

DISSERTATION TITLE

**MORPHOLOGICAL/DEVELOPMENTAL CHARACTERISTICS AND
OXIDATIVE STRESS RESILIENCE OF PORCINE OOCYTES DERIVED
FROM ANTRAL FOLLICLES WITH VARIOUS DIAMETERS AND
CLASSIFIED BY BRILLIANT CRESYL BLUE STAINING**

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VAN NGOC PHONG

Graduate School of Environmental and Life Science

(Doctor's Course)

OKAYAMA UNIVERSITY

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GENERAL ABSTRACT

Oocyte-cumulus complexes (OCCs) that are derived from the ovaries of prepubertal gilts, slaughtered commercially before sexual maturity, are an essential material in both in vitro maturation (IVM) and in vitro fertilization (IVF) systems due to their availability. Prepubertal gilt's ovaries contain various sizes of follicles, of which the number of small follicles (SF, with 1-3 mm in diameter) and very small follicles (VSF, less than 1 mm in diameter) are larger than medium follicles (MF, with 3-6 mm in diameter). MF-derived OCCs were primarily used as materials for in vitro embryo production due to their superior meiotic and developmental competence compared to SF-derived OCCs. With the development of embryonic gene editing technology, research on creating gene-edited pigs for xenotransplantation and pig models for use in studies of human disease requires a large amount of embryonic material. Thus, maximizing the utilization of material from each ovary, including the OCCs derived from SF and even VSF, could be necessary. Brilliant cresyl blue (BCB) staining, which can approximately assess the activity of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway (PPP), has proven effective in selecting porcine MF-derived BCB-positive (BCB+) oocytes with both meiotic and developmental competences. However, BCB+ oocytes (considered to have relatively low G6PD activity) are observed even in SF; oocytes are believed to still be in the growing phase and possess higher meiotic competence compared to BCB-negative (BCB-, considered to have relatively high G6PD activity) oocytes. Therefore, it is essential to reanalyze whether the characteristics of BCB+ oocytes have long been regarded as grown ones with meiotic and developmental competences. Furthermore, BCB staining has been demonstrated to cause oxidative stress to OCCs, especially BCB+ ones, through increasing reactive oxygen species (ROS) levels. However, the damage to oocytes through IVM culture has not been evaluated yet. Notably, the number of cumulus cells surrounding the oocyte is less in SF-derived OCCs than in MF-derived ones; the resilience of OCCs against oxidative stress through BCB staining may differ among those derived from VSF, SF, and MF.

The objectives of the current study were to determine whether porcine OCCs derived from various antral follicle sizes (VSF, SF, and MF) exhibit differences in BCB staining status, meiotic and developmental competences, oocyte diameters, number of surrounding viable cumulus cells, and expression of genes related to the PPP, as well as to evaluate their resilience to BCB staining-induced oxidative stress by analyzing intracellular GSH, ROS, and NADPH levels in oocytes during IVM.

The first experiment series sought to determine the characteristics of OCCs derived from VSF and SF in comparison with controls from MF. Additionally, the present study examined the utility of BCB staining for assessing these OCCs. The incidence of BCB- oocytes in VSF- and SF-derived OCCs was higher than in MF-derived OCCs. Although the meiotic and developmental competences of BCB+ oocytes from MF were superior to those from VSF and SF, blastocysts were successfully obtained from BCB+ oocytes, even derived from VSF. The mean numbers of both total and viable cumulus cells surrounding an oocyte were significantly affected not only by the origin of the OCCs but also by the BCB status of the oocytes (largest in MF-derived OCCs containing BCB+ oocytes). Although the outer and inner diameters of zona pellucida were affected by the origin of OCCs and the BCB status of oocytes (largest in MF-derived oocytes), the ooplasmic diameter of BCB+ oocytes did not differ among those derived from VSF, SF, and MF. Regardless of the BCB status, the transcriptional levels of G6PD and TKT in cumulus cells decreased during follicular development from VSF to MF, whereas the RPIA mRNA level in cumulus cells of MF-derived BCB+ OCCs was lower than in the others. These results underscore the utility of BCB staining for selecting MF-, SF-, and even VSF-derived OCCs containing oocytes with relatively higher meiotic and developmental competences, as well as the importance of having a sufficient number of healthy cumulus cells expressing genes related to the pentose phosphate pathway at lower levels.

The second experimental series aimed to determine the resilience of OCCs derived from three different follicle sizes, including MF, SF, and VSF, to BCB staining. BCB staining affected

transcriptional levels of oxidative stress-related genes in both oocytes and cumulus cells. Regardless of the origins of OCCs, even in BCB⁺ oocytes, the blue dye disappeared around 1 h in an IVM culture condition. Within 1 h after BCB staining, distinct responses in the NADPH regulation were observed between MF-, SF-, and VSF-derived OCCs and were affected by both the origins of OCCs and the BCB status. A transient increase in ROS and a decrease in GSH in BCB⁺ oocytes were also observed. After BCB staining, the ROS levels in both BCB⁺ and BCB⁻ oocytes were increased, although the level was affected by both the origin of OCCs and BCB status until 4 h of IVM and remained at low levels during IVM. The GSH levels of MF-, SF-, and VSF-derived BCB⁺ oocytes were significantly lower than those of BCB⁻ ones and non-stained controls, but the difference disappeared during IVM. These results demonstrate that, although BCB staining induces transient oxidative stress in porcine OCCs, especially those including BCB⁺ oocytes, OCCs rapidly restore redox balance by regulating and utilizing NADPH to produce reduced GSH, with different resilience between the origins of OCCs.

Through current studies on OCCs derived from MF, SF, and VSF of prepubertal ovaries, an understanding of the characteristics of OCCs according to the follicular development process and the meiotic and developmental competence of oocytes during IVM and IVC has been achieved. BCB staining effectively classified VSF- and SF-derived OCCs contained a higher proportion of BCB⁻ oocytes and showed lower meiotic and developmental competences compared to those from MF; however, it is still possible to obtain oocytes capable of developing into blastocysts from even VSF-derived BCB⁺ OCCs. Furthermore, the sufficient number of healthy cumulus cells surrounding the oocytes, as well as the low expression of PPP-related genes (G6PD, TKT, RPIA) in the surrounding cumulus cells during follicle development from VSF to MF, were closely associated with the meiotic and developmental competences of oocytes. Although BCB staining induced transient oxidative stress, characterized by increased ROS and decreased GSH, especially those including BCB⁺ oocytes, these redox imbalances were rapidly corrected within the first hours of IVM via NADPH-mediated GSH regeneration.

In conclusion, these findings support the usefulness of BCB staining as a selection tool for identifying developmentally competent OCCs even from VSF as potential biotechnological materials and emphasize the critical role of cumulus cell quality and redox regulation in supporting meiotic and developmental competences of oocytes. Future research should focus on optimizing pre-IVM culture conditions to increase the number of surrounding cumulus cells and prolong the cytoplasmic maturation time of oocytes, especially for VSF-derived BCB⁺ oocytes, under conditions that temporarily maintain oocyte meiotic arrest.

DECLARATION

I certify that the content of this dissertation was conducted in strict accordance with the regulations of Okayama University. This research is my original work, except that indicated or acknowledged through cited references. This thesis has not been presented to any other academic or non-academic institution for any other degree or qualification.

Furthermore, I acknowledge receipt of the Academic Rules and Regulations for the Graduate School of Environmental and Life Science, which outline the standards for the conduct of my study and research.

Name of the Student: VAN NGOC PHONG

Student's ID No: 77D22951

Graduate School: Graduate School of Environmental and Life Science

Division: Agricultural and Life Science

Thesis Title: Morphological/developmental characteristics and oxidative stress resilience of porcine oocytes derived from antral follicles with various diameters and classified by brilliant cresyl blue staining

Signature of Student:

Date:

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PUBLICATIONS ARISING FROM THIS THESIS

- 1. Characteristics of porcine oocyte-cumulus complexes derived from various sizes of antral follicles and classified by brilliant cresyl blue staining, and developmental competence of the oocytes**

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1. Relation between pentose phosphate pathway activity according to follicle size and meiotic and developmental competence of porcine oocytes.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
%	Percentage
°C	degree(s) Celsius
µg	microgram(s)
ng	nanogram(s)
mL	milliliter(s)
µL	microliter(s)
mm	millimeter(s)
nm	nanometer(s)
µm	micrometer(s)
M	Molar
mM	milimolar(s)
µM	micromolar(s)
nM	nanomolar(s)
h	hour(s)
min	minute(s)
s	second(s)
µs	microsecond(s)
kV/cm	kilovolt/cm
v/v	volume/volume
w/v	weight/volume
A-I	anaphase I
ANOVA	analysis of variance
Bax	B cell lymphoma/leukemia-2 associated X
BCB	brilliant cresyl blue

Caspase-3	cysteine-aspartic acid protease-3
CAT	catalase
CC	cumulus cells
cDNA	complementary deoxyribonucleic acid
Ct	cycle threshold
db-cAMP	dibutyryl cyclic adenosine monophosphate
DO	denuded oocyte
dpc	days post coitum
eCG	equine chorionic gonadotrophin
EDTA	ethylenediaminetetraacetic acid
EM	electroporation medium
F	forward
FSH	follicle-stimulating hormone
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLYT1	glycine-specific transporter 1
GPX1	glutathione peroxidase 1
GSH	glutathione
GSSG	glutathione disulfide
GV	germinal vesicle
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IU	International Units
IVC	in vitro culture
IVF	in vitro fertilization

IVM	in vitro maturation
LH	Luteinizing Hormone
M-I	metaphase I
M-II	metaphase II
MF	medium follicle(s)
mM199	modified medium 199
mPOM	modified porcine oocyte medium
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
No.	number
NRF2	nuclear factor, erythroid 2 like 2
OCCs	oocyte-cumulus complexes
PBS	phosphate buffered saline
PI	propidium iodide
PPP	pentose phosphate pathway
PVA	polyvinyl alcohol
PZM-5	porcine zygote medium
R	reverse
RNA	ribonucleic acid
ROS	reactive oxygen species
RPIA	ribulose 5-phosphate isomerase A
RT-PCR	real-time polymerase chain reaction
SEM	standard error of the mean
SF	small follicle(s)
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2

T-I	telophase I
TKT	transketolase
TL-HEPES-PVA	Tyrode Lactate-HEPES-polyvinyl alcohol buffered medium
T _m	melting temperature
VEGF	vascular endothelial growth factor
vs.	versus
VSF	very small follicle(s)

CHAPTER 1

GENERAL INTRODUCTION

1.1. Preface

In vitro breeding, including gamete/embryo production, is one of the promising reproductive biotechnologies that aim to increase reproductive efficiency, genetic improvement, genome editing, and the conservation of unique genetic resources (Okere & Nelson, 2002). Pigs are an excellent model for investigating in vitro maturation (IVM), as the timing and underlying mechanisms of oocyte maturation in pigs are also similar to those in humans. This similarity makes the porcine IVM system a valuable platform for evaluating and refining human IVM protocols. Research using the porcine IVM model provides critical insights that can enhance culture systems, boost oocyte competence, and ultimately improve the success rates of assisted reproductive technologies (ART) in humans. Furthermore, pigs are also widely recognized as one of the most promising models for xenotransplantation because of the similarity in size, structure, and function of their organs to humans (Ibrahim et al., 2006). Research on the generation of gene-edited pigs for xenotransplantation (Anand et al., 2023; Pan et al., 2024) and pig models for use in human disease studies (Hou et al., 2022) using embryonic gene editing technology (Takebayashi et al., 2022; Tanihara et al., 2023) is urgently needed and of interest. Oocyte-cumulus complexes (OCCs) are collected from the ovaries of prepubertal gilts, slaughtered commercially before sexual maturity and used as materials for in-vitro production of embryos due to their availability, although the quality of the oocytes may be less capable as compared with those of cyclic gilts (Pawlak et al., 2011) and sows (Bagg et al., 2007; Marchal et al., 2001). Therefore, the selection of immature oocytes before IVM affects the meiotic and developmental competences of oocytes. In addition to selection based on the morphological analysis of the oocytes (Areekijseeree & Chuen-Im, 2012; Yuan & Krisher, 2012), BCB staining has been utilized to select porcine MF-derived BCB-positive (BCB+) oocytes with both meiotic and developmental competences (Ericsson et al., 1993; Fonseka et al., 2024; Roca et al., 1998). However, BCB+ oocytes (considered to have relatively low G6PD activity) are

observed even in SF; oocytes are believed to still be in the growing phase and possess higher meiotic competence compared to BCB-negative (BCB-, considered to have relatively high G6PD activity) oocytes (Fonseka et al., 2024). Therefore, it is essential to reanalyse whether the characteristics of BCB+ oocytes have long been regarded as grown ones with meiotic and developmental competences. Furthermore, BCB staining has been demonstrated to cause oxidative stress to OCCs, especially BCB+ ones, through increasing reactive oxygen species (ROS) levels (Fonseka et al., 2024). However, the damage to oocytes through IVM culture has not been evaluated yet. Notably, the number of cumulus cells surrounding the oocyte is less in SF-derived OCCs than in MF-derived ones; the resilience of OCCs against oxidative stress through BCB staining may differ among those derived from VSF, SF, and MF.

In this context, the current study determines whether porcine OCCs derived from various antral follicle sizes (VSF, SF, and MF) exhibit differences in BCB staining status, meiotic and developmental competences, morphological characteristics, and expression of genes related to the PPP, as well as to evaluate their resilience to BCB staining-induced oxidative stress by analysing intracellular GSH, ROS, and NADPH levels in oocytes during IVM.

1.2. Ovary: Anatomy and physiological development

1.2.1. The ovary formation and oogenesis

In mammals, the ovary is a key reproductive organ responsible for producing germ cells, or oocytes, which are essential for fertilization and the continuation of the species. Beyond its reproductive role, the ovary also functions as a major endocrine gland, secreting steroid hormones that drive the development of female secondary sexual characteristics and support pregnancy. The ovary in pigs originates from the genital ridge, a bipotential structure located alongside the mesonephros. Around 20 to 24 days post coitum (dpc), primordial germ cells migrate from the yolk sac endoderm to the genital ridge. Upon arrival, primordial germ cells undergo rapid mitotic proliferation to expand the germ cell population, while adjacent coelomic epithelial and mesonephric cells differentiate into pre-granulosa cells, which later play a key role in follicle

formation. At this point, the gonad remains undifferentiated. In the absence of testis-determining signals, the gonad begins to differentiate into an ovary, structurally dividing into an outer cortex and an inner medulla. During this phase, germ cells initiate meiosis around 40 dpc and arrest at prophase I (diplotene stage, namely germinal vesicle) until 47 dpc (Bielańska-Osuchowska, 2006). Between days 60 and 90, oogonia become surrounded by flattened pre-granulosa cells, forming primordial follicles that are stored in the ovarian cortex as the lifelong reserve of oocytes. By day 100 until birth, the basic architecture of the ovary is completed and ready for postnatal development. The cortex becomes densely populated with primordial follicles, while the medulla contains connective tissue, blood vessels, lymphatics, and nerves. The ovary weight of pre-pubertal gilts also increased threefold after 20 days (from 2 g in 110 days old to 6 g in 130 days old) to prepare for puberty (Erickson, 1967). The porcine ovary is oval-shaped, approximately 24 mm long and 16 mm wide before puberty. By puberty, the size increases to about 33 mm long and 22 mm wide (Bagg et al., 2004).

1.2.2. Folliculogenesis

Folliculogenesis in pigs is a complex process with sequential development of ovarian follicles from the primordial follicle to the preovulatory (Graafian) follicle, capable of releasing a mature oocyte. This progression includes primordial, primary, secondary, tertiary (antral) follicles and preovulatory follicles (Figure 1.1). In pigs, about 500,000 primordial follicles are present at birth, gradually decreasing to approximately 400,000 by puberty (Black & Erickson, 1968). The ovaries of 77-day-old pigs averaged 74% primordial, 3% primary, and 22% secondary follicles and some tertiary follicles (Figure 1.2) (Oxender et al., 1979).

During folliculogenesis, primordial follicles are seen in the ovaries at 68 days post-coitum. In the primordial follicles stage, the oocyte is surrounded by flattened pre-granulosa cells. Up to 30 days after birth, almost all (99%) oocytes have been reported to proceed to the diplotene stage in primordial follicles (Black & Erickson, 1968). Primordial follicles stay dormant until activated or released from local inhibitory signals (Guthrie, 2005; Matsuda et al., 2012). The transition from

flattened to cuboidal granulosa cells marks the initiation of primary follicles, which are first observed in ovaries about 70 days post-coitum (Oxender et al., 1979). In these primary follicles, the oocyte is surrounded by a single layer of cuboidal granulosa cells and proliferates (Eppig, 2001). Follicular recruitment from dormant primordial follicles occurs continuously, starting with the initiation of primary follicle formation. When multiple layers of granulosa cells enclose the oocyte, the follicle is classified as a secondary follicle, which begins sensitivity to the gonadotrophins (follicle-stimulating hormone - FSH and luteinizing hormone - LH). A complex capillary network forms throughout this phase, commencing blood circulation to the follicle. Secondary follicles began to appear around birth, and their proportion gradually increased, reaching nearly 30% in the ovary by 90 days after birth. During the early antral stage, the follicles develop a basement membrane, distinct theca cell layers, and capillary networks (Shimizu et al., 2003). Additionally, an antral cavity forms and progressively enlarges due to fluid accumulation. Progression beyond the early antral follicle stage is strongly dependent on gonadotropins. Antral follicles, also known as tertiary follicles, typically contain meiotically competent oocytes. At this stage, proper levels of gonadotropins are essential for maintaining follicular growth. Under the influence of FSH, granulosa cells differentiate and begin synthesizing oestrogen (Fortune & Eppig, 1979). Antral follicles are primarily dependent on FSH for growth up to the 2-3 mm stage, become jointly dependent on both FSH and LH at 4-5 mm, and shift to relying predominantly on LH once they exceed 5 mm in diameter (Knox, 2023). Well-developed antral follicles are called Graafian follicles. The development of the follicular antrum results in the segregation of granulosa cells into two primary populations: cumulus cells, which closely surround the oocyte, and mural granulosa cells, which form the lining of the follicular wall (Figure 1.3). Following the preovulatory surge of gonadotropins, these follicles are designated as preovulatory follicles. In the preovulatory phase, granulosa cell proliferation accelerates, involving both cumulus and mural granulosa cells. Triggered by the preovulatory gonadotropin surge, oocytes continue their maturation process, eventually gaining the capacity to resume meiosis and progress to metaphase II before ovulation.

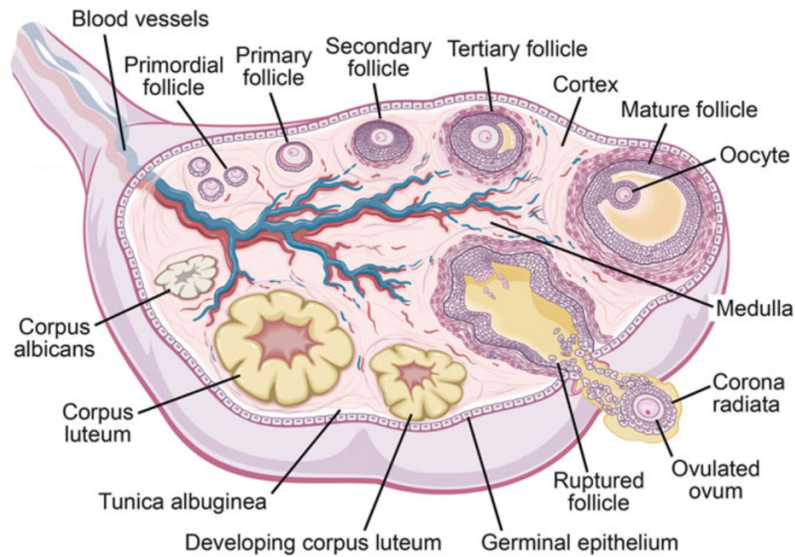


Figure. 1.1. The schematic of the ovary depicts the developing follicle and oocyte in the ovarian cortex (Desai et al., 2013)

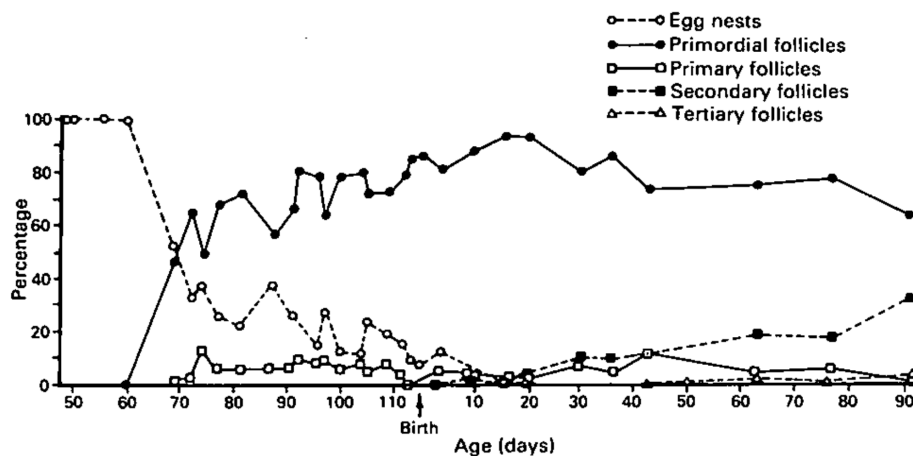


Figure 1.2. Changes in the proportion of ovarian follicle types in pig ovaries from pigs 49 days post-coitum to 92 days post-partum (Oxender et al., 1979)

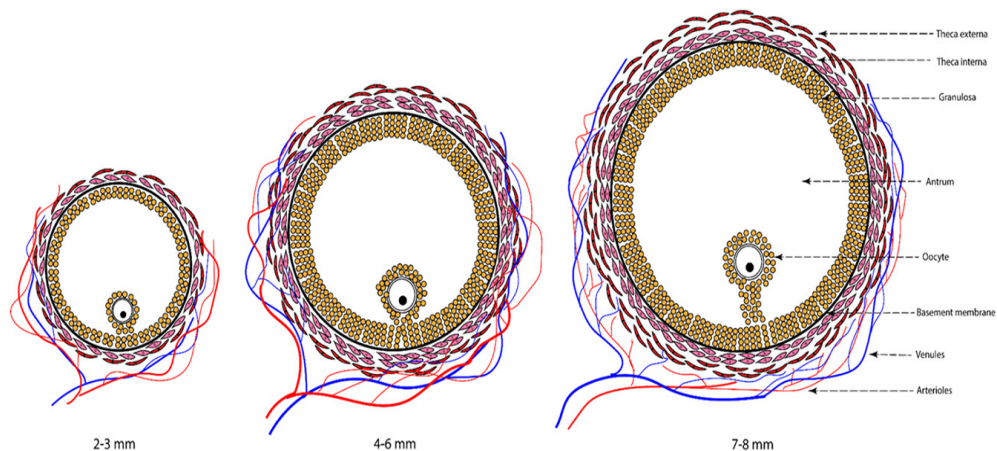


Figure 1.3. Healthy follicles grow from small (2–3 mm), to medium (4–6 mm) and large (7–8 mm) ovulatory size in the pig (Knox, 2023).

1.2.3. Follicle development in prepubertal gilts

Along with the development of the follicle, the diameter of the oocyte also increases gradually (Figure 1.4). Primordial follicles consist of an immature oocyte (40.1 μm) surrounded by one to two layers of flattened pre-granulosa cells. As development proceeds, the follicle progresses to the primary stage (approximately 0.12 mm in diameter), characterized by an oocyte of about 50 μm and one to three layers of granulosa cells. It then transitions into the secondary follicle stage with a diameter ranging from 0.14 to 0.40 mm (Christenson et al., 1985), featuring 3 to 20 layers of granulosa cells and an oocyte ranging from 70 to 100 μm in diameter (Morbeck et al., 1992). Once the follicle grows beyond 0.4 mm, an antral cavity begins to form, and its further development becomes dependent on gonadotropin stimulation. A primary follicle (less than 0.1 mm in diameter) takes a total of 84 days to grow to the early antral stage with an antrum formation (follicle over 0.4 mm in diameter) and then this follicle requires only 14 days to reach a diameter of 3.13 mm (Morbeck et al., 1992). Tertiary follicles containing an antrum were absent from the ovaries of pigs under 60 days old, and only a small number of ovaries from pigs aged 60 to 90 days exhibited one or more tertiary follicles (Oxender et al., 1979). Follicle size varies continuously, with the maximum size sometimes varying weekly (Grasso et al., 1988) within groups of 40-60 follicles (Schwarz et al., 2013). When antral follicles grow beyond 1 mm in diameter, they become visible on the ovarian surface (Knox, 2005), and approximately 95% of them are healthy (non-atretic) and capable of steroid production (Dufour et al., 1988; Guthrie et al., 1995). However, at any point thereafter, these follicles may either continue to develop or undergo atresia. In most cases, atresia occurs before the follicles reach a diameter of 6 mm. Although follicles larger than 6 mm have been observed in prepubertal gilts, they are few, do not ovulate, and regress rapidly. During preovulatory maturation, less than 5% of follicles larger than 6 mm in diameter are atretic follicles. On days 1 to 3 of preovulatory maturation, only 17% of follicles with 3 to 6 mm in diameter are atretic, while this rate is 87% atretic follicles on day 5 (Guthrie et al., 1995). The largest follicles typically have an average diameter of 6.5 to 8.0 mm

before ovulation (Knox, 2023). During the early luteal phase, the number of atretic follicles increase from 6% on day 5 to 50% on day 7 after oestrus (Guthrie et al., 1995).

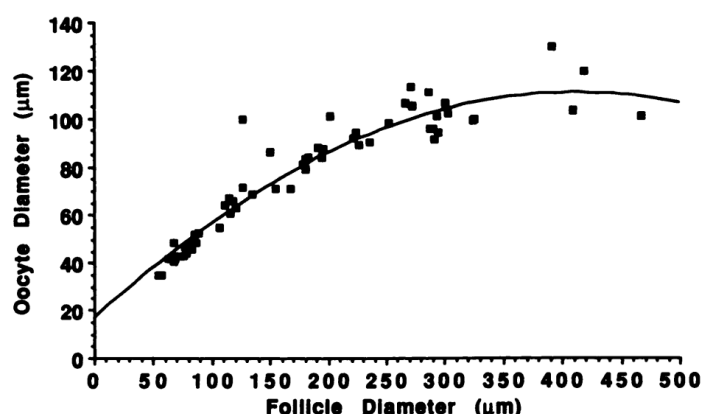


Figure 1.4. Oocyte diameter in relation to follicle diameter in preantral follicles (Morbeck et al., 1992)

1.3. OCCs as a material for in vitro maturation in pigs

An oocyte-cumulus complex is structured with an oocyte surrounded by some layers of cumulus cells and exists inside antral follicles. OCCs derived from medium follicles (MF; with 3–6 mm in diameter) of prepubertal gilts, slaughtered commercially before sexual maturity at 150–175 days old for pork production in many countries, are essential material in both IVM and IVF systems, even though the quality of the oocytes may be less capable as compared with those of cyclic gilts (Pawlak et al., 2011, 2015) and sows (Bagg et al., 2007; Marchal et al., 2001). Techniques to produce embryos in vitro from MF-derived OCCs have been well established in the pigs (Funahashi & Day, 1997) due to the superior meiotic and developmental competence compared to small follicles (SF; with 1–3 mm in diameter)-derived OCCs (Kohata et al., 2013; Okudaira et al., 2017). However, smaller antral follicles with a diameter of less than 3 mm are more numerous than MF in the ovaries of prepubertal gilts (Morbeck *et al.*, 1992). Oocytes obtained from SF have fewer surrounding cumulus cells (Matsunaga & Funahashi, 2017) and are smaller in diameter (Matsunaga & Funahashi, 2017; Yoon et al., 2000) compared to those derived from MF. Recently, successful outcomes in several studies using OCCs from SF (Bui *et al.*, 2017; Matsunaga & Funahashi, 2017; Nguyen *et al.*, 2020) have opened new possibilities for utilizing this plentiful source in porcine in vitro embryo production. Moreover, applying a pre-IVM under

optimal conditions may effectively promote oocyte growth and enhance the meiotic and developmental competence of SF-derived OCCs (Cayo-Colca et al., 2011; Jitjumnong & Tang, 2024; Nguyen et al., 2020).

1.4. Cumulus cells: Functions, proliferation, and morphological change during IVM

Granulosa cells are classified into distinct lineages based on their location within the follicle, including steroidogenic mural granulosa cells located along the follicular wall and cumulus cells surrounding the oocyte (Uslu & Johnson, 2013). In addition, considering differences in gene expression and hormonal responsiveness, granulosa cells can be further categorized into three subtypes: mural granulosa cells, antral granulosa cells, and cumulus cells (Russell & Robker, 2018). Cumulus cells form direct connections with the oocyte's plasma membrane via gap junctions that span the zona pellucida. Moreover, communication among cumulus cells is facilitated by numerous microvilli that enable interaction between adjacent cumulus cells (Areekijseer & Chuen-Im, 2012).

1.4.1. The proliferation of cumulus cells in vitro culture

Proliferation and differentiation of somatic cells occur throughout the development of the follicle from the primordial to the preovulatory follicle, and they are mainly controlled by FSH and LH, especially from the secondary follicle stage onwards. In vitro, the proliferative capacity of cumulus cells is influenced by the expression of key markers such as follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), connexins (Cx), and the enzyme aromatase (CYP19A1) (Ciesiółka et al., 2017; Kempisty et al., 2014). During IVM of porcine OCCs, progesterone produced by FSH- and LH-stimulated cumulus cells has inhibited the proliferation of cumulus cells (Okazaki et al., 2003). A high level of progesterone receptor-B is associated with enhanced proliferation of cumulus cells within the first 10 h of IVM. This receptor is expressed during the early phase of IVM (peaking between 4-12 h) when gonadotropins (LH and FSH) are present in the culture medium. After 12 h, progesterone receptor-B expression declines, while progesterone receptor-A becomes more dominant in cumulus cells. Treatment with

RU486, a progesterone inhibitor, helps maintain cumulus cell proliferation between 10-20 h of culture by preventing the downregulation of progesterone receptor-B mRNA and protein (Shimada et al., 2004). Cumulus cells collected from follicles larger than 5 mm show a reduced proliferation index at the onset of in vitro culture (Kempisty et al., 2015). Nevertheless, after 10 and 20 h of IVM, the number of cumulus cells per OCC derived from both MF and SF is significantly higher compared to 0 h (Okudaira et al., 2017). In the presence of gonadotropins (FSH and LH), the number of cumulus cells per intact OCC can increase by over 3,000 cells, whereas in hormone-free conditions, the count may decrease by approximately 1,000 cells (Alvarez, Dalvit, et al., 2012).

1.4.2. Morphological change of cumulus cells during IVM

During IVM, cumulus cells undergo a morphological transformation from a round shape to an elongated or columnar form. As a result, microvilli-mediated communication between cumulus cells is no longer observed after 24-48 h of culture (Areekijserree & Chuen-Im, 2012). This morphological shift marks the onset of oocyte maturation. Additionally, an increase in the proliferation index during the first 12 h of IVM indicates the initiation of cumulus cell expansion (Kempisty et al., 2013). The expansion of cumulus cells in intact OCCs becomes significant after 20 h of culture and remains at this size until 48 h of IVM (Appeltant et al., 2015). OCCs derived from large follicles (LF, 5-8 mm) begin expanding earlier - around 11 h after the start of IVM - than those from MF (3-5 mm). Furthermore, the extent of cumulus expansion in LF-derived OCCs is greater than in MF-derived ones. After 22 h of IVM, expansion of the cumulus cells extends to all the layers of the cumulus cells (Bagg et al., 2009).

Somfai *et al.* (2004) classified granulosa-cumulus-oocyte complexes (GCOCs) and OCCs into four categories based on the degree of somatic cell expansion observed after 30 h of IVM. By 30-48 h, most GCOCs and OCCs exhibit complete cumulus expansion with a bright appearance, occurring in 74.3-82.8% of GCOCs and 48.3-65.9% of OCCs. However, the morphological type

of cumulus expansion does not significantly influence the rates of germinal vesicle breakdown or progression to metaphase II between the GCOC and OCC groups (Somfai et al., 2004).

1.4.3. The roles of cumulus cells in IVM

Cumulus cells support oocyte growth and maturation mainly through metabolic processes. Among essential metabolites, glucose is a vital metabolite for the maturation of oocytes. It is metabolized via four main pathways: glycolysis, the pentose phosphate pathway (PPP), the polyol pathway, and the hexosamine biosynthesis pathway (HBP). During maturation, a significant portion of glucose is converted to pyruvate through glycolysis, providing energy for the oocyte. Additionally, glucose contributes to other critical functions during in vitro maturation (IVM), such as regulating the redox balance and nuclear maturation via ribose 5-phosphate synthesis by the PPP, facilitating cumulus cell expansion via extracellular matrix synthesis, and participating in cell signalling via O-linked glycosylation in the HBP (Sutton-McDowall et al., 2010).

Porcine oocytes themselves have limited capacity to utilize glucose (Alvarez, Ferretti, et al., 2012; Wen et al., 2020). Supplementation of glucose in the culture medium significantly enhances the maturation of OCCs, but not denuded oocytes (DOs), indicating that glucose is primarily metabolized by cumulus cells to produce substrates such as pyruvate and lactate that oocytes can effectively use (B. Yuan et al., 2016). In both oocytes and cumulus cells, pyruvate enters the mitochondrial electron transport chain, while lactate is converted back to pyruvate via LDH-catalyzed oxidation. Metabolites derived from glycolysis and the PPP in cumulus cells thus support oocyte maturation (Wen et al., 2020). The efficiency of glycolysis and PPP is closely linked to the maturation competence of OCCs (Alvarez, Ferretti, et al., 2012). These pathways promote the cytoplasmic maturation of porcine oocytes by releasing energy (ATP), controlling maturation-promoting factor (MPF) activity, and regulating maternal gene expression (B. Yuan et al., 2016). The PPP is vital for maintaining the redox state and supporting cytoplasmic maturation in porcine oocytes than glycolysis (Wen et al., 2020; B. Yuan et al., 2016).

Carbohydrate metabolism is associated with cumulus cell morphology during IVM under gonadotropin stimulation. OCCs with densely packed cumulus cells show higher glucose uptake and lactate production than OCCs with partial or poor cumulus layers. A strong positive correlation ($r = 0.85$) has been found between glucose uptake and lactate production per OCC, with a molar ratio of glucose to lactate of approximately 1:2-3.5. In contrast, denuded oocytes do not exhibit detectable glucose uptake or lactate production after 48 h of IVM (Alvarez, Dalvit, et al., 2012).

Moreover, a positive relationship exists between oocyte lipid and ATP levels and the number of surrounding cumulus cells, which is regulated by glucose uptake in oocyte-granulosa cell complexes (OGCs). Additionally, variations in cumulus cell morphology are linked to amino acid metabolism in OCCs during IVM. Notably, OCCs with a dense cumulus structure exhibit higher protein content at the start and end of IVM than OCCs with poor morphology (Alvarez, Dalvit, et al., 2012).

1.5. Selecting good-quality oocytes by the BCB staining method

Oocyte selection based on morphological characteristics, such as oocyte size, shape, and the structure of surrounding cumulus cell layers, is the main method for selecting high-quality oocytes. Morphological assessment of OCCs can improve fertilization efficiency and enhance subsequent embryo development outcomes (Jackowska et al., 2009). However, distinguishing high-quality oocytes remains challenging due to the morphological similarities between high- and low- developmentally competent oocytes (Ozturk, 2020). Further development of selection methods is needed to improve the assessment of immature oocyte quality before IVM. These approaches include biochemical tests to measure metabolic activity and molecular markers to analyse gene expression patterns to assess the developmental competence of oocytes.

Brilliant Cresyl Blue (BCB) staining is a widely used technique in assisted reproductive technologies, particularly for selecting developmentally competent oocytes for in vitro embryo production. This technique was first established and validated in porcine oocytes by Ericsson *et al.* (1993) and has since become a reliable tool for identifying high-quality oocytes in pigs and other species. BCB staining is a simple but effective method to improve the outcome of IVM and

IVF. BCB staining, which can approximately and noninvasively assess the activity of the key rate-limiting enzyme in PPP, glucose-6-phosphate dehydrogenase (G6PD), has been utilized to select OCCs containing oocytes with high meiotic and developmental competences in various mammalian species (Catalá et al., 2011; Ericsson et al., 1993; Fonseka et al., 2024; Manjunatha et al., 2007; Roca et al., 1998; Rodríguez-González et al., 2002; Silva et al., 2011). Oocytes with partial and complete decolorization of the BCB dye (light blue or colorless, respectively; BCB-negative) are believed to be still growing and have a high G6PD activity, whereas those with a lower G6PD activity are fully grown and retain the BCB dye (dark blue; BCB-positive). BCB+ porcine oocytes consistently exhibit superior meiotic and developmental competences compared with BCB- oocytes (Ericsson et al., 1993; Fonseka et al., 2024; Roca et al., 1998). However, BCB+ oocytes are observed even in SF, oocytes are believed to still be in the growing phase and possess higher meiotic competence compared to BCB- oocytes, and the proportion of SF-derived BCB- oocytes was significantly higher than that obtained from MF (Fonseka et al., 2024). Furthermore, BCB+ oocytes showed a markedly greater extent of cumulus cell expansion and significantly improved IVF outcomes, including higher monospermy rates, enhanced fertilization efficiency, increased blastocyst formation rates and reduced apoptosis in blastocysts, relative to BCB- oocytes (S. Lee et al., 2020).

1.6. Oxidative stress

1.6.1. Source and effect of oxidative stress to oocytes during IVM and IVC

Oxidative stress arises from an imbalance between the generation of ROS and their neutralization by antioxidant defences. The main sources of oxidative stress in IVM are elevated reactive oxygen species (ROS) from environmental factors (light, temperature, and oxygen-rich culture conditions), metabolic activity, and chemical inducers in IVM medium, which damage cellular structures and impair developmental competence.

- Environmental factors:

+ Exposure to light, originating from microscopes and surrounding environmental sources, is a frequent stressor during the *in vitro* manipulation of oocytes and embryos. Ambient light exposure can negatively impact embryo development, especially when the embryos are exposed to direct light for more than an hour, as well as when culture medium is exposed to light for long periods of time (R. Li et al., 2014). Furthermore, cool white fluorescent light, which emits a higher percentage of short-wavelength visible light and is widely used in most research and clinical laboratories, generates more ROS in mouse and hamster zygotes than warm white fluorescent light (Takenaka et al., 2007). Eliminating white light exposure to oocytes during all work stages by applying red filters on laboratory lamps and UV or infrared filters in the microscopes positively affects fertilization rate and increases blastocyst development (Bódis et al., 2020). Another simple and cost-effective green filter (498-563 nm wavelength) appears to lessen light-induced stress during bovine *in vitro* production (IVP) procedures, without affecting the accuracy of embryo observation or requiring increased light intensity (Korhonen et al., 2009).

+ Heat stress increases ROS levels in oocytes in IVM and IVC (Ascari et al., 2017; S. Lee et al., 2021) (Amaral et al., 2020). Heat stress is known to impair porcine oocyte maturation via ceramide-mediated apoptosis. More detailed, heat stress conditions (41.5°C) during IVM significantly increased ceramide levels in porcine OCCs, leading to increased ROS production and mitochondrial dysfunction, thereby inducing apoptosis compared with the control group (38.5°C). However, supplementation with specific inhibitors (fumonisin B1 and desipramine) of ceramide formation under heat stress notably decreased ceramide levels, ROS production, cytochrome C expression, and apoptosis, while enhancing mitochondrial membrane potential in OCCs (S. Lee et al., 2021). The exposure of bovine oocytes to elevated temperature (40.5°C in laboratory incubator, sustained for 6 h) disrupts the meiotic and developmental competence, reduces blastocyst yield, increases ROS, and significantly alters the coordinated pattern of gene expressions (Amaral et al., 2020; Stamperna et al., 2020). In addition to the effects of heat stress, lower culture temperature

(35°C vs. 39°C) delayed nuclear maturation and reduced blastocyst rate in porcine oocytes (Abeydeera et al., 2001).

+ High oxygen concentration: The oxygen concentration within the lumen of the female reproductive tract ranges from 1.5% to 9% depending on part of the reproductive tract, with a maximum level of one-third of the 20% typically used under in vitro conditions (Fischer & Bavister, 1993; Mastroianni & Jones, 1965). Although no significant difference was found in maturation rates of bovine oocytes during IVM at 20% and 5% oxygen levels, maturation under 20% oxygen conditions showed increased levels of both ROS and GSH compared with 5% oxygen (Cruz et al., 2023).

- Metabolic activity: Oocytes and cumulus cells generate ROS as byproducts of metabolism. A variety of ROS, both free radicals and nonradicals, are present in cells, including oocytes and cumulus cells. Among the most damaging are superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), hydroperoxyl, peroxy, and alkoxy radicals (Halliwell & Whiteman, 2004; Phaniendra et al., 2015). Nonradical forms of oxygen include hydrogen peroxide (H_2O_2), hypobromous acid, hypochlorous acid, ozone, singlet oxygen, organic peroxides, nitric oxide ($NO\bullet$), peroxynitrite ($ONOO^-$), and peroxynitrous acid (Halliwell & Whiteman, 2004; Phaniendra et al., 2015). These ROS can be produced in different cell parts, but mitochondria are the primary site of their formation (Di Meo et al., 2016). Mitochondria are essential for producing adenosine triphosphate (ATP) through oxidative phosphorylation in aerobic cells. During this process, several electrons consistently leak from the electron transport chain, reacting rapidly with oxygen to form superoxide anion (He et al., 2017). Superoxide anion is converted to H_2O_2 by superoxide dismutase (SOD), an enzyme localized primarily in the matrix or inner membrane of the mitochondria. Hydroxyl radicals are produced when superoxide anions react with metal ions and iron-sulphur clusters in the presence of H_2O_2 (Liochev & Fridovich, 1994).

- In addition, several chemicals were added in porcine IVM medium culture such as hydrogen peroxide (H_2O_2) (H. J. Park et al., 2022; Shi et al., 2025), tunicamycin (C. Li et al., 2025;

H. J. Park et al., 2022), as well as environmental toxins such as arsenic (Kang et al., 2022) for experimental use to model oxidative stress.

1.6.2. Antioxidant defense systems in oocyte and cumulus cells

Antioxidants play a vital role in neutralizing oxidants and combating free radicals. The endogenous antioxidant defense system includes enzymatic antioxidants and non-enzymatic antioxidants.

Glutathione (GSH) is a key intracellular antioxidant, and its production and storage in oocytes are heavily supported by surrounding cumulus cells, which help shield oocytes from DNA damage and apoptosis triggered by ROS (Maedomari et al., 2007; Tatemoto et al., 2000). Glycine, a precursor of GSH, enhances GSH levels and promotes the expression of antioxidant-related genes, leading to reduced lipid peroxidation and lower risk of ferroptosis (Gao et al., 2023).

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are upregulated in both oocytes and cumulus cells, particularly in response to antioxidant supplementation like oleanolic acid (Wang et al., 2024), equol (Xiao et al., 2025), pyrroloquinoline quinone (PQQ) (Zhang et al., 2025), and β -cryptoxanthin (a carotenoid) (Y. G. Park et al., 2018). These enzymes play a direct role in neutralizing ROS. In addition, triggering the Nrf2/HO-1 signaling pathway enhances the production of antioxidant enzymes and improves cellular resistance to oxidative stress (Wang et al., 2024; Xiao et al., 2025).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Chemicals and media

Sodium chloride, KCl, KH_2PO_4 , NaOH, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, potassium penicillin, gentamicin sulfate, and paraffin oil were purchased from Nacalai Tesque (Kyoto, Japan). Human chorionic gonadotrophin (hCG; Gonatropin) and equine chorionic gonadotrophin (eCG; Serotropin) were obtained from ASKA Pharmaceutical (Kyoto, Japan). Except for specific cases, all other chemicals were sourced from Sigma-Aldrich (St. Louis, MO, USA).

The collection medium used was Modified Tyrode's Lactate HEPES buffer, supplemented with 0.1% (w/v) polyvinyl alcohol (TL-HEPES-PVA). The electroporation medium (EM) contained 0.25 M mannitol, 0.5 mM HEPES, 0.1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.01% (w/v) polyvinyl alcohol (pH 7.2). The transit culture medium of parthenogenetically activated oocytes was based on Medium 199 (M199) modified with 3.05 mM glucose, 2.92 mM calcium L-lactate hydrate, 26.2 mM NaHCO_3 , 0.91 mM sodium pyruvate, 12 mM sorbitol, 75 $\mu\text{g/mL}$ penicillin G potassium, and 75 $\mu\text{g/mL}$ gentamicin sulfate (mM199, pH 7.4). The IVM medium for OCCs was Porcine Oocyte Medium (POM, Research Institute for Functional Peptides, Yamagata, Japan), modified to include 50 $\mu\text{M/L}$ 2-mercaptoethanol (Akaki et al., 2009). The medium used for IVC of parthenogenetically activated oocytes to the blastocyst stage was chemically defined as Porcine Zygote Medium (PZM-5, Research Institute for Functional Peptides, Yamagata, Japan). All media were stored at 4 °C and pre-warmed overnight at 39 °C before use. Except for TL-HEPES-PVA and EM, the other media were equilibrated in a sterile, humidified incubator at 39°C with 5% CO_2 in the air.

2.2. Preparation of OCCs

Ovaries from pre-pubertal gilts (any corpus lutea) were obtained at a local slaughterhouse, transported to the laboratory, and washed as previously described (Fonseka et al., 2024). MF- and SF-derived OCCs were aspirated separately from the surfaces of the ovaries using an 18-gauge

needle (1.2 mm in diameter) connected to a 10-mL disposable syringe at room temperature (25 °C). In contrast, VSF-derived OCCs were aspirated using a 21-gauge needle (0.8 mm in diameter). The aspirated follicular fluid containing OCCs was placed in separate 15-mL tubes and washed three times with TL-HEPES-PVA. Only OCCs containing oocytes with uniform cytoplasm and surrounded by at least two compact layers of cumulus cells for VSF or three compact layers for MF and SF were used for IVM culture and subsequent experiments.

2.3. BCB staining

After collection, the OCCs were incubated in mPOM containing 26 μ M BCB for 90 min at 39°C in a humidified atmosphere with 5% CO₂ in air (Fonseka et al., 2024). Subsequently, the OCCs were washed twice in mPOM and divided into two groups based on the presence or absence of BCB coloration in the ooplasm within 5 min (BCB+ and BCB- groups, respectively). Oocytes exhibiting dark blue coloration in the ooplasm were classified as BCB+, whereas oocytes with colorless or slightly blue coloration were designated as BCB-. After classification, the OCCs were washed twice with mPOM for IVM or TL-HEPES-PVA for subsequent measurements.

2.4. Culture for in vitro maturation of oocytes

After BCB staining, forty to sixty OCCs from MF, SF, and VSF were cultured separately in 500 μ L mPOM supplemented with gonadotropins (10 IU eCG/mL and 10 IU hCG/mL) and 1 mM dibutyryl cAMP (db-cAMP) in a four-well culture plate (Thermo Fisher Scientific, Waltham, MA, USA) for 20 h at 39 °C in 5% CO₂ (Akaki et al., 2009; Funahashi et al., 1997). The OCCs were washed three times and then cultured for an additional 24 h in fresh medium without gonadotropins and db-cAMP. After a total of 44 h for IVM culture, the oocytes were denuded by pipetting in TL-HEPES-PVA containing 0.01% (w/v) hyaluronidase. Some of the denuded oocytes were stained with 25 μ g/mL Hoechst 33342 in TL-HEPES-PVA for 15 min at 39 °C and then washed three times with TL-HEPES-PVA to evaluate meiotic progression under a fluorescence microscope (BZ-X700, Keyence, USA) at 200X magnification. Besides, mature oocytes were determined by

the presence of the first polar body in hypertonic TL-HEPES-PVA supplemented with 0.25 M sucrose.

2.5. Assessment of the expression of genes

A total of 30 denuded oocytes and their surrounding cumulus cells were collected separately and stored in 10 μ L of RNeasy lysis solution (Qiagen, USA) at -80 °C. Total RNA samples were extracted from the oocytes and cumulus cells using TRIzol Reagent (Thermo Fisher Scientific, Belgium). Briefly, after homogenizing the samples with TRIzol, chloroform (Nacalai Tesque, Japan) was added to induce phase separation. RNA was then precipitated with cold 2-propanol (Nacalai Tesque, Japan) and co-precipitated with GlycoBlue (Thermo Fisher, USA) to enhance the pellet size and visibility of the pellet. Before air-drying, RNA pellets were washed twice with 75% ethanol (Sigma-Aldrich, Belgium). The RNA pellets were then dissolved in 13 μ L of nuclease-free water before quantification using the NanoDrop OneC (Thermo Fisher Scientific, Belgium). For reverse transcription, the high-capacity of cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Belgium) was utilized. An equal amount of RNA (100 ng) from each sample was used for reverse transcription (Ståhlberg et al., 2004), following the manufacturer's instructions, and subsequently diluted fourfold with nuclease-free water. Quantitative RT-PCR was conducted using the LightCycler 96 (Roche, Belgium) and GeneAmp SYBRTM qPCR Mix α as the detection dye. The protocol included a pre-incubation step at 95 °C for 10 min, followed by 40 amplification cycles consisting of 15 s at 95 °C and 60 s at 60 °C. The specificity of the PCR products was verified using melting curve analysis. The expression levels of specific genes in cumulus cells and oocytes were normalized to *GAPDH*. Fold changes in expression levels were calculated using $2^{-\Delta\Delta C_t}$ method. Each experiment was replicated four times, with duplicate PCR reactions performed for each sample.

CHAPTER 3

CHARACTERISTICS OF PORCINE OOCYTE-CUMULUS COMPLEXES DERIVED FROM VARIOUS SIZES OF ANTRAL FOLLICLES AND CLASSIFIED BY BRILLIANT CRESYL BLUE STAINING, AND DEVELOPMENTAL COMPETENCE OF THE OOCYTES

Abstract

The present study sought to determine the characteristics of porcine oocyte-cumulus complexes (OCCs) derived from very small and small antral follicles (with less than 1 mm and 1-3 mm in diameter, respectively; VSF and SF) in comparison with controls from medium ones (with 3-6 mm in diameter; MF). Additionally, the present study examined the utility of brilliant cresyl blue (BCB) staining for assessing these OCCs. The incidence of BCB- oocytes in VSF- and SF-derived OCCs was higher than that in MF-derived OCCs. Although the meiotic and developmental competences of BCB+ oocytes from MF were superior to those from VSF and SF, blastocysts were successfully obtained from BCB+ oocytes, even derived from VSF. The mean numbers of both total and viable cumulus cells surrounding an oocyte were significantly affected not only by the origin of the OCCs, but also by the BCB status of the oocytes (largest in MF-derived OCCs containing BCB+ oocytes). Although the outer and inner diameters of zona pellucida were affected by the origin of OCCs and the BCB status of oocytes (largest in MF-derived oocytes), the ooplasmic diameter of BCB+ oocytes did not differ among those derived from VSF, SF, and MF. Regardless of the BCB status, the transcriptional levels of *G6PD* and *TKT* in cumulus cells decreased during follicular development from VSF to MF, whereas the *RPIA* mRNA level in cumulus cells of MF-derived BCB+ OCCs was lower than in the others. These results underscore the utility of BCB staining for selecting MF-, SF-, and even VSF-derived OCCs containing oocytes with relatively higher meiotic and developmental competences, as well as the importance of having a sufficient number of healthy cumulus cells expressing genes related to the pentose phosphate pathway at lower levels.

3.1. Introduction

The importance of in vitro maturation (IVM) and fertilization (IVF) systems that utilize porcine oocytes derived from developing follicles has increased significantly with the focus on genome editing to create disease models and xenograft donors (Takebayashi et al., 2022; Tanihara et al., 2023; Watanabe & Nagashima, 2023). For decades, oocyte-cumulus complexes (OCCs) derived from medium follicles (MF; with 3-6 mm in diameter) have been collected from the ovaries of prepubertal gilts and used as materials for in-vitro production of embryos (Funahashi & Day, 1997; Martinez et al., 2023; Romar et al., 2016), even though the quality of the oocytes may be less capable as compared with those of cyclic gilts (Pawlak et al., 2011) and sows (Bagg et al., 2007; Marchal et al., 2001). This could be because a majority of prepubertal gilts are commercially slaughtered at 150-175 days old for pork production in many countries, and the ovaries are readily available. The meiotic and developmental competences of MF-derived oocytes are also well known to be higher than those from small follicles (SF; with 1-3 mm in diameter) (Kohata et al., 2013; Marchal et al., 2001; Okudaira et al., 2017). However, smaller antral follicles with less than 3 mm in diameter, as well as preantral and primordial follicles, are more numerous than MF in the ovaries of prepubertal gilts (Almeida et al., 2017). If the meiotic and developmental competences of oocytes derived from smaller antral follicles could be improved somehow, a greater number of oocytes would be available for the in-vitro production of porcine embryos. Although characteristics, such as the number of granulosa cells in the follicles, the status of gonadotropin receptors expressed in those cells, and the diameter of the oocytes-appear to differ significantly between MF and SF (Knox, 2023), recent efforts have improved some of the competences of SF-derived oocytes through the presence of VEGF or by co-culture with healthy cumulus cell masses (Bui et al., 2017; Fonseka et al., 2024; Matsunaga & Funahashi, 2017). Nonetheless, the characteristics of OCCs from very small antral follicles less than 1 mm in diameter (VSF) and the meiotic and developmental competences of the oocytes within these follicles remain unclear.

Additionally, the ovaries of prepubertal gilts appear to contain a proportion of atretic follicles (Almeida et al., 2017).

Since the brilliant cresyl blue (BCB) staining method, which enables a preliminary assessment of intracellular glucose-6-phosphate dehydrogenase (*G6PD*) activity without causing toxicity, has proven effective in selecting porcine MF-derived oocytes with both meiotic (Ericsson et al., 1993) and developmental competences (Roca et al., 1998), this non-invasive method has been applied across various mammalian species (Catalá et al., 2011; Manjunatha et al., 2007; Rodríguez-González et al., 2002; Silva et al., 2011). The principle of BCB staining is as follows: when the activity of G6PD, the key enzyme in the intracellular pentose phosphate pathway (PPP), is high, its metabolite NADPH reduces the intracellular BCB dye, converting it to a colorless form. Conversely, when G6PD activity is low, the dye remains blue. It is widely believed that BCB staining is a straightforward method for selecting fully grown oocytes, G6PD activity appears to be elevated in growing oocytes during folliculogenesis but decreases in grown ones. However, BCB-positive (BCB+; considered to have relatively low G6PD activity) oocytes are observed even in SF, oocytes are believed to still be in the growing phase and possess higher meiotic competence compared to BCB-negative (BCB-; considered to have relatively high G6PD activity) oocytes. Furthermore, OCCs containing BCB- oocytes exhibited a higher proportion of dead cumulus cells than those with BCB+ ones (Fonseka et al., 2024).

Therefore, it is essential to reanalyze whether the characteristics of BCB+ oocytes have long been regarded as grown ones with meiotic and developmental competences. The objective of the current study was to examine if the characteristics of VSF- and SF-derived porcine OCCs, including BCB staining assessment, meiotic and developmental competences, the number of surrounding viable cumulus cells, the size of oocytes, and the expression of genes related to the PPP, differ from those of MF-derived ones.

3.2. Materials and Methods

3.2.1. Culture for early development of parthenotes

Only mature oocytes containing the first polar body were selected in hypertonic TL-HEPES-PVA supplemented with 0.25 M sucrose. After washing the oocytes three times with EM, they were transferred to an activation chamber (with a 1 mm distance between the two electrodes) within the same solution and activated by a single electrical pulse (direct current, 1.2 kV/cm, 30 μ s) using the BTX-Electro-Cell Manipulator 2001M (BTX, San Diego, CA, USA). To inhibit the extrusion of the second polar body, the activated oocytes were incubated in 500 μ L of mM199 supplemented with 0.4% (w/v) BSA and 5 μ M cytochalasin B for 4 h at 39 °C in a 5% CO₂ atmosphere, following three times washes with the same medium. Subsequently, the oocytes were washed three times with PZM-5 and cultured in 500 μ L of PZM-5 under paraffin oil in a four-well culture plate for 5 days at 39 °C in an atmosphere of 5% CO₂. Cleavage and blastocyst formation rates were assessed 2 and 5 days after the initiation of culture, respectively. To determine the number of blastomeres per embryo, blastocysts were incubated in TL-HEPES-PVA containing 25 μ g/mL of Hoechst 33342 at 39 °C for 30 min. Following incubation, the blastocysts were washed three times with TL-HEPES-PVA and mounted on a glass slide using an antifade mounting medium (VECTASHIELD; Vector Laboratories, CA, USA) before being covered with a coverslip. The specimens were examined under a fluorescence microscope (BZ-X700, Keyence, USA) at a magnification of 200X.

3.2.2. Measurement of oocyte diameters

After BCB staining, oocytes were denuded by pipetting in TL-HEPES-PVA. The diameter of the ooplasm (calculated as the average of the largest and smallest diameters) and the outer and inner diameters of the zona pellucida (determined as the average of two orthogonal diameters) were measured using an inverted microscope (IX73; Olympus, Tokyo, Japan) at a magnification of 200X with RI Viewer Imaging Software. Since the perivitelline space between the zona pellucida and the ooplasm was newly formed after denudation, and the size of the perivitelline

space was calculated from the outer and inner diameters of the zona pellucida and the diameter of the ooplasm, respectively (J. Lee et al., 2013).

3.2.3. Assessment of the viability of cumulus cells surrounding the oocyte

Just after collection from MF, SF, and VSF, the viability of cumulus cells from OCCs was assessed using SYBR Green and Propidium Iodide (PI) staining (Invitrogen, Carlsbad, CA, USA). MF-, SF-, and VSF-derived BCB+ or BCB- OCCs were incubated separately in TL-HEPES-PVA containing 0.4 μ M SYBR Green and 7.5 μ M PI for 30 min at 39 °C. After washing three times with TL-HEPES-PVA, the OCCs were mounted on glass slides and covered with coverslips. Images of each OCC were captured using a fluorescence microscope (BZ-X700, Keyence, Osaka, Japan) at a magnification of 200X. Images of 29-36 COCs from each experimental group were analyzed using ImageJ software, version 1.53 (National Institutes of Health, Bethesda, MD, USA), to quantify the number of viable (green) and non-viable (orange) cumulus cells per OCC.

3.2.4. Assessment of the expression of genes associated with the PPP

The sequences of the forward and reverse primers used in this experiment are presented in Table 3.1. The expression levels of three genes associated with the PPP, including *glucose 6-phosphate dehydrogenase (G6PD)*, *transketolase (TKT)*, *ribulose 5-phosphate isomerase A (RPLA)*, and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* were analyzed in both oocytes and cumulus cells.

3.2.5. Experimental designs

3.2.5.1. The meiotic and developmental competences of BCB+ and BCB- OCCs derived from antral follicles with various sizes (Experiment 1)

Just after collection, OCCs derived from MF, SF, and VSF were subjected to BCB staining and divided into two groups: BCB+ and BCB-. These complexes were then maintained in TL-HEPES-PVA. Forty to sixty BCB+ and BCB- OCCs from each follicle type were cultured separately for IVM, and meiotic progression was assessed 44 h after the initiation of IVM.

Additionally, only mature oocytes were electrically activated and cultured for IVC for 5 days. Cleavage and blastocyst formation rates were evaluated two and five days after the initiation of IVC, respectively. The number of blastomeres per blastocyst was also recorded. All experiments were replicated seven times.

3.2.5.2. Characteristics of BCB+ and BCB- OCCs derived from antral follicles with various sizes (Experiment 2)

Just after BCB staining and the selection of OCCs based on the staining status of the oocytes, some OCCs were stained with SYBR Green and PI to determine the number of viable and non-viable cumulus cells surrounding the oocytes. Other OCCs were denuded by pipetting, and the diameters of the ooplasm, as well as the outer and inner zona pellucida of the oocytes, were measured to assess the morphological characteristics of the oocytes. All experiments were replicated five times.

3.2.5.3. PPP-related gene expressions of BCB+ and BCB- OCCs derived from antral follicles with various sizes (Experiment 3)

The expression of genes associated with PPP in oocytes and cumulus cells was examined using quantitative RT-PCR. The relative expression levels of *G6PD*, *TKT*, and *RPIA* in BCB+ and BCB- oocytes derived from both SF and VSF, as well as those in MF-derived BCB- oocytes, were compared to those in MF-derived BCB+ oocytes. All experiments were replicated four times.

3.2.6. Statistical analyses

Data from 4 to 7 replications were processed to examine the variables using one-way (experimental groups) and/or two-way (origins and BCB status of OCCs) ANOVA employing IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). Percentage data were subjected to arc-sine transformation before statistical analyses if the data contained percentages greater than 90% or less than 10%. All data are presented as mean $\pm$ SEM. Findings were considered statistically

significant at $P < 0.05$, and when a significant effect was observed, values were compared using Tukey's post hoc test.

3.3. Results

3.3.1. The meiotic and developmental competence of BCB+ and BCB- OCCs derived from antral follicles with various sizes

The percentages of MF-derived OCCs with BCB+ and BCB- oocytes were significantly higher and lower ($P < 0.05$), respectively, compared to those derived from SF and VSF (Table 3.2). The incidence of BCB- OCCs was particularly 1.5- and 2-fold higher than that of BCB+ ones in SF- and VSF-derived OCCs, respectively.

As shown in Table 3.3, the meiotic competence of oocytes was significantly affected ($P < 0.01$) by both the origins of OCCs and the status of BCB staining. The percentage of oocytes that matured to the metaphase-II stage was highest in MF-derived BCB+ oocytes, followed by SF- and VSF-derived BCB+ oocytes, which ranked second and third, respectively. In contrast, the lowest percentage was observed in VSF-derived BCB- oocytes. There were no significant differences in the maturation rates between SF- and VSF-derived BCB+ oocytes.

The developmental competence of oocytes to reach the blastocyst stage after parthenogenetic activation was highest in MF-derived BCB+ oocytes, followed by MF-derived BCB- oocytes (Table 3.4). Some SF-derived BCB+ and BCB- oocytes, as well as VSF-derived BCB+ oocytes, demonstrated the ability to form blastocysts. The number of blastomeres in a blastocyst was influenced solely by the origin of OCCs ($P < 0.01$) and not by the BCB staining status. The number of blastomeres did not differ significantly between MF- and SF-derived BCB+ and BCB- oocytes.

3.3.2. Characteristics of BCB+ and BCB- OCCs derived from antral follicles with various sizes

As shown in Table 3.5, the total number of cumulus cells, both live and dead, surrounding the oocytes was significantly influenced ($P < 0.01$) by the origin of the OCCs and the BCB status of the oocytes. Specifically, the total and live cumulus cell counts in MF-derived OCCs were significantly higher ($P < 0.01$) than those in VSF-derived OCCs. Additionally, MF-, SF-, and VSF-derived OCCs containing BCB+ oocytes had significantly live cumulus cells compared to those with BCB- oocytes ($P < 0.01$). Moreover, the percentage of dead cumulus cells was higher in SF- and VSF-derived OCCs with BCB- oocytes than in those with BCB+ oocytes, and the percentages in VSF-derived OCCs were also greater than those in MF-derived OCCs.

The outer and inner diameters of the zona pellucida were significantly influenced ($P < 0.01$) by the origin of the OCCs (MF, SF, and VSF) and the BCB status of the oocytes (Table 3.6). No differences were observed in the outer diameter of the zona pellucida, ooplasmic diameter, and perivitelline space formed after denuding between BCB+ and BCB- MF-derived oocytes, except for the inner diameter of the zona pellucida. However, the outer and inner diameters of the zona pellucida and the ooplasmic diameter of BCB- oocytes derived from SF and VSF were significantly smaller than those of BCB+ oocytes from the same-sized follicles ($P < 0.01$, Table 3.6). Among BCB+ oocytes derived from MF, SF, and VSF, there were no differences in the ooplasmic diameter. However, the outer and inner diameters of the zona pellucida and the perivitelline space formed after denuding were significantly smaller ($P < 0.01$) in oocytes derived from VSF.

3.3.3. PPP-related gene expression of BCB+ and BCB- OCCs derived from antral follicles with various sizes

According to the results of the two-way analysis of variance, the expression status of the *G6PD* gene in oocytes (DO-G6PD in Figure 3.1) was significantly influenced by the BCB staining status of the oocytes, but not by the origins of OCCs. Conversely, the expression status of the *TKT*

gene in oocytes appeared to be affected by the origin of the OCCs (DO-TKT in Figure 3.1). However, no significant differences were observed when all experimental groups were compared using one-way ANOVA and post-hoc tests.

In contrast to oocytes, the expression of *G6PD* and *TKT* genes in the surrounding cumulus cells was significantly influenced by the origin of the OCCs, showing higher levels in cells derived from smaller follicles (CC-G6PD and CC-TKT in Figure 3.1).

However, no clear effect on the expression status of the *RPIA* gene in oocytes or cumulus cells was detected in the experimental section due to significant interactions. Nevertheless, a significantly higher expression level was observed in MF-derived BCB- oocytes compared to BCB+ oocytes (see DO-RPIA and CC-RPIA in Figure 3.1). The expression of the *RPIA* gene in cumulus cells was significantly higher ($P < 0.05$) not only in SF- and VSF-derived BCB+ OCCs, but also in MF-, SF-, and VSF-derived BCB- OCCs, when compared with MF-derived BCB+ OCCs (see CC-RPIA in Figure 3.1).

Table 3.1. Primer sequences for PPP genes

Gene	NCBI Accession No.	Primer Sequence (5' to 3')	Length	T _m (°C)
<i>G6PD</i>	XM_02180744.1	F: CCTCCTGCAGATGCTGTGTCT	21	66.9
		R: CGCCTGCACCTCTGAGATG	19	67.3
<i>TKT</i>	NM_001112681	F: AAGCGATGGTGTGGCTACAGAGAA	26	70.0
		R: GGACCACCTTGGCTTGTCCAATTT	26	71.7
<i>RPIA</i>	NM_001097495.1	F: TCAACAAGGCAGGACCTGTG	20	66.8
		R: TCCTGCAGCTTCGGTTTAGA	20	65.2
<i>GAPDH</i>	NM_001206359.1	F: GAGTCCACTGGTGTCTTCACG	21	65.3
		R: ATGAGTCCCTCCACGATGC	19	65.7

G6PD, Glucose-6-phosphate dehydrogenase; *TKT*, Transketolase; *RPIA*, Ribose 5-phosphate isomerase A; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase

Table 3.2. Percentages of BCB-positive and BCB-negative oocytes derived from various antral follicle sizes

Origin of COCs	No. of examined oocytes	No. (%) of oocytes showing the status of:	
		BCB+	BCB-
MF	2410	1,166 (47.9 ± 1.8 ^a)	1,244 (52.1 ± 1.8 ^b)
SF	2482	961 (39.8 ± 2.2 ^b)	1,521 (60.2 ± 2.2 ^a)
VSF	2298	731 (32.7 ± 2.6 ^b)	1,567 (67.3 ± 2.6 ^a)

Percentage data are shown as the mean ± SEM from 8 replicated trials. Values with different superscripts differ significantly ($P < 0.05$) within the same column. MF, medium follicles with 3-6 mm in diameter; SF, small follicles with 1-3 mm in diameter; VSF, very small follicles with less than 1 mm in diameter.

Table 3.3. Meiotic competence of oocytes derived from follicles with various sizes and classified by BCB staining

Origin of OCCs	BCB status of oocytes	No. of oocytes examined	No. (%) of oocytes proceeded to the stage of:					
			GV	GVBD	M-I	A-I/T-I	M-II	Degenerated
MF	BCB+	276	0 (0 ± 0)	0 (0 ± 0 ^b)	18 (6.5 ± 1.3 ^c)	9 (3.2 ± 1.3)	247 (89.5 ± 1.8 ^a)	2 (0.7 ± 0.5 ^b)
	BCB-	377	0 (0 ± 0)	0 (0 ± 0 ^b)	52 (14.2 ± 1.3 ^{bc})	16 (4.3 ± 0.7)	212 (56.9 ± 2.6 ^{cd})	97 (24.6 ± 4.1 ^a)
SF	BCB+	289	1 (0.3 ± 0.3)	0 (0 ± 0 ^b)	59 (20.4 ± 1.7 ^{ab})	4 (1.3 ± 0.7)	210 (72.8 ± 2.3 ^b)	15 (5.2 ± 1.2 ^b)
	BCB-	395	1 (0.2 ± 0.2)	3 (0.7 ± 0.3 ^b)	78 (20.7 ± 3.0 ^{ab})	13 (3.1 ± 1.3)	194 (49.4 ± 3.6 ^d)	106 (25.9 ± 4.7 ^a)
VSF	BCB+	276	3 (0.9 ± 0.6)	0 (0 ± 0 ^b)	62 (22.7 ± 1.8 ^{ab})	12 (4.5 ± 2.0)	174 (63.5 ± 3.4 ^{bc})	25 (8.4 ± 2.7 ^b)
	BCB-	406	3 (0.7 ± 0.5)	8 (2.3 ± 0.8 ^a)	106 (27.0 ± 3.4 ^a)	13 (3.2 ± 1.1)	138 (34.1 ± 3.3 ^c)	138 (32.8 ± 4.3 ^a)
Results of 2-way ANOVA								
The origin of OCCs			0.08	<0.01	<0.01	0.35	<0.01	0.07
BCB status of OCCs			0.77	<0.01	<0.05	0.64	<0.01	<0.01
Interaction			0.96	<0.01	0.26	0.42	0.28	0.84

Data are shown as the mean ± SEM from 7 replicated trials. Within columns, values with different superscripts differ significantly ($P < 0.05$). MF, medium follicles; SF, small follicles; VSF, very small follicles.

Table 3.4. Developmental competence of oocytes derived from follicles with various sizes and classified by BCB staining

Origin of OCCs	BCB status of oocytes	No. of mature oocytes examined	No. (%) of parthenotes		No. of blastomeres per blastocyst
			Cleaved	Formed blastocysts	
MF	BCB+	246	192 (77.5 ± 3.2)	51 (20.4 ± 2.5 ^a)	27.8 ± 1.1 ^a
	BCB-	243	193 (76.4 ± 6.4)	30 (12.1 ± 1.6 ^b)	25.9 ± 1.6 ^{ab}
SF	BCB+	215	172 (79.3 ± 4.2)	7 (5.5 ± 1.0 ^{cd})	24.5 ± 1.7 ^{ab}
	BCB-	166	132 (79.8 ± 2.2)	8 (4.9 ± 0.6 ^{cd})	23.6 ± 1.2 ^{ab}
VSF	BCB+	207	162 (78.8 ± 3.7)	14 (6.7 ± 0.9 ^{bc})	21.8 ± 1.3 ^b
	BCB-	111	86 (75.9 ± 6.0)	0 (0 ± 0 ^d)	-
Results of 2-way ANOVA					
The origin of OCCs			0.83	<0.01	<0.01
BCB status of OCCs			0.75	<0.01	0.31
Interaction			0.93	<0.05	0.72

Data are shown as the mean ± SEM from 7 replicated trials. Within columns, values with different superscripts differ significantly ($P < 0.05$). MF, medium follicles; SF, small follicles; VSF, very small follicles.

Table 3.5. The number of cumulus cells per OCC derived from follicles with various sizes and classified by BCB staining

Origin of OCCs	BCB status of oocytes	No. of oocytes examined	No. of cumulus cells per OCC	No. of live cumulus cells per OCC	No. of dead cumulus cells per OCC	% of live cumulus cells per OCC
MF	BCB+	29	3,300.6 ± 149.7 ^a	3,069.1 ± 125.4 ^a	231.6 ± 39.4 ^b	93.6 ± 0.9 ^d
	BCB-	34	2,780.2 ± 185.9 ^{ab}	2,527.3 ± 176.3 ^b	252.9 ± 41.3 ^b	92.3 ± 1.5 ^{cd}
SF	BCB+	36	2,819.7 ± 114.8 ^{ab}	2,598.2 ± 117 ^{ab}	221.5 ± 30.1 ^b	91.7 ± 1.2 ^{cd}
	BCB-	35	2,400.7 ± 113.5 ^{bc}	1,898.2 ± 104.5 ^c	502.5 ± 62.9 ^a	79.4 ± 2.2 ^{ab}
VSF	BCB+	29	2,115.5 ± 118.1 ^{cd}	1,798 ± 106.9 ^c	317.6 ± 50.9 ^{ab}	85.5 ± 2.1 ^{bc}
	BCB-	31	1,767.4 ± 81.3 ^d	1,290.1 ± 67.7 ^d	477.3 ± 55 ^a	73.7 ± 2.6 ^a
Results of 2-way ANOVA						
The origin of OCCs			<0.01	<0.01	<0.01	<0.01
BCB status of OCCs			<0.01	<0.01	<0.01	<0.01
Interaction			0.81	0.71	<0.05	<0.05

Data are shown as the mean ± SEM from 5 replicated trials. Within columns, values with different superscripts differ significantly (P < 0.05).

MF, medium follicles; SF, small follicles; VSF, very small follicles.

Table 3.6. Diameters of oocytes derived from follicles with various sizes and classified by BCB staining

Origin of OCCs	BCB status of oocytes	No. of oocytes examined	Outer diameter (μm) of zona pellucida	Inner diameter (μm) of zona pellucida	Ooplasmic diameter (μm) after denuding	Perivitelline space (μm) formed after denuding
MF	BCB+	96	151.8 ± 0.4^a	123.0 ± 0.3^a	112.2 ± 0.5^a	5.4 ± 0.2^a
	BCB-	92	150.9 ± 0.6^a	121.3 ± 0.5^b	111.8 ± 0.5^a	4.8 ± 0.2^{ab}
SF	BCB+	101	150.9 ± 0.4^a	121.6 ± 0.3^{ab}	112.5 ± 0.4^a	4.5 ± 0.2^b
	BCB-	126	149.0 ± 0.4^b	119.2 ± 0.4^c	109.6 ± 0.5^b	4.8 ± 0.2^{ab}
VSF	BCB+	82	148.9 ± 0.4^b	120.2 ± 0.4^{bc}	112.0 ± 0.5^a	4.1 ± 0.2^{bc}
	BCB-	94	146.3 ± 0.4^c	116.6 ± 0.4^d	109.4 ± 0.5^b	3.6 ± 0.1^c
Results of 2-way ANOVA						
The origins of OCCs			<0.01	<0.01	<0.05	<0.01
BCB status of OCCs			<0.01	<0.01	<0.01	0.07
Interaction			0.14	0.06	<0.05	<0.05

Data are shown as the mean $\pm$ SEM from 5 replicated trials. Within columns, values with different superscripts differ significantly ($P < 0.05$). MF, medium follicles; SF, small follicles; VSF, very small follicles.

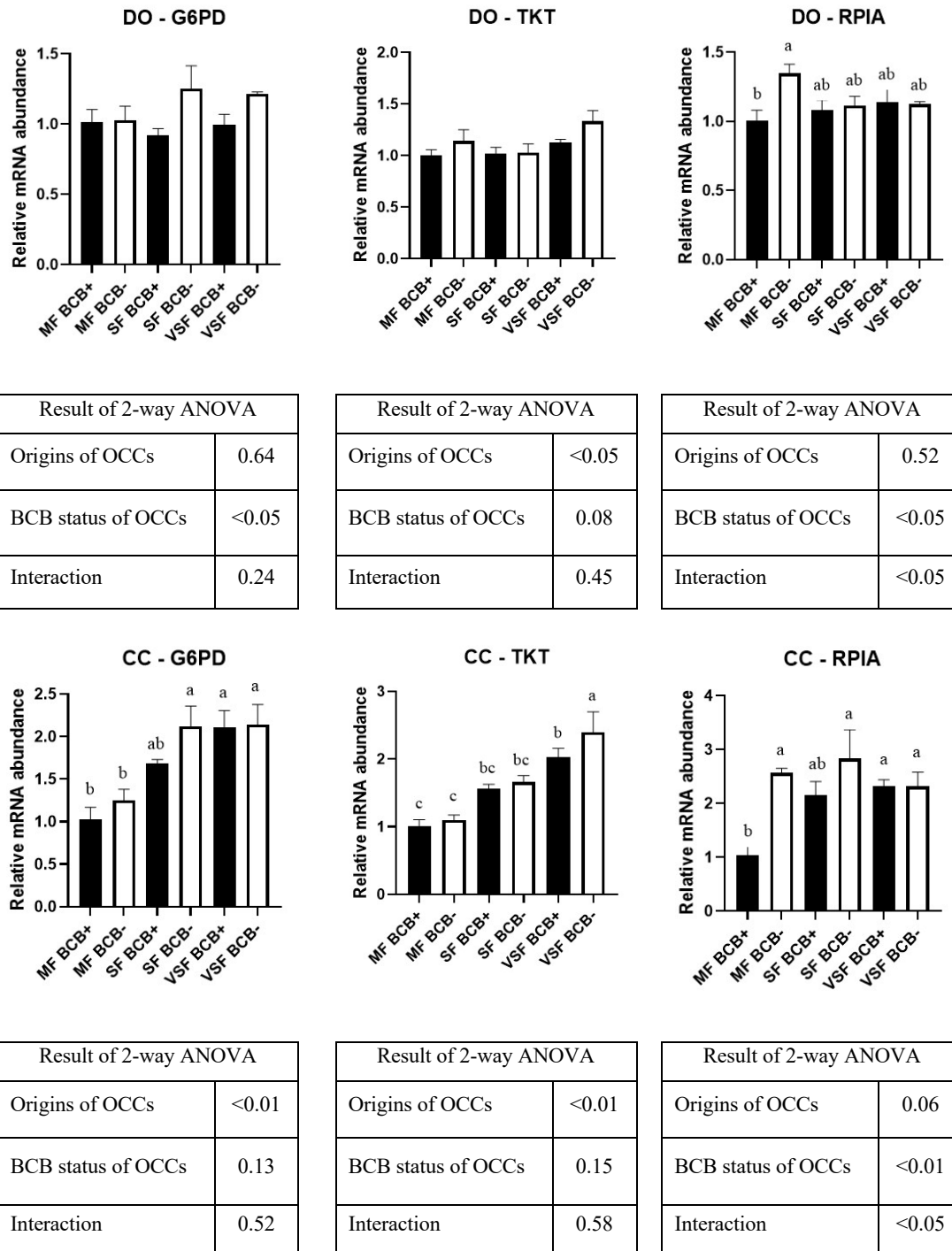


Figure 3.1. Gene expression in BCB-positive (black bars) and BCB-negative oocytes (white bars) collected from medium (MF), small (SF), or very small (VSF) follicles. The ratio to the mean value in BCB-positive oocytes obtained from MF-derived OCCs is represented. Data from 4 replicated trials and expressed as mean $\pm$ SEM. Different letters indicate significant differences between groups ($P < 0.05$).

3.4. Discussion

In the present study, we found that the incidence of MF-derived OCCs containing BCB+ oocytes was significantly higher than that of SF- and VSF-derived OCCs. Surprisingly, even VSF-derived OCCs contained BCB+ oocytes. Additionally, we confirmed that BCB+ oocytes from MF-derived OCCs exhibited greater meiotic and developmental competences compared to those from SF- and VSF-derived OCCs. These findings align with previous studies that compared MF-derived OCCs with SF-derived ones (Kohata et al., 2013; Marchal et al., 2001; Okudaira et al., 2017) and BCB+ oocytes with BCB- oocytes (Catalá et al., 2011; Ericsson et al., 1993; Manjunatha et al., 2007; Roca et al., 1998; Rodríguez-González et al., 2002; Silva et al., 2011). However, we found here that not only SF-derived oocytes, but also VSF-derived BCB+ oocytes demonstrated meiotic and developmental competences up to the metaphase-II and blastocyst stages, respectively, although the percentages were lower than those of MF-derived oocytes. Given that the number of smaller antral follicles, measuring less than 3 mm in diameter, is greater in the ovaries of prepubertal gilts compared to MF (Almeida et al., 2017), our results suggest the potential to utilize oocyte resources from smaller follicles, even those less than 1 mm in diameter, for in vitro embryo production. Although we did not assess the meiotic and developmental competences of VSF-derived BCB+ oocytes following pre-culture to complete their growth, additional in vitro growth procedures (Wu et al., 2001) may further enhance these competences.

The current study demonstrates that the total number of cumulus cells per OCC did not differ between BCB+ and BCB- groups across all follicle sizes. However, the number of viable cells was significantly higher in OCCs containing BCB+ oocytes compared to those with BCB- oocytes. Additionally, regardless of BCB status, both the total and viable cumulus cell counts were greater in OCCs derived from MF sources than in those derived from VSF sources. Furthermore, we have shown that, in addition to BCB staining, co-culturing with healthy cumulus cells or supplementing with VEGF, a secreted factor from these cells, significantly enhanced the meiotic and developmental competences of SF-derived oocytes (Bui et al., 2017; Fonseka et al., 2024;

Matsunaga & Funahashi, 2017). Cumulus cells are known to play critical roles by providing metabolites and creating favorable conditions for the development of growing oocytes (Mori et al., 2000; Tatemoto et al., 2000). Therefore, the presence of a sufficient number of healthy cumulus cells influences the metabolic system of the oocytes and may consequently enhance their meiotic and developmental competences. Some oocytes derived from SF and VSF may exhibit reversible characteristics. However, we also observed that BCB⁺ oocytes were present in VSF-derived OCCs that contained fewer cumulus cells than those derived from MF with BCB⁻ oocytes. This result suggests that the BCB status may be affected not only by the number of surrounding viable cumulus cells but also by other factors. Furthermore, there were no differences in the incidence of live cumulus cells between MF-derived OCCs containing BCB⁺ and BCB⁻ oocytes. However, the percentages of viable cells in SF- or VSF-derived BCB⁻ oocytes were significantly lower than those in BCB⁺ oocytes. These findings suggest that at least some SF- and VSF-derived OCCs containing BCB⁻ oocytes may originate from early atretic follicles.

The ooplasm of all healthy OCCs appeared to be in close contact with the inner surface of the zona pellucida and connected to the cumulus cells via gap junctions (Sasseville et al., 2009). However, in the present study, we unexpectedly observed the formation of a perivitelline space beneath the zona after denudation. When we measured the outer and inner diameters of zona pellucida, as well as the ooplasmic diameter and perivitelline space after denuding oocytes collected from various sizes of antral follicles, we found that the outer and inner diameters of zona pellucida were significantly larger in both OCCs derived from larger follicles and those containing BCB⁺ oocytes. This observation aligns with previous reports comparing the diameters of MF- and SF-derived oocytes (Matsunaga & Funahashi, 2017), as well as BCB⁺ and BCB⁻ oocytes (Murin et al., 2019; Roca et al., 1998). In contrast, our study revealed that the diameters of the ooplasm of MF-, SF-, and VSF-derived BCB⁺ oocytes were similar, while the diameters of SF- and VSF-derived BCB⁻ oocytes were significantly smaller. Consequently, a larger perivitelline space was observed in MF-derived oocytes compared to VSF-derived oocytes following denudation. These

results demonstrate that the ability of oocytes to form a perivitelline space after detachment from cumulus cells varies among OCCs derived from antral follicles of different sizes. Furthermore, the cell volume of oocytes and embryos is known to be regulated by glycine accumulation via the glycine-specific transporter, *GLYT1*, with a secondary contribution from betaine accumulation through the *SIT1* transporter (Baltz & Zhou, 2012). However, *GLYT1* is suppressed by the presence of cumulus cells (Richard & Baltz, 2017) and is activated after the oocyte detaches from the zona pellucida (Baltz & Tartia, 2010; Baltz & Zhou, 2012; Ortman & Baltz, 2023). Additionally, the variation in the number of live cumulus cells surrounding the oocytes in OCCs derived from different sizes of antral follicles may influence the ability to form a perivitelline space. Therefore, further studies are needed to clarify whether the differences observed in meiotic and developmental competences among MF-, SF-, and VSF-derived oocytes in this study are to the surrounding live cumulus cells and, consequently, related to variations in their ability to form a perivitelline space, including *GLYT1* expression.

In the current study, we assessed the expression of PPP-related genes in both oocytes and cumulus cells. The results demonstrated that, regardless of the BCB status of the oocytes, the transcriptional levels of *G6PD* and *TKT* in the surrounding cumulus cells of MF-derived OCCs were significantly lower than those in SF- and VSF-derived OCCs. In contrast, the mRNA levels of *G6PD* and *TKT* in oocytes were influenced only by the BCB status of the OCCs or their origin, respectively. These findings indicate that the expression of PPP-related genes in cumulus cells, but not clearly in oocytes, gradually changes during follicular development from VSF to MF. *G6PD* is the rate-limiting enzyme of the oxidative branch of the PPP, responsible for producing NADPH, while *TKT* is a crucial enzyme in the non-oxidative branch of the PPP. Our current results suggest that the activities of both the oxidative and non-oxidative branches of the PPP, represented by *G6PD* and *TKT*, respectively, are decreased in cumulus cells rather than in oocytes as follicular development processes from VSF to MF. However, while BCB staining is considered an effective method for roughly assessing *G6PD* levels, the levels of *G6PD* mRNA in oocytes and

cumulus cells did not correspond with the results of BCB staining in the present study. A detailed investigation is needed to determine the factors that critically affect *G6PD* activity and its metabolite NADPH levels in oocytes derived from follicles of various diameters, including the state of gap junctions between oocytes and cumulus cells. Furthermore, the mRNA level of *RPIA*, a key enzyme responsible for synthesizing ribose 5-phosphate in the PPP, was found to be lower only in BCB+ cumulus cells derived from MF. In this study, meiotic and developmental competences were higher in MF-derived BCB+ oocytes and lower in those derived from VSF and SF. These results indicate that meiotic and developmental competences are greater in BCB+ oocytes surrounded by cumulus cells with lower expression levels of PPP-related genes, highlighting the significance of the PPP-related gene expression in cumulus cells rather than in oocytes. Recent studies have demonstrated that gene expression levels in cumulus cells positively correlated with FSH receptor, insulin-like growth factor-1, anti-Mullerian hormone, and epidermal growth factor receptor, and negatively correlated with B cell lymphoma/leukemia-2 associated X (*Bax*) and cysteine-aspartic acid protease-3 (*Caspase-3*) and are associated with the developmental competence of bovine oocytes (Anazawa et al., 2025). Our current results appear to align with this previous report (Anazawa et al., 2025).

In conclusion, the current study demonstrates the effectiveness of BCB staining in selecting OCCs that contain oocytes with relatively higher meiotic and developmental competences, derived not only from MF but also from SF and even VSF. The significance of having a sufficient number of healthy cumulus cells surrounding the oocytes was also recognized. Additionally, the expression of PPP-related genes in the surrounding cumulus cells decreases during follicular development from VSF to MF, indicating that this may significantly impact the meiotic and developmental competences of the oocytes.

CHAPTER 4

RESILIENCE OF OOCYTE-CUMULUS COMPLEXES DERIVED FROM ANTRAL FOLLICLES WITH VARIOUS DIAMETERS TO OXIDATIVE STRESS BY BCB STAINING

Abstract

The present study aimed to determine the resilience of oocyte-cumulus complexes (OCCs) derived from three different follicle sizes, including medium follicles (with 3–6 mm in diameter; MF), small follicles (1–3 mm in diameter; SF), and very small follicles (with less than 1 mm in diameter; VSF) to brilliant cresyl blue (BCB) staining. BCB staining affected transcriptional levels of oxidative stress-related genes in both oocytes and cumulus cells. Regardless of the origins of OCCs, even in BCB+ oocytes, the blue dye disappeared around 1 h in an IVM culture condition. Within 1 h after BCB staining, distinct responses in the NADPH regulation were observed between MF-, SF-, and VSF-derived OCCs, and were affected by both the origins of OCCs and the BCB status. A transient increase in ROS and a decrease in GSH in BCB+ oocytes were also observed. After BCB staining, the ROS levels in both BCB+ and BCB- oocytes were increased, although the level was affected by both the origin of OCCs and BCB status until 4 h of IVM and remained at low levels during IVM. The GSH levels of MF-, SF-, and VSF-derived BCB+ oocytes were significantly lower than those of BCB- ones and non-stained controls, but the difference disappeared during IVM. These results demonstrate that, although BCB staining induces transient oxidative stress in porcine OCCs, especially those including BCB+ oocytes, OCCs rapidly restore redox balance by regulating and utilizing NADPH to produce reduced GSH, with different resilience between the origin of OCCs.

4.1. Introduction

Oocyte-cumulus complexes (OCCs; although COCs may be common, here we use OCCs to highlight oocytes under BCB stain) that are derived from the ovaries of prepubertal gilts, slaughtered commercially before sexual maturity, are an essential material in both in vitro maturation (IVM) and in vitro fertilization (IVF) systems, due to their availability. Prepubertal gilts' ovaries contain various sizes of follicles, of which the number of small follicles (SF, with 1-3 mm in diameter) and very small follicles (VSF, less than 1 mm in diameter) are larger than medium follicles (MF, with 3-6 mm in diameter) (Almeida et al., 2017). MF-derived OCCs were primarily used as materials for in vitro embryo production due to the superior meiotic and developmental competence compared to SF-derived OCCs (Kohata et al., 2013; Okudaira et al., 2017). With the development of embryonic gene editing technology (Takebayashi et al., 2022; Tanihara et al., 2023), research on creating gene-edited pigs for xenotransplantation (Anand et al., 2023; Pan et al., 2024) and pig models for use in studies of human disease (Hou et al., 2022) requires a large amount of embryonic material. Thus, it could be necessary to maximize the utilization of material from each ovary, including the OCCs derived from SF and even VSF, which contains a smaller number of surrounding cumulus cells (Matsunaga & Funahashi, 2017). In recent years, research has been conducted on improving the meiotic and developmental competences of SF-derived OCCs through vascular endothelial growth factor supplementation (Bui et al., 2017), co-culture with healthy cumulus cell masses (Bui et al., 2017; Fonseka et al., 2024; Matsunaga & Funahashi, 2017), pre-IVM culture system (Jitjumnong & Tang, 2024; Nguyen et al., 2020; Tonai et al., 2023) or selection of immature porcine OCCs by brilliant cresyl blue (BCB) staining (Ericsson et al., 1993; Fonseka et al., 2024; Van et al., 2025).

Brilliant cresyl blue staining, which can approximately and noninvasively assess the activity of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway (PPP), has been utilized to select OCCs containing oocytes with high meiotic and developmental competences in various mammalian species (Catalá et al., 2011; Ericsson et

al., 1993; Fonseka et al., 2024; Manjunatha et al., 2007; Roca et al., 1998; Rodríguez-González et al., 2002; Silva et al., 2011; Van et al., 2025). Oocytes with partial and complete decolorization of the BCB dye (light blue or colorless, respectively; BCB-negative) are believed to be still growing and have a high G6PD activity, whereas those with a lower G6PD activity are fully grown and retain the BCB dye (dark blue; BCB-positive). The percentage of BCB-positive (BCB+) OCCs increases during follicular development from VSF to MF (Van et al., 2025), and the oocytes had superior meiotic (Ericsson et al., 1993; Fonseka et al., 2024; Van et al., 2025) and developmental competences (Fonseka et al., 2024; Roca et al., 1998; Van et al., 2025). These competences may be reflected by the differential characteristics such as the number of surrounding live cumulus cells, oocyte diameter, and the expression levels of PPP-related genes in cumulus cells (Van et al., 2025) compared to BCB-negative (BCB-) OCCs. In our previous experiments, however, BCB staining itself demonstrated to cause oxidative stress to OCCs, especially BCB+ ones (Fonseka et al., 2024), through increasing reactive oxygen species (ROS) levels (Fonseka et al., 2024; Santos et al., 2014), although the damage of oocytes through IVM culture has not been evaluated yet. During the stress, the presence of nicotinamide adenine dinucleotide phosphate (NADPH) is essential to reduce oxidised glutathione (GSSG) to reduced one (GSH), the major intracellular antioxidant, it seems to be more dependent on the synthesis in the cumulus cells rather than the oocyte (Geshi et al., 2000). Since the number of cumulus cells surrounding the oocyte is less in SF-derived OCCs than in MF-derived ones, the resilience of OCCs against oxidative stress through BCB staining may differ among those derived from VSF, SF, and MF. Therefore, the objective of the current study was to evaluate the resilience of BCB-stained OCCs derived from different follicle sizes through changes in GSH, ROS, and NADPH levels in oocytes during IVM culture.

4.2. Materials and Methods

4.2.1. Assay to determine the intracellular NADPH concentration

For sample preparation, oocytes were denuded by pipetting in the presence of 0.05% (w/v) Trypsin-EDTA within 3 min before removing the zona pellucida by digesting in 0.1% (w/v)

protease in TL-HEPES-PVA within 10 seconds. After washing twice with 0.1% (v/v) PVA in PBS, approximately 120-150 denuded zona-free oocytes were collected into 5 μ L of the same buffer, frozen, and stored at -80°C. To measure the intracellular NADPH concentration, the oocyte samples were thawed and incubated with 20 μ L lysis buffer for 15 min at room temperature. After adding 100 μ L PBS, the samples were centrifuged (3,000 g, 5 min), and the supernatant was collected to measure NADPH concentration. NADPH assays were performed following the protocol of Abcam's Colorimetric NADPH Assay Kit (ab186031; Abcam Limited, Cambridge, UK) using a microplate reader (BIO-RAD, Hercules, CA, USA) to measure absorbance at 450 nm. The intracellular NADPH concentration per oocyte was the total NADPH concentration of the sample divided by the number of oocytes.

4.2.2. Measurement of glutathione (GSH) and reactive oxygen species (ROS) levels

To assess the glutathione (GSH) and reactive oxygen species (ROS) levels, denuded oocytes were incubated in TL-HEPES-PVA containing 10 μ M ThiolTracker™ Violet (glutathione detection reagent; Thermo Fisher Scientific, Waltham, MA, USA) or 10 mM CM-H₂DCFDA (a general oxidative stress indicator; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 39°C in the dark. After washing three times with TL-HEPES-PVA, oocytes were immediately captured using a fluorescence microscope (BZ-X700, Keyence Corp., Osaka, Japan) under a blue filter for GSH and a green filter for ROS at a magnification of 200X with an exposure of 1/4 s. Each group of oocytes was photographed only once to avoid photobleaching. GSH or ROS levels of oocytes in each group were quantified by measuring fluorescence intensity using ImageJ software, version 1.53 (National Institutes of Health, Bethesda, MD, USA) according to the previous formula (Tiwari & Chaube, 2016). The corrected total cell fluorescence (CTCF) = integrated density - (area of selected cell $\times$ mean fluorescence of background readings).

4.2.3. Assessment of the expression of genes associated with oxidative stress

The sequences of the forward and reverse primers are presented in Table 4.1. The expression levels of five genes associated with oxidative stress, including *superoxide dismutase 1*

and 2 (*SOD1* and *SOD2*), catalase (*CAT*), glutathione peroxidase 1 (*GPX1*), nuclear factor, erythroid 2-like 2 (*NRF2*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were analyzed in both oocytes and cumulus cells.

4.2.4. Experimental designs

4.2.4.1. Loss of BCB blue color in the ooplasm of MF-, SF-, and VSF-derived OCCs during the first 1 h of IVM culture after BCB staining (Experiment 1)

MF-, SF- and VSF-derived OCCs with BCB+ oocytes just after staining were cultured in IVM medium for 1 h. The number of BCB+ OCCs that changed to colorless was observed 30 and 60 min after the start of IVM culture. Experiments were replicated 8 times.

4.2.4.2. Change of intracellular NADPH concentration in BCB+ and BCB- oocytes derived from antral follicles with various sizes during the first 1 h of IVM culture after BCB staining (experiment 2)

Approximately 120-150 zona-free oocytes were used to determine intracellular NADPH concentration in oocytes. The intracellular NADPH concentration of BCB+, BCB-, and unstained (control) oocytes derived from MF, SF, and VSF were measured just after BCB staining and 1 h after the start of IVM by using the NADPH Assay Kit. All experiments were replicated four times.

4.2.4.3. Change of GSH and ROS levels in BCB+ and BCB- oocytes derived from antral follicles with various sizes during IVM culture (experiment 3)

Just after collection, some OCCs derived from MF, SF, and VSF were kept in TL-HEPES-PVA as the controls, while other OCCs were stained with BCB to separate into two groups: BCB+ and BCB-. At 0 h (just after BCB staining), 1 h, 4 h, 20 h, and 44 h after the start of IVM, oocytes in three groups (BCB+, BCB-, and control) were denuded by pipetting and stained with ThiolTracker™ Violet (to determine GSH level) or CM-H₂DCFDA (to determine ROS level). Experiments were replicated five times.

4.2.4.4. GSH and ROS levels in mature and immature oocytes derived from antral follicles with various sizes, exposed to BCB dye, and then cultured for IVM (Experiment 4)

Effects of BCB staining on the oxidative stress of mature and immature oocytes at the end of IVM culture were examined. Some OCCs from MF, SF, and VSF were kept in TL-HEPES-PVA as the controls, and other OCCs were exposed to BCB dye to separate into BCB+ and BCB- groups. All OCCs were separately cultured for IVM for a totally 44 h. After denuding, these oocytes of three groups (BCB+, BCB-, and controls) were divided into mature and immature ones according to the presence or absence of the first polar body before ROS and GSH levels of those were examined. Experiments were replicated five times.

4.2.4.5. Transcriptional levels of oxidative stress-related genes in oocytes and the surrounding cumulus cells derived from antral follicles with various sizes just after BCB staining (Experiment 5)

The expression levels of genes associated with oxidative stress in oocytes and cumulus cells were examined using quantitative RT-PCR. The relative expression levels of *SOD1*, *SOD2*, *CAT*, *GPX1*, and *NRF2* in BCB-stained and unstained oocytes and the surrounding cumulus cells, or BCB+ and BCB- oocytes, derived from MF, SF, and VSF were compared to determine the effects of BCB staining and OCCs' origins, or the effects of BCB status and OCCs' origins. All experiments were replicated four times.

4.2.5. Statistical analyses

Data from four to five replicated trials were evaluated using one-way (experiment groups) and/or two-way (origins and BCB status of OCCs) ANOVA employing IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). Percentage data were subjected to arcsine transformation before statistical analyses if the data contained percentages greater than 90% or less than 10%. All data are presented as mean $\pm$ SEM. Findings were considered statistically significant at $P < 0.05$, and when a significant effect was observed, values were compared using Tukey's post hoc test.

4.3. Results

4.3.1. Loss of BCB blue color in the ooplasm of MF-, SF-, and VSF-derived OCCs during the first 1 h of IVM culture after BCB staining (Experiment 1)

Regardless of the origin of OCCs (between MF, SF and VSF; $P = 0.41$ and $P = 0.26$ at 30 and 60 min after the start of IVM culture, respectively), the dark blue coloration in the ooplasm of close to 80% or more and more than 90% of BCB+ ones changed to colorless within 30 and 60 min after the start of IVM culture, respectively (Table 4.2).

4.3.2. Change of intracellular NADPH concentration in BCB+ and BCB- oocytes derived from antral follicles with various sizes during the first 1 h of IVM culture after BCB staining (experiment 2)

When NADPH concentration per oocyte was examined just after BCB staining, the concentration was influenced ($P < 0.01$) by both the origins of OCCs and the BCB status (Table 3). The concentrations in BCB+ and BCB- oocytes did not differ significantly in the same origins of OCCs. However, MF-derived BCB-exposed (BCB+ and BCB-) oocytes had significantly higher NADPH concentrations than unstained controls and SF- and VSF-derived BCB- ones (Table 4.3). After 1 h from the start of the IVM culture, NADPH concentrations did not differ in all groups of oocytes examined (Table 4.3).

4.3.3. Change of GSH and ROS levels in BCB+ and BCB- oocytes derived from antral follicles with various sizes during IVM culture (Experiment 3)

When GSH levels were observed during IVM culture, the levels in control (unstained) oocytes significantly decreased regardless of the origin of OCCs (between MF, SF, and VSF) during the first half period of IVM culture, in the presence of gonadotropins and db-cAMP, and then re-increased during the second half period, in the absence of those (Table 4.4). Just after BCB staining, the GSH level in oocytes was significantly affected ($P < 0.01$) by both the origins of OCCs and the BCB status (Table 4.4 and Fig. 4.1 A). The GSH levels in MF-, SF-, and VSF-derived BCB+ oocytes were significantly lower ($P < 0.05$) than those of BCB- and control ones,

while no difference was found in the GSH level of BCB- and control oocytes in each follicle size. However, their differences in GSH levels between BCB+ and BCB- or control (unstained) oocytes were resolved partially by 1 h (Fig. 4.2 A) and completely by 4 h after the start of IVM culture and remained so for the first half of the culture period (Table 4.4).

When ROS levels in oocytes were examined during IVM culture, the levels were significantly influenced ($P < 0.01$) by both the origins of OCCs and the BCB status of the oocytes at least until 4 h after the start of IVM culture (Table 4.5). Regardless of the origin of OCCs, ROS levels in oocytes exposed to BCB staining (both BCB+ and BCB-) were significantly higher than controls (unstained ones), whereas the level was significantly higher in BCB+ oocytes than BCB- ones and lower in VSF-derived BCB+ oocytes than MF-derived BCB+ ones (Table 4.5 and Fig. 4.1 B, 4.2B). By 4 h after the start of IVM culture, ROS were no longer detected in all MF-derived oocytes but in a few SF- and VSF-derived BCB-exposed ones at low levels. After 20 h from the start of IVM culture and thereafter, ROS were no longer detected in all oocytes examined (Table 4.5).

4.3.4. GSH and ROS levels in mature and immature oocytes collected from antral follicles with various sizes, exposed to BCB dye, and then cultured for IVM (Experiment 4)

As shown in Table 6, the maturation rate of oocytes was significantly influenced ($P < 0.01$) by both the origins of OCCs and the status of BCB staining. The highest maturation rate was for MF-derived BCB+ OCCs ($78.0 \pm 0.9\%$), while the lowest was for VSF-derived BCB- ones ($37.6 \pm 3.2\%$). No significant differences were observed in the maturation rates of SF-derived OCCs compared with MF-derived BCB- and VSF-derived BCB+ OCCs.

When ooplasmic GSH levels were observed after IVM culture, interestingly, the levels in MF-, SF-, and VSF-derived control (unstained) mature oocytes were significantly lower ($P < 0.05$) than those in immature ones (Table 4.6). Similar results were obtained in MF- and SF-derived BCB+ oocytes, as well as MF-derived BCB- ones, whereas it was not clear in SF-derived BCB-

and VSF-derived BCB⁺ ones. Furthermore, surprisingly, intracellular GSH levels in MF- and SF-derived BCB⁺ mature oocytes were not significantly different from MF- and SF-derived control (unstained) mature ones. In contrast to GSH levels, ROS levels in oocytes after IVM culture were barely detectable in both mature and immature oocytes, regardless of the origin of OCCs and the status of BCB staining (Table 4.7).

4.3.5. Transcriptional levels of oxidative stress-related genes in oocytes and the surrounding cumulus cells derived from antral follicles with various sizes just after BCB staining (Experiment 5)

As shown in Fig. 4.3, BCB staining significantly affected the transcriptional levels of all oxidative stress-related genes examined in both oocytes and the surrounding cumulus cells. In oocytes (DO-SOD1, DO-SOD2, DO-CAT, DO-GPX1, and DO-NRF2 in Fig. 4.3), the expression levels of all genes examined (*SOD1*, *SOD2*, *CAT*, *GPX1*, and *NRF2*) were negatively affected ($P < 0.01$) by the BCB staining, but not among the origins of OCCs. In cumulus cells (CC-SOD1, CC-SOD2, CC-CAT, CC-GPX1, and CC-NRF2 in Fig. 4.3), however, the expression levels of all genes examined (*SOD1*, *SOD2*, *CAT*, *GPX1*, and *NRF2*) were positively affected ($P < 0.05$) by the BCB staining, the expression levels of these genes, without *NRF2*, were higher ($P < 0.05$) in the cells derived from smaller follicles.

When we compared the transcriptional levels of oxidative stress-related genes in BCB⁺ and BCB⁻ oocytes (DO-SOD1, DO-SOD2, DO-CAT, DO-GPX1, and DO-NRF2 in Fig. 4.4), BCB status (BCB⁺ vs. BCB⁻) significantly affected the expression levels of *SOD1*, *SOD2*, and *NRF2*, whereas the effects of the origins of the oocytes were significant ($P < 0.05$) in only *SOD2* and *NRF2*. In cumulus cells (CC-SOD1, CC-SOD2, CC-CAT, CC-GPX1, and CC-NRF2 in Fig. 4.4), BCB-status (BCB⁺ vs. BCB⁻) of the oocytes did not affect the transcriptional levels of all genes examined (*SOD1*, *SOD2*, *CAT*, *GPX1*, and *NRF2*). However, the expression levels of these genes, without *NRF2*, were significantly different and higher ($P < 0.01$) in the cells derived from smaller follicles.

Table 4.1. Primer sequences for oxidative stress genes

Gene	NCBI Accession No.	Primer Sequence (5' to 3')	Length	T _m (°C)
<i>SOD1</i>	NM_001190422.1	F: GTTGGAGACCTGGGCAATGT	20	63.3
		R: CGGCCAATGATGGAATGGTC	20	62.1
<i>SOD2</i>	NM_214127.2	F: AATCTGAGCCCTAACGGTGG	20	62.4
		R: GACGGATACAGCGGTCAACT	20	61.5
<i>CAT</i>	NM_214301.2	F: AGCCAGTGACCAGATGAAGCATTG	24	63.7
		R: ATGTCGTGTGTGACCTCAAAGTAGC	25	62.6
<i>GPXI</i>	NM_214201.1	F: GGTCTCCAGTGTGTCGCAAT	20	62.3
		R: TCGATGGTCAGAAAGCGACG	20	62.1
<i>NRF2</i>	XM_013984303.2	F: AGCGGATTGCTCGTAGACAG	20	60.9
		R: TTCAGTCGCTTCACGTCGG	19	62.5
<i>GAPDH</i>	NM_001206359.1	F: GAGTCCACTGGTGTCTTCACG	21	65.3
		R: ATGAGTCCCTCCACGATGC	19	65.7

SOD1, superoxide dismutase 1; *SOD2*, superoxide dismutase; *CAT*, catalase, *GPXI*, glutathione peroxidase 1; *NRF2*, nuclear factor, erythroid 2 like 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Table 4.2. Percentages (No.) of MF-, SF-, and VSF-derived BCB+ OCCs changed to colorless 0, 30 and 60 min after the start of IVM culture

Origin of OCCs	No. of BCB+ OCCs examined	Percentage (No.) of BCB+ OCCs changes to colorless at		
		0 min	30 min	60 min
MF	448	0 ± 0 ^c (0)	79.9 ± 3.7 ^b (358)	93.7 ± 1.9 ^a (419)
SF	532	0 ± 0 ^c (0)	81.7 ± 5.5 ^b (437)	93.2 ± 2.7 ^a (499)
VSF	479	0 ± 0 ^c (0)	87.8 ± 3.2 ^b (419)	97.7 ± 1.5 ^a (469)

Data are shown as the mean ± SEM from 8 replicated trials. Values with different superscripts differ significantly within the same row ($P < 0.05$). MF, medium follicles with 3-6 mm in diameter; SF, small follicles with 1-3 mm in diameter; VSF, very small follicles with less than 1 mm in diameter.

Table 4.3. NADPH concentrations in MF-, SF-, and VSF-derived oocytes after classification with BCB staining

Origin of OCCs	BCB status of oocytes	NADPH conc. (nM) per oocyte	
		Just after BCB staining	1 h after the start of IVM
MF	BCB+	77.3 ± 2.1 ^{a,A}	50.5 ± 4.4 ^B
	BCB-	76.8 ± 2.6 ^{a,A}	55.4 ± 3.4 ^B
	Control (unstained)	61.6 ± 4.4 ^b	54.7 ± 3.2
SF	BCB+	68.3 ± 2.3 ^{ab,A}	50.3 ± 2.7 ^B
	BCB-	57.0 ± 3.6 ^b	52.1 ± 3.4
	Control (unstained)	61.7 ± 3.0 ^b	52.9 ± 3.1
VSF	BCB+	62.7 ± 2.8 ^{ab,A}	53.8 ± 1.2 ^B
	BCB-	58.7 ± 2.1 ^{b,A}	49.4 ± 1.3 ^B
	Control (unstained)	58.6 ± 3.9 ^b	49.3 ± 2.9
Results of 2-way ANOVA			
Origin of OCCs		<0.01	0.54
BCB status of OCCs		<0.01	0.94
Interaction		0.03	0.52

Data are shown as the mean ± SEM from 4 replicated trials. Values with different lowercase and uppercase superscripts are significantly different within the same columns and rows, respectively ($P < 0.05$). MF, medium follicles; SF, small follicles; VSF, very small follicles.

Table 4.4. Total fluorescence intensity of GSH in oocytes derived from follicles with various sizes (MF, SF, and VSF) and classified by BCB staining (BCB+, BCB-, and unstained control) during IVM

Origin of OCCs	BCB status of oocytes	Just after BCB staining		1 h after IVM		4 h after IVM		20 h after IVM		44 h after IVM	
		No. of oocytes examined	Fluorescence intensity of GSH	No. of oocytes examined	Fluorescence intensity of GSH	No. of oocytes examined	Fluorescence intensity of GSH	No. of oocytes examined	Fluorescence intensity of GSH	No. of oocytes examined	Fluorescence intensity of GSH
MF	BCB+	100	54.8 ± 1.1 ^{e,BC}	78	55.7 ± 1.0 ^{b,B}	95	50.8 ± 1.0 ^{bc,CD}	105	50.0 ± 0.9 ^{ab,D}	144	61.3 ± 1 ^{def,A}
	BCB-	100	78.2 ± 1.1 ^{bc,A}	83	59.6 ± 1.05 ^{ab,B}	95	52.7 ± 1.1 ^{ab,C}	90	47.2 ± 1.3 ^{ab,D}	116	58.7 ± 1.4 ^{f,B}
	Control (unstained)	90	77.6 ± 1.3 ^{c,A}	85	58.5 ± 1.6 ^{ab,B}	88	51.0 ± 0.8 ^{bc,C}	90	48.4 ± 1.5 ^{ab,C}	134	57.5 ± 0.7 ^{f,B}
SF	BCB+	90	60.8 ± 1.3 ^{d,B}	80	56.0 ± 1.1 ^{b,C}	85	48.0 ± 0.9 ^{c,D}	100	51.8 ± 1.1 ^{a,CD}	131	65.6 ± 1.3 ^{bcd,A}
	BCB-	100	76.8 ± 1.0 ^{c,A}	70	61.9 ± 1.4 ^{a,C}	90	55.6 ± 0.8 ^{a,D}	90	51.8 ± 1.2 ^{a,D}	111	69.6 ± 0.8 ^{b,B}
	Control (unstained)	90	79.9 ± 1.5 ^{abc,A}	80	58.3 ± 1.1 ^{ab,B}	90	51.7 ± 0.7 ^{abc,C}	89	49.7 ± 1.5 ^{ab,C}	128	60.6 ± 0.7 ^{ef,B}
VSF	BCB+	90	62.7 ± 1.4 ^{d,A}	90	55.2 ± 1.1 ^{b,B}	100	51.6 ± 0.9 ^{bc,BC}	95	48.9 ± 0.8 ^{ab,C}	101	66.0 ± 1.1 ^{bc,A}
	BCB-	100	83.4 ± 1.0 ^{ab,A}	82	61.6 ± 1.1 ^{a,C}	85	53.8 ± 1.1 ^{ab,D}	100	49.8 ± 0.9 ^{ab,D}	102	76.1 ± 1.2 ^{a,B}
	Control (unstained)	90	84.6 ± 1.3 ^{a,A}	95	59.9 ± 1.1 ^{ab,C}	90	54.1 ± 0.8 ^{ab,D}	90	46.3 ± 0.8 ^{b,E}	115	63.9 ± 1.0 ^{cde,B}
Results of 2-way ANOVA											
Origin of OCCs			<0.01	0.56		0.06		<0.01		<0.01	
BCB status of OCCs			<0.01	<0.01		<0.01		0.07		<0.01	
Interaction			<0.05	0.66		<0.01		0.29		<0.01	

Data are shown as the mean ± SEM from 5 replicated trials. Values with different lowercase and uppercase superscripts are significantly different within the same columns and rows, respectively (P < 0.05). MF, medium follicles; SF, small follicles; VSF, very small follicles.

Table 4.5. Total fluorescence intensity of ROS in oocytes derived from follicles with various sizes (MF, SF, and VSF) and classified by BCB staining (BCB+, BCB-, and unstained control) during IVM

Origin of OCCs	BCB status of oocytes	Just after BCB staining		1 h after IVM		4 h after IVM		20 h after IVM		44 h after IVM	
		No. of	Fluorescence	No. of	Fluorescence	No. of	Fluorescence	No. of	Fluorescence	No. of	Fluorescence
		oocytes examined	intensity of ROS	oocytes examined	intensity of ROS	oocytes examined	intensity of ROS	oocytes examined	intensity of ROS	oocytes examined	intensity of ROS
MF	BCB+	90	66.9 ± 0.5 ^{a,A}	95	2.9 ± 0.5 ^{b,B}	100	0 ± 0 ^{b,B}	79	0 ± 0 ^B	126	0 ± 0 ^B
	BCB-	90	17.5 ± 2.0 ^{cd,A}	73	0 ± 0 ^{c,B}	68	0 ± 0 ^{b,B}	59	0 ± 0 ^B	107	0 ± 0 ^B
	Control (unstained)	90	2.1 ± 1.3 ^{e,A}	89	0 ± 0 ^{c,B}	100	0 ± 0 ^{b,B}	71	0 ± 0 ^B	126	0 ± 0 ^B
SF	BCB+	100	64.6 ± 0.7 ^{a,A}	95	4.9 ± 0.7 ^{a,B}	100	0.1 ± 0.1 ^{ab,C}	93	0 ± 0 ^C	106	0 ± 0 ^C
	BCB-	90	22.4 ± 1.5 ^{c,A}	52	0.1 ± 0.0 ^{c,B}	75	0.1 ± 0.0 ^{ab,B}	70	0 ± 0 ^B	97	0 ± 0 ^B
	Control (unstained)	90	5.0 ± 1.8 ^{e,A}	90	0 ± 0 ^{c,B}	100	0 ± 0 ^{b,B}	79	0 ± 0 ^B	110	0 ± 0 ^B
VSF	BCB+	100	38.2 ± 0.6 ^{b,A}	69	0.9 ± 0.4 ^{b,B}	86	0.3 ± 0.1 ^{ab,B}	96	0 ± 0 ^B	93	0 ± 0 ^B
	BCB-	90	12.5 ± 1.8 ^{d,A}	81	0.5 ± 0.2 ^{c,B}	85	0.4 ± 0.2 ^{a,B}	81	0 ± 0 ^B	74	0 ± 0 ^B
	Control (unstained)	90	4.4 ± 0.8 ^{e,A}	95	0 ± 0 ^{c,B}	100	0 ± 0 ^{b,B}	93	0 ± 0 ^B	95	0 ± 0 ^B
Results of 2-way ANOVA											
Origin of OCCs			<0.01	<0.01			<0.01	-			-
BCB status of OCCs			<0.01	<0.01			<0.05	-			-
Interaction			<0.01	<0.01			0.16	-			-

Data are shown as the mean ± SEM from 5 replicated trials. Values with different lowercase and uppercase superscripts are significantly different within the same columns and rows, respectively (P < 0.05). MF, medium follicles; SF, small follicles; VSF, very small follicles.

Table 4.6. Total fluorescence intensity of GSH in oocytes derived from follicles with various sizes (MF, SF, and VSF) and classified by BCB staining (BCB+, BCB-, and unstained control) at the end of IVM culture

Origin of OCCs	BCB status of oocytes	No. of oocytes examined polar body extrusion	No. (%) of mature oocytes	Mature		Immature	
				No. of oocytes examined	Fluorescence intensity of GSH	No. of oocytes examined	Fluorescence intensity of GSH
MF	BCB+	344	268 (78.0 ± 0.9 ^a)	100	57.4 ± 1.2 ^{d,B}	44	70.0 ± 1.1 ^{bcd,A}
	BCB-	236	151 (64.0 ± 0.2 ^{bc})	75	54.3 ± 1.6 ^{d,B}	41	66.8 ± 2.1 ^{cd,A}
	Control (unstained)	295	204 (69.1 ± 0.8 ^{ab})	85	56.0 ± 0.9 ^{d,B}	49	60.3 ± 1.0 ^{e,A}
SF	BCB+	271	174 (64.4 ± 1.7 ^{bc})	87	60.1 ± 1.3 ^{cd,B}	44	76.4 ± 1.9 ^{a,A}
	BCB-	223	125 (56.0 ± 1.9 ^c)	61	68.3 ± 1.2 ^b	50	71.1 ± 1.0 ^{abc}
	Control (unstained)	258	147 (57.0 ± 3.0 ^c)	70	57.9 ± 1.1 ^{d,B}	58	64.0 ± 0.8 ^{de,A}
VSF	BCB+	199	119 (59.6 ± 4.2 ^{bc})	57	66.1 ± 1.3 ^{bc}	44	65.7 ± 2.0 ^{cde}
	BCB-	196	76 (37.6 ± 3.2 ^d)	42	80.0 ± 1.7 ^{a,A}	60	73.4 ± 1.6 ^{ab,B}
	Control (unstained)	248	106 (42.6 ± 2.6 ^d)	48	59.3 ± 1.6 ^{d,B}	67	67.2 ± 1.0 ^{bcd,A}
Results of 2-way ANOVA							
Origin of OCCs			<0.01		<0.01		<0.01
BCB status of OCCs			<0.01		<0.01		<0.01
Interaction			0.20		<0.01		<0.01

Data are shown as the mean ± SEM from 5 replicated trials. Values with different lowercase and uppercase superscripts are significantly different within the same columns and rows, respectively (P < 0.05). MF, medium follicles; SF, small follicles; VSF, very small follicles.

Table 4.7. Total fluorescence intensity of ROS in oocytes derived from follicles with various sizes (MF, SF, and VSF) and classified by BCB staining (BCB+, BCB-, and unstained control) at the end of IVM culture

Origin of OCCs	BCB status of oocytes	Mature		Immature	
		No. of oocytes examined	Fluorescence intensity of ROS	No. of oocytes examined	Fluorescence intensity of ROS
MF	BCB+	88	0.0001 ± 0.0001	32	0 ± 0
	BCB-	73	0.00001 ± 0.00001	34	0.001 ± 0.001
	Control (unstained)	82	0 ± 0	44	0 ± 0
SF	BCB+	66	0 ± 0	40	0 ± 0
	BCB-	55	0.0009 ± 0.0009	42	0.003 ± 0.003
	Control (unstained)	59	0 ± 0	51	0 ± 0
VSF	BCB+	57	0 ± 0	36	0 ± 0
	BCB-	35	0.0004 ± 0.0004	39	0.0003 ± 0.0003
	Control (unstained)	45	0 ± 0	50	0 ± 0
Results of 2-way ANOVA					
Origin of OCCs			0.47	0.51	
BCB status of OCCs			0.15	0.20	
Interaction			0.40	0.63	

Data are shown as the mean $\pm$ SEM from 5 replicated trials. Values with different lowercase and uppercase superscripts are significantly different within the same columns and rows, respectively ($P < 0.05$). MF, medium follicles; SF, small follicles; VSF, very small follicles.

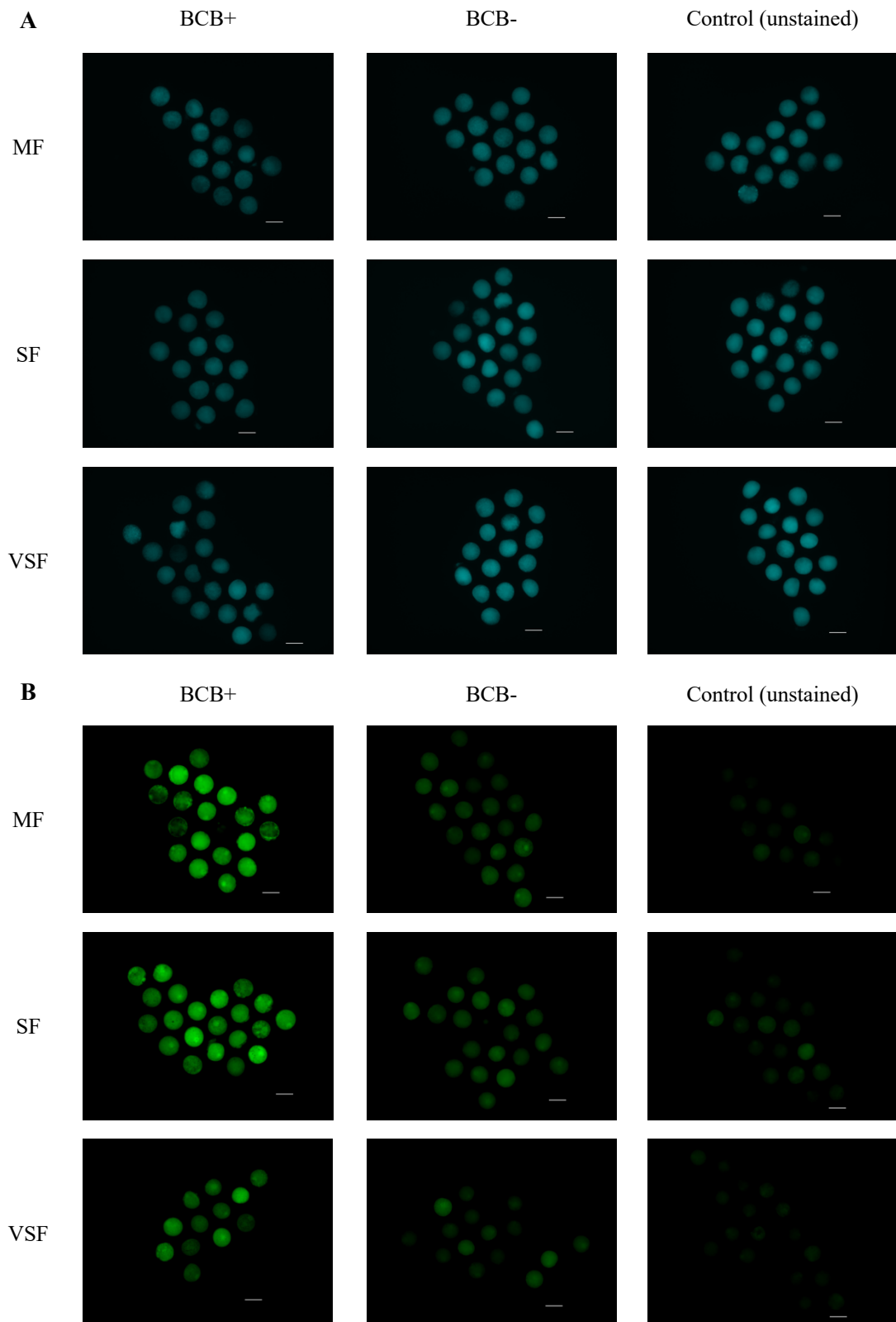


Figure 4.1. Ooplasmic fluorescence demonstrating GSH (A) and ROS (B) levels in oocytes derived from follicles with various sizes (MF, SF, and VSF) just after BCB staining. Scale bar = 100 μ m. Exposure time: $\frac{1}{4}$ second.

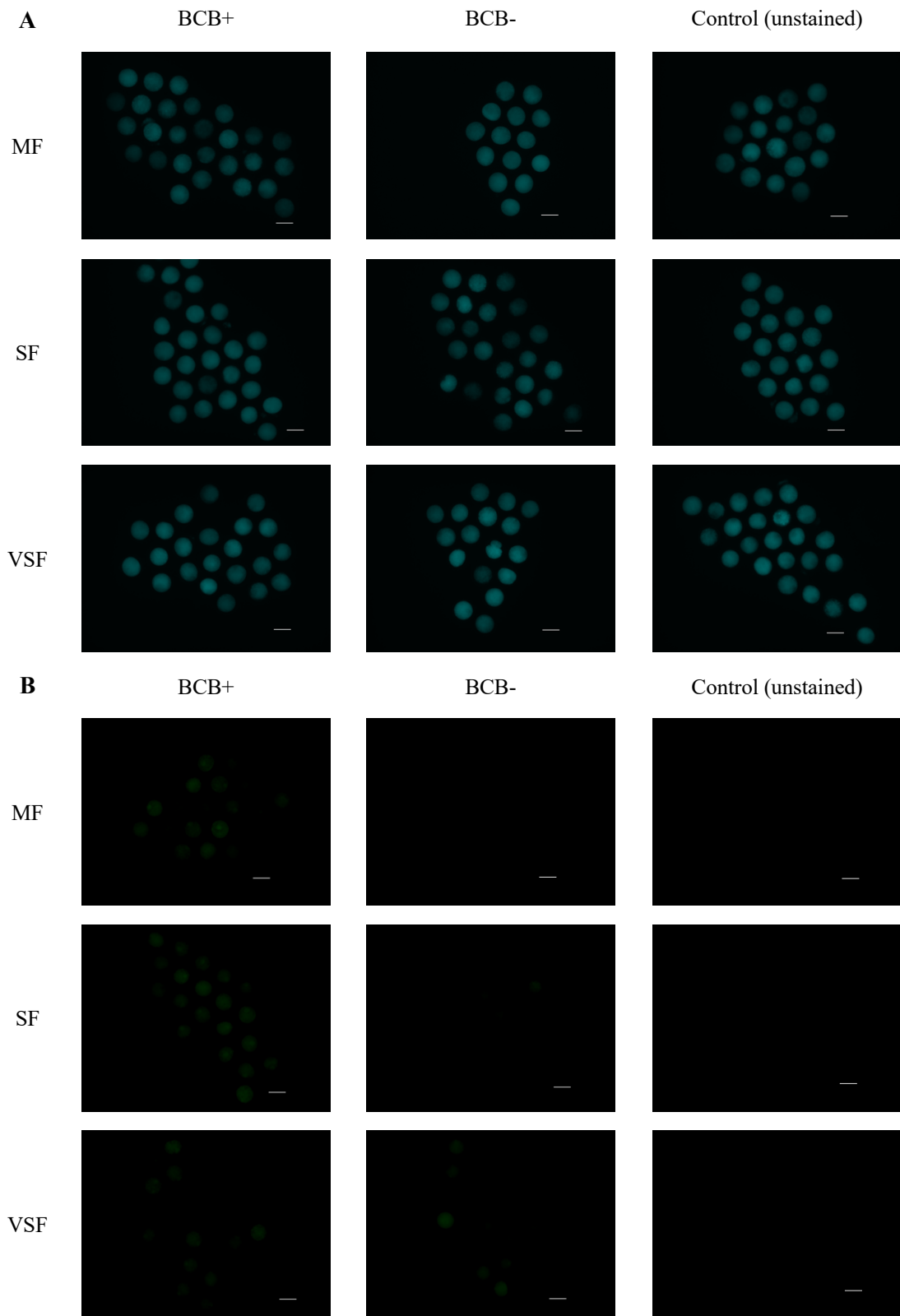


Figure 4.2. Ooplasmic fluorescence demonstrating GSH (A) and ROS (B) levels in oocytes derived from follicles with various sizes (MF, SF, and VSF) and classified by BCB staining (BCB+, BCB- and unstained control) after 1 h of IVM. Exposure time: $\frac{1}{4}$ second. Scale bar = 100 μ m.

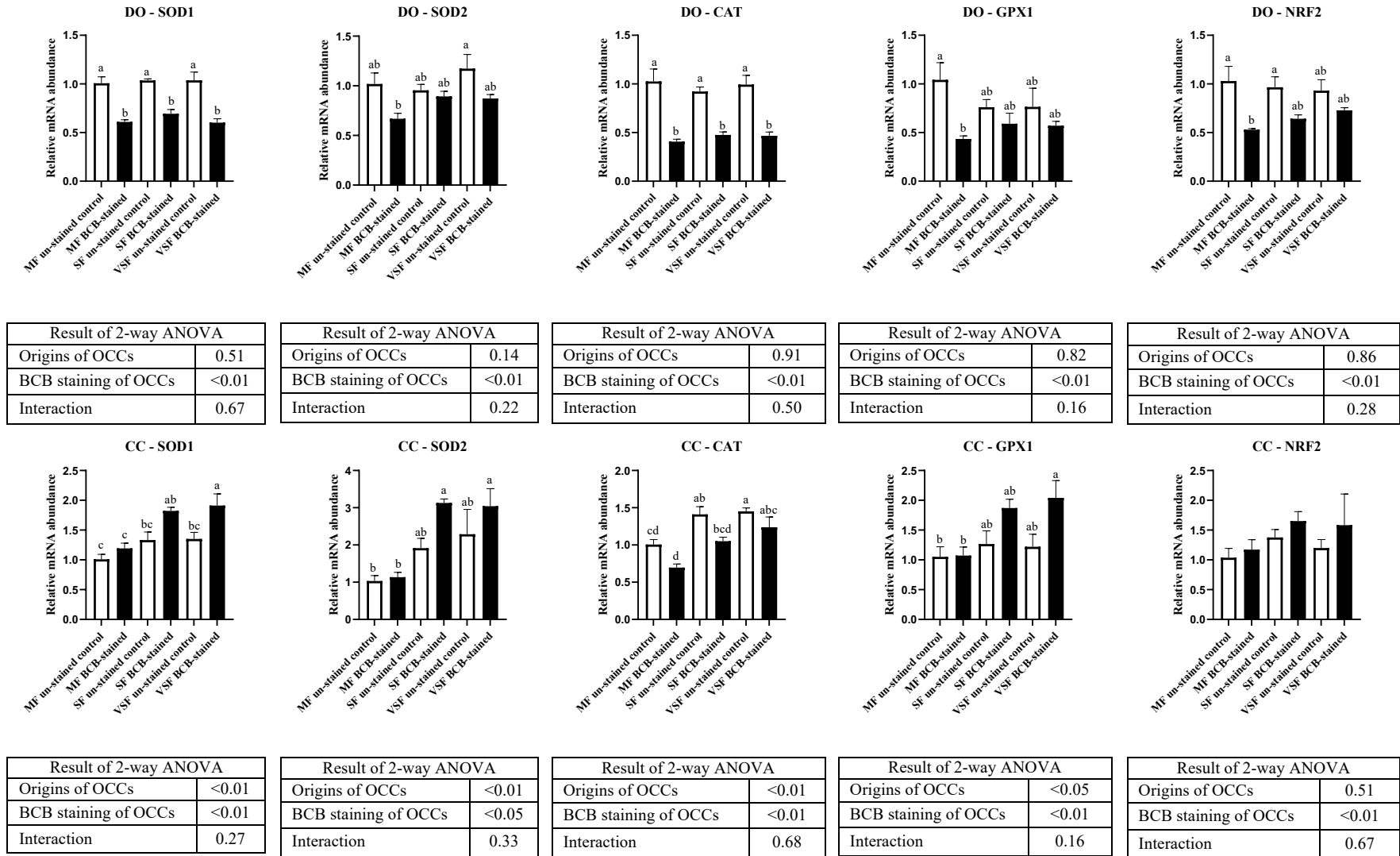


Figure 4.3. Gene expression in BCB-stained (black bars) and unstained control (white bars) OCCs collected from medium (MF), small (SF), or very small (VSF) follicles. The ratio to the mean value in control oocytes obtained from MF-derived OCCs is represented. Data from 4 replicated trials and expressed as mean $\pm$ SEM. Different letters indicate significant differences between groups ($P < 0.05$).

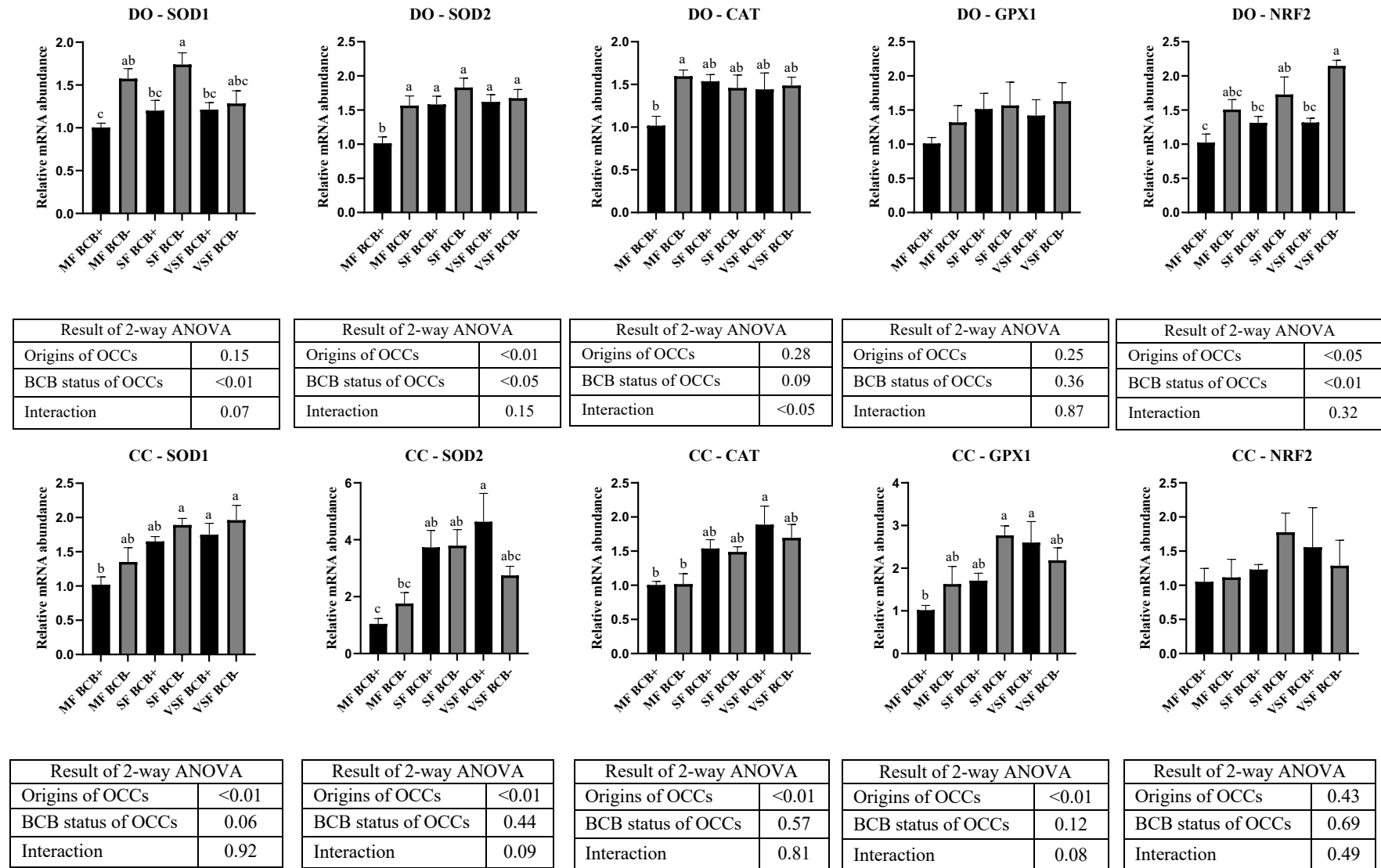


Figure 4.4. Gene expression in BCB-positive (black bars) and BCB-negative (grey bars) OCCs collected from medium (MF), small (SF), or very small (VSF) follicles. The ratio to the mean value in BCB-positive oocytes obtained from MF-derived OCCs is represented. Data from 4 replicated trials and expressed as mean $\pm$ SEM. Different letters indicate significant differences between groups ($P < 0.05$).

4.4. Discussion

In the present study, regardless of the origin of OCCs (from MF, SF and VSF), the dark blue color in the ooplasm of a majority of BCB⁺ oocytes rapidly disappeared and changed to colorless within 1 h after BCB staining, confirming that the G6PD activity, which is an enzyme that catalyzes the production of NADPH at PPP, is also present in BCB⁺ oocytes, although not as much as in BCB⁻ ones (Alm et al., 2005). NADPH provides electrons, reducing BCB from its blue (oxidized) form to its colorless (reduced) form. Therefore, along with the decrease in NADPH concentration in BCB⁺ oocytes after 1 h of IVM, there is a loss of BCB dye in the cytoplasm in most BCB⁺ oocytes. Alternatively, since our IVM condition contained 2-mercaptoethanol (Akaki et al., 2009), it may be possible that the presence of the potent antioxidant counteracted partially this disappearance and against oxidative stress by BCB stain. Within 1 h after BCB staining, we also observed different responses in the NADPH regulation between MF-, SF-, and VSF-derived OCCs, even in the presence of 2-mercaptoethanol. NADPH levels in oocytes just after BCB staining were affected by both the origins of OCCs (from MF, SF, and VSF) and the BCB status (BCB⁺ and BCB⁻), although the levels did not differ between MF-, SF-, and VSF-derived unstained controls. Exposure of MF-derived OCCs to BCB dye significantly increased the NADPH levels in both BCB⁺ and BCB⁻ oocytes. This may be due to the response of oocytes to oxidative stress by BCB staining (Fonseka et al., 2024; Santos et al., 2014) with enhanced NADPH production in the PPP. Furthermore, the differences in response among the oocytes of various follicular origins may also reflect the supplied levels of NADPH, GSH, and other substances to the oocytes due to disparity in the number of surrounding healthy cumulus cells (Fonseka et al., 2024; Van et al., 2025) and transzonal projections (El-Hayek et al., 2018). However, these significant differences disappeared 1 h after the start of IVM culture, even in the presence of 2-mercaptoethanol as an antioxidant. Therefore, these results indicate that oocytes respond to BCB staining as seen in NADPH levels, but this stress is transient and varies depending on the origin of the OCCs (MF, SF, and VSF), even in the presence of an antioxidant. On the other hand, regardless of the origin of OCCs (from MF, SF vs. VSF), there were no significant

differences in NADPH levels between BCB⁺ oocytes, which are considered to have low G6PD activity, and BCB⁻ oocytes, which are assessed to have high. Since these results were consistent with the transcriptional levels of *G6PD* in MF-, SF-, and VSF-derived BCB⁺/BCB⁻ oocytes just after BCB staining (Van et al., 2025), the NADPH levels in oocytes observed in this study may include both the result of G6PD activity in the oocytes before staining and the response to oxidative stress during BCB staining.

In the present study, along with a brief increase in NADPH levels, we found a transient increase in ROS and a decrease in GSH in BCB⁺ oocytes, which largely returned to a similar level within 1 h after the start of IVM culture (the end of BCB staining). This result is consistent with our previous report (Fonseka et al., 2024) that ROS levels just after BCB staining were significantly higher only in MF-, SF-, and VSF-derived BCB⁺ oocytes than in BCB⁻ ones. NADPH is required to regenerate GSH from GSSG via glutathione reductase. Since ROS levels in BCB⁻ oocytes were not as elevated as those in BCB⁺ oocytes, and GSH levels did not significantly differ from unstained controls in the present study, the presence of BCB dye, acting as an electron acceptor (El-Hayek et al., 2018), may induce transient oxidative stress on the oocytes. It has been known that exposure to oxidative stress rapidly increases the activity and expression of *G6PD* in cells to maintain the levels of NADPH required for the enzymatic ROS removal pathway (Ursini et al., 1997). In our experiment, furthermore, one hour after the end of BCB staining, when more than 90% of BCB⁺ oocytes became colorless, the decrease in GSH and increase in ROS in BCB⁺ oocytes were restored. These results indicate that the presence of BCB dye (blue color) in the ooplasm is the cause of oxidative stress in the oocytes and that the oxidative stress seems to be resolved once the blue dye is reduced to colorless and the reaction ceases. In the present study, slight differences in ROS loss from oocytes derived from different-sized follicles were observed at 1 and 4 h into IVM culture. These differences in oocyte resilience to oxidative stress by BCB staining may also be due to metabolites supplied from the cumulus cells (Khatun et al., 2024) via gap junctional communication (Bagg et al., 2009), and the consequent amount of

NADPH in the oocytes. We also found that the ROS levels of MF-, SF-, and VSF-derived BCB+ oocytes were significantly higher than those of BCB- ones, whereas the GSH levels were opposite. These results appear to be due to relatively lower G6PD levels in BCB+ oocytes. Furthermore, the ROS level of VSF-derived BCB+ oocytes was significantly lower than that of MF- and SF-derived BCB+ ones just after BCB staining. This is consistent with a previous report showing high levels of *G6PD* mRNA in VSF-derived OCCs with BCB+ oocytes (Van et al., 2025).

The GSH levels in both BCB+ and BCB- oocytes decreased after the start of IVM culture and then increased until the end of the second half of IVM culture without gonadotropins and db-cAMP. In contrast, ROS levels in oocytes were not detected in all samples at the end of the first half of IVM culture in the presence of gonadotropin and db-cAMP, nor at the end of the second half in their absence, although a transient increase was observed in BCB-stained OCCs. Glutathione, which is an essential scavenger against excessive ROS generation, appears to be regulated at the ooplasmic level of OCCs by the transportation of metabolites, such as GSH and NADPH, from surrounding somatic cells into the oocytes via transzonal projections (Khatun et al., 2024), of which the number also gradually increased during oocyte growth (El-Hayek et al., 2018). Exposure of OCCs to gonadotropins and db-cAMP during the first half of IVM culture takes place to disconnect the gap junction of transzonal projections between oocytes and cumulus cells (Bagg et al., 2009), prohibiting the supply of metabolites, such as GSH and NADPH, from cumulus cells. Consequently, it may decrease the GSH level in the oocytes. This speculation is also consistent with our current results, showing that GSH levels in mature oocytes were lower than those in immature ones at the end of IVM culture.

We also demonstrated that, regardless of the origin of OCCs, the ROS levels were not different between BCB+, BCB-, and unstained control oocytes, and even between mature and immature ones, at the end of IVM culture. Although it has been reported that BCB staining of porcine OCCs increases ROS levels in oocytes immediately after staining as well as after IVM culture (Santos et al., 2014), under the culture conditions of this study, it only affected them about 1 h after

staining when BCB dye was present and not after IVM. Again, we observed higher maturation rates in BCB+ oocytes from various sizes of follicles, therefore confirming the utility of BCB staining for selecting MF-, SF-, and VSF-derived OCCs containing oocytes with relatively higher meiotic and developmental competences (Van et al., 2025).

In the current study, the expression levels of oxidative stress-related genes in oocytes and their surrounding cumulus cells just after BCB staining were examined separately. We found that BCB staining significantly affected the transcriptional levels of *SOD1*, *SOD2*, *CAT*, *GPX1*, and *NRF2* negatively in oocytes and positively in the surrounding cumulus cells. The effects of BCB staining on the expression levels of oxidative stress-related genes in oocytes and surrounding cumulus cells remain unclear, whereas there is a recent report that the expression levels of *SOD2* and *GPX1* in buffalo's OCCs significantly increase after BCB staining (M. Q. Li et al., 2024). Our current results are not only consistent with their report but also provide more detailed information. Especially, it is very interesting that the expression of oxidative stress-related genes, without *NRF2*, examined here in cumulus cells, regardless of BCB staining and the BCB-status (BCB+ and BCB-) of the oocytes, is significantly affected by the size of the follicles from which the OCCs are derived, which seems to be reflected in the resilience of the OCCs examined here. Furthermore, the expression of *NRF2*, a key regulator of oxidative stress response, remained stable in cumulus cells, regardless of the origins of OCCs and the BCB status of the oocytes. This result demonstrates that cumulus cells could maintain a more stable redox regulation than oocytes.

In conclusion, the current study demonstrates that although BCB staining induces transient oxidative stress in porcine OCCs, especially those including BCB+ oocytes, and different transcriptional reactions of oxidative stress-related genes in oocytes and the surrounding cumulus cells, ROS in the ooplasm become undetectable with the disappearance of the BCB blue dye, in our IVM culture condition. There are differences in the resilience among OCCs derived from MF, SF, and VSF to oxidative stress, but the OCCs seems to rapidly restore redox balance by regulating and utilizing NADPH to produce reduced GSH.

CHAPTER 5

GENERAL DISCUSSION

In this study, BCB staining was used as a predictive marker of meiotic and developmental competence of porcine oocytes derived from VSF, SF, and MF through an approximate assessment of G6PD activity based on retained dye (dark blue, BCB+) or partial and complete decolorized dye (slight blue and colorless, BCB-) in the cytoplasm. Our findings demonstrated that the incidence of MF-derived OCCs containing BCB+ oocytes was significantly higher than that of SF- and VSF-derived OCCs. Surprisingly, even VSF-derived OCCs contained BCB+ oocytes. Additionally, we confirmed that SF- and VSF-derived BCB+ oocytes demonstrated meiotic and developmental competences up to the metaphase-II and blastocyst stages, although the percentages were lower than those of MF-derived BCB+ oocytes. Our results suggest the potential to utilize oocyte resources from SF and even VSF, which are more abundant in the ovaries of prepubertal gilts, for in vitro embryo production. Future research should focus on assessing the meiotic and developmental competences of VSF-derived BCB+ oocytes following pre-IVM to complete their growth; additional in vitro growth procedures may further enhance these competences.

Cumulus cells play critical roles by providing metabolites and facilitating the growth of developing oocytes. Therefore, the presence of a sufficient number of healthy cumulus cells influences the metabolic system of the oocytes and may consequently improve and enhance their meiotic and developmental competences. Our observations indicated that the total number of cumulus cells per OCC did not differ between BCB+ and BCB- groups across all follicle sizes. However, the number of viable cells was significantly higher in OCCs containing BCB+ oocytes than in those with BCB- oocytes. In addition, we also observed that BCB+ oocytes were present in VSF-derived OCCs that contained fewer cumulus cells than those derived from MF with BCB- oocytes, and there were no differences in the incidence of live cumulus cells between MF-derived OCCs containing BCB+ and BCB- oocytes. This result suggests that the BCB status may be affected by the number of surrounding viable cumulus cells and other factors. The percentages of

viable cells in SF- or VSF-derived BCB- oocytes were significantly lower than those in BCB+ oocytes. These findings suggest that some SF- and VSF-derived OCCs containing BCB- oocytes may originate from early atretic follicles.

When we measured the outer and inner diameters of zona pellucida, as well as the ooplasmic diameter and perivitelline space after denuding oocytes collected from various sizes of antral follicles, we found that the outer and inner diameters of zona pellucida were significantly larger in both OCCs derived from larger follicles and those containing BCB+ oocytes. In contrast, the diameters of the ooplasm of MF-, SF-, and VSF-derived BCB+ oocytes were similar, while the diameters of SF- and VSF-derived BCB- oocytes were significantly smaller.

The current study assessed the expression of PPP-related genes in both oocytes and cumulus cells. The results demonstrated that, regardless of the BCB status of the oocytes, the transcriptional levels of G6PD and TKT in the surrounding cumulus cells of MF-derived OCCs were significantly lower than those in SF- and VSF-derived OCCs. In contrast, the mRNA levels of G6PD and TKT in oocytes were influenced only by the BCB status of the OCCs or their origin, respectively. These findings indicate that the expression of PPP-related genes in cumulus cells, but not clearly in oocytes, gradually changes during follicular development from VSF to MF. Furthermore, the activities of both the oxidative and non-oxidative branches of the PPP, represented by G6PD and TKT, are decreased in cumulus cells rather than in oocytes as follicular development processes from VSF to MF. Besides, the mRNA level of RPIA, a key enzyme responsible for synthesizing ribose 5-phosphate in the PPP, was lower only in BCB+ cumulus cells derived from MF. These results indicate that meiotic and developmental competences are greater in BCB+ oocytes surrounded by cumulus cells with lower expression levels of PPP-related genes, highlighting the significance of the PPP-related gene expression in cumulus cells rather than in oocytes.

Within 1 h after BCB staining, we also observed different responses in the NADPH regulation between MF-, SF-, and VSF-derived OCCs. NADPH levels in oocytes just after BCB

staining were affected by both the origins of OCCs (from MF, SF, and VSF) and the BCB status (BCB+ and BCB-), although the levels did not differ between MF-, SF-, and VSF-derived unstained controls. Exposure of MF-derived OCCs to BCB dye significantly increased the NADPH levels in both BCB+ and BCB- oocytes. Furthermore, the differences in response among the oocytes of various follicular origins may also reflect the supplied levels of NADPH, GSH, and other substances to the oocytes due to disparity in the number of surrounding healthy cumulus cells and transzonal projections. However, these significant differences disappeared 1 h after the start of IVM culture, even in the presence of 2-mercaptoethanol as an antioxidant. These results indicate that oocytes respond to BCB staining as seen in NADPH levels, but this stress is transient and varies depending on the origin of the OCCs (MF, SF, and VSF), even in the presence of an antioxidant.

Along with a brief increase in NADPH levels, a transient increase in ROS and a decrease in GSH in BCB+ oocytes, which largely returned to a similar level within 1 h after the start of IVM culture (the end of BCB staining), and ROS levels just after BCB staining were significantly higher only in MF-, SF-, and VSF-derived BCB+ oocytes than in BCB- ones. Furthermore, one hour after the end of BCB staining, when more than 90% of BCB+ oocytes became colorless, the decrease in GSH and increase in ROS in BCB+ oocytes were restored. These results indicate that the presence of BCB dye (blue color) in the ooplasm is the cause of oxidative stress in the oocytes and that the oxidative stress seems to be resolved once the blue dye is reduced to colorless and the reaction ceases.

The slight differences in ROS loss from oocytes derived from different-sized follicles were observed at 1 and 4 h into IVM culture. These differences in oocyte resilience to oxidative stress by BCB staining may also be due to metabolites supplied from the cumulus cells via gap junctional communication and the consequent amount of NADPH in the oocytes. We also found that the ROS levels of MF-, SF-, and VSF-derived BCB+ oocytes were significantly higher than those of BCB- ones, whereas the GSH levels were the opposite. These results are due to relatively lower G6PD

levels in BCB⁺ oocytes. Furthermore, the ROS level of VSF-derived BCB⁺ oocytes was significantly lower than that of MF- and SF-derived BCB⁺ ones just after BCB staining. This is consistent with high levels of G6PD mRNA in VSF-derived OCCs containing BCB⁺ oocytes. Notably, the GSH levels in both BCB⁺ and BCB⁻ oocytes decreased after the start of the IVM culture and then increased until the end of the second half of the IVM culture. In contrast, ROS levels in oocytes were not detected in all samples at the end of the first half of IVM culture, nor at the end of the second half, although a transient increase was observed in BCB-stained OCCs.

The expression levels of oxidative stress-related genes in oocytes and their surrounding cumulus cells were examined separately after BCB staining. BCB staining significantly affected the transcriptional levels of SOD1, SOD2, CAT, GPX1, and NRF2 negatively in oocytes and positively in the surrounding cumulus cells. It is especially exciting that the expression of oxidative stress-related genes, without NRF2, examined in cumulus cells, regardless of BCB staining and the BCB-status (BCB⁺ and BCB⁻) of the oocytes, is significantly affected by the size of the follicles from which the OCCs are derived. Furthermore, the expression of NRF2, a key regulator of oxidative stress response, remained stable in cumulus cells, regardless of the origins of OCCs and the BCB status of the oocytes. This result demonstrates that cumulus cells could maintain a more stable redox regulation than oocytes.

In conclusion, the current study demonstrates the effectiveness of BCB staining in selecting OCCs that contain oocytes with relatively higher meiotic and developmental competences, derived not only from MF but also from SF and even VSF. The significance of having sufficient healthy cumulus cells surrounding the oocytes was also recognized. Additionally, the expression of PPP-related genes in the surrounding cumulus cells decreases during follicular development from VSF to MF, indicating that this may significantly impact the meiotic and developmental competences of the oocytes. Moreover, although BCB staining induces transient oxidative stress in porcine OCCs, especially those including BCB⁺ oocytes, and different transcriptional reactions of oxidative stress-related genes in oocytes and the surrounding cumulus cells, ROS in the ooplasm

become undetectable with the disappearance of the BCB blue dye, in our IVM culture condition. There are differences in the resilience among OCCs derived from MF, SF, and VSF to oxidative stress, but these OCCs seem to rapidly restore redox balance by regulating and utilizing NADPH to produce reduced GSH.

CHAPTER 6

SUMMARY

Prepubertal gilt's ovaries contain various sizes of follicles, of which the number of SF and VSF is larger than MF. Thus, maximizing the utilization of material from each ovary, including the OCCs derived from SF and even VSF, could be necessary for the development of embryonic gene editing technology. BCB staining has proven effective in selecting porcine MF-derived BCB+ oocytes with both meiotic and developmental competences. However, BCB+ oocytes (considered to have relatively low G6PD activity) are observed even in SF; oocytes are believed to still be in the growing phase and possess higher meiotic competence compared to BCB- (considered to have relatively high G6PD activity) oocytes. Therefore, it is essential to reanalyse whether the characteristics of BCB+ oocytes have long been regarded as grown ones with meiotic and developmental competences. Furthermore, BCB staining has been demonstrated to cause oxidative stress to OCCs, especially BCB+ ones, through increasing ROS levels. However, the damage to oocytes through IVM culture has not been evaluated yet. The objectives of the current study were to determine whether porcine OCCs derived from various antral follicle sizes (VSF, SF, and MF) exhibit differences in BCB staining status, meiotic and developmental competences, oocyte diameters, number of surrounding viable cumulus cells, and expression of genes related to the PPP, as well as to evaluate their resilience to BCB staining-induced oxidative stress by analysing intracellular GSH, ROS, and NADPH levels in oocytes during IVM.

The first experiment results showed that the incidence of BCB- oocytes in VSF- and SF-derived OCCs was higher than in MF-derived OCCs. Although the meiotic and developmental competences of BCB+ oocytes from MF were superior to those from VSF and SF, blastocysts were successfully obtained from BCB+ oocytes, even derived from VSF. The mean numbers of both total and viable cumulus cells surrounding an oocyte were significantly affected not only by the origin of the OCCs but also by the BCB status of the oocytes (largest in MF-derived OCCs containing BCB+ oocytes). Although the outer and inner diameters of zona pellucida were affected by the origin of

OCCs and the BCB status of oocytes (largest in MF-derived oocytes), the ooplasmic diameter of BCB+ oocytes did not differ among those derived from VSF, SF, and MF. Regardless of the BCB status, the transcriptional levels of G6PD and TKT in cumulus cells decreased during follicular development from VSF to MF, whereas the RPIA mRNA level in cumulus cells of MF-derived BCB+ OCCs was lower than in the others.

Furthermore, BCB staining affected transcriptional levels of oxidative stress-related genes in both oocytes and cumulus cells. Regardless of the origins of OCCs, even in BCB+ oocytes, the blue dye disappeared around 1 h in an IVM culture condition. Within 1 h after BCB staining, distinct responses in the NADPH regulation were observed between MF-, SF-, and VSF-derived OCCs and were affected by both the origins of OCCs and the BCB status. A transient increase in ROS and a decrease in GSH in BCB+ oocytes were also observed. After BCB staining, the ROS levels in both BCB+ and BCB- oocytes were increased, although the level was affected by both the origin of OCCs and BCB status until 4 h of IVM and remained at low levels during IVM. The GSH levels of MF-, SF-, and VSF-derived BCB+ oocytes were significantly lower than those of BCB- ones and non-stained controls, but the difference disappeared during IVM.

In conclusion, the current study demonstrates the effectiveness of BCB staining in selecting OCCs that contain oocytes with relatively higher meiotic and developmental competences, derived not only from MF but also from SF and even VSF. The significance of having sufficient healthy cumulus cells surrounding the oocytes was also recognized. Additionally, the expression of PPP-related genes in the surrounding cumulus cells decreases during follicular development from VSF to MF, indicating that this may significantly impact the meiotic and developmental competences of the oocytes. Although BCB staining induces transient oxidative stress in porcine OCCs, especially those including BCB+ oocytes, and different transcriptional reactions of oxidative stress-related genes in oocytes and the surrounding cumulus cells, ROS in the ooplasm become undetectable with the disappearance of the BCB blue dye, in our IVM culture condition. There are differences in the

resilience among OCCs derived from MF, SF, and VSF to oxidative stress, but these OCCs seem to rapidly restore redox balance by regulating and utilizing NADPH to produce reduced GSH.

Future research should focus on optimizing pre-IVM culture conditions to increase the number of surrounding cumulus cells and prolong the cytoplasmic maturation time of oocytes, especially for VSF-derived BCB⁺ oocytes, to enhance their meiotic and developmental competences to meet the needs for embryonic gene editing technology.

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