CHARACTERIZATION OF GILT'S OVARIES AND ATTEMPTS TO ACHIEVE A SUCCESSFUL IN VITRO MATURATION OF OOCYTES FROM SMALL AND MEDIUM FOLLICLES

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Pilar FERRÉ PUJOL

GRADUATE SCHOOL OF

ENVIRONMENTAL AND LIFE SCIENCE

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OKAYAMA UNIVERSITY

Dedication

To both my parents, for giving me life.

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Abstract

In the developed countries, pigs are usually slaughtered for pork production at 5-6 months old when those gilts have still not reached puberty. Porcine oocytes are easily prepared, as cumulus-oocyte complexes (COCs), from ovaries of slaughtered females at local abattoirs. Although there are, at least, three different types of ovaries; smooth surfaced ovaries (the follicles do not protrude through the ovarian cortex thus presenting a smooth appearance), bubbled-like surface ovaries (the follicles protruded strongly through all the ovarian surface) and mixed surface ovaries (the ovaries showed a mixed pattern with some smooth appearance areas and some with protruding follicles), it is not clear whether the morphology of the ovaries affects the meiotic competence of the oocytes in each ovary type. For in vitro production of mammalian embryos, furthermore, usually COCs are collected from middle-sized (3-6 mm in diameter; MF) or larger follicles of the ovaries, due to a higher developmental competence of the oocytes as compared with those from small-sized follicles (less than 3 mm in diameter; SF). Since larger numbers of SF exist in the ovary, if we can develop a new IVM system for achieving higher developmental competence of oocytes from SF, we may be able to further improve the efficiency to utilize female gametes available in the ovaries for their use in reproductive technologies. Through three experiments described below, therefore, the morphological differences of ovaries and the conditions for in-vitro maturation (IVM) were examined for oocytes from SF and MF to achieve a more efficient meiotic and developmental competence.

1. Characterization of prepubertal gilt ovaries with different patterns in the follicular morphology on the surface and the meiotic competence of the oocytes.

Oocytes from middle-size follicles have been commonly utilized in domestic animals for in vitro production of embryos, but their maturation and developmental competence capacities depend on extrinsic and intrinsic factors, which are unknown in ovaries obtained from slaughterhouses. And so, in this study we evaluated if ovaries from prepubertal gilts have any morphological differences according to their follicular populations. For this reason, ovaries were classified into three categories; ovaries with smooth (SSO), bubbled (BSO) or mixed (uneven) surface (MSO), weighted, and the number of small (SF; < 3 mm), medium (MF; 3-6 mm) and large (> 6 mm) follicles present on their surface were counted. Cumulus-oocyte complexes from SF and MF of SSO or MSO were cultured in vitro to assess their meiotic competence capacity. Based on the follicular numbers, a significantly higher number of SF was observed in MSO than SSO and BSO (138.8 vs. 116.6 and 94.4, respectively) and the number of

MF per ovary was significantly higher in BSO and MSO than SSO (18.44 and 14.18 vs. 11.04, respectively). When the meiotic competence of oocytes was assessed, MSO showed a significantly higher percentage of metaphase-II stage oocytes than SSO in SF (55.6% vs. 50.0%, respectively) and MF (80.2% vs. 75.6%, respectively). In conclusion, we propose that MSO can suppose a good source of oocytes for improving the efficiency of the actual reproductive technologies.

2. Effect of removing cumulus cells from porcine cumulus-oocyte complexes derived from small and medium follicles during in vitro maturation on the apoptotic status and meiotic progression of the oocytes.

The present study was undertaken to examine the apoptotic status and meiotic progression of oocytes from small (SF; 0.5-2 mm in diameter) and medium follicles (MF; 3-6 mm in diameter) when the oocytes were denuded before, during and after in-vitro maturation (IVM). Cumulus-oocyte complexes (COCs) from SF or MF of prepubertal gilt ovaries were cultured for IVM, in the presence of 1 mM dibutyryl cAMP, 10 IU/ml eCG and 10 IU/ml hCG for 20 h and then in the absence of these supplements in the same medium for another 24 h. Before or 20 h after the start of IVM culture, some of the oocytes were isolated from COCs and continued the culture for IVM. At the end of IVM, the apoptotic status and meiotic progression of the oocytes were compared with oocytes cultured in the presence of cumulus cells (CCs) by Annexin-V/PI assay and DAPI staining. At the start of IVM, there was not a significant difference in the viability between oocytes from SF and MF, but the incidence of oocytes at the GV_0 stage was higher in the specimens from SF than MF, and that of oocytes at the GV_I stage was the opposite. Apoptotic status of the oocytes was only affected by the time when the oocytes were denuded. The percentage of intact oocytes was significantly higher when CCs were removed at 20 and 44 h after the start of IVM in both, oocytes from SF and MF. The incidence of early and late apoptotic oocytes was significantly higher when the CCs were removed before IVM in both, SF and MF. The incidence of mature oocytes was significantly affected by both, the origin of COCs and decumulation timing. Although the percentage of mature oocytes was higher in MF, maturation rates were significantly higher when oocytes were denuded at 20 h, as compared with 0 and 44 h after the start of IVM. However, the percentage of mature oocytes with a morphologically normal spindle was significantly higher when oocytes were denuded at 44 h, rather than 20 h of IVM. In conclusion, removing CCs at 20 h after the start of IVM seems to promote meiotic progression of the oocytes to the metaphase-II stage even when the COCs were collected from SF, but factor(s) from or communication with CCs during IVM may be needed to obtain a morphologically normal spindle in mature oocytes.

3. Effect of removing cumulus cells from porcine cumulus-oocyte complexes derived from small and medium follicles during IVM in the presence or absence of GDF9 or VEGF on the meiotic and developmental competences of the oocytes.

Strategies to induce meiotic resumption of oocytes could be reducing the cAMP levels by interruption of communication between oocyte and cumulus cells, or by supplementation of the IVM medium with exogenous hormones and growth factors. Vascular endothelial growth factor (VEGF) and growth-differentiation factor 9 (GDF9) have been proved to improve the meiotic competence of oocytes. Firstly, the effect of timing of removing cumulus cells during IVM on meiotic and developmental competences of oocytes was assessed, and it was also examined if the addition of GDF9 or VEGF could further improve IVM rates of oocytes denuded during maturation culture. In all experiments, it was observed that SF oocytes had lower maturation, cleavage and blastocyst rates than those from MF. Denuding oocytes before the start of IVM had detrimental effects on all the parameters studied, whereas culturing the oocytes as COCs during the first 10 h was enough to induce meiotic resumption in the same levels as the controls. When intact COCs were denuded 20 h after the start of IVM, maturation rates of the oocytes from SF (64.1%) and MF (82.5%) were significantly higher than SF (51.2%) and MF controls (76.0%), whereas developmental competence to the blastocyst stage of those oocytes was similar with SF and MF controls, respectively. When an exogenous growth factor, VEGF or GDF9, was supplemented to the IVM medium, any significant effects were not observed in oocytes denuded 20 h after the start of IVM. For this, we concluded that oocytes from MF have a higher quality than those from SF and that denuding the oocytes 20 h after the start of IVM could be a good method to promote the meiotic resumption of oocytes, without any reduction in the developmental competence.

Results from current experiments demonstrate that using COCs from MSO or by removing cumulus cells of COCs 20 h after the start of IVM can promote the meiotic resumption of oocytes, without any reduction in the developmental competence. This information may contribute to improve the efficiency to prepare female resources for in-vitro embryo production.

Declaration

This dissertation contains no materials which have been accepted for the award of any other degree or diploma in any other tertiary institution and to the best of my knowledge and belief, contains no materials previously published or written by another person, except for the references that have been included in this text.

Signature.

Date.

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Publications arising from this thesis

1. **Ferré P**, Bui TMT, Wakai T, Funahashi H (2016). Effect of removing cumulus cells from porcine cumulus-oocyte complexes derived from small and medium follicles during in vitro maturation on the apoptotic status and meiotic progression of the oocytes. Theriogenology. [In Press] doi: 10.1016/j.theriogenology.2016.05.024

Conference proceedings

1. **Ferré P** and Funahashi H. Effect of cumulus cell removal during in vitro maturation of porcine cumulus-oocyte complexes on the apoptotic status and meiotic progression of the oocytes. Poster was presented at the 41st International Embryo Transfer Society meeting in Versailles, France (2015).

2. **Ferré P**, Bui TTM, Wakai T and Funahashi H. Effect of cumulus cell removal at different timings during IVM on nuclear maturation of porcine oocytes. Poster was presented at the 108th Meeting of the Society for Reproduction and Development in Miyazaki, Japan (2015).

3. **Ferré P**, Bui TTM, Tran MT, Wakai T and Funahashi H. Characterization of prepubertal gilt ovaries with different patterns in the follicular morphology on the surface and the meiotic competence of the oocytes. Poster was presented at the 5th International Conference on Sustainable Animal Agriculture for Developing Countries in Pattaya, Thailand (2015).

4. **Ferré P**, Bui TTM, Tran MT, Wakai T and Funahashi H. Effect of addition of follicular fluid or growth differentiation factor-9 on in vitro maturation of porcine oocytes denuded 20 h after the start of in vitro maturation. Poster was presented at the 42nd International Embryo Transfer Society meeting in Louisville, USA (2016).

5. **Ferré P**, Wakai T and Funahashi H. Effect of cumulus cell removal during in-vitro maturation on the meiotic competence of oocytes derived from small and medium follicles. Poster was presented at the 18th International Congress on Animal Reproduction in Tours, France (2016). Poster was also presented at the 11th Research and exchange meeting of high school students and graduate students, Okayama University, Japan (2016).

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Abbreviations

%	Percentage
°C	degree(s) Celsius
µg	microgram(s)
µl	microliter(s)
µm	micrometer(s)
µM	micromole(s)
µsec	microsecond(s)
AC	alternating current
AI	anaphase I
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCs	cumulus cells
COCs	cumulus-oocyte complexes
Da	Dalton
DAPI	4',6-Diamidino-2-phenylindole
dbcAMP	dibutyryl cyclic adenosine monophosphate
DC	direct current
Deg	degenerated oocyte
DMSO	dimethyl sulfoxide
DO	denuded oocyte
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
FF	follicular fluid
g	gram(s)
GDF9	growth differentiation factor-9
GV	germinal vesicle
GV_0	germinal vesicle stage 0
GV_I	germinal vesicle stage I
GV_{II}	germinal vesicle stage II
GV_{III-IV}	germinal vesicle stage III-IV
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotropin
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IVC	in vitro culture
IVF	in vitro fertilization

IVM	in vitro maturation
LF	large follicle
M	Molar
MF	medium follicle
mg	milligram(s)
MI	metaphase I
MII	metaphase II
mM	milimolar
mM199	modified medium 199
mPOM	modified porcine oocyte medium
ng	nanogram(s)
nM	nanomolar
PI	propidium iodide
PMI	prometaphase I
PVA	polyvinyl alcohol
SF	small follicle
TI	telophase I
TL-HEPES	HEPES buffered Tyrode Lactate solution
VEGF	vascular endothelial growth factor
ZP	zona pellucida

CHAPTER 1: GENERAL INTRODUCTION

1.1 Preface

The history of oocyte maturation in-vitro started long ago, with the pioneer studies of Evans (1931) and of Pincus and Enzmann (1935). In the reproductive field, in-vitro maturation (IVM) consists in the culture of reproductive cells (most commonly oocytes or germinal stem cells) outside the body in controlled conditions which allow them to grow and develop in an optimal manner. Actually, reproductive technologies are important for the maintenance of the genetic diversity in many farm and endangered species, and they are also basic for the application of other technologies such as the creation of transgenic animal models, xenografting and the creation of bioreactors.

The success of the in-vitro technologies is influenced by many factors in which the ones with most importance would be the source of samples and the quality of reproductive cells, so it is of great interest to research new methods that will allow us to obtain great numbers of high quality gametes. For this, it is important to understand the factors that affect the reproductive performance of animals, since it will affect the quality of gametes and also we must understand the pathways for their growth and maturation in vivo, so we can improve and develop new systems to culture oocytes in vitro in a more efficient way.

1.2 The pig as a research model

The domestic pigs are closely related to humans in terms of anatomy, genetics and physiology (Hart et al., 2007; Dyce et al., 1987; Swindle et al., 2012) making them into a good model for the study of many infectious and metabolic disorders or illnesses as an alternative to the rodent models (Meurens et al., 2012). Pigs can also be used for the creation transgenic animals that can be used as bioreactors for producing proteins of pharmacologic interest (Park et al., 2006; Lee et al., 2009) and they are also important for the creation and development of new medical therapies, such as xenotransplants (Cozzi and White, 1995; Levy, 2000) which have had relative success (Reinholt et al., 1998; Deacon et al., 1997) giving hope into improving human life quality. For being able to apply these innovative technologies, reproductive in vitro techniques are of crucial importance, since they constitute the first step for their successful achievement.

1.3 Factors influencing reproduction performance in pigs

Animal reproductive performance and efficiency is affected by many elements that will influence the quality of the gametes and they can be classified into two main groups: extrinsic factors; which depend on the environment and the management conditions and intrinsic factors; those which depend on the individual (Rodríguez FPC., 2005).

1.3.1 Extrinsic factors

The intense selection of the growth and productivity characters for the domestic pig breeding has developed a continuous polyestric female (Love et al., 1981; Falceto, 1992), but there are still influences of seasons (and photoperiod) on prolificacy in both, male and female pigs (Greer, 1983). In males, in the summer, heat stress affects negatively to spermatogenesis (Wettemann et al., 1979). During this period of stress, the semen quality parameters, such as concentration of sperm and total number of sperm in the ejaculate lower (Wettemann et al., 1976, 1979) and the proportion of morphologically abnormal spermatozoides increases (Cameron and Blackshaw, 1980). In females, sows can present heat stress anoestrus during the summer or in elevated ambient temperatures (Einarsson et al., 2008) and mating the animals in these conditions can lead to lower conception rates and smaller litters (Edwards et al., 1968; Nelson et al., 1970).

Zak et al., (1997) detected that sows with delayed heat had low levels of insulin, so nutrition can be used as a tool for improving fertility. It has been reported that providing gilts and sows with abundant feed after mating can alleviate these negative effects caused by seasonality (Peltoniemi and Virolainen, 2006). Supplementation of exogenous insulin in the cyclic gilt increases ovulation and stimulates LH secretion (Cox et al., 1987) which can reverse the negative effects of feed restriction by affecting the brain glucose levels (Almeida et al., 2001; Ubeda, 2003).

1.3.2 Intrinsic factors

There are differences among animal species, for example, cows (monoovulatory species) have lower ovulation rates than pigs or sheep (polyovulatory species) (Kumar Pramod et al., 2013). Differences in fertility can also be detected between different breeds of the same species, as Miller et al. (1998) showed that Meishan sows have higher ovulation rates than Large White hybrids (27.7 vs 17.6 corpora lutea) probably due to differences in follicle recruitment and atresia. Finally, there can also be differences among individuals, since many reproductive anomalies and illnesses are heritable, producing malformations in males and females that can lead to gonad inactivity such as having ovotestes or ovarian hypoplasy (Baker JR., 1925; Falceto et al., 1990).

1.4 The porcine ovary

The ovary is the main organ at a reproductive level; they are found in pairs, but their placement and size varies among species. They are responsible for the supply of germ cells (oocytes) and the production of steroidogenic hormones from circulating cholesterol for the production of estrogen and progesterone that control many aspects of the female development and physiology (Edson et al., 2009).

The reproductive system in pigs is situated dorsal to the intestines in the pelvic cavity. Macroscopically, the ovaries from prepubertal gilts have a blackberry-like shape with abundant pink follicles of different diameters with less than 6 mm in diameter. In these young females, there are continuous waves of follicular growth and atresia that never overcome the intermediate follicular size, so the ovaries from these young gilts do not possess corpora albicans (Falceto, 1987). In post-pubertal females presenting anestrous, the ovaries look flattened and smooth with few numbers of follicles of variable size on the surface with less than 6 mm in diameter that will regress by atresia and not ovulate, but, unlike the ovaries of prepubertal gilts, these ovaries possess corpora albicans, indicating that these females reached puberty even if their estrous was not detected (Falceto et al., 2004).

1.5 Problematic about the differences in the ovarian samples for experimentation.

We indicated that the differences in the reproductive performance of the pigs used commercially for the production of meat can be due to numerous factors such as breed, age, season, nutrition and housing conditions that can result in differences on the quality of oocytes and sperms.

Since the ovaries are highly-available and because they constitute an unwanted sub product of the meat industry, most of the actual studies in reproductive biotechnologies using the pig as a model are performed with ovaries obtained from animals daily abated for meat production. Among these animals, we can find prepubertal gilts, pubertal gilts or gilts that spend a long time in anestrous. As we said, anestrous is a symptom of ovarian inactivity, which is a cause of infertility and sterility in the swine (Falceto et al., 2004). For this, the samples will be from a variable origin, making it difficult to find a method for standardizing samples for making the IVM protocols more efficient.

1.6 Oocyte maturation in vivo

The mammalian oocytes develop and mature inside the ovarian follicles. The growth and development of these follicles requires a series of coordinated events that induce morphological and functional changes within the follicle that lead to cell differentiation and subsequent oocyte growth and development (Palma et al., 2012).

1.6.1 Oogenesis in vivo

In the mammalian species, the process of oogenesis is initiated early during the fetal development and it ends when the female becomes sexually mature and happens synchronically with the folliculogenesis process. The oogenesis begins with the formation of the primordial germ cells (PGCs) during the gastrulation process. These oocyte precursors have motility and can respond to chemotactic stimuli making them able to migrate from the site of origin in the embryonic epiblast through the genital ridge until they arrive to the undifferentiated but developing gonad. During and after their migration, the PGCs proliferate by mitoses and, when they become surrounded by somatic cells, are called oogonia (Picton et al., 1998; Palma et al., 2012).

In a determined moment, the oogonia will enter the meiosis process and will become arrested at the diplotene stage of the first meiotic prophase (dictyate stage) surrounded by a single layer of flat pre-granulosa cells forming the primordial follicles. The oocytes will remain at this stage until the female reaches puberty, when a surge of LH will induce the resumption of meiosis and the ovulation of the oocytes arrested at the MII stage (Huang et al., 2010; Zuccotti et al., 2011).

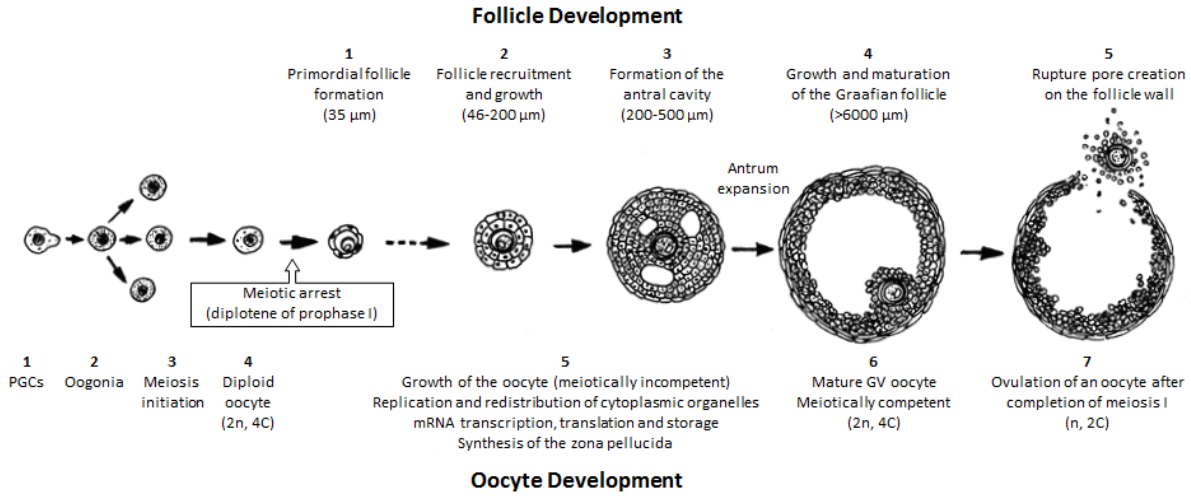


Fig. 1.1: Mammalian oogenesis and folliculogenesis stages from the development of the primordial germ cells (PGCs) to the ovulation of a mature oocyte from a Graaffian follicle. Follicular diameter, chromosomal (n) and DNA content (C) of the oocyte are shown in the scheme.
(modified from Picton et al., 1998).

1.6.2 Folliculogenesis in vivo

An ovarian follicle is a multicellular structure consisting in a meiotically arrested oocyte surrounded by one or several layers of somatic cells, pre-granulosa or granulosa cells. Pre-granulosa cells are the primary cell type in the ovary and are cells that provide the physical support and the microenvironment required for the developing oocyte. These granulosa cells establish strong connections with the oocyte (intermediate junctions and gap junctions) which ensure a bi-directional communication pathway for the delivery of signals necessary for the production of hormones and the growth of the oocytes (Nicholson et al., 1997; Huang et al., 2010; Palma et al., 2012).

In the pig, follicular growth from the primary to nearly pre-ovulatory status (also called Graafian follicle) takes more than three months, and the process can be divided into two distinct phases:

- A slow growth phase, which is independent on gonadotropin stimulation. During this phase, pre-antral follicles grow by increasing the number of granulosa cells and the size of the oocyte.
- A rapid growth phase, which is dependent on gonadotropin stimulation. The size of the follicle augments due to a dramatic increase in the proliferation of granulosa cells and the formation of the antrum occurs (Morbeck et al., 1992).

1.6.2.1 Activation of primordial follicles

Oocytes can control follicular formation through the activity of transcription factor in germ cell α (TNFα), which regulates the initial organization of primordial follicles and modulates the survival of the germ cells (Zuccotti et al., 2011; Sánchez et al., 2012). Numerous factors have been demonstrated to arrest or induce the recruitment of primordial follicles in an experimental way, but the exact role of these factors is not yet fully understood, but they can be classified according to their effect on the follicular development as inhibiting or activating factors. (Skinner, 2005; Zuccotti et al., 2011; Palma et al., 2012).

1.6.2.2 Pre-antral and early antral stages

When a primordial follicle leaves the resting pool, the pre-granulosa cells secrete kit-ligand (KL) that will interact with the oocyte promoting its growth. During this growth phase, the oocyte will secrete glycoproteins which will condense around it to form a translucent layer, the zona pellucida (ZP; Gupta et al., 2012). When the ZP synthesis starts, the granulosa cells will become cuboid and they will extend processes through the newly synthetized structure to the ooplasm where they will form gap junctions allowing a bi-directional communication

between the oocyte and the cumulus cells (Nicholson et al., 1997; Sugiura et al., 2005; Gilchrist et al., 2008; Palma et al., 2012).

Gap junctions present between oocyte and surrounding cumulus cells will allow the last ones to support oocyte development and to play an essential role during its growth and its metabolism by exchanging molecules of less than 1kDa, inorganic ions and electrical impulses (Kidder and Mhawi, 2002). The cumulus cells will also provide the oocyte with aminoacids and substrates derived from glycolysis, such as pyruvate, that will be used for nutrition and energy production (Biggers JD, 1967), thanks to the oocyte secretion of paracrine factors that will stimulate the glycolytic activity of cumulus cells by promoting the expression of genes encoding glycolytic enzymes (Sugiura et al., 2005; Zuccotti et al., 2011; Palma et al., 2012). During this time of oocyte growth, the stromal cells outside the basement membrane will become organized into the theca cell layers by the presence of soluble factors secreted by the granulosa cells (Young and McNeilly, 2010).

The regulation of the follicular cell proliferation during the recruitment is due to the cooperative effect of the oocyte secreted factors from the TGF-β superfamily growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15). The first has a dominant effect during the early phases, while the activity of the second is performed during more advanced phases (Hunter et al., 2005). The correct growth of the follicle is balanced trough the regulation of the KL expression, factor that co-ordinates the growth of the oocyte and the proliferation of the granulosa cells. Follicle growth is inhibited by GDF-9 and is activated by BMP-15. BMP-15 also inactivates itself by the KL expression in a negative feedback loop (Hunter et al., 2005; Zuccotti et al., 2011). Growth differentiation factor-9, BMP-15 and also BMP-6 act through receptors to activate the intracellular signal transducer molecules (SMAD) cascade by binding to a type-I receptor (ALK) or a type II receptor, that leads to ALK phosphorylation of numerous SMAD molecules necessary for inducing the gene transcription pathway (Gilchrist et al., 2008).

1.6.2.3 Follicular antral stage

The antral stage of follicular development is characterized by its dependence on gonadotrophins (FSH and LH) secreted cyclically by the pituitary gland, and since the oocyte lacks the receptors, their action on oocyte development will be exerted via cumulus cells (Schoevers et al., 2007; Zuccotti et al., 2011).

When a follicle reaches to 300 μm in diameter, the oocyte has acquired its final size and it is in this moment when the follicle undergoes pre-antral to antral transition. In this phase, the granulosa cells will continue to proliferate, and when the follicle reaches a diameter of 400 μm,

follicular fluid will accumulate and it will end up coalescing to form a single follicular antrum (Morbeck et al., 1992; Huang et al., 2010). With the formation of the antral cavity, the granulosa cells differentiate into two anatomically and functionally distinct lineages: the mural granulosa cells that line the follicle wall and have principally a steroidogenic role, and the cumulus cells, which form an intimate association with the oocyte that will gradually acquire meiotic and developmental competence (Sugiura et al., 2005). This differentiation is due to the suppressive effect that the oocyte exerts on the protein expression in cumulus cells, maintaining their phenotype by down-regulating several mural granulosa cell markers such as: LHCGR, CYP11A1 and CD34 (Gilchrist et al., 2008; Paradis et al., 2010; Zuccotti et al., 2011).

The response of cumulus cells to the gonadotropin surge is mediated by a high concentration of FSH that accompanies the LH surge together with the action of local paracrine effectors such as epidermal growth factor-like ligands from the granulosa cells, interleukin-1 from the thecal compartment and oocyte-derived signals. In the mural granulosa cells, LH binds to its receptor and stimulates the adenyl cyclase (AC) causing an increase in the intracellular concentration of cAMP, which activates the protein kinase A (PKA) and the downstream extracellular-signal-regulated kinases 1 and 2 (ERK1, ERK2 or MAPK3/1) signaling cascade. Subsequently, the ERK1/2 pathway induces the down-regulation of genes related to follicular development and the up-regulation of the ovulation-related genes, concretely those required for cumulus expansion. The products of these genes, will act in an autocrine/juxtacrine way within the mural granulosa cells or they will diffuse to activate the cumulus cells (Conti et al., 2005; Shimada et al., 2006; Procházka et al., 2011). The ERK1/2 pathway also mediates the immediate effect of LH on gap junctional closure in granulosa cells, stopping the somatic influx of cAMP to the oocyte, which will lead to a drop of the ooplasm level of cAMP allowing meiotic resumption and germinal vesicle breakdown (GVBD; Sánchez et al., 2012).

Finally, the process of follicular development will be completed with the creation of a rupture pore in the apical follicle wall and the ovulation of a MII arrested oocyte surrounded by a surrounding mass of expanded cumulus cells (Akison et al., 2012).

1.7 Oocyte maturation in vitro

In vitro maturation is an important reproductive biotechnology that allows the production of large numbers of viable oocytes capable to be fertilized and generate embryos with the ability of developing to term and it can also reduce the generation intervals and increase the quality of the offspring obtained by this process. This technology allows to use many of the oocytes present in the ovaries that otherwise would generally be lost due to atretic processes in the ovarian follicles and by allowing the use of ovaries derived from

slaughterhouses that otherwise would be wasted. These oocytes can be used in basic research and also for advanced animal husbandry and that is basic to the application of other biotechnological applications such as cloning (Kim et al., 2005) or the production of transgenic animals to be used as xenotransplants or bioreactors (Cozzy and White, 1995; Lee et al., 2009).

A very important point for the IVM technology is to be able to succeed into obtaining oocytes that can achieve both, nuclear and cytoplasmic maturation. Since many years ago, it is known that mammalian oocytes can resume meiosis when they are liberated from the follicular environment (Pincus and Enzmann, 1935), and that they can be matured in vitro in defined mediums supplemented with exogenous agents like bovine serum (Edwards, 1965). Currently, the mechanisms responsible for oocyte maturation are still not completely understood causing the actual IVM systems to be unable to mimic the in-vivo conditions of follicular environment before and during culture.

1.7.1 Nuclear maturation

In oocytes, nuclear maturation involves the condensation and redistribution of chromatins during meiosis.

In pigs, the oocytes have a high variability in the GV morphologies after collection (Nagai et al., 1997) and also during culture (Funahashi et al., 1997a). This heterogeneity is responsible for observable differences in the meiotic and developmental competences of the oocytes collected for IVM purposes (Mcgaughey and Polge, 1971) and might also be responsible for the oocytes to induce meiotic resumption on different times, thus causing variations in their maturation at the end of the culture. It is known that at the end of IVM culture, not all oocytes are able to complete their nuclear maturation. Some oocytes remain at the GV or MI stages, whereas others simply degenerate through apoptosis (Miao et al., 2009). These differences not only affect oocyte maturation but also their developmental ability and can be caused by numerous factors such as coming from oocytes and follicles with a different size in their diameters (Szybek, 1972; Motlik and Fulka, 1986; Eppig et al., 1994; Grabowska et al., 2016), stress caused by the usage of inadequate culture media (Laurinick et al., 1994; Fischer Russell et al., 2006) or by differences in the age of the donor (Revel et al., 1996; O'Brien et al., 1996; Marchal et al., 2001; Khatun et al., 2011).

A way of partly solving the previously stated problems is by a temporary inhibition of the meiotic maturation during the first part of the IVM culture, fact that also increases oocyte developmental competence (Funahashi et al., 1997b; Hashimoto et al., 2002) because this process does not inhibit the protein synthesis during the GV stages (Marchal et al., 2001). The inhibition of the GVBD can be achieved with different methods: the inhibition of the maturation

promoting factor (MPF) activity (Mermillod et al., 2000; Kulbeka et al., 2000) or with the supplementation of agents capable of incrementing the intracellular levels of cAMP such as dibutyryl cyclic AMP (dbcAMP), a cAMP analogue that is permeable to cell membranes and can also increase the activity of the PKA (Kim et al., 2008). It has been demonstrated that the addition of this molecule during the first part of IVM, can inhibit oocyte GVBD, has a synchronization effect on the nuclear stages of pig oocytes (Funahashi et al., 1997b) and also has a positive effect on promoting MI to MII transition thus enhancing the meiotic potential of the oocytes (Somfai et al., 2003). Furthermore, it has been suggested that the lower developmental capacity of oocytes from prepubertal gilts might be due to their incapacity of accumulating cAMP in their cytoplasm during IVM culture (Bagg et al., 2006).

1.7.2 Cytoplasmic maturation

The cytoplasmic maturation of an oocyte, also known as oocyte capacitation, occurs during the oocyte growth phase and it comprises a series of events that imply ultrastructural and molecular changes occurring in the cytoplasm of the oocytes (Hyttel et al., 1997).

It is believed that the storage of a determined amount of transcripts or proteins is required for the oocytes to acquire higher meiotic and developmental competence. Pig oocytes are known to require a phase of protein synthesis before the GVBD occurs for a normal progression of meiosis (Fulka et al., 1986), and that after 12-16 h after the LH peak takes place, a new synthesis period begins, being necessary for breaking the nuclear envelope (Kulbeka et al., 1988).

Ultrastructural changes during the period of cytoplasmic maturation can suppose the relocation of organelles such as cortical granules or mitochondria. In the oocyte, the cortical granules develop from the Golgi apparatus and, when the oocyte has finished its maturation they get positioned just under the oolema for, after fertilization, undergo exocytosis and release their contents into the perivitelline space for blocking polyspermy (Liu, 2011). An irregular distribution of the oocyte cortical granules might be responsible for a high incidence of polyspermy after IVF in vitro (Cran and Cheng, 1986).

Mitochondria relocation seems to be important for a normal development in many species. In immature oocytes, their shape is round or ovoid, possess few cristae and can contain vacuoles (Motta et al., 2000), and they gradually will become more elongated and the number of transverse cristae will increase (Hyttel and Niemann, 1990). Mitochondria are distributed throughout all the cytoplasm in mature bovine oocytes, but they can be not present in the cortical region (Van Blerkom, 1990; Plante and King, 1994). In oocytes at the GV stage, mitochondria are situated mostly in the cortex but during maturation they are relocated

depending on the nutrients present in the medium (Krisher and Bavister, 1997). If the mitochondria remain in the cortex, it is a sign of low developmental competence. The functionality of this redistribution is not understood, but it is speculated that they might supply energy to the nucleus or reduce the concentrations of oxygen, thus acting as an antioxidant agent (Bavister and Squirrell, 2000).

1.8 Problematic of the IVM protocols

The oocytes obtained in the ovarian follicles present heterogenic characteristics that will affect to their maturation and developmental competence capacities. Furthermore, the mechanisms implicated in the synchronous nuclear and cytoplasmic maturation of oocytes in vivo are not completely understood, fact that causes difficulties in the development of good methods for maturing oocytes in vitro. Protocols based in the inhibition of an early meiotic resumption have been developed to improve the quality and number of mature oocytes, but even if the actual maturation protocols allow similar maturation rates of oocytes derived from both stimulated large and unstimulated middle or small follicles, the blastocyst rates of the later ones are still much lower, fact that reduces significantly the effectivity of the reproductive technologies.

Strategies to characterize the oocyte quality by morphologic and biochemical criteria have recently gotten attention with the objective of improving oocyte meiotic potential and developmental competence.

1.9 Conclusion

The pig is a farm species with a high research interest for its use in numerous reproductive technologies. Many factors unknown to researchers affect to the quality of the oocytes from ovaries of slaughtered pigs for pork production, and the in vivo mechanisms implied in the synchronic nuclear and cytoplasmic maturation of oocytes is not totally understood due to the complexity of the follicular environment and the pathways implicated.

Technique for IVM has been developed to allow the maturation of oocytes from different sources, but as the oocyte samples are highly heterogenic, current technologies are limited to obtain large proportions of mature oocytes with a good quality, especially if they are derived from small follicles. It is of great importance to develop new methods for improving the efficiency of the actual IVM technologies by obtaining large numbers of highly competent oocytes.

1. 10 OBJECTIVES AND HYPOTHESES

After having analyzed the actual status of porcine in vitro maturation protocols, two main objectives were proposed for this doctoral thesis.

- Firstly, to find an ovarian intrinsic factor that may be used for the improvement on the efficiency of the actual in vitro maturation protocols.
- Secondly, to find a method for improving the in vitro maturation protocols for the obtainment of high-quality mature oocytes from small and medium-sized ovarian follicles.

For the achievement of these main objectives, the following working hypotheses were prepared:

1. Reproductive efficiency in farm animals, such as pigs, depends on two main factors; intrinsic and extrinsic. As most of ovaries used for experimentation purposes are obtained from slaughtered animals, extrinsic factors which influence reproduction such as nutrition, the environment and the sanitary conditions are factors which are unknown and cannot be controlled by the experimenter. To be able to **select a criterion** that is **intrinsic on the sample**, (which means that must be a characteristic which has to be observed on the sample, in this case the ovaries from slaughtered gilts), the morphology of ovarian surface was selected as the main criterion that will allow to select high quality cumulus-oocyte complexes with the objective of **improving the efficiency of culture protocols for IVM**.

   For this, firstly ovaries were divided, according to their surface characteristics, into 3 groups: smooth-surfaced ovaries (SSO), bubbled-like surfaced oocytes (BSO) and mixed-surface ovaries (MSO), and their weight and the number of small, medium and big follicles present on the surface were assessed for each type. Afterwards, groups of COCs from small and medium follicles of SSO and MSO types were in vitro cultured and their nuclear status was subsequently observed under a microscope to detect if the efficiency of the maturation culture can be improved by the criterion.

2. In the actual protocols used for the IVM of oocytes, these cells are usually matured in the presence of meiotic inhibitors that act in a reversible manner. After co-incubating the oocytes with the inhibitor for some time, they are afterwards transferred to a medium without the inhibitor supplements to complete their maturation. During the culture, cumulus cells surrounding oocytes decrease their communication as the time

passes, diminishing the transport of inhibiting substances such as cAMP and cGMP to the ooplasm. That is the main reason why it was hypothesized that a way to **improve** the **nuclear maturation** of oocytes can be performed by diminishing the intracellular levels of these inhibiting molecules such as cAMP **by removing the cumulus cells during IVM**.

For this, it was firstly investigated the oocyte nuclear and apoptotic status of oocytes from small and medium follicles before IVM. The second experiment was to assess the maturation rates and the apoptotic status of oocytes after IVM when they were denuded after the first part of IVM (after 20 h) when compared with the controls (before IVM, after 0 h; and at the end of the culture, after 44 h). It was also examined the quality of meiotic spindle of mature oocytes by observing the morphology under a fluorescence microscope.

3. The third part of this study was centered on the **maturation rates** and the **developmental competence** capacity of oocytes from small and medium follicles when cumulus cells were removed before, during and after IVM. It is known that when growth factors are added to the in vitro maturation culture media can increase nuclear and developmental competence in oocytes. This time it was assessed if **maturation rates could be further improved when cumulus cells were removed after 20 h**, when the culture media were **supplemented** with two different growth factors, growth differentiation factor 9 (GDF9) or vascular endothelial growth factor (VEGF).

   For this objective, firstly it was examined the maturation rates of oocytes from SF and MF when denuded at 0, 20, 32 and 44 h of IVM, and chose the timing considered as the optimum for maturation according to the results. After that, the maturation media were supplemented with exogenous GDF9 or VEGF at concentrations which had been proven to increase maturation rates in standard IVM conditions (100 ng/mL for GDF9 and 200 ng/mL for VEGF) and analyzed nuclear maturation of the oocytes after treatments. Finally, as an observable maturation increase was not obtained, oocytes denuded at 0, 20 and 44 h were parthenogenetically activated to assess their developmental competence ability and the blastocyst quality.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Chemicals and culture media.

The reagents NaCl, NaOH, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, KH_2PO_4, gentamicin-sulphate and paraffin liquid were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Mannitol, potassium chloride, $NaH_2PO_4 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$ were purchased from Ishizu Pharmaceutical Co., Ltd. (Osaka, Japan). Equine chorionic gonadotropin (eCG; Serotropin) and human chorionic gonadotropin (hCG; Gonatropin) were purchased from ASKA Pharmaceutical Co., Ltd. (Tokyo, Japan). Unless specified, all the other reagents were obtained from Sigma-Aldrich Japan K. K. (Tokyo, Japan).

2.1.1 Ovary transport and COCs collection mediums.

The ovaries were transported in the laboratory in a 0.9% (w/v) saline solution supplemented with 75 mg/L of potassium penicillin G and 50 mg/L of streptomycin-sulphate at 25 °C (Funahashi, 2005). The medium used for the collection and the washing of the COCs was modified TL-HEPES-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2.0 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10.0 mM sodium lactate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 10.0 mM HEPES, 0.2 mM sodium pyruvate, 12.0 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 25 µg/mL gentamycin and 65 µg/mL potassium penicillin G (Funahashi et al., 1997).

2.1.2 In vitro maturation medium.

The medium used for IVM culture was a BSA-free chemically defined medium, porcine oocyte medium which was composed of (Research Institute for the Functional Peptides, Yamagata, Japan), this medium was supplemented with 50 µM β-mercaptoethanol (mPOM; Akaki et al., 2009), and it has been shown to support successful development to the blastocyst stage following IVF. This IVM medium has been showed to support successful development to the blastocyst stage following IVF (Yoshioka et al., 2008) and piglet production (Akaki et al., 2009).

2.1.3 Parthenogenesis and early embryo culture mediums.

The mediums used for the parthenogenetic activation of the oocytes were an Electroporation solution composed of 0.25 M mannitol, 0.5 mM HEPES, 100 µM $CaCl_2 \cdot 2H_2O$, 100 µm $MgCl_2 \cdot 6H_2O$ and 0.01% (w/v) polyvinylalcohol (PVA) at a pH of 7.2 (Patel et al., 2014).

The other medium used just after the parthenogenetic activation of the oocytes was a basic fertilization medium, Medium-199 with Earle's salts (Gibco, Grand Island, NY) modified with 3.05 mM D-glucose, 2.92 mM hemi-calcium lactate, 0.91 mM sodium pyruvate, 12 mM sorbitol, 75μg/L potassium penicillin G and 25 μg/L gentamicin at a pH of 7.4 (mM199; Funahashi and Day, 1993).

The medium used for early embryo culture was porcine zygote medium (PZM) which consisted in 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H2O$, 25 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 2 mM L(+)-lactic acid, 2 mM L-glutamine, 5 mM hypotaurine, 10 μg/mL gentamycin, 2% (v/v) BME amino acids solution, 1% (v/v) MEM non-essential amino acids solution and 3% (w/v) polyvinylalcohol.

All the mediums (except the Electroporation solution) were equilibrated overnight under paraffin oil at 39°C in a wet atmosphere of 5% CO_2 in air before their usage. The Electroporation solution was simply pre-warmed at 39°C overnight.

2.2 Animals

The animals used for obtaining the ovaries were prepubertal gilts which proceeded from farms in the Okayama prefecture which were sacrificed when their total corporal live weight was of approximately 110 kg in a local slaughterhouse (Okayama Prefectural Meat). The samples were obtained over four years and, as the pig is a continuous polyoestric species, the animals were continuously in different stages of the estrous cycle at the moment of the sacrifice.

2.3 Ovary collection and COCs selection

Ovaries from prepubertal gilts were collected at a local abattoir and placed in a 0.9% (w/v) NaCl solution supplemented with 75 mg/L of potassium penicillin G and 50 mg/L of streptomycin-sulphate. They were transported to the laboratory within 2 h at 25°C. On arrival, the ovaries were washed twice in new pre-warmed transport solution and they were classified according to their external morphology into two main groups: early follicular phase and late follicular phase ovaries.

- Early follicular phase ovaries included those ovaries which did not show signals of having hemorrhagic bodies, *corpora lutea* and/or *corpora albicans* visible on the surface.
- The ovaries classified as late follicular phase, were those with the presence of hemorrhagic bodies, *corpora lutea* and/or *corpora albicans* visible on their surface.

The late follicular phase ovaries were discarded, and they were not used for any experiment in this study. Ovaries which showed the presence of illnesses as the ones with the presence of cysts were also discarded for all the experiments (Fig 1.1).

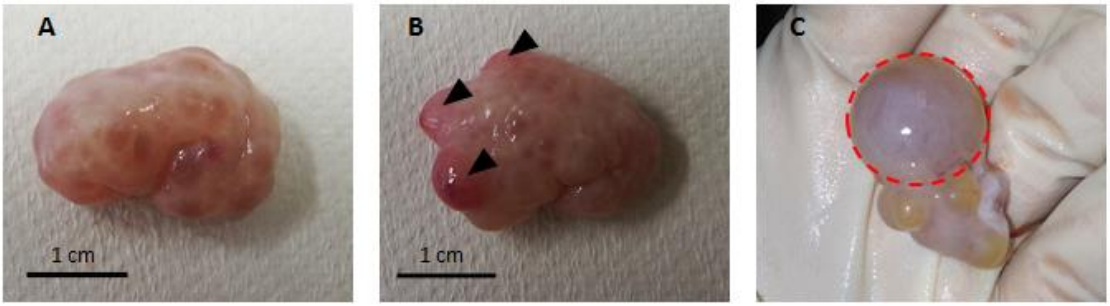


Fig. 1.1: Classification of ovaries according to the morphology of their cortex.
A. Early follicular phase ovary. The cortex looks pink without signs of having had an estrous cycle. B. Late follicular phase ovary, we can appreciate 3 corpora lutea (black arrowheads) on the surface. C. Ovary with a cyst on the surface (red circle).

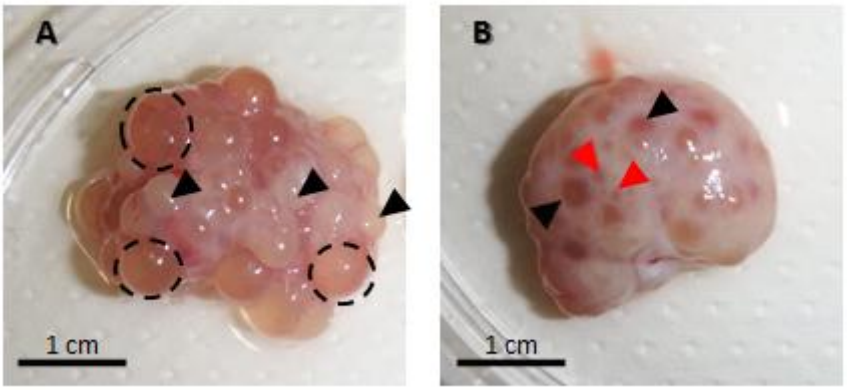


Figure 1.2: Ovaries with the presence of follicles with different diameters on their surface.
A: Ovary with big follicles (black circles) and medium follicles (black arrowheads) on the cortex.
B: Ovary with medium (black arrowheads) and small follicles (red arrowheads) present.

COCs were obtained from the follicles present in the surface of the ovaries. They were classified as small follicles (SF) if they had a diameter of < 3 mm, as medium follicles (MF) if their diameters were between 3-6 mm and as large follicles if their diameters were > 6 mm (Fig 1.2). For our in vitro maturation experiments, COCs were aspirated only from SF and MF by using a disposable 10 mL plastic syringe attached to an 18-gauge needle, separately. The pooled follicular contents were then placed in a 50 mL centrifuge tube and washed 3 times with TL-HEPES-PVA to remove any remaining follicular fluid and endogenous hormonal activity. COCs were selected only if the oocytes had a uniform ooplasm and were surrounded completely

by at least 3 layers of clear and compact cumulus cells. Those oocytes incompletely surrounded by CCs or those COCs in which the cumulus cells were already expanded were not used for our experiments (Fig 1.3).

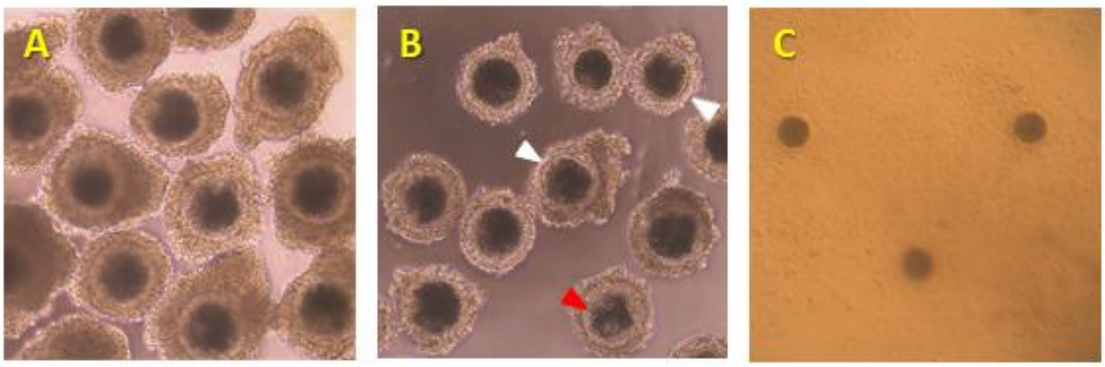


Figure 1.3: Different types of COCs according to the quality of the surrounding cumulus cells. A: Good quality COCs oocytes have a uniform dark cytoplasm and are surrounded by numerous layers of a clear compact cumulus cell mass. B: Low quality COCs, oocytes present areas without cumulus cells (white arrow head), and some oocytes have granulated cytoplasm (red arrow head). C: Oocytes surrounded by an expanded cumulus cell mass.

2.4 Routine protocol for in vitro maturation of COCs

Groups of approximately 50 COCs were cultured in a well of a 4-well dish (Nunclon Delta Surface, Thermo Scientific) with 500 µL mPOM supplemented with 1 mM dibutyryl cyclic AMP (dbcAMP), 10 IU/mL equine chorionic gonadotropin (eCG) and 10 IU/mL human chorionic gonadotropin (hCG) for 20 h at 39°C and 5% CO_2 in a wet atmosphere. After that, the oocytes were washed 3 times in pre-warmed mPOM and changed into a new dish with fresh pre-warmed medium without the hormone supplements and dbcAMP to continue maturation for another 24 h at 39°C and 5% CO_2 in air.

2.5 Parthenogenetic activation of the oocytes after in vitro maturation.

After oocyte IVM, the oocytes from the experimental groups were denuded of cumulus cells with a solution of 0.1% (w/v) hyaluronidase in TL-HEPES by pipetting with the help of a glass narrow-bore pipette. Then the oocytes were examined under a confocal microscope and they were only selected for parthenogenetic activation if they showed signs of nuclear maturation by the detection of the first polar body (1^{st} PB) extruded to the perivitelline space.

After selection, the mature oocytes were washed three times with 2 ml of Electroporation solution and the equilibrated oocytes were placed in a drop of electroporation medium between the two parallel wire electrodes of an electroporation chamber connected to a BTX electro cell manipulator ECM 2001M (BTX, San Diego, CA, USA) and a single DC pulse

of 120V for 30 µsec was given to the oocytes for their activation. Next, the oocytes were washed 3 times in mM199 supplemented with 0.4% BSA and 5 µM cytochalasin B at a pH of 7.8 under paraffin oil and they were kept in a drop of the same medium for 4 h at 39°C and 5% CO_2 in air. Finally, the oocytes were washed 3 times thoroughly with PZM medium and transferred to a drop of the same medium for embryo culture.

2.6 Early embryo culture

The oocytes were cultured for a total of 5 days in a drop of PZM under paraffin oil at 39°C and 5% of CO_2 in a wet atmosphere. Cleavage and blastocyst formation rates were observed at day 2 and day 5 after the start of culture. Non-cleaved oocytes were removed from the culture on day 2; and on day 5, the blastocysts present in the culture were fixed for at least 24 h in a drop of 4% PFA at 4°C, washed in a 1x PBS solution supplemented with 0.1% (w/v) PVA to avoid attachment to the dish, stained with Vectashield™ mounting medium with 4’,6-Diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) and finally observed under a fluorescence microscope for counting the number of cells in the blastocysts.

CHAPTER 3: Characterization of gilt's ovaries with different patterns in the follicular morphology and the meiotic competence of the oocytes.

3.1 Introduction

In many farm species, it is of general knowledge that the reproductive efficiency is affected by extrinsic factors (those that depend on the environment and the management) and intrinsic factors (those depending on the animal) (Rodríguez FPC, 2005). Amongst the extrinsic ones we can find environmental factors such as heat stress (Usui and Koketsu, 2005); nutritional factors (Peltoniemi and Virolainen, 2006); and those derived from the manipulation and housing conditions of the animals (Caton et al., 1986). In the factors that are considered intrinsic, we can distinguish factors such as the breed (Yen et al., 1987) and the age (Faillace et al, 1994) which affect greatly to the animal fertility.

Prepubertal animals suppose a potential important stock of ovarian follicles and oocytes, because their ovaries contain higher numbers of growing follicles than in pubertal ones (Reynaud et al., 2002). Gilts generally reach puberty between 150 and 220 days of age (Soede et al., 2011) but they are usually slaughtered before its onset, so their ovaries, which contain high numbers of developing follicles, can constitute a good source of oocytes for reproductive biotechnologies such as in vitro maturation (IVM), fertilization (IVF) and embryo production.

In the ovaries, the population of follicles shows non-homogenic characteristics. There are not only differences on their diameter sizes, but also in their hormonal, lipid and protein contents (Fahimiya et al., 2010). Amongst this heterogenic population, oocytes destined to IVM are mainly classified according to the size of the follicle of origin in small (SF; less than 3 mm in diameter) and medium follicles (MF; 3-6 mm in diameter). It has been observed that oocytes from MF are capable to achieve higher degrees of nuclear maturation and have improved developmental competence than those derived from SF in the pig (Marchal et al., 2002; Kohata. et al., 2013).

A way of optimizing reproduction protocols for reproductive biotechnologies in pigs could be performed by obtaining elevated numbers of high quality oocytes derived from MF in ovaries from prepubertal slaughtered animals. As the extrinsic and intrinsic factors of the abated animals are usually unknown to the investigators, the aim of the present study was to evaluate if we could find any differences in the numbers of the follicular populations of gilt ovaries when classified according to the morphology of their surface. We also intended to assess if any

differences in the maturation capacity of oocytes obtained from SF and MF of ovaries with different morphologies could be detected.

3.2 Materials and methods

3.2.1 Ovary collection

Ovaries were randomly collected from prepubertal gilts of approximately 110 kg of live body weight at a local abattoir, immediately separated from the reproductive tract and placed in a 0.9% (w/v) saline solution supplemented with 75 mg/L of potassium penicillin G and 50 mg/L of streptomycin sulphate and transported to the laboratory within 1 hour according to our laboratory routine protocol (Funahashi, 2005). On arrival, the ovaries were washed twice and only those ovaries which had no presence of corpora lutea on their surface were selected for use in this study.

3.2.2 Experiment 1: Ovarian weight, surface patterns and follicular population assessment

A total of 250 ovaries were trimmed from excess mesovaric tissue, blotted with a paper towel and individually weighted on an electronic digital scale (H120, Sartorius, Germany). According to the surface morphology, ovaries were classified into three types according to those previously described (Grasso et al., 1988); briefly; smooth surfaced ovaries (SSO) if the follicles did not protrude through the ovarian cortex thus presenting a smooth appearance, bubbled-like surface ovaries (BSO) if the follicles protruded strongly through all the ovarian surface, or mixed surface ovaries (MSO) if the ovaries showed a mixed pattern with some smooth appearance areas and some with protruding follicles (see Fig. 3.1). After that, follicular

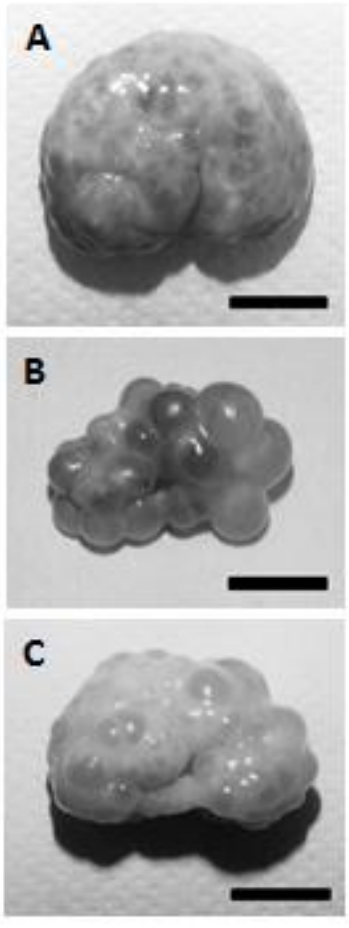


Figure 3.1: Three categories of porcine ovaries according to their surface morphology. A. Smooth surfaced ovary (SSO), the ovarian cortex presents a smooth appearance without follicles protruding through it. B. Bubbled surface ovary (BSO), the cortex is totally covered with follicles strongly protruding through it. C. Mixed surface ovary (MSO), the cortex presents some areas with protruding and non-protruding follicles. Scale bars represent 1 cm in all the pictures.

populations on the ovarian cortex were classified according to their diameter size in: small follicles (SF; < 3 mm in diameter), medium follicles (MF; 3 to 6 mm in diameter) and large follicles (LF; > 6 mm in diameter). To avoid counting a follicle more than once, following the count of a follicle, its contents were aspirated with a disposable syringe. When no follicles were visible on the ovarian surface, the ovaries were strongly pressed with a paper towel for removing the remaining follicular fluid and weighed for a second time to know the remaining ovarian weight. The difference between the ovarian weight with and without follicles represents the follicular fluid weight, as shown in a previous study (Edey et al., 1972).

3.2.3 Experiment 2: Oocyte maturation in vitro

For this experiment, we selected 30 SSO and MSO each time. Due to the low numbers of BSO specimens in the sample, we were unable to include this type of ovary in our experiment. Cumulus-oocyte complexes (COCs) were aspirated only from SF and MF separately. After washing 3 times with TL-HEPES-PVA, only groups of 50 COCs with a uniform ooplasm and completely surrounded by a compact cumulus cell mass of at least 3 layers were cultured in a well of a 4-well dish (Nunclon Delta Surface, Thermo Scientific) with 500 µL mPOM supplemented with 1 mM dibutyryl cyclic AMP (dbcAMP), 10 IU/mL equine chorionic gonadotropin (eCG) and 10 IU/mL human chorionic gonadotropin (hCG) for 20 h and then without eCG, hCG and dbcAMP for another 24 h at 39°C and 5% CO_2 in a wet atmosphere (Funahashi et al., 1997).

After the IVM, cumulus cells were completely removed by pipetting with 0.1% (w/v) hyaluronidase in TL-HEPES-PVA, the oocytes were fixed in a solution of 4 % (w/v) paraformaldehyde in PBS for 20 min at room temperature, washed with PBS and mounted on glass slides with Vectashield™ mounting medium with 4',6-Diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) to stain the nucleus. Meiotic status of the oocytes (see Fig. 3.2) was examined under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan).

3.2.4 Statistical analysis

All statistical analyses were performed with the IBM SPSS Statistics 21.0 for Windows software. Statistical analyses with 250 ovaries (Exp. 1) and 5 replicated trials (Exp. 2) were conducted with a one-way and two-way ANOVA tests, respectively. If the P value was inferior to 0.05 in ANOVA, a Tukey post-hoc test was performed. The data of the ovarian follicular numbers were subjected to an analysis with a Kruskal-Wallis test. Independently of the test used for the analysis, all the data in Exp. 1 are expressed as means ± SD and $P < 0.05$ was considered

to be statistically significant in all the cases. Before the statistical analysis, all the percentage data for the IVM experiment were subjected to arc-sine transformation if there were values > 90% or < 10%. All data were posteriorly transformed back into percentages for the tables and figures and are expressed as means ± SEM.

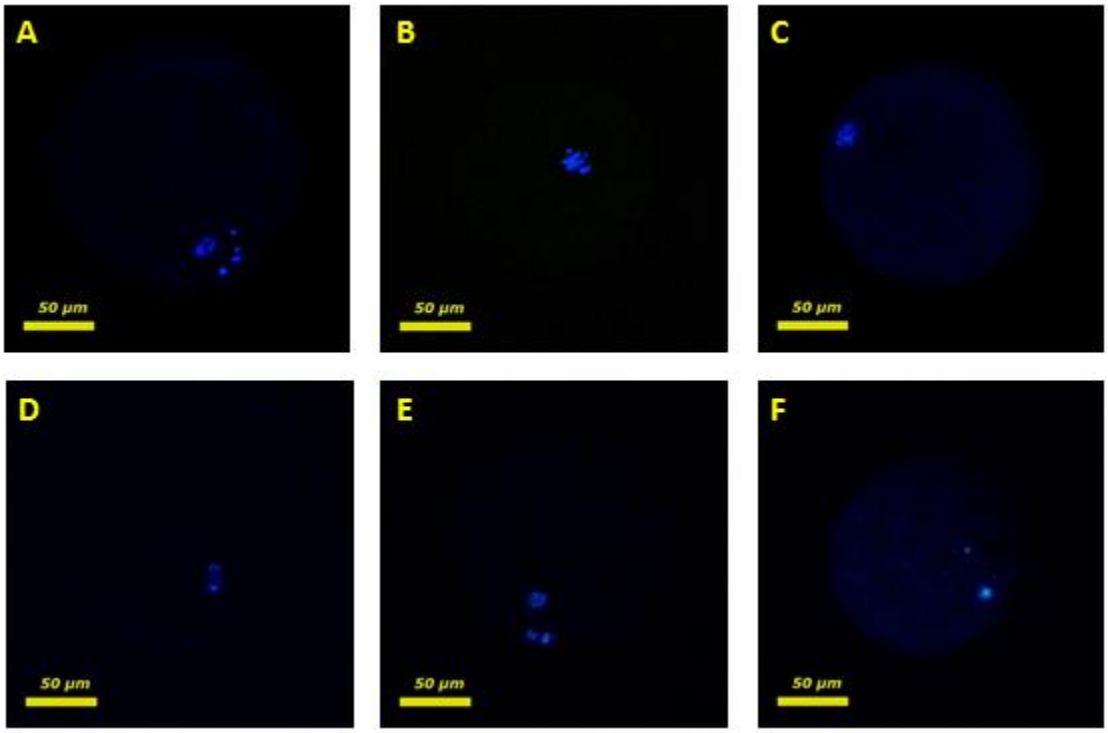


Figure 3.2: Nuclear morphologies of oocytes after 44 h of IVM culture.
A: GV stage oocyte. B: PM-I stage oocyte. C: M-I stage oocyte. D: A-T stage oocyte. E: M-II stage oocyte. F: Degenerated oocyte.

3.3 Results

3.3.1 Ovarian weight and ovarian surface patterns

A total of 250 ovaries were analyzed and classified into 3 groups according to the patterns of the follicles on their surface. Totally 114 (45.6%) ovaries were SSO, 120 (48.0%) were MSO and only 16 (6.4%) were BSO, being this last one the scarcest type of ovary. The mean ovarian weight of the sample was 3.728 g, and after follicle aspiration, the remaining ovarian weight was of only 2.072 g. When the ovary weight was assessed, MSO had a significantly higher mean weight as compared with the SSO and BSO (Table 3.1; 3.944 g vs. 3.586 g and 3.126 g, respectively), whereas the weight after follicular aspiration was significantly lower in BSO than SSO and MSO (Table 3.1; 1.452 g vs. 2.043 g and 2.182 g respectively).

Table 3.1: Weight of prepubertal porcine ovaries according to their surface morphological characteristics

Ovary type	Number (%)	Weight (g; Mean ± SD) of		
		Ovary	Ovary after aspiration	Follicular fluid
SSO	114 (45.6 %)	3.586 ± 1.167^{a}	2.043 ± 0.823^{a}	1.543 ± 0.050^{a}
MSO	120 (48.0 %)	3.944 ± 0.965^{b}	2.182 ± 0.69^{a}	1.762 ± 0.412^{b}
BSO	16 (6.4 %)	3.126 ± 1.257^{a}	1.452 ± 0.66^{b}	1.674 ± 0.166^{ab}
Total	250 (100 %)	3.728 ± 1.101	2.072 ± 0.77	1.657 ± 0.032

SSO: smooth surfaced ovary, MSO: mixed surfaced ovary, BSO: bubbled surfaced ovary.

[a,b] Different superscript letters indicate significant differences amongst the values present in the same column ($P < 0.05$).

Table 3.2: Number of follicles present in prepubertal porcine ovaries with different morphologies.

Ovary type	No. of ovaries examined	Total no. of follicles/ovary (mean ± SD)	Number (mean) of follicles (% ± SD)		
			SF	MF	LF
SSO	114	$128.4^{a} \pm 58.0$	116.6^{b} (87.2 ± 12.5)	11.0^{b} (12.0 ± 12.6)	0.8 (0.8 ± 2.1)
MSO	120	$153.6^{b} \pm 58.7$	138.8^{a} (88.2 ± 9.7)	14.2^{a} (11.1 ± 9.6)	0.6 (0.8 ± 2.7)
BSO	16	$113.6^{a} \pm 72.6$	94.4^{b} (73.5 ± 19.8)	18.4^{a} (24.7 ± 17.6)	0.8 (1.8 ± 4.5)
Total	250	139.6 ± 60.7	125.8 (86.8 ± 12.3)	13.0 (12.4 ± 12.1)	0.7 (0.8 ± 2.6)

SF, small follicles with less than 3 mm in diameter; MF, middle follicles with 3-6 mm in diameter; LF, large follicles with a diameter larger than 6 mm; SSO, smooth surfaced ovary; MSO, mixed surfaced ovary; BSO, bubbled surfaced ovary.

[a,b] Different superscript letters indicate significant differences amongst the values present in the same column ($P < 0.05$).

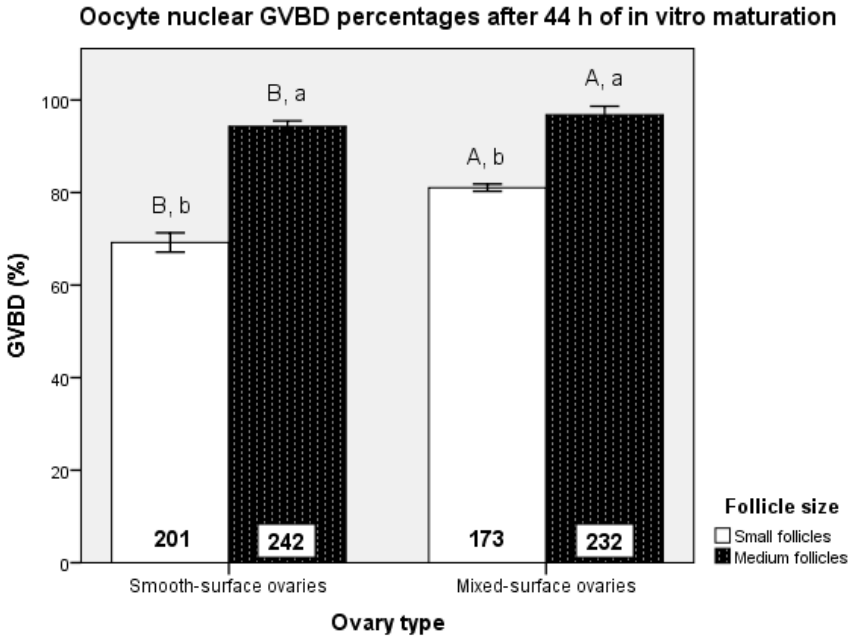
3.3.2 Follicular population

As shown in Table 3.2, the mean number of follicles per ovary was 139.6. When we analyzed these data according to the ovarian morphology type, the total number of follicles was significantly higher in MSO than in SSO and BSO (153.6 vs. 128.4 and 113.6 respectively). MSO also had a significantly higher number of SF per ovary than the SSO and BSO (138.81 vs 116.58 and 94.44, respectively). The MSO and BSO types had a significantly higher number of MF per ovary, although the percentage of MF was lower in MSO than that of BSO (11.1% vs. 24.7%), because MSO was also the type which possessed the most follicles.

3.3.3 Oocyte maturation in vitro

The two-way ANOVA analysis (Figure 3.2) showed that there were significant differences in the germinal-vesicle break down (GVBD) capacity between SSO and MSO oocytes, especially in those derived from SF. Independently of the ovary type, oocytes from MF always had a significantly higher rate of GVBD than those from SF. The meiotic stages of oocytes after IVM are shown in table 3. The analysis revealed that there were significant differences in the percentages of oocytes at the GV and M-II stages between the ovarian types and the follicular size was found to have a significant effect on all the oocyte nuclear stages.
When the type of ovary was taken into account, oocytes presented significant differences only in the incidence of GV and M-II nuclear configurations. The incidence of oocytes from MF and SF at the GV stage was significantly higher in SSO than in MSO (16.4% and 4.1% vs. 7.7% and 2.1% respectively). The analysis revealed also a significant effect of the ovarian type on the M-II oocyte achievement, being slightly higher in MSO than SSO in both, SF and MF (55.6% and 80.2% vs. 50.0% and 75.6% respectively). When the nuclear stages according to the follicular type were analyzed, oocytes at the GV stage always showed a significantly higher number when those were derived from SF (SSO: 7.7% vs. MSO: 16.4%) as compared with the MF derived ones (SSO: 4.1% vs. MSO: 2.1%). Significantly higher percentages of oocytes at the M-I stage and degenerated configurations were observed in oocytes from SF than those from MF, which in contrast, had significantly higher rates of oocytes at the M-II stage (SSO: 50.0% MSO: 55.6% vs. SSO: 75.6% MSO: 80.2%, respectively).

Figure 3.3: Percentages of GVBD oocytes derived from SF and MF of different morphologies of ovaries (SSO vs. MSO).



[a,b] Different superscripts indicate significant differences (P<0.05) between oocytes from SF and MF. [A,B] Different superscripts indicate significant differences (P<0.05) between oocytes collected from SSO and MSO.

Table 3.3: Nuclear stage of oocytes from SF and MF of smooth (SSO) and mixed surface ovaries (MSO) after 44 h of IVM culture.

Ovary type		Total No. of oocytes examined	Number (% ± SEM) of oocytes at the stage of:					
			GV	ProM-I	M-I	A-I/T-I	M-II	Deg
MSO	SF	248	19 (7.7 ± 0.7)^{Ba}	4 (1.6 ± 0.8)	55 (22.2 ± 3.2)^{a}	4 (1.6 ± 0.8)	138 (55.6 ± 3.2)^{Ab}	28 (11.3 ± 0.5)^{a}
	MF	248	5 (2.1 ± 1.1)^{Bb}	1 (0.4 ± 0.4)	35 (14.1 ± 1.6)^{b}	7 (2.8 ± 0.8)	199 (80.2 ± 1.8)^{Aa}	1 (0.4 ± 0.4)^{b}
SSO	SF	250	44 (16.4 ± 0.8)^{Aa}	4 (1.6 ± 1.0)	43 (16.8 ± 1.5)^{a}	9 (2.8 ± 0.8)	117 (50.0 ± 1.5)^{Bb}	31 (12.0 ± 3.2)^{a}
	MF	246	10 (4.1 ± 1.5)^{Ab}	2 (0.8 ± 0.5)	31 (12.6 ± 2.4)^{b}	13 (5.3 ± 0.5)	186 (75.6 ± 2.5)^{Ba}	4 (1.6 ± 0.8)^{b}

$^{AB, ab}$ Different superscripts indicate significant differences among the values in the same column ($P < 0.05$).

Result of 2-way ANOVA.

	GV	ProM-I	M-I	A-I/T-I	M-II	Deg
MSO vs SSO	< 0.01	-	0.179	-	< 0.05	0.368
SF vs MF	< 0.001	-	< 0.05	-	< 0.001	< 0.001
Interaction	0.361	-	0.549	-	0.835	0.339

3.4 Discussion

Ovaries from prepubertal gilts were classified into 3 types according to their cortex morphology characteristics. Our result of macroscopic classification was consistent with data collected from 105 days-old gilts in a previous report (Dufour et al., 1985). In the current experiment, porcine ovaries were collected from gilts slaughtered when they reached a corporal weight of approximately 110 kg, which is a common time for pork production in Japan. For reaching this weight, these gilts could be estimated around 165 days of age (Kusec et al., 2008), which approximately coincides with the time of puberty (Oxender et al., 1979). Here we demonstrated that SSO and MSO are majority of the morphology of ovaries in these gilts, and that the ratios were similar in both. In the present study, we also observed the mean weight of follicular fluid (1.657 $\pm$ 0.032 g) accounted for about 44% of the weight of the ovary (3.728 $\pm$ 1.101 g), whereas the weight was significantly lower than a previous report (Clark et al., 1982), probably because we aspirated follicular fluid from follicles only located in the cortex. In this experiment, we could characterize the morphology of porcine ovaries as COC-resource for embryo production in vitro.

In the present study, the mean number of follicles was 139.6 $\pm$ 60.7, and the value was similar to a previous study (Dufour et al., 1993) with Meishan gilts near puberty. This result shows that there are many follicles on the surface of ovaries. In addition, classification of the ovarian morphology allowed us to find that total number of follicles in MSO was significantly larger than those in SSO and BSO. The number of SF in MSOs was significantly larger than those of SSO and BSO, whereas the number of MF in MSO was larger than that of SSO, MSO and BSO and no differences could be detected in the numbers of LF. These results demonstrate the superiority of MSO in the number of SF and MF to SSO. Although Grasso et al. (1988) showed that the number of SF was lower in BSO than SSO and MSO, our result showed a similar trend, but not significant. Therefore, the ovarian cortex morphology seems to significantly affect the numbers of SF and MF in their cortex, and hence it may be a good characteristic to take into account for improving the efficiency of COC collection for IVM protocols.

Oocyte meiotic competence is known to be acquired gradually during the follicular growth, until it reaches the early antral stages (Armstrong et al., 2001). In the pig, follicles with 0.5 mm in diameter have a fully differentiated antrum, but the oocytes have not yet reached their final size. These small oocytes have very limited ability to initiate nuclear IVM (Motlik et al., 1984) because growing oocytes are unable of undergoing GVBD thus being meiotically incompetent (Szybek et al., 1972; Combelles et al., 2002). In the present study, we found that the meiotic competence of oocytes was significantly higher when the COCs were collected from

MSO than SSO, whereas the competence was significantly higher when the COCs were derived from MF than SF in both MSO and SSO. This result shows not only that MSO has a larger number of MF and SF as compared with SSO but also that the oocytes in MSO have a higher meiotic competence. According to a previous study (Dufour et al., 1985), the number of follicles with 2.05-3.56 mm in diameter was larger in MSO than SSO, whereas the number of those with 1.13-2.00 mm in diameter was smaller. Furthermore, a recent study by Töpfer et al. (2016) has demonstrated that those follicles with a diameter superior than 4 mm have better nuclear and cytoplasmic maturation capacities than those oocytes with a smaller diameter. In the present study, the follicles were considered to be MF if the diameter of follicles was 3-6 mm and SF if that was less than 3 mm. It is possible that the difference in the meiotic competence observed in this study, would be because the mean diameters in each criterion were smaller in SSO than MSO.

3.5 Conclusion

In conclusion, the total number of follicles (MF plus SF) was larger in MSO than SSO and BSO and the meiotic competence of oocytes was also higher when the COCs were collected from MSO than SSO. We propose that MSO can constitute a good source of oocytes for the improvement of the efficiency of the actual in vitro reproductive technologies. Further studies on the developmental competence of the oocytes and on oocyte viability and apoptosis should be performed to have a better insight on this matter.

CHAPTER 4: Effect of removing cumulus cells from porcine cumulus-oocyte complexes derived from small and medium follicles during in vitro maturation on the apoptotic status and meiotic progression of the oocytes.

4.1 Introduction

Usually pigs for pork production have been slaughtered before the onset of puberty in developed countries. The ovaries are a common resource of follicles and oocytes for their use in reproductive biotechnologies (Day and Funahashi, 1996; Funahashi and Day, 1997) and many studies have shown that oocytes in the cumulus-oocyte complexes (COCs) obtained from prepubertal gilts have significantly lower meiotic and cytoplasmic competence than those from cycling sows. For in vitro embryo production, routinely COCs from medium follicles (MF) with 3-6 mm in diameter of prepubertal gilt's ovaries are used, because oocytes from MF have a relatively higher rate of meiotic competence as compared with those from small follicles (SF; with less than 2 mm in diameter), though the number of MF in the ovary is significantly lower than that of SF (Bolamba et al., 1991; Knox, 2005; Marchal et al., 2002; Kohata et al., 2013).

The oocytes recovered from antral follicles are surrounded by multiple layers of cumulus cells (CCs) which are in deep communication with the oocyte via cytoplasmic projections that go through the zona pellucida and contact with the oolema via gap junctions forming a structure known as COC (Albertini et al., 2007). It has been well known that COCs not only secrete paracrine factors but they also interact through the gap junctions (passing molecules only smaller than 1 kDa, (Kidder and Mhawi, 2002) in a bi-directional manner, which is essential for oocyte nutrition, growth and regulation of meiotic progression (Hunter, 2000; Sanchez-Lazo et al., 2014; Gilchrist et al., 2004). Also, CCs contribute partially to maintain the oocyte into meiotic arrest by the production and transfer of cAMP via gap-junctions, which accumulates into the ooplasm (Mao et al., 2013). The communication through the gap-junctions between the oocyte and the CCs decreases progressively during IVM (Ozawa et al., 2008; Bagg et al., 2009). This disconnection between the cells seems to be responsible for meiotic resumption in fully grown oocytes, due to a reduction in the intracellular cAMP level (Thomas et al., 2004; Sela-Abramovich et al., 2006), which will activate CDC2/CDK1 and MAP kinases (Shimada et al., 2002; 2006), and consequently permit the oocytes to achieve maturation to the metaphase-II stage in vitro (Edry et al., 2006; Norris et al., 2009) and in vivo (Sela-Abramovich et al., 2006). In the pig, this communication between the oocyte and the CCs during the first 4 hours of IVM appears to be most important when the COCs were collected from MF (Sasseville et al., 2009).

However, there are no studies about if removing CCs or breaking off the communication between oocyte and CCs affects the nuclear maturation of oocytes derived from SF. Therefore, the aims of the present study were to analyze the viability and meiotic ability of porcine oocytes from SF and MF when they were denuded before, during or after IVM.

4.2 Materials and methods

4.2.1 Preparation of COCs

Ovaries without any evidences of corpora lutea were collected at a local abattoir and placed in a 0.9% (w/v) NaCl solution supplemented with 75 mg/L of potassium penicillin G and 50 mg/L of streptomycin-sulphate. They were transported to the laboratory within 2 h at 25°C. Cumulus-oocyte complexes were aspirated from SF (0.5-2 mm in diameter) and MF (3-6 mm in diameter) located on the ovarian surface using a disposable 10-mL syringe with an 18-gauge needle and collected into a 50-mL centrifuge tube. The pooled follicular contents were washed 3 times with modified TL-HEPES-PVA medium at room temperature to remove any traces of follicular fluid and thus any endogenous gonadotropic activity and growth factor activity. Thereafter, only COCs from SF and MF were selected if they had a uniform ooplasm and a compact cumulus cell mass of at least 3 layers of CCs.

4.2.2 In vitro maturation and decumulation of the oocytes.

Cumulus-oocyte complexes derived from SF and MF were randomly distributed into groups (30 to 35 COCs per group). One group from each, SF and MF, was used to analyze the apoptotic status and nuclear stage of the oocytes before IVM. The oocytes were denuded with TL-HEPES-PVA containing a 0.1% (w/v) hyaluronidase by using a glass narrow-bore pipette, and washed 3 times with TL-HEPES-PVA and then used for fluorescence assays.

The remaining groups of COCs from SF and MF were cultured separately in 300 µL of mPOM supplemented with 1mM dibutyryl cyclic AMP (dbcAMP), 10 IU/mL eCG and 10 IU/mL hCG for 20 h at 39°C in an atmosphere of 5% CO_2 in air. The COCs were then washed 3 times in equilibrated mPOM without the dbcAMP and gonadotropins, transferred to 300 µL of the same medium and continued culture for an additional 24 h at 39°C in an atmosphere of 5% CO_2 in air. Before (0 h) and after the start 20 h of IVM, the oocytes from SF and MF COCs were denuded as described above and continued the IVM culture after washing three times with mPOM. At the end of IVM culture (totally 44 h from the start), the remaining oocytes were also denuded, washed 3 times and then used for the fluorescence assays.

4.2.3 Fluorescence assays for the apoptotic status, viability and meiotic progression of oocytes

Denuded oocytes were processed for an apoptotic/viability assay before (0 h) and after IVM for totally 44 h of culture, according to the manufacturer's instructions by using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, USA). Briefly, after washing 3 times with cold PBS, the denuded oocytes were incubated in a 100 µL drop of 1x Annexin-binding buffer, in which 5 µL of Annexin V conjugate and 2 µL of 100 µg/mL propidium iodide were added, at room temperature for 15 min. After washing twice with 1x Annexin-binding buffer, the oocyte specimens were mounted on a glass slide with Vectashield™ mounting medium with 4',6-Diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA). The slide was immediately observed under a fluorescence microscope with excitation (450-490 nm) and emission (520 nm) filters (Nikon eclipse 80i, Tokyo, Japan).

For the apoptosis/viability assessment, oocytes were considered as normal if they showed no fluorescence, in a state of early apoptosis if they showed a partial green fluorescence in the oolema, in a state of late apoptosis if they showed a strong green fluorescence which surrounded the oolema forming a complete fluorescent circle and dead if they had a positive red signal of propidium iodide in the chromatin or in the cytoplasm, respectively (see Fig. 4.1).

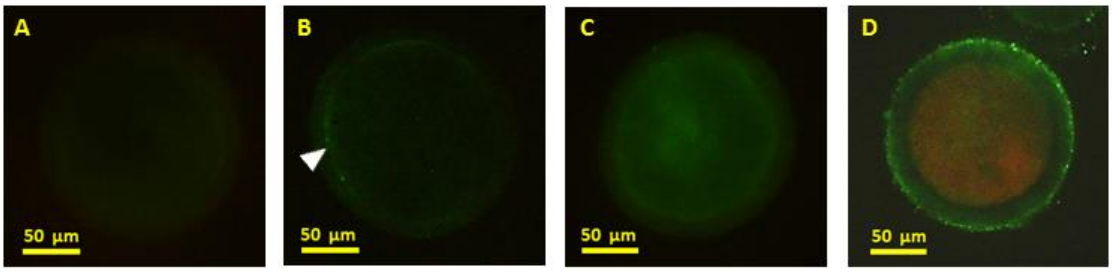


Figure 4.1: Apoptotic status of the oocytes stained with Annexin V/PI.
A: Normal live oocyte, there is no fluorescence in the cell. B: Early apoptotic oocyte with the membrane partially stained green (white arrowhead). C: Late apoptotic oocyte, all the membrane is strongly stained in green. D: Dead oocyte. The cytoplasm is stained in red.

For the assessment of meiotic progression, the oocytes were classified by the morphology of DAPI fluorescence. For the oocytes analyzed before IVM culture, the morphology of germinal vesicle was determined according to the modified criteria of previous reports (Motlik and Fulka, 1976; Funahashi et al., 1997). Briefly, GV_0 stage is that characterized by the presence of a nucleolus surrounded by filamentous chromatin which occupies all the nuclear space, the GV_I stage is that with the presence of a nucleolus surrounded by chromatin in

the form of a ring or a horseshoe, the GV_{II} stage is the one with the presence of a nucleolus as a ring or horseshoe and a few well stained clumps located near it, the GV_{III} stage is characterized

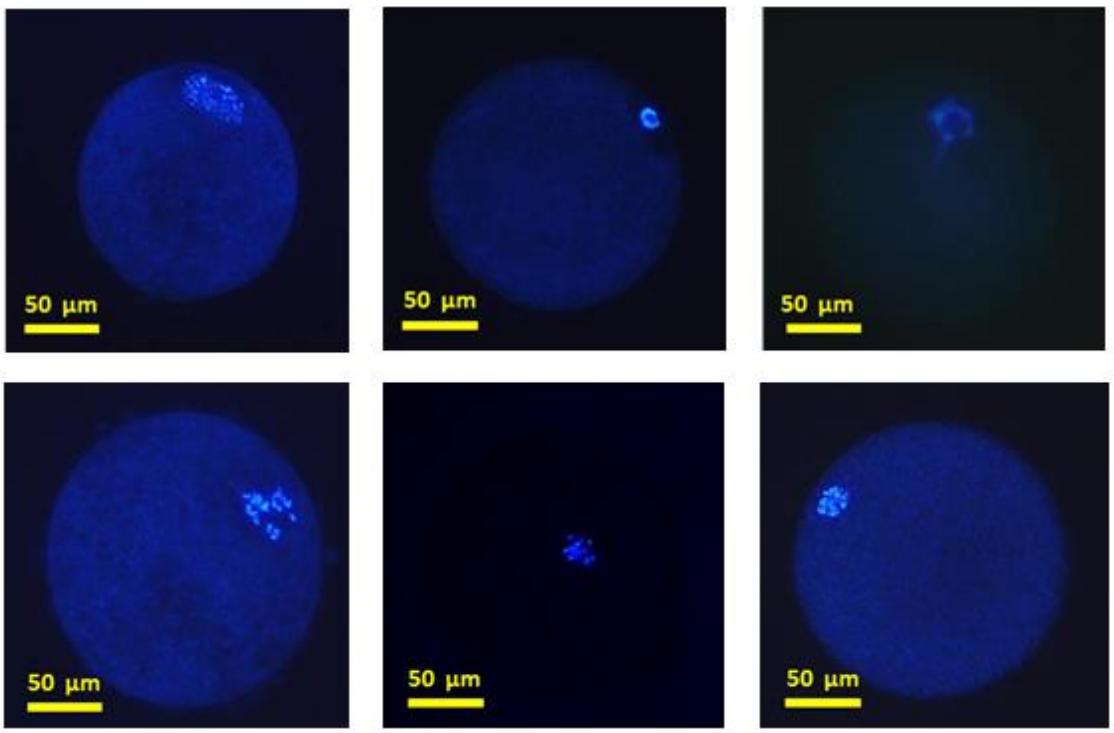


Figure 4.2: Nuclear morphologies of GV stage oocytes.
A: GV_0 stage oocyte. B: GV_I stage oocyte. C: GV_{II} stage oocyte. D: GV_{III-IV} stage oocyte. E: PM-I stage oocyte. F: M-I stage oocyte.

by the presence of a nucleolus surrounded by an irregular network of filamentous bivalents and clumps in the whole area of the GV, and the GV_{IV} stage is characterized by the presence of filamentous bivalents and no nucleolus or by the presence of diakinesis chromatin (See figure 4.2). After IVM culture, mature oocytes at the metaphase-II stage were classified into two groups according to the spindle morphology. Oocytes were considered as having a normal spindle if the revolver configuration could be seen, and as having an abnormal configuration if the spindle DNA appeared highly compacted or fragmented (see Fig. 4.3).

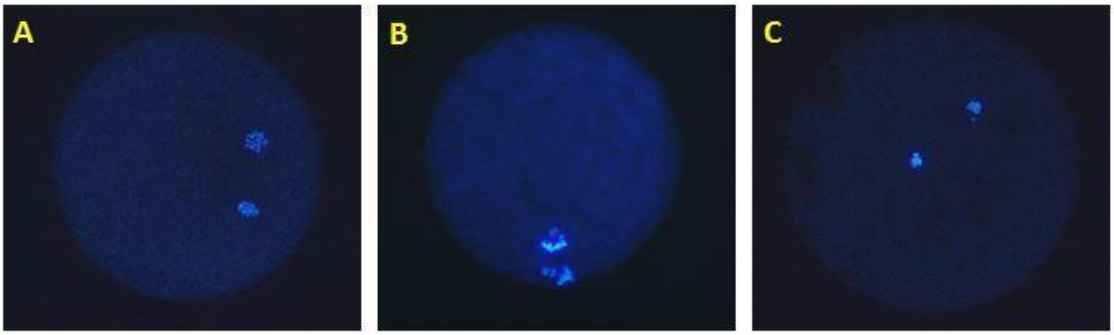


Figure 4.3: Nuclear morphologies of MII stage oocytes.
A: Normal M-II stage oocyte, the revolver configuration can be seen clearly. B-C: Abnormal configurations of the MII spindle. Chromosomes are disorganized (B) or highly compacted (C).

4.2.4 Statistical Analysis

Data obtained from 5 replicated trials were processed by one-way or two-way ANOVA using StatView 4.0 statistical software (Abacus Concepts, Inc., Berkeley, CA). All the data were expressed as mean ± SEM. Findings were considered to be significantly different at $P<0.05$, and when there was a significant effect, values were compared with a Tukey's multiple comparisons test.

4.3 Results

4.3.1 Apoptotic status, viability and nuclear stage of oocytes before IVM

As shown in Table 4.1, there were no significant differences in the apoptotic status and viability between oocytes derived from SF and MF just after collection from ovaries. However, when the nuclear stage of the oocytes was examined (Table 4.2), the incidence of oocytes at the GV_0 stage was significantly higher in those from SF (24.8 ± 2.4%) than MF (3.3 ± 1.4%), whereas the incidence of GV_I stage oocytes was the opposite, being significantly higher in MF (57.8 ± 5.9%) than SF (22.7 ± 4.4%). No significant differences were observed in any of the other nuclear stages between the MF and SF oocytes.

Table 4.1: Apoptotic status of oocytes just after collection of the COCs from SF and MF.

Origin of COCs	**No. of oocytes examined**	**No. (% ± SEM) of oocytes at the status of:**			**No. (% ± SEM) of dead oocytes**
		Normal	Early apoptotic	Late apoptotic	
SF	198	78 (39.4 ± 3.8)	99 (49.8 ± 5.5)	10 (5.2 ± 3.0)	11 (5.6 ± 2.6)
MF	191	81 (42.6 ± 6.3)	103 (53.6 ± 7.4)	5 (2.8 ± 1.8)	2 (1.0 ± 0.6)

Experimental data were obtained from trials replicated 5 times.

4.3.2 Viability, nuclear stage of the oocytes after 44 h of IVM and MII spindle quality.

When the apoptotic status of the oocytes was assessed, the status was not different between oocytes from SF and MF, but it was affected by the time when the oocytes were denuded (Table 4.3). The percentage of intact oocytes was significantly higher when CCs were removed after 20 h (39.7 ± 2.8% in SF oocytes and 45.4 ± 3.1% in MF oocytes) or 44 h (39.3 ± 3.9% in SF oocytes and 36.8 ± 3.3% in MF oocytes) after the start of IVM, as compared with when the CCs were removed 0 h after the start of IVM (17.7 ± 3.8% in SF oocytes and 22.2 ± 2.3% in MF oocytes). Denuding the oocytes 20 h after the start of IVM did not affect the

incidence of the oocytes at early apoptotic stage (57.4 ± 1.9% in SF oocytes and 52.8 ± 2.6% in MF oocytes) as compared with controls (when oocytes were denuded 44 h after the start of IVM; 57.2 ± 4.0% in SF oocytes and 62.6 ± 3.4% in MF oocytes), whereas denuding the oocytes at the start of IVM significantly increased the incidence of early apoptotic oocytes in the same origin of COCs (74.3 ± 5.4% in SF oocytes and 69.4 ± 3.4% in MF oocytes). The percentage of late apoptotic oocytes was also significantly higher when oocytes were denuded before IVM (7.4 ± 2.5% in SF oocytes and 6.7 ± 3.3% in MF oocytes), as compared with when the oocytes were denuded 20 h (2.9 ± 1.3% in SF oocytes and 1.8 ± 0.8% in MF oocytes) and 44 h after the start of IVM (3.5 ± 1.7% in SF oocytes and 0.6 ± 0.6% in MF oocytes).

When the nuclear stages of oocytes were examined 44 h after the start of IVM, the incidence of oocytes at the metaphase-II stage was significantly affected by both, the origin of the COCs and by the time when the oocytes were isolated from the COCs (Table 4.4). In both, oocytes derived from SF and MF, denuding the oocytes 20 h after the start of IVM significantly increased the percentage of mature oocytes 44 h after the start of IVM (65.4 ± 2.3% in SF oocytes and 83.1 ± 1.4% in MF oocytes), whereas denuding 0 h after the start of IVM significantly decreased their incidence (27.9 ± 3.6% in SF oocytes and 32.3 ± 2.5% in MF oocytes), as compared with control oocytes which were denuded at the end of IVM for 44 h (41.0 ± 2.1% in SF oocytes and 68.5 ± 1.9% in MF oocytes). As shown in Table 4.5, however, the percentage of oocytes with a morphologically normal spindle at the metaphase-II stage was significantly higher in those derived from MF (91.5 ± 1.8%) than SF (70.7 ± 5.4%) when the CCs were not removed until the end of IVM. In both SF and MF oocytes, the percentage was significantly lower when oocytes were denuded 20 h after the start of IVM (66.8 ± 3.9% and 73.0 ± 3.0%, respectively). Regardless of the type of follicle the oocytes were derived from, SF and MF, the incidence of oocytes with a normal morphological spindle at the metaphase-II stage was lowest when the oocytes were denuded just before the start of IVM culture.

Table 4.2: Nuclear stage of live oocytes just after collection of the COCs from SF and MF.

Origin of COCs	No. of oocytes examined	No. (% ± SEM) of live oocytes at each nuclear stage before IVM					
		GV_0	GV_I	GV_{II}	GV_{III-IV}	ProM-I	Deg.
SF	187	46 (24.8 ± 2.4)[a]	43 (22.7 ± 4.4)[a]	24 (12.9 ± 2.3)	72 (38.5 ± 4.1)	-	2 (1.1 ± 1.1)
MF	189	6 (3.3 ± 1.4)[b]	109 (57.8 ± 5.9)[b]	18 (9.6 ± 1.5)	55 (28.7 ± 6.0)	1 (0.5 ± 0.5)	-

[a, b] Different superscript letters indicate significant differences between the values in the same column ($P < 0.05$).

Experimental data were obtained from trials replicated 5 times.

Table 4.3: Effect of removal of cumulus cells from COCs derived from SF and MF before or during IVM on the apoptotic status of oocytes at the end of IVM

Origin of COCs	Time (h) when CCs were removed	No. of oocytes examined	No. (% ± SEM) of oocytes in the status of:			No. (% ± SEM) of dead oocytes
			Normal	Early apoptotic	Late apoptotic	
SF	0	174	31 (17.7 ± 3.8)[a]	129 (74.3±5.4)[a]	13 (7.4 ± 2.5)[a]	1 (0.6 ± 0.6)
	20	171	68 (39.7 ± 2.8)[b]	98 (57.4 ± 1.9)[bc]	5 (2.9 ± 1.3)[b]	-
	44	173	68 (39.3 ± 3.9)[b]	99 (57.2 ± 4.0)[bc]	6 (3.5 ± 1.7)[b]	-
MF	0	170	38 (22.2 ± 2.3)[a]	118 (69.4 ± 3.4)[ab]	11 (6.7 ± 3.3)[a]	1 (0.7 ± 0.7)
	20	165	75 (45.4 ± 3.1)[b]	87 (52.8 ± 2.6)[c]	3 (1.8 ± 0.8)[b]	-
	44	168	62 (36.8 ± 3.3)[b]	105 (62.6 ± 3.4)[abc]	1 (0.6 ± 0.6)[b]	-
Significant effect(P-values)						
	Origin of COCs		0.34	0.64	0.33	-
	Time when CCs were removed		<0.001	<0.001	<0.05	-

[a,b,c] Different superscript letters indicate significant differences between the values in the same column ($p < 0.05$).

Experimental data were from trials replicated 5 times.

Table 4.4: Effect of removal of CCs from COCs derived from SF and MF before and during IVM of COCs on meiotic competence of the oocytes 44 h after the start of IVM.

Origin of COCs	Time (h) when CCs were removed	No. of oocytes examined	No. (% ± SEM) of oocytes at the meiotic stage of:					
			GV	ProM-I	M-I	A-I/T-I	M-II	Deg.
SF	0	173	82 (47.3 ± 3.9)[a]	8 (4.7 ± 1.2)	9 (5.2 ± 1.4)[ab]	8 (4.5 ± 2.8)	48 (27.9 ± 3.6)[a]	17 (9.9 ± 2.4)[a]
	20	171	38 (22.2 ± 2.8)[b]	4 (2.34 ± 1.5)	4 (2.3 ± 0.6)[a]	6 (3.5 ± 1.1)	112 (65.4 ± 2.3)[b]	7 (4.1 ± 1.5)[a]
	44	173	61 (35.2 ± 1.3)[c]	5 (2.9 ± 1.3)	21 (12.2 ± 2.4)[b]	7 (4.1 ± 1.5)	71 (41.0 ± 2.1)[c]	8 (4.6 ± 1.1)[a]
MF	0	169	75 (44.7 ± 3.1)[a]	10 (5.9 ± 1.5)	12 (6.8 ± 2.6)[ab]	7 (4.2 ± 1.2)	55 (32.3 ± 2.5)[a]	8 (4.7 ± 1.1)[a]
	20	165	17 (10.3 ± 1.1)[d]	1 (0.6 ± 0.6)	3 (1.8 ± 1.2)[a]	5 (3.0 ± 0.1)	137 (83.1 ± 1.4)[d]	2 (1.2 ± 0.7)[b]
	44	167	18 (10.7 ± 1.1)[d]	6 (3.5 ± 1.1)	19 (11.4 ± 2.3)[b]	6 (3.7 ± 1.5)	115 (68.5 ± 1.9)[b]	2 (1.1 ± 0.7)[b]
Result of 2-way ANOVA (P-values)								
Origin of COCs			< 0.001	0.970	0.938	0.756	< 0.001	< 0.05
Time when CCs were removed			< 0.001	0.184	< 0.001	0.795	< 0.001	< 0.005

[a, b, c, d] Different superscript letters indicate significant differences between the values in the same column ($p < 0.05$).
Experimental data were obtained from trials replicated 5 times.

Table 4.5: Effect of removal of CCs during IVM on the normal morphology of metaphase spindle in the mature oocytes.

Origin of COCs	Time (h) when CCs were removed	No. of mature oocytes examined	No. (% ± SEM) of oocytes with a morphologically normal MII spindle
SF	0	48	32 (56.6 ± 2.4)[a]
	20	112	75 (66.8 ± 3.9)[b]
	44	71	50 (70.7 ± 5.4)[c]
MF	0	55	31 (56.0 ± 3.8)[a]
	20	137	100 (73.0 ± 3.0)[c]
	44	115	105 (91.5 ± 1.8)[d]

Result of 2-way ANOVA

Origin of COCs	< 0.05
Time when CCs were removed	< 0.001

[a, b, c, d] Different superscript letters indicate significant differences between the values in the same column ($p < 0.05$).

Experimental data were obtained from trials replicated 5 times.

4.4 Discussion

In the present study we found that, at the beginning of IVM, the apoptotic status of COCs was not different between those derived from SF and MF, whereas the percentage of oocytes at the GV_0 stage was significantly higher in those derived from SF than MF, as described previously (Funahashi et al., 1997). It has been reported that the meiotic and developmental oocyte competences are significantly lower when the COCs were collected from SF, as compared with MF (Marchal et al., 2002; Kohata et al., 2013). Our results suggest that the lower competence of the oocytes derived from SF could be due to the differences in the morphological GV stages, but not in the apoptotic status at the start of IVM.

We also found in this study that the time when CCs were removed from COCs during IVM significantly affected the apoptotic status of the oocytes at the end of IVM, but the origin of the COCs did not affect it. In the present study, although we did not observe any reductions in the incidence of live oocytes by denuding during IVM, which is different from results presented recently (Lin et al., 2015), removing CCs before the start of IVM significantly decreased the incidence of intact oocytes and increased the incidence of early and late apoptotic ones. Cumulus cells seem to act as a barrier, which protects the oocytes from in vitro induced lipotoxic effects, such as reactive oxygen species formation, caspase 3 activation, and mitochondria deterioration (Lolicato et al., 2015). Denuding cumulus cells has been known to severely damage oocytes, leading to their apoptosis or degeneration in rabbits (Lu et al., 2010). On the other hand, in the present study, any differences in the state of apoptosis of oocytes were not observed when the CCs were removed 20 h and 44 h after the start of the culture. The current results, therefore, demonstrate that denuding oocytes at the first half of IVM (20 h after the start of IVM), at least, can maintain the oocytes intact in relation to apoptosis.

In the present study, the percentage of oocytes matured to the metaphase-II stage was significantly affected not only by the origins of the COCs but also by the time when CCs were removed from the COCs. Although the maturation rate was lower in oocytes derived from SF than MF, we found that the denudation of oocytes at 20 h after the start of IVM significantly increased the maturation rate of the oocytes from both, SF and MF. Bidirectional communication between the oocytes and the surrounding somatic cells is required for the maintenance of the meiotic arrest in mammalian oocytes (Wigglesworth et al., 2013). It has been known that the C-type natriuretic peptide – natriuretic peptide receptor 2 signaling pathway is involved in meiotic arrest and cumulus oophorous formation in mice (Zhang et al., 2010; Kiyosu et al., 2012) and porcine oocytes (Santiquet et al., 2014). Oocyte-derived paracrine factor (Hiradate et al., 2014) and estradiol (Zhang et al., 2011) seem to participate in the regulation and expression of natriuretic peptide receptor 2 in cumulus cells. The present

results demonstrate that disruption of the communication between CCs and oocyte 20 h after the start of IVM is effective even in COCs derived from SF to induce the resumption of meiosis arrest and maturation to the metaphase-II stage.

However, the percentage of oocytes having a normal metaphase-II spindle was affected not only by the COCs precedence but also the time when the surrounding CCs were removed from the COCs. The percentage was significantly higher in oocytes from MF than SF and also in oocytes denuded after the IVM culture. A recent paper showed that the percentages of mature oocytes having a normal spindle, as well as the blastocyst formation rate, were significantly lower in oocytes denuded 22 h than 44 h after the start of IVM (Lin et al., 2015). The metaphase-II spindle assembly has been reported to be improved by elevated intra-ooplasmic ATP levels following either COC preincubation or ATP microinjection into the oocytes prior to IVM (Xu et al., 2014).

4.5 Conclusion

The current results obtained in this study suggest that disruption of the communication between CCs and the oocyte 20 h after the start of IVM may have a detrimental effect on the intra-ooplasmic content of ATP. Our results also suggest that secretion(s) from CCs and/or communication through the gap-junctions between CCs and the oocyte are required to obtain a morphologically normal spindle after IVM. Further studies will be required to elucidate if the factor carried into the oocyte from CCs and/or an increased ooplasmic ATP content somehow can improve the spindle quality of the oocytes derived from SF.

CHAPTER 5: Effect of removing cumulus cells from porcine COCs derived from small and medium follicles during IVM in the presence or absence of GDF9 or VEGF on the meiotic and developmental competences of the oocytes.

5.1 Introduction

Porcine ovaries used for experimentation purposes are usually procured from commercial slaughterhouses resulting in the obtainment of a sample with highly heterogenic characteristics that will affect oocyte quality (Ding and Foxcroft, 1992). Many strategies have been developed to improve oocyte maturation, fertilization and embryo development during the past years (Zhang et al, 2012). Some of these strategies for improving oocyte maturation consist in using factors present in the follicular environment for somehow mimic the in-vivo conditions of the follicle. Some factors secreted from oocytes and CCs have been commonly used as supplements in the in vitro maturation mediums.

Growth differentiation factor 9 (GDF9) is a member of the transforming growth factor-β (TGF-β) superfamily. In follicles, it is mainly expressed in the oocyte, and the expression is necessary for female fertility (Juengel and McNatty, 2005; Hanrahan et al., 2004). When this factor is added to granulosa or cumulus cells in vitro, it can mimic oocyte control on their actions by the activation of the SMAD cascade pathways that regulate proliferation, cumulus expansion and apoptosis (Gilchrist et al., 2004a; 2004b). The IVM treatment of COCs with GDF9, improves their capacity to develop to the blastocyst stage and their embryo quality (Hussein et al., 2006; Yeo et al., 2008).

Vascular endothelial growth factor (VEGF) was originally identified as a tumor-secreted protein, known for its ability to increase vascular permeability which results in the extravasation of proteins and other molecules (Dvorak et al., 1995). VEGF regulates angiogenesis in the developing ovarian follicles (Tempel et al., 2000; Barboni et al., 2000) and can also interact with the receptors present on the surface of the follicular somatic cells acting as a mitogenic factor in developing follicles (Bruno et al., 2009; Greenaway et al., 2004). The expression and production of this protein are both upregulated as the follicle develops (Shimizu et al., 2003; Greenaway et al., 2004). Supplementation of VEGF protein in the oocyte IVM medium has been reported to have a stimulating effect by improving the maturation and developmental competence capacity in the pig (Kere et al., 2014).

Oocytes are usually matured in vitro in the form of cumulus-oocyte complexes (COCs). These structures consist in an oocyte surrounded by several layers of cumulus cells that communicate with the central oocyte via gap junctions (Albertini et al., 2001) and can transfer small molecules and ions to the oocyte cytoplasm through them (Huang and Wells, 2010). Amongst these molecules, the continuous flow of high concentrations of cyclic adenosine 3'-5' monophosphate (cAMP) into the oocyte cytoplasm is believed to maintain the oocyte meiotic arrest and that a transient increase of the cytoplasmic cAMP levels is associated with the initiation of meiotic resumption in the pig (Chaube SK, 2000). Several studies have shown that the interruption of the communication via gap junctions by using inhibitors (Mao et al., 2013) or just by the removal of the cumulus cells during IVM culture in the presence of gonadotropins can induce meiotic resumption of the oocytes (Sasseville et al., 2009).

The objective of this study was to evaluate the effect of CCs removal on different times during IVM of prepubertal gilt oocytes from SF and MF and to evaluate their quality by the evaluation of their developmental competence capacity and blastocyst formation ability after parthenogenetic activation. In addition, it was evaluated if the exogenous supplementation of GDF9 or VEGF into the culture medium was able to further improve the nuclear maturation of the denuded oocytes.

5.2 Materials and methods

5.2.1 Ovary transport and COCs collection.

Oocytes were obtained from prepubertal gilts at a local slaughterhouse and brought into the laboratory into a 0.9% (w/v) saline solution supplemented with 75 mg/L of potassium penicillin G and 50 mg/L of streptomycin-sulphate within 2 h at 25°C (Funahashi, 2005).

Only ovaries without any visible *corpora lutea* were used for this experiment. Cumulus-oocyte complexes (COCs) were aspirated from small follicles (SF; < 3 mm in diameter) and medium follicles (MF; 3-6 mm in diameter) located on the ovarian surface using a disposable 18-gauge needle and collected into a 50 mL centrifuge tube separately. The pooled follicular contents were washed 3 times with modified TL-HEPES-PVA medium at room temperature, and only COCs with a uniform ooplasm and with at least 3 layers of clear and compact cumulus cells were selected for each type.

5.2.2 Experiment 1: Effect of the removal of cumulus cells during in vitro maturation on oocyte nuclear maturation.

SF and MF COCs were randomly distributed into groups (30-35 COCs per group) and cultured separately in a 300µL drop of mPOM supplemented with 1 mM dibutyryl cyclic AMP (dbcAMP), 10 IU/mL eCG and 10 IU/mL hCG under paraffin oil for 20 h at 39 °C in an atmosphere of 5% CO_2 in air. Then, the COCs were washed 3 times in equilibrated mPOM without the dbcAMP and the gonadotropins and transferred into a new dish with a 300 µL drop of the same medium and continued culture for another 24 h in the same conditions. At 0, 10, 20, 32 and 44 h of IVM the CCs were removed with a 0.1% (w/v) hyaluronidase solution and a glass narrow-bore pipette and, after washing 3 times in equilibrated mPOM solution, the denuded oocytes continued to culture. At the end of IVM (after 44 h), denuded oocytes were mounted on glass slides, fixed in 0.25% (v/v) acetic acid in ethanol solution for at least 72 h, stained with 1% (w/v) orcein in acetic acid solution and the nuclear configurations were evaluated under a phase-contrast microscope according to the criteria of Motlik and Fulka (1976; see Fig. 5.1).

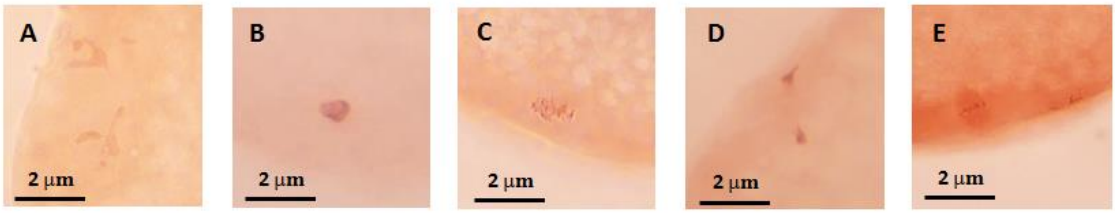


Figure 5.1: Oocyte nuclear stages after 44 h of IVM when stained with orcein.
A: GV stage oocyte, the DNA is disorganized inside the nuclear envelope; B: PM-I stage oocyte, the DNA looks highly compacted and there is no nuclear envelop; C: M-I stage oocyte, the DNA is organized into a configuration revolver-like; D: A/T stage oocyte, the chromosomes are being separated by the microtubules in opposite ways; E: MII stage oocyte, there is a meiotic spindle smaller than the one in MI stage accompanied with a polar body.

5.2.3 Experiment 2: Effect of the supplementation of GDF9 on meiotic progression of oocytes

Groups of approximately 30 COCs from SF and MF were cultured in a 300 µl drop of mPOM (as described in experiment 1) with or without 100 ng/mL GDF9 (Biovision, Milpitas, CA, USA) at 39 °C and 5% CO_2 in air for 20 h in the presence of dbcAMP, eCG and hCG. After the first period of IVM, CCs surrounding oocytes were removed as described previously and the denuded oocytes continued culture for IVM with or without GDF9 in the absence of dbcAMP and gonadotropins in the same medium for another 24 h. At the end of IVM culture, all oocytes were denuded and the oocytes were fixed at room temperature for 20 min in a solution of 4% (w/v) PFA in PBS solution and meiotic progression of the oocytes was examined after DAPI staining under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan).

5.2.4 Experiment 3: Effect of the supplementation of VEGF on oocyte in vitro maturation

As in the previous experiments, groups of approximately 30 COCs from SF and MF were cultured in a 300 µl drop of mPOM in the presence of dbcAMP, eCG and hCG for 20 h at 39 °C in a wet atmosphere of 5% CO_2 in air. During this first half of the culture, the medium was supplemented or not with a final concentration of 200 ng/mL of VEGF (V4512-5UG, Sigma-Aldrich), concentration that has been proven to promote maturation of porcine oocytes from SF in our laboratory (Bui et al., 2015). After the first half of the IVM protocol, oocytes were denuded and they continued the culture in a new pre-warmed drop of mPOM without any supplements. As in experiment 2, after the IVM period had finished; all the oocytes were denuded, fixed and stained with DAPI for the evaluation of the nuclear status under a fluorescence microscope.

5.2.5 Experiment 4: Developmental competence of oocytes denuded during IVM

Groups of 60 (30 COCs/dish) COCs were cultured for IVM in a 300 µL drop of mPOM and the oocytes were denuded at 0, 20 and 44 h of IVM as explained in experiment 1. At the end of the IVM culture, after CCs were removed from the remaining COCs, the cumulus-free oocytes were observed under a stereomicroscope for the presence of the first polar body (PB) extruded in the perivitelline space and only those with PB were selected for the parthenogenetic activation.

The selected oocytes were washed 3 times with electroporation solution (pH 7.2) and then transferred in a drop of the same medium situated between the electrodes of an activation chamber. A single electrical pulse (DC: 1.2 kV/cm, 30 µs) was given to the oocytes for their activation by using a BTX Electro-Cell Manipulator 2001M (BTX, San Diego, CA, USA). Next, the stimulated oocytes were washed three times in mM199 solution supplemented with 0.4% (w/v) BSA and 5 µmol/L cytochalasin B at 39 °C in an atmosphere of 5% CO_2 in air for 4 h. After that, the oocytes were washed three times in PZM and cultured in a drop of the same medium covered with paraffin oil in the same ambient conditions for 5 days. Cleavage and blastocyst formation rates were observed at day 2 and day 5 after the start of culture, respectively. Non-cleaved oocytes were removed from the culture on day 2; and on day 5, the blastocysts were fixed for at least 24 h in a drop of 4% PFA at 4°C, washed in a 1x PBS solution supplemented with 0.1% (w/v) PVA to avoid attachment to the dish and stained with Vectashield™ mounting medium with 4',6-Diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) and observed under a fluorescence microscope for

counting the blastocyst cell number. Embryos were considered to be pseudo-blastocysts they had less than 20 cells.

5.2.6 Statistical analysis

Data obtained from at least 5 replicated trials were processed by one-way or two-way ANOVA using IBM SPSS Statistics 21.0 software for Windows. Findings were considered to be significantly different at $P<0.05$, and when there was a significant effect, values were compared with a Tukey's multiple comparisons test. Before performing the statistical analysis, all percentage data in the experiments were subjected to arc-sine transformation if there were values > 90% or < 10%. All data were posteriorly transformed back into percentages for the tables and figures and are expressed as means ± SEM, except for the blastocyst cell count that was expressed as means ± SD.

5.3 Results

5.3.1 Experiment 1

The percentage of oocytes which achieved the germinal vesicle break down (GVBD), was significantly lower when the oocytes from SF and MF were denuded just before the start of IVM culture (51.6% and 73.6%, respectively) as compared with the other experimental groups (see Fig. 5.2).

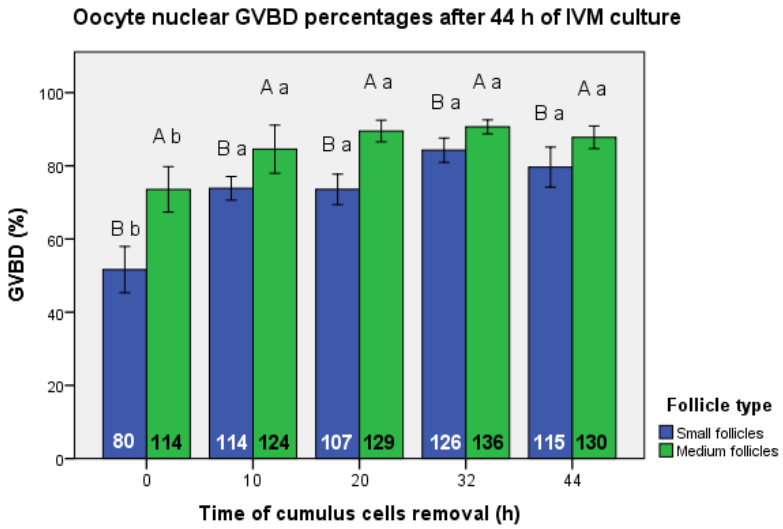


Figure 5.2: Oocyte GVBD rates after 44 h of IVM culture.
The experiment was replicated 5 times.
A,B: Different superscript letters indicate significant differences among the size of the follicles ($P<0.05$).
a, b: Different letters indicate significant differences among the different times of decumulation ($P<0.05$).

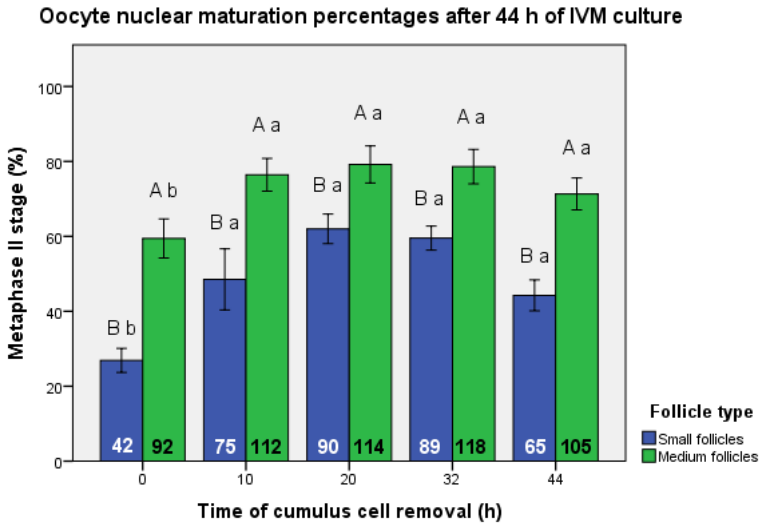


Figure 5.3: Oocyte nuclear maturation rates after 44 h of IVM culture.
The experiment was replicated 5 times.
A,B: Different superscript letters indicate significant differences among the size of the follicles (P<0.05).
a, b: Different letters indicate significant differences among the different times of decumulation (P<0.05).

The percentage of oocytes reaching to the metaphase-II (MII) stage was always significantly higher in those oocytes obtained from MF than in the SF ones (see Fig. 5.3 and supplementary table 1 in the Annex) and in both, COCs from SF and MF, the maturation rates of oocytes denuded at 10 h, 20 h and 32 h of IVM culture were similar with those which the CCs were removed after IVM. Even if there were no significant differences in the maturation rates of these groups, as the group we denuded after 20 h had the highest IVM rates, we used this timing for removing CCs in the following experiments.

5.3.2 Experiments 2 and 3

Regardless of the supplementation with GDF9 or VEGF, the maturation rates were always significantly higher in the denuded oocytes from MF than SF for both experiments (see Fig. 5.4 and Fig. 5.5, and supplementary tables 2 and 3 in the Annex).

In both cases, removing CCs 20 h after the start of IVM improved meiotic resumption rates in SF oocytes (Fig. 5.6; 59.2% vs 41.6% in GDF9 and Fig. 5.7; 61.3% vs 40.2% in VEGF), whereas no differences were detected in the MF groups (see Fig. 5.8 and Fig. 5.9). Despite of the origin of COCs (SF or MF), maturation rates of oocytes denuded 20 h after the start of IVM were not affected by supplementation with GDF9 or VEGF during the first or/and second half of IVM.

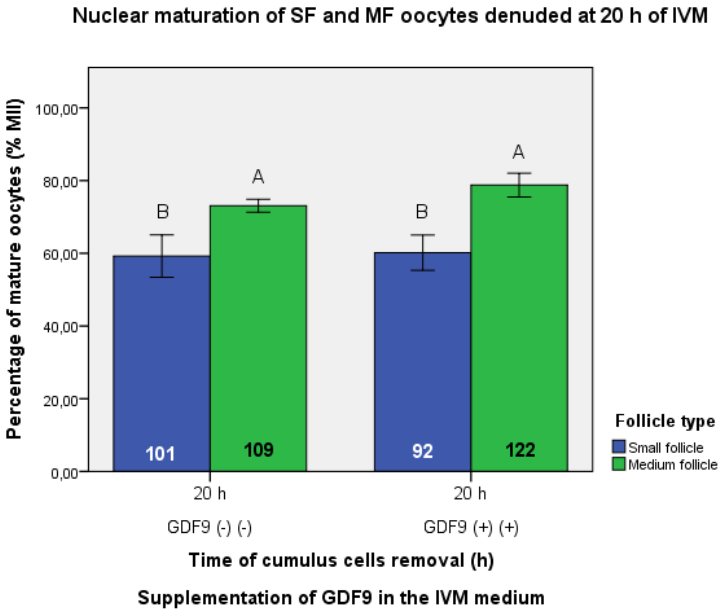


Figure 5.4: Oocyte nuclear maturation rates of SF and MF denuded at 20 h of IVM culture and supplemented or not with GDF9
The experiment was replicated 5 times.
A,B: Different superscript letters indicate significant differences among the size of the follicles (P<0.05).

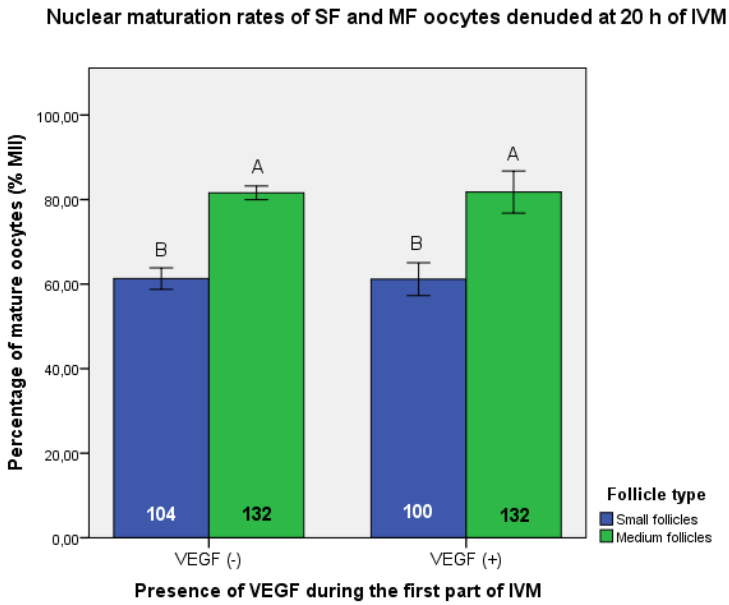


Figure 5.5: Oocyte nuclear maturation rates of SF and MF denuded at 20 h of IVM culture and supplemented or not with VEGF
The experiment was replicated 5 times.
A,B: Different superscript letters indicate significant differences among the size of the follicles (P<0.05).

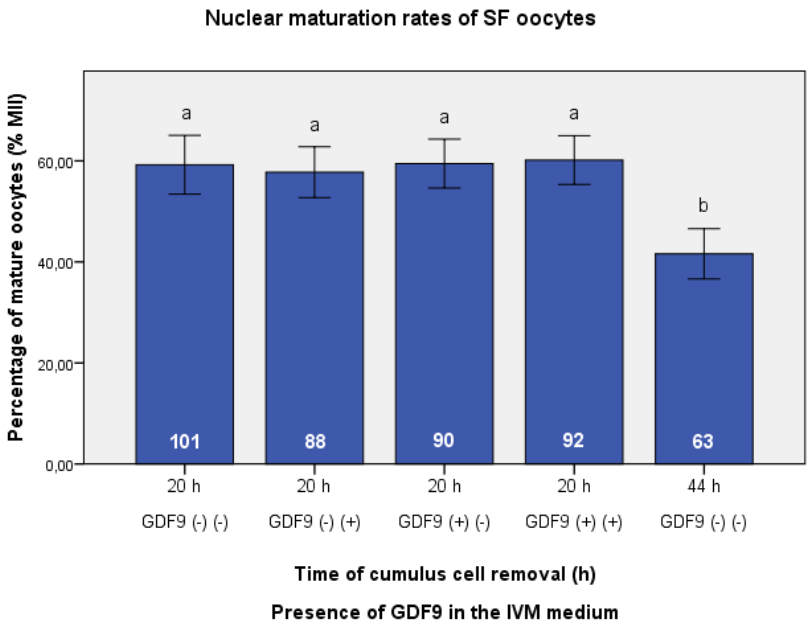


Figure 5.6: Nuclear maturation rates of SF oocytes denuded at 20 h of IVM culture and supplemented or not with GDF9

The experiment was replicated 5 times.

a, b: Different superscript letters indicate significant differences among the time of CCs removal (P<0.05).

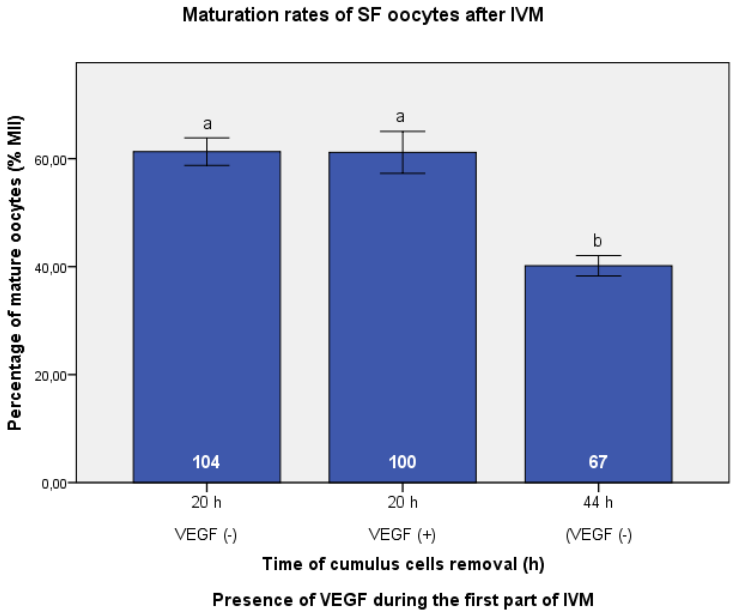


Figure 5.7: Nuclear maturation rates of SF oocytes denuded at 20 h of IVM culture and supplemented or not with VEGF

The experiment was replicated 5 times.

a, b: Different superscript letters indicate significant differences among the time of CCs removal (P<0.05).

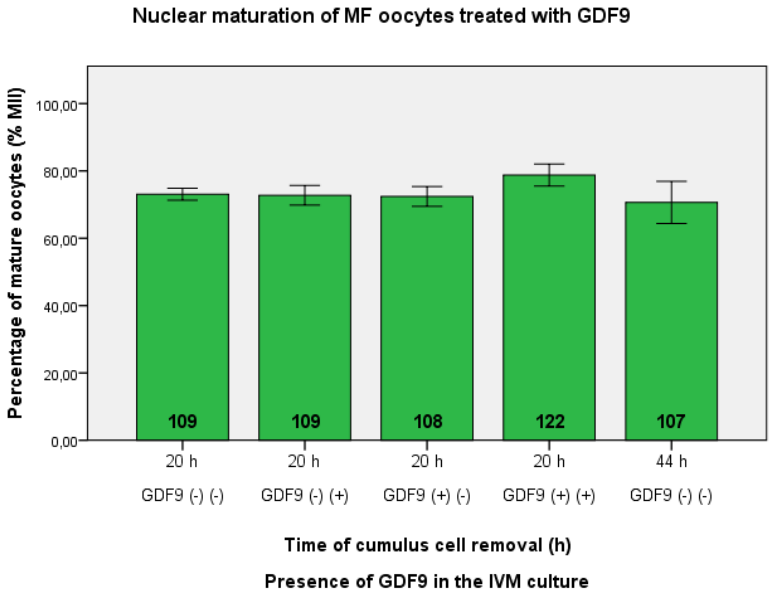


Figure 5.8: Nuclear maturation rates of SF oocytes denuded at 20 h of IVM culture and supplemented or not with GDF9
The experiment was replicated 5 times.

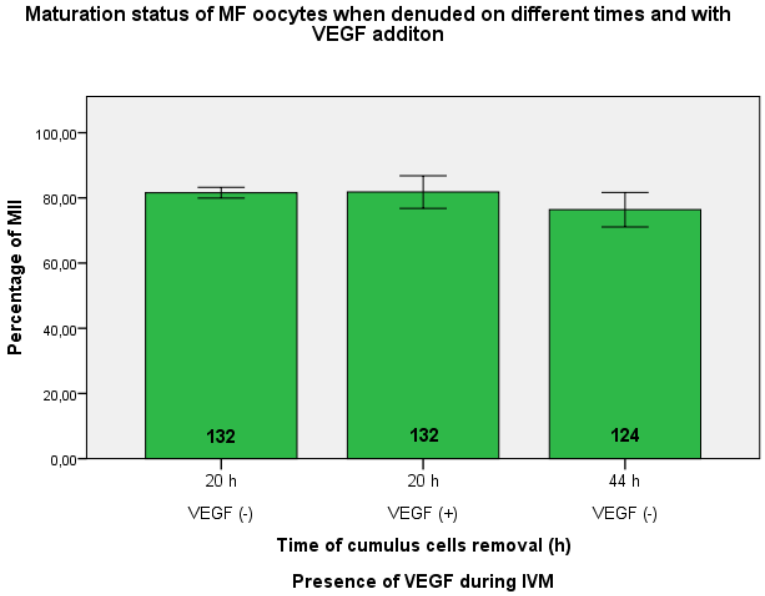


Figure 5.9: Nuclear maturation rates of MF oocytes denuded at 20 h of IVM culture and supplemented or not with VEGF
The experiment was replicated 5 times.

5.3.3 Experiment 4

Independently of the time of CCs removal, oocytes from MF had always significantly higher rates of maturation (Table 5.1), cleavage and blastocyst rates (Table 5.2) than those from SF, but there were no differences in the blastocyst cell number.

When oocytes were before the start of IVM, the oocytes caused significantly lower rates of maturation in both groups, SF (37.6% vs 51.2%) and MF oocytes (50.8% vs 76%) and also had negative effects on the blastocyst rates in both groups (2.7% vs 25.8% in SF; 27.3% vs 48.5% in MF). When CCs were removed 20 h after the start of IVM, it significantly promoted nuclear maturation in both, SF (64.1% vs 51.2%) and MF oocytes (82.5% vs 76.0%) and there were no significant differences on cleavage or blastocyst rates in both groups when compared with the controls.

Table 5.1: Maturation of SF and MF oocytes by polar body selection when CCs are removed at 0, 20 and 44 h of IVM.

Follicle type	CCs removal time (h)	Num. oocytes examined	Maturation rate (n (% ± SEM))
MF	0	180	92 (50.82 ± 4.59) ^{Ac}
	20	183	151 (82.49 ± 4.18) ^{Aa}
	44	188	143 (76.00 ± 3.35) ^{Ab}
SF	0	186	70 (37.56 ± 1.81) ^{Bc}
	20	181	116 (64.08 ± 2.60) ^{Ba}
	44	182	93 (51.15 ± 1.92) ^{Bb}

Result of 2-way ANOVA	
Follicle type	< **0.001**
CCs removal time	< **0.001**
Interaction	0.225

$^{A, B}$: Different superscript letters indicate significant differences amongst oocytes from different follicle size.

$^{a, b, c}$: Different superscript letters indicate significant differences among the time when were denuded.

Table 5.2: Embryo developmental capacity of SF and MF oocytes denuded at 0, 20 and 44 h of IVM after parthenogenetic activation.

Follicle type	CCs removal time (h)	Mature oocytes (n)	Embryo culture (Day 2)	Embryo culture (day 5)	
			Cleavage rate (n (% ± SEM)	Blastocyst rate (n (% ± SEM)	Blastocyst cell number ($\bar{X}$ ± SD)
MF	0	92	82 (88.78 ± 2.24) ^{A}	27 (27.34 ± 5.77) ^{Ab}	32.19 ± 8.42
	20	151	139 (91.79 ± 2.35) ^{A}	69 (44.88 ± 5.62) ^{Aa}	32.75 ± 3.90
	44	143	130 (90.92 ± 1.23) ^{A}	69 (48.52 ± 4.53) ^{Aa}	34.02 ± 2.63
SF	0	70	57 (81.66 ± 4.28) ^{B}	2 (2.68 ± 1.65) ^{Bb}	25.00 ± 1.41
	20	116	100 (85.92 ± 4.84) ^{B}	27 (23.34 ± 4.94) ^{Ba}	31.88 ± 1.70
	44	93	74 (79.07 ± 7.00) ^{B}	24 (25.79 ± 7.99) ^{Ba}	32.01 ± 2.54

Result of 2-way ANOVA

Follicle type	< **0.05**	< **0.001**	0.077
CCs removal time	0.410	< **0.001**	0.169
Interaction	0.881	0.275	0.392

$^{A, B}$: Different superscript letters indicate significant differences amongst oocytes from different follicles.

$^{a, b}$: Different superscript letters indicate significant differences among the time when oocytes were denuded.

5.4 Discussion

Mammalian oocytes that have reached their final size are arrested at the prophase of the first meiotic division indicated by the presence of a germinal vesicle (GV). Arresting oocytes at the GV stage is maintained by inherent factors correlated with low activity levels of cell cycle regulatory proteins (Yang et al., 2012) and also by the transference of cAMP from the CCs to the oocyte cytoplasm via gap junctions (Dekel, 1988). In some species, meiosis can be resumed spontaneously when oocytes are removed from the follicle and introduced in an adequate culture medium (De Loos et al., 1994; Edwards, 1965), so it has been suggested that cAMP derived from the follicle is the responsible for maintaining oocyte meiotic arrest (Tsafriri and Channing, 1975) by activating the cAMP dependent protein kinase A (PKA) (Conti et al., 2002). Meiosis also can be resumed by a reduction in the oocyte gap-junctional coupling of its surrounding somatic cells (Kalous et al., 1993; Larse WJ, 1986). In the pig, COCs maintain this gap-junctional communication during the first 24-32 of culture, and even if the CCs expansion starts after 16 h of IVM (Motlik and Fulka, 1986), the oocytes do not become disconnected from them until they reach a stage between the MI and MII a result of the CCs expansion process (Suzuki et al., 2000).

Our results indicate that oocytes from MF had higher maturation, cleavage and blastocyst rates than those from SF. Many studies also corroborate existing differences on these parameters (Kohata et al., 2013; Bagg et al., 2007; Marchal et al., 2002). Oocyte developmental competence is acquired during the process of folliculogenesis and, with the increasing size of the ovarian follicle, the oocytes will obtain a more adequate cytoplasmic maturation thanks to their companion somatic cells (Gilchrist et al., 2008). Oocytes from SF must have a poorer cytoplasmic maturation than those from MF, and, as follicles of less than 2 mm in diameter depend on the ovarian endogenous activity for their growth (Driancourt et al., 1995).

Culturing denuded oocytes impaired meiotic resumption and had negative effects on the developmental capacity of oocytes, especially in those from SF, whereas that when they were cultured as a COC during the first part of IVM, their nuclear maturation rates improved significantly. It has been widely reported that CCs provide the oocytes with nutrients and other molecules such as ions or transcripts necessary for achieving a good cytoplasmic maturation (Eppig, 1991; Park et al., 2013), and an early detachment of the CCs might cause the lack for some of these factors provided during the first hours of culture.

The pronounced improvement in the maturation rates of porcine oocytes from SF denuded at 20 h might be related to a transient raise in the levels of cAMP during the first part of IVM by the addition of the homologous molecule dbcAMP. That, together with the CCs

removal and the culture of the denuded oocytes in a medium without the modulator, might have caused a rapid decrease on the ooplasmic levels of cAMP. This diminution on the cAMP concentration levels, together with the activity of the PD3A into further reducing the levels of cAMP (Sun et al., 2015) and the phosphorylation of MAPK (Liang et al., 2007) caused the initiation of meiotic resumption.

The exogenous addition of GDF9 or VEGF had no effect in promoting oocyte maturation when supplemented during the first part of IVM culture when the CCs were removed at 20 h, but high concentrations of VEGF have been proven to affect negatively to oocyte maturation and developmental capacity in the human due to COC hypoxia (Malamitsi-Puchner et al., 2001). Many studies in cumulus-enclosed oocytes thorough IVM culture have shown a positive effect in maturation rates and developmental competence when growth factors were added into the maturation medium (Xia et al., 1994; Hussein et al., 2006; Singal and Prasad, 2009; Toori et al., 2014). The lack of response in our experiment indicates that the CCs probably exert their beneficial effect during the second part of culture, and the positive effects are not seen due to CCs removal. As oocyte maturation is correlated with the follicular stages in vivo (Gilchrist et al., 2001), developing pre-culture conditions with hormones or growth factors might be a good way for testing if the maturation rates when CCs are removed after the first half of IVM can be improved further by letting the factors to exert their beneficial action before CCs removal.

5.5 Conclusions

In conclusion, oocytes from MF have higher meiotic resumption and developmental competence than those from SF probably because they have reached a higher phase of maturation in vivo. Denuding oocytes 20 h after the start of IVM can be a good method for increasing the efficiency of IVM protocols since it can improve oocyte meiotic resumption rates without any reduction in the developmental competence of oocytes from SF. The addition of 100 ng/mL of GDF9 or 200 ng/mL of VEGF, were unable to further increase the percentages of nuclear maturation in both types of oocytes. We suggest that culturing the COCs with growth factors and/or hormones prior to IVM might help the oocytes by promoting maturation and developmental competence when denuded after the first part of culture.

CHAPTER 6: GENERAL DISCUSSION

The ovarian samples used for experimenting purposes are usually obtained from commercial slaughterhouses, in there, young animals and those with the presence of anestrous will be sacrificed, so the ovaries from slaughtered animals will have different characteristics which will affect to the quality of the gametes. Furthermore, even if the available protocols for IVM have improved greatly for the past years, the available protocols in the porcine species are still deficient, especially when researchers try to mature oocytes from small follicles.

The first aim of this study was to find an ovarian factor that could be used for reducing the variability of the samples and would help to improve the efficiency of oocyte IVM protocols. Firstly, the samples were classified into 3 categories according to the characteristics of their surface, weighed the ovaries and counted the numbers of different-sized follicles present on their surface. Cumulus-oocyte complexes from SF and MF of ovaries with a smooth or a mixed surface were cultured in vitro to assess their meiotic competence capacity. The number of small and medium follicles was higher in mixed surface ovaries than in the other ovarian types and this type of ovaries also presented significantly higher maturation rates in oocytes from small and medium follicles. This could be because the ovaries would be in the recruitment follicular phase, characterized by high proliferation rates of the ovarian follicles, which would also affect to the oocyte quality by having less numbers of atretic follicles, or by having higher oocyte quality.

As researchers generally have a limited amount of samples available for experiments, a method to improve maturation rates and the quality of oocytes from SF and MF was tried to develop, independently of the type of ovarian morphology. It is known that the decrease of the levels of cAMP in the ooplasm by cutting the communication between the oocyte and its surrounding cumulus cells can lead to GVBD and to the meiotic resumption of oocytes. To test removing cumulus cells can be used as a meiotic promotion factor, in the second experiment, the apoptotic and nuclear status of oocytes from SF MF were examined after removing CCs during IVM. Before IVM, the incidence of oocytes at the GV_0 and GV_I stage was different according to the follicle of origin, being higher in oocytes from SF and MF respectively; whereas no differences could be detected in the apoptotic status. Researchers have proposed that GV_I oocytes have started to receive maturation stimuli and are in a more advanced phase of maturation than those arrested in the GV_0. This fact could be one of the reasons why oocytes from MF reach higher rates of maturation than the ones derived from SF. After culture, the time when oocytes were denuded affected the oocyte apoptosis rates. The percentage of intact oocytes was significantly higher when cumulus cells were removed at 20 and 44 h and the incidence of early and late apoptotic oocytes was significantly higher when we cultured

denuded oocytes, because CCs act as a barrier that protects the oocytes against reactive oxygen species and in a final instance apoptosis. Maturation rates were affected by the size of the follicle of origin and by the time the oocytes were denuded. By denuding the oocytes after 20 h of culture we improved their maturation rates, due to the activation of the meiotic resumption process, but the percentages of oocytes with normal spindles were lower than the controls, meaning that there are probably differences in the energy contents when CCs are removed early.

The above concept led me to perform a third experiment for further investigating on the quality of the oocytes denuded at 20 h of IVM. Since VEGF and GDF9 have been proved to improve maturation of oocytes when cultured in vitro, it was examined if oocyte maturation rates could be further improved by the supplementation of these factors. Firstly it was assessed if denuding oocytes 20 h after the start of IVM was optimal and, after confirmation, developmental competence of the oocytes was examined. Afterwards, GDF9 or VEGF were supplemented to elucidate if maturation rates could be further improved. SF oocytes had lower maturation, cleavage and blastocyst rates than those from MF, and this coincides with current previous findings in which oocytes from SF possess lower quality because they have not become meiotically competent, and they are probably lacking in cytoplasmic maturation. Oocytes denuded after 20 h of IVM, had higher rates of maturation by maintaining the developmental competence in the same conditions as the controls. The exogenous addition of growth factors in the medium did not significantly improve oocyte maturation of the experimental groups, probably, because of the lack of cumulus cells during the second part of the IVM, they could not exert their beneficial effect on the oocytes, so it is suggested that, to further improve maturation rates, the addition of hormones and growth factors should be performed as a culture prior to the IVM protocol.

The work presented in this thesis shows that ovaries and oocytes from slaughtered animals have heterogenic characteristics and the efficiency of maturation protocols can be improved by selecting ovaries with a mixed surface that will provide elevated numbers of oocytes with high nuclear maturation capacity. Or, when the samples are limited, denuding oocytes after 20 h of IVM maintains their viability and promotes meiotic progression in both, SF and MF oocytes. Furthermore, even if the rates of abnormal spindles are higher in the treated oocytes, parthenogenetic activation does not reduce their developmental competence.

General conclusions

In conclusions, removing the cumulus cells 20 h after the start of IVM allow the obtainment of higher numbers of good quality oocytes because of the improvement in

maturation rates while maintaining the developmental competence of oocytes, especially in those derived from SF.

CHAPTER 7: SUMMARY

Currently, female gametes are easily collected from slaughtered or live ovaries and used for various reproductive biotechnologies to improve the efficiency and value of mammalian embryos. Pigs have been utilized as a model in biomedical research, whereas the gametes and embryos are quite unique from other species. In the developed countries, although pigs are usually slaughtered for pork production at 5-6 months old when those gilts have neared or still not reached to the puberty, ovarian morphologies have not been considered for the resource of cumulus-oocyte complexes (COCs). Furthermore, the effect of interrupting communication between oocyte and the surrounding cumulus cells has not been examined in porcine COCs derived from small follicles (SF; < 3 mm in diameter).

In the present studies, firstly, gilt's ovaries with different follicular morphologies were characterized and the meiotic competence of oocytes was compared among the criteria. Porcine ovaries were able to be classified into three categories; ovaries with smooth (SSO), bubbled (BSO) or mixed (uneven) surface (MSO). A significantly higher number of SF was observed in MSO than SSO and BSO and the number of middle follicles (MF; 3-6 mm in diameter) per ovary was significantly higher in BSO and MSO than SSO. Since MSO showed a significantly higher percentage of metaphase-II stage oocytes than SSO in SF and MF, it was concluded that MSO could suppose a good source of oocytes for improving the efficiency of the actual reproductive technologies.

In the second experiments, the apoptotic status and meiotic progression of oocytes from SF and MF were examined when the oocytes were denuded during culture for in-vitro maturation (IVM). Apoptotic status of the oocytes was only affected by the time when the oocytes were denuded (0, 20 and 44 h after the start of IVM). Although the percentage of mature oocytes was higher in MF, maturation rates were significantly higher when oocytes were denuded at 20 h, as compared with 0 and 44 h after the start of IVM. Therefore, it was concluded that removing CCs at 20 h after the start of IVM seemed to promote meiotic progression of the oocytes to the metaphase-II stage even when the COCs were collected from SF.

In the last experiments, the effect of the timing of removing cumulus cells during IVM on developmental competences of oocytes was assessed, and it was also examined if the addition of GDF9 or VEGF could further improve IVM rates of oocytes denuded during maturation culture. Regardless of the presence of GDF9 or VEGF, denuding oocytes 20 h after the start of IVM did not affect their developmental competence to the blastocyst stage, concluding that denuding the oocytes 20 h after the start of IVM could be a good method to promote the meiotic resumption of oocytes, without any reduction in the developmental competence.

Results from the current experiments demonstrate that using COCs from MSO or by removing the cumulus cells of COCs from SF and MF 20 h after the start of IVM can promote the meiotic resumption of oocytes, without any reduction in the developmental competence. This information may contribute to improve the efficiency to prepare female resources for in-vitro embryo production.

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APPENDICES

Supplementary table 1. Oocyte nuclear maturation and GVBD rates when CCs are removed during IVM in SF and MF oocytes.

Follicle type	Time of CCs removal (h)	N. of oocytes examined	Oocyte nuclear stage after 44 h of IVM ((n (% ± SEM))						
			GV	PMI	MI	A/T	MII	Deg	GVBD
SF	0	154	59 (38.8 ± 6.3) $^{A\ a}$	9 (5.9 ± 0.7)	19 (12.3 ± 3.8) $^{A\ bc}$	9 (5.9 ± 1.3)	42 (26.9 ± 3.2) ^{Bb}	16 (10.3 ± 3.5) ^{A}	80 (51.6 ± 6.3) $^{B\ b}$
	10	154	27 (17.6 ± 1.8) $^{A\ b}$	18 (11.6 ± 2.5)	15 (9.8 ± 3.7) $^{A\ b}$	6 (4.0 ± 1.6)	75 (48.5 ± 8.2) ^{Ba}	13 (8.6 ± 2.4) ^{A}	114 (73.8 ± 3.3) $^{B\ a}$
	20	143	26 (18.7 ± 4.2) $^{A\ b}$	8 (5.5 ± 1.7)	4 (2.8 ± 1.3) $^{A\ b}$	5 (3.2 ± 1.4)	90 (62.0 ± 4.0) ^{Ba}	11 (7.7 ± 1.4) ^{A}	107 (73.6 ± 4.1) $^{B\ a}$
	32	149	15 (10.2 ± 1.9) $^{A\ b}$	10 (6.6 ± 2.2)	18 (12.1 ± 2.2) $^{A\ bc}$	9 (6.1 ± 2.4)	89 (59.5 ± 3.2) ^{Ba}	8 (5.6 ± 1.9) ^{A}	126 (84.3 ± 3.3) $^{B\ a}$
	44	146	20 (13.8 ± 3.0) $^{A\ b}$	7 (4.8 ± 2.1)	40 (27.4 ± 3.9) $^{A\ ac}$	3 (2.2 ± 0.9)	65 (44.3 ± 4.1) ^{Ba}	11 (7.6 ± 2.6) ^{A}	115 (79.7 ± 5.4) $^{B\ a}$
MF	0	155	33 (21.2 ± 4.5) $^{B\ a}$	3 (2.0 ± 1.3)	17 (10.9 ± 4.3) $^{B\ bc}$	2 (1.3 ± 0.8)	92 (59.4 ± 5.2) $^{A\ b}$	8 (5.2 ± 2.3) ^{B}	114 (73.6 ± 6.2) $^{A\ b}$
	10	146	14 (9.9 ± 5.2) $^{B\ b}$	5 (3.4 ± 1.1)	3 (2.1 ± 0.9) $^{B\ b}$	4 (2.6 ± 1.8)	112 (76.4 ± 4.4) $^{A\ a}$	8 (5.6 ± 1.9) ^{B}	124 (84.5 ± 6.6) $^{A\ a}$
	20	144	9 (6.2 ± 2.8) $^{B\ b}$	6 (4.2 ± 0.7)	7 (4.8 ± 2.5) $^{B\ b}$	2 (1.4 ± 0.8)	114 (79.2 ± 5.0) $^{A\ a}$	6 (4.3 ± 2.6) ^{B}	129 (89.5 ± 3.0) $^{A\ a}$
	32	150	8 (5.2 ± 2.4) $^{B\ b}$	3 (2.0 ± 1.3)	13 (8.8 ± 2.6) $^{B\ bc}$	2 (1.3 ± 1.3)	118 (78.6 ± 4.6) $^{A\ a}$	6 (4.2 ± 2.1) ^{B}	136 (90.6 ± 1.9) $^{A\ a}$
	44	147	13 (8.9 ± 3.0) $^{B\ b}$	4 (2.8 ± 1.4)	20 (13.6 ± 1.5) $^{B\ ab}$	0	105 (71.3 ± 4.3) $^{A\ a}$	5 (3.3 ± 1.1) ^{B}	130 (87.8 ± 3.1) $^{A\ a}$
Result of 2-way ANOVA									
Follicle type			< 0.001	-	< 0.05	-	< 0.001	< 0.05	< 0.001
Time of CCs removal			< 0.001	-	< 0.001	-	< 0.001	0.791	< 0.001
Interaction			0.928	-	0.433	-	0.678	0.980	0.703

$^{A, B,C}$: Different superscript letters indicate significant differences amongst oocytes from different follicles.

$^{a, b, c}$: Different superscript letters indicate significant differences amongst oocytes decumulated on different times.

Supplementary table 2. Effect of CCs removal time and GDF9 supplementation on the nuclear maturation of SF oocytes.

Follicle type	Time of CCs removal	GDF9 1st part IVM	GDF9 2nd part IVM	Oocytes examined (n)	Nuclear stage of oocytes after 44 h of IVM (n (% ± SEM))					
					GV	PMI	MI	A/T	MII	Deg
SF	20 h	-	-	151	28 (18.38 ± 3.34)	4 (0.65 ± 0.65)	13 (10.48 ± 2.11)	3 (2.07 ± 1.41)	101 (59.24 ± 5.83)[a]	14 (9.18 ± 4)
		-	+	151	30 (19.7 ± 2.87)	3 (2 ± 1.33)	12 (7.88 ± 1.64)	3 (2.02 ± 0.83)	88 (57.76 ± 5.05)[a]	16 (10.64 ± 3.56)
		+	-	153	23 (14.94 ± 3.2)	3 (1.96 ± 1.3)	16 (10.67 ± 1.91)	8 (5.21 ± 1.23)	90 (59.47 ± 4.84)[a]	12 (7.74 ± 2,41)
		+	+	153	21 (13.67 ± 2.07)	3 (1.92 ± 1.29)	16 (10.38 ± 3.09)	5 (3.31 ± 1.06)	92 (60.16 ± 4.84)[a]	15 (9.87 ± 4.76)
	44 h	-	-	151	37 (24.56 ± 5.02)	1 (0.67 ± 0.67)	26 (17.52 ± 4.48)	4 (2.69 ± 1.25)	63 (41.58 ± 4.97)[b]	20 (12.98 ± 3.4)

Result of 2-way ANOVA						
Time of CCs removal	0.267	-	0.194	-	**< 0.05**	0.387
GDF9 supplementation	0.542	-	0.880	-	0.989	0.918
Interaction	-	-	-	-	-	-

[a, b, c]: Different superscript letters indicate significant differences amongst oocytes decumulated on different times.

Supplementary table 3. Effect of CCs removal time and GDF9 supplementation on the nuclear maturation of MF oocytes

Follicle type	Time of CCs removal	GDF9 1st part IVM	GDF9 2nd part IVM	Oocytes examined	Nuclear stage of oocytes after 44 h of IVM					
					GV	PMI	MI	A/T	MII	Deg
MF	20 h	-	-	149	12 (8.03 ± 3.04)	1 (0.65 ± 0.65)	13 (8,78 ± 2.07)	7 (4.81 ± 2.10)	109 (73.06 ± 1.79)	6 (4.00 ± 1.64)
		-	+	150	18 (12.16 ± 3.56)	0	15 (9.88 ± 3.35)	2 (1.29 ± 0.79)	109 (72.75 ± 2.92)	6 (3.92 ± 1.20)
		+	-	149	19 (12.94 ± 4.01)	1 (0.74 ± 0.74)	13 (8.53 ± 5.97)	3 (2 ± 2)	108 (72.41 ± 2.95)	4 (2.74 ± 1.95)
		+	+	155	14 (9.01 ± 3.88)	1 (0.63 ± 0.63)	12 (7.72 ± 2.99)	4 (2.61 ± 1.24)	122 (78.77 ± 3.29)	2 (1.27 ± 0.78)
	44 h	-	-	151	14 (9.32 ± 1.66)	1 (0.69 ± 0.69)	18 (12.06 ± 4.21)	6 (3.98 ± 1.64)	107 (70.64 ± 6.26)	4 (2.65 ± 1.94)

Result of 2-way ANOVA

Time of CCs removal	0.551	-	0.652	-	0.653	-
GDF9 supplementation	0.582	-	0.845	-	0.592	-
Interaction	-	-	-	-	-	-

Supplementary table 4. Effect of follicle size and GDF9 addition on oocyte nuclear maturation after IVM of SF and MF oocytes when denuded at 20 h of culture.

Follicle type	GDF9 1st part IVM	GDF9 2nd part IVM	Oocytes examined (n)	Nuclear stage of oocytes after 44 h of IVM (n (% ± SEM))					
				GV	PMI	MI	A/T	MII	Deg
SF	-	-	151	28 (18.38 ± 3.34)^{A}	4 (0.65 ± 0.65)	13 (10.48 ± 2.11)	3 (2.07 ± 1.41)	101 (59.24 ± 5.83)^{B}	14 (9.18 ± 4)
	+	+	153	21 (13.67 ± 2.07)^{A}	3 (1.92 ± 1.29)	16 (10.38 ± 3.09)	5 (3.31 ± 1.06)	92 (60.16 ± 4.84)^{B}	15 (9.87 ± 4.76)
MF	-	-	149	12 (8.03 ± 3.04)^{B}	1 (0.65 ± 0.65)	13 (8,78 ± 2.07)	7 (4.81 ± 2.10)	109 (73.06 ± 1.79)^{A}	6 (4.00 ± 1.64)
	+	+	155	14 (9.01 ± 3.88)^{B}	1 (0.63 ± 0.63)	12 (7.72 ± 2.99)	4 (2.61 ± 1.24)	122 (78.77 ± 3.29)^{A}	2 (1.27 ± 0.78)
Result of 2-way ANOVA									
Effect of follicle size				< **0.05**	-	0.518	-	< **0.01**	0.065
Effect of GDF9 addition				0.686	-	0.518	-	0.445	0.489
Interaction				0.551	-	0.874	-	0.579	0.473

$^{A, B}$: Different superscript letters indicate significant differences amongst oocytes from different size of follicles.

Supplementary table 5. Nuclear maturation status of oocytes from SF when denuded after 20 h of IVM and supplemented with VEGF protein and denuded at the end of IVM.

CCs removal time (h)	VEGF addition	Oocyte number	Nuclear stage after IVM						
			GV	PMI	MI	A/T	MII	Deg	GVBD
20	-	170	30 (17.82 ±3.71)	5 (3.01 ± 1.02)	15 (8.48 ± 5.38) [b]	5 (2.86 ± 1.28)	104 (61.30 ± 2.56) [a]	11 (6.54 ± 1.22)	129 (75.64 ± 3.69)
20	+	163	29 (17.80 ± 2.66)	7 (4.37 ± 1.24)	13 (8.08 ± 3.02) [b]	3 (1.86 ± 0.76)	100 (61.16 ± 3.89) [a]	11 (6.72 ± 1.47)	123 (75.48 ± 3.48)
44	-	167	30 (17.99 ± 0.98)	8 (4.84 ± 1.99)	49 (29.34 ± 4.49) [a]	7 (4.13 ± 1.92)	67 (40.17 ± 1.89) [b]	6 (3.59 ± 0.58)	131 (78.43 ± 1.45)

Results of 2-way ANOVA

Cumulus cell removal	0.849	-	**< 0.005**	-	**< 0.001**	-	0.529
VEGF addition	0.946	-	0.640	-	0.975	-	0.971
Interaction	-	-	-	-	-	-	-

Supplementary table 6. Nuclear maturation status of oocytes from MF when denuded after 20 h of IVM and supplemented with VEGF protein and denuded at the end of IVM

CCs removal time (h)	VEGF addition	Oocyte number	Nuclear stage after IVM						
			GV	PMI	MI	A/T	MII	Deg	GVBD
20	-	162	8 (4.85 ± 1.53)	3 (1.78 ± 0.73)	11 (6.83 ± 0.73)	1 (0.67 ± 0.67)	132 (81.59 ± 1.64)	7 (4.27 ± 0.66)	147 (90.87 ± 1.81)
20	+	161	17 (10.73 ± 4.14)	2 (1.27 ± 0.78)	8 (4.96 ± 2.13)	0	132 (81.79 ± 4.98)	2 (1.25 ± 0.77)	142 (88.02 ± 4.34)
44	-	162	11 (6.85 ± 1.23)	2 (1.20 ± 0.73)	20 (12.46 ± 2.88)	2 (1.27 ± 0.78)	124 (76.38 ± 5.29)	3 (1.84 ± 0.76)	149 (91.31 ± 1.86)

Results of 2-way ANOVA

Cumulus cell removal	0.400	-	0.167	-	0.504	-	0.903
VEGF addition	0.150	-	0.268	-	0.813	-	0.694
Interaction	-	-	-	-	-	-	-

Supplementary table 7. Nuclear maturation status of oocytes from SF and MF when denuded after 20 h of IVM and supplemented with VEGF protein

Follicle type	VEGF addition	Oocyte number	Nuclear stage after IVM						
			GV	PMI	MI	A/T	MII	Deg	GVBD
SF	-	170	30 (17.82 ±3.71)	5 (3.01 ± 1.02)	15 (8.48 ± 5.38)	5 (2.86 ± 1.28)	104 (61.30 ± 2.56) ^{B}	11 (6.54 ± 1.22)	129 (75.64 ± 3.69)
	+	163	29 (17.80 ± 2.66)	7 (4.37 ± 1.24)	13 (8.08 ± 3.02)	3 (1.86 ± 0.76)	100 (61.16 ± 3.89) ^{B}	11 (6.72 ± 1.47)	123 (75.48 ± 3.48)
MF	-	162	8 (4.85 ± 1.53)	3 (1.78 ± 0.73)	11 (6.83 ± 0.73)	1 (0.67 ± 0.67)	132 (81.59 ± 1.64) ^{A}	7 (4.27 ± 0.66)	147 (90.87 ± 1.81)
	+	161	17 (10.73 ± 4.14)	2 (1.27 ± 0.78)	8 (4.96 ± 2.13)	0	132 (81.79 ± 4.98) ^{A}	2 (1.25 ± 0.77)	142 (88.02 ± 4.34)

Results of 2-way ANOVA

Follicle size	< **0.005**	-	0.966	-	< **0.001**	-	< **0.005**
VEGF addition	0.286	-	0.940	-	0.831	-	0.764
Interaction	0.316	-	0.302	-	0.829	-	0.797