both cumulus oocyte complexes and denuded oocytes were used to study whether possible effects of estradiol are exerted via cumulus cells or directly on the oocyte. The role of estradiol on the cytoplasmic maturation was also investigated.

To study whether the effects of estradiol described in chapter 3 were mediated via a genomic or nongenomic pathway, we cultured oocytes in the presence of estradiol either before or after GVBD (the period when transcription ceased) (chapter 4). Additionally, an estradiol membrane impermeable conjugate was used to investigate the presence of a putative membrane receptor for estradiol and RT-PCR was performed to study the presence of mRNA for ERα and ERβ in oocyte and cumulus cells. Since induction of nuclear aberrations was one of the most prominent effects of estradiol during IVM (chapter 3), we further studied the involvement of estradiol on the meiotic spindle organization by confocal laser scanning microscopy.

In chapter 5, we investigated the possible role of estradiol during prematuration with an inhibitor of MPF kinase activity using follicles from different size groups.

Finally, the results of these studies and their implications for further research are summarized in chapter 6.

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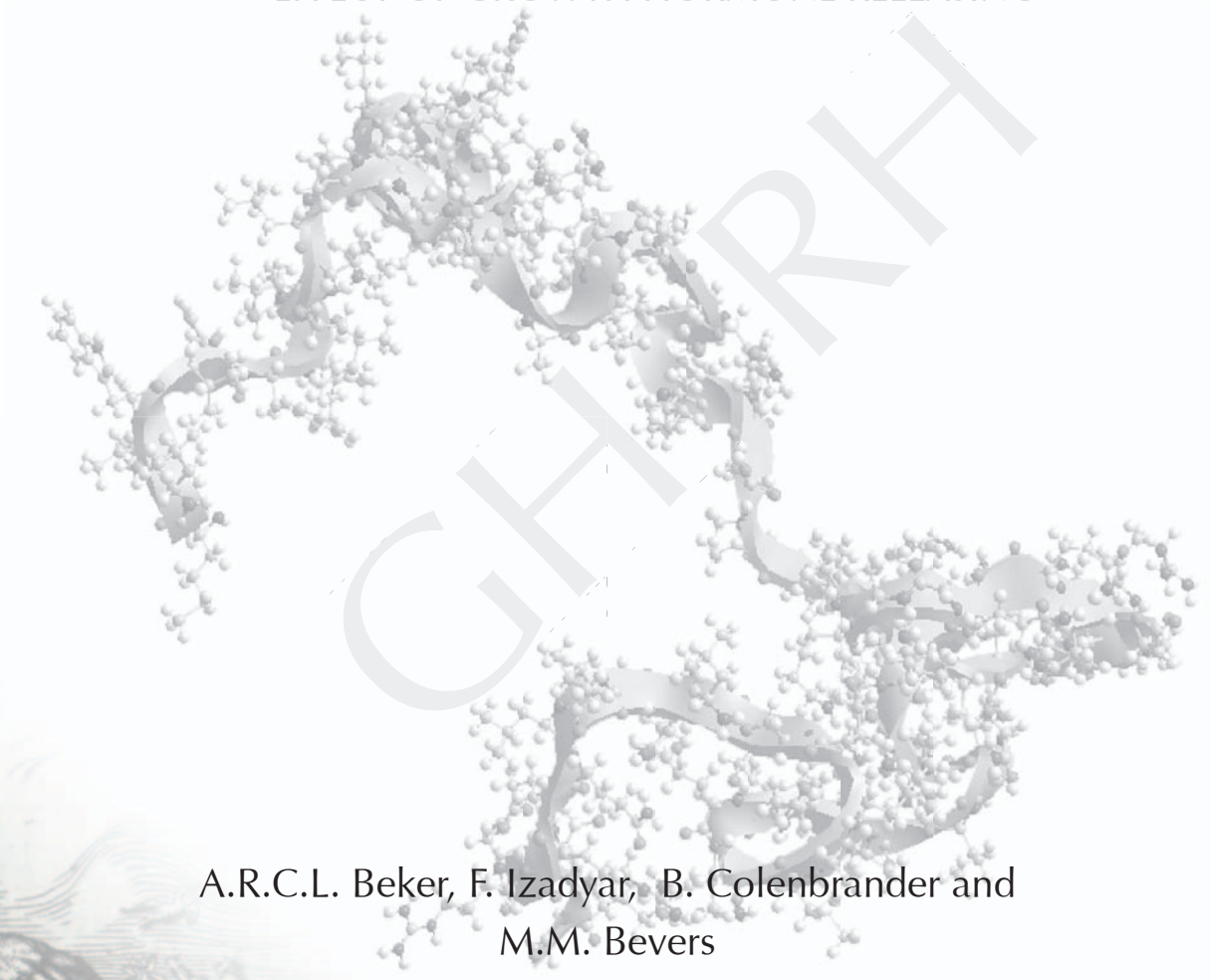
CHAPTER 2

EFFECT OF GROWTH HORMONE RELEASING HORMONE (GHRH) AND VASOACTIVE INTESTINAL PEPTIDE (VIP) ON IN VITRO BOVINE OOCYTE MATURATION



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Theriogenology 2000; 53: 1771-1782

ABSTRACT

The aim of this study was to investigate the effects of growth hormone releasing hormone (GHRH) and the structural-related peptide vasoactive intestinal peptide (VIP) on nuclear maturation, cortical granule distribution and cumulus expansion of bovine oocytes. Bovine cumulus oocyte complexes (COCs) were cultured in M199 without FCS and gonadotropins and in the presence of either 100 ng/ml bovine GHRH or 100 ng/ml porcine VIP. The COCs were incubated at 39ºC in a humidified atmosphere with 5% CO_2 in air, and the nuclear stage was assessed after 16 or 24 h of incubation using DAPI staining. Cortical granule distribution was assessed after 24 h of incubation using FITC-PNA staining. To assess the effects of GHRH and VIP on cumulus expansion, COCs were incubated for 24 h under the conditions described above. In addition, 0.05 IU/ml recombinant human FSH was added to GHRH and VIP groups. Cultures without GHRH/VIP/FSH or with only FSH served as negative and positive controls, respectively.

At 16 h neither GHRH (42.9%) nor VIP (38.5%) influenced the percentage of MII stage oocytes compared with their respective controls (44.2 and 40.8%). At 24 h there also was no difference in the percentage of MII oocytes between GHRH (77.0%), VIP (75.3%) and their respective controls (76.0 and 72%). There was no significant cumulus expansion in the GHRH or VIP group, while FSH induced significant cumulus expansion compared with the control groups, which were not inhibited by GHRH or VIP. Distribution of cortical granules was negatively affected by GHRH and VIP. The percentage of oocytes showing more or less evenly dispersed cortical granules in the cortical cytoplasm aligning the oolemma (Type 3) was lower in the GHRH (2.7%) and VIP (7.8%) groups than in the control group (15.9%). In conclusion, GHRH and VIP have no effect on nuclear maturation or cumulus expansion of bovine COCs but retard cytoplasmic maturation, as reflected by delayed cortical granule migration.

Key words: IVM, GHRH, VIP, nuclear maturation, cortical granules, cumulus expansion

INTRODUCTION

Although it has been recognized for many years that oocytes resume meiosis spontaneously in a suitable culture medium (Edwards,1965; Eppig and Downs,1984; Xu et al.,1986), the in vitro production of bovine embryos remains hampered by a great loss of embryos during early development. Embryo development rates, in terms of number of embryos per 100 cumulus oocytes complexes (COCs) cultured, are inferior to those of in vivo production systems (King et al.,1986; Leibfried-Rutledge et al.,1987; Greve and Madison,1991; Van de Leemput et al.,1996).

Hormones like LH, FSH and estrogens are frequently used as additives in culture media to enhance the quality of the maturation process (Fukui et al.,1982; Younis et al.,1989; Zuelke and Brackett,1990; Keefer et al.,1993). Supplementation of maturation media with these hormones originates from the presumptive involvement of these hormones in the in vivo maturation process. However, folliculogeneses and oocyte maturation is regulated not only by gonadotropins and steroids but also by other hormones such as growth hormone (GH) and a variety of locally produced paracrine acting cytokines. Among these regulators there are several growth factors such as insuline-like growth factors (IGFs); epidermal growth factor (EGF); transforming growth factor α (TGFα), TGFβ and activin (for review see Bevers et al.,1997).

Culture of bovine cumulus oocyte complexes (COCs) in the presence of GH accelerates the process of nuclear maturation and increases cumulus expansion (Izadyar et al.,1996). This stimulatory effect is mediated by cumulus cells (Izadyar et al.,1997). An effect of GH on in vitro maturation has also been demonstrated in other species such as the rat (Apa et al.,1994)) rabbit (Yoshimura et al.,1993) and pig (Hagen and Graboski,1990). Not only nuclear maturation is affected by GH, but also the rate of fertilization, of cleaved embryos and of blastocysts (Izadyar et al.,1996). This appeared to be due to an effect on cytoplasmic maturation, as demonstrated by the improved distribution of cortical granules (CGs) (Izadyar et al.,1998).

The hypothalamic polypeptide growth hormone releasing hormone (GHRH) is the major physiological stimulus of GH secretion from the anterior pituitary gland (Frohman and Jansson,1986); GHRH has also been detected in several extraneural tissues (Frohman et al.,1990; Mayo et al.,1995), suggesting that it has a regulatory role in several peripheral tissues. In the rat, specific binding of GHRH by granulosa cells has been demonstrated (Moretti et al.,1990; Bagnato et al.,1992). Apa et al.(1995) showed a stimulatory effect of

GHRH on the in vitro maturation of rat oocytes which was mediated by GH, indicating local production of the hormone.

Growth hormone releasing hormone belongs to the same family as glucagon, secretin and vasoactive intestinal peptide (VIP) (Bell,1986). Rat VIP has a striking amino acid sequence homology with GHRH (Spicer et al.,1992). The homology between these 2 peptides also extends to their ability to elicit similar biological responses in several target tissues. Investigations using rat ovarian granulosa cells suggest that VIP stimulates FSH-induced progesterone production in a manner similar to that of GHRH (Moretti et al.,1990) and that VIP can compete with GHRH for the same receptor on rat granulosa cells (Bagnato et al.,1991). The presence of VIP immunoreactive fibres in the bovine ovary and of VIP-positive cells in the granulosa layer of bovine preovulatory follicles have been demonstrated (Hulshof et al.,1994).

In the bovine, the influence of GHRH and VIP on oocyte maturation remains to be demonstrated. Therefore, the aim of this study was to investigate the effects of GHRH and VIP on nuclear maturation, cortical granule distribution and cumulus expansion of bovine oocytes.

MATERIALS AND METHODS

Collection and Culture of Oocytes

Bovine ovaries were collected at a slaughterhouse and transported in a thermosflask to the laboratory. Follicles between 2 and 8 mm were punctured by means of a suction pump under low vacuum. The follicle aspirates were pooled in a conical tube and allowed to settle for 15 min. The COCs, containing oocytes surrounded by a compact, multilayered cumulus investment were selected, washed in HEPES buffered M199 (Gibco BRL, Paisley, UK), and randomly allocated in groups of 35 per well to a 4-well culture plate (Nunc A/S, Roskilde, Denmark). Culture of the COCs was performed in 500 µL M199 per well supplemented with 2.2 g/L $NaHCO_3$ (defined as M199) at 39°C in a humidified atmosphere of 5% CO_2 in air for 24 h unless otherwise indicated. For each replicate of a particular experiment, another batch of ovaries was used.

Experiments

To investigate the effects of GHRH and VIP on nuclear maturation, 6 experiments were performed: 1) COCs were cultured for 16 h in the presence of 100 ng/ml bovine GHRH (ICN Biomedicals, OH, USA) under the conditions described above. Cultures without GHRH served as controls. The percentage of oocytes at MII was assessed at the end of culture. The experiment consisted of 9 replicates. 2) COCs were cultured for 16 h in the presence of 1000 ng/ml bovine GHRH under the conditions described. Cultures without GHRH served as controls. The percentage of oocytes at MII was assessed at the end of culture. The experiment consisted of 3 replicates. 3) COCs were cultured for 24 h in the presence of 100 ng/ml bovine GHRH under the conditions described. Cultures without GHRH served as controls. The percentage of oocytes at MII was assessed at the end of culture. The experiment consisted of 6 replicates. 4) COCs were cultured for 16 h in the presence of 100 ng/ml porcine VIP (Sigma Chemical Co., St. Louis, MO, USA) under the conditions described. Cultures without VIP served as controls. The percentage of oocytes at MII was assessed at the end of culture. The experiment consisted of 9 replicates. 5) COCs were cultured for 16 h in the presence of 1000 ng/ml porcine VIP under the conditions described. Cultures without VIP served as controls. The percentage of oocytes at MII was assessed at the end of culture. The experiment consisted of 3 replicates. 6) COCs were cultured for 24 h in the presence of 100 ng/ml porcine VIP under the conditions described. Cultures without VIP served as controls. The percentage of oocytes at MII was assessed at the end of culture. The experiment consisted of 6 replicates.

To investigate the effects of GHRH and VIP on cumulus expansion and on FSH-induced cumulus expansion, 2 experiments were performed: 1) COCs were cultured under the conditions described for the 24 h protocol: a) with 100 ng/ml GHRH; b) with 100 ng/ml GHRH plus 0.05 IU/ml recombinant human FSH (hFSH; gift from Organon, Oss, The Netherlands); c) with 0.05 IU/ml hFSH (positive control); and d) without GHRH/FSH (negative control). The experiment consisted of 3 replicates. 2) COCs were cultured under conditions described for the 24 h protocol: a) with 100 ng/ml VIP; b) with 100 ng/ml VIP plus 0.05 IU/ml hFSH; c) with 0.05 IU/ml hFSH (positive control); and d) without VIP/FSH (negative control). The experiment consisted of 3 replicates.

To investigate the effects of GHRH and VIP on cortical granule distribution, 1 experiment was performed: COCs were cultured under the conditions described for the 24 h protocol: a) with 100 ng/ml GHRH; b) with 100 ng/ml VIP and c) without GHRH/VIP (control). Cortical granule distribution was assessed in the MII oocytes. The experiment consisted of 7 replicates.

Assessment of Nuclear Maturation

After culture of the COCs, the cumulus cells were removed by vortexing. The oocytes where then fixed for 15 min in 2.5% w/v glutaraldehyde (Merck, Darmstadt, Germany) in PBS, washed twice with phosphate-buffered saline (PBS), and stained with 0.1μg/ml 4,6-diamino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, MO, USA) in PBS and mounted on slides (Mori et al.,1988). The evaluation of nuclear status was done by epifluorescence microscopy (BH2-RFCA; Olympus, Tokyo, Japan). The oocytes were classified into 3 categories: germinal vesicle (GV), oocytes with diffuse or slightly condensed chromatin; Metaphase I (MI), oocytes with clumped or strongly condensed chromatin that formed an irregular network of individual bivalents or a metaphase plate but no polar body ; and Metaphase II (MII), oocytes with either a polar body or 2 shiny chromatin spots.

Assessment of Cumulus Expansion

Cumulus expansion was assessed by measurement of the diameter of the COCs using a calibrated stage micrometer. Each COC was measured before (0 h) and after (24 h) maturation and in 2 perpendicular directions.

Assessment of Cortical Granule (CG) Distribution

After in vitro maturation, the COCs were denuded as decribed previously (Izadyar et al.,1998). The oocytes were permeabilized in 0.1% (v/v) Triton X-100 (Sigma) in PBS for 5 min at 39°C, fixed in a solution of 2% paraformaldehyde in PBS, and kept in the refrigerator (4°C) until the day of the analysis. After fixation the oocytes were rinsed 3 times with PBS and then incubated at 38°C for 30 min in a solution of PBS supplemented with 0.1% Triton X-100 (v/v), 1% BSA (w/v; Sigma), and 100 μg/ml FITC-conjugated peanut agglutinin (FITC-PNA; E-Y Laboratory Inc., San Mateo, CA). After staining, the oocytes were washed 3 times for 5 min in PBS supplemented with 0.1% Triton X-100 (v/v). Subsequently, the oocytes were stained with DAPI for nuclear assessment, as described above. They were mounted under a coverslip and the slides were evaluated under epifluorescence microscopy. The CG distribution was classified into 3 types: Type 1, large aggregates of CGs distributed over the entire cytoplasm; Type 2, CGs localized in the cortical cytoplasm and distributed as individual particles as well as small aggregates; and Type 3, CGs more or less evenly dispersed in the cortical cytoplasm aligning the oolemma (Izadyar et al.,1998).

Statistical Analysis

The results were analyzed by the Chi-square test. For the cumulus expansion, analysis of variance was applied; treatment differences were determined using Bonferroni multiple comparison test. Differences were considered significant when $P < 0.05$.

RESULTS

Culture of COCs for 16 h in the presence of either 100 ng/ml GHRH or 100 ng/ml VIP did not affect the percentage of MII oocytes compared with the control (Tables 1 and 2, respectively). A high concentration of GHRH and VIP (1000 ng/ml) also did not affect the percentage of MII oocytes after a 16 h culture period (data not shown). Maturation for 24 h in M199 supplemented with 100 ng/ml GHRH or VIP had no effect on the percentage of MII oocytes compared with the control (Tables 3 and 4).

Table 1. The effect of GHRH (100 ng/ml) present during IVM of bovine COCs on the nuclear maturation after 16 hours of incubation.

	Total number of COCs	Number of oocytes at		
		GV (%)	MI (%)	MII (%)
Control	1016	76 (7.5)	491 (48.3)	449 (44.2)
GHRH	1073	83 (7.7)	530 (49.4)	460 (42.9)

Table 2. The effect of VIP (100 ng/ml) present during IVM of bovine COCs on the nuclear maturation after 16 hours of incubation.

	Total number of COCs	Number of oocytes at		
		GV (%)	MI (%)	MII (%)
Control	1009	92 (9.1)	506 (50.1)	411 (40.8)
VIP	1311	105 (8.0)	701 (53.5)	505 (38.5)

Table 3. The effect of GHRH (100 ng/ml) present during IVM of bovine COCs on the nuclear maturation after 24 hours of incubation

	Total number of COCs	Number of oocytes at		
		GV (%)	MI (%)	MII (%)
Control	492	6 (1.2)[a]	112 (22.8)	374 (76.0)
GHRH	502	16 (3.3)[b]	82 (19.7)	304 (77.0)

[a,b] Values differ significantly ($P < 0.05$)

Table 4. The effect of VIP (100 ng/ml) present during IVM of bovine COCs on the nuclear maturation after 24 hours of incubation

	Total number of COCs	Number of oocytes at		
		GV (%)	MI (%)	MII (%)
Control	432	13 (3.0)	108 (25.0)	311 (72.0)
VIP	396	10 (2.5)	88 (22.2)	298 (75.3)

There was no significant expansion of the cumulus cells of bovine COCs after 24 h maturation in M199 supplemented with 100 ng/ml GHRH or VIP (Figures 1 and 2, respectively). Culture of COCs for 24 h in the presence of 0.05 IU/ml FSH resulted in a marked expansion of the cumulus cells as previously described (Izadyar et al.,1997). The FSH-induced cumulus expansion was not inhibited when GHRH (Figure 1) or VIP (Figure 2) was used to supplement the medium.

The effect of GHRH or VIP on cortical granule distribution is shown in Table 5. The percentage of MII oocytes that showed Type 3 cortical granule distribution was significantly higher in the control group than in the GHRH or VIP group (15.9, 2.7 and 7.8 %, respectively).

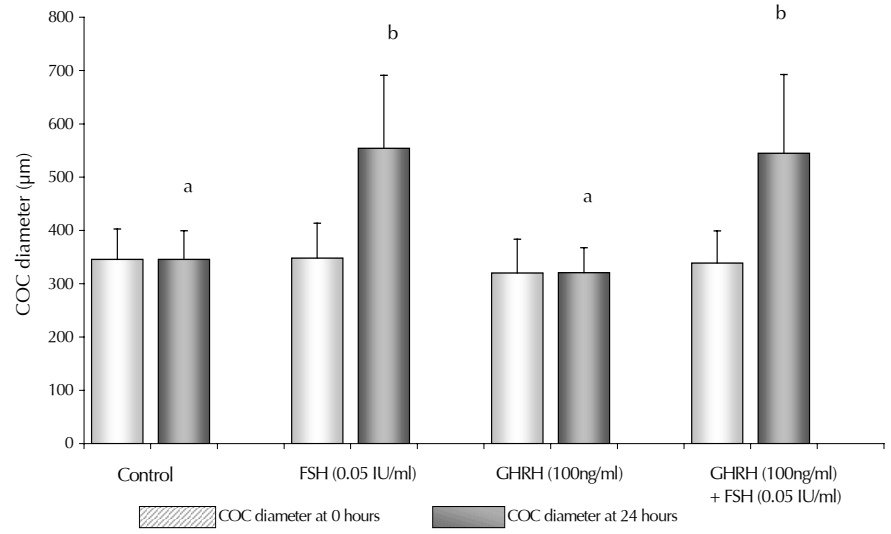

Fig. 1. Cumulus expansion of cumulus-enclosed bovine oocytes after 24 hours of culture in the presence of GHRH (100ng/ml) and /or FSH (0.05 IU/ml). (Between groups a,b: P< 0.05). Cultures without GHRH and FSH or with only FSH served as negative and positive controls, respectively. Number of oocytes per group = 110.

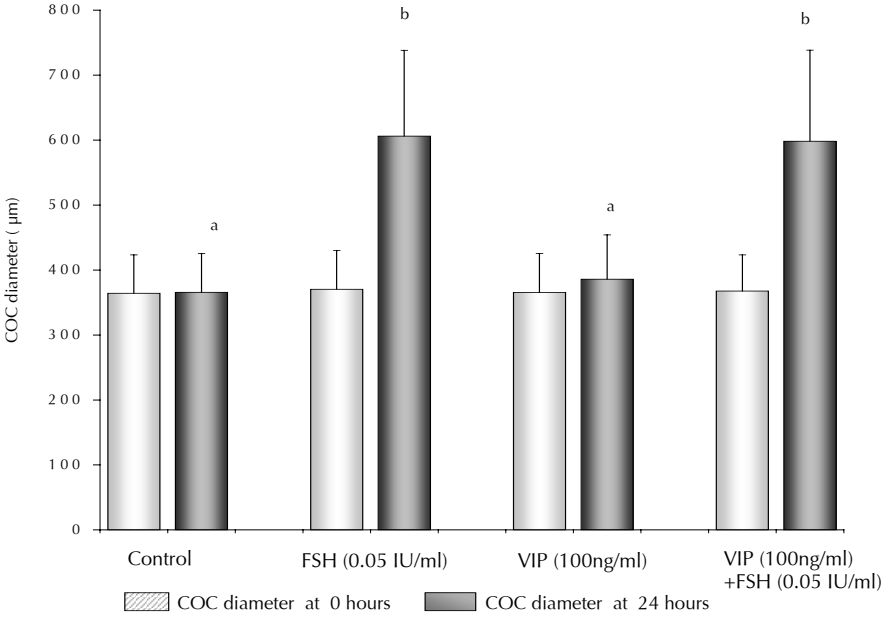

Fig. 2. Cumulus expansion of cumulus enclosed bovine oocytes after 24 hours culture in the presence of VIP (100 ng/ml) and /or FSH (0.05 IU/ml). (Between groups a,b: P <0.05). Cultures without VIP and FSH or with only FSH served as negative and positive controls, respectively. Number of oocytes per group = 120.

Table 5. Effects of GHRH (100 ng/ml) and VIP (100 ng/ml) on cortical granule distribution of bovine COCs, after culture for 24 hours

	Total number of COCs at MII	Number of MII oocytes showing cortical granule distribution of		
		Type 1 (%)	Type 2 (%)	Type 3 (%)
Control	383	22 (5.7)[ab]	300 (78.4)[a]	61 (15.9)[a]
VIP	477	15 (3.1)[a]	425 (89.1)[b]	37 (7.8)[b]
GHRH	403	31 (7.7)[b]	361(89.6)[b]	11 (2.7)[c]

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

DISCUSSION

This study clearly demonstrate that GHRH and VIP had no effect on cumulus expansion or nuclear maturation of bovine oocytes; however, both delayed cytoplasmic maturation as reflected by retarded cortical granule distribution. Regarding nuclear maturation, neither GHRH nor VIP affected the proportion of matured oocytes at the end of a 24 h culture period. Moreover, the progression of meiosis was also unaffected, as the proportion of MII oocytes at 16 h of culture was similar to the control. In the rat, GHRH significantly accelerated maturation of the follicle- and cumulus-enclosed oocytes (Apa et al.,1995), while VIP was effective only on follicle-enclosed oocytes (Tornell et al.,1988; Apa et al.,1997). Addition of VIP to the culture medium did not improve in vitro maturation and fertilization of sheep oocytes (Ledda et al.,1996).

Recently we showed that bovine oocytes contain mRNA for GH, but the capacity to synthesize GH seem does not to be regulated by GHRH since no mRNA for GHRH-receptor could be detected (Izadyar et al.,1999). The absence of an effect of GHRH on nuclear maturation supports that observation. On the other hand, the observed shift in Type 2/Type 3 ratio of cortical granule distribution by GHRH points to the existence of a receptor that mediates the action of GHRH on the cytoplasm of the oocyte.

Growth hormone releasing hormone may exert its action on cortical granule distribution via the VIP receptor. Both GHRH and VIP belong to the same peptide family, and their receptors share 40 to 47 % homology (Mayo,1992; Gaylinn et al.,1993; Hsiung et al.,1993). In rat pancreatic and liver

membranes (Robberecht et al.,1986), human and rat intestinal epithelial cells (Laburthe et al.,1983), rat leydig cells (Ciampani et al.,1992), and rat granulosa cells (Moretti et al.,1990) GHRH binds and exerts its effects through VIP receptors. On the other hand, GHRH and VIP may exert their effect on the oocyte cytoplasm via the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor. The PACAP is isolated from ovine hypothalamus, and recently the presence of its receptors in the rat ovary (Scaldaferri et al.,1996) and its effect on rat granulosa/lutein cells (Gras et al.,1999) and on rat oocyte maturation (Apa et al.,1997) have been demonstrated. The amino acid sequences of rat PACAP receptors showed a remarkable similarity with rat receptors for VIP, secretin, glucagon and GHRH (Hosoya et al.,1993). There are 2 major subtypes of PACAP receptors: the Type I receptor (PACAP-I-R) has much higher affinity for PACAP than VIP, while the Type II receptor (PACAP-II-R) has similar affinity for both peptides (Shivers et al.,1991; Arimura,1992). In a binding study of PACAP and its related peptides with rat lung membrane, GHRH was more potent than VIP in the displacement of PACAP from its receptors (Bitar and Coy,1993). Therefore, the observation that GHRH has a more pronounced effect on the cortical granule distribution, could be due to the higher affinity of PACAP receptors for GHRH than for VIP. Species specificity of bovine GHRH and porcine VIP is not an explanation for this observation, since the amino acid sequence of porcine VIP shows 100 % homology with bovine VIP (Nussdorfer and Malendowicz,1998).

Both the VIP (Christophe et al.,1986; Rosselin,1986; Ishihara et al.,1992) and PACAP (Ogi et al.,1993) receptors are members of the secretin family of G-protein-coupled receptors using cyclic AMP (c-AMP) as their signal transduction molecule (Robberecht et al.,1991). The absence of any effect of VIP and GHRH on cumulus expansion is in contrast to the cumulus expansion induced by FSH and GH (Izadyar et al.,1998), which both also use the c-AMP signal transduction system. This observation suggests that VIP and GHRH exert their effects on oocyte cytoplasm via a membrane receptor located on the oolemma. The fact that GHRH and VIP had no effect on FSH-induced cumulus expansion further supports the absence of a receptor on the cumulus cells.

Cortical granule distribution has been used as a reliable indicator for the evaluation of cytoplasmic maturation and developmental competence of bovine oocytes (Damiani et al.,1996; Izadyar et al.,1998). The assessed cortical granule distribution as presented in Table 5 showed that most of the MII oocytes exhibited some delay in migration and dispersal of the cortical granules (Type 1 and 2). This indicates lack of coordination between nuclear and cytoplasmic maturation of the oocytes. Cortical granules remained associated in aggregates

instead of being dispersed evenly below the plasma membrane. Addition of VIP or GHRH to the maturation medium further retarded the process of cortical granule migration, resulting in an even lower proportion of MII oocytes showing Type 3 cortical granule distribution. This effect opposes the stimulation of cortical granule migration by GH (Izadyar et al.,1998), and can be considered as additional proof for the absence of a GHRH receptor in bovine COCs.

In conclusion, culture of bovine COCs in the presence of GHRH or VIP did not affect nuclear maturation or cumulus expansion, but it retarded cytoplasmic maturation as reflected by delayed cortical granule migration. It is suggested that the effects of GHRH and VIP are exerted via a VIP/PACAP receptor localized on the plasma membrane of the oocyte.

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CHAPTER 3

EFFECT OF 17β-ESTRADIOL ON THE IN VITRO MATURATION OF BOVINE OOCYTES

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ABSTRACT

Although 1µg/ml of 17β-Estradiol (E2) is often used in routine in vitro maturation (IVM) and in vitro fertilization (IVF), its effect remains controversial. The objective of our study was to investigate the effects of E2 on bovine oocyte IVM and subsequent embryo development, using a defined medium. Bovine cumulus oocyte complexes (COCs), aspirated from 2 to 8 mm follicles of slaughterhouse ovaries, were matured in TCM199 in the presence of 1µg/ml E2 with or without 0.05 IU/ml recombinant hFSH. Cultures without E2, FSH or both served as controls. COCs were matured for 22 h at 39ºC in a humidified atmosphere of 5% CO_2 in air. To investigate the effect of E2 with and without FSH on nuclear maturation, COCs were fixed after maturation and the nuclear stage was assessed following DAPI staining. Similarly, denuded oocytes (DO) were matured in the presence of E2 and the nuclear stage assessed after 22 h. To investigate the effect of E2 with and without FSH during IVM on subsequent embryo development, in vitro matured COCs were fertilized in vitro and after removal of the cumulus cells, the presumed zygotes were cocultured on BRL monolayer for 11 days. At Day 4, the number of cleaved embryos, and at Days 9 and 11 the number of blastocysts, were assessed.

Addition of 1µg/ml E2 to TCM199 significantly decreased the percentage of Metaphase II (MII) compared to control (56.3 and 74.0% respectively), and increased the percentage of nuclear aberrations compared to control (13.3% and 2.1% respectively). The negative effect of E2 on nuclear maturation was stronger when DO were matured; 25.1 and 60.0% of the oocytes reached MII stage for the E2 and control groups respectively. When COCs were matured in TCM199 supplemented with FSH, the addition of 1µg/ml E2 did not influence the proportion of MII oocytes, although a higher percentage of nuclear aberrations as compared to control was observed. Presence of E2 during IVM also decreased the blastocyst rate (14.4 and 10.0% for control and E2 groups, respectively). However, when FSH was present, the addition of E2 had no effect on the cleavage rate and blastocyst formation (20.3 and 21.7% for control and E2 groups, respectively).

In conclusion, supplementation of 1µg/ml E2 to a serum free maturation medium negatively affects bovine oocyte nuclear maturation and subsequent embryo development. Although these effects are attenuated in the presence of FSH, we strongly suggest omission of E2 in routine maturation protocols of bovine oocytes.

INTRODUCTION

In the cow the LH peak inhibits androstedione production by the theca cells and induces luteinization of the mural granulosa cells. This process is reflected by the steroid concentrations in the follicular fluid of the preovulatory follicle which show a high estradiol concentration of about 1 µg/ml before and until 6 h after the maximum of the LH peak, whereafter a sharp decline of estradiol concentration occurs. This is followed by a gradual increase in progesterone beginning about 20 h after the LH peak (Dieleman et al.,1983; Fortune and Hansel,1985). Although the decline in estradiol takes place concurrently with germinal vesicle breakdown (GVBD) (Dieleman et al.,1983), there is no evidence that estradiol is involved in the resumption of meiosis. In the follicular fluid of isolated hamster follicles, addition of high amounts of estradiol and antibody against estradiol did not affect the resumption of meiosis (Racowsky and Baldwin,1989).

Although 1µg/ml estradiol is often used in maturation protocols, the role of estradiol in the in vitro maturation (IVM) of oocytes remains controversial. Addition of estradiol to the maturation medium reduced the maturation rate of cumulus-enclosed (Smith and Tenney,1980) and denuded (Eppig and Koide,1978) mouse oocytes. Richter and McGaughey (1979) and Eroglu (1993) reported an inhibitory effect of estradiol on the maturation of pig oocytes. IVM of porcine oocytes in the presence of estradiol and FSH reduced the percentage of porcine oocytes undergoing GVBD, while estradiol alone had no effect (Singh et al.,1993). In contrast, Bing et al. (2001) reported an inhibition of GVBD of porcine oocytes cultured in the presence of estradiol alone and no effect on the progression of meiosis when both estradiol and FSH were added to the maturation medium. The presence of estradiol in the culture medium of in vitro matured human oocytes had no effect on the progression of meiosis, but improved the fertilization and cleavage rate, suggesting that estradiol supports cytoplasmic maturational changes necessary for fertilization and early postfertilization development (Tesarik and Mendoza,1995). In sheep, addition of estradiol to maturation medium supplemented with gonadotrophins resulted in an increased percentage of blastocysts (Moor and Trounson,1977; Guler et al.,2000). IVM of bovine oocytes in the presence of estradiol increased the maturation rate (Fukui et al.,1982; Younis et al.,1989) but had no effect on the percentage of cleaved and 4-8 cell stage embryos (Younis et al.,1989). Gliedt et al. (1996) reported a negative effect of estradiol on cumulus expansion of bovine oocytes, and a decrease in blastocyst formation rate following in vitro fertilization (IVF). Maturation of bovine oocytes in the presence of high

concentrations of estradiol had a negative effect on spindle formation and polar body extrusion (Kruip et al.,1988).

The aim of our study was to investigate the effect of 17β-estradiol (E2) added to the maturation medium, on bovine oocyte maturation and subsequent embryo development using a defined medium.

MATERIAL AND METHODS

Collection and Culture of Oocytes

Bovine ovaries were collected at a slaughterhouse and transported in a thermoflask to the laboratory. Cumulus oocyte complexes (COCs) were obtained by aspiration of 2-8 mm follicles and selected based on the presence of a multilayered compact cumulus investment. Selected COCs were washed in HEPES buffered M199 (Gibco BRL, Paisley, UK), and, in groups of 35, were randomly allocated in each well of a 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 µL M199, with or without estradiol. Culture was performed for 22 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

In Vitro Fertilization and Embryo Culture

Both IVF and in vitro embryo culture (IVC) took place at 39°C in a humidified atmosphere of 5% CO_2 in air. The semen used throughout this study was from one ejaculate. Frozen-thawed spermatozoa, were centrifuged over a percoll gradient for 30 min at 27ºC at 700 x g. The sperm sample was collected by removing the gradient except for the last 150 µL containing the sperm pellet. Groups of 35 COCs were washed twice in M199 and transferred to a well of the 4-well culture plate (Nunc A/S) containing 430 µL of fertilization medium (Fert-Talp), as described by Parrish et al. (1988) and modified by Izadyar et al. (1996). Twenty microliters of sperm suspension, final concentration 12.5 x 10^6 spermatozoa/ml, plus 20 µL of heparin (final concentration 10 µg/ml) (Sigma Chemical Co., St. Louis, MO, USA) and 20 µL PHE (consisting of 20 µM D-penicillamine, 10 µM hypotaurine, 1 µM epinephrine) were added. After 20 h of incubation, the cumulus cells were removed by vortexing for 3 min. Groups of 35 presumptive zygotes were randomly placed in a coculture system of 500 µL M199 supplemented with 10% FCS (Gibco BRL) on a monolayer of Buffalo Rat Liver (BRL) cell in each well of a 4-well culture plate. At the fourth day of culture the noncleaved embryos were removed. Embryos were transferred to fresh coculture wells on the fourth and eighth day of culture.

BRL-Cell Culture

Buffalo Rat Liver cells separated from the BRL cell line from the American Type Culture Collection (ATCC) were cultured routinely in a 1:1 mixture of Ham's F12 medium (Gibco BRL) and Dulbecco's Modified Eagle's medium (Gibco BRL) supplemented with 7.5% FCS (Gibco BRL) and 0.1% penicillin/streptomycin (Gibco BRL). These cells differ from those currently available from ATCC in that they exhibit contact inhibition of growth.

Assessment of Nuclear Maturation

After culture of the COCs, the cumulus cells were removed by vortexing. The oocytes were then fixed for 15 min in 2.5% (w/v) glutaraldehyde (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS), washed twice with PBS, stained with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI; Sigma) in PBS and mounted on slides (Mori et al.,1988). The evaluation of nuclear status was done by epifluorescence microscopy (BH2-RFCA; Olympus, Tokyo, Japan). The oocytes were classified into four categories: germinal vesicle (GV), oocytes with diffuse or slightly condensed chromatin; Metaphase I (MI), oocytes with clumped or strongly condensed chromatin that formed an irregular network of individual bivalents or a metaphase plate but no polar body; Metaphase II (MII), oocytes with either a polar body or two shiny chromatin spots; and aberrations, oocytes with an abnormal chromosomal organization.

Assessment of Embryo Development

At Day 4 of culture (Day 0= day of fertilization), the total cleavage rate and the number of cleaved embryos consisting of ≥ 8 cells were evaluated. The blastocyst formation was evaluated on Day 9 and Day 11 of culture. The number of hatched blastocysts was evaluated on Day 11 and expressed as a percentage of the total number of zygotes at the beginning of the culture.

Experiments

A stock solution was prepared by dissolving 200 µg/ml estradiol (Sigma) in ethanol (Merck). The stock solution was stored at -20ºC. On the day of the experiment the stock solution was diluted in M199 to the final concentration of 1 µg/ml estradiol. Since membrane filters bind steroids, estradiol was added aseptically from each stock solution after the sterilization of the culture medium by membrane filters. The final concentration of ethanol in the maturation medium supplemented with estradiol was 0.5 % (v/v), therefore the control maturation medium was supplemented with 0.5 % (v/v) ethanol.

To investigate the effects of estradiol on nuclear maturation during bovine IVM three series of experiments were performed:

1) COCs were matured in the presence of 1 μg/ml estradiol under the conditions described above. Cultures without estradiol served as controls. The percentage of oocytes at GV, MI, MII and the percentage of nuclear aberrations were assessed at the end of the culture. The experiment consisted of seven replicates.

2) COCs were matured in M199 supplemented with 0.05 IU/ml recombinant human FSH (Organon, Oss, The Netherlands) in the presence of 1 μg/ml estradiol. Cultures of M199 supplemented with FSH and without estradiol served as control. The percentage of oocytes at GV, MI, MII and the percentage of nuclear aberrations were assessed at the end of the culture. The experiment consisted of four replicates.

3) COCs were vortexed for 3 min to remove cumulus cells and the denuded oocytes (DO) were cultured in the presence of 1 μg/ml estradiol. Cultures without estradiol served as controls. The percentage of oocytes at GV, MI, MII and the percentage of aberrations were assessed at the end of the culture. The experiment consisted of three replicates.

To investigate the effects of estradiol added during IVM on subsequent embryo development three series of experiments were performed:

1) COCs were matured in M199 in the presence of 1 μg/ml estradiol, fertilized in vitro and subsequently cultured for 11 days under the conditions described above. Cultures without estradiol served as controls. The percentage of cleaved zygotes, ≥8 cell embryos, blastocysts and hatched blastocysts were assessed. The experiment consisted of five replicates.

2) COCs were matured in M199 in the presence of 1 μg/ml estradiol under the conditions described above. Prior to in vitro fertilization, the COCs were denuded and only oocytes clearly showing a polar body (PB) were fertilized. Subsequently the presumptive zygotes were cultured for 11 days. Cultures without estradiol served as controls. The percentage of cleaved zygotes, ≥8 cell embryos, blastocysts and hatched blastocysts were assessed. The experiment consisted of three replicates.

3) COCs were matured in M199 supplemented with 0.05 IU/ml FSH and 1 μg/ml estradiol, fertilized in vitro, and subsequently cultured for 11 days under the conditions described above. Cultures in M199 supplemented only with FSH served as controls. The percentage of cleaved zygotes, ≥8 cell embryos, blastocysts and hatched blastocysts were assessed. The experiment consisted of five replicates.

Statistical Analysis

Treatment was considered as independent variable. For the nuclear maturation experiments, the dependent variable was the number of oocytes per stage (GV, MI, MII and aberrations). For the embryo development experiments, the dependent variables were: the number of cleaved embryos at Day 4, the number of embryos with ≥8 cells at Day 4, the number of blastocyst at Day 9 and 11 and the number of hatched blastocyst at Day 11. The results were analyzed by the chi-square test. Differences were considered significant when $P \leq 0.05$.

RESULTS

Culture of COCs for 22 h in the presence of 1 µg/ml estradiol decreased ($P<0.0001$) the percentage of MII oocytes as compared to the control group (56.3 and 74.0% respectively). Moreover, the proportion of oocytes presenting nuclear aberrations (Fig. 1) was also significantly higher ($P<0.0001$) in the presence of estradiol (2.1 and 13.3% for control and estradiol group respectively)(Fig. 2).

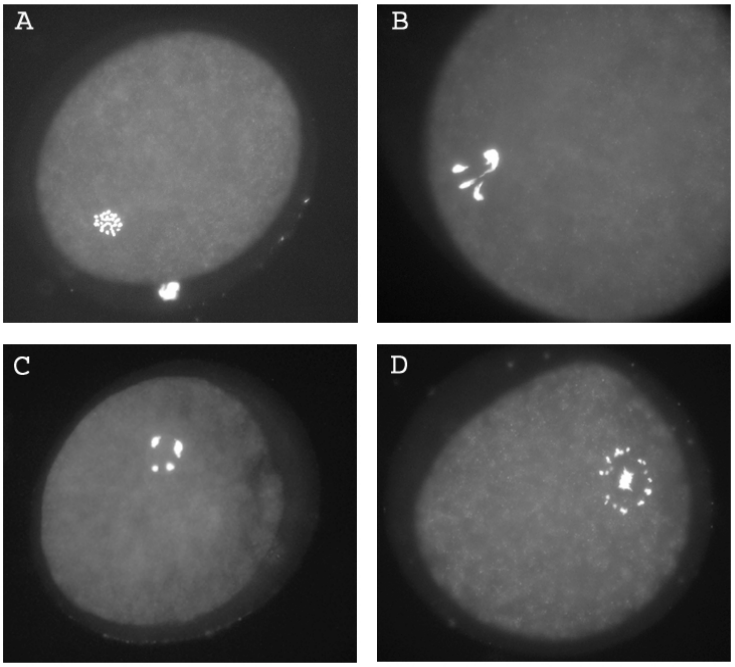

Fig. 1. DAPI staining of COCs after culture of 22 h in M199 supplemented with 1 µg/ml estradiol. Normal Metaphase II (A). Abnormal dispersion of the chromosomes defined as nuclear aberrations (B,C,D).

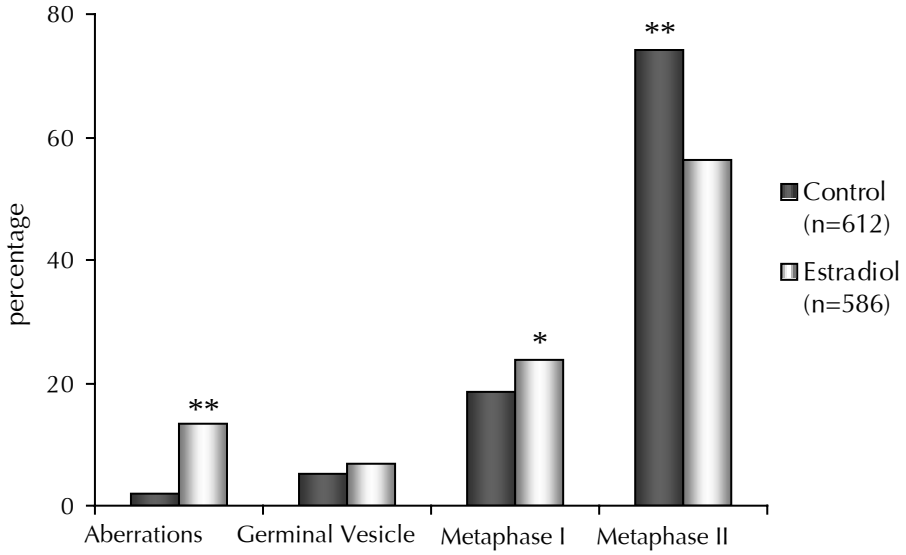

Fig. 2. Effect of estradiol (1 µg/ml) on the nuclear maturation of bovine COCs, after 22 h of in vitro maturation in M199. (Between groups: * P<0.05; ** P<0.0001)

There was no significant difference in the percentage of GV, MI and MII oocytes when bovine COCs were cultured for 22 h in M199 supplemented with 0.05 IU/ml FSH and 1 µg/ml estradiol, but the percentage of nuclear aberrations was higher (P=0.0001) when estradiol was present (1.4% in the control group versus 6.4% in the estradiol group) (Fig. 3).

The effect of 1 µg/ml estradiol during IVM of DO is shown in Fig. 4. The percentage of MII oocytes was significantly lower (P<0.0001) for the estradiol group as compared to the control group (25.1 and 60.0% respectively). In addition, the estradiol group showed higher percentages of oocytes in GV and MI (11.6 and 46.0% respectively) when compared with the control group (6.3 and 26.0% respectively). A significantly higher (P=0.0001) percentage of oocytes showing nuclear aberrations was observed when estradiol was present (7.7 and 17.3%; control and estradiol group, respectively).

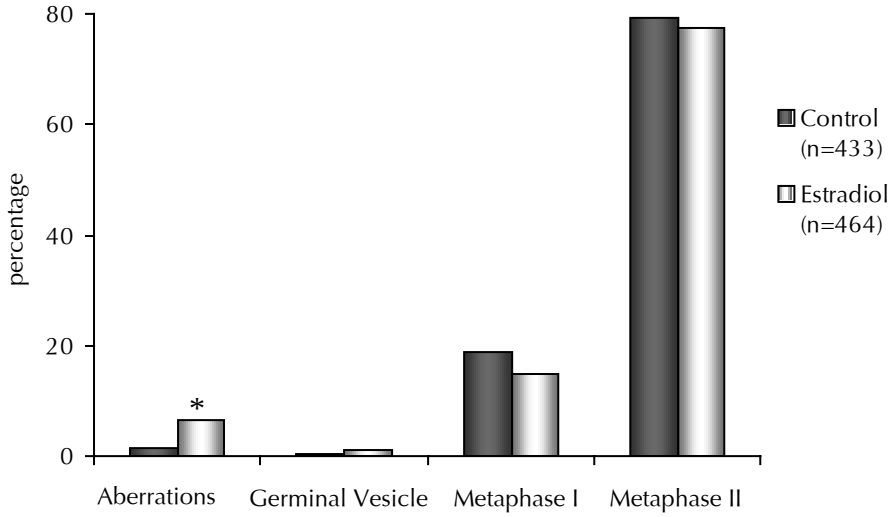

Fig. 3. Effect of estradiol (1 µg/ml) on the nuclear maturation of bovine COCs, after 22 h of in vitro maturation in M199 supplemented with 0.05 IU/ml FSH. (Between groups: * P=0.0001)

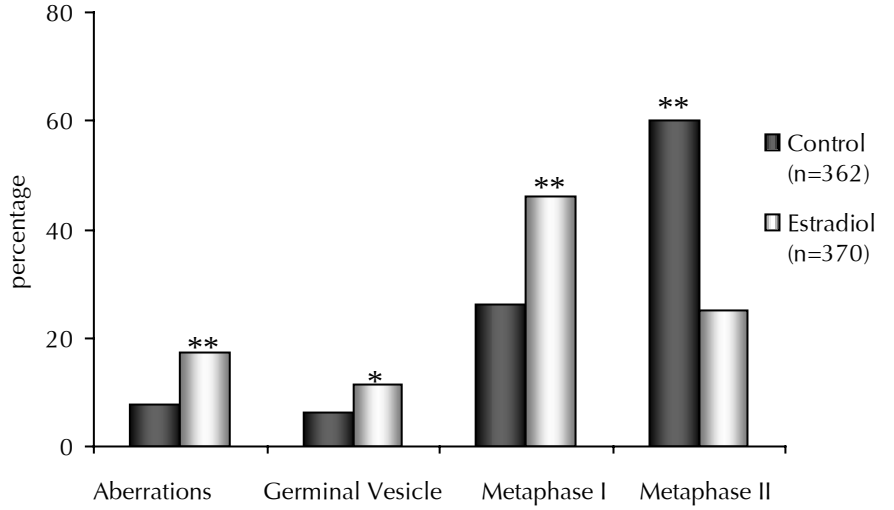

Fig. 4. Effect of estradiol (1 µg/ml) on the nuclear maturation of bovine denuded oocytes after 22 h of in vitro maturation in M199. (Between groups *: $P < 0.05$; **: $P \leq 0.0001$).

The presence of 1 µg/ml of estradiol during IVM negatively affected embryo development following fertilization since the percentage of blastocysts at Day 9 was lower (P<0.05) compared to the control group (Table 1).

Table 1.Effect of estradiol (1 µg/ml) during IVM, on bovine embryo development.

Oocytes N		Embryos at Day 4		Blastocysts		
		Cleaved n (%)	≥8cells n (%)	Total Day 9 n (%)	Total Day 11 n (%)	Hatched Day 11 n (%)
Control	521	278 (53.4)	130 (24.9)	75 (14.4) [a]	81 (15.5)	59 (11.3)
Estradiol	521	295 (56.6)	121 (23.2)	52 (10.0) [b]	63 (12.1)	42 (8.1)

Within columns: a, b P<0.05

Compared with the control group, the extrusion of the polar body after 22 h culture was significantly reduced (P<0.05) when estradiol was present in the maturation medium (74.7 and 68.6% respectively). There was no effect of 1 µg/ml of estradiol during IVM on subsequent embryo development when only oocytes with a clear polar body were fertilized in vitro (Table 2).

Table 2. Effect of estradiol (1 µg/ml) during IVM on bovine embryo development of oocytes, with extruded polar body, selected before IVF.

Oocytes N		Embryos at Day 4		Blastocysts		
		Cleaved n (%)	≥8cells n (%)	Total Day 9 n (%)	Total Day 11 n (%)	Hatched Day 11 n (%)
Control	396	188 (47.5)	100 (25.3)	59 (14.9)	61 (15.4)	42 (10.6)
Estradiol	377	177 (47.0)	93 (24.7)	53 (14.1)	63 (15.7)	42 (11.9)

Embryo development was not influenced by the addition of estradiol during IVM when FSH was present, since no significant difference was found between control and estradiol groups for cleavage rate and percentage of embryos with ≥ 8 cells at Day 4, blastocyst formation at Day 9 and 11, and hatched blastocysts at Day 11 (Table 3).

Table 3. Effect of estradiol (1 µg/ml) during IVM, in M199 supplemented with FSH (0.05 IU /ml) on bovine embryo development.

	Oocytes N	Embryos at Day 4		Blastocysts		
		Cleaved n (%)	≥8cells n (%)	Total Day 9 n (%)	Total Day 11 n (%)	Hatched Day 11 n (%)
Control	680	479 (70.4)	194 (28.5)	138 (20.3)	143 (21.0)	118 (17.4)
Estradiol	672	477 (71.0)	203 (30.2)	146 (21.7)	152 (22.6)	125 (18.6)

DISCUSSION

This study clearly shows that estradiol negatively affects bovine oocyte nuclear maturation as reflected by the decrease of the proportion of MII oocytes, and by the increase of occurrence of nuclear aberrations. Various studies (McGaughey,1977; Richter and McGaughey,1979; Eroglu,1993), also report an inhibitory effect of estradiol on the progression of meiosis of cumulus enclosed and DO. However, Fukui et al. (1982) and Younis et al. (1989) report that the addition of 1 µg/ml of estradiol to the maturation medium enhanced the maturation rate. Although we observed that 1 µg/ml of estradiol in a serum free medium clearly reduces the maturation rate, it also appeared that supplementation of the medium with FSH suppresses inhibition by estradiol, restoring the percentage of MII oocytes to control levels. This may explain why in culture media supplemented with serum, which contains gonadotrophins, no negative effect of estradiol on the percentage of MII oocytes is observed. FSH appeared to suppress the negative effect of estradiol on nuclear maturation as shown in the proportion of MII oocytes, although it was not a complete suppression, since nuclear aberrations could still be observed when oocytes were cultured in the media containing FSH and estradiol.

The lower number of blastocysts obtained when oocytes were matured in the presence of estradiol is probably due to the lower proportion of MII oocytes at the end of the culture. This is supported by the finding that the percentage of blastocysts was similar for control and estradiol groups when only oocytes having an extruded polar body were fertilized. Moreover, when oocytes were cultured in the presence of estradiol and FSH, resulting in the same percentage of MII oocytes at the end of culture, a similar proportion of

blastocysts formed was observed, as compared to oocytes cultured only in the presence of FSH. Those findings indicate that estradiol does not affect cytoplasmic maturation in terms of blastocyst formation, which is in contrast with the observations of Tesarik and Mendoza (1995).

The observation that the treatment of DO with estradiol resulted in the strongest effect in terms of nuclear aberrations, indicates that estradiol exerts its effect on the oocyte, and not via the cumulus cells. Since the effect was less in COCs and even weaker in COCs cultured in the presence of FSH, it can be concluded that the cumulus cells counteract to reduce the action of estradiol on the oocyte. This may be due to uptake of estradiol by the cumulus which may be enhanced in the presence of FSH, resulting in a lower concentration of estradiol, or a signal generated by cumulus cells which is amplified in the presence of FSH, that counteracts the effect of estradiol.

We show that for oocytes cultured in serum free media, the addition of 1 μg/ml of estradiol was high enough to induce a significant increase of the percentage of metaphase plates with misaligned chromosomes (nuclear aberrations), and also some oocytes with only a polar body without visible nuclear material (data not shown). Kruip et al. (1988), using a maturation medium containing FCS and gonadotrophins, reported a higher percentage of abnormal meiotic configurations when estradiol was added at a concentration of 10 μg/ml. McGaughey (1977) also described a high incidence of numerical chromosomal aberrations in pig COCs and DO. Similar effects are observed in somatic cells (Liehr,2000) where estradiol induces numerical chromosome aberrations in Syrian hamster embryo cells (Tsutsui et al.,1987), and in lung fibroblasts (Ochi,1999) and misaligned chromosomes in Chinese hamster cells (Wheeler et al.,1987). Treatment of Syrian hamsters with estradiol also leads to structural chromosomal aberrations such as deletions, inversions, and translocations in kidney cells (Banerjee et al.,1994). There is some evidence that estradiol affects the microtubule organization (Aizu-Yokota et al.,1995). A high incidence of aberrant spindles, such as tripolar and multipolar spindles occurred when cells were cultured with estradiol, and formation of aberrant spindles may play a role in the unequal segregation of the chromosomes (Ochi,1999), leading to aneuploidy and misalignment of chromosomes as observed in our study. Although the mechanism by which estradiol exerts its action is still unclear, studies regarding the involvement of estrogen on microtubule dynamics, and its carcinogenicity on somatic cells (Tsutsui et al.,1987; Wheeler et al.,1987; Singh et al.,1993; Ochi,1999), point to a non-genomic effect of the steroids (Kaji et al.,1987; Singh et al.,1993; Ochi,1999).

In summary, our results show that 1 µg/ml of estradiol added to a serum free maturation medium negatively affects bovine oocyte nuclear maturation and subsequent embryo development. This is reflected by a lower percentage of oocytes in MII stage, a higher percentage of nuclear aberrations, and a lower percentage of blastocysts. Although in the presence of FSH the effect is attenuated, we strongly suggest omission of estradiol in routine maturation protocols of bovine oocytes.

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CHAPTER 4

ESTRADIOL AND ITS MEMBRANE IMPERMEABLE CONJUGATE (ESTRADIOL-BOVINE SERUM ALBUMIN) DURING IN VITRO MATURATION OF BOVINE OOCYTES: EFFECTS ON NUCLEAR AND CYTOPLASMIC MATURATION, CYTOSKELETON AND EMBRYO QUALITY

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ABSTRACT

In various cell types, there is increasing evidence for non-genomic steroid effects, i.e., effects that are not mediated via the classical steroid receptors. However, little is known about the involvement of the nongenomic pathway of estradiol (E2) on mammalian oocyte in vitro maturation (IVM). The aim of this study was to investigate whether the effects of E2 on bovine oocyte IVM are mediated via a plasma membrane receptor (nongenomic). First, we investigated the expression of estradiol (classical) receptor alpha (ERα) and beta (ERβ) mRNA in oocytes and cumulus cells (CC). We also studied the effects of different exposure times to E2 (before and after germinal vesicle breakdown, GVBD) on nuclear maturation. To study the possible involvement of the putative estradiol plasma membrane receptor on the IVM of oocytes, we used E2 conjugated with bovine serum albumin (E2-BSA), which cannot cross the plasma membranes. Our results demonstrate that oocytes expressed ERβ mRNA, while CC expressed both ERα and ERβ mRNA. Exposure to E2 during the first 8h of culture (before GVBD) induced a block at the metaphase I stage (MI). However, the presence of E2 after GVBD induced an increase of oocytes with nuclear aberrations. Meiotic spindle organization was severely affected by E2 during IVM and multipolar spindle was the most frequently observed aberration. Exposure of oocytes to E2-BSA did not affect nuclear maturation, blastocyst formation rate, nor embryo quality. Our results suggest that the detrimental effects of E2 on in vitro nuclear maturation of bovine oocyte are not exerted via a plasma membrane receptor.

Key Words: Apoptosis, Embryo, Estradiol, Estradiol Receptor, In vitro Fertilization, Meiosis

INTRODUCTION

In vivo, bovine oocytes resume meiosis after the pre-ovulatory LH peak, while resumption of meiosis occurs spontaneously when cumulus-oocyte complexes (COCs) are removed from their follicle and cultured in vitro under suitable conditions (Pincus and Enzmann,1935; Edwards,1965). During meiotic maturation, the oocyte undergoes germinal vesicle breakdown (GVBD), which involves a gradual chromatin condensation, disappearance of the nucleolus and disintegration of the nuclear membrane (Hyttel et al.,1987). In bovine oocytes matured in vitro, GVBD occurs around 6-8h after the onset of the culture (Motlik et al.,1978; Khatir et al.,1998). Before GVBD, transcription and translation can take place, but after GVBD, transcription strongly declines (Tomek et al.,2002). It is suggested that, when the chromatin is condensed, the transcription system is practically inactive (Curtis et al.,1995). Therefore, it is likely that cellular changes that occur after GVBD are not dependent on transcription and are consequently considered as nongenomic effects.

It is well known that steroids, including estradiol (E2), exert their function by binding to intracellular receptors with subsequent direct activation of gene transcription and protein synthesis, a so-called classical genomic pathway (for review see Carson-Jurica et al.,1990; Hall et al.,2001). The classical estrogen receptor (ER) exists as two subtypes: ER-alpha (ERα) and ER-beta (ERβ) and these subtypes exhibit different tissue localization patterns and can have distinct biological functions. ERα mRNA is predominantly expressed in the uterus, mammary gland, testis, pituitary, liver, kidney, heart, and skeletal muscle; whereas ERβ transcripts are significantly expressed in the ovary and prostate (for review see Couse and Korach,1999). Additionally, a third type of ER (gamma) has been described in teleost fish (Hawkins et al.,2000) although it has not been found in mammals.

In addition to the genomic effect, indication of nongenomic effects exerted by steroids has been described in a wide variety of cell types, such as pancreatic cells, neurons, sperm, endometrial cells, granulosa cells, and oocytes (for reviews see Revelli et al.,1998; Falkenstein et al.,2000; Levin,2002). Involvement of non-genomic steroid pathways in oocytes was first described in amphibians and fish, providing some of the most convincing examples known of rapid, nontranscriptional regulation by steroid hormones (Masui and Markert,1971; Thomas et al.,2002). In *Xenopus*, for instance, progesterone binding to a plasma membrane-receptor triggers oocyte maturation (Lutz et al.,2000). However, although these mechanisms are well described in lower vertebrates, little is known about the plasma membrane-mediated effects of E2

during in vitro maturation of bovine oocytes. Additionally, specific nongenomic responses seem to depend on type of steroid, cell, tissue, and species.

Many of the steroid-mediated pathways are suggested to be initiated at the plasma membrane and involve conventional second messengers cascades (Falkenstein et al.,2000). For instance, a rapid transient increase in the intracellular free calcium concentration ([Ca2+]i) occurs in human oocytes (Tesarik and Mendoza,1995) as well as in sperm (Luconi et al.,1999) shortly after exposure to E2. However, the nature and characteristics of the mediating plasma membrane receptor remains controversial. In addition, direct steroid-membrane interactions without receptor involvement have also been described, e.g., an intercalation of the steroid into the membrane of target tissues, which might alter physicochemical membrane properties such as the fluidity and the microenvironment of membrane receptors (Whiting et al.,2000).

A complete meiotic maturation includes the extrusion of the first polar body, which requires, apart from other events, proper meiotic spindle formation. These structural changes are associated with the reorganization of microtubules and microfilaments, which are the major cytoskeletal components in mammalian oocytes that provide the framework for chromosomal movement and cell division (Albertini,1992; Albertini et al.,1993; Kim et al.,2000). Defects of the microtubular system in the oocyte can induce loss or gain of chromosomes, leading to aneuploidy (Edwards,1958), resulting in abnormal embryonic development following fertilization. Recently, we reported that, in bovine, the presence of E2 during in vitro maturation (IVM) has a detrimental effect (both in denuded oocytes and COCs) on the nuclear maturation, including abnormal dispersion of chromosomes and on subsequent embryo development (Beker et al.,2002). The adverse effects of E2 on nuclear maturation might be due to an improper spindle organization during IVM. Disruption of the meiotic spindle by synthetic estrogen analogues has been reported (Ochi,1999; Can and Semiz,2000). One of the reasons for impairment of embryo development is an inadequate cytoplasmic maturation, resulting in an increase of apoptosis during postfertilization development. Although studies in endothelial cells (Alvarez et al.,1997), male germ cells (Pentikainen et al.,2000) and cardiac myocytes (Pelzer et al.,2000) suggested that E2 inhibits apoptosis, little is known about the effect of the presence of E2 during IVM on apoptosis in subsequent bovine embryo development.

Estradiol is often used in maturation protocols, but the mechanisms of action of E2 on the oocyte IVM are poorly understood. The aim of this study was to examine whether the observed detrimental effects of the presence of E2 during IVM (Beker et al.,2002) is a consequence of binding to the classical

receptor (genomic), which regulates transcription, or via a transcription-independent manner (nongenomic) by binding to a plasma membrane-receptor. For this, E2 conjugated with bovine serum albumin (E2-BSA), which cannot cross the plasma membranes of living cells (Zheng et al.,1996), was used during IVM to assess whether E2 acts via a plasma membrane-bound receptor. In addition, we investigated the effect of exposure of oocytes to E2 either before or after GVBD, i.e., before or after transcription had ceased. We also studied the expression of ERα and ERβ mRNA in germinal vesicle stage (GV) oocytes, by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, to elucidate the nuclear abnormalities and low embryo development (percentage of blastocyst formation) induced by E2, we investigated the effect of E2 during IVM on meiotic spindle organization and on DNA fragmentation/apoptosis following subsequent in vitro embryo development.

MATERIALS AND METHODS

Collection and Culture of Oocytes

Bovine ovaries, obtained at a local slaughterhouse, were transported to the laboratory in a thermoflask within 2 h after slaughter. Antral follicles, between 2 and 8 mm in diameter, were aspirated and COCs were recovered from the follicular fluid aspirates after sedimentation for 15 minutes. Only intact COCs with a compact and multilayered cumulus investiment were used in this study. In experiments requiring denuded oocytes, those COCs had their cumulus cells removed by vortexing. Subsequently, the oocytes were washed in HEPES buffered M199 with Earle salts and glutamine (Cat.# 20011-011; Gibco BRL, Paisley, UK) then in M199 with Earle salts and glutamine (Cat.# 31100-027; Gibco BRL) supplemented with 26.2 mM $NaHCO_3$ (defined as M199) and randomly allocated in groups of 35 per well in four-well culture plates (Nunc A/S, Roskilde, Denmark). Culture of the oocytes was carried out in 500 µl M199 per well, at 39°C in a humidified atmosphere of 5 % CO_2 in air, for 22h.

In Vitro Fertilization and Embryo Culture

Both in vitro fertilization (IVF) and in vitro embryo culture (IVC) took place at 39°C in a humidified atmosphere of 5% CO_2 in air. All experiments were carried out using frozen semen from the same batch of the same bull. Spermatozoa were thawed in a 37°C water bath for 1 min and washed in a discontinuous Percoll gradient prepared by adding 1 ml of 90% (v/v) Percoll under 1 ml of 40% (v/v) Percoll in a 15 ml centrifuge tube (Greiner Bio-One,

Frickenhausen, Germany). The semen samples were added on top of the Percoll gradient and centrifuged for 30 min at 27ºC at 700 x g. The pellet was resuspended in 70 µl modified Tyrode medium. Groups of 35 oocytes were washed twice in M199 and transferred to a well of four-well culture plates containing 430 µl of fertilization medium (Fert-Talp), as described by Parrish et al. (1988) and modified by Izadyar et al. (1996). Twenty microliters of sperm suspension (final concentration 0.5x 10^6 spermatozoa/ml) plus 20 µl of heparin (final concentration 1.8 IU/ml) (Sigma Chemical Co., St. Louis, MO, USA) and 20 µl PHE (20 µM D-penicillamine, 10 µM hypotaurine, 1 µM epinephrine) were added. After 20 h of incubation, groups of 35 presumptive zygotes were randomly placed in a coculture system of 500 µl M199 supplemented with 10% (v/v) fetal calf serum (FCS; Gibco BRL) on a monolayer of buffalo rat liver (BRL) cell in each well of four-well culture plates. At the fourth day of culture the non-cleaved embryos were removed. At the fourth and eight day of culture, embryos were transferred to fresh cocultures.

BRL-Cell Culture

Buffalo rat liver cells separated from the BRL cell line from the American Type Culture Collection (ATCC) were cultured routinely in a 1:1 mixture of Ham F12 medium (Gibco BRL) and Dulbecco modified Eagle medium (Gibco BRL) supplemented with 7.5% (v/v) FCS (Gibco BRL) and 0.1% (v/v) penicillin/streptomycin (Gibco BRL). These cells differ from those currently available from ATCC in that they exhibit contact inhibition of growth.

Assessment of Nuclear Maturation

After culture, the oocytes were fixed for 15 min in 2.5% (w/v) glutaraldehyde (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS), washed twice with PBS, stained with 0.285µM 4,6-diamino-2-phenylindole (DAPI; Sigma) in PBS and mounted on microscope slides (Mori et al.,1988). Evaluation of the nuclear status was done by epifluorescence microscopy (BH2-RFCA; Olympus, Tokyo, Japan). The oocytes were classified into four categories: 1) germinal vesicle (GV), oocytes with diffuse or slightly condensed chromatin; 2) metaphase I (MI), oocytes with clumped or strongly condensed chromatin that formed an irregular network of individual bivalents or a metaphase plate but no polar body; 3) metaphase II (MII), oocytes with either a polar body or two chromatin spots; and 4) aberrations, oocytes with an abnormal chromosomal organization, e.g., when chromosomes were disperse (not aligned along the metaphase plate) or when chromatin was persistent in the central region at the time of the late anaphase/telophase.

Assessment of Embryo Development

At Day 4 of culture (Day 0= day of fertilization), the total cleavage rate and the number of cleaved embryos consisting of eight or more cells were evaluated. The embryos were examined by morphology at Day 9 and the percentage of blastocysts and hatched blastocyst was expressed on basis of the number of oocytes at the onset of the culture.

TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay was used as a way of evaluating bovine blastocyst quality and performed using a commercial kit (In Situ Cell Death Detection Kit, Fluorescein; Roche, Mannheim, Germany) as described by Gavrieli et al. (1992). Day 9 embryos were collected and rinsed twice in PBS + 0.1% (w/v) polyvinyl alcohol (PBS-PVA), stained for 5 min with 0.004 mM ethidium homodimer-1 (EthD-1; Molecular Probes, Inc, Eugene, OR, USA) in PBS, then rinsed in PBS-PVA and fixed in 4% (w/v) paraformaldehyde in PBS, overnight. Embryos were rinsed twice in PBS-PVA, permeabilized for 5 min on ice in PBS + 0.1% (v/v) Triton X-100 (Sigma) and 0.1% (w/v) sodium citrate. Following washing in PBS-PVA, embryos were incubated in prewarmed (37°C) microdrops (30 µl drop per 5 embryos) of the TUNEL solution, partially covered with mineral oil (Sigma) for 1 h, at 37°C, under a humidified atmosphere in the dark. Subsequently, embryos were rinsed twice in PBS-BSA and stained for 10 min with 0.285 µM DAPI in PBS. Finally, embryos were rinsed twice in PBS-PVA, once in plain PBS, mounted on a microscope slide with antifade mounting medium (Vectashield-Vector Lab. Inc., Burlingame, CA, USA) and sealed under the coverslip with nail polish. Slides were evaluated under an epifluorescent microscope using filters at 500/580/525 nm.

Cells showing DNA-fragmented nuclei (TUNEL-positive staining) were termed apoptotic and cells with only plasma membrane damaged (ethidium-positive staining) were considered as dead. The total number of cells per embryo was determined by counting DAPI-positive nuclei. Apoptotic and dead cell indexes were calculated as the percentage of apoptotic or dead cells relative to the total number of cells, respectively. Only embryos showing at least one apoptotic or dead cell were included in the indexes.

Microtubules/Microfilaments Staining

All chemicals were purchased from Sigma Chemical Co. unless specified otherwise.

Staining of microtubules/microfilaments was performed as described by Tremoleda et al. (2001). Briefly, denuded oocytes were washed three times in PBS and permeabilized for 1 h at 39ºC with medium M (Simerly and Schatten,1993). Prior to the microtubule staining, oocytes were washed three times in PBS + 0.1% (w/v) BSA (PBS-BSA). Microtubules were then labeled by incubating fixed oocytes for 90 min at 37ºC in a 1:250 solution of a mouse monoclonal anti-α-tubulin antibody (catalog # T-5168 ; Sigma) in PBS-BSA + 0.01% (v/v) Triton-X (PBT). After this incubation, the oocytes were washed three times in PBT and then incubated at 37°C for 1 h in a blocking solution as described by Albertini et al. (1984). Next, the oocytes were exposed for 1 h at 37ºC to a goat anti-mouse antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC), diluted 1:250 in PBT. After washing twice in PBT and then twice more in PBS, the oocytes were incubated for 1 h at 37°C with Alexa Fluor 488 phalloidin (15 IU/ml; Molecular Probes) to enable detection of the microfilaments. Finally, the oocytes were washed twice in PBS-BSA and, to visualize DNA, oocytes were incubated with monomeric cyanine nucleic acid stain (TO-PRO3; 1 mM in PBS: Molecular Probes) for 15 min.

Spindles were classified as bipolar, having two intact poles equidistant from the metaphase plate, or multipolar, in which greater than two microtubule arrays were detected in a single oocyte (Mailhes et al.,1999).

Confocal Laser Scanning Microscopy

Oocytes were examined using a laser scanning confocal microscope (CLSC; Leica TCS MP; Leica, Heidelberg, Germany) mounted on an inverted microscope (Leica DM IRBE) equipped with 40 and 100x oil immersion objectives. The CLSM was equipped with a krypto-argon ion laser for simultaneous excitation of Alexa Fluor 488 (microfilaments), TRITC (microtubules) and TO-PRO3 (DNA) using 488/568/650-nm excitation/barrier filter combinations.

Extraction of Total RNA and Reverse Transcription

Cumulus cells were separated from the oocytes using a narrow-bore Pasteur pipette. Denuded oocytes and cumulus cells were washed four times in PBS, collected in Eppendorf tubes, and stored at -80°C until RNA extraction. Ten denuded oocytes or cumulus cells from 10 COCs were collected per tube. Five independent replicates of each tissue sample were made.

Isolation of total RNA combined with on-column DNAse digestion was performed using the RNeasy Mini Kit and the RNAse-free DNAse Set (Qiagen, Valencia, USA) according the manufacturer's instructions. Briefly, 300 µl lysis

buffer was added to the frozen samples and vortexed. The lysates were then diluted (1:1) with 70% ethanol and applied to the mini column. After binding of the RNA to the column, DNA digestion was performed for 15 min at room temperature with RNAse-free DNAse (340 Kunitz units/ml). After washing of the membrane-bound RNA three times, the RNA was eluted with 30 µl RNAse-free water.

Prior to the reverse transcription reaction, the RNA samples were incubated for 5 min at 70 °C, vortexed, and chilled on ice. Reverse transcription was done in a total volume of 20 µl containing 10 µl of the sample RNA, 4 µl 5X reverse transcriptase buffer, 8 U RNAsin (Promega, Leiden, The Netherlands), 150 U Superscript II reverse transcriptase (Invitrogen, Breda, The Netherlands), 0.036 U random primers (Invitrogen) and final concentrations of 10 mM DTT and 0.5 mM of each dNTP (Promega). The mixtures were incubated for 1 h at 42°C, for 5 min at 95°C, and stored at –20°C. Minus RT blanks were prepared under the same conditions but without reverse transcriptase.

Amplification of ERα and ERβ cDNA by PCR

PCR reactions were carried out in a total volume of 25 µl as described previously by Tremoleda et al. (2003). Amplification with ER-alpha specific primers was performed in two stages; for the first round of amplification the primers were ER-alpha L1 (5'-CATGATCAGGTCCACCTTC-3'; sense, GenBank Identification number 334, position 9-28) and ER-alpha R1 (5'-GTGATCTTGTCCAGGACTCG-3'; antisense, position 322-341). To increase the recovery and specificity of the final product, a second round of heminested PCR was performed using primer ER-alpha L2 (5'-GCCTGGCTAGAGATCCTGA-3'; sense, position 36-55) and ER-alpha R1. Amplification with ER-beta-specific primers was performed in one round with primers ER-beta L1 (5'-ATCCATTGCCAGCCGTCAG-3'; sense, GenBank identification number 27806640,position 102-121) and ER-beta R1 (5'-TGTCGGCCAGCTTGGTGAG-3'; antisense, position 724-743).

The thermal cycling profile for the first round was initial denaturation and activation of the polymerase for 15 minutes at 94°C, followed by 40 cycles of 15 sec at 94°C, 30 sec at 55°C and 45 sec at 72°C. Final extension was for 10 min at 72°C. For nesting, 1 µl of the first round product was transferred to 24 µl amplification mix and amplified for 30 cycles according to the same profile. A standard sequencing procedure (ABI PRISM 310 Genetic analyser; Applied Biosystems, Foster City, CA, USA) was used to verify the analytical specificity of the PCR products.

Estradiol Preparation

A 734μM estradiol (Sigma) stock solution was prepared in ethanol (Merck) and stored at -20ºC. Because membrane filters bind steroids, E2 was added aseptically from the stock solution after sterilization of the culture medium. On the day of the experiment, the stock solution was added to a Petri dish, the ethanol was evaporated, and only then M199 was added to the Petri dish. The dish was kept stirred for at least 5 h at 4°C. Then the medium was transferred to a four-well plate (500 μl per well) and put in the incubator (39°C in a humidified atmosphere of 5 % CO_2 in air) for at least 2 h before use. The final concentration of E2 was 3.67 μM and was determined by validated solid-phase ^{125}I RIA method (TKE: Diagnostic Products Corporation, Los Angeles, CA, USA) as described previously (Dieleman and Bevers,1987). Control media underwent the same treatment without E2.

Estradiol-BSA Conjugate Preparation

A 1 μM stock solution of estradiol-BSA conjugate (E2-BSA; Sigma) was prepared in M199 and stored at -20ºC. On the day of the experiment, the stock solution was diluted in M199 to a final concentration of 0.1 μM of E2-BSA (equivalent to 3.67 μM of free E2, since 1 molecule BSA is conjugated with 35 molecules E2). Because the final concentration of BSA in the maturation medium supplemented with E2-BSA was 0.1 μM, the control maturation medium was supplemented with BSA to obtain an equivalent concentration.

Experiment 1: Different Time Exposure of Estradiol During Oocyte Maturation

Transcription is practically absent in oocytes after GVBD (Tomek et al.,2002). Therefore, to examine whether the observed impairment on nuclear maturation in the presence of E2 during IVM (Beker et al.,2002) is a consequence of binding to the classical receptor (genomic), which regulates transcription, or it is via a transcription-independent manner (nongenomic), denuded oocytes were cultured in the presence of 3.67 μM estradiol at different time of exposure: a) during the whole culture period of 22h (E2: 0-22h), b) from 0 to 8h of the culture (E2: 0-8h, i.e., before GVBD), or c) from 8 to 22h of the culture (E2: 8-22h, i.e., after GVBD). After 8 h of culture, the oocytes were washed twice in M199 and then transferred to a new four-well dish containing the subsequent medium (according to the treatment). Media were prepared as described above. Cultures without E2 (during 22 h) served as controls. The percentage of oocytes at GV, MI, MII and the percentage of nuclear aberrations were assessed at the end of the culture. Three independent experiments were performed.

Experiment 2: Effect of Estradiol on Meiotic Spindle

Denuded oocytes were cultured in the presence of 3.67 µM estradiol in the conditions above described. Cultures without E2 served as controls. At the end of the culture, the oocytes were stained for microtubule, microfilament, and DNA and analysed by confocal laser microscopy as described previously. Three independent experiments were performed.

Experiment 3: Effect of Estradiol-BSA Conjugate on Nuclear Maturation of Bovine Oocytes

Estradiol conjugated with BSA cannot cross the plasma membrane and therefore cannot exert an effect via binding to the classical intracellular ER. However, it can bind to a receptor on the plasma membrane, if present, with specificity and affinity similar to the free steroid form (Zheng and Ramirez,1997). To examine how E2 could affect oocyte maturation, denuded oocytes were matured in the presence of a) 0.1 µM BSA, b) 0.1 µM E2-BSA conjugate, or c) 3.67 µM estradiol + 0.1 µM BSA under the conditions described above. Cultures without (free or conjugated) estradiol and BSA served as controls. The percentage of oocytes at GV, MI, MII and the percentage of nuclear aberrations were assessed at the end of the culture. Five independent experiments were performed.

Experiment 4: Estradiol-BSA Conjugate During IVM and Its Effect on Subsequent Embryo Development and Embryo Quality

To determine whether exposure of COCs to E2 during IVM has an effect on subsequent embryo development and embryo quality and if those effects are exerted via a plasma membrane receptor, COCs were matured in M199 in the presence of a) 0.1 µM BSA, b) 0.1 µM E2-BSA conjugate, or c) 3.67 µM estradiol under the conditions described above. Prior to in vitro fertilization, the COCs were denuded and only oocytes showing an extruded polar body (PB) were fertilized. Subsequently, the presumptive zygotes were cultured for 9 days. Cultures in only M199 served as controls. The percentage of cleaved zygotes, embryos of eight or more cells, blastocysts, and hatched blastocysts were assessed. In addition, TUNEL-staining was performed on the blastocysts at the end of the culture. Five independent experiments were performed.

Statistical Analysis

The number of oocytes per stage (GV, MI, MII and aberrations), of cleaved and embryos of ≥ 8 cells at Day 4 and of blastocysts at Day 9 were analysed by the chi-square test (SPSS for windows version 10.0.05). Cell

numbers of embryos were analyzed by ANOVA (SPSS). Apoptotic and dead cell indexes were analysed by logistic regression with overdispersion (Statistical software:R version 1.6.2).Differences were considered significant when $P< 0.05$.

RESULTS

Expression of mRNA for ERα and ERβ on Bovine Oocyte and Cumulus Cells

Amplification of cDNA from cumulus cells primed with specific primers for ERα resulted in all samples in a specific DNA product after two rounds of amplification, while amplification of cDNA from oocytes yielded no specific product (Fig. 1). PCR analysis also demonstrated the presence of a specific band with expected size for ERβ after one round of amplification in all five replicates generated from, respectively, oocytes and cumulus cells. Amplification of RT blanks yielded no specific products (Fig. 1).

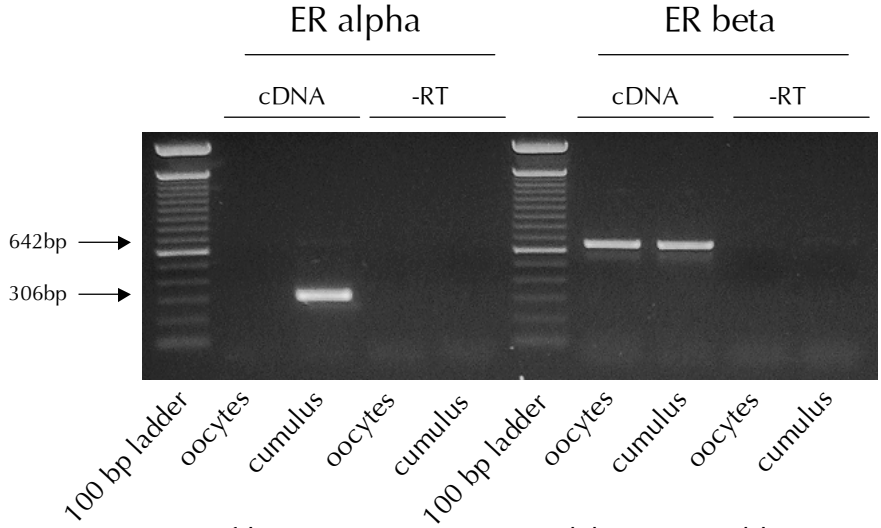

Fig. 1. Expression of bovine estrogen receptor alpha (ERα) and beta (ERβ) mRNA in oocytes and cumulus cells as detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Samples are indicated at the bottom and a 100bp DNA ladder is indicated as the marker for fragment size.

Therefore, our results demonstrate that bovine cumulus cells express both ERα and ERβ mRNA, but bovine oocytes express only ERβ mRNA. This different pattern of mRNA expression for ER might have implications on the mechanisms regulated by E2, because ERα and ERβ can have distinct biological functions (Couse and Korach,1999).

Effect of Estradiol on Nuclear Maturation Depends on Timing of Exposure

When COCs were cultured in the presence of E2 or E2-BSA during the whole period of culture (22 h), there was a significant decrease of the percentage of oocytes that reached MII stage, compared with control oocytes (Table 1). The low percentage of MII oocytes resulted from a significant higher percentage of MI oocytes and also from a significant higher percentage of aberrations compared with controls. However, when E2 was present only for the first 8 h of culture (Table 1; group 3) the decrease of the percentage of MII oocytes resulted from a high percentage of MI oocytes without a significant increase in the percentage of oocytes with nuclear aberrations. In addition, E2 present only after the first 8 h of culture (Table 1; group 4) also showed a significant decrease in the percentage of MII oocytes compared with control oocytes, but it was mostly caused by an increased percentage of aberrations compared with control oocytes. These results suggest that, although we cannot rule out that, during the first 8 h of culture, E2 can affect the in vitro maturation via a genomic pathway, we concluded that E2 is affecting nuclear maturation also in a nongenomic manner, since effects were observed after condensation of chromatin (GVBD), when the transcription system in oocytes is practically inactive.

Table 1. Effect of different periods of exposure to 17β-Estradiol (3.67 μM) on in vitro nuclear maturation of bovine denuded oocytes.

Group	n	Aberr (%)	GV (%)	MI (%)	MII (%)
1 (Control; 0-22h)	266	19 (7.1) [a]	6 (2.3) [a]	75 (28.2) [a]	166 (62.4) [a]
2 (E2; 0-22h)	248	32 (12.9) [b]	16 (6.5) [b]	123 (49.6) [b]	77 (31.0) [b]
3 (E2; 0-8h) [d]	323	15 (4.6) [a]	12 (3.7) [a,b]	174 (53.9) [b]	122 (37.8) [b]
4 (E2; 8-22h) [e]	307	42 (13.7) [b]	20 (6.5) [b]	103 (33.6) [a]	142 (46.2) [c]

[a, b, c] Values within column with different superscripts differ significantly ($P<0.05$).

[d] from 8-22 h, oocytes cultured in only M199.

[e] from 0-8 h, oocytes cultured in only M199.

Aberr = aberrations; GV = germinal vesicle; MI = metaphase I; MII = metaphase II

Effect of Estradiol on the Meiotic Spindle Organization

Control oocytes at the GV stage did not have a detectable microtubule network in the cytoplasm, and microfilaments were seen in the cortex where the vesicle was located (Fig.2A). During prometaphase stage, microtubules were

observed in association with each chromatin particle (Fig. 2B). MI spindles were barrel shaped and were often located at the periphery of the cell (not shown). During anaphase I and telophase I (Fig. 2, C-E), microtubules were found in the central part of the spindle and a microfilament furrow was observed between the two sets of chromatin (Fig. 2D, arrow), indicating a role of microfilaments in polar body extrusion. MII spindles were often located close to the polar body and slightly rotated compared with MI spindles, yet they maintained a typical barrel shape with chromosomes lined up on midplate (Fig. 2F).

However, when oocytes were matured in the presence of E2, spindle morphology was impaired in 35% of the oocytes (n=52). Multipolar spindles (Fig. 3, A-E,G) and the persistence of chromatin in the central region of late anaphase/telophase (Fig. 3F, arrows) were the principal forms of anomaly observed in E2-treated oocytes. Some oocytes showed multipolar spindles even when chromosomes were properly aligned on the metaphase plate (Fig. 3E). Occasionally, spindles were fragmented into several small pieces distributed throughout the cytoplasm (Fig. 3, H-I). These results demonstrate that presence of E2 during IVM has adverse effects on the spindle organization of bovine oocytes.

Estradiol-BSA Conjugate Does Not Affect Nuclear Maturation

Although (free) E2 + BSA (Table 2; group 4) induced a significant decrease of the percentage of MII oocytes and a significant increase of the percentage of GV, MI and aberrations compared with oocytes exposed to E2 in the conjugate form (Table 2; group3), there was no significant difference in the percentage of GV, MII and aberrations when bovine oocytes were cultured for 22 h in M199 supplemented with E2-BSA conjugate, compared with oocytes cultured in the presence of BSA. Even when a 10-fold higher concentration of E2-BSA (1µM) was used, there was no significant difference in the percentage of aberrations, GV, MI or MII compared with oocytes cultured in the presence of 10-fold concentrated BSA (control)(4.5, 4.5, 12.0, 79.0 and 3.1, 3.6, 14.7, 78.6%, respectively). These results indicate that the observed effects of E2 on in vitro nuclear maturation are not exerted via a putative membrane receptor, because no significant effect was observed when E2 was conjugated with BSA, which cannot cross the plasma membrane.

Table 2. Effect of estradiol (free or BSA conjugated) during IVM on nuclear maturation of bovine denuded oocytes.

Group	n	Aberr (%)	GV (%)	MI (%)	MII (%)
M199	445	20 (4.5) [a]	21 (4.7) [a]	107 (24.0) [a]	297 (66.8) [a]
BSA	588	30 (5.1) [a]	30 (5.1) [a]	161 (27.4) [a]	367 (62.4) [a,b]
E2-BSA Conj.	641	20 (3.1) [a]	34 (5.3) [a]	218 (34.0) [b]	369 (57.6) [b]
E2+BSA	336	29 (8.6) [b]	83 (24.7) [c]	177 (52.7) [c]	47 (14.0) [c]

[a, b] Values within column with different superscripts differ significantly ($P<0.05$); [c] ($P \leq 0.0001$).
Aberr = aberrations; GV = germinal vesicle; MI = metaphase I; MII = metaphase II

Estradiol-BSA Conjugate, During IVM, Does Not Affect Subsequent Embryo Development

When COCs were cultured in the presence of E2 or E2-BSA for 22 h, denuded and only oocytes with an extruded polar body were fertilized in vitro, the presence of E2 (free or conjugated) did not affect the subsequent embryo development in terms of cleavage rate, eight or more cells, and (hatched) blastocyst at D9 (Fig. 4), because there were no significant differences between groups for all of the parameters studied. This suggests that E2, either free or conjugated, does not affect oocyte cytoplasmic maturation.

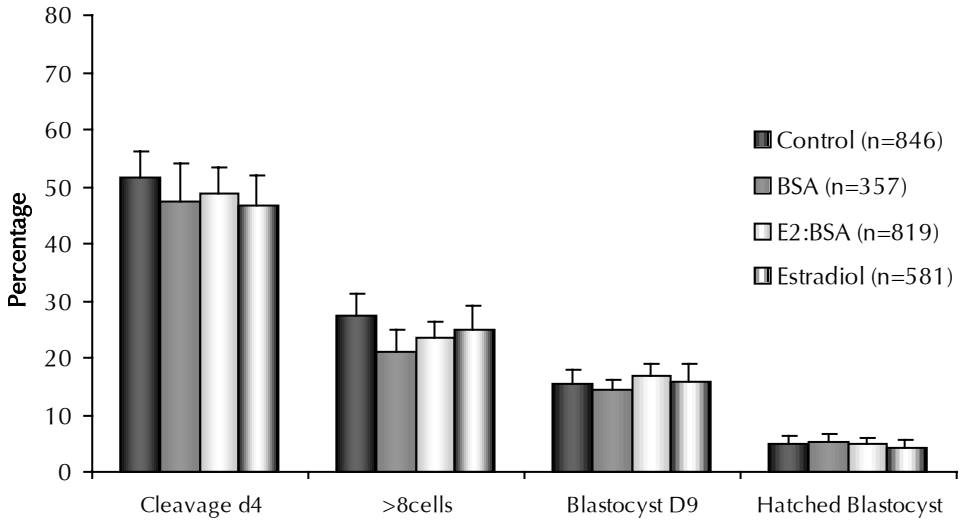

Fig. 4. Effect of estradiol (free or conjugated) during IVM on subsequent bovine embryo development of oocytes. (IVF was performed only with oocytes showing extruded polar body; Mean ± SEM)

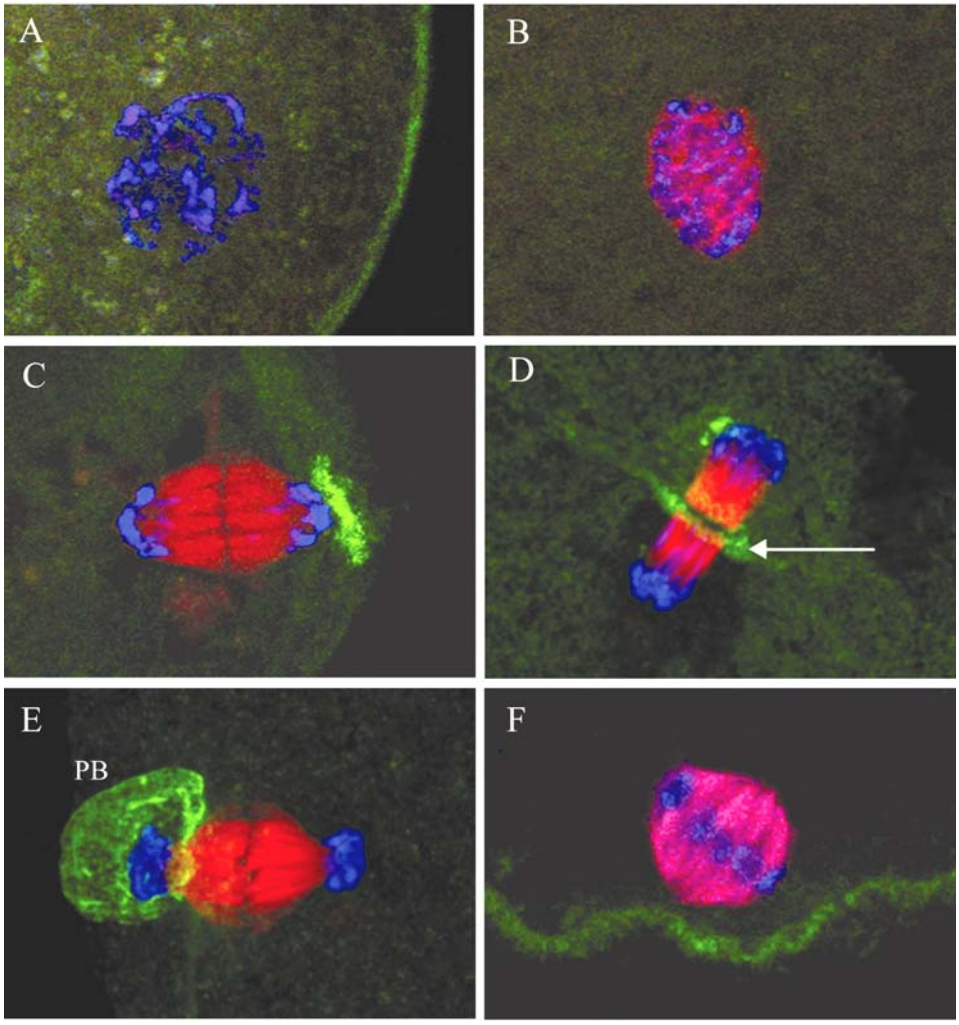

Fig. 2. Confocal laser scanning photomicrographs of normal organization of microtubules/microfilaments in bovine oocytes. Green: microfilaments; red: microtubules; blue: chromatin. A) At germinal vesicle stage, an organized microtubule-network was not detectable; B) At prometaphase I, microtubules were tightly associated with the condensed chromatin; C-E) At Late-Anaphase/Telophase I stage, microtubules were seen in the central part of the spindle. Note the microfilament furrow between the two separated set of chromosomes (arrow); F) At metaphase II stage, a barrel-shaped spindle with chromosomes aligned up on mid-plate. All fig's were taken at magnification of x1000.

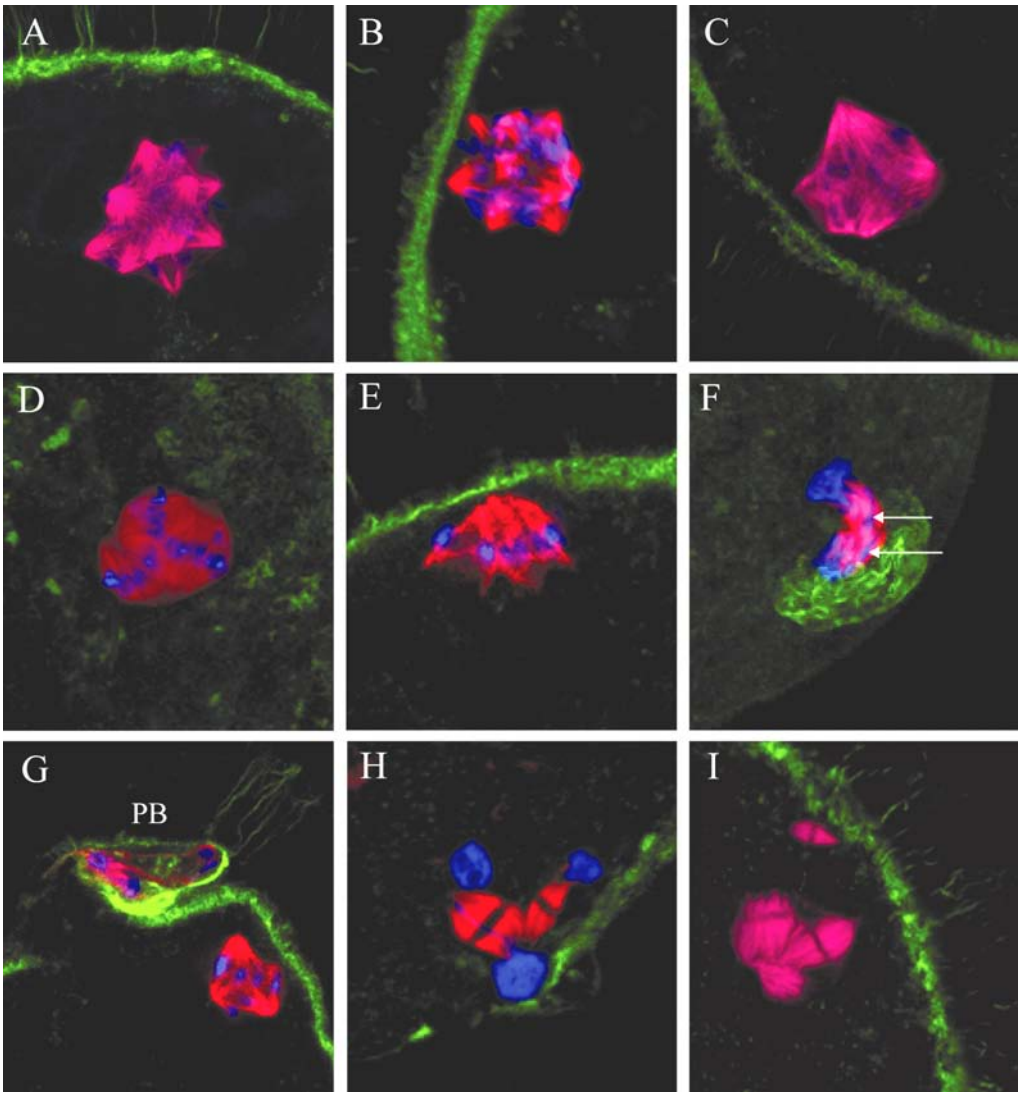

Fig. 3. Confocal laser scanning photomicrographs of the aberrant spindle configuration of bovine oocytes cultured in the presence of estradiol. Green: microfilaments; red: microtubules; blue: chromatin. A-E) Multipolar spindles in metaphase I stage oocytes; F) Late anaphase/telophase I with lagging chromatin between microtubule array (arrows); G) Multipolar spindle in metaphase II stage oocytes (PB: Polar Body); H-I) Spindle Fragmentation. Fig's A, B, C, D and E were taken at magnification of x800 and fig's F, G, H, I at x400.

Apoptotic and Dead Cell Indexes Were Not Increased by the Presence of Estradiol

At least one cell with DNA-fragmented nucleus and/or plasma membrane damage was detected in 94% of all the Day 9 embryos studied (n=141), and it was similar for all groups. There was no significant difference between different groups in the percentage of embryos showing DNA fragmentation (TUNEL positive) or membrane damage (dead cells). The number of cells per embryo was also not affected by any treatment. In addition, the average apoptotic cell and dead cell indexes were not different between groups (Table 3). These results indicate that exposure to E2 during IVM does not affect the embryo quality in terms of apoptotic/dead cells when only MII oocytes with an extruded polar body were fertilized.

Table 3. Effect of estradiol (free or BSA conjugated) during IVM, on the incidence of apoptosis and dead cells of Day 9 blastocyst originated from IVF of selected oocytes (only MII oocytes with an extruded polar body).

Group	Embryos evaluated (n)	Cells per embryo*	Apoptotic cell index*	Dead cell index*
M199	46	147 ± 9	2.2 ± 0.5	2.0 ± 0.5
BSA	32	154 ± 11	2.6 ± 0.7	1.7 ± 0.3
E2-BSA	39	134 ± 9	5.9 ± 2.1	2.1 ± 0.4
E2	24	131 ± 9	2.1 ± 0.9	3.2 ± 0.6

*Mean ± SEM

DISCUSSION

The present study demonstrates that the presence of estradiol in the culture media, either partially or during the whole period of IVM, had a negative effect on the in vitro nuclear maturation of bovine denuded oocytes. The pattern of this negative effect was dependent on the timing of exposure of the oocytes to E2. Exposure of bovine oocytes to E2 during the first 8 h of IVM (before GVBD) induced a block at MI stage for a high proportion of the oocytes without increasing the percentage of nuclear aberrations. Because transcription

still can occur during the first 8 h of culture (Tomek et al.,2002), a genomic effect of E2 during this period cannot be excluded. On the other hand, addition of E2 to the culture medium after the first 8 h of culture (after GVBD) causes a significant increase of the percentage of oocytes showing nuclear aberrations. The aberrations varied from multipolar spindles to metaphase plates showing outliners chromosomes, indicating damage to the meiotic spindle apparatus. This result, associated with the assumption that transcriptional nuclear activities are absent from 8 h after resumption of meiosis (after GVBD) (Tomek et al.,2002), indicates that the effect of E2 on the metaphase spindle is mediated nongenomically, possibly by a direct binding of E2 to the microtubules and not via a plasma membrane because E2-BSA conjugated failed to induce those aberrations. Various studies with 2-Methoxyestradiol (2MOE2), an endogenous metabolite of estradiol, demonstrated that 2MOE2 inhibits tubulin polymerization by direct binding at the colchicine site (D'Amato et al.,1994; Brueggemeier et al.,2001), suggesting that interactions of estrogens and/or estrogen metabolites with tubulin and microtubule assembly may play important epigenetic roles. In addition, Aizu-Yokota et al. (1995) demonstrated that the microtubule-disruptive effect of E2 is not associated with newly synthesized proteins and mRNA because cycloheximide and actinomycin D had no preventive action on the effect of E2. Furthermore, microtubule disruption can be induced by estradiol in estrogen receptor-negative human breast cancer cell lines, clearly indicating that estradiol can induce microtubule disruption independent of its binding to estrogens receptors (Aizu-Yokota et al.,1994).

Exposure of oocytes to E2-BSA conjugate during IVM affected neither the nuclear maturation of bovine oocytes (in terms of percentage of mature oocytes), the embryo development (in terms of the percentage of blastocysts), nor the embryo quality (in terms of cell number and apoptotic index). Our results are in agreement with Tesarik and Mendoza (1995), who, using human oocytes, also did not find significant differences in percentage of MII oocytes when E2-BSA conjugate was present during IVM compared with the control. In contrast with our results, however, an increase of cleavage rates was described after IVM in the presence of E2-BSA conjugate. Differences in results might be explained by the fact that, in our study, oocytes were used from 2- to 8-mm follicles, which did not include large (preovulatory) follicles, while Tesarik and Mendoza (1995) used GV oocytes originating from ovum-pick-up of gonadotrophin-stimulated women undergoing micromanipulated-assisted fertilization. In addition, differences between the species might be relevant for the observed differences.

Here we demonstrated the expression of both ERα and ERβ mRNA in cumulus cells. In addition, the present study is, to our knowledge, the first report about the expression of ERβ mRNA in bovine oocytes. Although ovaries express both ERα and ERβ mRNA, different expression patterns can occur within the heterogeneous cell types composing the tissue (see review Couse and Korach,1999). In rats, ERβ is preferentially localized in granulosa cells, whereas ERα is detectable in the surrounding thecal cells (Sar and Welsch,1999). In bovine, however, Schams and Berisha (2002) demonstrated the expression of ERα and ERβ mRNAs in both theca and granulosa cells. Because, in our present study, all maturation experiments were done using denuded oocytes, it is likely that all genomic effects of E2 on those oocytes were mediated via ERβ. However, in vivo, the importance of ERα mRNA expression in cumulus cells should not be underestimated. We previously demonstrated that the adverse effects of E2 on nuclear maturation of bovine in vitro-cultured oocytes were more pronounced in denuded oocytes compared with COCs, suggesting that cumulus cells could have a protective role (Beker et al.,2002). In addition, ERα and ERβ form both heterodimers and homodimers (Cowley et al.,1997) and these forms may interact differentially with response elements on genes, suggesting that ERα and ERβ may play different roles in gene regulation (Paech et al.,1997). Moreover, indication for certain distinct biological functions of the ER subtypes is presented by different phenotypes of ERα and ERβ knockout mice (Couse and Korach,1999).

In a previous study, nuclear maturation was impaired when COCs and denuded oocytes were cultured in the presence of E2, although E2 did not affect the cytoplasmic maturation in terms of blastocyst formation (Beker et al.,2002). In the present study, we extended this information by demonstrating that also embryo quality, in terms of apoptotic and dead cell indexes, was not affected by the presence of E2 during IVM when only oocytes showing extruded polar body were fertilized. The apoptotic cell index was relatively low for all treatment groups (range of 2-6%), which is in agreement with other studies (Byrne et al.,1999; Knijn et al.,2003). However VanSoom et al. (2002) reported a higher average of apoptotic cell index (16.5%) for Day 9 blastocysts. The differences in the level of apoptosis might be due to the different in vitro culture conditions. In our study, despite the low number of apoptotic cells per embryo, almost all blastocysts showed at least one cell with DNA-fragmentation and/or plasma membrane damage. Our results are in agreement with other studies which also showed some cell death in almost all in vitro-produced embryos (Byrne et al.,1999; Matwee et al.,2000; VanSoom et al.,2002). Not only in vitro but also in vivo embryos can show signs of apoptosis. Hardy (1997) reported over 80%

of mouse blastocysts freshly flushed from the uterus on Day 4 or 5 had one or more fragmented nuclei. Although apoptosis may result from suboptimal culture conditions or may be involved in the elimination of abnormal cells (see review Hardy,1999), the presence of cells in vivo with classic features of apoptosis (Gjorret et al.,2003) indicates a role for apoptosis in normal development.

In summary, the present study demonstrates that the pattern of the negative effect of E2 on in vitro nuclear maturation of bovine oocytes depends on the timing of exposure of the oocytes to E2. When E2 was present in the first 8 h of culture, a higher percentage of oocytes arrested at MI stage was the main feature observed. While, when oocytes were cultured in the presence of E2 only after the first 8 h of culture, the main effect observed was the increase of the percentage of nuclear aberrations. We also showed that E2 severely affects the meiotic spindle organization by increasing the percentage of multipolar spindles, possibly in a nongenomic manner. However, we did not find evidence for a membrane bound receptor, because E2 conjugate with BSA affected neither the oocyte nuclear maturation, the subsequent embryo development, nor embryo quality. In addition, we demonstrated that the presence of E2 during IVM neither affects cytoplasmic maturation in terms of blastocysts formation nor affects embryo quality in terms of apoptosis. Our results suggest that the negative effect of E2 on in vitro nuclear maturation of bovine oocytes is not exerted via a plasma membrane receptor.

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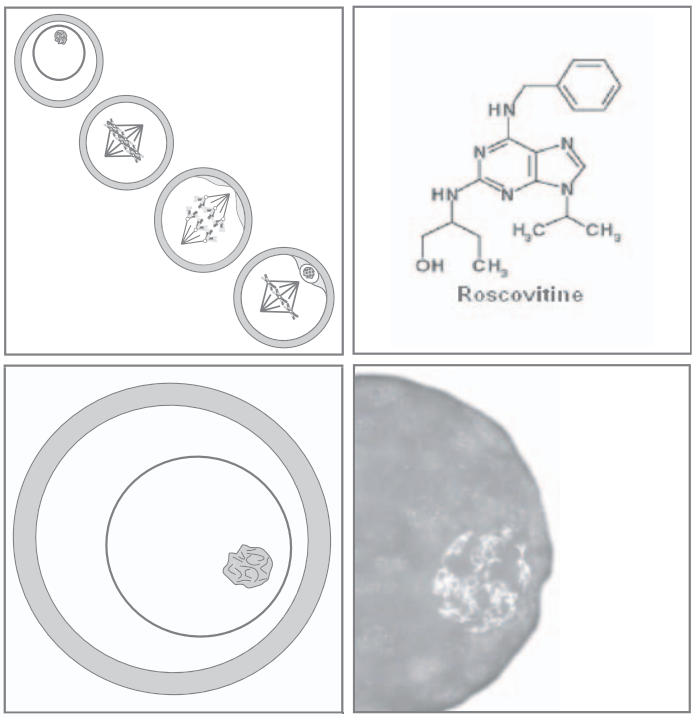
CHAPTER 5

DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES AFTER SPECIFIC INHIBITION OF MPF KINASE ACTIVITY: EFFECT OF ESTRADIOL SUPPLEMENTATION AND FOLLICLE SIZE



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(to be submitted)

ABSTRACT

The concentration of 17β-estradiol (E2) in the follicular fluid of the dominant follicle is high, indicating a possible role of E2 on the cytoplasmic maturation that occurs before the LH surge. The aim of this study was to investigate the role of E2 on the developmental competence of bovine oocytes originating from different sized follicles and temporarily maintained at the germinal vesicle stage with roscovitine (ROS).

We investigated the efficiency of ROS to inhibit germinal vesicle breakdown (GVBD) in oocytes harvested from small (3-4mm diameter) and medium (5-8mm) sized follicles. Oocytes were cultured in M199 with 0.05UI/ml FSH and with or without 25 μM ROS for 24 h. Nuclear stage was evaluated at the end of the culture. Next, the effect of E2 during temporary inhibition of GVBD by ROS (a prematuration period) on the subsequent nuclear maturation was evaluated. Oocytes from small and medium sized follicles were cultured in the presence of ROS, FSH and with or without 1 μg/ml E2 for 24 h. After this period, oocytes were cultured for another 24h in M199 supplemented with FSH but without ROS and E2. At the end of culture the nuclear stage was assessed. We also investigated the effect of E2 supplementation during prematuration period on the developmental competence of oocytes from different follicle sizes. Oocytes were cultured as described above and subsequently fertilized in vitro. The presumptive zygotes were cultured for 9 days. At Day 4, the number of cleaved and ≥ 8 cell embryos, and at Day 9 the number of blastocysts were assessed.

Oocytes from small and medium sized follicles cultured in the presence of ROS resulted in 95.2 and 90.5% of the oocytes arrested at germinal vesicle (GV), respectively. In the control group, 100% of the oocytes from both follicle size groups progressed beyond GV. The presence of E2 during prematuration did not affect the percentage of oocytes at GV, MI and MII stages in the group from small follicles (1, 5 and 93%, respectively) nor in the group from medium follicles (0, 2 and 98%, respectively) as compared with control groups. Supplementation of E2 during prematuration did not affect the subsequent percentage of cleaved, ≥8cell embryos and blastocysts developed from oocytes from small (63, 37, 10%, respectively) nor from medium sized follicles (88, 53, 21%, respectively), as compared with controls (66, 29, 7% and 86, 65, 23%, from small and medium sized follicles, respectively).

In conclusion, we demonstrate that exposure to E2, during temporary inhibition of the GVBD with ROS, affected neither nuclear nor cytoplasmic maturation of oocytes originating from small and medium sized follicles. It might be that in vivo, the increase of E2 during follicular growth is more related to selection of the dominant follicle than to the cytoplasmic maturation of the oocyte as such.

INTRODUCTION

In vivo, meiotically competent oocytes are maintained at the germinal vesicle (GV) stage by the follicular environment until the preovulatory LH surge. Apart from meiotic competence, the oocyte has to complete its cytoplasmic maturation and finally obtain the ability to be successfully fertilized and develop into a viable embryo (Marchal et al.,2002). This process, referred to as prematuration or capacitation, occurs from the time that a follicle is selected to become dominant until shortly before the LH surge (Hyttel et al.,1997). This period of prematuration is bypassed when oocytes are collected from small/medium sized antral follicles (about 3 to 8 mm diameter) and subsequently cultured in vitro. Although those oocytes are considered to be meiotically mature, i.e., able to resume meiosis, there is a large heterogeneity in the stages of cytoplasmic maturation (Hendriksen et al.,2000). Consequently, the blastocyst yield obtained with such oocytes is low compared to that obtained with in vivo matured oocytes. To mimic the in vivo prematuration period, it is hypothesized that chemically preventing germinal vesicle breakdown (GVBD) until the time of initiation of the in vitro maturation might allow the oocyte more time to undergo cytoplasmic maturation and consequently acquire a higher developmental competence.

In mammalian oocytes, germinal vesicle breakdown is regulated by the activation of maturation promoting factor (MPF), a member of the cyclin-dependent protein kinases involved in the regulation of G2/M cell cycle transition (for review see Whitaker,1996; Sirard et al.,1998). Prevention of MPF activation by nonspecific inhibition of protein synthesis with cycloheximide (Kastrop et al.,1991) or by inhibition of protein phosphorylation by 6-dimethylaminopurine (Avery et al.,1998) can artificially maintain meiotic arrest in bovine oocytes. Although these agents effectively inhibit MPF activation, their nonspecific mechanisms of action might be harmful for the subsequent developmental competence. Roscovitine (ROS) is a selective inhibitor of the cyclin-dependent kinases by competing for the ATP-binding domain on p34cdc2 (Meijer et al.,1997). With roscovitine GVBD can be prevented by specifically inhibiting MPF activation without suppressing protein synthesis, phosphorylation, or MPF accumulation, all of which are required to complete oocyte maturation (Wu et al.,2002).

Various studies demonstrated that oocytes derived from large follicles are developmentally more competent than those derived from smaller follicles following in vitro fertilization (Pavlok et al.,1992; Lonergan et al.,1994; Mermillod et al.,1999; Marchal et al.,2002). A higher percentage of pig oocytes

from medium (3-5mm) and larger (>5mm) follicles developed into blastocysts compared with those originating from small (<3mm) follicles (Marchal et al.,2002). In bovine, a blastocyst rate of 66% was reported with oocytes from >6 mm follicles, while a blastocyst rate of 34% was obtained with oocytes from 2-6 mm follicles (Lonergan et al.,1994).

Follicular fluid composition also changes during follicle growth. 17β-Estradiol (E2) concentrations in follicular fluid for instance, are higher in large follicles than in small follicles (Shailaja et al.,1985; Gastal et al.,1999; Belin et al.,2000). In bovine, just before the LH surge, the E2 level in the follicular fluid is high (about 1µg/ml) but it sharply decreases after the LH surge (Dieleman et al.,1983; Fortune and Hansel,1985). Although there is no evidence that E2 is involved in the resumption of meiosis (Racowsky and Baldwin,1989) it might be that E2 is involved in the cytoplasmic changes that occur before the LH surge.

The aim of this study was to investigate the role of E2, during temporary inhibition of GVBD by roscovitine, on the subsequent nuclear maturation and developmental competence of bovine oocytes originating from different sized follicles.

MATERIAL AND METHODS

Collection and Culture of Oocytes

Bovine ovaries, obtained at a local slaughterhouse, were transported to the laboratory in a thermoflask within 2h after slaughter. Cumulus oocyte complexes (COCs) were recovered separately from small (3-4mm) and medium (5-8mm) sized antral follicles. Only intact COCs with a compact and multilayered cumulus investiment were used in this study. Subsequently, the COCs were washed in HEPES buffered M199 (Gibco BRL, Paisley, UK) and randomly allocated in groups of 35 per well in 4-well culture plates (Nunc A/S, Roskilde, Denmark). Culture of the oocytes was carried out in 500 µl M199 per well supplemented with 26.2 mM $NaHCO_3$ (defined as M199) at 39°C in a humidified atmosphere of 5% CO_2 in air, for 22 h.

In Vitro Fertilization and Embryo Culture

Both in vitro fertilization (IVF) and in vitro embryo culture (IVC) took place at 39°C in a humidified atmosphere of 5% CO_2 in air. All experiments were carried out using frozen semen from the same bull. Spermatozoa were thawed in a 37°C water bath for 1 min and washed in a discontinuous percoll (Sigma Chemical Co., St. Louis, MO, USA) gradient prepared by adding 1 ml of

40% (v/v) percoll over 1 ml of 90% (v/v) percoll in a 15 ml centrifuge tube (Greiner Bio-One, Frickenhausen, Germany). The semen samples were added on top of the percoll gradient and centrifuged for 30 min at 27ºC at 700 x g. The pellet was resuspended in 70 µl modified Tyrode medium. Groups of 35 oocytes were washed twice in M199 and transferred to a well of 4-well culture plates containing 430 µl of fertilization medium (Fert-Talp), as described by Parrish et al. (1988) and modified by Izadyar et al. (1996). Twenty microliters of sperm suspension (final concentration 0.5x 10^6 spermatozoa/ml) plus 20 µl of heparin (final concentration 1.8 IU/ml) (Sigma) and 20 µl PHE (20 µM D-penicillamine, 10 µM hypotaurine, 1 µM epinephrine) were added. After 20 h of incubation, groups of 35 presumptive zygotes were randomly placed in a coculture system of 500 µl M199 supplemented with 10% (v/v) fetal calf serum (FCS; Gibco) on a monolayer of Buffalo Rat Liver (BRL) cells in each well of 4-well culture plates. At the fourth day of culture the noncleaved embryos were removed. At the fourth and eight day of culture embryos were transferred to fresh cocultures.

Assessment of Nuclear Maturation

After culture, the oocytes were fixed for 15 min in 2.5% (w/v) glutaraldehyde (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS), washed twice with PBS, stained with 0.285 µM 4,6-diamino-2-phenylindole (DAPI; Sigma) in PBS and mounted on microscope slides (Mori et al.,1988). Nuclear status was evaluated with epifluorescence microscopy (BH2-RFCA; Olympus, Tokyo, Japan). The oocytes were classified into 4 categories: 1) germinal vesicle (GV): oocytes with diffuse or slightly condensed chromatin; 2) metaphase I (MI): oocytes with clumped or strongly condensed chromatin that formed an irregular network of individual bivalents or a metaphase plate but no polar body; 3) metaphase II (MII): oocytes with either a polar body or 2 chromatin spots; and 4) aberrations: oocytes with an abnormal chromosomal organization.

Assessment of Embryo Development

At Day 4 of culture (Day 0= day of fertilization), the total cleavage rate and the number of cleaved embryos consisting of ≥ 8 cells were evaluated. The embryos were scored morphologically at Day 9 and the percentage of blastocysts was calculated based on the number of oocytes at the onset of the culture.

Estradiol and Roscovitine Preparation

All chemicals were purchased from Sigma unless specified otherwise. A 734 µM 17β-estradiol stock solution was prepared in ethanol (Merck). A 10 mM of 2-(1-Ethyl-2-hydroxyethylamino-6-benzylamino-9-isopropylpurine; roscovitine) stock solution was diluted in DMSO. Stock solutions were stored at -20ºC untill the day of the experiment, when they were diluted in M199 (Gibco). The final concentration of E2 was 3.67 µM and that of ROS was 25 µM.

Experiment 1: Effect of Roscovitine on the Inhibition of GVBD of Bovine Oocytes Harvested From Different Sized Follicles.

COCs recovered separately from small (3-4mm) and medium (5-8mm) sized antral follicles were cultured for 24 h in the presence of 25 µM ROS and recombinant human FSH (FSH; 0.05UI/ml; Organon, Oss, The Netherlands) under conditions as described above. Cultures without ROS served as controls. The percentages of oocytes at GV, MI, MII stages and nuclear aberrations were assessed at the end of culture. The experiment consisted of three independent replicates.

Experiment 2: Effect of the Presence of E2 During Prematuration of Bovine Oocytes Harvested From Different Sized Follicles on Subsequent In Vitro Nuclear Maturation.

COCs recovered separately from small and medium sized antral follicles were cultured for 24 h in the presence of ROS, E2 and FSH under conditions as described above. Cultures without E2 served as controls. The oocytes were then washed three times in M199 and cultured for another 24 h in M199 supplemented with FSH but without ROS and E2. The percentages of oocytes at GV, MI, MII stages and nuclear aberrations were assessed at the end of culture. The experiment consisted of four independent replicates.

Experiment 3: Developmental Competence of Oocytes, Obtained From Different Sized Follicles, Following a Prematuration Period in the Presence of E2.

COCs were cultured as described in Exp. 2. At the end of the culture, oocytes were co-incubated with sperm for 20 h as described above. Subsequently the presumptive zygotes were cultured for 9 days. The percentages of cleaved embryos, ≥8 cell embryos, blastocysts were assessed. Four independent replicates were performed.

Statistical Analysis

The number of oocytes per stage (GV, MI, MII and aberrations); of cleaved and ≥ 8 cell embryos at Day 4 and of blastocysts at Day 9 were analysed by the chi-square test (SPSS for Windows version 10.0.05). Differences were considered significant when $P < 0.05$.

RESULTS

Roscovitine Inhibits GVBD in Oocytes Originating From Small and Medium Sized Follicles

When cultured in vitro under standard conditions (control group), a great majority of the oocytes were meiotically mature at the end of the culture and no differences were observed in the percentages of oocytes that reached the MII stage between COCs obtained from small (3-4mm) or medium (5-8mm) sized follicles (Table 1). To delay nuclear maturation, COCs were matured in vitro in the presence of ROS for 24 h. Indeed, with ROS the percentage of oocytes that progressed to the MII stage was markedly decreased, with most of the oocytes remaining at the GV stage. Additionally, also in the presence of ROS there was no difference in the nuclear maturation between oocytes obtained from small and medium sized follicles (Table1).

Table 1. Effect of 25 μM roscovitine (ROS) on the in vitro nuclear maturation of bovine oocytes originated from small (3-4mm) and medium (5-8mm) sized follicles.

	n	GV n (%)	MI n (%)	MII n (%)
Control (3-4mm)	126	0 (0.0) [a]	8 (6.4)	118 (93.6) [a]
Control (5-8mm)	80	0 (0.0) [a]	3 (3.7)	77 (96.3) [a]
ROS (3-4mm)	206	196 (95.2) [b]	10 (4.8)	0 (0.0) [b]
ROS (5-8mm)	63	57 (90.5) [b]	5 (8.0)	1 (1.5) [b]

Within columns, superscripts a and b differ significantly ($P < 0.05$)

Estradiol During Prematuration Does Not Affect Further Nuclear Maturation

After exposure to ROS for 24 h (alone or in the presence of E2) and an additional 24 h of culture in M199, the majority of oocytes (≥90%) reached the MII stage in all groups, indicating the reversibility of the inhibitory effect of ROS (Fig. 1). The presence of E2 during prematuration did not affect the percentages of oocytes at GV, MI and MII stages in the group from small follicles (1, 5 and 93%, respectively) nor in the group from medium follicles (0, 2 and 98% respectively) as compared with oocytes cultured in medium without E2 (Fig. 1). Furthermore, when compared within the same meiotic stage, there were no significant differences in the percentages of oocytes originating from small sized follicles compared with of oocytes originating from medium sized follicles, independent of the presence of E2.

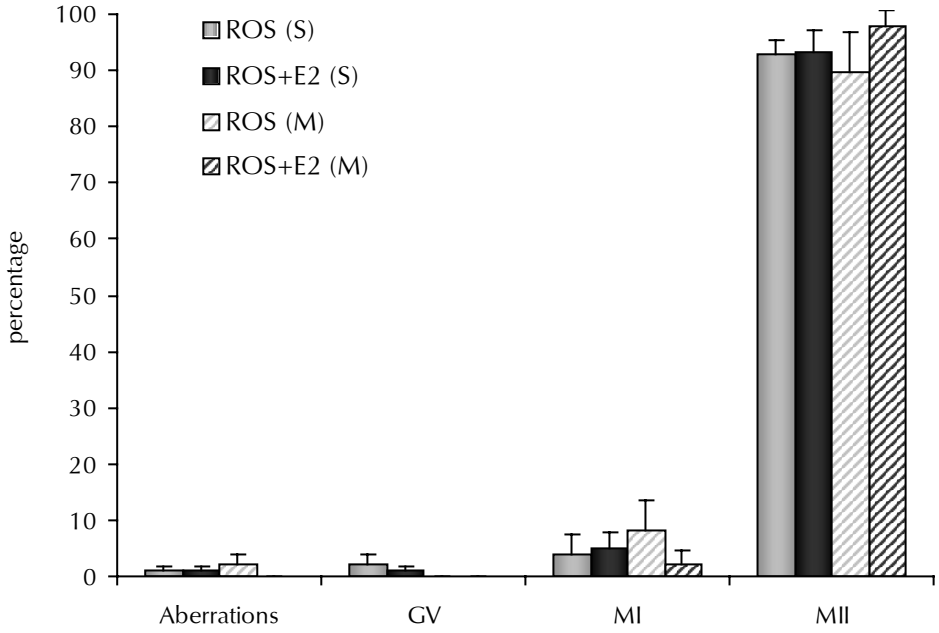

Fig. 1- Meiotic stages of bovine oocytes cultured for 24 h in the presence of roscovitine (ROS), FSH and with or without E2, followed by an additional culture of 24 h in permissive media (M199+FSH). Oocytes were obtained from small (3-4mm; S) and medium (5-8mm; M) sized follicles. Mean ± SEM.

Developmental Capacity of Oocytes is Not Affected by the Presence of Estradiol During Prematuration.

Although the meiotic maturation was similar between oocytes originated from small and medium sized follicles (Fig. 1), the cleavage rate, and percentages of oocytes forming ≥8 cell embryos at Day 4 and blastocysts at Day 9 were significantly higher for the oocytes originated from medium sized follicles (Fig. 2). Since the ability to develop to the blastocyst stage is an important indicator of the completion of cytoplasmic maturation (Eppig,1996) our results indicate that oocytes from the larger follicles have a better developmental capacity (cytoplasmic maturation) compared with oocytes originated from smaller follicles. However, the presence of E2 during prematuration did not affect the subsequent embryo development, in both follicle size groups studied (Fig. 2).

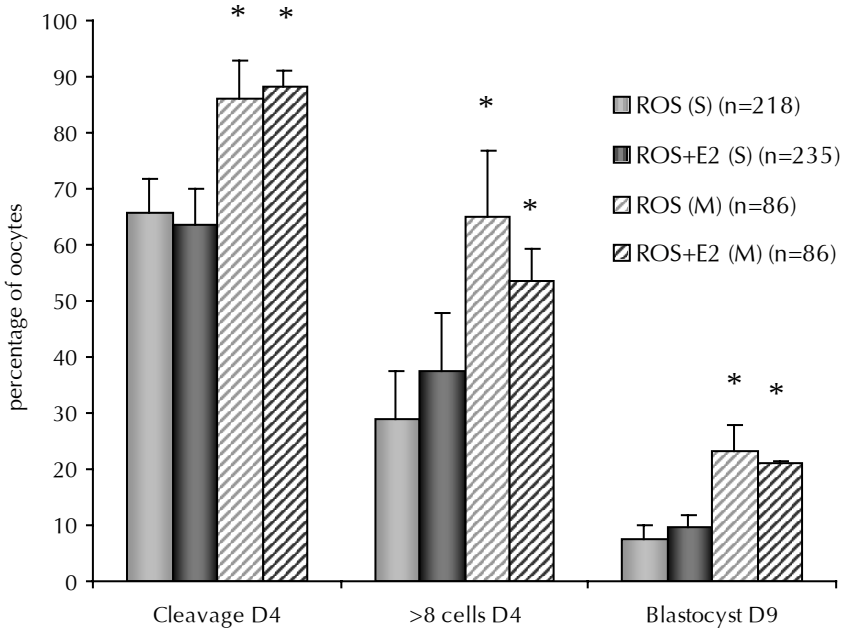

Fig. 2. Effect of follicle size and estradiol supplementation during prematuration on the subsequent embryo development. Oocytes originated from small (3-4mm; S) and medium (5-8 mm; M) sized follicles were cultured for 24 h in the presence of roscovitine (ROS), FSH and with or without E2, followed by an additional culture of 24h in a permissive media (M199+FSH) and subsequent IVF and IVC. Mean ± SEM

* = differ ($P < 0.05$) within the same developmental stage

DISCUSSION

In the present study, we demonstrated that the presence of estradiol during prematuration (using ROS to prevent GVBD) affected neither nuclear nor the cytoplasmic maturation of oocytes originated from small (3-4mm) and medium (5-8mm) sized follicles.

During the final growth of bovine follicles before the LH surge, the concentration of E2 in the follicular fluid increases (Dieleman et al.,1983; Fortune and Hansel,1985; Wise et al.,1986) and is higher in preovulatory than in atretic follicles (Carolan et al.,1996). Thus, we hypothesized that if E2 is present for a period before the resumption of meiosis, it may promote cytoplasmic maturation and consequently improve subsequent embryo development. Additionally, we expected that oocytes originating from small sized follicles, which still had not acquired complete developmental competence, would profit more from supplementation of E2 than oocytes originating from medium sized follicles. Surprisingly however, in both follicle-size groups studied, the presence of E2 during prematuration did not affect the further nuclear maturation of bovine oocytes, nor the subsequent embryo development. Saeki et al. (1997) using bovine oocytes from small/medium follicles (2-5mm), studied the effects of supplementation of FSH and E2 during prematuration, using cycloheximide to inhibit protein synthesis, on resumption of meiosis and developmental competence. They reported that E2+FSH enhanced development to the blastocyst stage compared to the respective control, although neither E2 nor FSH alone affected the blastocyst rate. In our study we did not observe this synergism between FSH and E2, since when oocytes were prematured in the presence of FSH alone (ROS+FSH) or supplemented with E2 (ROS+FSH+E2), the subsequent blastocyst formation rate was similar between both groups and in both follicle-size groups studied. Different protocols used for meiotic inhibition (cycloheximide versus ROS) and/or the absence of FCS in the culture media in our study may have caused the divergent results.

In the present study, ROS was able to maintain bovine oocytes at the GV stage during 24 h of culture. This inhibition was reversible, since the majority of oocytes reached the MII stage after further culture for 24 h in an inhibitor-free medium. This is in agreement with Mermillod et al. (2000), who demonstrated that 89% of the ROS-treated oocytes, originating from 2-6mm diameter follicles, reached the MII phase after a second 24 h culture in the absence of the inhibitor. Additionally, the concentration of roscovitine (25 μM) used in our study was sufficient to inhibit resumption of meiosis in the majority

of the oocytes. Dose-effect studies demonstrated that when bovine oocytes were cultured for 24 h in M199 supplemented with increasing concentrations of roscovitine (12.5, 25, 50 or 100 μM), the lowest concentration able to inhibit GVBD in more than 80% of the oocytes was 25 μM (Mermillod et al.,2000). Moreover, Lonergan et al. (2003) demonstrated by using electron microscopy that exposure of bovine oocytes to 125 μM roscovitine caused damage of the nuclear membrane, swelling of the mitochondria and degeneration of the cortical granules.

Estradiol during prematuration did not affect the percentage of meiotically matured oocytes, irrespective of the size of the follicle from which the oocyte was retrieved. In previous studies we demonstrated that E2 during IVM has detrimental effects on nuclear maturation and spindle organization, inducing high percentages of aberrations and multipolar spindles (Beker et al.,2002; Beker-Van Woudenberg et al.,2004). Interestingly, in the present study (where E2 was present during prematuration but absent during IVM) the occurrence of nuclear aberrations was very low and not significantly different from the control group. This indicates that the time frame during which E2 is present might be more important than the duration of the exposure to E2 as such.

Although GV oocytes express mRNA for estradiol receptor beta (ERβ) (Beker-Van Woudenberg et al.,2004), in the present study we clearly demonstrated that even if oocytes are artificially kept in the GV stage for 24 h, exposure to E2 during this period does not affect subsequent cytoplasmic maturation of those oocytes. In a previous study we also demonstrated that E2 present during a later time frame (during IVM) also did not affect cytoplasmic maturation (Beker-Van Woudenberg et al.,2004). Similar results have been reported for pigs (Dode and Graves,2003). In monovular species, one or two follicles become dominant in the final growth phase, leading to a marked increase in the ratio of estrogen:androgen of the dominant follicle(s). The remaining cohort of antral follicles stay androgen dominated and ultimately undergo atresia (Trounson et al.,2001). It might be that the increase of E2 during follicular growth is more related to selection of the dominant follicle than to the final maturation of the oocyte as such.

In conclusion, our results clearly demonstrate that exposure to estradiol during artificial inhibition of GVBD for 24 h did not affect the subsequent nuclear maturation nor the developmental competence of bovine oocytes originating from small and medium sized follicles.

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CHAPTER 6

SUMMARIZING DISCUSSION

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Maturation of oocytes is a fascinating but rather complex process. During this process, the oocyte needs to pass through extensive nuclear and cytoplasmic modifications, which require a harmonic coordination of numerous factors (such as: kinases, gonadotropins, steroids and growth factors) in a cascade of events involving different pathways. If all factors involved are present in the correct concentration and at the correct time and place, the oocyte maturation will culminate with an oocyte not only having a proper metaphase II but also having enough baggage to continue its voyage through fertilization and early embryo development. Although during the last decades a vast number of studies has been done and our understanding about the maturation process including in vitro maturation (IVM) has significantly improved, still many aspects need to be elucidated. The work presented in this thesis focuses on the fundamental aspects of the effect of estradiol, growth hormone releasing hormone (GHRH) and vasoactive intestinal peptide (VIP) on the in vitro maturation of bovine oocytes in order to enhance knowledge of the maturation process and the consequences for early embryo development. Additionally, we studied aspects of the mechanisms of action of estradiol and the influence of estradiol during the prematuration period on the developmental competence of bovine oocytes. This chapter summarizes the major findings and suggests prospects for future studies.

THE ROLE OF GHRH AND VIP ON BOVINE IVM

The presence of growth hormone (GH) during IVM accelerates the kinetics of meiosis, increases cumulus expansion (Izadyar et al.,1996) and improves cytoplasmic maturation in bovine oocytes (Izadyar et al.,1998). GH also stimulates in vitro nuclear maturation of oocytes in rat (Apa et al.,1994), rabbit (Yoshimura et al.,1993) and pig (Hagen and Graboski,1990). Furthermore, involvement of GH on early in vitro embryo development has been described in bovine (Izadyar et al.,2000) and pigs (Kidson et al.,2004).

Growth hormone releasing hormone (GHRH) is the major physiological stimulus of GH secretion from the pituitary (Frohman and Jansson,1986). However, the expression of GHRH has been described in various extraneural tissues such as gastrointestinal tract (Laburthe et al.,1983), placenta (Lacroix et al.,1996) , testis (Berry and Pescovitz,1988) and ovary (Bagnato et al.,1992) suggesting that GHRH may play a local regulatory role in several peripheral

tissues. In rat, the presence of GHRH stimulates in vitro oocyte maturation via a GH-mediated pathway (Apa et al.,1995). Therefore, we postulated in chapter 2 that GHRH also plays a role in the in vitro maturation of bovine oocytes. Additionally, the role of VIP, another member of the GHRH family that has a remarkable amino acid-sequence homology with GHRH (Gaylinn et al.,1993) was investigated.

Culture of bovine cumulus oocyte complexes (COCs) in the presence of GHRH or VIP did not affect nuclear maturation, based on the percentage of metaphase II stage (MII) oocytes at the end of the culture. In addition, supplementation of GHRH and VIP did not induce cumulus expansion (chapter 2). Similar results in ruminants have been described, for instance, addition of VIP to the culture medium did not improve in vitro maturation and fertilization of sheep (Ledda et al.,1996) and buffalo oocytes (Nandi et al.,2003). The absence of an effect of GHRH on nuclear maturation might be explained by the observation that bovine COCs do not express mRNA for GHRH receptor (Izadyar et al.,1999). On the other hand, cortical granule dispersion was retarded by the presence of GHRH and VIP during IVM (chapter 2). Since the receptors for GHRH, VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) are highly homologous (Nussdorfer and Malendowicz,1998; Sherwood et al.,2000; Vaudry et al.,2000) one cannot exclude that GHRH could exert its effect via the VIP or PACAP receptor. This is supported by studies in extra-pituitary tissues, which showed that GHRH can bind and exert its effects through VIP receptors, for instance in intestinal epithelium (Laburthe et al.,1983), pancreatic and liver cells (Robberecht et al.,1986), Leydig cells (Ciampani et al.,1992) and granulosa cells (Moretti et al.,1990). However, to our knowledge, the presence of VIP and PACAP receptor in the bovine oocyte has not been yet reported.

Although it is known that GHRH/VIP/PACAP receptors are G-protein-coupled receptors, the mechanism of those peptides involved in oocyte maturation is not clearly understood. When rat oocytes were cultured in the presence of GHRH and increasing concentrations of anti-GH antibody, the percentage of oocytes reaching germinal vesicle breakdown (GVBD) concomitantly decreased. From these results, it was concluded that in rats, GHRH stimulates oocyte maturation in a GH-dependent manner (Apa et al.,1995). In addition, it has been shown that the stimulatory effect of GH on rat oocyte maturation is dependent on IGF-I (Apa et al.,1994). However, in bovine, the effect of GH on in vitro maturation is not IGF-I dependent (Izadyar et al.,1997) and is not regulated by GHRH, since mRNA for GHRH was not expressed in bovine COCs. A considerable number of studies support the

concept that cAMP is of central importance for the regulation of meiosis and that increased levels of cAMP in the oocyte inhibit maturation, while an increase of the concentration of cAMP in granulosa cells sends a positive signal that activates the oocyte, triggering GVBD (for review see Conti et al.,2002). In rat, VIP but not GHRH was able to increase cAMP levels in granulosa cells (Apa et al.,1995). However, in our study neither VIP nor GHRH affected cumulus expansion, which is also mediated via a cAMP dependent signal transduction pathway. The mechanism of GHRH and VIP action might differ between species. Moreover, it is possible that cAMP is not involved in the signaling pathway for VIP in bovine granulosa cells. Depending on the tissues, other signaling pathway downstream of cAMP or independent of cAMP have been reported to be associated with VIP/PACAP receptor activation (Laburthe and Couvineau,2002).

VIP has been described as an important regulator of growth in early postimplantation mice embryos (Spong et al.,1999) and acts as a potent glial mitogen and neuron survival factor in vitro (Waschek et al.,1996). Ovine expanded blastocysts cultured in the presence of VIP exhibited an increased cell number, diameter and hatching rate (Leoni et al.,2000). Furthermore, immunoneutralization of exogenous VIP in culture with anti-VIP antibody caused a decrease in the blastocyst hatching rate. PACAP was demonstrated to suppress apoptotic DNA fragmentation, for instance in preovulatory follicles (Lee et al.,1999) and rat cerebellar cells (Vaudry et al.,1998). Taking those aspects together it might be that the presence of VIP/PACAP/GHRH during in vitro bovine embryo culture would increase the rate and/or quality of blastocyst development. Therefore, it would be interesting to study the role of GHRH, VIP and PACAP during different periods of bovine (in vitro) embryo development, as well as the presence of receptors for VIP and PACAP in the bovine oocyte and cumulus cells; and for GHRH/VIP/PACAP in bovine early embryo.

ESTRADIOL DURING IVM: PROS AND CONS

Bovine oocytes within the ovaries are exposed to a steroid-rich environment, especially during the final follicular growth. Up to the preovulatory gonadotropin surge, the estrogen concentration in the follicular fluid increases and then sharply declines whereas the concentration of progesterone in follicular fluid increases (Dieleman et al.,1983; Fortune and Hansel,1985; Komar et al.,2001). The physiological relationship between this

change in the steroidal content of follicular fluid and resumption of meiosis of the oocyte is a matter of debate.

A lower percentage of oocytes reached the MII stage when cultured in the presence of estradiol, as compared to oocytes cultured without estradiol. Additionally we observed a significant increase in the occurrence of nuclear aberrations when estradiol was present during IVM **(chapter 3).** Complete meiotic maturation includes the extrusion of the first polar body (PB) which requires, apart from other events, proper meiotic spindle formation (Albertini et al.,1993). Therefore, selecting oocytes with an extruded PB before in vitro fertilization (IVF) will, besides selection of only nuclear matured oocytes (MII), result in exclusion of the majority of nuclear aberrations that originate from spindle malformations. In **chapter 3** we demonstrated that embryo development was reduced when oocytes were matured in the presence of estradiol, but this negative effect of estradiol on blastocyst formation was not observed when only oocytes showing an extruded PB were selected. Thus, we concluded that the detrimental effects of estradiol during IVM on blastocyst formation probably resulted mainly from the occurrence of nuclear aberrations, which can be responsible for the arrest of subsequent embryo development, rather than from an effect of estradiol on cytoplasmic maturation. This suggestion is further supported by the results described in **chapter 4**, where we observed that estradiol, during IVM, affected neither the incidence of apoptosis and cell death, nor the total cell number of embryos. Although estradiol did not affect cytoplasmic maturation, we strongly suggest omission of estradiol in routine IVM protocols of bovine oocyte since estradiol has a potent detrimental effect on spindle organization, which consequently reduces blastocyst yield.

Oocytes that originate from follicles ≥ 3mm are able to resume meiosis, although there is a large heterogeneity in the stages of cytoplasmic maturation (Hendriksen et al.,2000). It was hypothesized that preventing GVBD until the time of initiation of the in vitro maturation might allow the oocyte more time to undergo cytoplasmic maturation and consequently acquire a better developmental competence. As described previously, in vivo, the concentration of estradiol in the follicular fluid increases during follicular growth. It is therefore possible that estradiol is involved in the cytoplasmic changes occurring in the oocyte during the preovulatory period before the LH surge, i.e., during prematuration. We also hypothesized that oocytes originating from small sized follicles, which still had not acquired complete developmental competence, would profit more from estradiol supplementation than oocytes originating from medium sized follicles. Therefore we cultured oocytes originating from two follicle size groups (small: 3-4mm and medium: 5-8mm

diameter) in the presence of roscovitine, which prevents GVBD, and estradiol during 24 h. Subsequently, the oocytes were cultured for an extra 24 h without estradiol and roscovitine. Additionally, we examined the effect of this culture system on the subsequent blastocyst formation **(chapter 5).** We observed that the developmental competence of oocytes obtained from different sized follicles was not affected by the presence of estradiol during prematuration. It might be that, in vivo, the increasing concentrations of estradiol in the follicular fluid during follicle growth are more associated with follicular growth and selection than with the development of competence of the oocyte within the follicle. Estradiol is known to play an essential role in the process of selection of the dominant follicle via inhibition of FSH secretion of the pituitary. The intrafollicular concentration of estradiol is a reliable biochemical marker for predicting which follicle within the growing cohort of similar-sized follicles would become dominant (Mihm et al.,2000). Indeed, the loss of capacity to produce estradiol is one of the first events distinguishing the future subordinate follicle from the future dominant follicle and it was found to precede cessation of follicular growth and the increase of granulosa cell apoptosis (Hendriksen et al.,2003). This is supported by a recent study in which bovine cDNA microarray and quantitative real-time PCR techniques were applied. High intrafollicular concentrations of estradiol in growing dominant follicles were found to be positively associated with enhanced expression of mRNAs in granulosa cells for aromatase, LH receptor and estradiol receptor β (ERβ). In contrast, the relatively low intrafollicular concentration of estradiol in growing subordinate follicles was positively associated with enhanced expression in granulosa cells of mRNA for genes associated with cell death and/or apoptosis, such as COX-1, TNFα and caspase 13 (Evans et al.,2004). Furthermore, the use of mice lacking genes for aromatase (ArKO), thus incapable of synthesizing endogenous estrogen, has been an important tool to explore the role of estrogen in folliculogenesis. Recently, Huynh et al. (2003) using young ArKO female mice demonstrated that following a standard superovulation protocol, ArKO oocytes did not ovulate. However, when recovered manually from the ovary, ArKO oocytes successfully progressed through in vitro maturation, fertilization and development to the blastocyst stage, at a similar rate as those obtained from wildtype littermates. Taken those observations together, one might conclude that estrogen is not essential to maintain developmental competence of the oocyte but is required for continued follicle growth and feedback regulation of ovulation.

MULTIFACETED MECHANISMS OF ESTRADIOL ACTION ON OOCYTE MATURATION

In **chapter 3** we observed that estradiol during IVM affects the rate of bovine oocyte maturation, both in COCs and denuded oocytes (DOs). However, the detrimental effects on nuclear maturation were much more pronounced in DOs, indicating that estradiol can acts directly on the oocyte and not necessarily via the cumulus cells. Nevertheless, cumulus cells might play an important role in this process. In **chapter 3,** we demonstrated that COCs cultured in the presence of estradiol and FSH showed a higher percentage of MII stage oocytes when compared with COCs cultured in the presence of only estradiol. This might be due to a higher uptake of estradiol by the cumulus cells in the presence of FSH, in this way preventing estradiol to reach the oocyte, and/or by a counteracting signaling system activated by FSH-stimulated cumulus cells. Additionally, in **chapter 4** we demonstrated that oocytes express mRNA only for ERβ, while cumulus cells express mRNA for both ERα and ERβ. It is known that ERα and ERβ can form both homodimers and heterodimers (Cowley et al.,1997). These dimers may interact differentially with response elements on genes thereby having different roles in gene regulation (Paech et al.,1997). Therefore, one can assume that the different patterns of expression for the two estrogen receptors in the oocyte and in the cumulus cells play an important role in the signaling regulation of estradiol during IVM. For instance, it might be that ERβ is responsible for negative signaling by suppressing the production of proteins important for the resumption of meiosis and/or completion of oocyte maturation. This would explain the reduced percentage of DOs reaching the MII stage observed in our study. On the other hand, when culture is performed with COCs, a possible positive signaling of the ERα present in the cumulus cells could counteract the negative signaling of the ERβ. Indeed, it has been proposed that in cells that expresses both receptors, the overall estrogen responsiveness may be determined by the ERα: ERβ ratio (Hall and McDonnell,1999). This might also be the case in bovine cumulus cells, although levels of expression of estrogen receptors in these cells are not known. Another possible mechanism is that binding of estradiol to ERβ in the oocyte triggers a different response than binding of estradiol to ERβ present in the cumulus cells. The expression of different levels and/or types of co-activators and co-repressors in the oocyte and cumulus cells may play a role in this mechanism. In summary, the physiological responses to steroids which involve direct transcriptional regulation of many genes, mediated via the so-called genomic pathway, is very complex and is determined by the structure of the

ligand, the ER subtype involved, the nature of the hormone-responsive gene promoter and the character and balance of coactivators and corepressors that modulate the cellular response to the ER-ligand complex (Katzenellenbogen et al.,2000; Castro-Rivera and Safe,2003).

In addition to the genomic pathway, indications for nongenomic effects exerted by steroids have been described in a wide variety of cell types (Revelli et al.,1998; Falkenstein et al.,2000; Levin,2002) . Involvement of nongenomic steroid pathways in oocytes was first described in amphibians and fish. For example in *Xenopus*, progesterone binding to a plasma membrane-receptor triggers oocyte maturation (Lutz et al.,2000). Although the identification of a putative plasma membrane-bound steroid receptor has been the subject of intense research for decades, only recently the cloning of a membrane progestin receptor has been described (Zhu et al.,2003). However, to our knowledge, the exact molecular characterization of a putative membrane-bound estrogen receptor has not been reported to date.

In **chapter 4** we designed a series of experiments to elucidate aspects of the mechanism of estradiol action on the oocyte IVM. Germinal vesicle breakdown occurs around 6-8 h after the beginning of in vitro maturation of bovine oocytes (Motlik et al.,1978; Khatir et al.,1998). Transcription can occur before GVBD, but it strongly declines after GVBD (Tomek et al.,2002). Therefore, possible effects of estradiol present only after GVBD are likely to be exerted via a nongenomic pathway. With this rationale, we investigated the effect of exposure of oocytes to estradiol either before or after the 8^{th} hour of culture (i.e. before or after GVBD). Another aspect investigated in **chapter 4** was the presence of a putative estradiol plasma membrane receptor. For this purpose, we cultured oocytes in the presence of estradiol conjugated with a high molecular weight protein (E2-BSA), which does not cross the plasma membrane. Using those strategies, we concluded that the detrimental effects of estradiol during IVM (decrease in the percentage of MII stage oocytes and increase in the percentage of oocytes showing nuclear aberrations) are not exerted via a plasma membrane-bound receptor, since no effects on nuclear maturation were observed when conjugate E2-BSA was used. In addition, the presence of estradiol in the media after 8 h of culture (when transcription is practically absent) did decrease the percentage of mature oocytes, mainly by an increased incidence of nuclear aberrations, suggesting a nongenomic effect. Moreover, nongenomic effects of estradiol other than via the plasma membrane can occur, for instance via direct binding to tubulin and consequently influencing the formation of the meiotic spindle. Studies with endogenous metabolites of estradiol, demonstrating that those metabolites inhibit tubulin

polymerization by direct binding at the colchicine site, support this idea (D'Amato et al.,1994; Brueggemeier et al.,2001).

ESTRADIOL AND MEIOTIC SPINDLE (DIS)ORGANIZATION

The resumption of meiosis in mammalian oocytes is a unique and complex process that involves germinal vesicle breakdown, chromosomal condensation, polar body extrusion and formation of metaphase structures (see chapter 1). These structural changes are associated with changes in the organization of microtubules and microfilaments during specific phases of the cell cycle. In chapter 4 we observed that when estradiol is present in the culture media on the last 2/3 of the in vitro maturation period (when meiotic spindle formation occurs) estradiol had a detrimental effect on the spindle organization, with a high percentage of the oocytes exhibiting multipolar spindles. Additionally, when estradiol was present only during the GV stage (first 8 h of culture and before spindle formation) no significant increase in the occurrence of nuclear aberrations was observed. Moreover, when estradiol was present in a period preceding the final maturation (during prematuration), but was absent during IVM, nuclear aberrations were not observed (chapter 5). This strongly suggests that the time frame at which estradiol is present is very important and might be more important than the duration of the exposure itself. In vivo, the physiological decrease in the levels of estradiol in the follicular fluid just after the LH peak (the time at which the oocyte resumes meiosis) could occur to prevent exposure of the oocyte to high concentrations of estradiol during final maturation, as that is deleterious for the formation of the spindle.

Further investigation on the mechanisms involved in the disorganization of the spindle by estradiol is of great importance since the incidence of misaligned chromosomes and a disorganized spindle in the presence of estradiol is not restricted to the oocyte. These features have been also described in somatic cells such as fibroblasts (Ochi,1999), Chinese hamster cells (Wheeler et al.,1987) and human breast cancer cell lines (Aizu-Yokota et al.,1994). The multipolar spindles are related to cell death, cell differentiation and malignant transformation via induction of an unequal chromosome distribution (aneuploidy) (Ochi,1999). A number of cancers in humans and rodents including breast cancer, endometrial cancer, ovarian cancer and liver cancer, is associated with exposures to estrogens (Barrett,1995) and the frequent occurrence of multipolar spindles in the presence of estradiol might be one of the mechanisms of the complex process of carcinogenesis in those tissues.

The technique of triple staining (for microtubule, microfilament and chromatin) of the oocyte, together with the use of confocal laser scanning microscopy (CLSM) used in this thesis offers an excellent opportunity to evaluate in detail the organization of the spindle, but has the disadvantage that it can only be done in fixed oocytes. Recently, new techniques of live imaging of oocytes by computer-assisted polarizing microscopy (PolScope; Liu et al.,2000) and by second harmonic generation microscopy (SHG; Mohler et al.,2003) permits the noninvasive analyses of spindle morphology, although less detailed compared with indirect immunofluorescence. Additionally, the development of green fluorescent protein (GFP) fusion proteins has added new dimensions to the capabilities of imaging live cells. This novel technique together with video-time-lapsed or multiphoton microscopy composes a promising protocol for evaluation of the bovine oocyte cytoskeleton.

ESTROGEN-LIKE COMPOUNDS IN THE ENVIRONMENT

The possible exposure of humans and animals to many industrial chemicals and pesticides has been a growing concern over the last decade for both the scientific community and the general public (Pocar et al.,2003). Environmental contaminants contain many estrogen-like substances which are considered as endocrine-disruptors, for example diethylstilbestrol (DES), pesticides such as dichlorodiphenyltrichloroethane (DDT), fertilizers such as polychlorinated biphenyls (PCBs) and plasticizers such as Bisphenol-A (McLachlan,2001; Alonso and Rosenfield,2002). Many of these compounds have the potential to compromise the health, welfare and productivity of domestic animals and, through accumulation in meat and milk, human health (Rhind,2002). Several studies have suggested that environmental estrogens could adversely affect reproductive function in a variety of vertebrates, including humans (for review see McLachlan,2001; Pocar et al.,2003). We demonstrated in **chapters 3 and 4** that the presence of estradiol during IVM increases the occurrence of nuclear aberration in bovine oocytes and in **chapter 4** we demonstrated that those nuclear aberrations were the result of disruption of a proper spindle organization. As described previously, even endogenous metabolites of estradiol, such as 2-Methoxyestradiol (2-MeOE2) and 2-Methoxymethylestradiol (2-MeOMeE2) can inhibit tubulin polymerization in vitro (D'Amato et al.,1994; Aizu-Yokota et al.,1995; Brueggemeier et al.,2001). Recently, Lattanzi et al. (2003) described marked alterations in the spindle assembly of bovine oocytes exposed to 2-MeOE2 during maturation, leading to

chromosomal aberrations after fertilization and developmental arrest at the morula stage. Similar results were obtained using PCBs during IVM (Pocar et al.,2001). Of the environmental estrogen contaminants, DES and Bisphenol are recognized as microtubule disruptive and aneugenic agents (Ochi,1999; Hunt et al.,2003). Therefore, it can be expected that exposure of women to high concentrations of environmental estrogens affecting microtubule polymerization will disturb spindle formation and chromosome segregation and pose a risk for errors in chromosome segregation at anaphase I and II stage, independent of maternal age (Eichenlaub-Ritter,2003). This could have serious consequences for the conceptus like trisomy or aneuploidy, which depending of the chromosome affected can result in abnormal development or fetal death. Female emancipation with increased participation in the labour force plays an important role in the delay in childbearing, especially in developed countries. However, it is known that the occurrence of aneuploidy increases dramatically with maternal age and is a major factor of reduced fertility in women (Eichenlaub-Ritter,2003). Battaglia et al. (1996) demonstrated that the meiotic spindle in older women (aged 40-45 years) was frequently abnormal with respect to chromosome alignment and the microtubule matrix, compared with the oocyte meiotic spindle of younger (aged 20-25 years) women. It was also suggested that the regulatory mechanisms responsible for the assembly of meiotic spindles are significantly altered in older women, leading to the high prevalence of aneuploidy. Changes in the endocrine environment, such as those that occur in the female in the decade preceding menopause, may influence meiotic chromosome behavior, creating age-related increase in aneuploidy occurrence (Hodges et al.,2002).

Another concern involving estrogenic effects on reproductive function is the worldwide contamination of foods and feeds with mycotoxins, secondary metabolites of molds (Hussein and Brasel,2001). It is estimated that 25% of the world's crops may be contaminated with mycotoxins (Fink-Gremmels,1999). Between the wide mycotoxins family, a specific group of zearalenone (ZEN), and its alcohol metabolites α zearalenol and β zearalenol, are brought into attention because of their estrogenic effects (Cheeke,1998). Decreased fertility, increased embryolethal resorptions, reduced litter size, teratogenic effects, and change in serum levels of progesterone and estradiol are some of the described estrogenic effects of ZEN in farm or laboratory animals. Although ruminants are apparently less sensitive than monogastrics, ZEN has been suggested as a causative agent of infertility, reduced milk production, and hyperestrogenism in cattle (Gajecki,2002). Accumulation of mycotoxins in animal tissues may cause indirect exposure to humans consuming products of animal origin (Trucksess et

al.,1983). In humans, Withanage et al. (2001) demonstrated the potential for ZEN to stimulate growth of human breast cancer cells containing estrogen receptor.

Since the availability of human oocytes for research is limited, in vitro maturation of bovine oocytes originated from slaughterhouse material is a valuable alternative to study the aneugenic potential of environmental contaminants, such as estrogen-like compounds, and their effects on fertility.

AND FINALLY….

"All living things come from eggs", was stated by William Harvey almost four hundred years ago….since then, we have made some relevant progress on the understanding of how the intriguing process of reproduction works. Although each day new discoveries are made….. to comprehend all fundamental aspects of oocyte maturation….certainly will demand another few hundred years.

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SAMENVATTING

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Rijping van eicellen, ook wel maturatie genoemd, is een fascinerend maar tevens complex proces. Gedurende de rijping ondergaat de eicel uitgebreide veranderingen van zowel het cytoplasma als de kern. Dit vereist een goede coördinatie van vele factoren zoals kinases, gonadotropine hormonen, steroïden en groeifactoren, in een cascade van gebeurtenissen waarbij verschillende signaaltransductiepaden betrokken zijn. Indien alle betrokken factoren aanwezig zijn in de juiste concentratie, op het juiste tijdstip en op de juiste plaats zal een eicel gevormd worden die niet alleen in het correcte stadium van meiose is, maar die tevens genoeg bagage heeft om bevrucht te worden en te ontwikkelen tot een volwaardig embryo. Hoewel gedurende de afgelopen tientallen jaren veel onderzoek is gedaan om een beter begrip te krijgen in het proces van eicelrijping, inclusief *in vitro* maturatie, zijn er nog vele aspecten van eicelmaturatie onbekend.

Het onderzoek dat beschreven is in dit proefschrift richt zich met name op de fundamentele aspecten van het effect van oestradiol, *growth hormone releasing hormone* (GHRH), en *vasoactive intestinal peptide* (VIP) op de *in vitro* maturatie van rundereicellen, om zodoende meer te weten te komen over de eicelmaturatie en de consequenties voor de vroege ontwikkeling van embryo's. Bovendien werden de werkingsmechanismen van oestradiol bestudeerd en het effect van oestradiol in de prematuratieperiode op de ontwikkelingscompetentie van rundereicellen.

Hoofdstuk 1 begint met een korte beschrijving van de historische aspecten van voortplantingsonderzoek, gevolgd door een inleiding over de verschillende fasen van de meiose, en een actueel overzicht van eicelrijping en de effecten van hormonen op dit proces, inclusief de werkingsmechanismen.

Van groeihormoon (GH), dat afgegeven wordt door de hypofyse na een stimulus van GHRH, is aangetoond dat het de kinetiek van de meiose versnelt, de expansie van cumuluscellen vergroot, en de cytoplasmatische rijping van rundereicellen versneld. Verscheidene onderzoeken hebben de expressie van GHRH beschreven in niet-neurale weefsels zoals het maag-darmkanaal, de placenta, de testis, en het ovarium. Deze expressie in weefsels buiten het zenuwstelsel suggereert dat GHRH ook een lokale regulerende rol speelt in verscheidene perifere weefsels. Daarom bestudeerden wij (in Hoofdstuk 2) de rol van GHRH en het gerelateerde eiwit VIP op de kernmaturatie en de expansie van cumuluscellen van cumulus-eicelcomplexen van het rund. Gevonden werd dat de kweek van cumulus-eicelcomplexen van het rund in de aanwezigheid van GHRH of VIP de kernmaturatie of cumulusexpansie niet

beïnvloedde, maar dat het de cytoplasmatische maturatie vertraagde, wat gereflecteerd werd door een vertraagde migratie van de corticale granula.

Follikelvloeistof van zoogdieren bevat steroïden. Het is aangetoond dat vloeistof in pre-ovulatoire follikels op het moment van de LH piek een verhoogde concentratie oestradiol bevat, maar dat 6 uur na de LH piek een sterke daling van de oestradiolconcentratie optreedt, die samenvalt met de hervatting van de meiose (gekenmerkt door *germinal vesicle breakdown*, of GVBD). Hoewel de meeste effecten van oestradiol *in vivo* bekend zijn is de rol van oestradiol in *in vitro* maturatie nog grotendeels onbekend. In Hoofdstuk 3 werden de effecten van oestradiol op de *in vitro* maturatie van rundereicellen in een gespecificeerd (serumvrij) kweekmedium bestudeerd. Zowel cumulus-eicelcomplexen als eicellen waarvan de cumuluscellen verwijderd waren werden gebruikt om te bestuderen of de mogelijke effecten van oestradiol via de cumuluscellen ontstonden of direct via de eicellen. Gevonden werd dat de aanwezigheid van oestradiol gedurende de *in vitro* maturatie het percentage van eicellen in het metafase II (MII) stadium significant deed verminderen en dat het percentage van eicellen met een afwijkend kernpatroon werd verhoogd. Dit effect van oestradiol was groter in eicellen waarvan de cumuluscellen verwijderd waren dan in intacte cumulus-eicelcomplexen. Blootstelling van eicellen aan zowel FSH als oestradiol gedurende de *in vitro* maturatie had geen invloed op het aantal eicellen in het MII stadium van de meiose, maar in dat geval werden wel kernafwijkingen waargenomen. De aanwezigheid van oestradiol gedurende de *in vitro* maturatie verlaagde ook het aantal blastocysten dat gevormd werd na *in vitro* bevruchting. Indien echter tevens FSH aanwezig was werd geen effect van oestradiol waargenomen op het percentage eicellen dat deelde na *in vitro* bevruchting, of op het aantal blastocysten dat gevormd werd. Geconcludeerd werd dat de toevoeging van oestradiol aan een serumvrij maturatiemedium de rijping van rundereicellen en daaropvolgende embryo-ontwikkeling negatief beïnvloedt. Hoewel deze effecten verminderd zijn in de gelijktijdige aanwezigheid van FSH wordt de toevoeging van oestradiol aan medium gebruikt voor de *in vitro* maturatie van rundereicellen sterk afgeraden.

Om te onderzoeken (Hoofdstuk 4) of de effecten van oestradiol zoals beschreven in Hoofdstuk 3 ontstonden via een genomisch of via een nongenomisch mechanisme werden eicellen gekweekt in de aanwezigheid van oestradiol vóór of ná GVBD (de periode waarin genomische transcriptie stopt). Ook werd gebruikt gemaakt van een oestradiolconjugaat (oestradiol-BSA) dat de plasmamembraan niet kon passeren om te onderzoeken of een mogelijke plasmamembraanreceptor aanwezig was, en werd met behulp van RT-PCR de aanwezigheid van mRNA van oestradiolreceptor alfa (ERα) en bèta (ERβ) in

eicellen en cumuluscellen bestudeerd. Omdat de inductie van kernafwijkingen een van de meest prominente effecten van oestradiol gedurende *in vitro* maturatie was, zoals beschreven in Hoofdstuk 3, werd verder het effect van oestradiol op de vorming van de meiotische spoelfiguur bestudeerd met behulp van confocale laser scanning microscopie (CLSM). In eicellen werd de expressie van ERβ mRNA aangetoond, terwijl gevonden werd dat cumuluscellen mRNA voor zowel ERα als ERβ tot expressie brachten. Blootstelling aan oestradiol gedurende de eerste 8 uur van kweek (vóór het optreden van GVBD) induceerde een blokkade op het metafase I (MI) stadium van de meiose. De aanwezigheid van oestradiol ná GVBD leidde echter tot een verhoging van het percentage van eicellen met kernafwijkingen. De vorming van de meiotische spoelfiguur was in ernstige mate verstoord door oestradiol gedurende de *in vitro* maturatie, met de vorming van een multipolaire spoelfiguur als de meest voorkomende afwijking. Blootstelling van eicellen aan oestradiol-BSA leidde niet tot afwijkingen in de kern, noch tot veranderingen in het percentage gevormde blastocysten of embryokwaliteit. Deze resultaten suggereren dat de nadelige effecten van oestradiol op de *in vitro* kernmaturatie van rundereicellen niet ontstaan via een receptor in de plasmamembraan.

Hoewel eicellen afkomstig van antrale follikels van kleine of middelmatige grootte (3-8 mm in doorsnede) voor wat de kernrijping betreft beschouwd worden als matuur, dat wil zeggen in staat om de meiose te hervatten, is er een grote heterogeniteit in de cytoplasmatische rijping van deze eicellen. Als gevolg hiervan is de opbrengst van blastocysten van zulke eicellen laag in vergelijking met die van *in vivo* gematureerde eicellen. Als hypothese werd opgesteld dat het voorkomen van de GVBD met behulp van roscovitine (ROS) een eicel meer tijd zou geven voor de cytoplasmatische maturatie, en zodoende een grotere ontwikkelingscapaciteit zou verkrijgen. In Hoofdstuk 5 werd de rol van oestradiol op de eicelrijping en ontwikkelingscapaciteit van eicellen afkomstig van follikels van verschillende grootte onderzocht gedurende een tijdelijke remming van GVBD met behulp van ROS. Gevonden werd dat blootstelling aan oestradiol gedurende prematuratie noch de kernmaturatie noch de cytoplasmatische maturatie van eicellen afkomstig van kleine follikels en follikels van middelmatige afmetingen beïnvloedde. Het kan zijn dat de verhoging van oestradiol *in vivo* gedurende de follikelgroei meer gerelateerd is aan de selectie en het onstaan van dominantie van een follikel dan aan de cytoplasmatische rijping van de eicel.

Ten slotte wordt in Hoofdstuk 6 een samenvattende discussie gegeven van de resultaten zoals gepresenteerd in dit proefschrift en wordt gespeculeerd over de implicaties van deze resultaten voor toekomstig onderzoek.

RESUMO

RESUMO

A maturação oocitária é um processo fascinante mas muito complexo. Durante este processo, o oócito necessita passar por extensivas modificações nucleares e citoplasmáticas, as quais requerem perfeita coordenação de inúmeros fatores tais como: quinases, gonadotrofinas, esteróides e fatores de crescimento, numa cadeia de eventos envolvendo diferentes mecanismos de ação. Se todos os fatores envolvidos estiverem presentes na concentração adequada, no momento e local apropriados, a maturação culminará em um oócito não só em uma fase meiótica apropriada, mas também com uma "bagagem" suficiente para continuar a sua trajetória pelos processos de fertilização e início do desenvolvimento embrionário. Apesar de inúmeros estudos terem sido realizados nas últimas décadas e o conhecimento sobre o processo de maturação oocitária *in vitro* ter avançado substancialmente, vários de seus aspectos ainda estão por serem esclarecidos.

Tendo como objetivo aprofundar o conhecimento sobre o processo de maturação oocitária e de suas consequências sobre os estágios iniciais do desenvolvimento embrionário, o trabalho apresentado nesta Tese concentrou-se no estudo dos aspectos fundamentais do papel do estrógeno (E2), do hormônio liberador do hormônio do crescimento (GHRH) e do peptídeo vasointestinal (VIP) na maturação *in vitro* de oócitos bovinos. Além disto, foram estudados alguns aspectos do mecanismo de ação do estrógeno, bem como sua influência, durante o período de prematuração de oócitos bovinos, na subsequente capacidade de desenvolvimento embrionário.

No capítulo 1, o processo de meiose em oócitos é ilustrado após uma breve descrição de alguns aspectos históricos da reprodução. Esse mesmo capítulo contém um resumo da revisão bibliográfica sobre a maturação oocitária em mamíferos e da influência de hormônios e de seus mecanismos de ação neste processo.

Estudos demonstraram que o hormônio do crescimento (GH), que é liberado pela pituitária através de estímulo do GHRH, acelera a cinética da meiose, aumenta a expansão do cumulus e melhora a maturação citoplasmática em oócitos bovinos. Outros trabalhos descreveram a presença de GHRH em tecidos extraneurais, tais como o trato gastrointestinal, a placenta, o testículo e o ovário, sugerindo que o GHRH possa ter um papel regulatório também em tecidos periféricos. No estudo do capítulo 2, foi investigado o papel do GHRH e do VIP na maturação nuclear e na expansão do cumulus de oócitos bovinos. Foi observado que o cultivo desses oócitos na presença de GHRH ou VIP não afetou nem a maturação nuclear nem a expansão do cumulus. No entanto, retardou a migração

dos grânulos corticais para a periferia do oócito, indicando atraso na maturação citoplasmática.

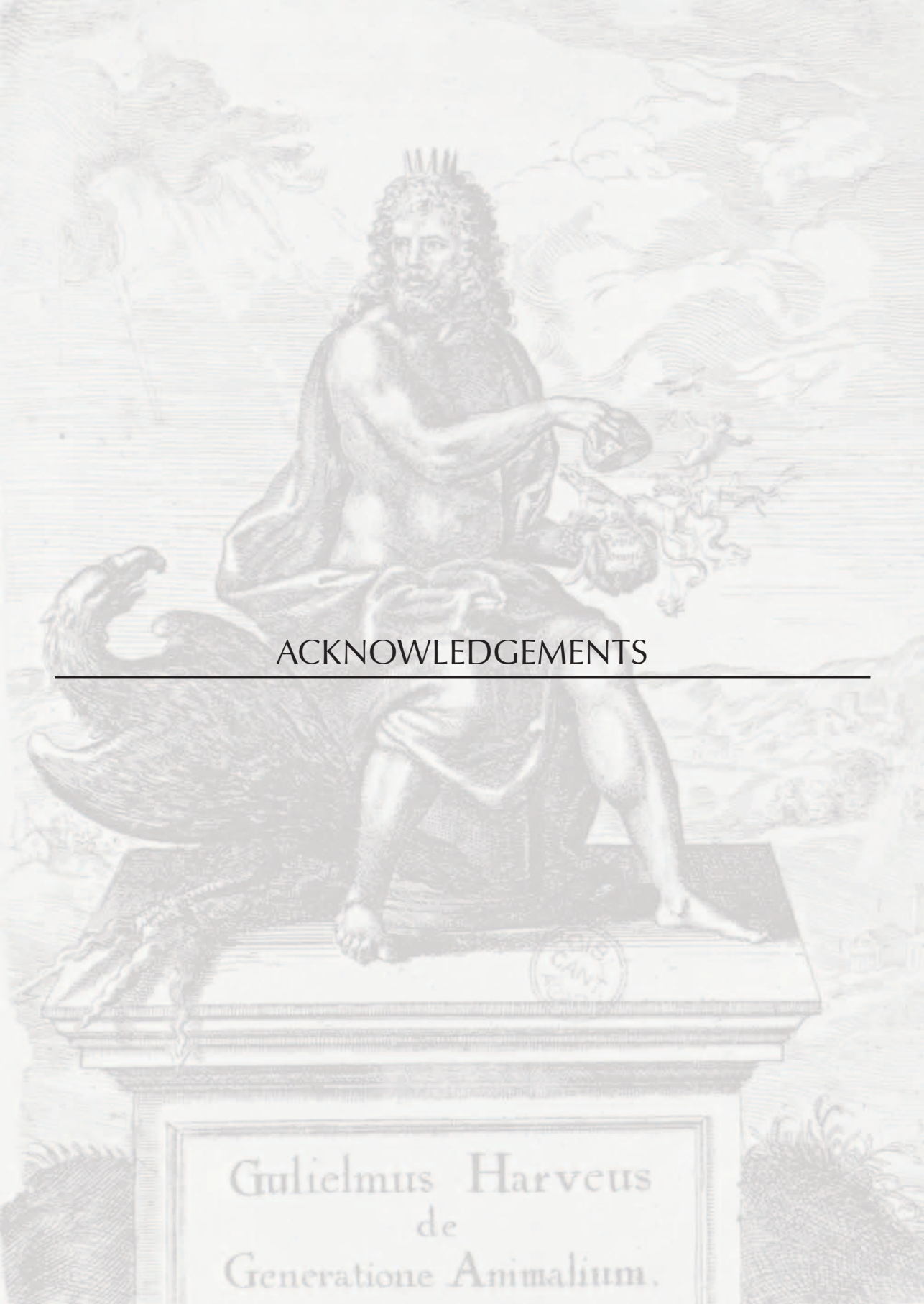
O fluido folicular de mamíferos contém hormônios esteróides. Trabalhos demonstraram que, em bovinos, no período do pico do hormônio luteinizante (LH), o fluido dos folículos pré-ovulatórios possui uma alta concentração de estrógeno, ocorrendo uma drástica redução deste hormônio 6 horas após o pico do LH, coincidindo com a ruptura da vesícula germinal (GVBD). Embora a maioria dos efeitos do estrógeno que ocorrem *in vivo* estejam bem definidos, o papel do estrógeno na maturação *in vitro* de oócitos bovinos ainda é bem contraditório.No capítulo 3, utilizando-se um meio de cultura definido (sem soro), foram estudados os efeitos do estrógeno (E2) na maturação *in vitro* de oócitos bovino e do subsequente desenvolvimento embrionário. Para pesquisar se os efeitos do E2 foram exercidos diretamente no oócito ou através do cumulus, neste estudo foram utilizados: complexo-oócito-cumulus (COCs) e oócitos desnudados (sem cumulus: DOs). Esse estudo demonstrou que a presenca de E2 durante a maturação *in vitro* diminuiu significativamente a percentagem de oócitos em metafase II (MII) e aumentou significativamente a percentagem de aberrações nucleares. O efeito negativo do E2 na maturação nuclear foi mais pronunciado em DOs que em COCs. Também neste estudo, a presenca concomitante do hormônio folículo estimulante (FSH) e do E2 durante a maturação *in vitro* não influenciou a proporção de oócitos maduros (no estágio de MII), embora aberrações nucleares ainda puderam ser observadas. A presença de E2 durante a maturação *in vitro* também diminuiu a taxa de formação de blastocistos, porém, na presenca de FSH, a adição de E2 não teve efeito nem na taxa de clivagem nem na taxa de blastocistos formados. Concluiu-se que, a suplementação de E2 no meio de cultura definido (sem soro) afeta negativamente a maturação nuclear de oócitos bovinos e o subsequente desenvolvimento embrionário. Apesar desses efeitos serem atenuados na presença de FSH, foi sugerida a omissão de E2 nos protocolos rotineiros de maturação *in vitro* de oócitos bovinos.

Com o objetivo de investigar se os efeitos do E2 descritos no capítulo 3 foram mediados por mecanismos genômicos ou não-genômicos, no estudo do capítulo 4, os oócitos foram cultivados na presença de E2 antes e depois da GVBD, tendo em vista que o sistema de transcrição é interrompido quando GVBD ocorre. Adicionalmente, para investigar a existência de receptores para estradiol na membrana plasmática foi utilizado um conjugado de estradiol com soroalbumina bovina (E2-BSA), ao qual a membrana é impermeável. A técnica de *reverse transcriptase-polymerase chain reaction* (RT-PCR) foi utilizada para estudar a presença de RNA mensageiro (mRNA) do receptor alfa (ERα) e beta (ERβ) para o estrógeno, no oócito e nas células do cumulus. Tendo em vista que, a ocorrência

de aberrações nucleares foi um dos mais proeminentes efeitos da presença do estrógeno durante a maturação *in vitro* de oócitos (capítulo 3), estudou-se também o envolvimento do E2 na organização do fuso meiótico através da técnica de microscopia laser confocal. Os resultados obtidos demonstraram que os oócitos expressam mRNA para ERβ, enquanto que o cumulus expressa ambos (ERα e ERβ). Observou-se também que a presença de E2 durante as primeiras horas do cultivo (antes da GVBD) induziu um bloqueio da meiose no estágio de metáfase I (MI), porém, a presença de E2 depois da GVBD induziu um aumento do número de oócitos exibindo aberrações nucleares. Quando foi realizada a coloração específica para o citoesqueleto, observou-se que a organização do fuso meiótico foi drasticamente afetada pela presença de E2 durante a maturação, sendo fusos com mais de dois polos (multipolares) a aberração mais frequentemente observada. Paralelamente, o cultivo de oócitos na presença de E2-BSA não influenciou a maturação nuclear, a taxa de formação de blastocistos, nem mesmo a qualidade dos embriões. Estes resultados sugerem que os efeitos deletérios do E2 na maturação nuclear *in vitro* de oócitos bovinos não foram exercidos através da membrana plasmática.

Oócitos bovinos originários de pequenos e médios folículos antrais (diâmetro aproximado de 3 a 8 mm) são considerados nuclearmente maduros, ou seja, capazes de reiniciar a meiose. Porém, existe entre eles uma grande heterogeneidade em relação ao grau de maturação citoplasmática. Como consequência, o número de blastocistos obtidos com a maturação *in vitro* desta classe de oócitos é menor quando comparado com oócitos amadurecidos *in vivo*. Para simular as condições de prematuração que ocorrem *in vivo*, foi sugerida a hipótese de que, ao prevenir-se GVBD por um certo período, estaríamos permitindo um tempo maior para a maturação citoplasmática e consequentemente possibilitando um aumento na capacidade de desenvolvimento do oócito. No estudo do capítulo 5, foi investigado o papel do E2 durante uma inibição temporária da GVBD, através do uso da roscovitine (ROS), na maturação nuclear subsequente e na capacidade de desenvolvimento dos oócitos bovinos originários de folículos de diferentes diâmetros. Foi observado que a presença de E2 durante o período de inibição da GVBD pela ROS, não afetou a maturação nuclear nem a citoplasmática de oócitos originários de folículos de pequeno e médio diâmetros. No entanto, é possivel que *in vivo*, o aumento da concentração de E2 durante o crescimento folicular esteja mais relacionado com os processos de seleção e de dominância folicular do que diretamente relacionado com a maturação citoplasmática do oócito.

Finalmente, o capítulo 6 apresenta uma discussão dos resultados dos vários estudos apresentados nesta Tese e de suas implicações para pesquisas futuras.



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CURRICULUM VITAE

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Anna Rita Costa Lage Beker van Woudenberg was born on the 14th of September 1966 in Belo Horizonte, Brazil. She studied veterinary medicine at the Federal University of Minas Gerais State-Brasil (UFMG) and received her D.V.M. degree in 1989. After graduation she practiced as specialist in horse reproduction in various stud farms in the state Minas Gerais during 8 years. In 1997 she obtained her Master of Science degree, in animal reproduction, under the supervision of Prof. Dr. Marc Henry, at the UFMG. The research project of her Masters focused on the improvement of the techniques of (cryo) preservation of stallion and donkey semen. In march 1996 she attended the 2nd International Symposium on Stallion Semen, in Amersfoort-The Netherlands and spend a month at the Department of Farm Animal Health-Utrecht University, where in 1998 she started her PhD research project under the supervision of Prof. Dr. Ben Colenbrander and Dr. Mart Bevers, resulting in this thesis.

LIST OF PUBLICATIONS

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Refereed papers

Anna R. Beker-vanWoudenberg, Helena T.A van Tol, Bernard A.J. Roelen, Ben Colenbrander, and Mart M. Bevers. Estradiol and its membrane impermeable conjugate (estradiol-bovine serum albumin) during in vitro maturation of bovine oocytes: effects on nuclear and cytoplasmic maturation, cytoskeleton and embryo quality. *Biology of Reproduction* 2004; 70:1465-1474.

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M.O. Gastal, M. Henry, **A.R. Beker** and E.L. Gastal - [Effects of season on jackass seminal characteristics]. *Proceedings of the 9th National Congress of Animal Reproduction* –1991- Belo Horizonte – Brazil - n:2, p.451.

"Cada coisa tem sua hora…
E cada hora o seu caminho"
Raquel de Queiroz