수의학박사학위논문

The improvement of porcine oocyte *in vitro* maturation systems through antioxidant and lipid metabolism upregulation

항산화제 및 지질대사 증진을 통한 돼지 난자 체외성숙 시스템 향상 연구

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The improvement of porcine oocyte *in vitro* maturation systems through antioxidant and lipid metabolism upregulation

by Jun-Xue Jin

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

Veterinary Biomedical Sciences

Department of Veterinary Medicine, Graduate School

Seoul National University

We accept this thesis as confirming to the required standard

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December 2017

Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Jun-Xue Jin

The improvement of porcine oocyte *in vitro* maturation systems through antioxidant and lipid metabolism upregulation

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ABSTRACT

In vitro maturation (IVM) is one of the routinely used systems for the *in vitro* production of embryos in pigs. It offers mature oocytes from immature status and then develops to blastocyst stage under *in vitro* condition. However, the maturation rate and the developmental competence of embryos derived from IVM oocytes are

significantly lower than those of oocytes matured *in vivo*. In this study, I attempted to investigate the oocyte maturation and their subsequent embryonic development were influenced by antioxidative and lipid metabolic functions.

Firstly, to determine the antioxidant property of spermine on IVM of porcine oocytes and their embryonic development after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT). As a result, treatment with various concentrations of spermine in IVM culture medium, there was no significant difference in nuclear maturation rate. Nevertheless, spermine treatment groups showed significantly increased intracellular GSH levels and decreased ROS levels compared to the control. Furthermore, 10 μM of spermine supported significantly higher blastocyst formation rates after PA and SCNT than the control group. Therefore, it can be concluded that 10 μM spermine supplementation during IVM improved the development of porcine PA and SCNT embryos by increasing intracellular GSH, scavenging ROS levels, and regulating gene expression.

Knockout serum replacement (KSR) is composed of small organic molecules, trace elements, and three proteins, namely insulin, transferrin, and lipid-rich albumin, without any undefined growth factors or differentiation-promoting factors. This study was conducted to examine the effects of KSR on oocyte maturation and embryonic development after PA in pigs. Although the cumulus cell expansion index was significant lower in 5% and 10% KSR compare to the control and 10% porcine follicular fluid (pFF), the 5% KSR supplementation was significantly increased the mRNA expression of *Ptgs1*, *Has2* and *Tnfaip6* than control group. Moreover, the mRNA expression of *GDF9*, *BMP15* and *Cdc2* were increased in 5%

KSR, in line with these results, the protein levels of GDF9 and BMP15 were also upregulated in 5% KSR and 10% pFF. In case of the *Bax/Bcl2* ratio, it was decreased in 5% KSR and 10% pFF both of oocytes and cumulus cells, in line with ROS, and GSH results in oocytes. In addition, rates of oocyte maturation and blastocyst formation after PA were significantly higher in the 5% KSR supplemented group than control group and more similar to those of the 10% pFF supplemented group. Finally, I conformed that 5% KSR has antioxidant property which played a crucial role in the acquisition of oocyte development and subsequent embryonic development.

Secondly, I analyzed the effect of lipid metabolism during porcine oocyte maturation by KSR and melatonin treatment. To investigate the mechanism of KSR during IVM, I focused on lipid metabolism and carried out experiments. In this study, I evaluated (i) the expression of lipid metabolism genes in cumulus cells and oocytes, (ii) measure the fluorescence staining intensity of BODIPY-LD, BODIPY-FA and BODIPY-ATP in oocytes and (iii) determine the influence of 5% KSR and 10% pFF on embryo development after SCNT and *in vitro* fertilization (IVF). Rates of blastocyst formation after SCNT and IVF were significantly higher in the 5% KSR supplemented group than in the control group and more similar to those of the 10% pFF supplemented group. Moreover, the intensity of BODIPY-LD, BODIPY-FA and BODIPY-ATP staining showed similar values between 5% KSR and 10% pFF, which were significantly higher than the control. Most of the gene expression related to lipid metabolism with both supplements exhibited similar patterns. In summary, 5% KSR increased blastocyst formation rate after

SCNT and IVF with providing an essential energy by upregulation of lipid metabolism. These indications support the idea that KSR used as a defined serum supplement for oocyte IVM.

The results of melatonin treatment as another supplementation showed that it significantly enhanced the number of lipid droplets (LDs) and upregulated gene expression related lipogenesis (*ACACA*, *FASN*, *PPARγ*, and *SREBF1*). Oocytes treated with melatonin formed smaller LDs and abundantly expressed several genes associated with lipolysis, including *ATGL*, *CGI-58*, *HSL* and *PLIN2*. Moreover, melatonin significantly increased the content of fatty acids, mitochondria and ATP, as indicated by fluorescent staining. Concomitantly, melatonin treatment upregulated gene expression related to fatty acid β-oxidation (*CPT1a*, *CPT1b*, *CPT2* and *ACADS*) and mitochondrial biogenesis (*PGC-1α*, *TFAM* and *PRDX2*). Overall, melatonin treatment not only altered both the morphology and amount of LDs, but also increased the content of fatty acids, mitochondria and ATP. Moreover, melatonin upregulated mRNA expression levels of lipogenesis, lipolysis, β-oxidation and mitochondrial biogenesis-related genes in porcine oocytes. These results indicated that melatonin promoted lipid metabolism and thereby provided an essential energy source for oocyte maturation and subsequent embryonic development. In addition, melatonin treatment increased SCNT efficiency for *in vitro* development, and one of 3 recipients was pregnant by melatonin treatment.

In conclusion, I demonstrated that supplementation with exogenous antioxidants such as spermine and KSR, which effectively contributed to oocyte maturation and embryonic development by increasing the intracellular GSH levels,

and decreasing the ROS levels. In addition, KSR and melatonin upregulated lipid metabolism in porcine oocytes thus provided an essential energy source to promote and improve oocyte quality and subsequent embryo development. It is feasible that upregulation of lipid metabolism and antioxidation are indispensable mechanisms for improving porcine oocyte *in vitro* maturation systems.

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Key word: pig, antioxidant, lipid metabolism, oocyte maturation, embryo development.

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LIST OF ABBREVIATIONS

AI	**Artificial insemination**
Ana-Telo	**Anaphase - Telophase I**
ANOVA	**Analysis of variance**
ATP	**Adenosine triphosphate**
BSA	**Bovine serum albumin**
cDNA	**Complementary DNA**
CEI	**Cumulus expansion index**
COCs	**Cumulus-oocyte complexes**
CRISPR	**Clustered regularly interspaced short palindromic repeats**
DMEM	**Dulbecco's modified Eagle's medium**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
DPBS	**Dulbecco's phosphate-buffered saline**
eCG	**Equine chorionic gonadotropin**
EDTA	**Ethylenediaminetetraacetic acid**
EGF	**Epidermal growth factor**
ESCs	**Embryonic stem cells**
FAO	**Fatty acid β-oxidation**
FAs	**Fatty acids**
FBS	**Fetal bovine serum**

GFP	**Green fluorescent protein**
GGTA1	**Alpha-1, 3-galactosyltransferase**
GSH	**Glutathione**
GV	**Germinal vesicle**
GVBD	**Germinal vesicle breakdown**
HSA	**Human serum albumin**
hCG	**Human chorionic gonadotropin**
ICSI	**Intra-cytoplasmic sperm injection**
iPS	**Induced pluripotent stem cell**
ITS	**Insulin transferrin selenium**
IVC	***In vitro* culture**
IVF	***In vitro* fertilization**
IVM	***In vitro* maturation**
IVP	***In vitro* production**
KSR	**Knockout serum replacement**
LDs	**Lipid droplets**
MI	**Metaphase I**
MII	**Metaphase II**
mRNA	**Messenger ribonucleic acid**
mTBM	**Modified Tris-buffered medium**
NCSU	**North Carolina State University**
NFW	**Nuclease-free water**
PA	**Parthenogenetic activation**

PB	**Polar body**
PBS	**Phosphate-buffered saline**
PCO	**Polycystic ovary**
PCOS	**Polycystic ovary syndrome**
PDX1	**Insulin promoter factor 1**
PERVs	**Porcine endogenous retroviruses**
pFF	**Porcine follicular fluid**
PVA	**Polyvinyl alcohol**
PZM-5	**Porcine zygote medium-5**
RFP	**Red fluorescent protein**
RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
SCNT	**Somatic cell nuclear transfer**
SEM	**Standard error of the mean**
TALP	**Tyrode's albumin lactate pyruvate**
TCM	**Tissue culture medium**
TG	**Triglycerides**
ZP	**Zone pellucida**

PUBLICATION LISTS

PUBLICATION PAPERS (* is co-first author)

1. **Jin JX**, Lee S, Khoirinaya C, Oh A, Kim GA, Ahn C, Lee BC. Supplementation with spermine during *in vitro* maturation of porcine oocytes improves early embryonic development after parthenogenetic activation and somatic cell nuclear transfer. Journal of Animal Science. 2016;51:870-6.
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PART I

GENERAL INTRODUCTION

1. Literature review

1.1. The application of pigs

1.1.1. Swine industry for meat production

Pigs have played a vital role in meat production for human consumption in many countries, especially in Asia (Nagai et al., 2006). Global pork markets are getting more competitive in the wind of a bilateral free trade agreement. The ability to keep economical viability is a role of providing optimal genetics, nutrition, facilities, and health programs to pigs in a systematic environment that addressed the cost of generating revenue and production from a high-quality product marketing (Moeller and Crespo, 2009). The pigs industries of the world are very dynamic and continue to evolve and change along with the external forces that shape agriculture world-wide. The factors influencing change are generally related to economic efficiency and competition from alternative protein sources (Giamalva, 2014). In Asia, they produced more than 5% of the protein sources and 25% of the energy sources that human acquired from pigs in early 1990s, and the percentage of requirements are getting higher nowadays (McGlone, 2013).

China is the largest pork producer with almost 50% of the total production of the world. A rapid growing economy in China has offered the country with a higher purchasing power, it perhaps allowed the Chinese swine industry to dilate very rapidly over the past a decade. It is also expected that China will continue high level of pork consumption in the coming years (Pan and Kinsey, 2002). South Korea is 11^{th} in world pork production ranking and 5^{th} for pork consumption. The

swine industry represents 30% from the total (35.5%) livestock industry (Oh and Whitley, 2011). In addition, high level of pork production should be genetically selected by technology, for instance, myostatin knockout pig produces high muscle mass (Wang et al., 2015). Because genetic improvement has played a role in improving nearly every production efficiency trait evaluated in livestock, including pigs. The rapid evolution of gene technology allows swine breeders and commercial pork producers to make breeding decisions based on gene marker technology (Cleveland and Hickey, 2013).

1.1.2. Pig as a scientific animal resource

Research in pigs is important not only for agricultural purposes, but also for their use as models in biomedical studies (Matsunari and Nagashima, 2009). Recently, it is being increasingly considered that pigs use as biomedical research models is also gaining ground (Jin et al., 2013). Because pigs are considered as a suitable animal model for human disease based on their similar to those of human in terms of physiology and size, providing an excellent source of xenotransplantation organs (Matsunari and Nagashima, 2009).

Moreover, pigs have another better reason for research area; first, multipara characteristic is fit for large reproducibility in industrial areas. As advantage of pigs compared with other animal models, it is highly reproductive displaying early sexual maturity with 5~8 months, a short generation interval of 12 months, parturition of multiple piglets, an average of over 10 offspring per litter, and all season breeding (Wolf et al., 2000). Second, due to pigs are industrial animal, less

ethical problems are emerged when it compares with others (Camara et al.; Rollin, 2001). Those reasons are enough to promise that pigs are utilized as transgenic animal in research areas.

After the first porcine cloning technique was successfully reported (Polejaeva et al., 2000), considerable research have been accomplished to produce gene inserted pigs, disease model, and xenotransplantation models.

First, in case of the reporter gene inserted pigs were largely reported. These genetically engineered pigs with fluorescent protein are highly useful in research that includes the tracking of transplanted cells or tissue. Green fluorescent protein (GFP) or red fluorescent protein (RFP) inserted transgenic pigs were produced by several research groups (Hyun et al., 2003; Kurome et al., 2006; Lai et al., 2002c; Lee et al., 2005b; Levy et al., 1996; Lu et al., 2013; Naruse et al., 2005; Park et al., 2001; Webster et al., 2005; Whitelaw et al., 2004).

Second, disease model pigs were established, such as porcine retinitis pigmentosa disease (Petters et al., 1997), Huntington disease model (Uchida et al., 2001), cardiovascular disease model (Lai et al., 2006), Alzheimer's disease model (Kragh et al., 2009), cystic fibrosis (Welsh et al., 2009) and polycystic kidney disease (He et al., 2015).

Third, xenotransplantation models were also produced with gene modification. The greatest challenge facing the field of organ transplantation today is increasing the number of allograft available for transplant (Saidi and Hejazii Kenari, 2014). Organ transplantation has proven to be highly effective in the treatment of various forms of end-stage organ failure (Saidi and Hejazii Kenari, 2014). Herein, in order

to accomplish successful organ graft pigs to human, the organ rejection should be solved. Moreover, the donation after circulatory death and expanded criteria donors are decreasing and the patients who need organ transplantation are increasing. This situation, together with the serious shortage of human organs for transplantation, results in the need for the development of xenotransplantation. Pigs share many similarities with humans in body size, anatomy, diet and their physiological and pathophysiological responses. Elimination of alpha-1, 3-galactosyltransferase (GGTA1) from pig is expected to be a solution to the problem of hyperacute and delayed vascular rejection (Lai and Prather, 2002). The GGTA1 KO pigs were produced by somatic cell nuclear transfer (SCNT) in 2002 (Lai et al., 2002b). After that, several groups rapidly modified other genes for xenotransplantation research in pigs (Le Bas-Bernardet et al., 2011; Mohiuddin et al., 2012; Takahagi et al., 2005). However, clinical xenotransplantation will require multiple genes modified pigs transmitting effective genetic modifications in a non-segregating manner. Another concern that emerged was the existence of dormant porcine endogenous retroviruses (PERVs), which are integrated into the porcine genome. Church and his colleagues used CRISPR to delete a gene common to all 62 of the PERV sequences in human kidney cell (Yang et al., 2015). Knockout a single gene used to take years, now CRISPR system could knockout 62 in a single pig generation. Deleting PERV genes removes the risk of infection, and inserting human genes substantially reduces the problem of rejection. In addition, deleting PDX1 gene created a ‘chimeric’ pig embryo with insertion of human induced pluripotent stem

cell (iPS) that was part-pig and part-human (Wu et al., 2017). It is expected that the pig chimera could mature and provide a pancreas that is compatible to patients.

Taken together, as a scientific research source, pig models developed to enhance human health; it is necessary efficiently increases embryo production systems for pigs are required to achieve all these purposes.

1.2. Porcine *in vitro* maturation system

The combined interest in porcine biotechnologies by both pig industry and biomedical field creates an aggrandized desire for the development of new technologies as well as for the implementation of the existing ones. The main objective of these procedures in pigs is to increase reproductive efficiency and rates of genetic improvement, while maintaining genetic diversity. The improvement and development of reproductive technologies are focusing on gamete and their collection, artificial insemination (AI), cryopreservation of gametes and embryos, *in vitro* production (IVP) of embryos, embryo transfer, and manipulation of embryos (Niemann and Rath, 2001).

The IVP of porcine embryos has been of particular interest to researchers for ages, and it enables us to produce a large number of embryos with less cost and in less time compared to *in vivo* embryo production in pigs (Gil et al., 2010). It includes three major technological steps: (i) *in vitro* maturation (IVM) of immature oocytes obtained from ovaries, (ii) activation of matured oocytes and (iii) *in vitro* culture (IVC) of embryo. These three steps comprise a complex set of physiological processes, each one conditioning *per se* the success or failure of the next step. IVM of oocyte provides mature eggs that are able to be materials as recipient oocytes for other reproductive technologies such as intra-cytoplasmic sperm injection (ICSI), *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT). These technologies using *in vitro* matured oocytes are now expected to be used to produce transgenic pigs. Overall, IVM has particular significance, being the platform technology for the abundant supply of mature, good quality oocytes for

applications such as reducing the generation of time interval and interspecies SCNT in important species, research to improve *in vitro* human reproduction and production of transgenic animals for cell therapies, protein production and for medical applications.

The success of oocyte maturation involves crucial nuclear and cytoplasmic modifications in the oocyte. Nuclear maturation refers to the changes that occur during the resumption of meiosis to ensure a haploid complement of chromosomes results from the previously diploid state. Cytoplasmic maturation refers to the changes that are essential for successful fertilization and embryo development. Incomplete cytoplasmic maturation of the oocyte appears to account for the majority of problem with subsequent embryos development (Heikinheimo and Gibbons, 1998).

The timing of oocyte maturation is also similar between the human and pig, making pig IVM an ideal platform for development of human IVM technologies (Ellederova et al., 2004). Moreover, differences and similarities of embryology between human and pig are shown in Table 1. Oocytes recovered from ovaries collected at the slaughter house were on immature stated in both nuclear and cytoplasmic aspects. Thus, it is important for immature oocytes to be matured to conduct further developmental process and usage. Three decades ago, Motli and Fulka first reported the ability of *in vitro* matured porcine oocytes to be fertilized (Motli and Fulka, 1974). And the first successful IVF of IVM oocytes in pigs was made (Nagai et al., 1988). Although a great deal of progress has been made over the last decade, current in IVM systems still suffer from major problems, such as a

low rate of embryo development to the blastocyst stages and the low quality of embryos as compared with *in vivo* production in pigs. To advance both the medical and reproductive research that benefit from IVP, the IVM systems for porcine oocyte maturation must be improved.

Table 1. Differences and similarities between human and pig in embryology.

Characteristic	Human	Pig
Volume of the zygote, metabolic reserves	-	Similar to humans
Cytoplamic lipid content	Moderate	Extremely high
Embryo genome activation	4-8 cell stage	4-8 cell stage
Development to blastocyst	On day 5-6	On day 5-6
Amino acid metabolism	-	Similar to humans
Development anomalies after *in vitro* culture	Very rare	Very rare
Time and location of embryo transfer	Flexible	Flexible
Demethylation and methylation during early embryo development	Probably moderate	Moderate
Genome structure	-	Close to humans
Genome sequencing	Complete	Almost completed
Overall sensitivity *in vitro*	High	Extremely high
Pyruvate/lactate versus glucose	No absolute need for glucose before hatching	No absolute need for glucose before hatching

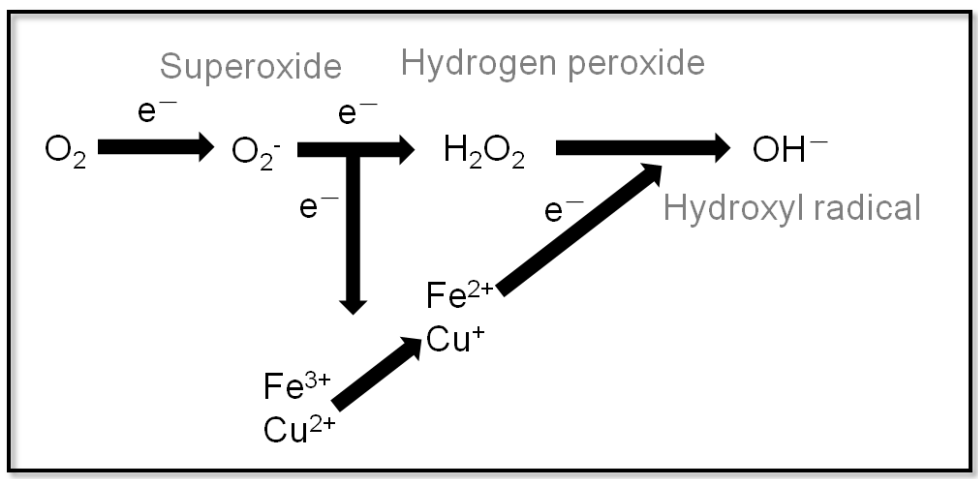
(Vajta et al., 2010)

1.3. Reactive oxygen species (ROS) and antioxidant

Intracellular and extracellular oxidative stress initiated by reactive oxygen species, which are generated in a wide range of normal physiology condition (Li et al., 2013). It means that ROS have important roles in regulating cell signaling and homeostasis in normal condition (Birben et al., 2012). ROS comprise both free radical and non-free radical oxygen containing molecules such as hydrogen peroxide (H_2O_2), superoxide ($\cdot O_2^-$) and the hydroxyl radical ($\cdot OH$) (Mates, 2000). There are also reactive nitrogen, iron, copper, and sulfur species which could attribute to increased ROS formation, oxidative stress and impair the redox balance (Mates, 2000). At low to moderate concentrations, they function in physiological cell processes, but at high concentrations, they produce adverse modification to cell components, such as lipid, protein and DNA (Blokhina et al., 2003). The oxidative stress also originates from embryo metabolism and/or embryo surroundings (Hu et al., 2014). The oxidative pathway is summarized in Figure 1. $\cdot O_2^-$ is produced, and is spontaneously converted to H_2O_2. The stable H_2O_2 permeates plasma membrane, and yields $\cdot OH$ in the presence of Fe^{2+} or Cu^+ through the Fenton reaction. High levels of ROS in the embryos might result in serious damage to cell membranes, nuclear and mitochondrial DNA of the cells, including apoptosis, DNA fragmentation, abnormal metabolism and aberrant gene expression (Guerin et al., 2001; Jimenez et al., 2003).

Oocytes and embryos seem to be protected against oxidative stress by oxygen scavengers that are present in follicular and oviductal fluids *in vivo* (Liu et al., 2017; Wang et al., 2002). Moreover, antioxidant enzymes (e.g. superoxide dismutase,

catalase, and glutathione peroxidase) are involved in cellular proliferation, amino acid, transport, DNA and protein syntheses and free radical scavenging in the oxidative microenvironment (Wang et al., 2002). However, when oocytes and embryos are removed from their natural environment for assisted reproduction techniques, this natural defense system is lost (Wang et al., 2002). In addition, the importance of protecting oocytes and embryos from oxidative stress *in vitro* is being increasingly recognized. It is necessary control the oxidative stress during *in vitro* culture. Therefore, numerous studies have been carried out to reduce ROS for producing porcine good quality oocytes and embryos using antioxidant treatment, such as spermine (Jin et al., 2016), quercetin (Kang et al., 2013), Ge-132 (Kim et al., 2015), 7,8-Dihydroxyflavone (Choi et al., 2013), zinc (Jeon et al., 2014), ascorbic acid (Kere et al., 2013), melatonin (Liang et al., 2017), canthaxanthin (Taweechaipaisankul et al., 2016) and vitamin C (Huang et al., 2011).



(Hu et al., 2014)

Figure 1. Among many ROS produced in the mammalian embryos, those that occur primarily by electrons transfer reactions.

1.4. Lipid metabolism

Energy metabolism is important for oocyte maturation because progression through all these dynamic processes requires large energy from numerous substrates including carbohydrates, amino acids, and lipids (Collado-Fernandez et al., 2012; Songsasen, 2012). To complete meiosis and to achieve full maturation, the oocyte requires energy from both carbohydrates and lipids (Downs and Mastropolo, 1994; Johnson et al., 2007). Glucose is considered the most important external energy source, mainly via glycolysis and pentose phosphate pathways, which provide the oocyte with directly usable oxidative substrates such as pyruvate and lactate for energy production (Cetica et al., 2002; Steeves and Gardner, 1999; Sutton-McDowall et al., 2010). Another important energy source for an oocyte may be lipid metabolism, it produces three times more than glucose (Sturmey et al., 2009).

Lipids are hydrophobic or amphipathic molecules with diverse biological roles that include being a rich source of energy, cell signaling mediators and the foundation of plasma and organelle membranes (Dunning et al., 2014b). Moreover, oocyte are renowned as large cells containing lipid stores and numerous studies have observed differences in the darkness of the oocyte cytoplasm, even in oocytes from the same ovary, and deemed it lipid (Dunning et al., 2014b). Oocyte intracellular lipids are mainly stored in lipid droplets (LDs), with the majority stored as neutral lipids (Sturmey et al., 2006), mainly triglyceride and cholesterol, which were threefold more abundant than phospholipids, but their function and importance are scarcely known (Homa and Brown, 1992; Prates et al., 2013). Pig

oocyte has the most triglyceride with ~74 ng per oocyte, about three times more than both cow and sheep, and also had the most total fatty acids (~160 ng per oocyte) (Dunning et al., 2014b).

Lipid droplets are organelles constituted primarily by triglyceride and cholesterol esters surrounded by monolayer of phospholipids with embedded integral and peripheral proteins, occupying a considerably mass in the eukaryotic cell, particularly in the mammalian oocytes and embryos (Fujihira et al., 2004; Walther and Farese, 2009; Zehmer et al., 2009). During the progress through oocytes meiotic maturation, the intracellular lipid (triglyceride) stores are decreased by lipolytic activity (Dunning et al., 2010). Lipolysis of intracellular triglyceride within lipid droplets is catalysed by intracellular lipases including hormone-sensitive lipase and adipocyte triglyceride lipase (Dunning et al., 2010). Intracellular triglyceride stored within lipid droplets is surrounded by a monolayer phospholipid and lipid droplet coat proteins of the perilipin family, which regulate droplet size and variously restrict access of intracellular lipases to the neutral core or promote lipolytic activity under the appropriate metabolic or hormonal conditions (Dunning et al., 2014b; Jin et al., 2017).

Energy from free fatty acids is produced via mitochondrial fatty acid β-oxidation. Carnitine palmitoyltransferases and their cofactor carnitine are critical for free fatty acid transport into mitochondria to undergo fatty acid β-oxidation pathway and thus to produce ATP (Bonnefont et al., 2004). Fatty acid β-oxidation pathway was shown to be indispensable for oocyte meiotic maturation and developmental competence in mice although oocyte lipid content in mice is

relatively low compared with farm animals (Downs et al., 2009; Dunning et al., 2010). In line with this, increasing fatty acid β-oxidation by supplementation of the culture medium with carnitine significantly improved oocyte developmental competence in terms of fertilization and embryo development in mice (Dunning et al., 2010), cow (Sutton-McDowall et al., 2012) and pigs (You et al., 2012). These are indicated that free fatty acids cleaved from triglyceride molecules stored in lipid droplets can be directly transported across the mitochondrial membrane and oxidized via β-oxidation that results in the production of acetyl CoA, a substrate for the TCA cycle (Sturmey et al., 2006).

1.5. Somatic cell nuclear transfer

Since the first mammal "Dolly", the sheep, was cloned from fully differentiated adult mammary cell by Wilmut in 1996 (Campbell et al., 1996), lots of species including, mouse (Wakayama et al., 1998), cattle (Cibelli et al., 1998), goat (Baguisi et al., 1999), pig (Polejaeva et al., 2000), gaur (Lanza et al., 2000), mouflon sheep (Loi et al., 2001), cat (Shin et al., 2002), rabbit (Chesne et al., 2002), deer (announcement, 2003), mule (Woods et al., 2003), rat (Zhou et al., 2003), horse (Galli et al., 2003), wildcat (Gómez et al., 2004), dog (Lee et al., 2005a), banteng (Sansinena et al., 2005), ferret (Li et al., 2006), swamp buffalo (Suteevun et al., 2006) and gray wolf (Kim et al., 2007) have been produced by SCNT (Table 2).

SCNT is a process by which animal are reproduced asexually. Application of SCNT is not confined to producing clones but has a variety of application. For instance, SCNT could be used to produce embryo-derived stem cells from a somatic cell by reprogramming somatic DNA (Tachibana et al., 2013). It can be a resource for regenerative medicine as patients with degenerative disease could potentially generate stem cells to cure their diseases. It can be also used to preserve valuable genetic backgrounds that may face extinction (Sherkow and Greely, 2013). Moreover, SCNT can be used to establish genetically engineered animals by using donor cells that have been modified (Lai et al., 2002a).

SCNT might be the best optional technology to establish cloned animals because of no existence of embryonic stem cells to date, especially in livestock.

Table 2. List of first cloned animals by somatic cell nuclear transfer.

Species	Country	First citation
Sheep	United Kingdom	(Campbell et al., 1996)
Mouse	United States	(Wakayama et al., 1998)
Cattle	United States	(Cibelli et al., 1998)
Goat	United States	(Baguisi et al., 1999)
Pig	United States	(Polejaeva et al., 2000)
Gaur	United States	(Lanza et al., 2000)
Mouflon sheep	Italy	(Loi et al., 2001)
Cat	United States	(Shin et al., 2002)
Rabbit	France	(Chesne et al., 2002)
Deer	United States	(Texas A&M announcement.,2003)
Mule	United States	(Woods et al., 2003)
Rat	France	(Zhou et al., 2003)
Horse	Italy	(Galli et al., 2003)
Wildcat	United States	(Gómez et al., 2004)
Dog	Korea	(Lee et al., 2005a)
Banteng	United States	(Sansinena et al., 2005)
Ferret	United States	(Li et al., 2006)
Swamp buffalo	United States	(Suteevun et al., 2006)
Gray wolf	Korea	(Kim et al., 2007)

2. General objective

The purpose of this study is to investigate the function of antioxidant and lipid metabolism during oocyte *in vitro* maturation influence their subsequent embryonic development in porcine. Thus this thesis is composed of 5 parts. In part I, as a general introduction, it was explained the reason of why this study have designed and performed. In part II, general methodology used in this study was described. In part III, I investigate the effect of antioxidants on nuclear maturation, intracellular levels of glutathione (GSH) and ROS in mature oocytes, embryonic development, and gene expression in oocytes, cumulus cells. In part IV, to determine the influence of lipid metabolism on porcine oocyte, I investigate the content of lipid droplets in mature oocytes; detect the several genes expression involved in lipogenesis, lipolysis, fatty acid β-oxidation and mitochondrial biogenesis after IVM; and also assess the content of fatty acid and ATP in porcine mature oocyte.

PART II

GENERAL METHODOLOGY

1. Chemicals and materials

All chemicals were obtained from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise stated.

2. Oocyte collection and *in vitro* maturation

Porcine ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory at 28-32 °C. The contents of follicles 3–6 mm in diameter were recovered by aspiration with an 18-gauge needle. Cumulus-oocyte complexes (COCs) were pooled and washed three times with tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA, USA), 2 mM of sodium bicarbonate, 10 mM N-(2-Hydroxyethyl) piperazine-N'- (2-ethanesulfonic acid) (HEPES), 5 mM sodium hydroxide, 1% Pen-Strep (Invitrogen), and 0.3% polyvinyl alcohol (PVA) (Table 3). Then, 50 COCs were placed into IVM medium comprising TCM-199 supplemented with 2 mM sodium pyruvate, 5 μl/mL insulin transferrin selenium solution (ITS) 100X (Invitrogen), 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 10% porcine follicular fluid (vol/vol), 10 IU/mL human chorionic gonadotropin (hCG), and 10 IU/mL equine chorionic gonadotropin (eCG) (Table 4). The selected COCs were incubated at 38.5 °C under 5% CO_2 in 95% humidified air for IVM. Following 22 h of maturation with hormones, the COCs were washed twice in fresh IVM medium and then cultured in hormone-free IVM medium for an additional 22 h.

Table 3. Components of porcine oocyte washing media

Component	Catalog No.*	M.W.**	Unit
Medium 199 powder***			1 pack/l
NaOH	S8045	40.00	2 tablets/l
$NaHCO_3$	S5761	84.01	2 mM
HEPES	H6147	238.31	10 mM
PVA****	P8136		0.30%
P/S*****			1%

* Sigma-Aldrich Co. LLC. (Missouri, USA)
** Molecular weight
*** Medium 199 powder (Invitrogen; 31100-035, California, USA)
**** Polyvinyl alcohol
***** Penicillin streptomycin (Invitrogen; 15070-063, California, USA)

Table 4. Components of porcine IVM media

Component	Catalog No.*	M.W.**	Unit
Medium 199 liquid***			500 ml
$NaHCO_3$	S5761	84.01	26.2 mM
Glucose	G7021	180.16	3.05 mM
Na pyruvate	P4562	110.04	0.91 mM
Ca lactate	L4388	103.10	2.92 mM
L-cystein	C7477	157.62	0.57 mM
Kanamycin	K1377	582.58	0.075 mM
pFF****			10% (v/v)
hCG*****			10 IU/mL
eCG******			10 IU/mL
ITS*******			5 µl/mL
EGF********			10 ng/mL

* Sigma-Aldrich Co. LLC. (Missouri, USA)

** Molecular weight

*** Medium 199 liquid (Invitrogen; 11150-059, California, USA)

**** Porcine follicular fluid

***** Human chorionic gonadotropin

****** Equine chorionic gonadotropin

******* Insulin transferrin selenium solution (Invitrogen; 41400045, California, USA)

******** Epidermal growth factor

3. Parthenogenetic activation (PA) of oocytes

The COCs were denuded with 0.1% hyaluronidase by pipetting and washed in Tyrode's albumin lactate pyruvate (TALP, Table 5) medium after 44 h of IVM. Denuded oocytes with homogeneous cytoplasm were selected and then gradually equilibrated in activation solution (Table 6) consisting of 0.28 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.5 mM HEPES. The oocytes were transferred to a chamber with two electrodes spaced 3.2 mm apart that was filled with activation solution and activated by electric stimulation with a single direct current (DC) pulse of 1.5 kV/cm for 60 μs utilizing a BXT Electro-Cell Manipulator 2001 (BXT Inc; San Diego, CA, USA). Activated oocytes were washed 3-4 times in porcine zygote medium-5 (PZM-5; Funakoshi Corporation, Tokyo, Japan) and transferred into wells containing 500 μL PZM-5 and cultured under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C for 7 days.

Table 5. Components of TALP media

Component	Catalog No.*	M.W.**	Unit
NaCl	S5886	58.44	114 mM
KCl	P5405	74.55	3.1 mM
$CaCl_2 \cdot 2H_2O$	C7902	147.02	2.1 mM
$MgCl_2 \cdot 6H_2O$	M2393	203.31	0.4 mM
$NaH_2PO_4 \cdot H_2O$	S3522	138.00	0.3 mM
$NaHCO_3$	S5761	84.01	2 mM
Na pyruvate	P4562	110.04	0.2 mM
Glucose	G7021	180.16	5 mM
HEPES	H6147	238.31	10 mM
Kanamycin	K1377	582.58	0.17 mM
PVP***	P0930	40.00	0.3%
BSA****	A6003		3 mg/ml
Na lactate	L7900	112.06	10 mM

* Sigma-Aldrich Co. LLC. (Missouri, USA)
** Molecular weight
*** Polyvinyl pyrrolidone
**** Bovine serum albumin (Fatty acid free, fraction V)

Table 6. Components of embryo activation media

Component	Catalog No. *	M.W. **	Unit
Mannitol	M1902	182.17	280 mM
$MgSO_4$	M2643	120.40	0.15 mM
PVA**	P8136	238.31	0.01% (w/v)
HEPES	H6147	238.30	0.5 mM
$CaCl_2$	C4901	111.00	0.05 mM

* Sigma-Aldrich Co. LLC. (Missouri, USA)
** Molecular weight
*** Polyvinyl alcohol

4. Donor cell preparation for SCNT

Porcine fibroblasts were isolated from ear tissue of an adult pig. The tissues were washed more than three times with phosphate-buffered saline (PBS; Life Technologies, Carlsbad, CA, USA), cut into small pieces with 0.25% trypsin/0.038% EDTA (Invitrogen), and then cultured at 38 ℃ in an atmosphere of 5% CO_2 in air in Dulbecco's modified Eagle's medium (DMEM; Gibco, culture medium) containing 10% fetal bovine serum (FBS; Gibco, culture medium) (v/v), 1 mM sodium pyruvate, and 1% of penicillin - streptomycin (Table 7). Cells from passages 3 and 7 were used as donors for SCNT. A single cell suspension was prepared by standard trypsinization procedures immediately before SCNT.

Table 7. Chemical information of primary culture and subculture for donor cell preparation

Component	Catalog No.*	M.W.**	Unit
PBS***	A20121		
0.25% trypsin/EDTA	25300054		
DMEM****	10938025		
FBS*****	12484028		10% (v/v)
P/S******			1%

* Sigma-Aldrich Co. LLC. (Missouri, USA)
** Molecular weight
*** Phosphate-buffered saline (Life Technologies, Carlsbad, CA, USA)
**** Dulbecco's modified Eagle's medium (Gibco, culture medium)
***** Fetal bovine serum (Gibco, culture medium)
******Penicillin streptomycin (Invitrogen; 15070-063, California, USA)

5. Somatic cell nuclear transfer

After IVM, COCs were denuded by gently pipetting with 0.1% hyaluronidase, and washed three times in Tyrode's albumin lactate pyruvate (TALP) medium. Denuded oocytes were incubated in TALP containing 5 μg/mL Hoechst 33342 for 10 min and observed under an inverted microscope equipped with epifluorescence. An oocyte was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. Enucleation was performed by aspirating the first polar body and adjacent cytoplasm containing the metaphase II chromosomes with an aspiration pipette in TALP medium containing 5 μg/mL cytochalasin B. A single donor cell was inserted into the perivitelline space of an enucleated oocyte, and the couplets were equilibrated in fusion solution (0.28 M mannitol solution containing 0.5 mM HEPES and 0.1 mM $MgSO_4$, Table 8.), and then fused in a 20 μL droplet of fusion solution with a single DC pulse of 1.2 kV/cm for 30 μs using an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). After 1 h, fused couplets were equilibrated with activation solution (0.28 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$), and then transferred to a chamber containing two electrodes overlaid with activation solution, and activated with a single DC pulse of 1.5 kV/cm for 30 μs using a BTX ElectroCell Manipulator 2001 (BTX Inc.). SCNT embryos were washed 3 times with fresh PZM-5, and transferred into 30 μL IVC droplets covered with mineral oil, and then cultured at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. To count total cell numbers of blastocysts, they were collected on Day 7, washed in Dulbecco’s

phosphate-buffered saline (DPBS; Invitrogen)-PVA (DPBS-PVA) and stained with 25 μg/mL of Hoechst 33342 for 10 min. After a final wash in DPBS-PVA, blastocysts were mounted on glass slides in a drop of 100% glycerol, compressed gently with a cover slip, and observed under a fluorescence microscope.

Table 8. Components of embryo fusion media

Component	Catalog No.*	M.W.**	Unit
Mannitol	M1902	182.17	280 mM
$MgSO_4$	M2643	120.40	0.1 mM
PVA***	P8136	238.31	0.01% (w/v)
HEPES	H6147	238.30	0.5 mM
$CaCl_2$	C4901	111.00	0.001 mM

* Sigma-Aldrich Co. LLC. (Missouri, USA)
** Molecular weight
*** Polyvinyl alcohol

6. Analysis of gene expression by quantitative real-time PCR

Total RNAs were extracted to analyze gene expression, using TRIzol reagent (Invitrogen), according to the manufacturer's protocol, and the total RNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was produced using amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT, Barker, TX, USA). A PCR plate (Micro-Amp Optical 96-Well Reaction Plate, Singapore) was made by adding 1 μL cDNA, 0.4 μL (10 pmol/μL) forward primer, 0.4 μL (10 pmol/μL) reverse primer, 10 μL SYBR Premix Ex Taq (TaKaRa, Otsu, Japan), and 8.2 μL of Nuclease-free water (NFW; Ambion, Austin, TX, USA) and then amplified on Applied Biosystems StepOneTM Real-Time PCR Systems (Applied Biosystems, Waltham, MA, USA). The amplification protocol included an initial denaturation step for 10 min at 95 ℃ followed by 40 cycles consisting of denaturation for 15 s at 95 ℃, annealing for 1 min at 60 ℃, and extension for 1 min at 72 ℃. The expression of each target gene was quantified relative to that of the internal control gene (GAPDH) using the equation, $R = 2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$. For ease of comparison, the average expression level of each gene from control group was set as 1.

PART III

ANTIOXIDANT REQUIREMENTS FOR PORCINE OOCYTES DURING *IN VITRO* MATURATION

Chapter I. Effects of spermine on porcine oocytes *in vitro* maturation and early embryonic development after parthenogenetic activation.

1. Introduction

To date, porcine SCNT has become a useful tool for basic biomedical research and xenotransplantation studies (Park et al., 2014b; Yeom et al., 2012). While the first successful SCNT was achieved more than a decade ago, mammalian embryos still have low *in vitro* developmental competence compared to those following *in vivo* development (Yang et al., 2007). Oocytes and embryos are vulnerable to adverse factors such as free radicals during IVM and IVC. It is well known that oxidative stress, mainly caused by reactive oxygen species (ROS), impairs IVP of porcine embryos (Choi et al., 2013). Consequently, antioxidants are required as a defence mechanism for cells to support their function in oxidative environments. Various antioxidants such as resveratrol (Lee et al., 2015), vitamin C (Hu et al., 2012), vitamin E (Tareq et al., 2012) and melatonin (Rodriguez-Osorio et al., 2007) have been added to IVM culture media to improve the capability of porcine oocytes to develop into preimplantation embryos. Spermine, known as polyamine, is a polybasic molecule ubiquitous in all living organisms and cells (Polticelli et al., 2012). It plays important roles in many cellular biochemical and physiological events including the regulation of transcription, modulation of kinase activities, functioning of protein synthesis, activity of ion channels (Pegg, 2014) and providing protection from oxidative damage in mammalian cells (Chattopadhyay et

al., 2006). However, none of the past studies elucidated the effects of spermine supplementation on porcine oocyte maturation and subsequent development of porcine parthenogenetic activation (PA) and SCNT embryos. Therefore, the current study was designed to investigate the effect of spermine on nuclear maturation, intracellular levels of GSH and ROS in mature oocytes, embryonic development after PA and SCNT, and gene expression in oocytes, cumulus cells and PA-derived blastocysts.

2. Materials and methods

2.1. Oocyte collection and *in vitro* maturation

Procedures for oocyte collection and *in vitro* maturation were described in general methodology.

2.2. Evaluation of porcine oocyte maturation

After 44h of IVM, cultured oocytes were denuded by gently pipetting with 0.1% hyaluronidase in TALP medium with HEPES buffer and then denuded oocytes were stained with 5 μg/mL of bisbenzimide (Hoechst 33342) in TALP-HEPES. The stained oocytes were evaluated using a fluorescence microscope (Nikon Corp., Tokyo, Japan). The experiment was repeated 3 times.

2.3. Measurement of intracellular ROS and GSH levels

Following IVM culture, oocytes at the metaphase II (MII) stage were sampled in medium supplemented with different concentrations of spermine or without it for determination of their intracellular ROS and GSH levels. Briefly, H_2DCFDA (2',7'-dichlorodihdrofluorescein diacetate; Invitrogen) and CellTracker Blue CMF2HC (4-chloromethy-6.8-difluoro-7-hydroxycoumarin; Invitrogen) were used to detect the intracellular ROS level as green fluorescence and the GSH level as blue fluorescence. Each treatment group was incubated (in the dark) for 30 min in DPBS-PVA containing 10 μM H_2DCFDA and 10 μM CellTracker Blue. After washing 3 times with DPBS, oocytes were placed into 4 μL droplets of TALP-

HEPES, and fluorescence was observed under an epifluorescence microscope (TE2000-S; Nikon) with UV filters (460 nm for ROS and 370 nm for GSH). Fluorescence intensities of the oocytes were analyzed using Image J software (Version 1.49q; National Institutes of Health, Bethesda, MD, USA) and normalized to control embryos.

2.4. Parthenogenetic activation of oocytes

Procedures for parthenogenetic activation of oocytes were described in general methodology.

2.5. Donor cell preparation

Procedures for donor cell preparation were described in general methodology.

2.6. Somatic cell nuclear transfer

Procedures for SCNT were described in general methodology.

2.7. Quantitative real-time PCR

Procedures for Quantitative real-time PCR were described in general methodology. All oligonucleotide primer sequences are presented in Table 9.

2.8. Statistical analysis

Each experiment was repeated at least 3 times. The data are expressed as the mean values ± standard error of the mean (SEM). The data were analyzed using

univariate analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software. Differences in gene expression and SCNT blastocyst rates were compared by Student's t-test. $P < 0.05$ was considered statistically significant.

Table 9. Primer sequences used for real-time PCR

Gene	Primer sequences (5'- 3')	Product size (bp)	GenBank accession number
GAPDH	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359
POU5F1	F: TTTGGGAAGGTGTTCAGCCAAACG R: TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060
FGFR2	F: ATTCTGGTGCCGGATGAAGAC R: GGTGTTGGAGTTCATGGAGG	121	NM_001099924
Bax	F: TGCCTCAGGATGCATCTACC R: AAGTAGAAAAGCGCGACCAC	199	XM_003127290
Bcl2	F: AGGGCATTCAGTGACCTGAC R: CGATCCGACTCACCAATACC	193	NM_214285

F, Forward primer; R, Reverse primer.

3. Results

3.1. Effect of spermine on nuclear maturation

In this part, I evaluated the effect of different concentrations (0, 10, 100, 500 and 1,000 μM) of spermine on oocyte nuclear maturation by measuring the rate of first polar body (PB) extrusion (Figure 2). A total of 469 oocytes were assessed in 3 replicates, and nuclear maturation rate ranged from 86.3% to 92.8%. However, there were no significant differences among the spermine treatment groups (Table 10).

3.2. Effect of spermine on intracellular levels of GSH and ROS

The results (Figure 3) showed that intracellular levels of GSH in spermine-treated oocytes were significantly higher (levels for the 10, 100 and 500 μM spermine groups were 1.28 ± 0.02, 1.23 ± 0.02 and 1.17± 0.03 pixels/oocyte, respectively) than that of the control group (1.00 ± 0.04). Consistent with this finding, levels of ROS were significantly lower in the spermine-treated oocytes (levels for the 10, 100 and 500 μM spermine groups were 0.70 ± 0.05, 0.80 ± 0.04 and 0.82 ± 0.05 pixels/oocyte, respectively) than that of the control group (1.00 ± 0.07 pixels/oocyte).

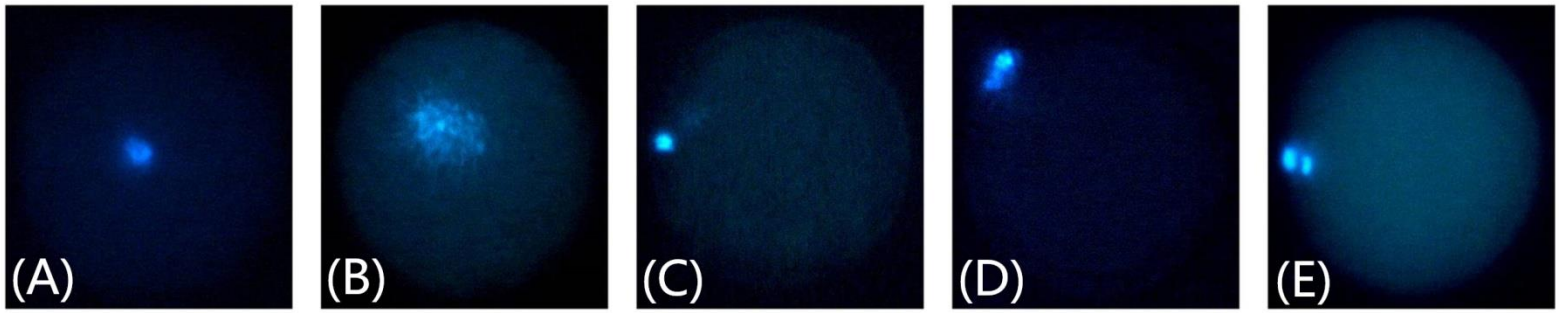


Figure 2. Chromatin configuration of porcine oocytes stained with Hoechst 33342 after 44 h of *in vitro* maturation. (A) Germinal vesicle, (B) Germinal vesicle breakdown, (C) Metaphase I, (D) Anaphase to Telophase I, (E) Metaphase II. Scale bar indicates 40 μm.

Table 10. Effect of spermine treatment during *in vitro* maturation (IVM) on nuclear maturation

Spermine concentration (μM)	No. of oocytes cultured for maturation[a]	No. (mean ± SEM, %) of oocytes at the stage of			
		GV - GVBD[b]	MI[c]	Ana - Telo[d]	MII[e]
0	88	0 (0.0 ± 0.0)	3 (3.5 ± 2.0)	4 (4.5 ± 2.9)	81 (92.1 ± 2.2)
10	98	0 (0.0 ± 0.0)	6 (5.7 ± 3.1)	2 (2.2 ± 1.1)	90 (92.1 ± 2.3)
100	84	0 (0.0 ± 0.0)	5 (5.7 ± 2.0)	1 (1.3 ± 1.3)	78 (93.0 ± 1.8)
500	97	0 (0.0 ± 0.0)	6 (6.3 ± 0.6)	6 (5.9 ± 3.6)	85 (87.8 ± 3.4)
1,000	102	2 (2.2 ± 2.2)	7 (6.7 ± 2.3)	5 (4.6 ± 4.6)	88 (86.5 ± 3.0)

[a] Experiment was replicated 3 times.

[b] GV - GVBD, Germinal vesicle - Germinal vesicle breakdown.

[c] MI, Metaphase I.

[d] Ana - Telo, Anaphase - Telophase I.

[e] MII, Metaphase II.

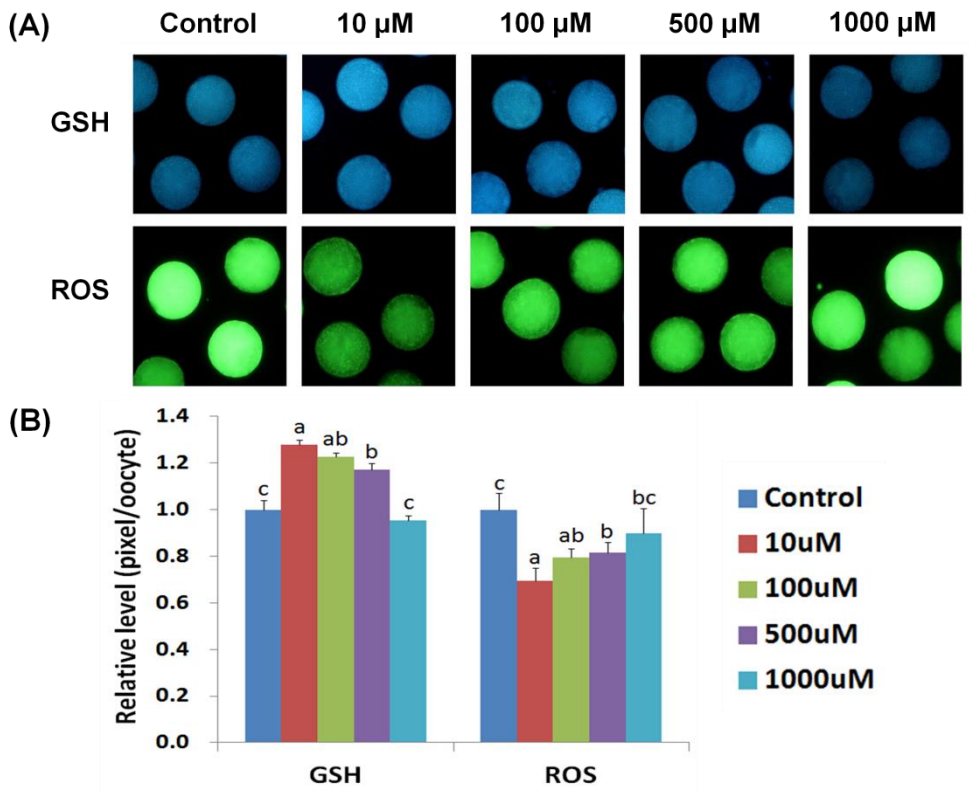


Figure 3. Epifluorescent photomicrographic images of *in vitro* matured porcine oocytes. **(A) Oocytes were stained with CellTracker Blue and H2DCFDA to detect intracellular levels of GSH and ROS, respectively. (B) Effect of Spm supplement during IVM on intracellular GSH and ROS levels *in vitro* matured porcine oocytes. Bars with different letters (a-c) represent significant differences (P < 0.05).**

3.3. Effect of spermine in IVM on embryonic development after PA and SCNT

I examined the effect of spermine treatment during IVM on their subsequent embryonic developmental competence of PA embryos. As shown in Table 11, the rate of blastocyst formation was significantly higher in the 10 μM spermine-treated group than in the control group (27.0% *vs.* 17.6%). However, spermine treatment (10 μM) did not affect cleavage rate (82.2% *vs.* 78.0%) and total cell numbers per blastocyst (51.6 ± 2.3 *vs.* 56.2 ± 8.2) in PA embryos.

According to the optimal concentration shown in Table 11, oocytes were treated with 10 μM spermine during IVM and subsequent development of SCNT embryos was compared with the control group. Table 12 shows a significant increase in blastocyst formation and total cell numbers in the treatment group compared with the control group (19.9% and 58.0 ± 14.3 vs. 11.0% and 41.2 ± 8.0, respectively).

3.4. Gene expression in mature oocytes, cumulus cells and PA-derived blastocysts after IVM with spermine

I evaluated the effect of spermine on *POU5F1*, *FGFR2*, *Bax*, and *Bcl2* gene expression in mature oocytes, cumulus cells and PA-derived blastocysts. As shown in Figure 4, 10 μM spermine increased *FGFR2* mRNA transcript levels significantly in oocytes, but not in cumulus cells. In cumulus cells, mRNA transcript levels of *Bax* and *Bcl2* were significantly different in the 10 μM spermine treatment group compared with the control group. Transcript levels for *POU5F1*

and *Bcl2* were significantly higher in PA blastocysts derived from 10 μM spermine-treated oocytes than in the control group.

Table 11. Effect of spermine supplementation during in vitro maturation (IVM) on embryonic development after parthenogenetic activation (PA).

Spermine concentration (μM)	No. of embryos cultured*	No. of embryos developed to (mean ± SEM, %)				Total cell no. (mean ± SEM) in blastocysts
		≥ 2-cells		Blastocyst		
0	165	127	(77.4 ± 3.3)	29	(17.9 ± 3.0)[b]	56.2 ± 8.2
10	174	143	(82.1 ± 2.0)	47	(27.2 ± 1.6)[a]	51.6 ± 2.3
100	173	139	(79.6 ± 3.4)	43	(24.4 ± 2.6)[a,b]	48.8 ± 4.2
500	176	141	(79.7 ± 3.2)	34	(19.1 ± 2.8)[a,b]	51.3 ± 6.1
1,000	164	111	(67.6 ± 8.4)	28	(17.7 ± 3.1)[b]	52.7 ± 5.6

* Experiment was replicated 6 times.

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

Table 12. Effect of spermine treatment during *in vitro* maturation (IVM) on embryonic development after somatic cell nuclear transfer (SCNT)

Spermine concentration (μM)	No. of embryos cultured*	No. of embryos developed to (mean ± SEM, %)				Total cell no. (mean ± SEM) in blastocysts
		≥ 2-cells		Blastocyst		
0	209	163	(79.6 ± 12.4)	23	(11.0 ± 1.0)[b]	41.2 ± 8.0[b]
10	196	151	(77.7 ± 16.0)	39	(20.2 ± 3.4)[a]	58.0 ± 14.3[a]

* Experiment was replicated 3 times.

[a,b] Values with different superscripts in the same column are significantly different ($P < 0.05$)

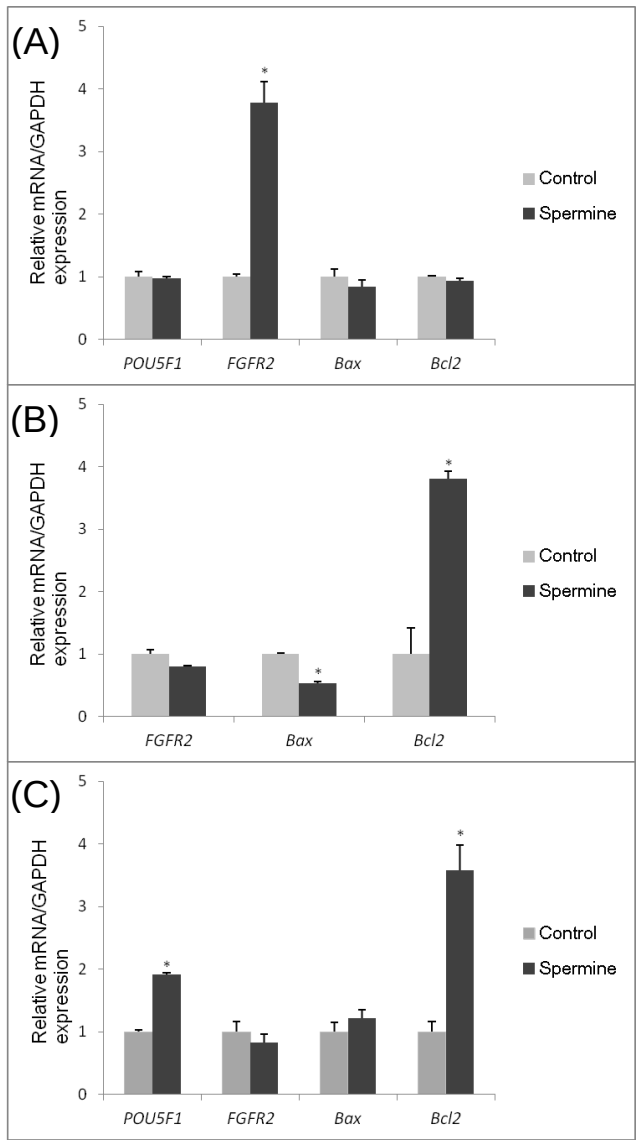


Figure 4. The mRNA expression levels (mean ± SEM) of *POU5F1*, *FGFR2*, *Bax*, and *Bcl2* by spermine treatment. Mature oocytes (A), cumulus cells (B), PA-derived blastocyst (C) after 10 μM spermine supplementation during *in vitro* maturation. Within the same mRNA, bars with asterisk are significantly different ($P < 0.05$). The experiment was replicated at least 3 times.

4. Discussion

Spermine belongs to polyamine and exerts various biological activities, including DNA synthesis, modulation of intracellular signal pathways, cell proliferation, and differentiation (Porat and Clark, 1990). As spermine play antioxidant function by direct scavenging of ROS, this is very active area with biochemical and physiological studies using mammals, plant, protozoan parasites, microorganisms and anti-inflammatory agents (Lovaas, 1995; Pegg, 2009). This is substantiated by the rapid induction of ornithine decarboxylase (DOC) by oxidative stress. Spermine is contained in the epidermis at high levels with a 30 times stronger antioxidant effect than vitamin E, and thereby delays skin aging by 20 % (Lovaas, 1995). Therefore, it is one of the natural antioxidant cosmetic ingredients in anti-aging creams. As a natural antioxidant, spermine added to blood preservative solutions provides better red blood cell storage and "survival" (Kucherenko and Bernhardt, 2015). In addition, a decrease in spermine directly influences embryonic development or embryonic cell blocks at early stages in mice (Nishimura et al., 2002).

In this study, I demonstrated that spermine treatment during IVM had a beneficial effect on cytoplasmic maturation and subsequent development of PA and SCNT embryos. Supplementation with 10 μM spermine during IVM increased GSH levels and decreased ROS levels in mature oocytes. Furthermore, 10 μM spermine treatments significantly improved subsequent *in vitro* development of PA

and SCNT embryos. Expression of some genes was also positively changed in mature oocytes, cumulus cells and PA blastocyst.

Intracellular GSH is one of the major antioxidants, and it plays a pivotal role in maintaining redox homeostasis, scavenging peroxides and detoxifying xenobiotics (Hayes et al., 2005). ROS plays an important role in modulating an entire spectrum of events in reproductive physiology, such as oocyte maturation, fertilization, embryo development and pregnancy (Agarwal et al., 2005). However, it is generally accepted that high levels of ROS have multiple adverse effects on mitochondria and nuclei, and can cause oxidative stress, change the membrane lipid composition, decrease the cellular concentration of ascorbic acid and the ratio of intracellular GSH/GSH disulfide (Gardiner et al., 1998; Tarin et al., 2002). Intracellular GSH is used as a molecular marker that predicts cytoplasmic maturation in porcine oocytes. Therefore, I speculated that the influence on GSH might result from changes in intracellular ROS levels in porcine oocytes. In my experiment, after culturing COCs in maturation media supplemented with spermine, although there were no significant differences in oocyte maturation rates, the levels of GSH with addition of 10 μM spermine was higher than that in other groups, and expression of ROS was decreased.

To investigate the effect of spermine on development and apoptosis of oocytes and cumulus cells, I analyzed gene expression of *POU5F1*, *FGFR2*, *Bax*, and *Bcl2*. Transcription factor *POU5F1* is an essential gene for early development that is overexpressed in good-quality oocytes and oocyte-derived blastocysts (Kwak et al., 2012). *FGFR2* is primary receptor partner for oocyte competent factor, such as

FGF10 and *FGF7* and its signaling is involved in regulating oocyte maturation, cumulus expansion and subsequent embryonic development (Zhang et al., 2010). My study showed that *FGFR2* gene expression of the 10 μM spermine treatment group was significantly higher than the control group of mature oocytes. *Bax* is a pro-apoptosis gene and *Bcl2* is an anti-apoptosis gene (Lowthert et al., 2012). *Bax* expression was reduced significantly, and *Bcl2* expression was significantly higher in cumulus cells derived from the 10 μM spermine-treated group. These results showed that spermine treatment reduced apoptosis in cumulus cells.

The beneficial effects of spermine as an antioxidant also contribute to embryo developmental competence, and this was reflected in blastocyst formation rates. The addition of 10 μM spermine to IVM medium increased blastocyst rates significantly after PA, but not in high concentration (500-1,000 μM). It is consistent with the previous study showing that relatively high concentration of exogenous spermine may induce cell death (Brunton et al., 1990). Therefore, with all the results, the optimal concentration for porcine oocyte maturation and embryo development was established as 10 μM. According to a previous study, spermine addition to the IVC medium did not enhance embryo developmental competence or the total number of nuclei after PA. The 10 μM concentration of polyamines in IVC reduced the percentage of blastocysts developing after PA (Cui and Kim, 2005). However, based on my results, 10 μM spermine treatments during IVM not only significantly improved blastocyst formation rates after both PA and SCNT, but also significantly increased the total cell numbers in SCNT blastocysts. Moreover, *POU5F1* expression was significantly increased in PA blastocysts after

10 μM spermine supplementation in IVM. The *Bcl2* anti-apoptotic transcript expression in blastocysts from the 10 μM spermine treatment group was also upregulated. Therefore, upregulated *POU5F1* and *Bcl2* expression was reflected in increased blastocyst formation of PA embryos. my results are consistent with previous studies showing that spermine could enhance the development of IVF embryos (Porat and Clark, 1990) and may act to prevent the generation of free radicals soon after IVF and protect the embryos from oxidative damage at the early stage (Lovaas and Carlin, 1991; Natsuyama et al., 1992).

The present study focused on the application of optimal conditions with spermine treatment during oocyte IVM on the *in vitro* development of porcine embryos. Based on my findings, I propose that treatment of porcine oocytes with 10 μM spermine has a beneficial effect on preimplantation development leading to enhanced PA and SCNT blastocyst formation rates by increasing the intracellular GSH levels, decreasing the ROS levels, and regulating gene expression related to development (*POU5F1* and *FGFR2*) and apoptosis (*Bax* and *Bcl2*). In the future, in order to use spermine routinely for the enhancement of *in vitro* conditions for mammalian oocytes and embryos, additional experiments such as, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for detecting DNA fragmentation and Western blotting assay of apoptosis-regulating proteins are required..

Chapter II. Antioxidant property of knockout serum replacement improves porcine oocyte maturation and their embryonic development.

1. Introduction

Up to now, due to physiological similarities of porcine oocyte to those of humans, researchers were particularly interested in improving the production of large quantities of porcine oocytes through IVP system (Prather et al., 2003). Although various researches for IVM of porcine oocytes have been simulated *in vivo* environment, their developmental competence is still lower compared to *in vivo* production (Abeydeera et al., 1998; Hong et al., 2004; Kishida et al., 2004). It is well known that two principal factors influence ability of oocyte and subsequent developmental competence to undergo IVM in many species is protein and hormonal supplements (Lonergan et al., 2003; Zheng and Sirard, 1992).

In general, porcine oocyte has been obtained from IVM by using North Carolina State University (NCSU) or TCM 199 solutions supplemented with 10% porcine follicular fluid (pFF) (Jin et al., 2016; Jin et al., 2013). The pFF contains a number of essential components such as hormones (Sluss and Reichert, 1984; Sluss et al., 1987), vitamins (Chew et al., 1984; Schweigert and Zucker, 1988), transport proteins (Daen et al., 1994) and attachment, spreading and growth factors (Oberlender et al., 2013; Reed et al., 1993). However, there are considerations during pFF supplemented to IVM. In the first place, pFF is considered a potential source of infectious agents (Sur et al., 2001). There is one more point, due to the

pFF is not available commercially, preparation of pFF from the abattoir-derived ovaries required time and effort, and there is substantial variation depends on batch; it may influence the efficiency of IVP (Suzuki et al., 2006). The last but not the least, pFF introduces a series of unknown factors to the medium, which cause difficulties in technical standardization and in the exact identification of the substances that are essential to the regulation of maturation (Yoshida et al., 1992). Therefore, I need further modification for new defined system.

Researchers have been developing new maturation media supplemented with FBS, which is commercially available potential protein source. The results suggested that porcine IVM supplemented with FBS reduced the maturational competence of oocyte, but once oocytes have matured, they have the same ability to develop to full term (Suzuki et al., 2006). Nevertheless, composition of FBS is not completely known.

Knockout serum replacement (KSR) is a protein source generally used for various applications of embryonic stem cells (ESCs), which directly replaces FBS in existing protocols (Sakurai et al., 2015). KSR was consisted by small organic molecules, trace elements, and three proteins, namely insulin, transferring, and lipid-rich albumin, but it does not contain any undefined growth factors or differentiation-promoting factors (Sakurai et al., 2015). To avoid the undefined components of FBS and pFF supplement to porcine IVM system, KSR may be a suitable supplement to replace FBS and pFF.

In addition, follicular fluid has antioxidant property (Park et al., 2014a). Therefore, the purpose of this study was to (i) conform KSR has antioxidant

property; (ii) determine the influence of KSR on cumulus expansion, oocyte maturation and embryo development after parthenogenetic activation; (iii) investigate the expression of genes involved in cumulus expansion and oocyte maturation; (iv) measure the fluorescence staining intensity of GDF9, BMP15, GSH and ROS in porcine oocytes.

2. Materials and methods

2.1. Oocyte collection and *in vitro* maturation

Procedures for oocyte collection and *in vitro* maturation were described in general methodology.

2.2. Cumulus expansion assessment

A degree of 0 indicated no expansion, characterized by detachment of cumulus cells from the oocyte to assume a flattened monolayer of fibroblastic appearance, leaving a partially or fully denuded oocyte. A degree of 1 indicated no expansion but cumulus cells are spherical, and remain compacted around the oocyte. For degree 2 complexes, only the outermost layers of cumulus cells have expanded, while degree 3 complexes have all cell layers except the corona radiata (cells most proximal to the oocyte) prominently expanded, and a degree of 4 indicated the maximum degree of expansion including the corona radiata. These values were expressed as the cumulus expansion index (CEI) (Vanderhyden et al., 1990).

2.3. Assessment of nuclear maturation

After 44 h of IVM, oocytes were sampled to analyze nuclear maturation. Samples of oocytes were denuded by gently pipetting with 0.1% hyaluronidase in TALP medium and washed three times in TALP medium. The denuded oocytes were evaluated with a microscope (TE2000-S, Nikon, Tokyo, Japan) and classified

as immature (without polar body extrusion), degenerate, or at metaphase II (MII; with first polar body extrusion).

2.4. Parthenogenetic activation

Procedures for parthenogenetic activation of oocytes were described in general methodology.

2.5. Measurement of intracellular GSH and ROS levels

Following IVM culture, oocytes at the metaphase II (MII) stage were sampled in medium supplemented with different concentrations of spermine or without it for determination of their intracellular ROS and GSH levels. Briefly, H_2DCFDA (2',7'-dichlorodihdrofluorescein diacetate; Invitrogen) and CellTracker Blue CMF2HC (4-chloromethy-6.8-difluoro-7-hydroxycoumarin; Invitrogen) were used to detect the intracellular ROS level as green fluorescence and the GSH level as blue fluorescence. Each treatment group was incubated (in the dark) for 30 min in DPBS-PVA containing 10 μM H_2DCFDA and 10 μM CellTracker Blue. After washing 3 times with DPBS, oocytes were placed into 4 μL droplets of TALP-HEPES, and fluorescence was observed under an epifluorescence microscope (TE2000-S; Nikon) with UV filters (460 nm for ROS and 370 nm for GSH). Fluorescence intensities of the oocytes were analyzed using Image J software (Version 1.49q; National Institutes of Health, Bethesda, MD, USA) and normalized to control embryos.

2.6. Immunofluorescence staining of oocytes

Porcine oocytes were washed three times in PBS containing 0.2% PVA, and fixed with 4% paraformaldehyde (w/v) in PBS for 30 min. All steps were performed at room temperature unless otherwise stated. Oocytes were transferred into PBS containing 1% Triton X-100 (v/v) for 30 min. After blocking nonspecific sites with 2% bovine serum albumin (BSA) in PBS overnight at 4°C, oocytes were incubated with primary antibodies (rabbit polyclonal antibody against GDF9 (ab93892, Abcam, City, Country) and BMP15 (PA5-34401, Invitrogen) diluted 1:200,) at 37°C for 3 h. Then, a goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was applied for 3 h at room temperature. Stained oocytes were then mounted on glass slides and evaluated under an epifluorescence microscope (TE2000-S; Nikon) with the same exposure times and adjustments. The intensities of GDF9 and BMP15 (green) were measured by analyzing the oocyte pictures with Image J software (version 1.46r; National Institutes of Health, USA).

2.7. Analysis of gene expression by quantitative real-time PCR

Procedures for Quantitative real-time PCR were described in general methodology. All oligonucleotide primer sequences are presented in Table 13.

2.8. Statistical analysis

All experiments were performed with a minimum of three independent replicates. Data are reported as the mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance followed by Tukey's *post hoc* test using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software. Differences were considered statistically significant if $P < 0.05$.

Table 13. Primer sequences lists

Gene	Primer sequences (5'- 3')	Product size (bp)	GenBank accession number
GAPDH	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359
Ptgs1	F: CAACACGGCACACGACTACA R: CTGCTTCTTCCCTTTGGTCC	121	XM_001926129
Ptgs2	F: ACAGGGCCATGGGGTGGACT R: CCACGGCAAAGCGGAGGTGT	194	NM_214321
Ptx-3	F: GGCCAGGGATGAATTTTAC R: GCTATCCTCTCCAACAAGTGA	185	NM_001244783
Has2	F:AGTTTATGGGCAGCCAATGTAGTT R: GCACTTGGACCGAGCTGTGT	101	AB050389
Tnfaip6	F: AGAAGCGAAAGATGGGATGCT R: CATTTGGGAAGCCTGGAGATT	106	NM_001159607
GDF9	F: CAGTCAGCTGAAGTGGGACA R: TGGATGATGTTCTGCACCAT	135	AY626786
BMP15	F: CCTCCATCCTTTCCAAGTCA R: GTGTAGTACCCGAGGGCAGA	112	NM_001005155
Cyclin B1	F: CAACTGGTTGGTGTCACTGC R: TTCCATCTGCCTGATTTGGT	126	L48205
C-Mos	F: GGGAGCAACTGAACTTGGAG R: AGAATGTTCGCTGGCTTCAG	115	NM_001113219
Cdc2	F: GGGCACTCCCAATAATGAAGT R: GTTCTTGATACAACGTGTGGGAA	260	AB045783
Bax	F: TGCCTCAGGATGCATCTACC R: AAGTAGAAAAGCGCGACCAC	199	XM_003127290

F, Forward primer; R, Reverse primer.

3. Results

3.1. Cumulus cell expansion index and expression of genes responsible for cumulus cell expansion

I evaluated the effects of 5%/10% KSR or 10% pFF supplements during IVM on cumulus cell expansion, and genes responsible for cumulus cell expansion (*Ptgs1, Ptgs2, Ptx-3, Has2* and *Tnfaip6*) and apoptosis (*Bax*/*Bcl2* ratio). Although the cumulus cell expansion values after 22 h IVM were not different among the groups (Figure 5), the highest expression of CEI was 10% pFF (3.73), and the CEI in the control group was significantly increased compared to the 5% KSR and 10% KSR (3.17 vs. 2.48 and 2.51, $P < 0.05$, Table 14) after 44 h IVM. In addition, transcription of cumulus expansion related genes (*Ptgs1, Ptgs2, Ptx-3, Has2*, and *Tnfaip6*) as the lowest in the control group (Figure 6A). Moreover, the *Bax*/*Bcl2* ratio was progressively reduced from the control to 10% pFF, 10% KSR and 5% KSR ($P < 0.05$).

3.2. Oocyte maturation rate and genes responsible for oocyte development

After 44 h of IVM, the rate of oocyte maturation was significantly lower in the control group compared to the 5% or 10% KSR and 10% pFF groups (75.7% vs. 86.0, 81.7% and 83.2%, respectively, $P < 0.05$, Figure 7). Additionally, I examined the expression of genes involved in oocyte development (*GDF9, BMP15, Cyclin B1, C-Mos* and *Cdc2*, Figure 6B). Expression of oocyte competence genes (*GDF9* and *BMP15*) was significantly increased in 5% KSR and 10% pFF than in other

groups ($P < 0.05$). Moreover, *Cyclin B1* and *C-Mos* showed the lowest expression in 10% KSR, and *Cdc2* was significantly increased in 5% KSR compared with other groups ($P < 0.05$). In the case of the *Bax*/*Bcl2* ratio, there was progressively decreased gene expression from the control to 10% KSR, 5% KSR and 10% pFF ($P < 0.05$).

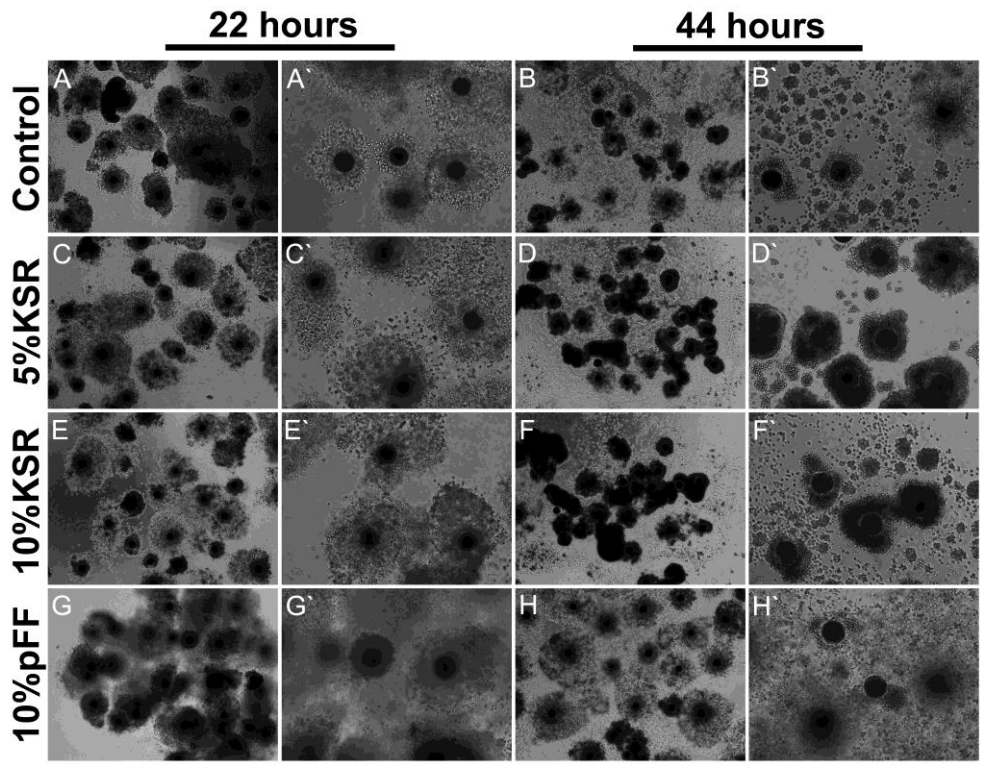


Figure 5. Cumulus expansion in cumulus oocyte-complexes (COCs) after 22 and 44 h of IVM culture. (A-B`) Control group; (C-D`) 5% KSR group; (E-F`) 10% KSR group; (G-H`) 10% pFF group. (A-H) Original magnification ×100; (A`-H`) Original magnification ×200.

Table 14. Evaluation of cumulus cell expansion in cumulus-oocyte complexes (COCs) after 44 h of *in vitro* maturation

Group	COCs maturated *in vitro* (n)	Degree of cumulus expansion					
		0	+1	+2	+3	+4	Cumulus cell expansion index
Control	187	0	5	27	87	68	3.17^{b}
5% KSR	189	0	21	73	78	17	2.48^{c}
10% KSR	190	0	24	69	73	24	2.51^{c}
10% pFF	186	0	0	9	32	145	3.73^{a}

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$). The experiment was replicated four times.

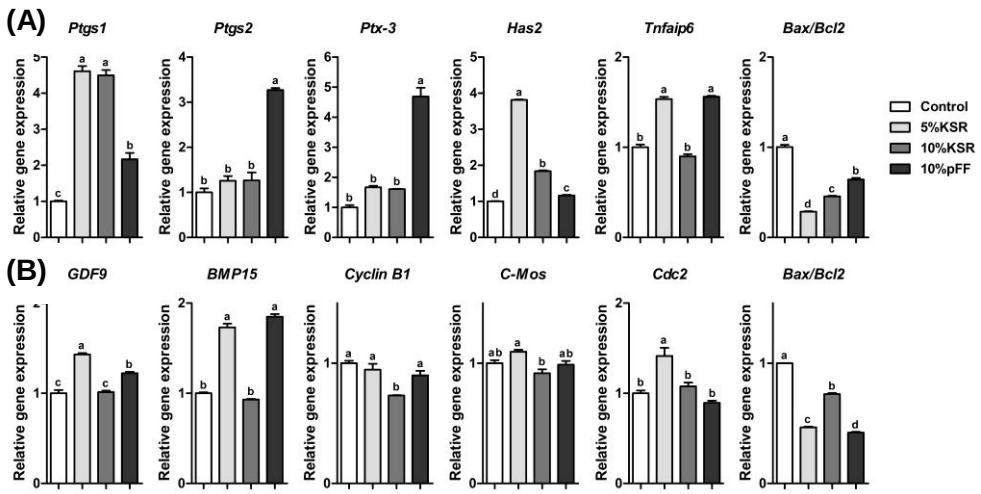


Figure 6. Expression of genes related to cumulus expansion, oocyte development and apoptosis. (A) Cumulus expansion and apoptosis factors in cumulus cells (*Ptgs1, Ptgs2, Ptx-3, Has2, Tnfaip6 and Bax*/*Bcl2*). (B) Oocyte development and apoptosis factors in oocytes (*GDF9, BMP15, Cyclin B1, C-Mos, Cdc2 and Bax*/*Bcl2*). Within the same mRNA, bars with different letters denote significant differences ($P < 0.05$). The experiment was replicated at least three times

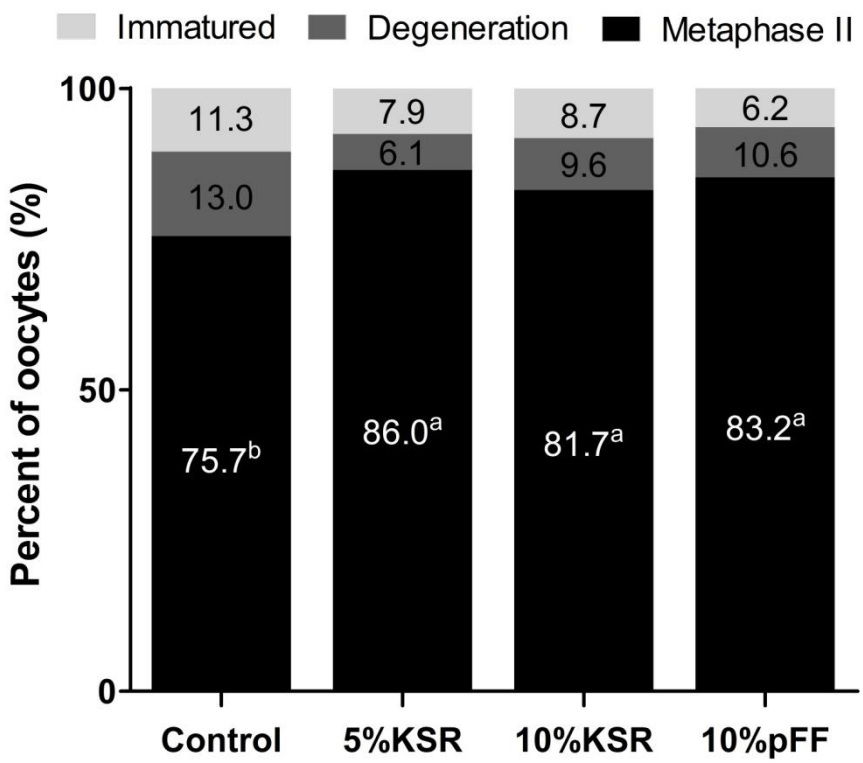


Figure 7. Effect of different types of supplementation on nuclear maturation of porcine oocytes. Bars with different letters denote significant differences ($P < 0.05$). The experiment was replicated four times.

3.3. Fluorescence intensity of GDF9, BMP15, GSH and ROS in oocytes

In concordance with the expression of oocyte competence genes (*GDF9* and *BMP15*), I found that protein levels of GDF9 and BMP15 showed similar patterns to expression of their respective genes, but there were significant differences between the control group and the other groups ($P < 0.05$). Using GSH as an oocyte quality marker, the levels of fluorescence were directly proportional to GDF9 and BMP15 levels. In contrast, ROS levels were directly inverse proportional to GSH levels (Figure 8).

3.4. Development embryos derived from parthenogenetic activation

After PA (Table 15), there was no significant difference in the cleavage rate among the groups. Blastocyst formation rates progressively increased from the control to 10% KSR, 10% pFF and 5% KSR groups (27.9 ± 1.1%, 28.7 ± 1.3%, 35.1 ± 0.7% and 41.0 ± 1.2%, respectively, $P < 0.05$). The total cell numbers in blastocysts significantly increased in the 5% KSR compared to the control group (64.9 ± 7.3 vs. 46.3 ± 2.4, $P < 0.05$). According to these results, the 5% KSR and 10% pFF treatments were chosen for comparison to the control in the following experiments

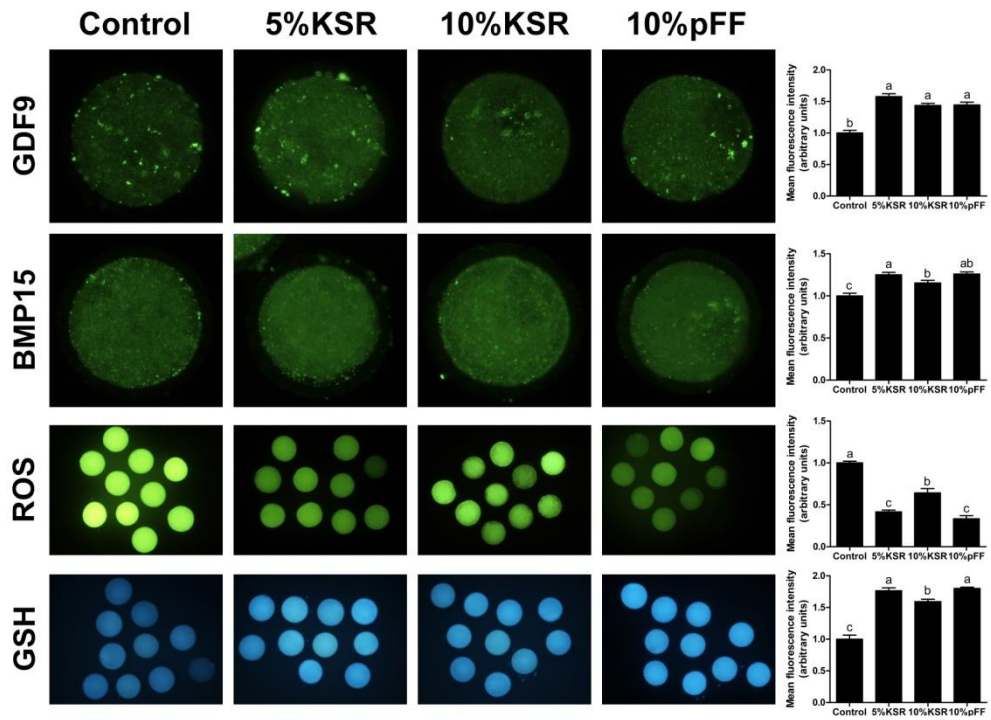


Figure 8. Analysis of GDF9, BMP15, GSH and ROS expression by fluorescence staining in porcine oocytes. Bars with different letters denote significant differences ($P < 0.05$). The experiment was replicated at least three times.

Table 15. Effect of different supplementation during *in vitro* maturation (IVM) on embryonic development after parthenogenetic activation (PA)

Group	No. of embryos cultured	No. of embryos developed to (mean ± SEM, %)		Total cell no. (mean ± SEM) in blastocysts
		≥ 2-cells	Blastocyst*	
Control	198	169 (85.8 ± 1.7)	56 (27.9 ± 1.1)^{c}	46.3 ± 2.4^{b}
5%KSR	203	177 (87.3 ± 2.0)	84 (41.0 ± 1.2)^{a}	64.9 ± 7.3^{a}
10%KSR	191	164 (86.2 ± 1.5)	54 (28.7 ± 1.3)^{c}	47.5 ± 3.0^{ab}
10%pFF	193	165 (86.2 ± 0.8)	68 (35.1 ± 0.7)^{b}	55.7 ± 3.8^{ab}

Values with different superscript letters within a column differ significantly ($P < 0.05$). Experiment was replicated six times.
The data represent means ± SEM. * Percentage of total cultured oocytes.

4. Discussion

My study demonstrated that supplementation of IVM medium with 5% KSR significantly enhanced oocyte maturation and embryo development up to blastocyst in PA when compared to the unsupplemented control. Moreover, the 5% KSR supplemented group significantly increased the intensity of GDF9, BMP15, GSH and decreased ROS compared to the unsupplemented control, and these results exhibited similar patterns to the 10% pFF supplemented group.

The present study exhibited that supplementation with KSR during IVM increased the tight compaction of cumulus cells, the migration of neighbor cells into a mass, or attachment of cumulus cells to the bottom of the dish, which was in line with supplementation of IVM medium using FBS (Moon et al., 2009; Suzuki et al., 2006). In contrast, good cumulus cell expansion was observed in the pFF supplement group. Even though the CEI with KSR supplementation was lower than that of the unsupplemented control and the pFF supplemented groups, there was significantly increased expression of *Ptgs1, Has2* and *Tnfaip6*, but not of *Ptgs2* and *Ptx-3*.

Oocyte maturation is a crucial step for the generation of good quality oocytes capable of being fertilized and undergoing normal embryonic development into blastocysts (Wang et al., 1997). Supplementation of IVM medium with FBS reduced the level of oocyte maturation (Suzuki et al., 2006). In contrast, in this study, the rates of oocyte maturation with 5% KSR were consistently over 85%, similar to the percentage obtained with 10% pFF supplementation. The mRNA

transcript levels of *GDF9*, *BMP15* and *Cdc2* were higher in the 5% KSR group than in the unsupplemented control, and these results are corroborated by the high protein level of GDF9 and BMP15. Moreover, there was a lower *Bax*/*Bcl2* expression ratio in cumulus cells and oocytes using 5% KSR, which supported higher GSH expression in oocytes. In the case of embryo development after PA, the 5% KSR and 10% pFF supplemented groups all showed higher blastocyst formation rates than the unsupplemented control group. These data supported the assumption that supplementation with 5% KSR was associated with oocyte maturation and apoptotic genes, and the intensities of GDF9, BMP15, GSH and ROS. These results showed that 5% KSR could support oocyte maturation ability during IVM and subsequent embryo development.

In summary, these results demonstrated that supplementation of the IVM culture medium with 5% KSR played a crucial role in the acquisition of oocyte developmental competence, with similar results to 10% pFF supplementation during IVM. This is evident is not only from the improvement in formation rates of blastocysts derived from PA but also from the regulation in fluorescence staining intensity of GDF9, BMP15, GSH and ROS. These indications support that 5% KSR supplementation has antioxidant property during IVM.

PART IV

LIPID METABOLISM REGULATING PORCINE OOCYTE MATURATION

Chapter I. Knockout serum replacement regulates lipid metabolism in porcine oocytes.

1. Introduction

It is well known that IVM of oocytes has become an ideal approach for treatment of polycystic ovary (PCO) and polycystic ovary syndrome (PCOS) patients who have absence of ovulation (Coticchio et al., 2012; Guzman et al., 2013; Lim et al., 2013). In general, numerous undefined supplements for oocyte culture media are used in IVM, such as follicular fluid (FF) (Jin et al., 2016), FBS (Suzuki et al., 2006), anestrous or estrous cow serum (Puri et al., 2015), estrous gilt serum (Son et al., 2013), BSA (Del Collado et al., 2015), and for human IVM, autologous patient serum (Chian et al., 1999) and human serum albumin (HSA) (Coticchio et al., 2013). These supplements have beneficial effects for nuclear and cytoplasmic maturation of oocytes due to the supply of vitamins, growth factors, nutrients, hormones and anti-oxidant compounds (Barnes and Sato, 1980; Zhang et al., 2007). Nevertheless, these components are highly undefined and present a risk of contamination of the culture environment. There has been a trend in recent years to use more defined components that have no batch-to-batch variability or risk of contamination.

As mentioned before, KSR is lipid-rich components. In addition, it is being increasingly recognized that the processes of lipogenesis and lipolysis are important for providing an essential energy source during oocyte maturation and embryo development (Jin et al., 2017a), which provide energy about three times

higher than glucose metabolism (Dunning et al., 2014b). Thus, to understand the mechanism of KSR during IVM, I focused on lipid metabolism and carried out researches.

The aims of this study were to (i) determine the influence of KSR on embryo development after SCNT and IVF; (ii) investigate the expression of genes lipogenesis, lipolysis, fatty acid β-oxidation, and mitochondrial biogenesis after IVM; (iii) measure the fluorescence staining intensity of BODIPY-LD, BODIPY-FA and BODIPY-ATP in porcine oocytes.

2. Materials and methods

2.1. Oocyte collection and *in vitro* maturation

Procedures for oocyte collection and *in vitro* maturation were described in general methodology.

2.2. Donor cell preparation for somatic cell nuclear transfer

Procedures for donor cell preparation were described in general methodology.

2.3. Nuclear transfer

Procedures for SCNT were described in general methodology.

2.4. *In vitro* fertilization

The COCs were denuded at 44 h following IVM by gently pipetting with 0.1% hyaluronidase and washed three times in TALP medium. Groups of 15 matured oocytes at the MII stage were randomly placed into 40 μL droplets of modified Tris−buffered medium (mTBM) in a 35x10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) covered with pre-warmed mineral oil. Subsequently, liquid semen supplied weekly from the DARBY Corporation (125-81-15252, An-Seong, Gyeonggi-do, Korea) was kept at 4˚C for 3 days prior to use. The semen sample was washed twice with DPBS supplemented with 0.1% BSA by centrifuging at 1,000 g for 2 min. After washing, the sperm pellet was re-suspended in mTBM. After appropriate dilution, 5 μl of the sperm suspension was

added to a 40 µl drop of fertilization medium (mTBM) to set a final sperm concentration of $1x10^6$ sperm/mL. Immediately before insemination, sperm motility was assessed and > 80% motile sperm was used in each experiment. The oocytes were co-incubated with spermatozoa for 20 min at 39˚C in a humidified atmosphere of 5% CO_2 and 95% air. After 20 min of co-incubation, loosely attached spermatozoa were removed from the zone pellucida (ZP) by gentle pipetting. Subsequently, the oocytes were washed three times in mTBM and incubated in mTBM without spermatozoa for 5-6 h at 39 ˚C in a humidified atmosphere of 5% CO_2 and 95% air. Thereafter, the gametes were washed three times in embryo culture medium and cultured in 25 µL microdrops (10 gametes/drop) of PZM-5 medium. The embryos with culture drops were incubated at 39˚C for 168 h under a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

2.5. Evaluation of lipid droplet, fatty acid and mitochondria content in oocytes

Denuded oocytes were fixed in 4% paraformaldehyde-PBS for 4 h at room temperature and washed in PBS before being stained in BODIPY-LD (BODIPY 493/503; D3922; Molecular Probes, Eugene, OR), BODIPY-FA (BODIPY 558/568 C12; D3835; Molecular Probes) and BODIPY-ATP (BODIPY FL ATP; A12410; Molecular Probes), and then incubated in PBS supplemented with 10 µg/mL BODIPY-LD, 6 µM BODIPY-FA and 500 nM BODIPY-ATP for 1 h at room temperature in the dark. Following staining, oocytes were washed in PBS three times and mounted on glass slides and gently compressed with cover slips. Images of each oocyte were captured using an epifluorescence microscope

(TE2000-S; Nikon). The fluorescence intensities and average sizes of lipid droplets were measured using Image J software (version 1.46r; National institutes of Health, USA).

2.6. Analysis of gene expression by quantitative real-time PCR

Procedures for Quantitative real-time PCR were described in general methodology. All oligonucleotide primer sequences are presented in Table 16.

2.7. Statistical analysis

All experiments were performed with a minimum of three independent replicates. Data are reported as the mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance followed by Tukey's *post hoc* test using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software. Differences were considered statistically significant if $P < 0.05$.

Table 16. List of real-time PCR primer

Gene	Primer sequences (5'- 3')	Product size (bp)	GenBank accession number
GAPDH	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359
ACACA	F: AACAAGGACCTGGTGGAGTG R: GTCATGTGCACGATGGAATC	170	NM_001114269
FASN	F: AACTTCCGAGACGTCATGCT R: GTGCTGAAGCAGCAGAACAG	180	NM_001099930
FADS1	F: ACTGGTTTGTGTGGGTGACA R: GCTCGATCTGGAAGTTGAGG	159	NM_001113041
PPARγ	F: AGAGCTGATCCAATGGTTGC R:GAGTTGGAAGGCTCTTCGTG	146	NM_214379
SREBF1	F: ACCCGCTTCTTCCTGAGTA R: ACGGAACAACTGAGTCACCT	207	NM_214157
LPL	F: AGGATGTGGCCACGTTTATC R: GGCTTGGAGCTTCTGCATAC	192	NM_214286
ATGL	F: CGAACTCAAGAGCACCATCA R: TTGCACATCTCTCGAAGCAC	189	NM_001098605
CGI-58	F: ATGCTCCATCGGATTGGTAA R: ATGTCCTGCTCCAAGAATGG	153	NM_001012407
HSL	F:TGTCTTTGCGGGTATTCG R:TTGTGCGGAAGAAGATGC	209	NM_214315
PLIN2	F: TGTGAGATGGCAGAGAAGGG R: CACAGCCCCTTTAGCATTGG	198	NM_214200
MGL	F: ACGTGGATGTCATGCAGAAG R: TGTTGCAGACTCAGGACTGG	164	NM_001143718

F, Forward primer; R, Reverse primer.

3. Results

3.1. Expression of lipid metabolism related factors in cumulus cells and oocytes

Although the relative expression of lipogenesis-related genes (*ACACA, FASN, FADS1, PPARγ* and *SREBF1*) were significantly increased in the control, lipolysis genes (*LPL, ATGL, CGI-58, HSL* and *PLIN2*) were significantly lower than in the 5% KSR and 10% pFF groups ($P < 0.05$). Moreover, fatty acid β-oxidation (*CPT1A, CPT1B, CPT2* and *ACADS*) gene expression was significantly increased in the 5% KSR group in cumulus cells (Figure 9).

In oocytes (Figure 10), the expression of genes for lipogenesis (*ACACA, FASN, FADS1, PPARγ* and *SREBF1*), lipolysis (*ATGL, HSL, PLIN2* and *MGL*), fatty acid β-oxidation (*CPT1A, CPT1B, CPT2* and *ACADS*) and mitochondrial biogenesis (*PGC-1α* and *PRDX2*) were significantly increased in the 5% KSR compared with the control group ($P < 0.05$). In the 10% pFF group, there was similar expression pattern to 5% KSR: there was a significant increase expression of genes *ACACA, FASN, FADS1, SREBF1, ATGL, HSL, MGL, CPT1B, CPT2, ACADS, PGC-1α* and *PRDX2* compared to the control group ($P < 0.05$). In addition, the fluorescence intensities of BODIPY-LD, BODIPY-FA and BODIPY-ATP in both supplementation groups (5% KSR and 10% pFF) were significantly different from the control group ($P < 0.05$, Figure 11).

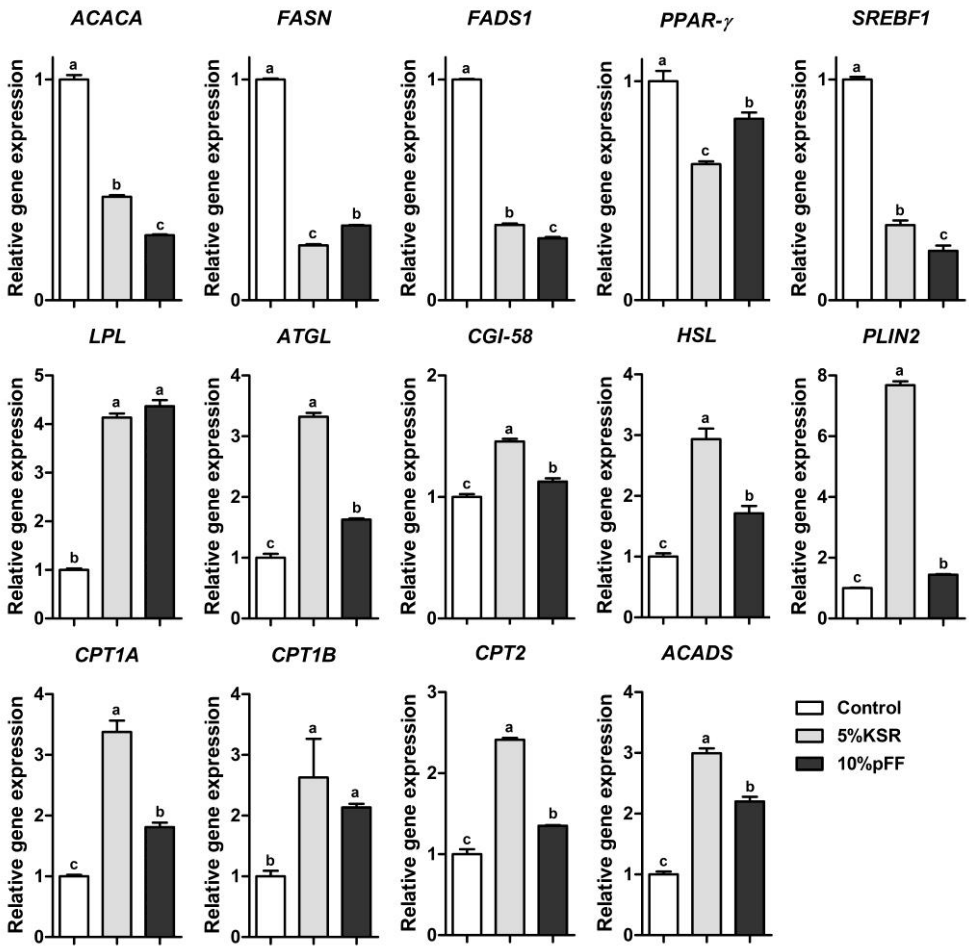


Figure 9. Expression of transcripts encoding lipid metabolism factors (lipogenesis, lipolysis and fatty acid β-oxidation) in porcine cumulus cells. Bars with different letters denote significant differences ($P < 0.05$). The experiment was replicated at least three times.

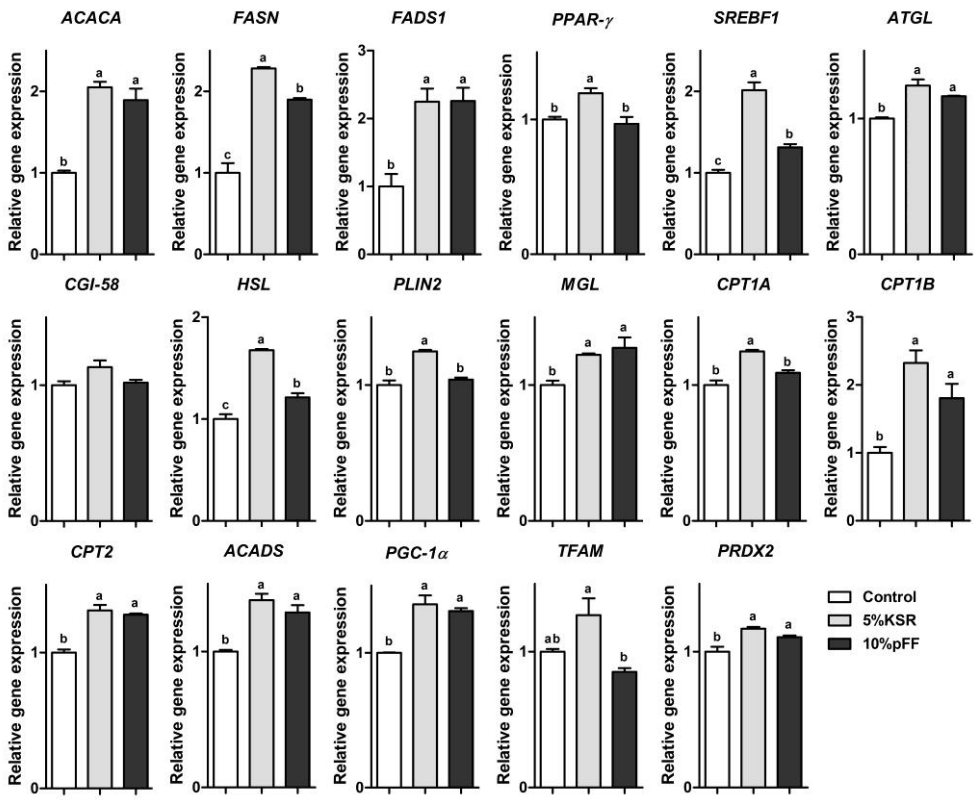


Figure 10. Expression of transcripts encoding lipid metabolism factors (lipogenesis, lipolysis, fatty acid β-oxidation, and mitochondrial biogenesis) in porcine oocytes. Bars with different letters denote significant differences ($P < 0.05$). The experiment was replicated at least three times.

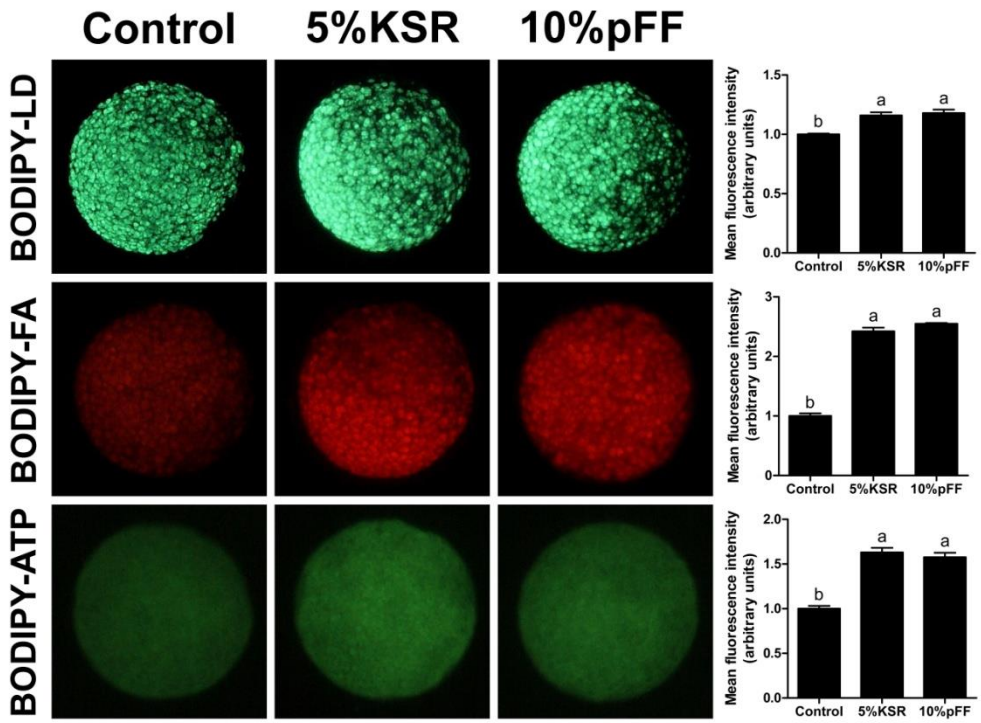


Figure 11. Analysis of BODIPY-LD, BODIPY-FA and BODIPY-ATP expression by fluorescence staining in porcine oocytes. Bars with different letters denote significant differences ($P < 0.05$). The experiment was replicated at least three times.

3.2. Development embryos derived from SCNT and IVF

In the case of SCNT (Table 17) and IVF (Table 18) embryo development, although there was no significant difference in cleavage rates and total cell numbers in blastocysts across all treatments, the rates of blastocyst formation with 5% KSR and 10% pFF were significantly higher than in the control group (23.8 ± 3.8 and 22.6 ± 2.4 vs. 11.1 ± 1.3 in SCNT; 20.1 ± 0.8 and 18.8 ± 0.9 vs. 11.8 ± 2.2 in IVF; $P < 0.05$, respectively).

Table 17. Effect of different supplementation during *in vitro* maturation (IVM) on embryonic development after somatic cell nuclear transfer (SCNT)

Group	No. of embryos cultured	No. of embryos developed to (mean ± SEM, %)				Total cell no. (mean ± SEM) in blastocysts
		≥ 2-cells		Blastocyst*		
Control	123	94	(75.0 ± 4.4)	13	(11.1 ± 1.3)[b]	41.6 ± 2.9
5% KSR	119	99	(82.6 ± 1.9)	27	(23.8 ± 3.8)[a]	54.0 ± 8.1
10% pFF	116	99	(85.7 ± 3.0)	26	(22.6 ± 2.4)[a]	46.6 ± 5.0

Values with different superscript letters within a column differ significantly ($P < 0.05$). Experiment was replicated six times.
The data represent means ± SEM.* Percentage of total cultured oocytes.

Table 18. Effect of different supplementation during *in vitro* maturation (IVM) on embryonic development after *in vitro* fertilization (IVF)

Group	No. of embryos cultured	No. of embryos developed to (mean ± SEM, %)				Total cell no. (mean ± SEM) in blastocysts
		≥ 2-cells		Blastocyst*		
Control	99	72	(73.0 ± 2.9)	12	(11.8 ± 2.2)[b]	54.7 ± 2.6
5% KSR	104	79	(75.1 ± 3.3)	21	(20.1 ± 0.8)[a]	62.2 ± 4.2
10% pFF	106	83	(78.6 ± 2.2)	20	(18.8 ± 0.9)[a]	62.5 ± 6.3

Values with different superscript letters within a column differ significantly ($P < 0.05$). Experiment was replicated three times.
The data represent means ± SEM. * Percentage of total cultured oocytes.

4. Discussion

In this study, I investigated that supplementation of IVM medium with 5% KSR significantly enhanced embryo development up to blastocyst in SCNT and IVF when compared to the control. In addition, the 5% KSR supplemented group significantly enhanced the intensity of BODYPI-LD, BODYPI-FA, BODYPI-ATP and the expression of lipid metabolism genes compared to the control, and these results exhibited similar patterns to the 10% pFF supplemented group.

To identify the role of KSR in oocyte maturation during IVM, I focused on lipid metabolism, and chose the 5% KSR and 10% pFF treatments compared with the control group for the subsequent experiments. In cumulus cells, the expression of lipogenesis-related genes (*ACACA*, *FASN*, *FADS1*, *PPAR-γ* and *SREBF1*) were significantly decreased in the 5% KSR and 10% pFF groups compared to the control group, and expression of lipolysis (*LPL*, *ATGL*, *CGI-58*, *HSL* and *PLIN2*) genes was significantly increased in the 5% KSR and 10% pFF groups compared to the control group. These data suggest that lipid droplets were not accumulated in cumulus cells during IVM. On the contrary, because lipolysis activities were more dynamic than lipogenesis in the 5% KSR and 10% pFF treatments, therefore the catalysis of fat hydrolysis was accelerated and fatty acid was subsequently released by these lipases (Dunning et al., 2014b). Additionally, it is well known that mitochondria play a vital role in oocyte and embryo development (Dumollard et al., 2007), and fatty acids are transported to the mitochondrial matrix by carnitine palmitoyltransferase I (*CPT1A* and *B*) and II (*CPT2*), which are located in the outer

and inner mitochondrial membranes, respectively (Downs et al., 2009; Paczkowski et al., 2014). My data revealed that transcription of *CPT1A* and *CPT2* was highly expressed in the 5% KSR and 10% pFF groups compared to the control group, and also acyl-CoA dehydrogenases (*ACADS*) which regulate β-oxidation pathway and ATP content.

In addition, lipid metabolism factors have been investigated in porcine oocytes after 44 h IVM. Lipogenesis and lipolysis activities were significantly increased in the 5% KSR and 10% pFF groups; these data are in line with expectations for the BODIPY-LD result. Thus, 5% KSR and 10% pFF treatment increased fatty acid content assayed by BODIPY-FA. The transcription of *CPT1B*, *CPT2* and *ACADS* was upregulated by 5% KSR and 10% pFF treatments. Until the end of the pathway, the transcription of mitochondrial biogenesis factors (*PGC-1α* and *PRDX2*) was more highly expressed by the Tricarboxylic acid Cycle in the 5% KSR and 10% pFF groups, in accord with results of ATP content determination (BODIPY-ATP). These data demonstrated that a balance between lipogenesis, lipid uptake, and intracellular lipolysis were required for maintaining lipid homeostasis in oocyte. The lipogenesis factors might be regulated by cluster of differentiation 36 (CD36) and fatty acid transport protein 4 (FATP4) transferred the fatty acids from cumulus cells to oocyte, and then accumulated neutral lipid in lipid droplets (Dunning et al., 2010). Simultaneously, generation of fatty acids by triglycerides utilization was indispensable process (Dunning et al., 2010). Therefore, β-oxidation pathway and production of ATP were important for their potential ability to

support and promote oocyte maturation and subsequent embryonic development by 5% KSR or 10% pFF supplements during IVM.

In summary, lipid metabolism-related genes were generally increased in the 5% KSR and 10% pFF groups compared to the control group, but not genes for lipogenesis in cumulus cells. Moreover, the intensity of BODIPY-LD, BODIPY-FA and BODIPY-ATP staining regulated by lipogenesis, lipolysis, β-oxidation and mitochondrial biogenesis factors were increased in the 5% KSR and 10% pFF groups. Therefore, I suggest that 5% KSR promotes lipid metabolism and thereby provides an essential energy source to sustain and improve oocyte quality and subsequent embryo development. These indications support the idea that KSR used as a defined serum supplement for oocyte IVM might be universally used in other species, especially for PCOS patients. Further research is needed to understand the pathway of fatty acid or ATP transfer from cumulus cells to oocytes.

Chapter II. Enhancement of lipid metabolism in porcine oocytes by melatonin treatment.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), synthesized from the mammalian pineal gland during the night (Stehle et al., 2011), has antioxidant properties (Reiter et al., 2016), and regulates physiological processes such as immune defence (Calvo et al., 2013), metabolic syndrome (Kitagawa et al., 2012), and lipid profiles (Kozirog et al., 2011). The functions of melatonin in reproduction are also associated with the quality of oocytes and optimal development of healthy embryos. Numerous studies have investigated the beneficial effects of melatonin on the development of oocytes and embryos, in various mammalian species, including mice (Banerjee et al., 2012; Coelho et al., 2015; Gao et al., 2012; Ishizuka et al., 2000; Tamura et al., 2008; Wang et al., 2013), sheep (Berlinguer et al., 2009), cattle (Papis et al., 2007; Wang et al., 2014; Zhao et al., 2015), and pigs (Kang et al., 2009; Li et al., 2015; Rodriguez-Osorio et al., 2007; Shi et al., 2009; Tian et al., 2014). However, no study has evaluated the effects of melatonin on lipid metabolism during porcine oocyte IVM.

Oocyte intracellular lipids are mainly stored in LDs, with majority in the triglycerides form (Prates et al., 2014; Sturmey et al., 2006), which provided energy for oocyte maturation and development (Prates et al., 2014). A balance between lipogenesis, lipid uptake and intracellular lipolysis are required for maintaining lipid homeostasis (Menendez, 2010). Therefore, accumulation of

neutral lipid in LDs (Carro et al., 2013) and generation of FAs by TG utilization (Dunning et al., 2014b) are indispensable processes. During oocyte maturation, LDs change their shape, volume and location by increasing lipolytic activity (Ferguson and Leese, 2006). These phenomena reflect the great requirement of FAs for biosynthetic processes that accompany resumption of meiosis and cytoplasmic maturation. Simultaneously, carnitine palmitoyltransferases (CPT1 and CPT2) and their cofactor carnitine are critical for transport of free FAs into mitochondria to undergo FA β-oxidation (FAO) pathway and thus to produce ATP, and are important for their potential ability to support and promote oocyte maturation and subsequent embryonic development (Dunning et al., 2010).

At present, the contribution of melatonin to lipid metabolism is debatable because investigation of their relationship has yielded contradictory results. Chronic supplementation with melatonin suppresses body weight and reduces adiposity in laboratory animals (Nduhirabandi et al., 2011). In sharp contrast, it was demonstrated that melatonin stimulates lipogenesis in 3T3-L1 fibroblasts (Gonzalez et al., 2012). Nevertheless, the function underlying these effects of melatonin on lipid metabolism has not been elucidated during porcine IVM. The aims of this study were to (i) determine the influence of melatonin on TG storage, basal lipolysis and ultimately size of LDs; (ii) investigate the expression of 17 genes involved in lipogenesis, lipolysis, FAO and mitochondrial biogenesis after IVM; (iii) assess the content of fatty acid, mitochondria and ATP in porcine oocytes; (iv) investigate the effect of melatonin on development of porcine SCNT embryos *in vitro* and *in vivo* with heterozygous knockout fetal fibroblasts.

2. Materials and methods

2.1. Oocyte collection and *in vitro* maturation

Procedures for oocyte collection and *in vitro* maturation were described in general methodology.

2.2. Parthenogenetic activation of oocytes

Procedures for parthenogenetic activation of oocytes were described in general methodology.

2.3. Lipid droplet staining

Using a variety of techniques, studies have quantified lipids in oocytes and determined that TG is a major constituent (Dunning et al., 2014b). BODIPY 493/503 (BODIPY-LD; D3922; Molecular Proves, Eugene, OR) is a neutral lipid dye that has recently been used to detect lipid droplets in oocytes (Dunning et al., 2014b). Denuded oocytes were fixed in 4% paraformaldehyde-PBS for 4 h at room temperature and washed in PBS before being stained in BODIPY-LD that was prepared by dissolving 10 mg BODIPY-LD in absolute dimethyl sulfoxide (DMSO) to a concentration of 2.5 mg/mL and further diluted to a final concentration of 10 μg/mL in PBS with 1% PVA to prevent adhesion among denuded oocytes, pipettes and dishes. Oocytes were stained for 1 h at room temperature in the dark. Following staining, oocytes were washed in PBS 3 times and mounted on cover slips. Images of each oocyte were captured using an epifluorescence microscope

(TE2000-S; Nikon, Japan). The fluorescence intensities and average sizes of lipid droplets were measured using Image J software (version 1.46r; National institutes of Health, USA).

2.4. Fluorescent fatty acid analog assays

Fluorescent fatty acid analog assays were performed as described by Lolicato *et al*. (Lolicato et al., 2015). Briefly, fixed oocytes were washed 3 times and incubated in PBS supplemented with 6 μM BODIPY 558/568 C12 (BODIPY-FA; D3835; Molecular Probes, Eugene, OR) for 1 h at room temperature in the dark. Oocytes were washed 3 times in PBS and mounted on cover slips. Images of each oocyte were captured using an epifluorescence microscope (TE2000-S; Nikon, Japan).

2.5. Evaluation of mitochondrial biogenesis in oocytes

The activities of mitochondria in oocytes were evaluated with MitoTracker@ Orange CMTMRos dye (M7510; Molecular Probes, Eugene, OR), which selectively stains live mitochondria. Matured oocytes were washed 3 times and incubated in PBS supplemented with 200 nM of MitoTracker@ Orange CMTMRos for 30 min under culture conditions. The oocytes were washed 3 times in PBS, and then fixed with 4% paraformaldehyde-PBS for 30 min at 37 ℃. After fixation, oocytes were washed 3 times in PBS and mounted on slides under cover slips. Images of each oocyte were captured using an epifluorescence microscope (TE2000-S; Nikon Japan).

2.6. ATP content in porcine oocytes

Denuded oocytes were washed 3 times in PBS and fixed with 4% paraformaldehyde-PBS for 1 h, washed 3 times and incubated in PBS supplemented with 500 nM BODIPY FL ATP (BODIPY-ATP; A12410; Molecular Probes, Eugene, OR) for 1 h at room temperature in the dark. Oocytes were washed 3 times in PBS and mounted on cover slips. Images of each oocyte were captured using an epifluorescence microscope. Images of each oocyte were captured using an epifluorescence microscope (TE2000-S; Nikon, Japan).

2.7. Donor cell preparation

Procedures for donor cell preparation were described in general methodology, and the cell line is heterozygous knockout fetal fibroblast.

2.8. Somatic cell nuclear transfer

Procedures for SCNT were described in general methodology.

2.9. Embryos transfer and pregnancy test

The surrogate mother was restrained and pre-anesthesia was induced by injection of ketamine (10 mg/kg; Yuhan, Seoul, Korea) and xylazine (1mg/kg; Bayer, Leverkusen, Germany) into an ear vein. The anesthetized pig was placed on a surgery table in a ventrodorsal posture. General anesthesia was maintained with isoflurane by concentration of 2.0 MAC (Hana Pharm, Seoul, Korea) under the supervision of a veterinarian. The recipient was ventrodorsally laid on surgical

table, and uterus and ovaries were taken out from abdomen through midline incision.

Reconstructed embryos were loaded into a Tomcat catheter (Catalog number; 8890703021, Covidien, Dublin, Ireland) with TALP medium equilibrated in 5% CO_2 with air. Only 2- to 4 –cell stage embryos were transferred into surrogate mothers those were 2 to 3 days after ovulation. The embryos were placed into the oviducts of each surrogate animal through a small puncture made with a suture needle (Catalog number; 6307-71, Covidien). The empty Tomcat catheter was removed carefully, and then put uterus and ovaries into the abdominal cavity, followed by suture the opened abdomen. The pregnancy status of recipients was determined by ultrasound on days 25.

2.10. Analysis of gene expression by quantitative real-time PCR

Procedures for Quantitative real-time PCR were described in general methodology. All oligonucleotide primer sequences are presented in Table 19.

2.11. Statistical analysis

Each experiment was repeated at least 3 times. The data were expressed as mean values ± standard error of the mean (SEM). The data were analyzed with univariate analysis of variance (ANOVA) followed by Tukey's test using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). Differences in gene expression and fluorescence intensity were compared by Student's t-test. $P < 0.05$ was considered statistically significant.

Table 19. List of primer for real-time PCR

Gene	Primer sequences (5'- 3')	Product size (bp)	GenBank accession number
GAPDH	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359
ACACA	F: AACAAGGACCTGGTGGAGTG R: GTCATGTGCACGATGGAATC	170	NM_001114269
FASN	F: AACTTCCGAGACGTCATGCT R: GTGCTGAAGCAGCAGAACAG	180	NM_001099930
FADS1	F: ACTGGTTTGTGTGGGTGACA R: GCTCGATCTGGAAGTTGAGG	159	NM_001113041
PPARγ	F: AGAGCTGATCCAATGGTTGC R:GAGTTGGAAGGCTCTTCGTG	146	NM_214379
SREBF1	F: ACCCGCTTCTTCCTGAGTA R: ACGGAACAACTGAGTCACCT	207	NM_214157
ATGL	F: CGAACTCAAGAGCACCATCA R: TTGCACATCTCTCGAAGCAC	189	NM_001098605
CGI-58	F: ATGCTCCATCGGATTGGTAA R: ATGTCCTGCTCCAAGAATGG	153	NM_001012407
HSL	F:TGTCTTTGCGGGTATTCG R:TTGTGCGGAAGAAGATGC	209	NM_214315
MGL	F: ACGTGGATGTCATGCAGAAG R: TGTTGCAGACTCAGGACTGG	164	NM_001143718
PLIN2	F: TGTGAGATGGCAGAGAAGGG R: CACAGCCCCTTTAGCATTGG	198	NM_214200

Gene	Primer sequences (5'- 3')	Product size (bp)	GenBank accession number
CPT1a	F: AAGGTGCTGCTCTCCTACCA R: CATCAGAGGCTTCACGGATT	204	NM_001129805
CPT1b	F: ATCAAGCCTGTGATGGCTCT R: GAGCCACACCTTGAAGAAGC	168	NM_001007191
CPT2	F: AGTTCCAGAGAGGAGGCAAAG R: GAGCATCTCTTGGTGAAGACG	199	NM_001246243
ACADS	F: CAGTCTGTGGAACTGCCTGA R: TGGTGTAGGCCAGGTAATCC	211	NM_213898
PGC-1α	F: TTCCGTATCACCACCCAAAT R: ATCTACTGCCTGGGGACCTT	137	NW_213963
TFAM	F: TCCGTTCAGTTTTGCGTATG R: TTGTACACCTGCCAGTCTGC	240	NM_001130211
PRDX2	F: TGTCCTTCGCCAGATCACT R: TCCACGTTGGGCTTGATT	156	NM_001244474

F, Forward primer; R, Reverse primer.

3. Results

A total of 658 oocytes (Figure 12A) were used in five replicates to evaluate the effects of melatonin at various concentrations during IVM on porcine embryo development after PA. The cleavage rate with 10^{-3} M melatonin was significantly lower than in the 0, 10^{-9}, 10^{-7} and 10^{-5} M melatonin (Figure 12B). The rates of blastocyst formation with 10^{-9}, 10^{-7} and 10^{-5} M melatonin were significantly higher than in the 10^{-3} M and control groups (Figure 12C). The mean cell numbers/blastocyst with 10^{-9}, 10^{-7} and 10^{-5} M melatonin during IVM were also significantly higher than in the 10^{-3} M and control groups (Figure 12D). According to these results, I chose the 10^{-9} M concentration of melatonin for the treatment in the following experiments.

To measure cytoplasmic lipid content and LDs sizes in mature oocytes, BODIPY-LD, a novel green fluorescent stain for neutral lipid, was used (Figure 13A) to examine the effect of melatonin on several genes associated with lipogenesis. As shown in Fig. 2B, the fluorescence intensity of BODIPY-LD with melatonin treatment was significantly higher than in the control group (Figure 13B). Moreover, gene expression of *ACACA*, *FASN*, *PPARγ* and *SREBF1* (Figure 14A) was also significantly upregulated in melatonin-treated oocytes.

To determine whether melatonin treatment promoted lipolysis, I analyzed the size of lipid droplets and lipolysis-related gene expression in oocytes. As shown in Figure 13C, LDs were smaller with melatonin treatment compared to the control

group. Moreover, melatonin treatment also enhanced lipolytic activity as judged by expression of lipase genes at the mRNA level, but not of *MGL* (Figure 14B).

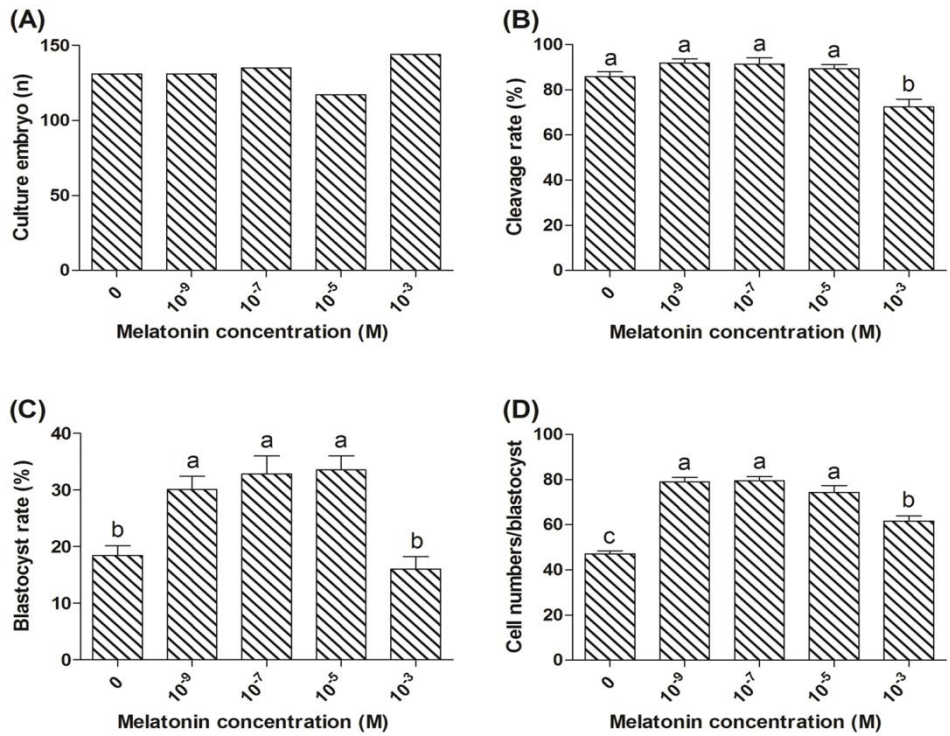


Figure 12. Effects of melatonin supplementation during *in vitro* maturation of parthenogenetically activated oocytes on embryonic development. (A) Number of embryos cultured; (B) Cleavage rate; (C) Blastocyst rate; (D) Total cell numbers/blastocyst. The experiment was replicated 5 times. The superscript letters (a-c) represent statistically significant differences ($P < 0.05$).

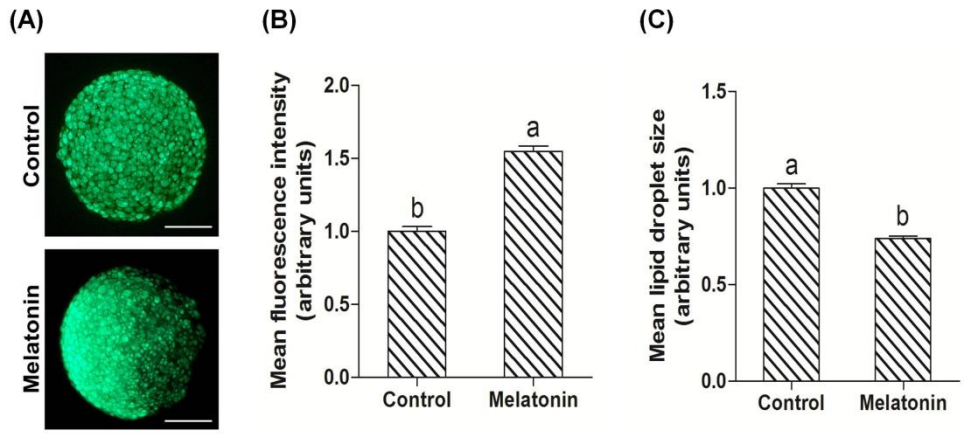


Figure 13. Effects of melatonin on lipid droplet distribution in porcine *in vitro* matured oocytes. (A) Images of oocytes obtained by fluorescence microscopy. Lipid droplets are stained with the lipophilic dye BODIPY 493/503. Scale bar indicates 50 μm. (B) BODIPY 493/503 fluorescence intensity quantitation in oocytes. (C) The size of lipid droplets, as measured with Image J. Data are mean ± SEM of three independent experiments. Different letters denote significant difference ($P < 0.05$).

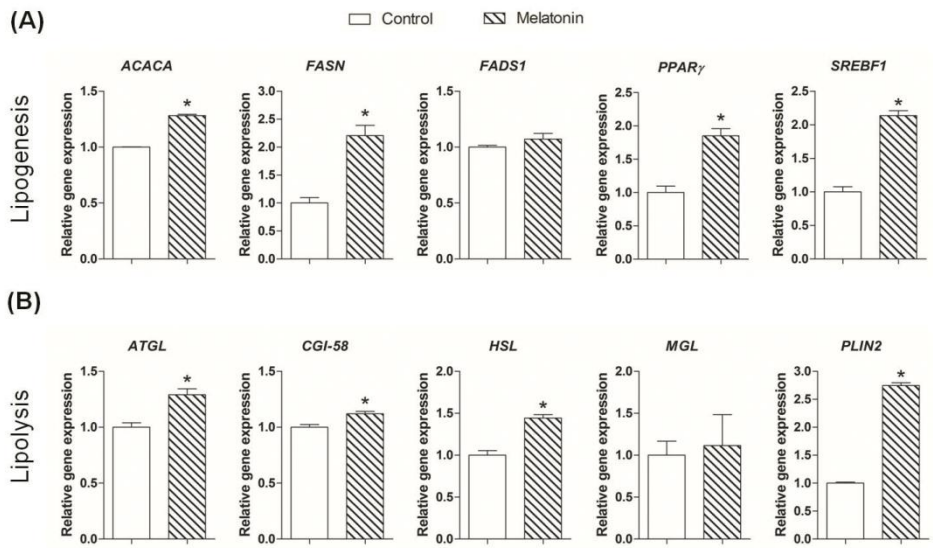


Figure 14. Effects of melatonin on expression of lipogenic and lipolytic genes in porcine *in vitro* matured oocytes. (A) Lipogenic factors (*ACACA*, *FASN*, *FADS1*, *PPARγ* and *SREBF1*). (B) Lipolytic factors (*ATGL*, *CGI-58*, *HSL*, *MGL* and *PLIN2*). Within the same mRNA, bars with asterisks are significantly different ($P < 0.05$). The experiment was replicated at least 3 times.

The role of melatonin in FAs uptake by oocytes was investigated by labeling with the fluorescent FA analog BODIPY-FA. Quantification of the BODIPY-FA stained areas of oocyte images is shown in Figure 15A. The uptake of BODIPY-FA was 2.03-fold greater in the melatonin treatment group compared to the control group (Figure 15B). Moreover, the expression levels of free FAO-related genes were increased in the melatonin treatment group (Figure 18A).

Recent advances suggest that LDs can be found in association with other organelles linked to cellular metabolism such as mitochondria (Prates et al., 2014). Consequently, I evaluated the content of mitochondria and ATP in oocytes with MitoTracker@ Orange CMTMRos dye and BODIPY-ATP, respectively. As shown in Figure 16, mitochondrial content was significantly increased in the melatonin-treated group compared to the control group. Moreover, the ATP content of oocytes treated with melatonin was also higher than in the control group (Figure 17). Additionally, melatonin treatment upregulated transcriptional activator of mitochondria related genes including, *PGC-1α, TFAM* and *PRDX2* (Figure 18B).

In addition, the SCNT embryos up to blastocyst were significantly increased by melatonin treatment group compare to control group (33.8 ± 3.0 vs. 20.3 ± 2.2, $P < 0.05$, Table 20), but not total cell numbers. Moreover, one of 3 recipients was pregnant in melatonin groups after embryo transfer (Table 21).

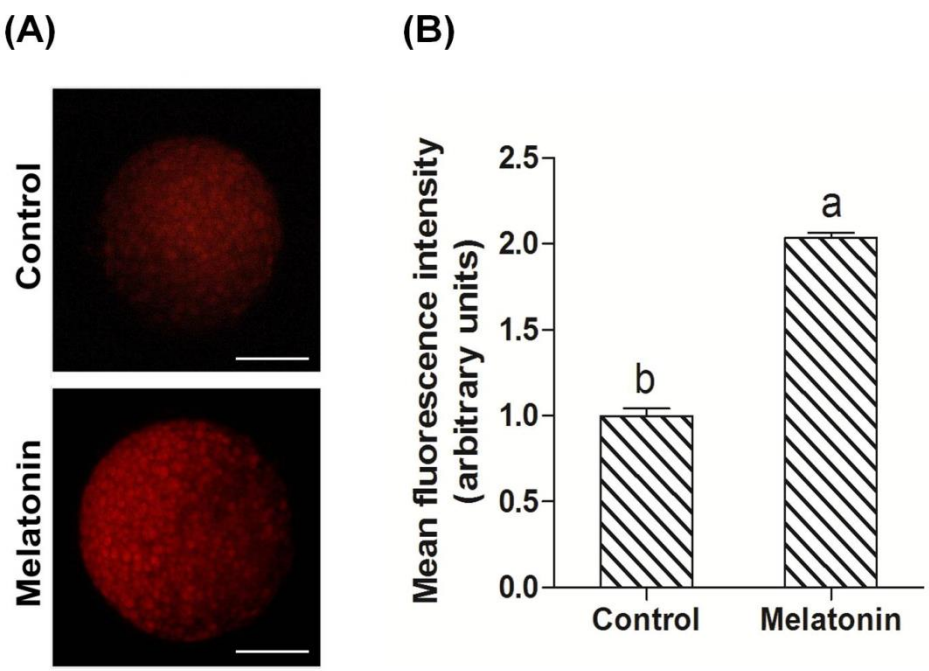


Figure 15. Fluorescent fatty acid analog in porcine *in vitro* matured oocytes. (A) Images of oocytes obtained by fluorescence microscopy. Fatty acids are stained with BODIPY-FA. Scale bar indicates 50 μm. (B) Content of fatty acid, as measured with Image J. Data are mean ± SEM of three independent experiments. Different letters denote significant difference ($P < 0.05$).

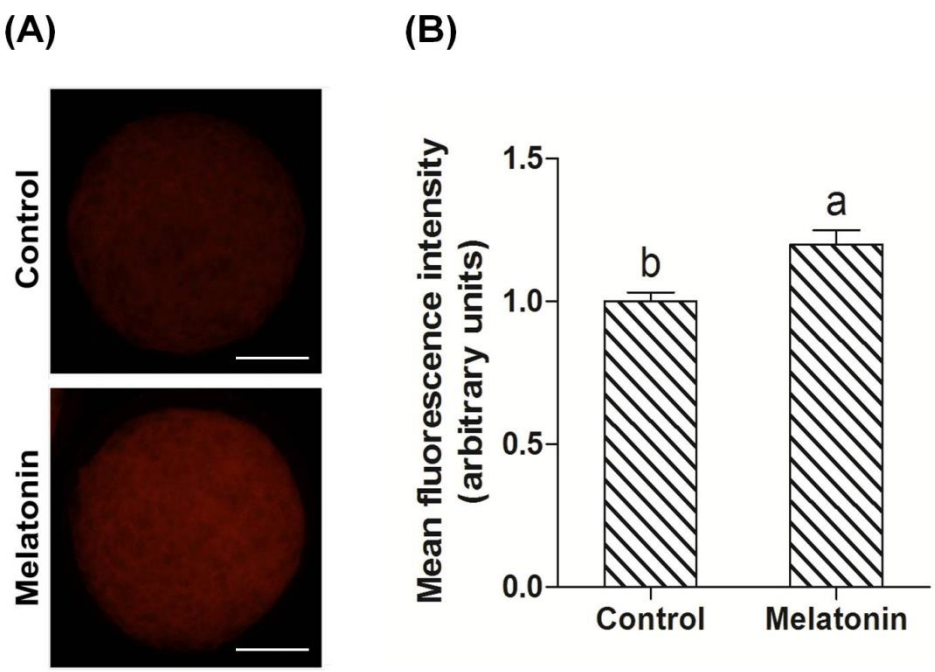


Figure 16. Effects of melatonin on numbers of mitochondria in porcine *in vitro* matured oocytes. (A) Oocytes were evaluated with MitoTracker@ Orange CMTMRos dye. Scale bar indicates 50 μm. (B) Fluorescence intensities of mitochondrial content, as measured with Image J. Data are mean ± SEM of three independent experiments. Different letters denote significant difference ($P < 0.05$).

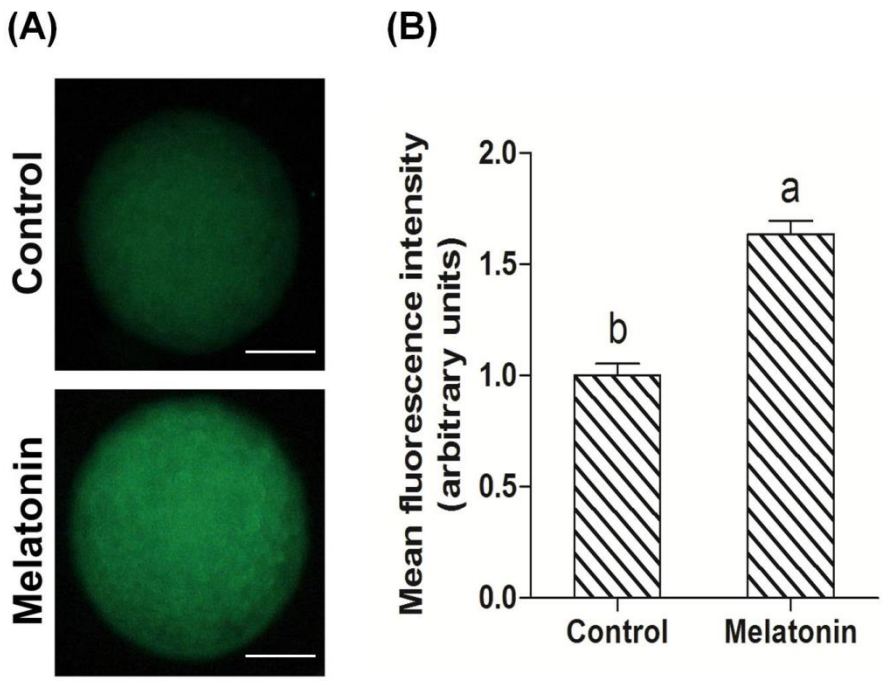


Figure 17. Melatonin treatment enhances ATP content in porcine oocytes. (A) ATP content stained with BODIPY-ATP. Scale bar indicates 50 μm. (B) Fluorescence intensities indicate ATP content. Data are mean ± SEM of three independent experiments. Different letters denote significant difference ($P < 0.05$).

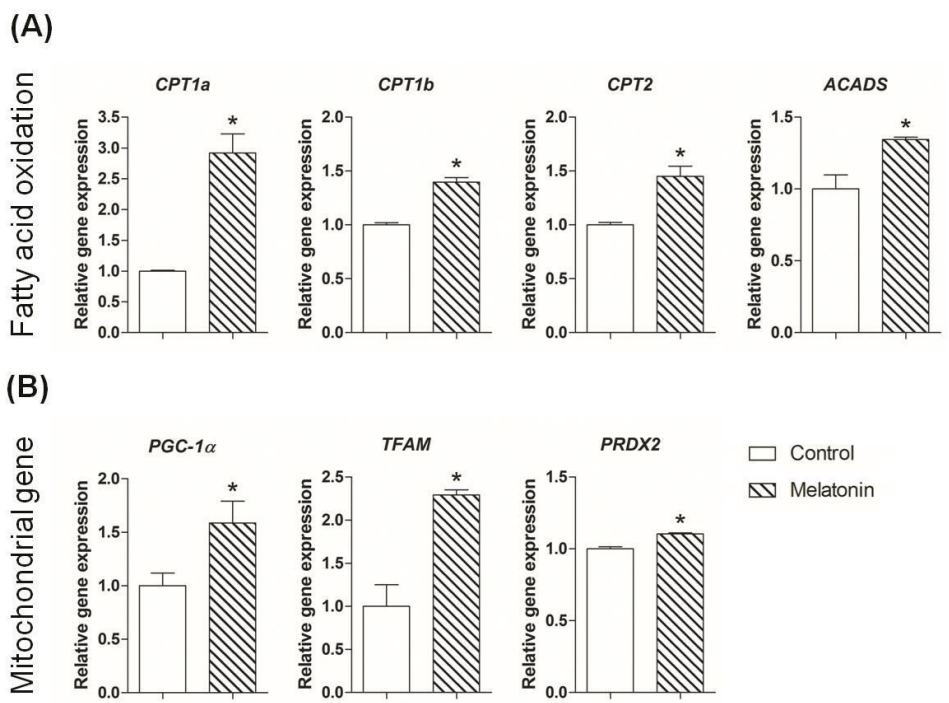


Figure 18. Effects of melatonin on fatty acid oxidation and mitochondrial biogenesis-related gene expression in porcine *in vitro* matured oocytes. (A) Fatty acid oxidation factors (*CPT1a*, *CPT1b*, *CPT2* and *ACADS*). (B) Mitochondrial biogenesis factors (*PGC-1α*, *TFAM* and *PRDX2*). Within the same mRNA, bars with asterisks are significantly different ($P < 0.05$). The experiment was replicated at least 3 times.

Table 20. Effect of melatonin treatment during IVM on embryonic development after somatic cell nuclear transfer (SCNT).

Group	No. of embryos cultured	No. of embryos developed to (mean ± SEM, %)				Total cell no. (mean ± SEM) in blastocysts
		≥2-cells		Blastocyst*		
Control	127	108	(84.5 ± 1.6)	13	(20.3 ± 2.2)[b]	48.3 ± 3.7
Melatonin	114	98	(86.1 ± 1.2)	27	(33.8 ± 3.0)[a]	53.4 ± 5.0

Values with different superscript letters within a column differ significantly ($P < 0.05$).
Experiment was replicated three times. The data represent means ± SEM. * Percentage of total cultured oocytes.

Table 21. Pregnancy status of nuclear transfer-derived embryos using heterozygous knockout fetal fibroblasts as donors after melatonin treatment

Melatonin treatment	No. of recipients	No. of transferred embryos	Pregnancy status
Melatonin-	Y168-70	102	-
	Y170-42	226	-
	Y166-62	141	-
Melatonin+	Y171-28	139	-
	Y171-6	139	-
	Y168-76	114	+

4. Discussion

In a previous study, Shi and colleagues found melatonin in follicular fluid at a concentration of approximately 10^{-11} M in pigs (Shi et al., 2009). They stated that the reason why the concentration of melatonin decreased inversely with the diameter of follicles during oocyte *in vivo* maturation is that melatonin is required to promote follicle development and oocyte maturation by inducing expression of luteinizing hormone receptor (Shi et al., 2009). Therefore, addition of 10% porcine follicular fluid to IVM medium is indispensable for oocyte maturation. However, the concentration of melatonin provided in this way is not enough to meet the requirements of IVM.

In this study, I found that 10^{-9}, 10^{-7} and 10^{-5} M concentrations of melatonin significantly improved the rate of blastocyst formation and total cell numbers compared to the control group. In line with this, Shi et al. also suggested that the peak value of melatonin treatment was observed with 10^{-9} M used in IVM (Shi et al., 2009). Thus, I indicated the idea that the required concentrations of melatonin are different between the *in vivo* and *in vitro* environments. In addition, 10^{-3} M melatonin treatment significantly reduced cleavage rates, and increased total cell numbers in blastocysts compared to the control group, but not blastocyst formation rates. In various *in vivo* and *in vitro* experiments, pharmacological levels of melatonin have been shown to have low levels of toxicity (Reiter, 1998). This was indicated by finding that embryo cleavage was delayed by melatonin in a dose-dependent manner. Although various concentrations of melatonin added to

IVM/IVC media influenced oocyte maturation and embryo development in different species (Banerjee et al., 2012; Berlinguer et al., 2009; Coelho et al., 2015; Gao et al., 2012; Ishizuka et al., 2000; Kang et al., 2009; Li et al., 2015; Papis et al., 2007; Rodriguez-Osorio et al., 2007; Shi et al., 2009; Tamura et al., 2008; Tian et al., 2014; Wang et al., 2014; Wang et al., 2013; Zhao et al., 2015), the precise mechanism of this effect remain unclear.

Several decades ago, because glucose is considered the most important exogenous energy source, studies were mostly focused on glucose metabolism, mainly glycolysis and the pentose phosphate pathway. These pathways provide energy to oocytes *via* directly usable oxidizable substrates such as pyruvate and lactate (Sanchez-Lazo et al., 2014). However, ATP produced by glucose metabolism is insufficient to maintain cellular redox. In contrast, it has recently been shown that FA produced from LDs is another important energy source, which provides several times more energy per molecule than glucose. For instance, oxidation of one molecule of FA can generate 106 ATP molecules compared with glucose oxidation which only yields ~30 ATP molecules (Dunning et al., 2014b). Thus, lipid metabolism is essential to provide energy for supporting and promoting oocyte maturation and embryo development (Dunning et al., 2010).

Recent reports suggested that expression of the key lipid metabolism regulators acetyl coenzyme A carboxylase α (*ACACA*) and FA synthase (*FASN*) supports lipogenic activity in oocytes (Auclair et al., 2013; Brisard et al., 2014). In addition, expression of sterol regulatory element binding transcription factor 1 (*SREBF1*) and peroxisome proliferator activated receptor gamma (*PPARγ*) regulates

lipogenesis and causes accumulation of lipids in oocytes (Al Darwich et al., 2010; Cui et al., 2002; Gu et al., 2015; Sampath and Ntambi, 2005; Sieber and Spradling, 2015). In this study, I found smaller sizes and larger numbers of LDs with melatonin treatment during IVM. The transformation of large areas of LDs in the cytoplasm into small ones was closely related to subsequent embryo development. In order to study this phenomenon, I examined key genes involved in lipogenesis and lipolysis with quantitative RT-PCR analysis. Melatonin treatment greatly increased the expression of *ACACA, FASN, PPARγ* and *SREBF1*, illustrating the importance of LDs accumulation in oocytes (da Costa et al., 2016; Sanchez-Lazo et al., 2014; Sieber and Spradling, 2015). The *ACACA* gene plays a vital role in lipid metabolism (Valsangkar and Downs, 2015). Uzbekova *et al.* suggested that greater expression of *ACACA* improved potential lipogenic activity of oocytes (Uzbekova et al., 2015). Consequently, the developmental competence of oocytes depends in part on their capacity to increase intracellular energy stores (Aardema et al., 2011).

Another aspect of this study showed that the genes encoding adipose triglyceride lipase (*ATGL*), comparative gene identification-58 (*CGI-58*), hormone-sensitive lipase (*HSL*) and perilipin 2 (*PLIN2*) were also upregulated by melatonin treatment, but not the gene for monoglyceride lipase (*MGL*). In the first place, *ATGL* catalyzes the initial step in the hydrolysis of TG (Zechner et al., 2009), which activity was associated with the co-activator, *CGI-58*. Another point is that *HSL* exhibits a much broader substrate spectrum, with a preference for DG (Holm, 2003), while last but not least, *MGL* degrades MG thereby generating FAs and glycerol (Morak et al., 2012). In addition, it was suggested that *PLIN2* seemed to

be a major LD-associated protein and was most likely involved in intracellular lipid storage and utilization processes during porcine oocyte maturation (Zhang et al., 2014). Activity of lipolysis genes induced the fragmentation of large LDs into small ones.

Using BODIPY-FA I also measured oocyte FA content which was significantly increased with melatonin treatment compared to the control group. These data support the hypothesis that melatonin reduced the size of lipid droplets and increased LDs utilization, which was associated with releasing a mass of FA by lipolysis. Therefore, melatonin treatment influenced the content of FAs which was related to oocyte quality in terms of maturation and embryo development competence.

FAs generated by lipolysis are further metabolized in mitochondria *via* β-oxidation for the production of ATP. FAs entering the mitochondrial matrix are catalyzed by carnitine palmitoyltransferase I (*CPT1a* and *b*) and II (*CPT2*), which are located in the outer and inner mitochondrial membranes, respectively (Paczkowski et al., 2014). Moreover, carnitine is an essential co-factor required for long-chain FAs to enter mitochondria. L-carnitine added to the IVM culture medium significantly increased β-oxidation in mouse COCs (Dunning et al., 2011; Dunning et al., 2014a). Other reports have demonstrated positive effects of L-carnitine on maturation of porcine oocytes and their developmental competence (Somfai et al., 2011; Wu et al., 2011; You et al., 2012). On the other hand, if β-oxidation was inhibited, oocyte maturation and embryo development were also impaired (Dunning et al., 2014b). My results demonstrated that melatonin

upregulated carnitine palmitoyltransferase and acyl-CoA dehydrogenases (*ACADS*). At the same time, the β-oxidation pathway was also activated by melatonin treatment.

It is well known that mitochondria play a vital role in oocytes and embryos. The numbers of mitochondria in oocyte/embryonic cells are also indicative of the energy and ion requirements associated with oocyte maturation, fertilization and early embryonic development (Niu et al., 2015). Peroxiredoxin-II (*PRDX2*) influenced the quality and mitochondrial activity of oocytes and embryos (Fakruzzaman et al., 2015). Mitochondrial transcription factor A (*TFAM*) is an autosomal mitochondrial gene that encodes a histone-like protein essential for the transcription and replication of mitochondrial DNA (Jiang et al., 2005). Furthermore, mitochondrial dysfunction is induced by silencing *TFAM*, which suppresses mitochondrial biogenesis and synthesis (Koh et al., 2007). Another report stated that mitochondrial biogenesis is believed to be triggered by increased expression of PPARγ coactivator-1α (*PGC-1α*), a master regulator of mitochondrial biogenesis and function (Liang and Ward, 2006). Notably, I found that melatonin significantly upregulated the expression of *PGC-1α, TFAM* and *PRDX2*, hence increasing the activity and content of ATP in oocytes.

In summary, these results demonstrated that melatonin plays an important role in the acquisition of porcine oocyte developmental competence when added to the IVM medium. This is evident from the improvement in blastocyst formation rates with the optimal concentration of melatonin (10^{-9} M) after PA and SCNT. In subsequent experiments, melatonin resulted in increased content and reduced size

of LDs, together with increased transcripts for genes related to lipogenesis and lipolysis. Moreover, melatonin also increased the content of FA, mitochondria and ATP together with upregulated gene expression. Finally, one of 3 recipients was pregnant by melatonin treatment after embryo transfer to surrogate mothers. Therefore, I suggest that melatonin treatment promotes lipid metabolism and thereby provides an essential energy source to support and improve oocyte quality and embryo development. Further research is needed to understand the upstream pathway underlying the mechanism by which melatonin promotes lipid metabolism in porcine COCs.

PART V

FINAL CONCLUSION

This thesis was conducted to introduce the role of antioxidant and lipid metabolism and then, apply these functions to IVM systems for improving porcine oocyte maturation and embryo developmental competence.

First, treatment of porcine oocyte with 10 uM spermine has a beneficial effect on preimplantation development leading to enhanced PA and SCNT blastocyst formation rates by increasing the intracellular GSH levels, decreasing the ROS levels, In the future, to use spermine routinely for the enhancement of in vitro conditions for mammalian oocytes and embryos.

Supplementation of the IVM culture medium with 5% KSR played a crucial role in the acquisition of oocyte developmental competence, with similar results to 10% pFF supplementation during IVM. These indications support that 5% KSR supplementation has antioxidant property, and is could be replaced to 10% pFF during IVM.

Second, 5% KSR promotes lipid metabolism and thereby provides an essential energy source to sustain and improve oocyte quality and subsequent embryo development. It will be used as a defined serum supplement for oocyte IVM might be universally used in other species, especially for PCOS patients.

Melatonin upregulates lipid metabolism during IVM and thereby produce essential energy to sustain and improve oocyte quality and their subsequent embryonic development.

It is feasible that upregulation of lipid metabolism and antioxidation are indispensable mechanisms for improving porcine oocyte IVM systems.

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국문초록

항산화제 및 지질대사 증진을 통한 돼지 난자 체외성숙 시스템 향상 연구

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돼지에서 수정란의 체외생산을 위해서는 체외성숙과정이 필요하다. 체외 성숙 과정을 거쳐 미성숙난자는 성숙난자로 발육되고, 체외배양을 통해 배반포로 발달하게 된다. 그러나 체외성숙 난자의 체외발달율은 체내성숙난자에 비해 유의적으로 낮다.

본 연구는 항산화제와 지질대사가 난자의 체외성숙과 이어지는 체외발달에 미치는 영향을 알아보고자 수행되었다.

첫 번째로, 항산화제로서 spermine 을 이용하여 돼지 난자의 체외성숙기간동안 처리하는 것이 난자의 체외성숙 이후 체외발달에 미치는 영향을 조사하였다. Spermine 의 처리 결과 돼지 난자의 체외성숙과 이후 단위발생 및 체세포복제수정란의 체외발달에 미치는 영향을 연구하였다. 그 결과 체외성숙배지에 다양한 농도의

spermine 을 처리하였을 때 핵 성숙에는 유의적인 차이가 없었다. 그러나 10uM 의 spermine 처리군에서는 대조군에 비해 세포내 glutathione (GSH) 농도가 증가하였고, reactive oxygen species (ROS) 농도는 유의적으로 감소되는 것을 알 수 있었다. 더욱이 단위발생란 및 복제수정란의 체외발달에 있어 체외성숙시 10uM 의 spermine 처리군은 대조군에 비해 유의적으로 높은 배반포 발달율을 보였다. 이를 통해 돼지 난자의 체외성숙시에 10uM 의 spermine 처리는 세포내 GSH 농도를 증가시키고, ROS 를 낮추어 주며, 그리고 유전자발현의 조절을 통해 이후 단위발생란 및 체세포복제란의 배반포발달율을 높이는 것으로 보인다.

Knockout serum replacement (KSR)은 작은 유기분자, 미량 원소, 그리고 3 가지 단백질 (인슐린, 트랜스페린 및 지질이 풍부한 알부민)로 구성되어 있으며, 불특정의성장인자 및 분화촉진인자가 포함되어 있지 않은 구성물이다. 그리하여 5% KSR 이 돼지난자의 체외성숙 시스템에서 일반적으로 사용하는 돼지난포액 (porcine follicular fluid, pFF) 을 대체할 수 있을지를 조사하였다. KSR 5% 및 10% 처리군은 대조군 및 10% pFF 첨가군보다 유의적으로 난구세포확장이 저하된 결과를 보였으나, KSR 5% 처리군은 대조군에 비해 *Ptgs1*, *Has2* 및 *Tnfaip6* 등 난구세포확장에 관여하는 유전자 발현은 유의적으로 높게 나타났다. 그리고 KSR 5% 처리군은 난자발달에 관여하는 유전자인 *GDF9*, *BMP15* 및 *Cdc2* 의 발현은 유의적으로 높았으며, 동시에 pFF 10% 처리군과 함께 GDF9 및 BMP15 의 단백질 발현량도 향상시켰다. 또한 KSR 5% 과 pFF 10% 처리군의 난자 및 난구세포에서 *Bax/Bcl2* 의 발현은 모두 감소됨을 밝혔고, 낮은 ROS 수준 및 높은 GSH 수준을 보였다. 이와 같은 조건에서 단위발생란의 발달에서도 유의적으로 높게 나타났다. 그러므로 5% KSR 의 항산화작용에 의해 난자 및 배아발달이 향상됨을 밝혔다.

둘째로는 KSR 과 멜라토닌의 처리에 의한 돼지 난자 성숙기간동안 지질대사 변화를 분석하였다. KSR 이 난자의 체외성숙 시스템에 어떠한 기전으로 영향을 미치는지를 조사하기 위하여 지질대사에 초점을 두어 분석을 진행하였다. 그 결과 KSR 5% 과 pFF 10% 처리군에서 체외수정 및 복제수정란의 배반포 형성이 모두 향상됨을 보여주었다. 그리고 KSR 5% 과 pFF 10% 처리군에서 BODIPY-LD, BODIPY-FA 및 BODIPY-ATP 가 높게 발현되었으며, 지질대사에 관여하는 대부분 유전자들도 높게 발현되었다. 그러므로 KSR 5% 처리는 배반포 형성을 위한 필요한 에너지를 돼지 난자에 제공함으로서 발달능에 좋은 영향을 미쳤다. 또한 돼지 난자의 체외성숙 시스템에서 일반적으로 사용하는 10% 돼지난포액을 대체할 수 있음을 밝혔다.

멜라토닌 처리가 돼지난자에 미치는 영향을 본 결과 돼지난자의 지질 함량을 유의적으로 향상시키고 지질형성에 관여하는 유전자 (*ACACA*, *FASN*, *PPARγ*, 및 *SREBF1*) 발현량을 유의적으로 높였다. 이와 동시에 멜라토닌을 처리한 난자 군에서 지질방울 (lipid droplets)의 크기가 작게 형성되었고, 지질분해효소에 관여하는 유전자인 *ATGL*, *CGI-58*, *HSL* 및 *PLIN2* 의 발현도 유의적으로 증가하였다. 또한 염색 후 분석결과 멜라토닌 처리군은 지방산, 미토콘드리아 및 adenosine triphosphate (ATP)의 함량이 증가됨을 알 수 있었다. 동시에 멜라토닌 처리결과 지방산 베타-산화 유전자 (*CPT1a*, *CPT1b*, *CPT2* 및 *ACADS*) 및 미토콘드리아 합성 유전자 (*PGC-1α*, *TFAM* 및 *PRDX2*)의 높은 발현을 확인하여, 지질대사에 영향을 준다는 것을 다시 한번 밝혔다. 이러한 결과로 볼 때 멜라토닌을 돼지 난자 체외성숙 시스템에 처리시 돼지 난자에서 지질방울의 형태적 변화를 볼 수 있을 뿐만 아니라, 지방분해효소에 의해 생성된 지방산은 미토콘드리아로 이송되고 ATP 를 생성하며, 생성된 ATP 는 돼지난자 및 배아발달의

에너지로 쓰인다는 것을 밝혔다. 그리고 멜라토닌은 복제수정란의 발달을 높여주고, 멜라토닌 처리군에서 대리모 한 마리가 임신되었다.

외인성 항산화제인 KSR 및 spermine 을 돼지 난자 체외성숙 시스템에 처리 하였을 때 난자 및 배아발달을 향상 시켰으며, 이는 GSH 향상 및 ROS 감소에 기여된것을 밝혔다. 그리고 KSR 및 멜라토닌은 지질대사를 향상시켜 돼지 난자 및 배아발달에 에너지원을 제공 함으로써 배반포 발달률을 향상시켰다. 이상의 결과로 보아 항산화 및 지질대사 작용은 돼지 난자 체외 성숙 시스템 향상의 필수적인 기전임을 알 수 있었다.

주요어: 돼지, 항산화제, 지질대사, 난자성숙, 배아발달
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